Journal of Clinical Tuberculosis and Other Mycobacterial Diseases 8 (2017) 6–12

Contents lists available at ScienceDirect

Journal of Clinical Tuberculosis and Other Mycobacterial Diseases

journal homepage: www.elsevier.com/locate/jctube

Front loading sputum microscopy – an alternative approach for diagnosis of pulmonary tuberculosis

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ARTICLE INFO

Article history:
Received 13 February 2017
Accepted 15 May 2017

ABSTRACT

Introduction: Until newer, rapid, economical tools are introduced for diagnosis of Pulmonary Tuberculosis in resource limited settings, optimization of sputum smear examination for increasing case detection remains of utmost priority. The aim of the study was to detect presumptive TB patients using Front Loading sputum microscopy and compare it with Standard method.
Methods: Three sputum specimens (Spot 1- on spot at the time of first visit, Spot 2- one hour after Spot 1 and early morning-next day early morning sample) from 552 TB suspect cases were collected. Zeihl Neelsen staining (spot 1, spot 2 and early morning respectively) and microscopy by Front Loading (spot 1, spot 2) and Standard method (spot 1, early morning) of sputum microscopy were done.
Results: Culture on LJ media being the gold standard, the sensitivity and specificity of the Front Loading and the Standard method of sputum microscopy were 68.65%, 94.43% and 70.14%, 93.6% respectively. The difference between two methods was not statistically significant. 91.1% patients gave preference for same day sampling process.
Conclusion: The sensitivity and specificity of sputum microscopy using an early morning sample followed by another sputum one hour later from the same day appears not to be inferior to using two early morning samples on subsequent days. The Front Loading sputum microscopy can be implemented in DOTS clinic on the day of first visit of patients to health care center to increase compliance of patients with diagnostic procedure and decrease drop-outs.

Introduction

Tuberculosis (TB) is an immense public health problem that causes ill health among millions of people every year. Although TB had been recognized since ancient times, it still remains a top infectious disease killer in present scenario globally. Worldwide, 9.6 million people are estimated to have fallen ill with TB in 2014 comprising of 5.4 million men, 3.2 million women and 1.0 million children. In 2014, TB killed 1.5 million people, the toll comprised 890 000 men, 480 000 women and 140 000 children [1]. Of all the countries that report their TB statistics to WHO, there are 22 countries, including India, that are referred to as the TB "high burden" countries; and they have been prioritized at a global level since 2000. They accounted for 82% of all estimated cases of TB worldwide in 2014 [2]. According to the WHO statistics for 2014, the estimated incidence of TB in India is 2.2 million cases which accounts for approximately 1/5th of global incidence [3]. In addition at least 2.7 lakh (270,000) Indians die of TB every year [4]. It is estimated that about 40% of the Indian population is infected with TB with an estimate of approximately 794,046 sputum smear-positive cases reporting to RNTCP in 2014 and an overall treatment success rate for new TB patients and retreatment patients success rate being 88% and 70% respectively [3]. Despite the low multi-drug resistance (MDR-TB) prevalence in India (2.2% among new cases and 15% among retreatment cases), the absolute number of cases happens to be a huge figure due to the size of the population and number of TB cases reporting annually,. Thus, India ranks first among the 27 MDR-TB high-burden countries worldwide, contributing to 21% of all MDR-TB cases estimated among notified cases [5].

To overcome the burden of this disease, India is implementing WHO endorsed Directly Observed Treatment Short course (DOTS) strategy under a national program-Revised National Tuberculosis Control Programme (RNTCP). RNTCP is the world's largest DOTS

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http://dx.doi.org/10.1016/j.jctube.2017.05.002

program achieving global targets of case finding and treatment success rates [1]. Although new technologies are under development, microscopic examination of clinical sputum specimens has been the main stay of TB case detection for over 100 years and is likely to remain the primary tool for the laboratory diagnosis of TB in resource poor countries for the foreseeable future. Microscopy is rapid, fairly inexpensive, and less labor intensive than other technologies. In addition, in the case of TB, a positive smear is indicative of a high risk of transmitting infection to others [6].

As per the RNTCP guidelines, presumptive TB patients are required to submit two sputum samples, i.e. one on the spot at the time of first visit and second specimen collected at home next day morning and brought to the laboratory [7]. This requires at least 2 visits for sample submission and subsequent visit for report collection. Due to repeated visits and the economic constraint involved in it, the patient often abandons the diagnostic procedure, the defaulting patients contributing to spreading the disease in the community. The WHO has recently indicated that "same-day diagnosis" or the "front-loading" method of specimen collection should be done according to a phased implementation plan with due consideration given to training, human resources, laboratory work-load, infection control, and monitoring issues. According to this scheme, the presumptive TB patients are required to submit two sputum samples on first day of visit one hour apart [8,9]. It reduces number of visits of patient and helps in preventing drop-outs.

In our study we aim to assess the accuracy of Front Loading sputum microscopy in case detection of presumptive TB patients. In view of the increase in TB cases, higher morbidity probably because of the high drop-outs and studies revealing an increased yield of acid fast bacilli (AFB) on serial sputum smear examination on same day, this study was designed to assess the feasibility of 2 serial sputum smear examination on the same day and compare the results with Standard method of sputum microscopy and gold standard *Mycobacterium tuberculosis* culture for diagnosis of presumptive TB patients.

