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# One-sample two-smear versus two-sample two-smear approach for the diagnosis of pulmonary tuberculosis

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## Abstract:

**BACKGROUND:** To evaluate the efficacy of one-sputum sample two-smear approach for the diagnosis of pulmonary tuberculosis (PT).

**MATERIALS AND METHODS:** Data from January 2012 to December 2015 were analyzed to find (1) number of smear positives (SPs) by spot (S) sample with one and two smears; (2) number of SPs by morning (M) sample with one and two smears; and (iii) number of SPs by two samples with two smears, that is, same-day (SS<sub>2</sub>) and spot morning (SM) approaches. The Chi-square test was used to evaluate the statistical difference in SP cases.

**RESULTS:** With one-sample two-smear approach, the smear positivity (SPT) was 87% and 87.5%, for S and M samples, respectively, for Ziehl–Neelsen (ZN) staining; whereas, SPT was 96% and 97%, respectively, for S and M samples, for fluorescent staining (FS) technique. With two-sample two-smear approach, for ZN staining, SPT was 89% each and for FS technique, SPT was 97% and 99%, respectively, for SS<sub>2</sub> and SM approaches. The difference was not statistically significant ( $P > 0.05$ ) between one- and two-sample approaches in the staining techniques.

**CONCLUSION:** Significant number of SP cases are identified by S sample two-smear approach. Thus, the World Health Organization/Revised National Tuberculosis Control Programme can initiate S sample two-smear approach for the diagnosis of PT.

## Key words:

Sample, Staining, Tuberculosis

## Introduction

Tuberculosis (TB) is more prevalent among poor people of low living standards.<sup>[1,2,3]</sup> India has the highest TB burden in the world.<sup>[4]</sup> In resource-limited countries such as India, rapid diagnostic tests for TB are not possible owing to their high cost.<sup>[5]</sup> Sputum smear examination (SSE) is the only alternative.<sup>[3,6]</sup> For SSE, three sputum samples are collected by spot morning spot (SMS) approach. The majority of smear positive (SP) TB cases were however identified in the first two samples.<sup>[7]</sup> The World Health Organization (WHO) and

Revised National Tuberculosis Control Programme (RNTCP) therefore reduced the minimum number of samples to two, that is, spot morning (SM) approach.<sup>[8]</sup>

Even with the SM approach, patients were required to visit the health-care center a minimum of two times, which led to patient dropouts (PDs), and 50% of patients failed to return to provide a second sample or obtain their results.<sup>[9,10]</sup> However, studies have proved that the diagnostic accuracy of the same-day (SS<sub>2</sub>) approach is on par with the SM approach.<sup>[6,11,12]</sup> In the SS<sub>2</sub> approach, two sputum samples are collected with a gap of 1 h.

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The SS<sub>2</sub> approach has many advantages: early initiation of treatment, no PDs, and same-day screening of smear-negative (SN) cases with GeneXpert. Due to less bacillary load compared to morning (M) samples, most of the clinicians are reluctant to rely on spot (S) sample results.<sup>[13]</sup> Another important issue with the SS<sub>2</sub> approach is the transmission of TB in the healthcare center as patients need to wait in the microscopy centers for a long time to collect the results. The WHO has responded to this issue in speedup management by providing separate waiting halls as well as face masks.<sup>[14,15]</sup> However, in high TB burden countries such as India, especially under field conditions, providing waiting halls and face masks is yet another hurdle. In addition to these, continuous motivation of patients for collection of two good-quality S samples is a significant hurdle in the SS<sub>2</sub> approach. The change to the SS<sub>2</sub> approach under field conditions therefore remains questionable. The current study was therefore done to determine the diagnostic utility of one sputum sample versus two samples, that is, SS<sub>2</sub> and SM approaches.

To stain acid-fast bacilli in sputum smears, different staining techniques were reported in the literature, Ziehl-Neelsen (ZN) staining,<sup>[6]</sup> modified ZN staining,<sup>[12]</sup> reverse ZN staining,<sup>[16]</sup> ZN staining with jar method,<sup>[17]</sup> and fluorescent staining (FS).<sup>[11]</sup> However, most of the TB control programmes recommend ZN staining and FS techniques. Hence, in this study also ZN staining and FS techniques were used to stain the sputum smears.

## Materials and Methods

The study was conducted in the Department of Microbiology, GSL Medical College, Rajahmundry, Andhra Pradesh, India, from January 2012 to December 2015. The study protocol was approved by the Institutional Research and Ethics Committee of GSL Medical College.

