International Journal of Community Medicine and Public Health
Barnes CE et al. Int J Community Med Public Health. 2018 Aug;5(8):3191-3196
http://www.ijcmph.com
pISSN 2394-6032 | eISSN 2394-6040

DOI: http://dx.doi.org/10.18203/2394-6040.ijcmph20183050

Original Research Article

Comparison of one versus two sputum specimens in the diagnosis of pulmonary tuberculosis

Carrie E. Barnes[1]*, Ramraj Balaji[2], K. R. John[2], Muthunarayanan Logaraj[2]

[1]School of Public Health and Community Medicine, University of New South Wales, Sydney, NSW, Australia
[2]Department of Community Medicine, SRM Medical College, Kattankulathur, Tamil Nadu, India

Received: 31 May 2018
Accepted: 05 July 2018

***Correspondence:**
Ms. Carrie E. Barnes,
E-mail: barnes103@hotmail.com

ABSTRACT

Background: In 2007, the number of sputum smears required for diagnosis of pulmonary tuberculosis was reduced from three to two. In high burden settings such as India, this practice continues to be the referenced standard whilst using smear microscopy. The objective of the study was to assess the plausibility of relying on one sputum smear rather than two serial sputum smears for tuberculosis diagnosis while also examining inter-specimen grading discrepancies.
Methods: A retrospective review of tuberculosis specimen registers (2012–2016) to compare sputum positivity and grading for incremental yield. Inter-specimen grading proportions were compared using McNemar's test.
Results: Incremental yield of the second specimen was 9.9% [95% CI 7.3%, 12.6%]. Differences in inter-specimen grading were not significant: nil (p=0.08), scanty (p=0.75), 1+ (p=0.66), 2+ (p=0.39), 3+ (p=0.15). Less than 25% of specimens showed a change in inter-specimen grading when classified as negative, low-grade, or high-grade results.
Conclusions: Two serial sputum specimens for the diagnosis of pulmonary tuberculosis remains a valid practice while continuing to rely on conventional smear microscopy. Inter-specimen grading congruencies support further exploration into same-day two-specimen collections to expedite diagnosis.

Keywords: Smear microscopy, Acid-fast bacilli, Serial

INTRODUCTION

Tuberculosis (TB) in India remains an issue of high public health importance. Consistently classified as one of the world's highest burden countries, India comprises 23% of the projected global total of TB incident cases accounting for an estimated 2.2 million people. Incidence estimates for all forms of TB in India have declined by 2-3% per year since 2005, with latest figures at 167 cases per 100,000 population.[1,2] Significant progress is required to approach global End TB strategy aims of a 95% reduction in tuberculosis deaths and an incidence below 10 cases per 100,000 population by 2035.[3] Undetected cases remain a significant concern, and as many as one-third of new cases are believed to be improperly diagnosed or receiving treatment outside of national TB programmes' guidelines.[4] High burden countries often rely on passive case finding through self-presentation as the present most cost-effective approach to diagnosis.[5,6] Sputum smear microscopy remains the most common and inexpensive diagnostic means of identifying acid-fast bacilli (AFB) for presumptive pulmonary TB.[1,7-9]

Current practice endorses the suitability of two serial sputum specimens, adapted in 2007 from the previous requirement of three serial sputum specimens, in low-resource high burden areas.[10] Changes to the policy were largely based on a review of 37 studies that found a collective 85.8% positivity in the first sputum specimen with an incremental yield of 11.9% in the second

specimen and 3.1% in the third specimen.[11] In light of this evidence, the definition for sputum smear positive TB was also adapted to include "the finding of a single AFB in at least one sputum examination" (i.e. ≥1 AFB/100 high power fields) in an effort to enhance case finding and expedite treatment initiation.[12] Decreasing the number of required smears has led to less patient visits, with both tangible and intangible cost savings to the individual, and decreased workload for laboratory staff at designated microscopy centres (DMCs).[13,14] Repeated visits to submit multiple specimens and obtain results also perpetuates high dropout rates, prompting further exploration of single sputum diagnosis methods or same-day multi-specimen collections.[14]