Materials and methods

A Cross-sectional Study was carried out at the Departments of Microbiology and Medicine, University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi. Five hundred and fifty two (552) suspected cases of pulmonary TB (new cases) that came to Medicine OPD/Chest clinic/wards/DOTS Clinic in Guru Teg Bahadur Hospital, Delhi were enrolled.

Patients of age 14 years and above with history of cough for 2 weeks or more were included in the study. Patients with past history of TB or past history of intake of anti-tubercular drugs for more than one month or history of chronic diseases like Diabetes Mellitus, Hypertension were excluded from the study.

Ethical approval for the study was received from the Institutional Ethical Committee-Human Research.

Informed consent was taken from all the cases. In a standardized form, patients' demographics, preference of patients for sputum sample submission on same day and any other relevant clinical history and information were collected.

Three sputum specimens from each patient were collected. The patient was provided with sterile, leak proof, disposable and appropriately labeled sputum container and was asked to provide sputum specimen. He/she was asked to take 2–3 deep breaths followed by deep cough in order to produce a good quantity of sputum, 2 ml–5 ml in volume. [10] As per the Front Loading sputum microscopy, the patient provided one sputum specimen at the time of first visit (Spot 1) and another sputum specimen on the same day one hour later (Spot 2). [11] As per the RNTCP guidelines of Standard method of sputum microscopy, in addition to Spot 1, the patient was asked to collect another sputum specimen the next day morning at home and bring it to the laboratory (early morning). Hence, a total of 1656 sputum specimens were collected from 552 patients.

Specimens were transported to the laboratory as soon as possible after collection. If delay was unavoidable, the specimens were refrigerated at 4 °C to inhibit the growth of unwanted microorganisms. [12] Biosafety cabinet II B2 was used for handling all the specimens and cultures. Personal protective measures like N95 mask, protective gowns, rubber gloves and disposable caps were used.

AFB microscopy by front loading and RNTCP conventional methods:-[10]

Using the jagged ends of a broken stick the larger, yellow, purulent portion of the sputum sample was picked and smear were prepared from spot 1, spot 2 and early morning sample respectively for each patient and stained with Ziehl Neelsen staining (ZN) technique to be examined under oil immersion (100x) for presence of AFB. Smears were examined on a daily basis. Smear from spot 1 and spot 2 samples were examined under the Front Loading sputum microscopy and smear from spot 1 and early morning samples were examined under the Standard method of sputum microscopy. Smears were graded and reported according to the recommended way of interpretation by RNTCP [7].

Tubercle bacilli appeared as red, long, slender, slightly curved rods with pointed ends and beaded appearance. The bacilli were arranged singly, in pairs, or in clumps and they stood out clearly against a blue background under 100x oil immersion. RNTCP guidelines require 300 fields to be read before a smear is reported negative, which took about 15 min [10].

The slides of sputum smear were provided separate study number and routine laboratory numbers, which were covered by plane stickers. The slides were mixed before examination. This process helped in blinding during reporting of sputum smears.

The three sputum specimens of each patient were homogenized and decontaminated separately by the NALC – 4% NaOH method [12]. 4% NaOH and Trisodium citrate solution was prepared, autoclaved and stored in refrigerator. 0.5% NALC was added just before processing of samples. Equal volumes of sputum specimen and NALC-4% NaOH were processed as per the standard protocol. After processing with NALC-NaOH solution, Phosphate Buffer Saline (PBS) was added to neutralize the action of alkaline NaOH.

For each patient, from 50 µl resuspended sediment of processed Spot 1, Spot 2 and early morning samples, three slopes of Lownstein Jensen (LJ) medium were inoculated. The McCartney bottles containing inoculated LJ media were labeled as A, B and C respectively. The bottles were incubated in slanted position at 37 °C with screw cap slightly loosened for 24 h to ensure even distribution of inoculum on the slope and evaporation of any water of condensation in the bottles. Thereafter, caps of bottles were tightened to prevent drying out of media and were further incubated at 37 °C in upright position. The growths in both LJ media were examined 48–72 h after inoculation to detect gross contaminants. Thereafter cultures were examined weekly for appearance of macroscopic colonies. After 8 weeks of incubation, LJ media slants showing no growth were reported as negative.

Preliminary identification from macroscopic examination of mycobacterial isolates on LJ media was done depending on the organism's rate of growth, colonial morphology, colonial texture and pigmentation. For microscopic identification, smears were prepared from suspected colonies of *M. tuberculosis* on LJ media, stained by ZN staining technique and examined for AFB. After the preliminary identification, growth of *M. tuberculosis* was identified by Para-nitrobenzoic acid (PNB) and Niacin test. For PNB test, the bacterial colonies were emulsified with 1 ml sterile distilled water and one

loopful of suspension was inoculated on LJ media containing PNB. If no growth was found in the media, the test culture was reported as *M. tuberculosis*. If growth was found in the media, the test culture was reported as NTM.

For Niacin test, sterile distilled water was added to culture bottle containing growth and kept horizontally for 20–30 min for extraction of niacin from the media which was examined by yellow color formation with addition of aniline in ethanol and cyanogen bromide.

