A NEW RESEARCH ON THE BIOLOGICAL ACTIVITY OF (2S,3S)-1,4-BIS-SULFANILBUTANE-2,3-DIOL

(***Note II***)♦

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Abstract: The universal sample processing multipurpose methodology was not developed for the diagnosis of tuberculosis.
We have recently described a sputum specimen processing method that involved homogenization and decontamination of the specimens by a treatment with dithiothreitol [(2S,3S)-1,4-bis-sulfanilbutan-2,3-diol] [1, 2]. This enable a highly sensitive smear microscopy and a better isolation of *M. tuberculosis*, showing, for the first time, the usefulness of dithiothreitol in TB laboratories. This report describes a study which was carried out to determine the effects of centrifugal force and centrifugation time on the concentration of mycobacteria in clinical specimens liquefied with dithiothreitol.
The effect of increasing centrifugal force was evaluated by AFB smear sensitivity in a study of 80 sputa that contained AFB, picked from routine

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submissions, and also by recovery rates of *M. tuberculosis* from sputa, in this case we performed 10 determinations using AFB smear negative sputa that were sterilized and artificially seeded with known concentrations of *Mycobacterium tuberculosis*, strain $H_{37}R_v$.

Keywords: *dithiothreitol (DTT), M. tuberculosis, centrifugal force, centrifugation time.*

INTRODUCTION

Concentration of acid-fast bacilli in clinical specimens is an important step in the laboratory diagnosis of tuberculosis. The buoyant density of *M. tuberculosis* (which ranges from 0.79 to 1.07) complicates collection by centrifugation due to inefficient sedimentation of bacilli. The net result is that the integrity of processed specimens is compromised following centrifugation because bacilli are poured off with the supernatant fraction. When bacilli clump or cord, large numbers of bacilli can partition as a single bacillus. In those specimens in whom few bacilli are present, these factors can leave the sediment virtually devoid of bacilli. Therefore, specimen processing has a significant impact on diagnostic sensitivity.
A relative centrifugal force of 1,800g to 2,400g and a centrifugation time of 15 to 30 min have been recommended for recovering mycobacteria [3].
This study is trying to find the possibility of enhancing smear/culture correlation of clinical specimens processed with DTT, under conditions of increasing the centrifugal force from 2,000g to 4,000g, at various centrifugation times.

EXPERIMENT DESIGN

For this we randomly collected AFB smear negative sputa from routine submissions. Sputa were sterilized by autoclaving and artificially seeded with test organisms. Seeded specimens were liquefied by mixing with a small volume of dithiothreitol. The digestant, dithiothreithol (DTT) without NaOH was prepared by dissolving 0.5 g of DTT in 50 mL of 2.9% sodium citrate and 50 mL of M/15 phosphate buffer and the *pH* was adjusted to 7. All reagents were brought to room temperature, and all procedures, including centrifugation, were performed at this temperature. 50 mL polypropylene conical tubes were used for all centrifugations.
Split samples of sterilized sputa were distributed into sets of nine centrifuge tubes (9 mL/tube) and each tube was seeded with 1 mL of mycobacterial suspension adjusted to contain 2 x 10^3 CFU/mL. DTT solution without NaOH was added in 10 mL quantities and then mixed on the vortex for 15 s. Phosphate buffer (*pH* = 7) was then added to the 40 mL mark, and the contents were thoroughly mixed. Each set of specimens was centrifuged at 2,000, 3,000 and 4,000g for 10, 15 and 20 min. The supernatant was poured off and the sediments were suspended in 2 mL phosphate buffer and thoroughly mixed. Three Löwenstein-Jensen slants were inoculated with 100 µL of

the each sediment. The slants were incubated at 37 °C and cultures were read after 60 days. It has been done 10 such of determinations. The recovery rate of mycobacteria was expressed as a percentage of the mean count of colonies obtained on Löwenstein-Jensen slants.

