A New Study on (2S, 3S)-1,4-bis-Sulfanylbutane-2,3-diol

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The objective of this study was to assess the performance of dithiothreitol [(2S,3S)-1,4-bis-sulfanylbutane-2,3-diol] – NaOH digestion-decontamination procedure versus N-acetyl-L-cysteine (NALC) - NaOH method in detection and recovery of M. tuberculosis in clinical practice. The study was made of two independent procedures. Each procedure used a different specimen processing method. For one procedure, all specimens were processed with DTT - NaOH, and for the other , all specimens were processed with NALC - NaOH. DTT - NaOH specimen processing method was compared with NALC - NaOH method. Processed sediments were split for analysis by fluorescente smear microscopy and by culture using solid media. A total of 823 sputum were processed in the DTT-NaOH procedure of the study and a total of 865 sputum were processed for NALC-NaOH procedure of the study. Statistical comparisons of the procedures were done and the implications of the results are discussed. Dithiothreitol sputum processing significantly increased the smear sensitivity.

Keywords: reduction agent, dithiothreitol (DTT), M. tuberculosis

There are myriad problems associated with detecting M. tuberculosis organisms from clinical materials.

The diagnosis of tuberculosis is hampered by the own characteristics of this versatile microorganism. M. tuberculosis is best known for its slow growth rate and its acid-fast lipid–rich cell wall.

The cell walls of mycobacteria consist of a basal structure of peptidoglycan covalently linked to an arabinogalactan-mycolate. The peptidoglycan has repeating units of β-1,4-linked pyranosides of N-acetylglucosamine (GlucNAc) – N-acetylmuramic acid (MurNAc) tied in to L-alanine (L-Ala) – D-glutamic acid-NH_2 (D-Glu-NH_2) – diaminopimelic acid- NH_2 (DAP- NH_2) – D-alanine (D-Ala) (fig.1).

Therefore, there is a need to detect new methods to achieve increased, rapid and accurate TB diagnosis. Appropriate digestion and decontamination procedures must be selected to facilitate optimal recovery of mycobacteria.

In previous studies [1,2] we tried new synthetised compounds, respectively 1,2,4-dithiazolium salts (fig. 2), as decontaminating agents for sputum specimens, but not all are acceptable for culturing of mycobacteria.The specimen processing method based on the use of (2S,3S)-1,4-bis-sulfanylbutane-2,3-diol (DTT) combined with NaOH is the preferred method for digestion step because it is the least toxic to the mycobacteria and therefore provides the highest yield of positives [3,4]. DTT perform the liquefaction step, and permits the use of a lower (2%) concentration of NaOH than that required when DTT is omitted.

DTT is an unusually strong reducing agent, owing to its high conformational propensity to form a six-membered ring with a internal disulfide bond.

DTT is oxidized to the cyclic disulfide and thereby ensures the reduction of other disulfides in solution (fig. 3):

Its usefulness as reducing agent stems from its water solubility and reduced odor compared to previous thiol compounds. The reducing power of DTT is limited to *p*H

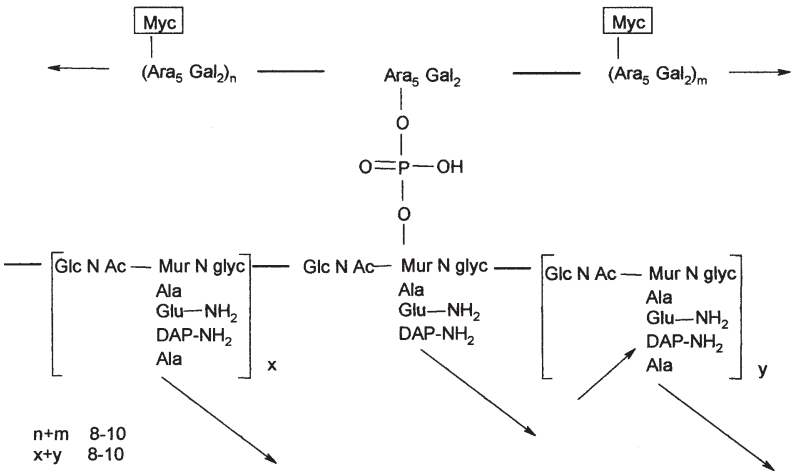

Fig.1. Possible structure of the mycobacterial cell wall. Myc-mycolic acid (The α-branched, β-hydroxylated long-chain fatty acids C_{60}-C_{90}).The arrows indicate interpeptide bridges between DAP residues

