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Evaluation of Hypertonic Saline-Sodium Hydroxide Method for Concentration of Sputum Samples for Mycobacterial Culture

Anugoon Bunkhong[1,2], Wijit Wonglumsom[3], and Duangnate Pipatsatitpong[1,*]

[1]*Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathum Thani 12120, Thailand*
[2]*The Office of Disease Prevention and Control No.,3 Nakhon Sawan 60000, Thailand.*
[3]*Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University, Nakhon Pathom 73170, Thailand.*

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ABSTRACT

Mycobacterium tuberculosis complex (MTBC) is a major causative agent of public health problems. Nontuberculous mycobacteria are also increasingly encountered worldwide. Acid-fast bacilli (AFB) staining and culture are conventional methods used for mycobacterial identification. The aim of the study was to compare the performance of the two methods for AFB staining and culture, *i.e.* The hypertonic saline-sodium hydroxide (HS-SH) technique was compared with the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method for AFB staining and cultivation. A cross-sectional study was conducted in a tuberculosis laboratory of the 3rd Office of Disease Prevention and Control, Nakhon Sawan Province, Thailand, during October 2015 to September 2016. Totally, 427 paired samples of sputum were digested and decontaminated by HS-SH and NALC-NaOH methods. After concentration, the processed samples were cultured in Löwenstein–Jensen (LJ) media and Mycobacteria Growth Indicator Tube (MGITs). The direct smear detection before and after concentration of the sputum by both methods was also examined. To evaluate HS-SH method, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy and kappa were analyzed. The positive rates of AFB smears by both concentration methods were significantly higher than the direct method (P=0.032). The HS-SH method was highly comparable to the traditional NALC-NaOH method for microscopy and culture in LJ media and MGITs (kappa=0.96, 0.59 and 0.45, respectively) with no statistically significant difference. Sensitivity, specificity, PPV, NPV and accuracy of the HS-SH method for cultivation was 96.3%, 100.0%, 100.0%, 94.5% and 97.7%, respectively. The conclusion, The

*Corresponding author: duangnat@tu.ac.th doi: 10.14456/scitechasia.2019.34

HS-SH method demonstrated good sensitivity, specificity, PPV, NPV and accuracy similar to NALC-NaOH method for sputum digestion and concentration of both microscopy and culture with low cost and workload.

Keywords: *Mycobacterium tuberculosis*; Hypertonic saline-sodium hydroxide; NALC-NaOH

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* complex (MTBC) that kills 1.3 million people annually around the world [1]. Some nontuberculous mycobacteria (NTM), predominantly *M. avium* complex, are emerging pathogens that cause pulmonary diseases similar to MTBC infection, mostly in immunocompromised hosts [2-3]. Thailand, as classified by the World Health Organization (WHO), is a country that has high TB and drug-resistant TB cases with about 108,000 new cases yearly and mortality of 9,300 cases [1]. Current laboratory testing of MTBC and NTM is based on molecular assays; however, sputum microscopy and culture, which is the gold standard, are essential for mycobacterial diagnosis and patient monitoring in low-income and middle-income countries. Cultivation of mycobacteria by conventional solid medium and automated system with liquid medium requires sputum digestion and decontamination steps with N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) as recommended by the WHO and the Centers for Disease Control and Prevention (CDC) [4,5]. This method needs costly reagents and special training. Therefore, a simpler and inexpensive procedure, hypertonic saline-sodium hydroxide (HS-SH) method, is interesting to be used in mycobacterial laboratories [6,7]. The Hypertonic saline, mucolysis reagent, breaks down hydrogen bonds and separates DNA from mucus. The sodium hydroxide has a decontaminating function [8,9,10]. To evaluate HS-SH method, it was compared with the standard NALC-NaOH method for sputum concentration examined by Acid-fast bacilli (AFB) microscopy and cultivation.

2. Materials and Methods

2.1 Study design and study population

A prospective study was carried out in the tuberculosis laboratory at the 3rd Office of Disease Prevention and Control, Nakhon Sawan Province, Thailand from October 2015 to September 2016. A total of 427 sputum samples were collected from patients with suspected re-treatment, on-treatment, pre-treatment and new cases of tuberculosis who had visited government hospitals located in the central part of Thailand. The re-treatment group included patients who relapsed and patients whose treatment was interrupted or lost to follow-up for at least 2 consecutive months. On-treatment cases are defined as patients were still being treated with anti-TB drugs after the two-month intensive treatment phase. Pre-treatment patients were suspected cases of tuberculosis including multidrug-resistant tuberculosis (MDR-TB) household contacts, patients with human immunodeficiency virus (HIV) infection, and prisoners in jails. New cases are defined as the suspected TB patients who have never received treatment for TB, or who have taken anti-TB drugs for less than one month [11,12]. The sputum samples were immediately transported to the tuberculosis laboratory at the 3rd Office of Disease Prevention and Control, Nakhon Sawan Province. This study was reviewed and approved by The Ethical Review Sub-Committee Board for Human Research Involving Sciences, Thammasat University, No. 3 (ECScTU 013/2558).