The effect of increasing centrifugal force on AFB smear sensitivity was determined with 80 clinical specimens that contained AFB obtained from patients with tuberculosis., the only criteria for entry into the study were sufficient specimen volume (> 5 mL). All sputum specimens were initially liquefied by mixing on the vortex with a small volume of DTT without NaOH, and then each specimen was distributed in equal volumes into a set of three tubes and treated in accordance with the DTT-NaOH method. Bloodstained, highly purulent and/or tenacious sputa were incubated for a few minutes at room temperature. Centrifugation was made at 2,000, 3,000 or 4,000g. Centrifugation time was constant and fixed at 20 min. Smears were prepared by spreading 25 μL of the sediment, before decontamination with NaOH on microscope slides on an aria of 1 × 2 cm. After decontamination, 200 μL samples of the sediments were cultured on Löwenstein-Jensen slants. Smears were stained by the auramine-rhodamine [4] and scanned at ×250 with a fluorescent microscope. Twenty fields per smear were searched independently by two workers to ensure maximum smear coverage. Smears were graded semi quantitative [2, 4]. The only specimens assessed were those for which both the smear microscopy results were in complete agreement (positive or negative). Presence of even a single mycobacterial colony constituted a positive culture to ensure that the maximum number of true positive results was detected. Another reason was that the sputum samples were divided and separated analyzed. The smear findings were correlated with culture results and the centrifugal force used. Barring 5 specimens out of the total of 80 (due to disagreements in microscopic examination) and another 3 that were excluded due to technical errors, all of the specimens were culture positive for *M. tuberculosis*. Cultures were considered to be truly positive only after confirmation of their identity by Ziehl-Neelsen staining and by the niacin test [4, 5]. The diagnostic accuracy of smear microscopy was assessed by their sensitivities, specificities and confidentiality. The culture results were considered the gold standard when calculating the sensitivity of smear microscopy. The test results were classified as true positive (T_p), true negative (T_n), false positive (F_p) and false negative (F_n). Sensitivities (S) were calculated as [6]:

$$S = \frac{T_p}{T_p + F_n} \times 100$$

RESULTS AND DISCUSSION

The effect of increasing centrifugal force was assessed by smear microscopy on a total of 80 sputa that were positive for AFB and also by culture of mycobacteria, making 10 determinations with sterilized sputa that were seeded with adjusted *M. tuberculosis* suspension, strain $H_{37}R_{v.}$

In experiments designed to determine the rate of recovery of mycobacteria in DTT liquefied and presterilized sputa, test results showed variations and overlap between

centrifugal forces and time of centrifugation. Recovery rate of mycobacteria ranged from 57 to 84.5 % (table 1). The data indicated a trend of higher recovery rate as centrifugal force and time were increased. The difference between 15 and 20 min were minimal. When centrifugation time was constant, the differences in recovery rate were relatively less between centrifugal forces of 3,000g and 4,000g, but greater than those between 2,000g and 4,000g.

Table 1. *Centrifugal force and centrifugation time effect on recovery of mycobacteria from sterilized sputa that were seeded with M. tuberculosis and liquefied with DTT*

Time (min)	Centrifugal force		
	2,000g	**3,000g**	**4,000g**
10	57.0	64.5	69.8
15	69.2	78.6	80.4
20	75.0	82.6	84.5

The test results are expressed as the percentage of the mean count of *M. tuberculosis* colonies on Löwenstein-Jensen slants, based on 10 determinations.

Table 2. *Centrifugal force effect on AFB smears sensitivity*

Centrifugal force	No. of specimens positive AFB				Smear sensitivity (%)
	±	1+	2+	3+	
2,000g	5	28	19	5	72.2
3,000g	6	30	20	7	79.1
4,000g	8	31	20	7	80.5

Smear sensitivity was based on 72 specimens yielding positive cultures.
Among three missed specimens by centrifugation at 3,000g, two were grade as scanty (±) and one had a score of 1+, by centrifugation at 4,000g. In other words, these specimens belonged to patients with low numbers of bacilli in their sputa. The fact that the centrifugation on 3,000g fails to detect all specimens with low bacterial loads may be due to technical errors during smear preparation or faulty reading of slides.