According to RNTCP case definitions, the total number of smear positive and smear negative presumptive TB patients were diagnosed [13].

Contaminated cultures which showed growth other than *M. tuberculosis* and Non-tubercular mycobacteria (NTM) were discarded in 1% sodium hypochlorite and were not included in the analysis.

Statistical analysis:-

McNemar Test was used to calculate *p*-value to compare sensitivity and specificity of Standard method of sputum microscopy and Front Loading method of sputum microscopy taking culture as gold standard. If *p*-value $<=0.05$ the difference was significant and if *p*-value $>=0.05$ the difference was not significant.

Results

Patient profile

Out of 552 participants in the study, 297 (53.8%) were males and 255 (46.2%) were females. The age and sex distribution of the enrolled patients is given in Fig. 1. The male to female ratio was 1.16 showing a male preponderance. 461 (83.51%) patients were from various OPDs and 91 (16.49%) were from various wards of Guru Teg Bahadur Hospital. 332 (60.1%) patients gave a H/O fever for more than 2 weeks of duration while 88 (15.9%) patients gave a H/O fever for less than 2 weeks duration. 112 (20.3%), 121 (21.9%), 323 (58.5%) and 216 (39.1%) patients complained of breathlessness, hemoptysis, chest pain and loss of appetite respectively. 137 (24.8%) patients showed radiographic abnormalities suggestive of active pulmonary TB. 134 (24.3%) patients had H/O contact with TB patients. The preference of patients for submission of sputum specimens on the same day which was recorded in case record forms showed that 503 (91.1%) of the patients had preference for this sampling process.

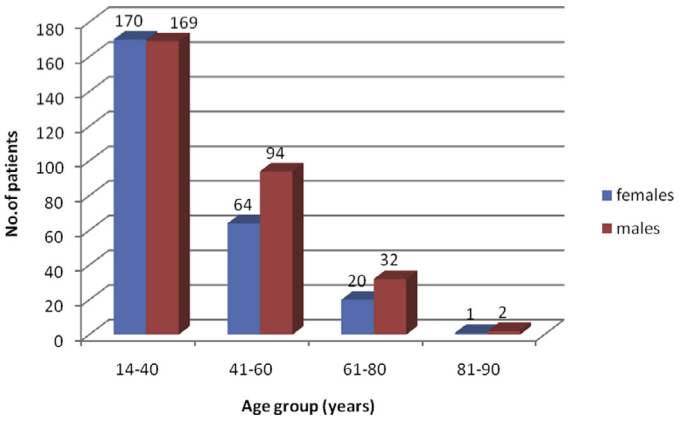

Fig. 1. Age and gender based distribution of patients.

Smears

Out of the 552 smears, 70 (12.7%), 66 (12%) and 76 (13.8%) of smears from spot 1, spot 2 and early morning were AFB positive while 482 (87.3%), 486 (88%) and 476 (86.2%) of smears from spot 1, spot 2 and early morning were AFB negative (Table 1). Whereas in 63 patients both smear from spot 1 and spot 2 were positive, 7 and 3 patients were positive in smear from spot 1 and spot 2 only respectively by Front Loading sputum microscopy. Smear positivity was 0.7% more in spot 1 in comparison with spot 2. While 68 patients had both spot 1 and early morning smear positive by Standard method of sputum microscopy, 2 and 8 patients had only spot 1 and early morning smear positive respectively. The difference in smear positivity between spot 1 and early morning was 1.1%. The difference in smear positivity between spot 2 and early morning was 1.8%. Considering one or both smear from spot 1 and spot 2 as AFB positive, 73 (13.2%) patients were detected as presumptive TB patients by Front Loading sputum microscopy method. Considering one or both smear from spot 1 and early morning as AFB positive, 78 (13.2%) patients were detected as presumptive TB patients by Standard method of sputum microscopy. The difference between the two methods was not statistically significant (*p* value $=0.063$) (Table 2). Five patients of presumptive TB who were missed by Front Loading sputum microscopy, were detected by Standard method of sputum microscopy (Fig. 2). In 62 (79.5%) patients all the 3 sputum smears were AFB positive. In 10 (12.8%)

Table 1
Sputum smear positivity per patient.

Sputum smears positivity per patient			Number of patients n (%)	
Spot 1 +	Spot 2 +	Early morning +	62 (11.23)	
Spot 1 +	Spot 2 +	Early morning -	1 (0.18)	10 (12.8)
Spot 1 +	Spot 2 -	Early morning +	6 (1.08)	
Spot 1 -	Spot 2 +	Early morning +	3 (0.54)	
Spot 1 +	Spot 2 -	Early morning -	1 (0.18)	6 (7.7)
Spot 1 -	Spot 2 +	Early morning -	0 (0)	
Spot 1 -	Spot 2 -	Early morning +	5 (0.91)	
Spot 1 -	Spot 2 -	Early morning -	474 (85.9)	
Total patients			**552 (100)**	

Table 2
Comparison of AFB microscopy by Front Loading and Standard method of sputum microscopy.