2.2 Comparison of HS-SH method versus NALC-NaOH method in tuberculosis identification

All samples underwent direct smear and staining with the Ziehl-Neelsen method. Each sample was then equally divided into two parts; one part was processed with NALC-NaOH technique and the other was concentrated by the HS-SH method.

For the NALC-NaOH method, at least 1 ml sputum was added to a 50 ml centrifuge tube with an equal volume of a mixture containing 0.1 grams of NALC, 10 ml of 2.9% sodium citrate and 10 ml of 4% NaOH. The tube was vortexed well and kept at room temperature for 15 min. For neutralization, sterile phosphate buffer saline (PBS) pH 6.8, was added to bring the total volume to 50 ml. After mixing, the tube was centrifuged at 3000xg for 15 min at 4ºC. The supernatant was discarded and the pellet was resuspended in 1 ml sterile PBS. For the last suspension, 500 µl was added into MGIT tube and 100 µl each was inoculated into two LJ media. One drop of the suspension was also used to prepare a smear for AFB microscopy. The LJ culture tubes were incubated at 37ºC for up to 8 weeks and the MGIT broth was incubated inside the MGIT 960 automated culture system at 37ºC for up to 6 weeks according to the current guideline [13]. Mycobacteria were identified using the criteria of time for growth detection, colony morphology, pigmentation and AFB smear. *M. tuberculosis* was identified by SD Bioline MPT64 rapid test (Standard Diagnostic, South Korea).

For the HS-SH method, at least 1 ml sputum was mixed with an equal volume of HS-SH solution containing 10 ml of 10% NaCl and 10 ml of 4.0% NaOH. The tube was mixed well and incubated at room temperature for 15 min. The rest of the process was as described in the NALC-NaOH procedure.

Statistical analysis: The data were analyzed using basic descriptive statistics and represented frequencies and percentages. The Chi-square or Fisher's exact test was used to compare proportions. To evaluate the HS-SH method, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) accuracy and kappa were calculated to measure the efficacy of the HS-SH method when compared to the NALC-NaOH method. The level of significance was at P-value < 0.05.

3. Results and Discussion

3.1 Results

Of the 427 patients, the median age was 53 years (range: 18-102 years) with male to female ratio of 3.5:1. The mean duration of specimens that were stored at 2-8°C until concentration processing was 4.8 days with the average sputum volume of 4.4 ml. Both HS-SH and NALC-NaOH concentration methods yielded about 7% more positive AFB microscopy than the direct smear method (Table 1) (kappa index = 0.4, $P < 0.032$), while agreement between the two concentration methods was excellent (kappa index = 0.96, $P = 0.998$).

Out of all smear-positive sputum processed by HS-SH and NALC-NaOH methods, 41.9% and 41.0% were AFB grades of 3 plus, respectively, whereas 21.8% was found for the direct smear method (Table 2). When patients were classified into 4 groups including re-treatment, on-treatment, pre-treatment and new case tuberculosis, the positive rates of AFB smears by the concentration methods were also higher than the unconcentrated direct method with P-value of 0.009, 0.001, 0.018 and 0.036, respectively.

Cultivation using the HS-SH method on LJ media and MGITs yielded slightly more positive results than the NALC-NaOH method. Contaminated cultures were detected by macroscopic and microscopic assessment. The appearances of

contaminated LJ media were liquefaction, media color change or growth of non-mycobacterial colonies, while contaminated MGITs generally showed heavy turbidity. Then AFB smears from both media were examined for distinguishing between mycobacterial growth and contamination. There were about 4% less contaminated cultures by HS-SH than by NALC-NaOH. Nevertheless, there were no statistically significant differences in culture results of LJ and MGITs with P=0.230 and 0.148, respectively (Table 1). The contamination rates for HS-SH and NALC-NaOH of re-treatment, on-treatment, pre-treatment and new case groups were not significantly different with *P*-value of 0.78, 0.24, 1.00 and 1.00 respectively (data not shown). Nontuberculous mycobacteria (NTM) were isolated in 10.8% of 427 patients. Both decontamination methods (HS-SH and NALC-NaOH) showed equal mean and median times to culture positive of 25 days and 21 days by LJ media, respectively. By MGITs, the coverage times to detect growth were 9.8 days for HS-SH and 9.6 days for NALC-NaOH, while the median times were equal to each other at 8.0 days.

