Same day sputum smear microscopy for the diagnosis of pulmonary tuberculosis: Ziehl-Neelsen versus fluorescent staining

T. Jaya Chandra¹, R. Selvaraj², Y. V. Sharma³

¹Departments of Microbiology and ²Pathology, GSL Medical College, Rajahmundry, Andhra Pradesh, ³Centre for Laboratory Animal Technology and Research, Sathyabama University, Chennai, Tamil Nadu, India

Abstract

Background: Sputum smear microscopy is the main tool for the diagnosis of pulmonary tuberculosis (TB), especially in low- and middle-income countries (LMICs). Limited sensitivity of smear microscopy and patient dropouts (PDs) are the important obstacles of national TB control programs. Objectives: (1) To assess the diagnostic utility of the same day (SS2) approach (2) To compare the smear results of the spot morning (SM) and the SS2 approaches. Materials and Methods: The study was conducted in the Department of Microbiology, GSL Medical College, Rajahmundry, Andhra Pradesh, India from January 2011 to February 2015. Three sputum samples were collected [spot (S), second spot (S₂) 1 h after S, and morning sample (M)] from the volunteers. The sputum smears were stained by Ziehl-Neelsen (ZN), modified ZN (MZN), and fluorescent staining (FS) techniques and the results were pooled and compared under SM and SS2 approaches. Results: Of the 3,186 study participants, sputum smear positivity (SSP) for SM approach was 9.6% and 10.8% and for SS2 approach, it was 9.4% and 10.6%, respectively, with ZN and FS and the results were statistically insignificant (Mann-Whitney U test, P > 0.05). Conclusion: Technically SSP was similar for both the approaches and no improvement was observed with the SS2 approach. Hence, there is an urgent need to improve SSP.

Keywords: Fluorescent staining, patient dropouts, spot morning approach, sputum smear positivity, same day (SS2) approach, Ziehl-Neelsen staining

Introduction

Over 90% of tuberculosis (TB) cases occur in low- and middle-income countries (LMICs).¹,² In high TB-burden countries, pulmonary TB is diagnosed by identifying acid-fast bacilli (AFB) in sputum smears by Ziehl-Neelsen (ZN) staining. Due to simplicity, rapidity, and low cost, ZN is the commonly used technique for the diagnosis of pulmonary TB in LMICs.³ Most of the national TB control programs (NTCPs) were collecting three sputum samples by the SMS2 approach, i.e. one spot sample (S) at the time of consultation, morning sample (M) on the next day, and second spot (S2) at the time of submitting the M. However, studies revealed that majority of the smear positive (SP) pulmonary TB cases were diagnosed by the first two sputum samples.⁴ Hence, as per the recommendations of the World Health Organization (WHO), the number of sputum samples was reduced from three to two, i.e., the spot morning (SM) approach.⁵,⁶

The advantage with change in the number of sputum samples is the significant reduction in the workload of laboratory technicians (LTs) and reduction in wastage of the sample containers, slides, and reagents, etc., that are of financial criteria. But there is no change in the number of visits because the patient has to attend the hospital/diagnostic center twice due to the submission of M on the next day.
Patient dropout (PD) is a strong and major drawback of the SM approach, especially in the LMICs. The dropouts were reported to be 4.3-13%[9] and were much more in the field conditions.[6,7] PDs will not only lead to delay in the diagnosis and treatment of TB but will also spread infection. The only way to overcome these PDs is rapid or same day diagnosis of TB if possible. In LMICs, people cannot afford rapid TB diagnostic tests due to their high cost; so, sputum smear microscopy (ssm) is the only feasible technique in these settings.[8]

With this background in the present study, ZN and fluorescent staining (FS) with light-emitting diode (LED) fluorescent microscope (FM) were used to identify pulmonary TB in sputum smears of the same day (SSD) approach and the standard SM approach. Smear results of the study were pooled and compared in the following two headings: Standard SM approach and SSD approach. In the SSD approach, two sputum samples (S and S2) were collected with a gap of 1 h.

**Materials and Methods**

This study was conducted in the Department of Microbiology, GSL Medical College, Rajahmundry, Andhra Pradesh, India from January 2011 to February 2015. The 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 in the local language and given demonstrations about the importance of submission of sputum sample, visual difference between the sputum and the saliva, and how to produce good quality sputum sample. Finally, they were asked to provide 5 mL of sputum sample. All the individuals were informed to provide three sputum samples, i.e. S at the time of the first visit to the hospital and S2 collected 1 h after S. M was collected after getting up from bed early in the morning. After collecting the two spot samples, the patients were provided prelabeled sample containers to collect M at home.

Immediately after collection, three smears were prepared with each sample on new glass slides. One smear was stained by the standard ZN technique as per the Revised National TB Control Programme (RNTCP) guidelines,[10] second smear was stained by the modified ZN (MZN) method,[11] and third smear was stained by FS[12] as per the RNTCP guidelines. After staining, the data on the slides were covered with a wrap so that the microscopist would not be aware of the smear staining technique, thus avoiding misinterpretation of the smear reading.