Individuals with signs and symptoms suggestive of TB were included in the study. The participants were informed to submit deeply coughed sputum sample, minimum 5 ml. How to produce good quality of sputum sample was explained in vernacular language, and it was demonstrated practically. Three sputum samples were collected from each patient. After collection of S and second spot (S<sub>2</sub>) samples, the participants were provided with pre-labeled sample containers to collect M samples on next day, early in the morning, after getting up from bed. Immediately after collection, two smears were prepared and stained by ZN staining and FS techniques, respectively. Procedure for collection of sputum samples, smear preparation, staining, screening under a microscope, grading of SP cases were done as per the RNTCP guidelines.<sup>[8,18]</sup> As a part of internal quality

control and study protocol, all the positive smears and randomly 25% negative smears were rechecked.

Using ZN staining and FS techniques, the data were analyzed as follows:

1. Number of SPs by S sample with one and two smears,
2. Number of SPs by M sample with one and two smears,
3. Number of SPs by two samples with two smears, that is, SS<sub>2</sub> and SM approaches.

## Statistical analysis

Data were analyzed using SPSS, version 16 (SPSS, Inc., Chicago, IL, USA), with patient as the unit of analysis; Chi-square test was used to find the statistical difference in the SP cases between the approaches, staining techniques and the smears.  $P < 0.05$  was considered statistically significant.

## Results

Of the 2799 participants, 182 (6.5%) did not submit the M sample. Hence, the data were analyzed with 2617 participants. The male:female ratio was 1.32:1. Among the study patients, 232 (100%) were identified as SP for TB. In the SP cases, the male:female ratio was 1.4:1 and mean age was 34.7 years.

With one-sample one-smear approach, for ZN staining, the smear positivity (SPT) was 80% (185) each, and for FS technique, the SPT was 89% (206) and 90% (208), respectively, for S and M samples as shown in Table 1. There was no statistical difference ( $P > 0.05$ ). In this approach, with ZN staining 21 and 23 SP cases were missed for S and M samples, respectively, when compared with the results using FS technique. When compared with the total SPT, in this approach, ZN staining technique missed 47 SP cases each and with FS technique, 26 and 24 SP cases were missed, respectively, for S and M samples.

With one-sample two-smear approach, for ZN staining, the SPT was 87% (202) and 87.5% (203) and for FS technique, 96% (223) and 97% (225), respectively, for S and M samples [Table 1]. Statistically, the difference was not significant ( $P > 0.05$ ). In this approach, with ZN staining technique, 21 and 22 SP cases were missed, respectively, for S and M samples when compared with the results using FS technique. In one-sample approach, with two smears in comparison with a single smear, 17 and 18 more SP cases were detected with ZN staining and 17 more SP cases each were detected with FS technique for S and M samples, respectively. When compared with the total SPT, in this approach, ZN staining technique missed 30 and 29 SP cases and with FS technique, 9 and 7 SP cases were missed, respectively, for S and M samples.

**Table 1: Findings of smear positives using Ziehl–Neelsen and fluorescent staining in one and two sputum sample and one and two smear approaches**

	One sample, n (%)				Two sample, n (%)	
	One smear		Two smear		SS <sub>2</sub> approach	SM approach
	S sample	M sample	S sample	M sample		
ZN staining	185 (80)	185 (80)	202 (87)	203 (87.5)	206 (89)	206 (89)
FS	206 (89)	208 (90)	223 (96)	225 (97)	226 (97.4)	230 (99)

SS<sub>2</sub> = Same-day, ZN = Ziehl–Neelsen, SM = Spot morning, FS = Fluorescent staining

With two-sample two-smear approach using ZN staining, the SPT was 89% (206) each whereas with FS technique, the SPT was 97.4% (226) and 99% (230), respectively, for SS<sub>2</sub> and SM approaches as shown in Table 1. Statistically, the difference was not significant ( $P > 0.05$ ). In this approach, with ZN staining technique, 20 and 24 SP cases were missed, respectively, for SS<sub>2</sub> and SM approaches when compared with the results using FS technique. When this approach was compared with total SPT, ZN staining technique missed 26 each and FS technique missed 6 and 2 SP cases, respectively, for SS<sub>2</sub> and SM approaches.

## Discussion

TB ranks as the second leading cause of death from an infectious disease worldwide, after HIV.<sup>[19]</sup> In addition to gender difference,<sup>[20–22]</sup> technique used to collect the sputum, that is, intervention,<sup>[23]</sup> quality and quantity of sputum,<sup>[24]</sup> type of staining technique,<sup>[12,25,26]</sup> and number of samples<sup>[27]</sup> submitted for diagnosis influence the sputum microscopy results. The SPT increase when multiple samples are screened.<sup>[28]</sup>

In this study, 182 participants did not submit the M sample, so PD was 6.3%. In our previous studies, PD was reported to be 6.3%,<sup>[6]</sup> 4.2%,<sup>[12]</sup> 4.3%.<sup>[12,26]</sup> Whereas the PD was reported to be 13% and 52%, respectively, by Chandrasekaran *et al.*,<sup>[29]</sup> and Botha *et al.*<sup>[10]</sup>

