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

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Abstract: One of the key points in tuberculosis control programs is the early confirmation of the diagnostic through Koch bacilli detection. Therefore, this study is trying to highlight the role of digestion-decontamination agents in microscopic detection of M-Tuberculosis, in order to determine the ability of a smear made from respiratory sample to identify which specimens would be culture positive for *M. tuberculosis*.
The research methodology has been based on comparison of three types of smears processed by standard technique and by NALC treatment respiratory specimens, respectively by a new tested mucolitic reagent, dithiothreitol. During each stage of preparation process, AFB microscopic sensitivity has been assessed by Ziehl-Neelsen and auramine-rhodamine staining methods.

Keywords: *dithiothreitol, digestion-decontamination, M. tuberculosis, AFB, microscopy.*

INTRODUCTION

The bacteriological testing for *M. tuberculosis* is the differential key element of abnormal chest x-ray results in order to confirm a presumptive diagnosis of tuberculosis. Moreover, the bacteriological testing is a very useful tool allowing the monitoring of disease progression, therapy efficacy, public health concern about transmission as well as medical economics and the general design of tuberculosis control program.

Bacteriological investigation in TB provides physicians with data concerning the presence of AFB in pathological specimens, by microscopy. This process is also important in order to assess the germens viability, by tubercle bacilli culturing, providing the support for drug susceptibility testing.

Microscopic examination of respiratory specimens for acid-fast bacilli (AFB) plays an important role in the initial diagnosis of tuberculosis and determination of eligibility for release from isolation [1]. In addition, in the hospitals the smear must be negative for AFB before a patient can be released from isolation.

The early confirmation of TB diagnosis is hampered by frequent lack of AFB during microscopic testing and slow or even no growth at all of bacilli on selective culture media. This fact can delay the start of specific chemical therapy.

For pulmonary TB, the most favored respiratory specimen to be bacteriological investigated for bK is the sputum. The majority of clinical specimens submitted to the tuberculosis culture laboratory are contaminated to varying degrees by normal flora organisms.

The difference between the growing rhythm of different other germens and tubercle bacilli can cause the "drowning process" of Mycobacteria colonies by more rapidly growing normal flora organisms [2, 3]. Mycobacteria, commonly, are more resistant to most harsh chemicals than are other microorganisms, due to the high concentration of lipids in the cell wall, therefore chemical decontamination is often successful for the recovery of acid-fast organisms.

Kubica and co. [4] showed that the decontamination process could be facilitated by treating the viscous sample with a mucolytic agent, N-acetyl-L-cysteine (NALC). This agent liquefies organic debris surrounding the microorganisms by breaking the disulfide bonds of mucoproteins, releases the captive *Mycobacterium* cells and facilitates the access of decontaminating agent to undesirable microorganisms.

An ideal digestant-decontaminant solution should liquefy the organic debris in the specimen, kill most of the undesirable contaminants and permit survival of most Mycobacteria. None of the most chemical agents used nowadays meets all these requirements, therefore a compromise is needed. As a general rule, a contamination rate of 3% is acceptable [2].

In our study, we used a new liquefying agent, the dithiothreitol (DTT), which is known as a superior reagent for the specific and total reduction of disulfide bonds. DTT is a strong reducing agent, owing to its high conformational propensity to form a six member ring with an internal disulfide bond. It has a redox potential of –0.33 V at pH 7. The reducing power of DTT is limited to pH values above 7, since only the negatively charged thiolate form $–S^-$ is reactive (the protonated thiol form –SH is not). The pK_a of thiol groups is ~ 8.3. DTT is oxidized to the cyclic disulfide, and thereby ensures the reduction of other disulfides in solution. The disulfide reduction is complete in minutes.

Its usefulnes as an reducing agent stems from its water solubility (50mg/mL), yielding a clear, colorless solution. Aqueous solutions are stable in air.
These properties suggest that dithiothreitol might be a better reagent than NALC for the routine digestion of sputum [9].
Despite its long history, the acid fast smear remains unstandardised. Technical variations in the preparation of clinical material mean that AFB smear sensitivity relative to culture may vary.
In order to enhance the microscopic examination efficacy we have tried different enriching techniques which are able to concentrate the specimens after their homogenization.