Smear preparation:[11] new unscratched slides were used for smear preparation. The smears were prepared with a sterile bacteriological loop. A good smear is spread evenly over a size of 2 cm × 3 cm and is neither too thick nor too thin. This was allowed to air-dry for 15-30 min and fixed by passing it over the blue flame of the bunsen burner three to four times.

ZN:[11] The 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% sulfuric acid was used to decolourise the smears for 2-4 min and if necessary, the decolourisation step was planned to be repeated for another 1-3 min. The slides were rinsed as per the above mentioned process and counterstained with 0.1% methylene blue for 30 s. The slides were then washed, air-dried, and examined under oil immersion. Minimum 100 fields were examined. The smears were graded as per the RNTCP technical manual.[11]

MZN:[13] This is very similar to that of the standard ZN technique except the primary staining step with 1% CF that was done for 15 min.

FS:[13] The slides were placed on the staining rack and it was made sure that they did not touch each other, flooded with freshly filtered auramine phenol, and left for 7-10 min. Then the slides were washed well with running water and care was taken to control the flow of water so as to prevent washing away of the smear. They were decolourised by completely covering with acid alcohol for 2 min twice and then washed well with running water as done previously to remove the acid alcohol. It was counterstained with 0.1% potassium permanganate for 30 s and washed as done previously with water. Then the slides were air-dried and observed under 25X objective. The smears were graded as per the RNTCP technical manual.[13]

Comparative grading was done as per the RNTCP guidelines,[13] i.e. by dividing the number of AFB observed in FS with the correction factor.

As a part of internal quality control, all the positive slides and randomly 25% of the negative slides were read by the senior author. In case of any discrepancy in smear reading, the senior author’s decision was final.

**Statistics**

The data were analyzed using SPSS version 16 (SPSS Inc., Chicago, IL, USA) with the patient as the unit of analysis. The Mann-Whitney U test was used to compare the smear results among the two methods. “P” value less than 0.05 was used to indicate statistical significance.

**Results**

During the study period, 3,186 patients were included. A total of 343 (10.8%) patients were SP by any staining technique and the remaining 2,843 (89.2%) were smear negative. Sputum smear positivity (SSP) was 307 (9.6%), 343 (10.8%) in the SM approach and 304 (9.4%) and 338 (10.6%) in the SSD approach for ZN and FS, respectively. The diagnostic accuracy of LED FM is better compared to ZN. The difference was statistically
insignificant [Mann-Whitney U test (P > 0.05), Table 1] at 95% significant level.

**Discussion**

Several investigators studied the SS2 approach.[12,14-16] Chandra et al. reported that with ZN, SSP was similar for SM and SS2 approaches and the difference was statistically insignificant.[12] However, Chandra et al. also reported that SSP was improved with the MZN technique and the difference was statistically insignificant among the schemes that were concerned.[14,15] Nayak et al. compared the smear results of SM and SS2 by using the ZN technique and the investigators reported that SSP was 14% and 17% for the SS2 and SM approaches, respectively, and the authors missed 17% (73 out of 433) of SP cases with the SS2 approach.[16]

The WHO Technical Advisory Group for TB recommended LED FM as an alternative for conventional ZN microscopy.[17] Hence, FS by LED FM is the current ssm technique under the RNTCP of the Government of India. Cuevas et al. reported that LED FM had a higher sensitivity than ZN.[18] In the current study, sensitivity of ZN and FS was 98.79-100% and 98.91-100%, respectively, at 95% significant level. But the specificity was 0.52.18% and 79.13-98.4% for FS and ZN, respectively.

Diagnosis of TB and initiation of treatment can occur on the same day with the SS2 approach. In addition to this, nil dropouts is the major asset of the SS2 approach because waiting for a few hours can be acceptable rather than coming on the second day. In the current study, all the volunteers submitted S and S2 but 143 volunteers did not submit M and PD was 4.3% (143 out of 3,328). In one of the South African studies, 16% (58 out of 373) of SP cases were reported to be initial defaults after submitting the first sputum sample.[19] But in the technical point of view, there is no improvement in SSP with the SS2 approach, hence, it is well-known that ssm is not a very sensitive diagnostic tool.[20] Because of similar sensitivity with the SM approach, the SS2 approach is also eligible to fit under the heading “diagnostic accuracy of SSM is limited.”

Diagnosis and treatment of the maximum number of cases are the only ways to make the world free of TB. Again, ssm is the only feasible diagnostic technique in high TB-burden and LMICS. Hence, there is an urgent need to improve SSP and our study can open a Pandora’s box in the field of phthisiology.

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**Conflicts of interest**

There are no conflicts of interest.

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