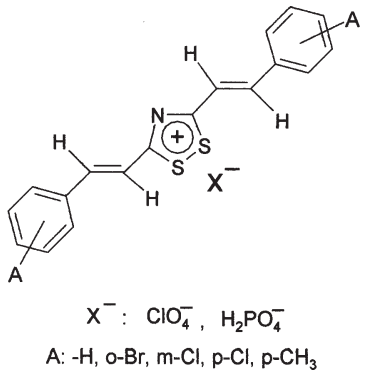

Fig. 2. Structures of tested 3,5-disubstituted 1,2,4-dithiazolium salts

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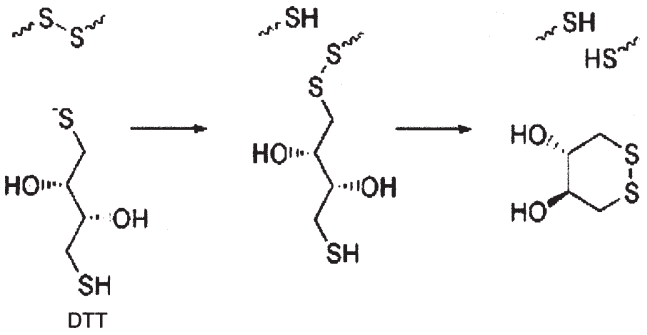

Fig. 3. Reduction reaction of disulfides by dithiothreitol

values above ~7, since only the negatively charged thiolate form S^- is reactive.

The present study was a follow-up to previous studies [3,4] that used split specimens in equal aliquots for processing by different methods and that had shown increased smear sensitivity following dithiothreitol (DTT) sputum processing but not significant difference in culture sensitivity. The DTT - NaOH treatment of sputum in culture of M.tuberculosis has similar positivity rate but higher decontamination capacity than the NALC - NaOH method of culturing.

In order to see if DTT specimen processing has a significant impact on diagnostic sensitivity, this study was made of two independent procedures in order to minimize the effects of specimens splitting.

Experimental part

All sputum specimens were collected from patients of Pneumology National Institute. The different processing methods were alternated on a daily basis and the specimens were selected randomly, the only one criteria for entry into the study were sufficient specimen volume (>4 ml). During the experimental phase of the study, all sample volumes, speed and duration of centrifugation were the same to allow a better comparison of the methods being tested.

Both decontamination procedures followed the same steps.

Briefly, all specimens were liquefied with mucolitic agent, decontaminated with 4% NaOH , incubated at room temperature for 15-20 min, neutralized with 67 mM phosphate buffer (*p*H 6.8) and then subjected to centrifugation at 3000 x g for 20 min [5]. The final concentration of NaOH in both solutions was approximately 2%.

The specimens were decanted after centrifugation and the sediments were resuspended in the remaining backwash. From the resuspended sediment, a portion was taken for smears, than to the remaining sediment was added 1 ml phosphate buffer. 200µL of each diluted sediment was used to inoculate 3 tubes of Löwenstein-Jensen medium.

For smear microscopy, acid-fast staining using auramine-rhodamine was performed. Specimens positive by fluorochrome staining were confirmed using Ziehl-Neelsen technique.

The smears have been prepared by a technician without acces to any information that could influence the results.

The smears were reported as positive or negative for acid-fast bacilli (AFB). Positive smears were further classified based on the American Thoracic Society scale [6] (table 1).

All inoculated Löwenstein-Jensen slants were incubated at 35 to 37^{0}C for 2 months and inspected for micobacteria growth at 21, 30, 45 and 60 days. Identification of mycobacterial isolates as M. tuberculosis used nitrate reductase and niacin test.

