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Same Day Sputum Smear Microscopy for the Diagnosis of Pumouary Tuberculosis: ZN Staining Versus Reverse ZN Staining

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In same day sputum smear microscopy two sputum samples are collected with a gap of 1 hour. Study was conducted in department of Microbiology, GSL Medical College, Rajahmundry from March 2014 to May 2015. Three sputum (S, S_2 and M) samples were collected from the pulmonary tuberculosis patients. Duplicate smears were prepared; one smear was stained by standard Ziehl Neelsen (ZN) staining technique and the other smear by reverse ZN (RZN) staining technique. In RZN staining technique, 0.1% methylene blue was used as primary staining reagent and 1% Carbol Fuchsin was the counter staining reagent. Of the 61 study participants, male/female ratio was 1.33:1, with mean age of 42.1 years. The smear positivity was 100%, 77% for spot morning (SM) approach and 92%, 72% for same day (SS_2) approach, respectively for ZN staining and RZN staining. Statistically the difference was significant between the staining techniques in SM and SS_2 approaches ($P < 0.05$). Due to the limited smear positivity, confusion in smear reading RZN staining technique neither used for teaching in tertiary hospitals nor implemented by the RNTCP.

Keywords: Ziehl Neelsen (ZN) staining, reverse Ziehl Neelsen (RZN) staining, acid fast bacilli (AFB).

Mycobacterium tuberculosis (MTB) complex, an acid fast bacilli (AFB), is the causative agent of TB. MTB can infect any organ but lung infection, pulmonary tuberculosis (PT) is very common. In PT patients AFB are identified by examination of sputum smears under microscope. Most of the national tuberculosis control programs (NTPs) are using Ziehl Neelsen (ZN) staining technique to identify AFB. Limited sensitivity and inability to detect drug resistance are the drawbacks of ZN staining [1]. But culture on LJ medium is the gold standard [2] for the diagnosis of TB. However ZN staining technique is simple, rapid and economical[3]. Inspite of the disadvantages, ZN staining technique is the main stay for TB diagnosis especially in low and middle income countries (LMICs) [4]. Not only for the diagnosis, ZN staining is also used to find the treatment prognosis and also used as one of the criteria's for patient classification under Revised National Tuberculosis Control Programme (RNTCP).

ZN staining technique contain three steps: primary staining, decolorisation and counter staining. The RNTCP recommend 1% Carbol Fuchsin (CF) as primary staining reagent, 25% sulphuric acid (H_2SO_4) for destaining and 0.1% methylene blue (MB) as counter staining reagent.

Currently most of the NTPs are collecting two sputum samples by SM approach [spot (S) sample and morning (M) sample] for the diagnosis of TB. Studies showed that the utility of the same

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day (SS_2) approach (collection of 2 sputum samples with a gap of one hour) is at par with the SM approach [4,5,6,7,8,9] in the diagnosis of TB.

With these, in the current study, smears of SM and SS_2 approaches were stained by ZN staining and reverse ZN (RZN) staining techniques with hypothesis that there is no change in smear positivity with RZN staining technique. RZN staining technique is very similar to ZN staining technique, except, 0.1% MB was used for primary staining and 1% CF for counter staining.

MATERIALS AND METHODS

The study was conducted in the department of Microbiology, GSL Medical College, Rajahmundry, from March 2014 to May 2015. The study protocol was approved by Institutional Research and Ethics Committee of GSL Medical College. An informed written consent in the presence of witness was taken from all the volunteers who participated in the study. Individuals aged 18 years and above were included in the study. All the individuals were explained in local language about the importance of submission of sputum sample. Visual difference between sputum and saliva and how to produce good quality sputum sample were demonstrated practically.

All the participants were confirmed PT patients, using DOTS therapy. They were informed to provide three sputum samples (S, S_2 and M), i.e., spot (S) sample at the time of first visit to the hospital, second spot (S_2) sample was collected 1 h after the S sample. Morning (M) sample was collected after getting up from bed early in the morning. After collecting two spot samples (S and S_2), patients were provided with prelabelled sample containers to collect morning sample at home.

Immediately after collection, two smears were prepared with each sample on new glass slide. One smear was stained by standard ZN staining technique as per RNTCP guidelines [4] and second smear was stained by RZN staining method. After staining, data on slides was covered with wrap, so that the microscopist would not be aware of smear staining technique, thus avoiding bias.

