

## Effect of relative centrifugal forces and centrifugation times on recovery of *Mycobacterium tuberculosis*

Sharma A<sup>1</sup>, Madaan I<sup>2</sup>, Shinu P<sup>3</sup>, Singh VA<sup>4</sup>, Garg A<sup>5</sup>

<sup>1</sup>Dr Amit Sharma  
PG Student

<sup>2</sup>Dr Isha Madaan  
PG Student

<sup>3</sup>Dr Pottathil Shinu  
Assistant Professor

<sup>4</sup>Dr Varsha A. Singh  
Professor & Head

<sup>5</sup>Dr Anu Garg  
Associate Professor

<sup>1,2,3,4,5</sup>Department of Microbiology  
M.M.I.M.S.R., MM University  
Mullana, Ambala, India-133207

Received: 07-09-2013

Revised: 18-10-2013

Accepted: 28-10-2013

Correspondence to:

Dr Amit Sharma  
91-999-183-5669  
91-173-130-4557  
amit801045@gmail.com

### ABSTRACT

**Background:** Tuberculosis (TB) is one of the major cause of morbidity and mortality worldwide, affecting one third of world's population and the incidence is much higher in South-East Asia (India and China together account for nearly 40% of the global TB cases)

**Objectives:** The current study was designed to evaluate the efficacy of relative centrifugal forces (RCFs) and various centrifugations times (CTs) on recovery of *Mycobacterium tuberculosis* (MTB) from sputum specimens pre-inoculated with MTB.

**Materials & Methods:** For this study, direct acid fast bacilli (AFB) smear negative sputum specimens were randomly collected, which were further subjected to sterilization ( by autoclaving at 121<sup>0</sup> C for 15 min ) and then seeded with MTB. Afterwards, these sputum specimens were subjected to centrifugation (in a cold centrifuge) at various RCFs and CTs. After centrifugation, supernatants and sediments were inoculated on LJ media and incubated at 37<sup>0</sup> C with daily monitoring for 8 weeks.

**Results:** In the current study, as the RCFs (6000xg) and CTs ( 25 min and 20 min) were increased, the growth detection time exponentially decreased (9 and 11 days) and culture grades (4+) were increased when cultured from the sediments. However, the recovery rates of MTB from the supernatant fluids were constantly decreased as RCFs and CTs increased.

**Conclusion:** The current study concluded that a higher RCF of 6000xg and CTs of 20 and 25 min could effectively detect the tubercle bacilli from the sputum specimens more efficiently than other lower RCFs and CTs.

**Keywords:** Pulmonary tuberculosis, *Mycobacterium tuberculosis*, acid fast bacilli, relative centrifugal force, centrifugation time

### Introduction

Tuberculosis (TB) is one of the major cause of morbidity and mortality worldwide, affecting one third of world's population. Geographically, the incidence is much higher in South- East Asia (India and China together account for nearly 40% of the global TB cases).<sup>[1]</sup> However, the prevalence and further epidemic of TB and other mycobacterial infections may be considerably minimized by detecting the disease in the early stage.<sup>[2]</sup> Global tuberculosis control programs uses microscopic examination for detection of Acid Fast bacilli (AFB).<sup>[3]</sup> However, sputum

direct microscopy has low sensitivity.<sup>[4]</sup> Therefore, the definitive diagnosis of TB could be attained using culture (detects 10-100 bacilli/ml).<sup>[5, 6]</sup> But isolation of mycobacterium from clinical specimens (for specimens containing normal commensal organisms) requires a decontamination procedure to remove these contaminating organisms.<sup>[7]</sup>

