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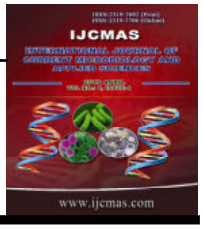

Original Research Article

Same day sputum smear microscopy for the diagnosis of pulmonary tuberculosis: modified ZN staining versus LED FM

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Keywords

ZN staining, modified ZN staining, LED FM, SM approach, SS2 approach, Tuberculosis

A B S T R A C T

Tuberculosis is public health emergency. Sputum smear microscopy is an economical, rapid method to identify tubercle bacilli, an acid fast bacterium. Sputum smears of Spot Morning (SM) and same day (SS_2) samples were stained by ZN, modified ZN and fluorescent staining by LED FM. Out of 3186 patients, sputum smear positivity for SM approach was 297 (9.3%), 311 (9.8%), 343 (10.8%) and for SS_2 approach smear positivity was 294 (9.2%), 311 (9.8%), 338 (10.6%) respectively for ZN, modified ZN and fluorescent staining by LED FM. Modified ZN staining and LED FM results were not significantly associated ($p<0.05$) for both SM and SS_2 approaches. For sputum smear microscopy, SS_2 approach with LED FM is strongly recommended in the diagnosis of lung tuberculosis.

Introduction

Mycobacterium tuberculosis (MTB), an acid fast bacterium (AFB), is the causative agent of pulmonary tuberculosis (PT). Several diagnostic tests are available to identify MTB. But, due to advantages like simplicity, rapidity, ease of technique, sputum smear microscopy (ssm) is considered to be the main diagnostic tool to identify this AFB especially in high tuberculosis (TB) burden and developing countries like India [1]. Most of the national tuberculosis control programmes (NTPs) follow spot morning (SM) scheme of sputum collection for the diagnosis of PT.

The available literature revealed that same day (SS_2) approach i.e. collection of two sputum samples with a gap of 1 hour is at par with SM approach in terms of sensitivity and specificity [2, 3, 4, 5].

It was reported by Jaya Chandra that sputum smear positivity (SSP) was 9.43% for SS_2 approach and 9.72% for SM approach, statistically there is no significant difference ($p>0.01$) [2]. Whereas in another study by Jaya Chandra et al SSP was reported as 9.43% and 9.37% respectively for SM and

SS_2 approaches, statistically not significant ($p>0.01$) [3]. Though statistically there is no significant difference, but ethically we should not miss even a single case of tuberculosis (TB). Van den et al reported that modified ZN (MZN) staining i.e. 15 minutes primary staining step with 1% basic fuchsin (BF) might be superior to 0.3% BF for 5 minutes [6]. This was confirmed by Jaya Chandra et al that SSP was 9.43%, 9.8% for SM approach and 9.37%, 9.8% for SS_2 approach respectively with ZN and MZN staining techniques [3].

High TB burden countries like India, the incidence is over 2 million annually [7]. With this high TB burden, the infrastructure and man power also has to be increased proportionately for better management of NTPs. Due to high work load, the lab technicians (LTs) may not spend sufficient time on ZN staining, this may influence the smear results. In addition to this, WHO technical advisory group for TB recommended that fluorescent microscopy (FM) using Light Emitting Diode (LED) is an alternative for conventional ZN microscopy [8]. With this back ground in the present study ZN staining, MZN staining, fluorescent staining (FS) with LED FM were used to identify PT in SM and SS_2 approaches.

Material and Methods

This study was conducted in the department of Microbiology, GSL Medical College, Rajahmundry from January 2011 to October 2014. Study protocol was approved by the Institutional Ethics and Research Committee. An informed written consent in the presence of witness was taken from all the volunteers who participated in the study. Individuals aged 18 years or above were included in the study.

All the individuals were explained about the importance of submission of sputum sample. The visual difference between sputum and saliva was demonstrated. They were also explained how to produce a good quality sputum sample in local language with practical demonstration. Finally they were explained to provide 5 ml of sputum sample. All the individuals were informed to provide three sputum samples, i.e. spot sample (S) at the time of first visit to the hospital, S_2 is second spot collected one hour after S sample. M sample was collected after getting up from bed early in the morning. After collecting 2 spot samples, patients were provided with pre labeled sample containers to collect M samples at home.

Immediately after collection, three smears were prepared with each sample on new glass slide and one slide was stained with standard ZN technique as per RNTCP guidelines [9]. Second smear was stained by MZN method [3] and third slide was stained with FS [10] as per RNTCP guidelines. After staining slides were covered with wrap around stickers, so that the microscopist is not aware of smear staining technique, thus avoiding bias.