	Front Loading sputum microscopy Positive *n* (%)	Front Loading sputum microscopy Negative *n* (%)	Total *n* (%)
Standard method of sputum microscopy Positive *n* (%)	73 (13.2)	5 (0.9)	78 (14.1)
Standard method of sputum microscopy Negative *n* (%)	0	474 (85.9)	474 (85.9)
Total *n* (%)	73 (13.2)	479 (86.8)	552 (100)
p value = 0.063 (not significant)			

Table 3
Grading of AFB positive smears of Front Loading and Standard method of sputum microscopy (by RNTCP AFB grading*).

Grading of AFB positive smears	Front Loading sputum microscopy		Standard method of sputum microscopy	
	Spot 1 *n* (%)	Spot 2 *n* (%)	Spot 1 *n* (%)	Early morning *n* (%)
3+	18 (25.7)	18 (27.3)	18 (25.7)	29 (38.2)
2+	16 (22.9)	17 (25.8)	16 (22.9)	24 (31.6)
1+	22 (31.4)	22 (33.3)	22 (31.4)	16 (21.1)
Scanty	14 (20)	9 (13.6)	14 (20)	7 (9.1)
Total	70 (100)	66 (100)	70 (100)	76 (100)

*3+ - >10 AFB/oil immersion field (oif)
2+ - 1 to 10 AFB/oif
1+ - 10 to 99 AFB/100oif
Scanty- 1–9 AFB/100oif

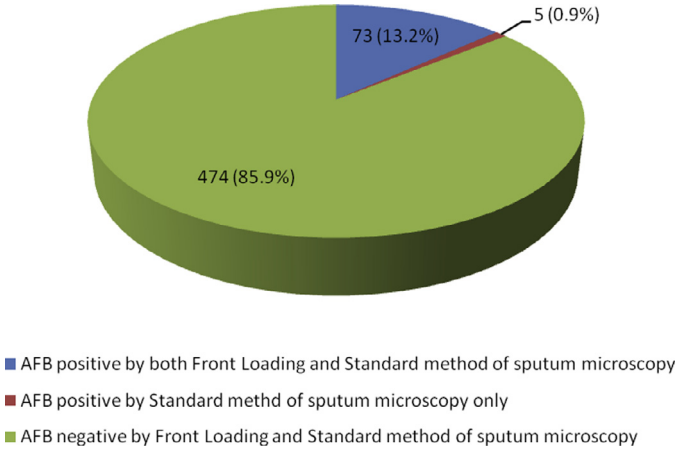

Fig. 2. Incremental yield in positivity by Standard method of sputum microscopy.

patients 2 sputum smears were positive. There were 6 (7.7%) patients in which either spot 1, spot 2 or early morning smear was only positive. These 6 patients with 1 sputum smear positive for AFB, had radiographic findings consistent with active pulmonary TB. Out of the 552 patients enrolled for our study, 78 (14.1%) were detected as smear positive presumptive TB patients and remaining 474 (85.9%) were smear negative presumptive TB patients.

Grading of 1656 sputum smears showed that the majority of 3+[29 (38.2%)] and 2+[24 (31.65)] graded AFB positive smears were seen in prepared from early morning specimen. Majority of 1+ and scanty graded AFB positive smears were seen in spot 2 specimen collected one hour after spot 1. Out of 78 patients detected as presumptive TB patients, 30 (38.5%) of patients were 3+ by Standard method of sputum microscopy as compared to 20 (27.4%) out of the 73 patients detected by Front Loading sputum microscopy. Grading of AFB smears of most of the patients diagnosed by Front Loading sputum microscopy was either 1+ [24 (32.8%)] or scanty [13 (17.8%)] respectively (Table 3).

Growth of *M. tuberculosis* was observed in spot 1 or spot 2 or early morning or in any two or in all the three sputum specimens of 67 (12.1%) patients. No growth of *M. tuberculosis* was observed in all the three specimens of 459 (83.2%) patients. NTM was isolated in samples of 26 (4.7%) patients. Out of the 67 patients with sputum specimen showing isolation of *M.tuberculosis* on LJ media, in 25 (37.4%) patients isolation of *M.tuberculosis* was seen by 4 weeks, in 13 (19.4%) patients by 5 weeks, in 15 (22.4%) patients by 6 weeks, in 7 (10.4%) patients by 7 weeks and another 7 (10.4%) patients by 8 weeks. Majority, 53 (79.2%) patients were diagnosed as cases of pulmonary TB by appearance of macroscopic colonies of *M.tuberculosis* on LJ media between 4–6 weeks of incubation. Out of the 67 patients diagnosed by isolation of *M.tuberculosis* on LJ media in 10 (14.9%) patients isolation was seen in all the three specimens, in 24 (35.8%) patients isolation was seen from two specimens and in 33 (49.3%) patients isolation of *M.tuberculosis* was seen from only one specimen. 12(17.9%) of the isolates were from spot 1, 7 (10.5%) from spot 2 and 14 (20.9%) from early morning specimen (Table 4). Out of the 1656 samples cultured on LJ media a total of 111 *M.tuberculosis* culture isolates were observed, out of which 37 (33.3%), 34 (30.6%) and 40 (36%) were from spot 1, spot 2 and early morning specimen respectively (Table 4).