Paired smear / culture specimens were compared according to the following relations and terminology (table 2).

Sensitivity $= a / (a+c)$
Specificity $= d / (d+b)$
Predictive value of positive smear $= a / (a+b)$
Predictive value of negative smear $= d / (c+d)$
False negative $= c / (a+c)$
False positive $= b / (b+d)$
Overall correlation $= (a+d) / N$

Results and discussions

Results from the DTT-NaOH study

A total of 823 sputum specimens were processed. This group produced 150 specimens positive for M. tuberculosis by culture (18.2%) and no positive specimen for

Table 1
METHOD OF REPORTING OF ACID-FAST BACILLI IN THE SMEAR

Number of bacilli seen	Report
0 in entire slide	No AFB seen
1-2 in entire slide	Repeat collection
3-9 per slide	Rare
10 or more per slide	Few
More than one per field	Numerous

Table 2
RELATIONS AND TERMINOLOGY FOR PAIRED SMEAR / CULTURE SPECIMENS

	Results of culture		
Smears	Positive	Negative	Total
AFB Positive	a	b	a+b
AFB Negative	c	d	c+d
Total	a+c	b+d	N

Table 3
SUMMARY OF SMEAR MICROSCOPY AND CULTURE IN THE DTT-NAOH STUDY ARM

Smear microscopy	No. of cultures			
	M.T. positive	N.M.T.positive	Negative	Total
AFB +	137	0	2	139
AFB -	13	0	671	684
Total	150	0	673	823

Table 4
SUMMARY OF TIME TO M. TUBERCULOSIS POSITIVE RESULTS ON LÖWENSTEIN-JENSEN MEDIUM

Group	Average time to positive results (days)	
	DTT-NaOH	NALC-NaOH
All samples	23,8	31,6
AFB positive samples	22,5	24,4
AFB negative samples	25,5	38,8

nontuberculous mycobacteria. 19 Löwenstein-Jensen slants (2.3%) were contaminated.

A total of 137 specimens were positive by auramine-rhodamine staining in the DTT-NaOH study (table 3) The overall sensitivity and specificity of the smear relative to culture were 91.3 % and 99.7 %, respectively. The predictive value of positive smear was 98.6%, and 98.1% for negative smears.

The overall correlation, which is a measure of accuracy, was 98.2%.

The average time to positive results was faster than in the NALC-NaOH study (table 4).

Results from the NALC-NaOH study

A total of 865 sputum specimens were processed in the NALC-NaOH study . This group produced 161 M.tuberculosis culture-positive specimens (18,6%) and one specimen positive for M. avium (0.1 %). 32 Löwenstein-Jensen slants (3.7%) were contaminated.

A total of 106 specimens were positive by staining (Table 5). Relative to culture, the overall sensitivity and specificity of the smear were 65.8% and 99.4%, respectively.

The predictive value of a positive and negative smears for all specimens were 96.4 %, and 92.6 %, respectively. The overall correlation was 93%.

Comparision between DTT-NaOH and NALC-NaOH studies

The two methods have been compared on the following criteria: sensitivity, specificity, predictive value of positive smear, predictive value of negative smear, false positive, false negative and accuracy.

The comparative results of the two techniques are tabulated in table 6.

The smear sensitivity of DTT-NaOH sputum processing was higher (91.3%) than NALC-NaOH method (65.8%), but the specificities for both techniques were the same. DTT-NaOH method had lower false positive (8.7% vs. 34.2%) and false negative results (0.3% vs. 0.6%). The overall correlation, which is a measure of accuracy, was better with DTT specimens processing (98.2% vs. 93%).

The false positive results of 8.7% for DTT-NaOH and 34.2% for NALC-NaOH could be higher than its true values because the probability of false negative cultures e.g. mycobacteria present in the specimen failed to grow in culture has to be taken into consideration.