EXPERIMENT DESIGN

In our study, 20 adult patients with a high clinical probability of tuberculosis but who were not under treatment at the time of sampling, have been included. Three respiratory specimens were collected from patients, in accordance with the norms of Tuberculosis National Control Program 2001-2005 (PNCT) and immediately processed or refrigerated no more than 24 hours.
Split sputum specimens were processed in parallel with the three methods :

- standard decontamination method with 4% NaOH [10];
- digestion-decontamination method that combine 5% *N*-acetyl-L-cysteine (NALC), 2% NaOH, 0.1M trisodium citrate and neutralisation with phosphate buffer pH 6.8 [2];
- digestion-decontamination method that combine 5% dithiothreitol (DTT), 2% NaOH, 0.1M trisodium citrate and neutralisation with phosphate buffer pH 6.8.

Using an additional mucolytic agent, routine processing comprises the following steps:

- NALC, respectively DTT liquefaction and centrifugation;
- Decontamination (2% NAOH);
- Neutralisation with phosphate buffer pH 6.8 in order to get the optimal pH for the mycobacteria growth (6.5-7);
- Centrifugation;
- Inoculation on isolation and detection specific culture media Löwenstein-Jensen.

In every method, the equal amount of sputum has been used. The proportion of sputum: decontamination agent: phosphate buffer has been established at 1:1:3. Centrifugation has been carried out at 3,000g for 20 minutes. All specimens were then cultured on Löwenstein-Jensen slants and incubated at 37^{o}C for 60 days, while the growth of colonies has been observed for intervals 21, 30, 45 and 60 days. All specimens that were culture positive for mycobacteria were tested by standard biochemical methods to distinguish *M. tuberculosis* from nontuberculous mycobacteria [11].
At each of the stages were made two parallel smears, one of which was stained by the ZN method and examined under oil immersion (x1000) and the other stained with auramine-rhodamine and scanned at x250 with an fluorescent microscope. To compensate for difference in field size, for auramine-rhodamine smears were examined 20 fields and for ZN smears, 100 field. The smears were examined by two workers [10] and was used the following quantitation (Table 1).

Table 1. *Quantitation results for microscopic examination*

Ziehl-Neelsen 1000x	**Results**	**Field size in fluorescent examination**
		250x
0 AFB	NEGATIVE AFB	0 AFB
1-9 AFB/100 fields	Number OF AFB/100 fields	AFB observed is divided by 10
10-99 AFB/100 fields	POSITIVE AFB 1+	
1-10 AFB/ fields	POSITIVE AFB 2+	
> 10 AFB/ fields	POSITIVE AFB 3+	

Note: Positive result of 1-3 must be prudential interpreted, another sputum sample examination being necessary.

Results were analysed and compared with the culture data. For each method AFB detection rates were calculated.

RESULTS AND DISCUSSION

A total of 60 respiratory specimens were included in this study. Out of these, 34 have been found with positive culture of M. tuberculosis by one of three methods. The samples with contaminated cultures and those whose smears were washing away during staining were excluded from the study. Only specimens that were *M. tuberculosis* culture positive have been considered for sensitivity assesment of smear for detection of AFB. 29 specimens were positive for AFB with one of the stained smears and no fals positive were detected. The results after each processing stages are shown in tables 2-7.

Table 2. *Numbers of acid fast bacilli detected in untreated samples*

		Auramine (59%)				
	n = 34	**0**	**1 – 9**	**1+**	**2+**	**3+**
ZN (45%)	**0**	17	1	3		
	1 – 9			2	1	
	1+			1	7	
	2+					1
	3+					1
17 positive results by any method from 29 positives in all; detection rates noted in parenthesis.						

Table 3. *Numbers of acid fast bacilli detected after 4% NAOH treatment of samples*

		Auramine (48%)				
	n = 34	**0**	**1 – 9**	**1+**	**2+**	**3+**
ZN (35%)	**0**	20	2	2		
	1 – 9		1	3	1	
	1+				4	
	2+					1
	3+					
14 positive results by any method from 29 positives in all; detection rates noted in parenthesis.						

Table 4. *Numbers of acid fast bacilli detected after NALC liquifaction and concentration of samples*

		Auramine (73%)				
	n = 34	**0**	**1 – 9**	**1+**	**2+**	**3+**
ZN (59%)	**0**	13	1	3		
	1 – 9				4	
	1+			2	7	
	2+					3
	3+					1
21 positive results by any method from 29 positives in all; detection rates noted in parenthesis.						

Table 5. *Numbers of acid fast bacilli detected after dithiotreitol liquifaction and concentration of samples*

		Auramine (97%)				
	n = 34	**0**	**1 – 9**	**1+**	**2+**	**3+**
ZN (80%)	**0**	6	4	1		
	1 – 9			1		
	1+			2	2	
	2+				3	4
	3+				10	2
28 positive results by any method from 29 positives in all; detection rates noted in parenthesis.						