Strategies

Isolation of *M.tuberculosis* on LJ media being the gold standard, the sensitivity, specificity, positive predictive value and negative predictive value of the Front Loading sputum microscopy was 68.65%, 94.43%, 63.01% and 95.61% respectively and that of Standard method of sputum microscopy was 70.14%, 93.6%, 60.25% and 95.78% respectively. The differences in the sensitivity and specificity of the two sputum microscopy methods were not statistically significant (P = 0.161 and P = 0.471 respectively) (Table 5). Standard method of sputum microscopy could identify 1 additional case of culture positive pulmonary TB (Table 5). The comparison of grading of positive smears by the two methods with isolation and turn around time is shown in Table 6 and Fig. 3.

Out of 479 patients reported negative by Front Loading sputum microscopy, 21/479 (4.4%) were diagnosed as culture positive cases of pulmonary TB and out of 474 patients reported negative by Standard method of sputum microscopy, 20/474 (4.2%) were pulmonary TB cases by isolation of *M.tuberculosis* on LJ media. Out of the 552 patients participating in our study, *M.tuberculosis* was not

Table 4
Number of cultures showing growth of *M.tuberculosis* on LJ per patient.

Culture results of LJ slopes per patient			Number of patients n (%)	
Spot 1 +	Spot 2 +	Early morning +	10 (14.9)	
Spot 1 +	Spot 2 +	Early morning -	8 (11.9)	24 (35.8)
Spot 1 +	Spot 2 -	Early morning +	7 (10.4)	
Spot 1 -	Spot 2 +	Early morning +	9 (13.5)	
Spot 1 +	Spot 2 -	Early morning -	12 (17.9)	33 (49.3)
Spot 1 -	Spot 2 +	Early morning -	7 (10.5)	
Spot 1 -	Spot 2 -	Early morning +	14 (20.9)	
TOTAL			**67 (100)**	

Table 5
Diagnostic comparison of Front Loading and Standard method of sputum microscopy with gold standard of culture on LJ media.

		Culture on LJ ($n=67$)		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
		Positive ($n=67$)	Negative ($n=485$)				
Front Loading sputum microscopy	Positive ($n=73$)	46	27	68.65	94.43	63.01	95.61
	Negative ($n=479$)	21	458				
Standard method of sputum microscopy	Positive ($n=78$)	47	31	70.14	93.6	60.25	95.78
	Negative (n=474)	20	454				
***p* value by McNemar test**				**0.161 (NS)**	**0.471 (NS)**		

NS-not significant

Table 6
Comparison of AFB grading of positive smears by Front Loading sputum microscopy and Standard method of sputum microscopy with *M.tuberculosis* isolation and TAT of isolation on LJ media.

		Front Loading sputum microscopy *n* (%)				Standard method of sputum microscopy *n* (%)						Total
		3+	2+	1+	Scanty	Neg	3+	2+	1+	Scanty	Neg	
Isolation	Growth on LJ *n* (%)	18 (90)	10 (62.5)	12 (50)	6 (46.2)	21 (4.4)	25 (83.3)	10 (43.5)	10 (58.9)	2 (25)	20 (4.2)	67 (12.1)
	No growth on LJ *n* (%)	2 (10)	6 (37.5)	12 (50)	7 (53.8)	458 (95.6)	5 (16.7)	13 (56.5)	7 (41.1)	6 (75)	454 (95.8)	485 (87.9)
	Total	20	16	24	13	479	30	23	17	8	474	552
TAT of culture on LJ	4 weeks *n* (%)	6 (24)	7 (28)	6 (24)	2 (8)	4 (16)	11 (44)	3 (12)	6 (24)	1 (4)	4 (16)	25 (37.3%)
	5 weeks *n* (%)	4 (31)	1 (7)	2 (15.3)	0	6 (1.3)	3 (23.1)	2 (15.3)	2 (15.3)	0	6 (46.8)	13 (19.4%)
	6 weeks *n* (%)	6 (40)	1 (6.7)	2 (13.4)	2 (13.4)	4 (26.8)	7 (46.7)	4 (26.8)	0	0	4 (26.8)	15 (22.3%)
	7 weeks *n* (%)	2 (28.6)	1 (14.3)	1 (14.3)	1 (14.3)	2 (28.6)	2 (28.6)	1 (14.3)	1 (14.3)	1 (14.3)	2 (28.6)	7 (10.4%)
	8 weeks *n* (%)	0	0	1 (14.3)	1 (14.3)	5 (71.5)	2 (28.6)	0	1 (14.3)	0	4 (57.2)	7 (10.4%)

isolated in 485 (87.9%) presumptive TB patients by 4–8 weeks of incubation on LJ media. Out of these 458 (94.4%) and 454 (93.6%) were reported AFB negative by Front Loading and Standard method of sputum microscopy respectively. According to case definitions, smear positive presumptive TB patients were 78 (14.1%) and smear negative culture positive presumptive TB patients were found to be 20 (13.6%). A total of 98 (17.7%) new presumptive TB patients were laboratory diagnosed in our study. Out of these 98 new presumptive TB patients, in 67 (68.4%) cases *M.tuberculosis* was isolated on LJ media.