The previous study of criteria of sputum quality for diagnosing tuberculosis found that quality of the sputum specimen should have > 25 white blood cells (WBCs) per low power field (LPF) and < 25 squamous epithelial cells per LPF [14]. The positive results of AFB microscopy determined by all methods were significantly higher in qualified specimens than unqualified specimens (P<0.001) with positive rates increasing to 20-24%. The good specimens were also associated with positive mycobacteria cultures by LJ media and MGITs in which positive culture rate increased to 10-16%.

About 60% of sputum specimens collected from re-treatment, pre-treatment and new case-patients were positive by AFB smear and culture. However, only 30-35% was detected as positive by both methods in on-treatment cases (Table 3). In on-treatment patients, the number of positive direct smear and negative mycobacteria growth was about 43% while only less than 5% was found in the other patient groups.

The sensitivity, specificity, predictive values and accuracy of AFB microscopy and cultures were calculated by using total culture results as the reference. These values of the HS-SH method had a high correlation with the NALC-NaOH method when they were used in the concentration of sputum samples for AFB smear and cultivation (Table 4). The sensitivity, NPV and accuracy of AFB microscopy prepared by both concentration methods were increased when compared with the direct microscopic method. The specificity and PPV of both concentration methods were lower than in the direct microscopic method because there were on-treatment samples in this study. Some AFB-positive samples of the on-treatment group were non-viable bacilli because of treatment which yield no growth in culture. This discordance affected both concentration methods to yield the low specificity and PPV in AFB microscopy.

The efficiency of 1-month-old HS-SH solution was compared with a freshly prepared solution for digestion and concentration of 60 sputum samples (Table 5). Both decontamination methods yielded similar results for AFB microscopy (kappa index = 1.0). Growth of mycobacteria on LJ media and MGITs using fresh solution was slightly better than the one-month storage solution, but showed no significant difference (kappa = 0.9 and 0.78, respectively, with *P*>0.90).

In Thailand, the cost of homemade HS-SH solution was about US$ 0.01 per sputum sample and US$ 0.06 when using in-house NALC-NaOH solution and US$ 1.01 when using commercial NALC-NaOH solution.

Table 1. Results of AFB microscopy and culture by HS-SH and NALC-NaOH methods.

Results	AFB microscopy			*P*	LJ media		*P*	MGIT 960 media		*P*
	n (%)				*n* (%)			*n* (%)		
	Direct smear	HS-SH	NALC-NaOH		HS-SH	NALC-NaOH		HS-SH	NALC-NaOH	
Negative	168 (39.3)	136 (31.9)	137 (32.1)		193 (45.2)	181 (42.4)		144 (33.7)	135 (31.6)	
Positive	259 (60.7)	291 (68.1)	290 (67.9)	0.032	197 (46.1)	194 (45.4)	0.230	238 (55.7)	228 (53.4)	0.148
Contaminated	-	-	-		37 (8.7)	52 (12.2)		45 (10.6)	64 (15.0)	

Table 2. Comparison of AFB microscopy grades and different preparation methods in various patients.

Patients	Methods	AFB Microscopy grading				
		Negative	Scanty	1+	2+	3+
Re-treatment	Direct smear	51	15	10	9	35
n = 120	HS-SH	42	3	17	9	49
	NALC-NaOH	42	3	18	8	49
On-treatment	Direct smear	37	11	34	24	11
n = 117	HS-SH	24	2	19	23	49
	NALC-NaOH	24	3	19	24	47
Pre-treatment	Direct smear	30	9	11	11	22
n = 83	HS-SH	28	1	10	6	38
	NALC-NaOH	28	1	10	7	37
New case	Direct smear	50	8	8	16	25
n = 107	HS-SH	42	5	12	5	43
	NALC-NaOH	43	5	11	6	42

Table 3. Comparison of mycobacterial culture with the concentration methods according to patient groups.