Smear preparation [4]: New unscratched slides were used for smear preparation. Smears were prepared with sterile bacteriological loop. A good smear is spread evenly, over a size of 2X3 cm and neither too thick nor too thin. This was allowed to air dry for 15 to 30 minutes and fixed by passing it over blue flame of bunsen burner, 3–4 times.

ZN staining [4]: Smears were flooded with filtered 1% carbol fuchsin (CF) and heated until they were steamed and left to steam for 5 min. After rinsing the slides with a gentle stream of water, 25% sulphuric acid (H_2SO_4) was used to decolorise the smears for 2–4 min, and if necessary, the decolorisation step was repeated for another 1–3 minutes. The slides were rinsed as mentioned earlier and counterstained with 0.1% methylene (MB) blue for 30 s. The slides were then washed, air dried and examined under oil immersion.

RZN staining: Smears were flooded with filtered 0.1% MB and heated until they were steamed and left to steam for 5 min. After rinsing the slides with a gentle stream of water, slides were dipped in coplin jar containing 25% H_2SO_4 for 2–4 min, and if necessary, decolorisation step was repeated for another 1–3 minutes. The slides were rinsed as mentioned earlier and counterstained with 1% CF blue for 30 s. The slides were then washed, air dried and examined under oil immersion.

After staining, minimum 100 fields were examined and smears were graded as per RNTCP technical manual. 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.

Statistical analysis

Data were analysed using of SPSS (version 16; SPSS, Inc., Chicago, IL, USA), with patient as the unit of analysis; χ^2-test was used to find the statistical difference in smear positive cases between the approaches and staining techniques. A *P* value of less than 0.05 was used to indicate statistical significance.

RESULTS

Of the 61 study participants, male/female ratio was 1.33:1, with mean age of 42.1 years. The smear positivity was 100%, 77% for SM approach and 92%, 72% for the SS_2 respectively for ZN staining and RZN staining techniques. Statistically the difference was significant in both SM and SS_2 approaches ($P < 0.05$) (Table 1).

Table 1. Smear results of SM and SS_2 approaches

Approach	Technique	Smear result, n (%)						Statistical analysis			
		Scanty	1+	2+	3+	Any positive	Negative	Total	χ^2	*P* value	Statistical significance
SM	ZN staining	13 (21.3)	16 (26.2)	25 (41)	7 (11.5)	61 (100)	0				
	RZN staining	8 (13.1)	12 (19.7)	20 (32.8)	7 (11.5)	47 (77)	14 (23)	61	13.636	0.002	significantly associated
SS_2	ZN staining	18 (29.5)	12 (19.7)	21 (34.4)	5 (8.3)	56 (92)	5 (8)				
	RZN staining	13 (21.3)	11 (18)	16 (26.2)	4 (6.6)	44 (72)	17 (28)		6.71	0.0096	

DISCUSSION

Microbiological diagnosis is the main stay for the effective treatment of PT, major public health problem in LMICs like India, Pakistan and Sub Saharan African countries. In these countries people are not affordable for rapid TB diagnostic tests. Hence ZN staining is the only alternative [1]. In one study, Chandra TJ et al., [10] mentioned that patient education is imperative for obtaining the correct sputum sample because sputum quality influences the smear results [7]. Hence in the current study extra efforts were put by the researchers for collection of good quality of sputum sample by explaining in local language and practical demonstration regarding how to produce good quality sputum sample because specimen quality is very important to get the optimum smear results.

It was reported in the literature that in ZN staining, the phenol concentration is crucial to get optimum smear results and Chandra TJ et al., [11] reported that the concentration of the ingredients should not be changed in ZN staining. With these, in this study, the concentrations of the reagents were not changed. And the mordant phenol was incorporated in primary staining reagent i.e. in MB, at a concentration of 5%.

With RZN staining technique, the smear positivity was reduced to 23% (14) and 28% (17) in SM and SS_2 approaches, respectively, the difference was statistically not significant ($\chi^2 = 0.3892$, P = 0.532). In the ZN staining technique, AFB appears in bright red, whereas under RZN staining AFB appear in blue. This leads to confusion and automatically require prolonged time period for smear reading. Non acceptability by laboratory technicians and increased false negatives are the added draw backs with the RZN staining. With the above drawbacks, RZN staining technique cannot be implemented in the RNTCP for the diagnosis of PT and the null hypothesis was rejected.

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