In previous years, various decontamination techniques have been attempted and most of the techniques use two steps; treatment of the clinical specimen with two chemical agents [one chemical agent that causes the lyses of

mucous (in sputum) and release of bacilli and the other one kills the commensal bacteria] followed by centrifugation (3000 x g) for 15-20 min to concentrate bacilli in the clinical specimen. [8-12] However, tubercle bacilli have a low specific gravity ranging from 1.07 to 0.79 and hence, requires high relative centrifugal force (RCF) to sediment the bacilli. If the RCF is not high enough, many tubercle bacilli could remain in suspension even after centrifugation and poured off while discarding the supernatant. [13] Therefore, the supernatant fluids of clinical specimens centrifuged at low RCFs (for instance; 2000xg and 3000xg), likely to be cultured for recovery of Mycobacterium tuberculosis (MTB). Further, the smear-culture correlation was found to be increased from 25 to 82% when the centrifugal force increased from 1260 x g to 3800 xg. [14] Furthermore, it is also reported that an increased recovery rate and decreased time to detection of MTB when the centrifugal forces and centrifugation times (CT) were increased. [7] The recommended CT is 15 to 20 minutes, but this range is very much vague for the recovery of mycobacterium when the practical aspects are considered. Therefore, the current study was designed to evaluate the effect various RCFs ranging from 2000xg to 6000xg and different CT (15, 20 and 25 min) on recovery of MTB from both the supernatant fluids and sediments.

## Material and Methods

### Study setting

This experimental study was conducted (between December 2012 and March 2013) in the department of microbiology, M. M. Institute of Medical Science and Research, Ambala, Haryana, which also operates as peripheral centre for Revised National Tuberculosis Control Programme. Further,

the study was approved by the Maharishi Markandeshwar University Ethical Committee (IEC-No/12/45). The study received a waiver of informed consent because the samples used were collected for routine microscopic examination in the peripheral centre for Revised National Tuberculosis Control Programme.

### Specimen collection and study duration

In this study, AFB smear negative sputum specimens (stained by Auramine O and examined by Light Emitting Diode fluorescent Microscopy, 400 x magnification, Ziess i LED, Primo star Gottingen, Germany) were randomly collected (between December 2012 and January 2013) from clinically suspected cases of pulmonary tuberculosis. After collection, all the sputum specimens (a total of 48 sputum specimens) were mixed in a container (150 ml) and subjected to the study.

### Digestion-decontamination

The sterilization of sputum samples were performed by autoclaving the sample at 121°C for 15 min and after autoclaving, a pinch of N-Acetyl L Cystiene was added to digest the sputum specimens.

### Preparation of Mycobacterial suspension

To a 14 ml BD Falcon™ tube (BD Gurgaon, India) containing glass beads (enhances equal distribution of bacilli while vortexing), 8ml of normal saline (pH: < 7.2) was added and mixed with 5 colonies of MTB(ATCC H37Rv). This mycobacterial suspension was mixed with 60 ml of predigested (using NALC) and decontaminated (by autoclaving) sputum. After that, a smear was prepared from the sputum-mycobacterial suspension, then stained using ZN staining technique and examined under 100 x oil immersion

objectives (Nikon E100 – magnification 1000×). After that, this sputum-mycobacterial suspension was further diluted by adding small quantities of decontaminated and digested sputum and direct AFB smears were prepared from each dilutions and the dilution was continued till one bacilli /oil immersion field (one bacilli per oil immersion field indicate that the specimen contains 10,000 bacilli/ml) was observed. Finally at 75 ml, the ZN stained direct smear showed one bacilli per oil immersion field and this particular quantity was used for carrying out all the experimental procedures.

### Experimental method

In this experimental study, a total of 5 sets were prepared and each set was comprised with three BD Falcon centrifuge tubes (for three different CT such as 15, 20 and 25 min). Then, each centrifuge tube was filled with 5 ml of decontaminated and digested

sputum containing MTB (contains one AFB per oil immersion field). Then, distilled water was added till the brim of the BD Falcon tube. After that, each set was centrifuged in a cold centrifuge (C-24 BL, Remi, Mumbai, India) at RCFs ranging from 2000xg to 6000xg and for CTs 15, 20 and 25min, respectively and kept at room temperature for 20 min to settle the sediments (minimizes the release of aerosols containing MTB into the atmosphere). Then, one loop full of supernatant was inoculated from each tube on LJ media. After that, the supernatant was poured off from all the tubes and sediments were suspended in 2.0 ml of PBS and thoroughly mixed in a vortex for 30 sec. Then, one loop full of sediment was inoculated on LJ media from each tube. All the LJ slants were incubated at 37°C and culture was monitored daily for 8 weeks.