Discussion

WHO and RNTCP insist sputum smear microscopy for diagnosing presumptive TB patients, initiating anti-tubercular drugs and monitoring disease progress in these patients, in developing countries with high prevalence of the disease like India. Although the sensitivity of AFB microscopy is reported to be low and varying from 20% to 80%, various steps to optimize this diagnostic method can increase its sensitivity [14]. To decrease the drop-out rate in presumptive TB patient diagnosis, the recently considered “same-day diagnosis” or the “front-loading method” for collection of sam-

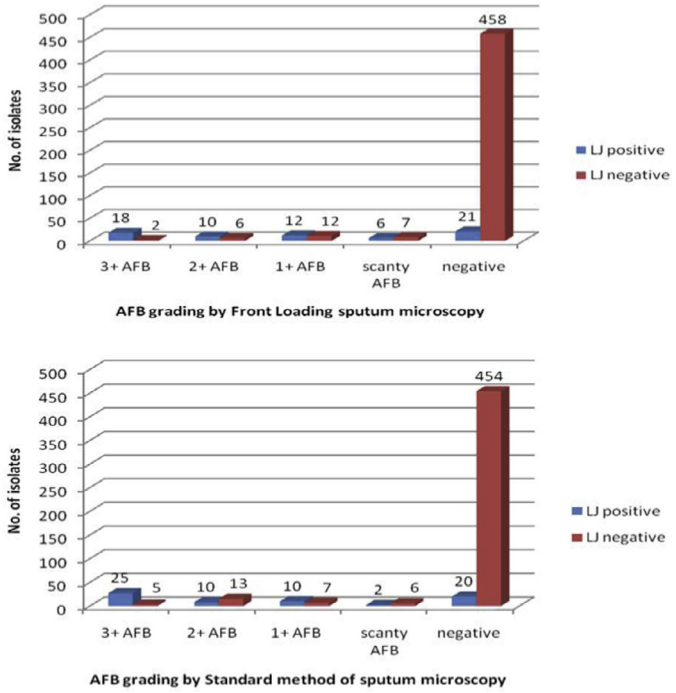

Fig. 3. Comparison of AFB grading of positive smears by front loading sputum microscopy and standard method of sputum microscopy with isolation of *M. tuberculosis* on LJ medium.

ples from patients completes the process of sample collection and reporting of AFB microscopy on the same day reducing the loss of time and income of the patients thus helping them to complete the diagnostic procedure and start treatment [9]. There is an urgent need to evaluate this new method and compare it with the standard method of RNTCP before this method can be implemented in laboratories routinely nationwide.

In our study 503 (91.1%) patients had preference for same day sampling process which was comparable with the study by Myneedu et al. in which 93.03% of patients preferred the new method of sample collection [11]. But in a study conducted in Chhattisgarh patient drop-out during diagnostic process was higher with same-day approach [15]. In our study, 8.9% of the patients did not prefer to wait for 1 h for submission of 2nd specimen due to inconvenience involved in waiting in overcrowded patient unfriendly health facilities and possible nosocomial exposure.

In our study 12.7%, 12% and 13.8% of smears from spot 1, spot 2 and early morning specimen were AFB positive, similar to findings by Myneedu et al. where the smear positivity of first, second and third sputum samples were 12.72%, 11.8% and 18.48% respectively [11]. The overall smear positivity rate in our study was 14.1% when compared to 16% and 18.48% smear positivity reported by Harrries et al. and Myneedu et al. respectively [11,16].

Maximum smear positivity was seen in smear from early morning specimen (13.8%) and least in spot 2 specimen in our study. An incremental increase in the smear positivity in the third smear has been reported to range from 0% to 11% depending on various variables such as study design, study population, processing of samples and staining method used [14]. In a systematic review Mase et al. quantified the diagnostic yield of each of the three sputum specimens and reported an incremental yield of third specimen as 3.1% when the first two specimens were negative, similar to Rieder et al. who reported increase in percentage in diagnosing presumptive TB patients from third specimen from 0.7% to 7.2% in 42 laboratories of four high-burden countries [17,18]. Unlike our findings, a study reported the incremental yield in positivity of second specimen as 4% compared to 20% in the third specimen [15]. Poor quality of sputum submitted by the patient at the spot may be the reason for failure of spot 2 specimen to show an increase in AFB positivity over spot 1 and early morning specimen.

In concordance with our findings several studies have shown higher positivity by Standard method of sputum microscopy than Front Loading method, but the difference in positivity not being statistically significant [11,15,19]. Majority of the sputum smear positive patients could be identified by the Front Loading sputum microscopy in our study which suggests that under routine conditions the incremental yield from a morning sample smear is relatively small, hence, examination of smears of two sputum specimens collected on the same day of first visit of patients to health care centers could be the basis for detection of presumptive TB patients in low-income, high prevalence countries like India. The cases missed by Front Loading sputum microscopy were paucibacillary cases and less than other reports [17]. The most probable reason for missing such cases could be the poor quality and quantity of the spot sputum specimens or the intermittent shedding of bacilli during the day. Similar to our findings other studies have reported higher bacillary load in morning specimen [20,21]. Since the mucociliary clearance is lower during the night, sputum gradually accumulates in the airways thus yielding better quality of sputum in the morning [22]. The slight increase in sensitivity and grading of AFB microscopy gained under the Standard method of sputum microscopy is outweighed by limitations of delay in reporting and repeated health center visits leading to drop-outs. The probable reason behind this could be the intermittent shedding of bacilli in the day when patients are active or poor quality of sputum submitted by patient on the spot.