Given that conventional smear microscopy is encumbered by suboptimal sensitivity varying from 50-80%, lacks specificity for *Mycobacterium tuberculosis*, and is only suitable for the diagnosis of pulmonary TB cases, liquid culture methods remain the referenced diagnostic gold standard for TB.[1,4,7,8] The current estimate of availability of culture facilities in India is 0.3 locations per 5 million population and only 0.2 locations per 5 million population offering drug susceptibility testing, while laboratories offering smear microscopy number approximately 1 per 100,000 population.[2] Mycobacterial culture, despite high levels of specificity and sensitivity, typically requires 6 weeks to procure results, hindering efforts for rapid diagnosis and initiation of treatment.[15] The most reliable, efficient and cost-effective means of testing in resource limited countries will continue to be the use of smear microscopy in the immediate future.[16] For this reason we have sought to investigate the plausibility of relying upon one sputum specimen, rather than the current recommendation of two serial sputum specimens on consecutive days, for the diagnosis of pulmonary TB in a high burden setting.[10]

METHODS

A retrospective study was conducted using hospital laboratory mycobacteriology registers containing a comprehensive list of all AFB smear results performed at a DMC of the private tertiary care medical centre, SRM Medical College Hospital and Research Centre (SRM MCHRC) in Kattankulathur, Kancheepuram district, Tamil Nadu. Records were obtained from the SRM MCHRC microbiology department and reviewed for the period between January 2012 and December 2015. Ethical approval was obtained for the study protocol from the Institute Ethics Committee of SRM MCHRC. Individual patient consent was deemed unnecessary as this study was a review of results routinely collected for suspected pulmonary TB cases. The reviewed data was analysed anonymously. Data was entered into Excel® version 14.1.0 and analysed using SPSS (Statistical Package for the Social Sciences) software version 23.0.0.2.

During the four-year period, all patient episodes for serial AFB results were recorded and categorised into binary groupings as positive results or negative results. The age and sex of the individuals returning positive samples was also recorded. Inclusion criteria for positive results were based on World Health Organization (WHO) standards and included any sputum specimen, either in the first, second or both supplied specimens, in which any bacilli were detected.[17] All sputum smears were processed in following with India's Revised National Tuberculosis Control Program (RNTCP) laboratory guidelines. Ziehl-Neelsen (ZN) staining and conventional bright field microscopy was routinely used by this DMC until 2013 when LED-FM with Auromine O (AO) staining became the preferred method for reviewing specimens and was used in conjunction with ZN over-staining when required. Positive results were recorded and classified by the microbiology technician using the accepted standard WHO/International Union against TB and Lung Disease (IUATLD) scale (i.e. scanty, 1+, 2+, 3+) for either staining technique.

Positive results were delineated into three categories: first specimen negative, second specimen positive (NP); first specimen positive, second specimen negative (PN); and both specimens positive (PP). Those individuals that supplied only one specimen for AFB smear were included in the summary statistics, but were removed from analysis in the instances where calculation of incremental yield and proportions required two serial specimens.

Based on the initial AFB grading, positive sputum specimens were also recoded into three simplified groups described as negative (i.e. nil bacilli seen), low-grade (i.e. either a scanty or 1+ result), and high-grade (i.e. either a 2+ or 3+ result).[9] Frequencies were recorded of occurrences where the sputum grade increased, decreased, or did not change from specimen one to specimen two. For the purpose of this study, the secondary groups were used to assess for meaningful fluctuations in AFB grading between the two serial specimens in an effort to examine any association concerning the quality of the specimen provided and the timing of collection.

Timing of the individual sputum specimen collections was not recorded in the register. Following RNTCP protocol and laboratory practice, specimens consisted of a spot collection on the day of initial presentation, with a second specimen collected from an early morning expectoration ideally the next day.[6,7] Variability of grading was assessed to attempt to highlight any differences between the specimen results based on the timing of collection. Total proportions of sputum grade per serial specimen were calculated and compared for differences using McNemar's chi-squared test and testing for marginal homogeneity in the interest of comparing intra- and inter-specimen variability.

RESULTS

A total of 6468 individuals provided sputum specimens to the DMC for examination of AFB during the reference period. Positive results were detected in at least one smear in 573, or 8.9% (95% CI 8.2%, 9.6%) of individuals, including those failing to provide a second specimen. Of the total sample population, 25.5% (1572 negative and 79 positive) individuals failed to return a second sample for analysis. Valid (n=4817) two-specimen serial samples yielding at least one positive smear (n=494) were 10.3% (95% CI 9.4%, 11.1%). Of those individuals that did not submit a second specimen, the 79 first specimen positive (PX) individuals were excluded from further calculation of proportions of the positive results based on the inclusion criteria of two serial specimens. The yield of positive results in the first of the two specimens (PP or PN) was 90.1% (n=445). In the PN category, all but 3 specimens (30 of 33) were low-grade positives before returning a negative second smear result. The incremental yield of the second serial specimen (i.e. those classified as NP) consisted of 49 positive smears or 9.9% [95% CI 7.3%, 12.6%].