Table 6. *Numbers of acid fast bacilli detected after NALC and 2% NaOH treatment of samples*

		Auramine (66%)				
	n = 34	**0**	**1 – 9**	**1+**	**2+**	**3+**
ZN (52%)	**0**	15	1	3		
	1 – 9		2	3	2	
	1+			1	5	
	2+				1	1
	3+					
19 positive results by any method from 29 positives in all; detection rates noted in parenthesis.						

Table 7. *Numbers of acid fast bacilli detected after dithiotreitol and 2% NaOH treatment of samples*

		Auramine (80%)				
	n = 34	**0**	**1 – 9**	**1+**	**2+**	**3+**
ZN (69%)	**0**	12		2		
	1 – 9			3	3	
	1+			2	4	
	2+				6	2
	3+					1
23 positive results by any method from 29 positives in all; detection rates noted in parenthesis.						

Comparing the results, it can be observed that liquefaction and concentration of sputum before staining improves microscopy sensitivity of acid-fast bacilli detection in sputum; in addition, an improvement of microscopic field could be noticed, making examination of smears rapid and convenient. There were more positive smears with the dithiothreitol reagent than with NALC (Table 8).

Table 8. *Results of processing smears following sputum digestion with NALC or dithiothreitol*

	NALC	DTT	Number of specimens
Auramine-rhodamine	POSITIVE	POSITIVE	21
	POSITIVE	NEGATIVE	0
	NEGATIVE	POSITIVE	7
	NEGATIVE	NEGATIVE	1

Comparing the quantities of AFB in the smears after sputum digestion and concentration by centrifugation, more AFB was scored on the smears from dithiothreitol digestion specimens.

Using culture growth of *M. tuberculosis* as the reference standard for diagnostic effectiveness evaluation, auramine-rhodamine staining proved to be more efficient than Ziehl-Neelsen staining for analyzing liquiefied specimens. Out of its low sensitivity, Ziehl-Neelsen staining methods poses some health risks.

It has been shown that dithiothreitol smears processing along with the use of fluorescent stains significantly improves the sensitivities of AFB smears for *M. tuberculosis.*

Poor smears results were obtained after 4% NaOH decontamination of sputum. Comparing the two digestion-decontamination methods, the recovery of AFB in 2% NaOH-dithiothreitol group was higher than in the 2% NaOH-NALC group. The results indicated that no decontamination method was clearly superior, however, a concentration of 2% NaOH effectively kill contaminating bacteria without significantly affecting the viability of *M. tuberculosis*.

According with the findings of this study, the 2% dithiothreitol method seems to be the most optimal for bacteriological confirmation of cultures media, the *M. tuberculosis* colonies could be visible after 21 days (table 9).

Table 9. *Results of liquefied specimens inoculation on solid culture media*

	NALC	DTT	Number of specimens
Culture on Löwenstein-Jensen media after 3 weeks	POSITIVE	POSITIVE	28
	POSITIVE	NEGATIVE	1
	NEGATIVE	POSITIVE	5
	NEGATIVE	NEGATIVE	0

CONCLUSIONS

Smear microscopy is currently the most feasible microbiological method for diagnosis of pulmonary TB in developing countries due to its rapidity, low cost and high positive

predictive value for *Mycobacterium tuberculosis*. Techniques to improve the sensitivity of smear microscopy would facilitate early tuberculosis diagnosis. Previous data [9] suggest that the liquefaction agents may increase the sensitivity of culture diagnostic testing.

The goal of the present study was to evaluate whether the sensitivity of the smear technique using microscopy could be improved by treating respiratory samples with a liquefaction agents and if the dithiothreitol is most effective for this presumption.

It has been shown that mucolitic agents sputum processing improves the sensitivities of AFB smears, while the decontamination methods affect the recovery of mycobacteria. Our results strongly suggest that the most effective method for the demonstration of acid fast organisms in sputum is the auramine-rhodamine stain applied to a liquefied with dithiothreitol and concentated sample by centrifugation, examined before the 2% NaOH decontamination process.

Our protocol showed an increase in tuberculosis diagnostic by using dithiothreitol - 2% NaOH compared to NALC - 2% NaOH digestion-decontamination and to 4% NaOH decontamination method. These results are consistent with our findings of another evaluation [9].

In spite of the cost of chemicals, the dithiothreitol digestion - 2% NaOH decontamination method was found to be the most accurate and the safest. The method is simple as the only extra reagent required is dithiothreitol that is easily available.

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