The average isolation rate on LJ media has ranged from 6.8% to 34.74% [23–26]. This depends on various factors like bacillary load, decontamination process used, time of contact with decontaminants, centrifugal force and heat produced during centrifugation during processing of specimen, nature of LJ media (its homogeneity, pH, concentration of Malachite green), temperature of incubation of LJ slants [12]. The low positivity rate due to decontamination process by 4% NaOH could be a possibility in our study.

The turn around time of *M. tuberculosis* culture on LJ media has ranged from 2 weeks to 8 weeks in various studies similar to ours [24,27–30]. The difference in sensitivity and specificity of the two methods of microscopy was not statistically significant in our study similar to others where sensitivity and specificity ranged from 63.6–91% & 86.2–97.4% for Front Loading and 64.8–91% & 91.7–97.8% for Standard method of sputum microscopy [11,19,31]. Culture positivity among AFB positive & negative cases by Front Loading microscopy was 63.01 and 4.4% whereas the same among AFB positive & negative cases by Standard method was 60.3 and 4.2% respectively. The increase in culture positivity and decrease in turn around correlated with higher AFB grading by both methods of sputum microscopy as evidenced in other studies too [30]. A study in Malawi has reported 15% of smear-positive cases dropping out of the diagnostic pathway between submitting specimens and being offered treatment [16]. In another study conducted in Uganda, the number of participants who failed to return with an early morning sample was high 301/2418 (12.4%) [31]. The new method has potential to reduce drop-out rates of patients, but the decision to adopt this needs to consider the greater bacillary yield of the morning sample, the potential burden on laboratory technicians, and the potential inconvenience and nosocomial exposure involved in having the patient wait at the health care facility. A limitation in our study was the paucity of clinically relevant data that could have been elicited from the patients to provide a better patient profile in the study. Several newer, recommended diagnostic techniques like Line Probe assay, CB-NAAT are being increasingly available for better results in less turn-around time, though

universal easy access to these is yet not there. Hence optimization in sputum microscopy remains the key to improving the diagnosis in such settings. In the recent times WHO has intended to implement the Front Loading sputum microscopy method in laboratories in a phased manner keeping in view few issues pertaining to training, human resources, laboratory work load, infection control and quality control [8,9].

Conclusion

Until newer, rapid and economical tools are introduced for diagnosis of pulmonary TB in resource limited settings, sputum smear examination remains the frontline method; hence optimization of this method for increasing its rate of pulmonary TB cases detection and reducing drop-out rate of patients from the diagnostic procedure is highly important. The sensitivity and specificity of sputum microscopy using an early morning sample followed by another sputum one hour later from the same day appears not to be inferior to using two early morning samples on subsequent days. The Front Loading sputum microscopy should be implemented in RNTCP laboratories to complete sample collection from patients on the day of first visit to health care centre thus increasing compliance and decreasing drop-outs. Proper implementation of DOTS Programme and strict adherence to treatment in new diagnosed pulmonary TB patients should be the foremost step to prevent MDR-TB.

References

[1] WHO. Global tuberculosis report Available from: www.who.int/tb/publications/global_report.

[2] TB statistics | Global, regional & drug resistant - TBFacts.org. Available from: www.tbfacts.org/tb-statistics/.

[3] TB Statistics for India | National and state statistics. Available from: www.tbfacts.org/tb-statistics-india.

[4] TB in India | TB burden, NSP, private TB care - TB Facts. Available from: www.tbfacts.org/tb-india.

[5] Tuberculosis control in the South-East Asia Region. Annual report Available from: http://www.searo.who.int/tb/annual-tb-report-2015.pdf.

[6] Parrish NM, Carroll KC. Role of the clinical mycobacteriology laboratory in diagnosis and management of tuberculosis in low-prevalence settings. J. Clin. Microbiol. 2011;49(3):772–6.

[7] Revised National tuberculosis Control Programme (RNTCP). Training module for medical practitioners. Central TB Division, Directorate General of Health Services. New Delhi India: Ministry of Health and Family Welfare; 2010.

[8] Proposed reduction of number of smears for the diagnosis of pulmonary TB-background document. Available from: www.who.int/tb/laboratory/reduction_of_smears.pdf

[9] Same-Day Diagnosis of Tuberculosis by Microscopy: WHO Policy Statement. Geneva: World Health Organization; 2011.

[10] Central TB Division, Directorate General of Health Services, Ministry of Health and Family Welfare, Nirman Bhavan, New Delhi. Revised National Tuberculosis Control Programme (RNTCP). Manual for Laboratory Technicians. Available from: http://ntiindia.kar.nic.in/cddistrictlevel/Ielearn%5CCATEGORY %5CRNTCP% 20Modules%5CLABMANUAL.pdf.