Demographic characteristics of individuals returning positive smears revealed a 4:1 male to female ratio with total participant mean age of 48.2 years. There was little difference in the mean ages between sexes (males=48.6 years, SD 15.12 years; females 46.6 years, SD 20.07 years), however sex-based age distribution was dissimilar. Male incidence cases (n=454) follow a fairly normal distribution pattern with a frequency peak in the 50 to 64 year old (34.6%) age range. Female incidence cases (n=113) reveal a non-parametric distribution amongst the age ranges, possibly due to the smaller sample size. Peak age range for case finding in females was in the less than 35 years (38.1%) demographic. Place of residence was also recorded in the register for individuals returning positive smears but was not considered for the study analysis.

Table 1: Comparison of sputum grade proportions and significance from 494 individuals, submitting two serial sputum specimens.

Sputum grade	First specimen: Spot collection	Second specimen: Early morning collection	Difference in proportions in specimen one vs. specimen two (%)	McNemar's x^2 test and P value[1]
Nil	n=49 (9.9%; 95% CI 7.3, 12.6)	n=33 (6.7%; 95% CI 4.5, 8.9)	3.2 (95% CI -0.2, 6.7)	3.12 (p=0.08)
Scanty	n=63 (12.8%; 95% CI 9.8, 15.7)	n=60 (12.1%; 95% CI 9.3, 15.0)	0.6 (95% CI -0.9, 2.1)	0.11 (p=0.75)
1+	n=92 (18.6%; 95% CI 15.2, 22.1)	n=87 (17.6%; 95% CI 14.3, 21.0)	1.0 (95% CI -3.8, 5.8)	0.20 (p=0.66)
2+	n=97 (19.6%; 95% CI 16.1, 23.1)	n=106 (21.5%; 95% CI 17.8, 25.1)	1.8 (95% CI -3.2, 6.9)	0.73 (p=0.39)
3+	n=193 (39.1%; 95% CI 34.8, 43.4)	n=208 (42.1%; 95% CI 37.8, 46.5)	3.0 (95% CI -3.1, 9.2)	2.10 (p=0.15)
Totals	N=494 (100%)	N=494 (100%)	-	-

1 Testing marginal homogeneity (P < 0.05, DF=1) for each sputum grade between specimens.

Table 2: Changes in sputum grade between serial specimens by year, 2012-2015.

Change in sputum grading[1]				
Year	**Increased**	**Decreased**	**No change**	**Total**
2012	27	14	74	115
2013	24	13	79	116
2014	24	22	78	124
2015	37	28	74	139
Total	112	77	305	494[2]

1-Grading categories defined as negative (i.e. no bacilli), low-grade (i.e. scanty, 1+), and high-grade (i.e. 2+, 3+).
2-Total excludes data from 79 positive specimens where a second specimen was not recorded.

Direct comparison of sputum grading (i.e. scanty, 1+, 2+, 3+) between the two specimens showed no significant differences between the paired proportions (Table 1).

Increased frequencies were noted with each increasing grade, and when categorised according to low- or high-grade results, the majority of sputum specimens for both samples were of high-grade quality (Table 2).

Nearly two-thirds of specimens showed no change in grading from specimen one to specimen two. Individuals providing specimens of differing grades, either increasing

or decreasing, divided the remaining serial specimens at 22.7% and 15.6% respectively.

DISCUSSION

The findings of this study indicated an overall case finding ratio of approximately 1 in 10, identifying one positive sputum smear for every ten sputum smears examined. Drop out rates were clinically significant, comprising approximately 25% of the individuals initially included in the analysis. High rates of attrition remains a long-standing multi-factorial problem of TB diagnosis with up to 37% of individuals lost to follow-up when three serial specimens were required.[14,18] The incremental yield of 9.9% from the positive second serial specimen translated into 49 newly diagnosed cases of pulmonary TB, all of which would have otherwise been missed if there were a reliance on only one sample. This outcome did not significantly differ from similar results by Islam et al quoting a 9% (p=0.47) positive second specimen yield and a comprehensive review by Mase et al reporting a cumulative incremental yield of 11.9% (p=0.19) for the second specimen.[7,11] Considering 1572 individuals submitted one negative specimen and failed to return a second specimen for analysis, the potential exists for approximately 156 undiagnosed cases during the four-year time period at this DMC alone. Based on such projections, this study endorses the necessity to analyse two serial sputum specimens for the presence of AFB whilst continuing to rely on smear microscopy for pulmonary TB diagnosis.