Suspected false-positive results must be analyzed in various ways. First, have been reported noncultivable bacteria from patients already under therapy after the first evaluation. Second, technical factors have to be taken into account. Since the sputum specimens were processed in the microbiological laboratory that handle a large number of specimens on a daily basis, cross-contamination at the sample level could not be reled out, as such settings could be a frequent source of contamination.

False negative smear results are not unusual because to see tubercle bacilli by microscopic examination of sputum smears, there must be approximately 10^4 organisms per ml of sputum, in contrast with culture technique that can detect 10 to 100 viable mycobacteria per mL of sputum. More commonly, false negative results are due to deficiencies in the preparation of the smear or inadequate sputum collection. Nevertheless, a considerable proportion of cases are missed by sputum microscopy alone. Fortunately, these patients have been shown to be discharging minimal amounts of tubercle bacilli and therefore not as infectious as patients with positive sputum microscopy.

Table 7 match the results of the different methods used following quantitative apreciation.

Table 5
SUMMARY OF SMEAR MICROSCOPY AND CULTURE IN THE NALC-NaOH STUDY

Smear microscopy	No. of cultures			
	M.T. positive	N.M.T. positive	Negative	Total
AFB +	106	0	4	110
AFB -	55	1	699	755
Total	161	1	703	865

Table 6
COMPARISON OF THE DTT-NaOH AND NALC-NaOH PROCESSING TECHNIQUES

	DTT-NaOH	NALC-NaOH
Sensitivity (%)	91,3	65,8
Specificity (%)	99,7	99,4
Predictive value of positive smear (%)	98,6	96,4
Predictive value of negative smear (%)	98,1	92,6
False positive (%)	8,7	34,2
False negative (%)	0,3	0,6
Accuracy (%)	98,2	93

Table 7
RESULTS OF MICROSCOPIC EXAMINATION IN SPUTUM SPECIMEN PREPARED BY DIFFERENT METHODS

Microscopic Examination Results				
	DTT-NaOH		NALC-NaOH	
Results	Nr.	%	Nr.	%
Negative	684	83,1	755	87,3
Rare	9	1,1	8	0,9
Few	41	5	63	7,3
Numerous	89	10,8	39	4,5
Total	823	100	865	100

When the results were lumped as either positive or negative alone, the percentage of positivity of the DTT sputum smear was 16.6 %, that of NALC was 12.2 %.

The results of the present study show that when specimens are processed with DTT, there is a statistically significant increase of the smear sensitivity ($p<0.05$). In this respect, we have chosen the chi-square test which has been computed using the SPSS. The outputs are given in the following tables.

Method * BAAR Crosstabulation

Count

		BAAR		
		BAAR+	BAAR-	Total
Method	DTT	137	686	823
	NALC	106	759	865
Total		243	1445	1688

Method * BAAR Crosstabulation

			BAAR		
			BAAR+	BAAR-	Total
Method	DTT	Count	137	686	823
		Expected Count	118,5	704,5	823,0
		% within Method	16,6%	83,4%	100,0%
		% within BAAR	56,4%	47,5%	48,8%
		% of Total	8,1%	40,6%	48,8%
		Residual	18,5	-18,5	
	NALC	Count	106	759	865
		Expected Count	124,5	740,5	865,0
		% within Method	12,3%	87,7%	100,0%
		% within BAAR	43,6%	52,5%	51,2%
		% of Total	6,3%	45,0%	51,2%
		Residual	-18,5	18,5	
Total		Count	243	1445	1688
		Expected Count	243,0	1445,0	1688,0
		% within Method	14,4%	85,6%	100,0%
		% within BAAR	100,0%	100,0%	100,0%
		% of Total	14,4%	85,6%	100,0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6,602[b]	1	,010		
Continuity Correction[a]	6,250	1	,012		
Likelihood Ratio	6,610	1	,010		
Fisher's Exact Test				,010	,006
Linear-by-Linear Association	6,598	1	,010		
N of Valid Cases	1688				

a. Computed only for a 2x2 table

b. 0 cells (,0%) have expected count less than 5. The minimum expected count is 118,48.