Smear preparation[9]: 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 2X3 cm and is neither too thick nor too thin. This was allowed to air dry for 15 to 30 minutes and fixed by passing it over a blue flame 3 – 4 times.

ZN staining[9]: Smears, flooded with filtered 1% carbol fuchsin (CF) were heated until it was steaming and left to steam for five minutes. After rinsing the slides with a gentle stream of water, 25% sulphuric acid was used to decolorize the smears for 2 to 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.

MZN staining[3]: This is very similar to that of standard ZN staining, except the primary staining step by using 1% CF was for 15 min.

FS[10]: Slides should be placed on the staining rack without touching each other. Initially slides were flooded with freshly filtered auramine-phenol, left for 7 – 10 minutes. Then slides were washed well with running water, taking care to control the flow of water so as to prevent washing away the smear. Decolorized by covering completely with acid-alcohol for 2 minutes, twice and washed well with running water, as before to remove the acid alcohol. Counter stained with 0.1% potassium permanganate for 30 seconds, washed as before with water. Then slides were air dried and observed under 40X objective.

Result and Discussion

During the study period 3328 patients were included in the study. Out of this 142 (4.3%) were dropped out. The remaining 3186 patients' results were given. For SM approach SSP was 297 (9.3%), 311 (9.8%), 343 (10.8%) and for SS_2 approach SSP was 294 (9.2%), 311 (9.8%), 338 (10.6%) respectively for ZN, MZN and FS by LED FM (Table I). MZN and FS techniques were not significantly associated in SM and SS_2 approaches ($p<0.05$) (Table: II, III).

More than 90% of TB cases occur in low and middle income countries (LMICs) [11] and around 70 – 90% of ssm examinations take place in 22 high TB burden countries [12]. In the developing countries like India usually people refuse to visit hospital / health care setup. Due to loss of wages they do not come for repeated ssm. So SM approach leads to default. In this context the diagnosis of PT may not possible, cause delay in initiation of treatment. It was reported by WHO that each individual with active TB can spread disease 10 – 12 members per annum[13]. This reflects the countries development and financial statuses. In the current study the dropout rate was 4.3% and it was 13% in one of the south Indian study [14]. Among the SSP patients, after submitting first sputum sample the default cases were reported to be 52% [15]. These dropout rates were much more in the field conditions [2].

To overcome the dropouts, TB is diagnosed by rapid / early diagnostic techniques like Gene X pert, Loop mediated isothermal Amplification (LAMP) etc. These rapid diagnostic techniques are very costly. Hence for most of NTPs, ssm is the only weapon to diagnose this white plague. At this juncture SS_2 is the only alternative for rapid diagnosis of PT especially in the developing countries like India.

In the current study SSP for SM approach was 9.8%, 10.8% and for SS_2 approach 9.8%, 10.6% respectively for ZN & LED FM, the difference was statistically not significant ($p<0.05$). The diagnostic accuracy is very similar (10.8% and 10.6% respectively) for SM and SS_2 approaches with LED FM (Chi square is 0.0411, $p<0.05$). However efforts to improve the ZN smear sensitivity by altering the concentration of reagents also did not worked[16]. Maryline bonnet[17] study stated that the detection yield of LED FM and ZN staining were 20.3% and 20.6% ($p=0.64$) respectively and the authors also coated that LED FM did not increase the sensitivity. But Ben J Marais et al reported the SSP as 14% and 17% respectively for ZN and LED FM [18].

Table.1 Staining results for SM and SS2 approaches

Approach	Staining	Scanty (%)	1+ (%)	2+ (%)	3+ (%)	Any positive (%)	Negative (%)	Total
SM	ZN	102 (3.2)	82 (2.5)	76 (2.4)	47 (1.5)	307 (9.6)	2879 (90.4)	3186
	MZN	98 (3.1)	63 (2)	87 (2.7)	63 (2)	311 (9.8)	2875 (90.2)	3186
	FS	117 (3.7)	76 (2.4)	83 (2.6)	67 (2.1)	343 (10.8)	2843 (89.2)	3186
SS_2	ZN	124 (3.9)	74 (2.3)	61 (1.9)	45 (1.4)	304 (9.6)	2882 (90.4)	3186
	MZN	110 (3.4)	76 (2.4)	66 (2.1)	59 (1.8)	311 (9.8)	2875 (90.2)	3186
	FS	101 (3.2)	89 (2.8)	83 (2.6)	65 (2)	338 (10.6)	2848 (89.4)	3186

SM: Spot Morning approach, SS2: Same day approach, ZN: Ziehl Neelsen, MZN: Modified Ziehl Neelsen, FS: Fluorescent staining