### Results

**Table: 1 Comparison of culture results of supernatants and sediments as indicated by various relative centrifugal forces and centrifugation times for the detection of Mycobacterium tuberculosis**

Methods		Relative Centrifugal forces(x g) and Centrifugation Time (min)														
		2000xg			3000xg			4000xg			5000xg			6000xg		
		15 min	20 min	25 min	15 min	20 min	25 min	15 min	20 min	25 min	15 min	20 min	25 min	15 min	20 min	25 min
Culture on LJ Media	Sediment	+1	1+	+1	+1	+2	+2	+2	+3	+3	+3	+3	+3	+3	+4	+4
	Supernatant	+2	+2	+1	+1	+1	+1	-	-	-	-	-	-	-	-	-

**+1:1-19 colonies, +2: 20-99, +3:100-200, +4 :> 200**

Table 1 demonstrates comparison of culture grades of supernatants and sediments as indicated by various RCFs and CTs for the detection of MTB. In the present study, the different centrifugal forces and CT were used to demonstrate earlier recovery of MTB. As the centrifugal forces and CTs were increased, the growth detection time decreased and culture grades were increased when cultured from the sediments. The higher culture grade 4+ was observed when the specimen was centrifuged at a RCF of 6000xg and CTs 20 and 25 min, respectively. Table - 2 illustrates growth detection time as indicated by various RCFs and CTs for the recovery of MTB from supernatants and sediments. For sputum specimens centrifuged at RCF of 6000xg for 20min and 25min, the growth detection times were 11 and 9 days, respectively. However, specimens centrifuged at RCF 2000xg, the

culture grades were found to be 1+ and growth detection times were 46, 46 and 45 days for CTs 15, 20 and 25 min, respectively. On the other hand, specimens centrifuged at a RCF of 3000xg, the culture grades were found to be 1+ (for 15 and 20 min CTs) and 2+ (for 25 min CT) and demonstrated growth within 39, 37 and 36 days, respectively. In contrast, the specimens centrifuged at a RCF of 4000xg, the culture grades were found to be 2+ (for 15 and 20 min CT) and growths were visible within 26 and 25 days. However, when specimens centrifuged at 4000 x g for 25 min, growth detection time was reduced from 26 days (4000 x g for 15 min) to 23 days. Further, the specimens centrifuged at RCF 5000xg, the culture grades were considerably increased (3+) and growth detection times were significantly reduced (19, 18, and 16 days for CT 15, 20 and 25 min respectively).

**Table 2: Growth detection time as indicated by various relative centrifugal forces and centrifugation times for the recovery of Mycobacterium tuberculosis from supernatants and sediments**

Methods		Relative Centrifugal forces(xg) and Centrifugation Time(min)														
		2000xg			3000xg			4000xg			5000xg			6000xg		
		15 min	20 min	25 min	15 min	20 min	25 min	15 min	20 min	25 min	15 min	20 min	25 min	15 min	20 min	25 min
Growth detection Time (in days) on LJ Media	Sediments	46	46	45	39	37	36	26	25	23	19	18	16	14	11	9
	Supernatants	32	33	35	39	40	42	-	-	-	-	-	-	-	-	-

The recovery rates of MTB from the supernatant fluid were constantly decreased as centrifugal forces and CT increased (Table 2). When the supernatant fluid was cultured after centrifugation (at 2000xg for 15 and 20 min), growth of Mycobacteria (2+) was observed within 32 and 33 days on the LJ slants. However, when the CT was increased from 20 min to 25 min, the culture grade was reduced to 1+ and growth was visible within 35 days. But when the centrifugal force further increased (3000xg) and the supernatant fluid was cultured, the culture grade was reduced to 1+ with delayed growth detection times of 39, 40, 42 days for CTs 15, 20 and 25 min, respectively. It is also evident from the table -1 that there was no growth observed from supernatant fluids after centrifugation at 3000xg and above.