Method * Cultures Crosstabulation

			Cultures				Total
			Mt+	nMt	Mt-	Contaminate	
Method	DTT	Count	150	0	654	19	823
		Expected Count	151,6	,5	646,0	24,9	823,0
		% within Method	18,2%	,0%	79,5%	2,3%	100,0%
		% within Cultures	48,2%	,0%	49,4%	37,3%	48,8%
		% of Total	8,9%	,0%	38,7%	1,1%	48,8%
		Residual	-1,6	-,5	8,0	-5,9	
	NALC	Count	161	1	671	32	865
		Expected Count	159,4	,5	679,0	26,1	865,0
		% within Method	18,6%	,1%	77,6%	3,7%	100,0%
		% within Cultures	51,8%	100,0%	50,6%	62,7%	51,2%
		% of Total	9,5%	,1%	39,8%	1,9%	51,2%
		Residual	1,6	,5	-8,0	5,9	
Total		Count	311	1	1325	51	1688
		Expected Count	311,0	1,0	1325,0	51,0	1688,0
		% within Method	18,4%	,1%	78,5%	3,0%	100,0%
		% within Cultures	100,0%	100,0%	100,0%	100,0%	100,0%
		% of Total	18,4%	,1%	78,5%	3,0%	100,0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3,878[a]	3	,275
Likelihood Ratio	4,299	3	,231
Linear-by-Linear Association	,016	1	,899
N of Valid Cases	1688		

a. 2 cells (25,0%) have expected count less than 5. The minimum expected count is ,49.

This suggests that either the smear following DTT processing was more sensitive or culture sensitivity among smear-negative specimens was enhanced following NALC processing. The previous studies [3,4] using split specimens for processing in the two methods reported higher smear sensitivity following DTT processing. Therefore, the latter explanation that DTT processing enhances smear sensitivity, is probably correct.

An increase in the total number of AFB isolated was not observed following DTT processing ($p>0.05$). Both the dithiothreitol and NALC methods showed comparable percentages of positivity in cultures. The isolation of M. tuberculosis from smear-negative samples was higher following NALC processing. A possible explanation is related to the disadvantage of study such as the two independent procedures and random introducing of specimens in the study, because DTT has not inhibitory effects on M. tuberculosis. If the DTT processing is more efficient at collecting bacilli by centrifugation and these bacteria are viable, then the population of specimens that would have positive cultures may be increased.

The time to positive results were shorter following DTT processing for M. tuberculosis positive specimens, a result consistent with previous findings [3,4]. However, due to the design of this study (not split specimens), direct comparisons of time to positive results were not posible.

The DTT specimen processing method proved to be very efficient at decontamination and yielded fewer contaminated cultures (n=19) than NALC method (n=32), thereby making it more suitable for using it during warm weather. The contamination rates were 2.3 and 3.7 %, respectively. This suggests that one of the factors might have been causing more efficient decontamination of specimens in DTT – NaOH method, or that as NALC solution is air instabile and fail to break down mucous components around bacteria, allowing therefore, the NaOH to get acces to the undesirable microorganisms.

Conclusions

In Romania where the prevalence rate for tuberculosis is high, the sputum stained smear for acid-fast bacilli is the major epidemiological tool in the diagnosis of pulmonary tuberculosis.

Smear positive tuberculous patients are given the highest priority in case finding and treatment since these patients are the most dangerous sources of infection. While a positive smear will be taken as a possible tuberculosis bacillum infection warning sign by the physician, a false negative result could lead to wrong security actions.

The extraordinary sensitivity of the DTT smear method will enable the correct identification of a sizeable population of infected individuals, particularly those with paucibacillary TB, who would otherwise escape detection by conventional methods.

In terms of its usefulness for M. tuberculosis culture, the DTT method was not significantly different from the NALC method.

Another positive feature of DTT processing is the reduced rate of contamination compared to that of NALC processing and, also the precocity growing of M. Tuberculosis colonies on culture media.

This is the reason why the DTT specimen processing method is more recommended during the summer time, when because of the high temperature many cultures are contaminated or in the case of specimens from patients having big pulmonary cavities bacterically contaminated.

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