Table.2 Smear results of SM approach

		MZN staining							Statistics
		Scanty (%)	1+ (%)	2+ (%)	3+ (%)	Any positive (%)	Neg (%)	Total (%)	
FS	Scanty (%)	84 (2.6)	5 (0.2)	2 (0.06)	0	91 (2.9)	26 (0.8)	117 (3.7)	Chi square value 1.7488, P value 0.186528
	1+ (%)	14 (0.4)	56 (1.8)	0	0	70 (2.2)	6 (0.2)	76 (2.4)	
	2+ (%)	0	0	78 (2.4)	5 (0.2)	83 (2.6)	0	83 (2.6)	
	3+ (%)	0	2 (0.06)	7 (0.2)	58 (1.8)	67 (2.1)	0	67 (2.1)	
	Any positive (%)	98 (3.1)	63 (2)	87 (2.7)	63 (2)	311 (9.8)	32 (1)	343 (10.8)	
	Neg	0	0	0	0	0	2843 (89.2)	2843 (89.2)	
	Total (%)	98 (3.1)	63 (2)	87 (2.7)	63 (2)	311 (9.8)	2875 (90.2)	3186 (100)	

As per the above table, FS and MZN techniques were significantly not associated in SM approach ($p<0.05$)

Table.3 Smear results of SS2 approach

		MZN staining							Statistics
		Scanty (%)	**1+ (%)**	**2+ (%)**	**3+ (%)**	**Any Positive (%)**	**Neg (%)**	**Total (%)**	
FS	**Scanty (%)**	103 (3.2)	3 (0.1)	0	0	106 (3.3)	24 (0.8)	130 (4.1)	Chi square value 1.2506 P value 0.263429
	1+ (%)	7 (0.2)	69 (2.2)	5 (0.2)	0	81 (2.5)	3 (0.1)	84 (2.6)	
	2+ (%)	0	4 (0.1)	61 (1.9)	8 (0.3)	73 (2.3)	0	73 (2.3)	
	3+ (%)	0	0	0	51 (1.6)	51 (1.6)	0	51 (1.6)	
	Any positive (%)	110 (3.4)	76 (2.4)	66 (2)	59 (1.9)	311 (9.8)	27 (0.8)	338 (10.6)	
	Neg (%)	0	0	0	0	0	2848 (89.4)	2848 (89.4)	
	Total (%)	110 (3.4)	76 (2.4)	66 (2)	59 (1.9)	311 (9.8)	2875 (90.2)	3186 (100)	

As per the above table, FS and MZN techniques were significantly not associated in SS2 approach ($p<0.05$).

Whereas Cuevas LE et al reported that LED FM has higher sensitivity than ZN smear, with two smears per patient, the sensitivity was 72.8%, 65.8% and with 3 smears per patient the sensitivity was 77% & 70.5% respectively for LED FM and ZN staining [19].

Lower specificity is the major drawback of LED FM. Cuevas LE et al reported that with two LED FM and 2 ZN smears per patient specificity was 90.9% & 98%, with three LED FM and three ZN smears per patient specificity was 88.1% & 96.5% [19]. Maryline Bonnet et al also reported that LED FM has low specificity (95.9%) when compared with ZN staining (96.7%) [17].

The meta analysis study on SM and SS_2 approaches by J.Lucian Davis reported that the pooled sensitivity for ZN staining was 64%, 63% respectively [5]. The authors also reported the pooled sensitivity for LED FM was 73%, 69% respectively for SM and SS_2 approaches. But the specificity was low (93%) for LED FM, than ZN (98%) [5]. Low specificity of LED FM resulted in significantly higher number of patients being treated. Not only the correct and rapid diagnosis of TB but also treatment to correct needy is also very important.

Manipulation of the sputum smear results is the major drawback of SS_2 especially in the field conditions. Because if first sputum smear results are negative, the LTs may not be motivated to screen the second smear by remembering the results of the first smear. But the current study is purely for research purpose, no involvement of LTs. As a part of internal quality control, all the positive slides and randomly 25% of the negative slides were rechecked by the senior author.

As per WHO policy Gene X pert is likely to be used for sputum smear negative (SSN) cases to confirm PT. With SS_2 approach SSN patients can be evaluated on the same day by Gene X pert. This is the added advantage of SS_2 approach among SSN cases.

Threat of nosocomial infections due to long time hospital stay, improvement of infrastructure facilities and low specificity are draw backs of SS_2 approach. In spite of minor disadvantages SS_2 approaches is strongly recommended due to significant operational advantages like increased sensitivity, reduction in laboratory work load, rapidity in smear reading. Hence NTPs / WHO may have to consider SS_2 approach with LED FM in the diagnosis of PT in areas where sufficient infrastructure and man power are available.

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