[11] Myneedu VP, Verma AK, Sharma PP, Behera D. A pilot study of same day sputum smear examination, its feasibility and usefulness in diagnosis of pulmonary TB. Indian J. Tuber. 2011;58:160–7.

[12] Revised National TB Control Programme Training Manual for Mycobacterium tuberculosis Culture & Drug susceptibility testing. Available from: tbcindia.nic.in/pdfs/Training%20Manual%20tuberculosis%20C%20DST.pdf.

[13] Revised National TB Control Programme (RNTCP) at glance - National Tuberculosis Institute. Central TB Division, directorate general of health services, Ministry of Health and Family Welfare, New Delhi India. Available from: ntiindia.kar.nic.in/cdphclevel/ielearn%5Ccategory%5CRNTCP% 20Modules%5CRNTCP%20at%20a%20 glance.pdf

[14] Steingart KR, Ramsay A, Pai M. Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis. Expert. Rev. Anti Infect. Ther. 2007;5(3):327–31.

[15] Nayak P, Kumar AM, Claassens M, Enarson DA, Satyanarayana S, Kundu D, et al. Comparing same day sputum microscopy with conventional sputum microscopy for the diagnosis of tuberculosis. PLoS One 2013;8(9):e74964.

[16] Harries AD, Mphasa NB, Mundy C, Banerjee A, Kwanjana JH, et al. Screening tuberculosis suspects using two sputum smears. Int. J. Tuberc. Lung Dis. 2000;4:36–40.

[17] Mase SR, Ramsay A, Ng V, Henry M, Hopewell PC, Cunningham J, et al. Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. Int. J. Tuberc. Lung Dis. 2007;11(5):485–95.

[18] Reider HL, Chiang CY, Rusen ID. A method to determine the utility of third diagnostic and second follow-up sputum smear examinations to diagnosis tuberculosis cases and failures. Int. J. Tuberc. Lung Dis. 2005;9(4):384–91.

[19] Ramsay A, Yassin MA, Cambanis A, Hirao S, Almotawa A, Gammo M, et al. Front-loading sputum microscopy services: an opportunity to optimise smear-based case detection of tuberculosis in high prevalence countries. J. Trop. Med. 2009;2009:398767.

[20] Andrews RH, Radhakrishna S. A comparison of two methods of sputum collection in the diagnosis of pulmonary tuberculosis. Tubercle 1959;40:155–62.

[21] Ramakrishna R, Chundari B. A comparative study of sputum smears by Ziehl Neelsen staining versus fluorescent staining in the diagnosis of pulmonary tuberculosis in patients suffering with cough for 2 weeks or more. J. Evid. Based Med. Healthcare 2015;2:1663–74.

[22] Parsons LM, Somoskövi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin. Microbiol. Rev. 2011;24(2):314–50.

[23] Piersimoni C, Scarparo C, Callegaro A, et al. Comparison of MB/Bact alert 3D system with radiometric BACTEC system and Löwenstein-Jensen medium for recovery and identii cation of mycobacteria from clinical specimens: a multicenter study. J. Clin. Microbiol. 2001;39:651–7.

[24] Naveen G, Peerapur BV. Comparison of the Lowenstein-Jensen medium, the middlebrook 7H10 medium and MB/BacT for the Isolation of mycobacterium tuberculosis (MTB) from clinical specimens. J. Clin. Diagn. Res. 2012;6(10):1704–9.

[25] Chihota VN, Grant AD, Fielding K, Ndibongo B, van Zyl A, Muirhead D, et al. Liquid vs. solid culture for tuberculosis: performance and cost in a resource-constrained setting. Int. J. Tuberc. Lung Dis. 2010;14(8):1024–31.

[26] Rodrigues C, Shenai S, Sadani M, Sukhadia N, Jani M, Ajbani K, et al. Evaluation of the bactec MGIT 960 TB system for recovery and identification of Mycobacterium tuberculosis complex in a high through put tertiary care centre. Indian J. Med. Microbiol. 2009;27(3):217–21.

[27] Muddaiah RK, James PM, Lingegowda RK. Comparative study of smear microscopy, rapid slide culture, and Lowenstein - Jensen culture in cases of pulmonary tuberculosis in a tertiary care hospital. J. Res. Med. Sci. 2013;18(9):767–71.

[28] Oberoi A, Aggarwal A. Comparision of the conventional diagnostic techniques,BACTEC and PCR. JK Sci. 2007;9:179–81.

[29] Negi SS, Khan SF, Gupta S, Pasha ST, Khare S, Lal S. Comparison of the conventional diagnostic modalities, bactec culture and polymerase chain reaction test for diagnosis of tuberculosis. Indian J. Med. Microbiol. 2005;23(1):29–33.

[30] Rishi S, Sinha P, Malhotra B, Pal N. A comparative study for the detection of mycobacteria by BACTEC MGIT 960, Lowenstein Jensen media and direct AFB smear examination. Indian J. Med. Microbiol. 2007;25(4):383–6.

[31] Miremba P, Kalyango JN, Worodria W, Mugerwa H, Nakakawa E, Asiimwe BB. Performance of frontloading for smear microscopy in the diagnosis of pulmonary tuberculosis: a cross-sectional study at a referral hospital in Uganda. PLoS One 2012;7(10):e48531.