Early morning specimens are believed to be superior to spot specimens, as they are likely to contain more bacilli, resulting in a higher grade result, and are associated with a greater case finding yield.[13] Chinnakali et al highlights the significance of sputum smear grading by linking it to important diagnostic and treatment indicators such as enhanced transmission capabilities, cure rates, and the quality of sputum smear microscopy.[9] Comparing the conventional grading of each specimen result in accordance with the timing of the specimen in this study (inferred by the ordering of the serial specimen), failed to show any significant differences between the two specimens (Table 1). In addition, less than one-quarter of the early morning second specimens actually showed an increase to a significantly higher grading using the secondary classification, which was only marginally higher than the proportion of second specimens that conversely showed a significant decrease in the simplified grade. The majority of specimens experienced no meaningful change in grading categories between samples. There was however a notably greater proportion of high-grade 3+ specimens compared to other grades. But given that the DMC in this study was a tertiary care centre that receives referrals from the surrounding locality, grading of specimens may have been biased towards high-grade cases deemed most symptomatic to require testing and treatment.[9]

From the distribution of sputum grades between samples in this study, it is difficult to ascertain whether an early morning specimen provides any greater diagnostic value over a randomly collected spot specimen on the first day. Although some studies report increased diagnostic sensitivity from morning specimens due to a higher bacilli count, a comprehensive review by Davis et al as well as studies by Cuevas et al and Ramsay et al provide strong evidence for the non-inferiority of two same-day spot specimens compared to the traditional *spot-morning* two day protocol.[13,19-21] Bonnet et al also supports the establishment of one-day collection [of two specimens] regimes in high burden countries, where such systems have the potential to connect passive case finding to greater efficiency while also lessening costs and productivity loss for individuals presenting for testing.[14,22]

The variable and suboptimal sensitivity of smear microscopy may generate difficulties with the application of the results to various global settings.[8] This study was limited by a lack of availability of culture confirmation for positive smear results as a means of establishing the sensitivity of smear microscopy at this particular facility and ascertain true case finding proportions. However, with only roughly 77 facilities across India possessing culture capabilities as opposed to just under 13,000 facilities offering smear microscopy, many DMCs in India operate under similar conditions.[2] It was noted the DMC of SRM MCHRC initially utilised primarily ZN staining methods with conventional bright field microscopy before adopting the improved method using LED-FM and AO staining. LED-FM may have been of particular importance in identifying the majority of low-grade (scanty, +1) smear results that fell under the PN category of results in this study.[23] With a reported improved performance in identifying low-grade smears and average increase in sensitivity of 10%, LED-FM and AO staining may have contributed to an estimated additional 50 positive cases in the study population over the four-year time period with an uncertain quantified impact on incremental yield.[8,23]

CONCLUSION

Smear microscopy remains the most commonly used and least expensive diagnostic method for TB in many high burden countries such as India. The examination of two serial sputum specimens for AFB, one collected on the spot and one submitted the following morning, has been the recommended protocol for nearly a decade in countries with a high TB burden and established quality assurance procedures. While the requirement of two sputum specimens has been shown to contribute to a clinically significant serial yield, there is inconsistent evidence to suggest the timing of the specimens is contributory to positivity. Further consideration into the use of same day, two specimen collections using smear microscopy analysis as well as other innovative methods for TB diagnosis are necessary to expedite case finding in

many low and middle-income countries whilst improving progression towards global TB eradication targets.[14,19-21]

ACKNOWLEDGEMENTS

We are thankful to the microbiology staff at SRM MCHRC for providing the necessary registers, facilities and support for the study.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: Not required