Patients	Methods	AFB smear/Culture					
		+/+	+/-	-/+	-/-	+/c	-/c
Re-treatment	HS-SH	70	5	3	35	3	4
n = 120	NALC-NaOH	72	5	2	35	1	5
On-treatment	HS-SH	39	50	8	16	4	0
n = 117	NALC-NaOH	35	51	7	16	7	1
Pre-treatment	HS-SH	51	2	4	23	2	1
n = 83	NALC-NaOH	50	2	3	24	3	1
New case	HS-SH	63	2	3	37	0	2
n = 107	NALC-NaOH	62	2	5	35	0	3

+: Positive results, -: Negative results, c: Contaminated results

Table 4. Sensitivity, specificity, PPV, NPV and accuracy of AFB microscopy and culture by HS-SH and NALC-NaOH methods.

Results	AFB microscopy			Culture	
	Direct smear	HS-SH	NALC-NaOH	HS-SH	NALC-NaOH
Sensitivity (%)	84.3	91.3	90.9	96.3	96.3
Specificity (%)	73.4	65.6	65.6	100.0	100.0
PPV (%)	83.3	80.7	80.6	100.0	100.0
NPV (%)	74.8	82.8	82.1	94.5	94.5
Accuracy (%)	80.1	81.3	81.1	97.7	97.7

PPV: Positive predictive value, NPV: Negative predictive value

Table 5. Results of AFB microscopy and culture of fresh and 1-month-old HS-SH solutions.

	AFB microscopy (%)			LJ (%)		MGIT (%)	
	Direct smear	Fresh	1-month-old	Fresh	1-month-old	Fresh	1-month-old
Negative	30 (50.0)	26 (43.3)	26 (43.3)	38 (63.3)	40 (66.7)	32 (53.3)	33 (55.0)
Positive	30 (50.0)	34 (56.7)	34 (56.7)	19 (31.7)	17 (28.3)	26 (43.3)	24 (40.0)
Contaminated	-	-	-	3 (5.0)	3 (5.0)	2 (3.3)	3 (5.0)

3.2 Discussion

Sputum culture is commonly used for TB diagnosis and monitoring treatment in cases with drug resistance, and useful for epidemiological surveillance. This report included four patient groups for efficiency comparison between an HS-SH decontamination method and a conventional NALC-NaOH method. Both methods showed concordance results of AFB smear microscopy and culture on LJ and MGIT media. On-treatment cases are patients receiving anti-TB drugs so an appearance of smear-positive and culture-negative results is frequently encountered [15]. The AFB smear-positivity with culture-positivity was 43% of on-treatment patients in this study that was related to a finding after 2 months of treatment in 45% of patients [16] and after 5 months of therapy in 80% of cases [17].

AFB microscopy is a simple inexpensive and rapid method for diagnosis of tuberculosis with lower sensitivity than a culture. Concentrated sputum by HS-SH and NALC-NaOH methods resulted in higher positive rates of AFB microscopy by about 7% compared to direct smear. With an 8% increase in re-treatment, 11% in on-treatment, 2% in pre-treatment and 7% in new case groups, our results were similar to previous studies that had increased positive rates of 2-19% [6,7]. Qualified sputum specimens could influent high positive rates of AFB smear and mycobacterial culture as shown in this study and the other that used a criteria of $\geq$ 25 WBC/LPF [18].

Mycobacterial culture needs decontamination and concentration procedures before inoculating sputum samples in liquid and solid media. Our results show that MGIT 960 liquid media provided higher recovery rate and rapid growth detection than LJ media which was similar to other studies [6,19]. However, an international guideline recommends using both media for mycobacterial culture [20]. In this study, overall contamination rates of HS-SH on LJ and MGIT media were less than those of NALC-NaOH which resembled other observations [6,7]. Additionally, contaminated cultures from the continuous monitoring system were slightly higher than those using LJ media, which was similar to some studies [21,22] and dissimilar to others [6,23]. However, the MGIT system definitely facilitates faster mycobacteria detection [6,21-24]. This study also demonstrated that the better the quality of sputum samples collected, the lower the contamination rates found by MGIT system in both concentration methods. While quality of sputum had no association with contaminated cultures by LJ media. The contamination rates by HS-SH and NALC-NaOH methods were 9-11% and 12-15%, respectively, in our study which is slightly higher than recent studies that range from 4-13% [6,25-27].

4. Conclusion

The NALC-NaOH method is a recommended standard by international organizations for sputum decontamination of mycobacterial cultivation. An obvious disadvantage of the method is the expense and instability of the NALC solution which may hinder the progression of a TB control program in non-developed countries. This study supports the HS-SH method as an alternative decontamination and concentration method for mycobacterial culture and AFB smear.

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