Smear positive specimens mostly yielded 50 to 150 colonies per slant, while smear negative specimens, no more than 30 colonies per slant. Increasing centrifugal force showed a slight increase in the number of colonies recovered in culture and occasionally in the number of AFB in smears. Although it had some effect on the degree of positivity of positive smears (figure 1), increasing centrifugal force failed to significantly increase smear sensitivity.
The highest rates of recovery of *M. tuberculosis* from DTT liquefied sputa were obtained with centrifugal forces of 3,000g and 4,000g, applied for 15-20 min, but not significantly lower than with 2,000g, for 20 min. This force applied for 10 min yielded a low recovery of organisms and is not adequate for concentrating *M. tuberculosis* in clinical specimens. Regardless, over 78% of mycobacteria can be recovered in the sediment with a centrifugal force of 3,000g to 4,000g for 15 to 20 min, by digestion of sputum with DTT. But the increase of centrifugation time may have adverse effects on the viability of *M. tuberculosis* in specimens treated by DTT – NaOH digestion-decontamination method. Moreover, centrifugation at high speed may generate heat, which could injure or kill *M. tuberculosis*.

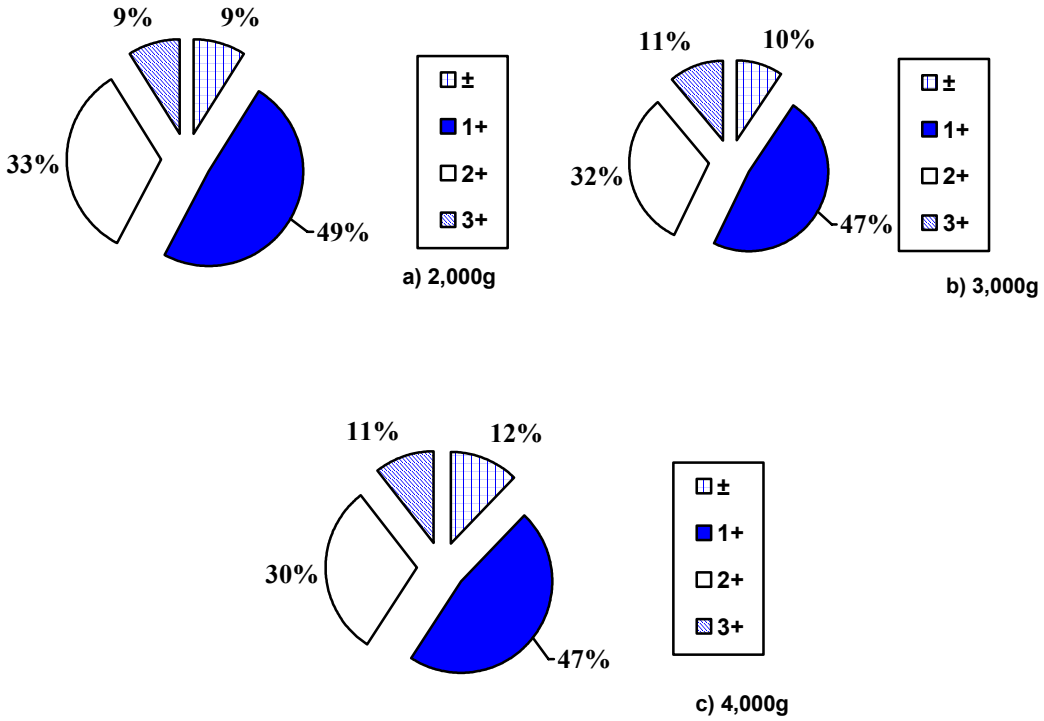

Figure 1. *Percent distribution of different grades of smear positivity following sputum digestion with DTT and centrifugation at a) 2,000g; b) 3,000g; c) 4,000g*

CONCLUSIONS

However, the increase of centrifugal force up to 3,000g would likely have facilitated isolation of a slightly larger number of *M. tuberculosis* from DTT liquefied sputa, it failed to increase smear sensitivity.

Centrifugation at 3,000g – 4,000g is an appropriate method to concentrate DTT liquefied sputa that contain small numbers of mycobacteria, when used before NaOH decontamination procedure.

Our date indicate that a centrifugal force of 3,000g applied for 20 min, is ideal to concentrate *M. tuberculosis* in DTT liquefied sputa, both technically and practically standpoint.

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