The sensitivity of light-emitting diode–fluorescent microscope (LED–FM) is higher compared to ZN staining in the diagnosis of TB.<sup>[30,31]</sup> In this study, for one-sample one-smear approach, FS technique identified 9% (21) and 10% (23) more SP cases, and for one-sample two-smear approach, 9% (21) and 9.5% (22) more SP cases, respectively, for S and M samples. In the two-sample approach, FS technique identified 8.6% (20) and 10.5% (24) more cases, respectively, for the SS<sub>2</sub> and SM approaches. Statistically, the difference was not significant ( $P > 0.05$ ) between the staining techniques neither within the one-sample approach nor between the one- and two-sample approaches.

Freiman and Pinner<sup>[32]</sup> reported that examination of second smear from the same specimen resulted in 12% increase in the proportion of SP specimens.

Cattamanchi *et al.*<sup>[33]</sup> reported that with ZN staining technique, the incremental gain in sensitivity was lower at 4% and 5%, respectively, for S and M samples when the second smear was prepared from the same sample. With LED–FM, the incremental gain in sensitivity was 3% and 6%, respectively, for S and M samples.<sup>[33]</sup> In this study too, when the second smear was examined from the same sputum, the incremental gain in SPT was 7% (17) and 7.5% (18) for ZN staining and 7% (17) each for FS technique, respectively, for S and M samples. The difference in incremental gain in SP cases was not statistically significant ( $P > 0.05$ ) in one-sample approach for S and M samples, respectively.

When two-smear approaches were considered, in S sample category, with FS technique, 3 (1.3%) and 7 (3%) SP cases were missed and for M sample category 1 (0.4%) and 5 (2%) SP cases were missed, respectively, for SS<sub>2</sub> and SM approaches. With ZN staining, in S sample category, 4 (2%) SP cases each were missed and for M sample category, 3 (1.5%) SP cases each were missed, respectively, for SS<sub>2</sub> and SM approaches. Statistically, the difference was not significant ( $P > 0.05$ ). Although statistically there was no significant difference among the SP cases in one-sample two-smear versus two-sample two-smear approaches ( $P > 0.05$ ), nondetection of even a single SP TB patient has consequences for the individual patient and for transmission as it has been shown that each individual with active TB can spread the disease to a minimum of 10–12 per annum.<sup>[34]</sup>

S sample two-smear approach has several advantages: (1) on par SP results, (2) patient need not come on second day, (3) patients need not wait for a long time in the microscopy centres, so reduction in cross infection, (4) reduction in expenditure in the form of sample containers, (5) ease in preparation of two smears, simultaneously. As per the new RNTCP diagnostic algorithm,<sup>[35]</sup> chest X-ray (CXR) and cartridge-based nucleic acid amplification test (CBNAAT) can also be considered for the diagnosis of TB. Hence, with S sample two-smear approach, SN patients can complete the diagnostic algorithm (CXR and CBNAAT) on the same day. Hence, the WHO/RNTCP can initiate S sample two-smear approach with FS technique for the diagnosis of TB.

Bacillary load was found to be more in the M sample.<sup>[12]</sup> Hence, with FS technique, 25% M samples showed 3+ grading and 20% showed scanty result, whereas with S sample, 16% showed 3+ grading and 24% showed scanty result. Hence, with the technical point of view, ease in smear reading as well as monitoring the patient response to the treatment are the only advantages with M samples. In addition, if SP patient is graded as 3+, he/she can be educated thoroughly, so that extracare will be taken to control the spread of TB. However, the smear grading has no role to initiate anti-TB treatment (ATT); SPT is the only criteria to initiate ATT.

If bacillary load is high in sputum, technically that may be easy to screen the smears under a microscope; this may be the drawback with the S samples. High cost is the drawback of FS technique. In India, FS is done at free of cost because, RNTCP in partnership with the International Union Against TB and Lung Disease, ZN staining was replaced by FS technique and LED FMs were installed<sup>[31]</sup> in all the Medical Colleges. However, the number of SP cases which were missed are also very important criteria. In S sample two-smear approach, with FS technique, 9 (3.9%) SP cases were missed when compared to the total SPT. As per Chandra,<sup>[6]</sup> ethically we should not miss even a single SP case of TB. However, this number can be considered when compared with the PDs, which were reported to be much more especially in the field conditions.<sup>[29]</sup>

## Conclusion

Significant number of SP cases are identified by S sample two smear approach. Thus, the World Health Organization/Revised National Tuberculosis Control Programme can initiate S sample two smear approach for the diagnosis of PT. However, a study in the field conditions in high TB burden countries is recommended.

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## Conflicts of interest

There are no conflicts of interest.

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