## Discussion

The most widely used method to diagnose pulmonary TB is the detection of AFB in sputum by direct microscopy. However, direct sputum microscopy has a low sensitivity often not more than 20 to 65%, in comparison with culture.<sup>[2,4]</sup> Further, the increasing frequency of smear-negative pulmonary TB cases could be due to the low sensitivity of the direct AFB smear. However, culture detects 10-100 bacilli /ml of sputum ensuring the isolation of tubercle bacilli more effectively than direct smear microscopy.<sup>[5,6]</sup> But isolation of mycobacterium from clinical specimens (for specimens containing normal commensal organisms) requires a decontamination procedure to kill the contaminating organisms.<sup>[7]</sup> Therefore, various decontamination techniques have been introduced in recent past and most of the techniques use a centrifugation step (3000 x g for 15-to 20 min) after treatment with

chemical agents.<sup>[8-12]</sup> However, due to the low specific gravity (0.79- 1.07) of tubercle bacilli, a relatively high centrifugal force is required to sediment the bacilli. Further if the RCF is not high enough, many tubercle bacilli could remain in suspension even after centrifugation and poured off while discarding the supernatant.<sup>[13]</sup> Hence, culturing of the supernatant fluid (specimens centrifuged at low RCFs), is recommended in the past for recovery of MTB. Further it is also reported that an increased recovery rate and decreased growth detection time of MTB when the centrifugal forces and CTs were increased.<sup>[7]</sup> Therefore, the current study was designed to assess the performance of various RCFs and CTs on recovery of MTB.

In the current study, the sterilized sputum specimen containing M. tuberculosis (one bacilli per oil immersion field) were divided into various aliquots of 5 sets (each set contains 3 aliquots for 15, 20 and 25 min centrifugations times). After that, all the aliquots were subjected to centrifugation at various RCFs and CTs (15, 20 and 25 min) and then inoculated on LJ media and culture was monitored daily for 8 weeks. The growth detection time (the time of inoculation of specimen and appearance of growth on LJ media) and the culture grades (the number of colonies formed) on LJ media were also noted. In the present study, when sediments were cultured on LJ media after centrifugation (using various RCFs and CTs), the culture grades and growth detection times were also varied. The higher culture grade (4+) was observed when specimens centrifuged at 6000xg for 20 and 25 min, indicating the potential of higher RCF along with increased CTs could effectively sediment the bacilli from clinical specimens. Further, this data was comparable with the study carried out

by Rickman et al<sup>[14]</sup> wherein culture positivity was increased from 7.1% to 11.6% when the centrifugal force was increased from 1260 x g to 3800xg. Further, it is also reported that a higher RCF could result in production of heat and that may likely to kill the bacilli. However, this difficulty can be avoided if a cold centrifuge is used for centrifugation of clinical specimens.<sup>[15]</sup>

Isolation of MTB in culture requires 4-8 weeks of incubation. However, in the present study, when the sputum specimens centrifuged at 6000 x g for 25 minutes and when the sediment cultured on LJ media, the growth was observed within 9 days. This could be substantiated due to the fact that the increase in the RCF may result in sedimentation of more number of viable bacilli, ensuing early growth. In contrast, when the supernatant fluid was cultured on LJ media ( after centrifugation of specimens at RCF 4000xg and above) , no growth was observed indicating the irrelevance of culturing the supernatant fluid after centrifugation at higher RCF .However , when the supernatant fluid was cultured on LJ media (for specimens centrifuged at low RCFs such as 2000xg and 3000xg), the growth was visible within 32,33,35 days ( for 2000xg for 15 ,20, 25 min) and 39,40,42 days , ( for 3000xg for 15 ,20, 25) respectively, and the culture grade was also reduced. This reduced culture grades from supernatant fluids could be due to splitting of bacillary load more specifically; as the RCFs and the CTs increases more number of bacilli settles down at the bottom of the tube, resulting in the low bacillary load in the supernatant fluid.

In summary, higher RCF of 6000xg for 20 and 25 min could effectively detect the tubercle bacilli from the sputum specimens more efficiently than other lower RCFs and CTs. However, the current

study was carried out under experimental conditions and therefore we recommend that further studies to be performed using RCF of 6000xg for 20 and 25 min in comparison with routinely used RCF of 3000 xg for 15 to 20 min.