REFERENCES

1. World Health Organization. Global tuberculosis report 2015, 20th ed. World Health Organization; 2015. Available at: http://apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf?ua=1. Accessed on 24 April 2016.
2. World Health Organization. India tuberculosis profile. Available at: http://www.who.int/tb/data. Accessed on 24 April 2016.
3. World Health Organization. The End TB Strategy: global strategies and targets for tuberculosis prevention, care and control after 2015. Available at: http://www.who.int/tb/post2015_strategy/en/. Accessed on 24 April 2016.
4. World Health Organization. Systematic screening for active tuberculosis: principles and recommendations. World Health Organization; 2013. Available at: http://www.who.int/tb/publications/tbscreening/en/ Accessed on 02 May 2016.
5. Azman AS, Golub JE, Dowdy DW. How much is tuberculosis screening worth? Estimating the value of active case finding for tuberculosis in South Africa, China, and India. BMC Med. 2014;12:216.
6. Parsons LM, Somoskövi Á, Gutierrez C, Lee E, Paramasivan CN, Abimiku A. Laboratory diagnosis of tuberculosis in resource-poor countries: Challenges and opportunities. Clin Microbiol Rev. 2011;24(2):314-50.
7. Islam MR, Khatun R, Uddin MKM, Khan MSR, Rahman MT, Ahmed T, et al. Yield of two consecutive sputum specimens for the effective diagnosis of pulmonary tuberculosis. PLoS One 2013;8(7):e67678.
8. Kulkarni HK, Wiseman MPJ, Jayaprakash T, Banur A, Basavarajappa KG, Jayasimha VL, et al. Evaluation of different staining methods for the detection of acid fast bacilli in sputum samples. Int J Curr Microbiol App Sci. 2015;4(12):536-40.
9. Chinnakali P, Selvaraj K, Thekkur P, Ramasamy G, Thulasingam M, Vasudevan K. Age and sex differences in sputum smear microscopy results for acid-fast bacilli in a tertiary care centre, south India. J Resp Med. 2014;674942:1-5.
10. World Health Organization. Proposed reduction of number of smears for the diagnosis of pulmonary TB: background document. World Health Organization; 2007. Available at: http://www.who.int/tb/areas-of-work/laboratory/policy_diagnosis_pulmonary_tb/en/. Accessed on 24 April 2016.
11. Mase S, Ramsay A, Ng N, 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.
12. World Health Organization. TB diagnostics and laboratory strengthening – WHO policy. World Health Organization, 2016. Available at: http://www.who.int/tb/laboratory/policy_diagnosis_pulmonary_tb/en/. Accessed on 03 May 2016.
13. 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;398767:1-6.
14. Bonnet M, Ramsay A, Gagnidze L, Githui W, Guerin PJ, Varaine F. Reducing the number of sputum specimens examined and thresholds for positivity: an opportunity to optimize smear microscopy. Int J Tuberc Lung Dis. 2007;11(9):953–8.
15. Ministry of Health and Family Welfare. Standards for TB care in India. Available at: http://www.tbcindia.nic.in/showfile.php?lid=3061. Accessed on 01 May 2016.
16. Dowdy DW, Steingart KR, Pai M. Serological testing versus other strategies for diagnosis of active tuberculosis in India: a cost-effectiveness analysis. PLoS Med. 2011;8(8):e1001074.
17. World Health Organization. Revision of the case definition for sputum smear positive pulmonary TB: background document. Available at: http://www.who.int/tb/laboratory/policy_sputum_smearpositive_tb_case/en/ Accessed on 28 April 2016.
18. Kemp J, Squire SB, Nyasulu I, Salaniponi F. Is tuberculosis diagnosis a barrier to care? [Abstract] Trans R Soc Trop Med Hyg. 1996;90:472.
19. Kirwan DE, Gilman RH. Same-day diagnosis and treatment of tuberculosis. The Lancet. 2013;13(2):102-4.
20. Davis JL, Cattamanchi A, Cuevas LE, Hopewell PC, Steingart KR. Diagnostotic accuracy of same-day microscopy versus standard microscopy for pulmonary tuberculosis: a systematic review and meta-analysis. Lancet Infect Dis. 2013;13(2):147-54.
21. Cuevas L, Yassin M, Al-Sonboli N, Lawson L, Arbide I, Al-Aghbari N, et al. A multi-country non-inferiority cluster randomized trial of frontloaded smear microscopy for the diagnosis of pulmonary tuberculosis. PLoS Med. 2011;8(7):e1000443.
22. Cambanis A, Yassin MA, Ramsay A, Squire SB, Arbide I, Cuevas LE. A one-day method for the

diagnosis of pulmonary tuberculosis in rural Ethiopia. Int J Tuberc Lung Dis. 2006;10(2):230–2.
23. Reza LW, Satyanarayna S, Enarson DA, Kumar AMV, Sagili K, Kumar S, et al. LED-fluorescence microscopy for diagnosis of pulmonary tuberculosis under programmatic conditions in India. PLoS ONE. 2013;8(10):e75566.

Cite this article as: Barnes CE, Balaji R, John KR, Logaraj M. Comparison of one versus two sputum specimens in the diagnosis of pulmonary tuberculosis. Int J Community Med Public Health 2018;5:3191-6.