## References

1. Global tuberculosis report 2012; WHO [Internet]. Available at: [www.who.int/tb/publications/global\\_report/gtbr12\\_main.pdf](http://www.who.int/tb/publications/global_report/gtbr12_main.pdf)
2. Saceanu CA, Pfeiffer NC, McLean T. Evaluation of sputum smears concentrated by cytocentrifugation for detection of acid-fast bacilli. *J Clin Microbiol* 1993;31(9): 2371-4.
3. WHO - World Health Organization 2012. Tuberculosis. [Internet] Available at: [who.int/mediacentre/factsheets/fs104/en/index.html](http://who.int/mediacentre/factsheets/fs104/en/index.html)
4. Shinu P, Nair A, Singh V, Kumar S, Bareja R. Evaluation of rapid techniques for the detection of mycobacteria in sputum with scanty bacilli or clinically evident, smear negative cases of pulmonary and extra-pulmonary tuberculosis. *Mem Inst Oswaldo Cruz* 2011;106(5):620-4.
5. Biotec Laboratories 2000a. Biotec FASTPlaque TB™ 2000. A rapid bacteriophage assay for detection of Mycobacterium complex in clinical samples. Instruction Manual, Biotec, Ipswich;2000.p. 15.
6. Biotec Laboratories 2000b. Biotec FASTPlaque TB™ 2000. An innovative breakthrough for the detection of tuberculosis. Technical data report, Biotec, Ipswich; 2000.p.9.
7. Ratnam S, March SB. Effect of relative centrifugal force and centrifugation time on sedimentation of mycobacteria in clinical specimens. *J Clin Microbiol* 1986;23(3):582-5.

8. Ganoza CA, Ricaldi JN, Chauca J, Rojas G, Munayco C, Agapito J et al. Novel hypertonic saline-sodium hydroxide (HS-SH) method for decontamination and concentration of sputum samples for MTB microscopy and culture. *J Med Microbiol* 2008;57:1094-8.
9. Chakravorty S, Tyagi JS. Novel multipurpose methodology for detection of Mycobacteria in pulmonary and extrapulmonary specimens by smear microscopy, culture and PCR. *J Clin Microbiol* 2005;43:2697-702.
10. Carricajo A, Fonsale N, Vautrin AC, Aubert G. Evaluation of BacT/Alert 3D Liquid Culture System for Recovery of Mycobacteria from Clinical Specimens Using Sodium Dodecyl (Lauryl) Sulfate-NaOH Decontamination. *J Clin Microbiol* 2001;39:3799.
11. Thornton CG, MacLellan KM, Brink TL Jr, Passen S. In vitro comparison of NALC-NaOH, tween 80, and C18-carboxypropylbetaine for processing of specimens for recovery of mycobacteria. *J Clin Microbiol* 1998;36:3558-66.
12. Farnia P, Mohammadi F, Zarifi Z, Tabatabaee DJ, Ganavi J, Ghazisaeedi K et al. Improving sensitivity of direct microscopy for detection of acid-fast bacilli in sputum: use of chitin in mucus digestion. *J Clin Microbiol* 2002;40(2):508-11.
13. Nadia Boujtita, Concentration of Mycobacteria in Clinical Samples using the Thermo Scientific General Purpose Centrifuge with 8x50 mL Individually Sealed Rotor [Internet] Available at: <https://static.thermoscientific.com/images/D01739>
14. Rickman TW, Moyer NP. Increased sensitivity of acid-fast smears. *J Clin Microbiol* 1980;11(6):618-20.
15. Selvakumar N, Govindan D, Chandu NA, Frieden TR, Narayanan PR. Processing sputum specimens in a refrigerated centrifuge does not increase the rate of isolation of Mycobacterium tuberculosis. *J Clin Microbiol* 2003;41(1):469-71.

Cite this article as: Sharma A, Madaan I, Shinu P, Singh VA, Garg A. Effect of relative centrifugal forces and centrifugation times on recovery of Mycobacterium tuberculosis. *Int J Med and Dent Sci* 2014; 3(1):257-263.

Source of Support: Nil  
Conflict of Interest: No