

## Comparison of N-Acetyl-L-Cysteine-Sodium Hydroxide-Based and Modified Petroff's Decontamination Method for *Mycobacterium tuberculosis* Culture

ARIYANI KIRANASARI<sup>1\*</sup> AND MUHAMMAD RAYHAN<sup>2</sup>

<sup>1</sup>*Microbiology Department, Faculty of Medicine, Universitas Indonesia  
Jalan Pengangsaan Timur No.16, Jakarta Pusat, 10320, Indonesia;*

<sup>2</sup>*Medical Science, Faculty of Medicine, Universitas Indonesia.*

Indonesia is one of 22 countries with a high incidence of tuberculosis in the world, particularly related to TB-HIV and MDR-TB cases. Contamination of normal flora from nasopharyngeal tract is the main problem to isolate *Mycobacterium tuberculosis* (MTB) from sputum. Therefore, a safe solution to decontaminate sputum without killing MTB bacilli is needed. N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH)-based method and Petroff's methods which were modified with NaOH (4%) are widely used in laboratories. In the present study, we will evaluate the different of these methods. Of the 110 sputum samples were collected from suspected cases of Pulmonary TB, and the decontamination of sputum by these methods was performed, after acid-fast bacillus (AFB) smear, then the samples were cultured in Lowensteins Jensen slant medium. The positive culture was validated by chromatography test for detecting the antigen of MPT-64 and PNB. Based on the investigation, it has been shown that neither NALC-NaOH (71%) nor modified Petroff's methods (66%) had a significant effect on the positivity rate of AFB smear. However, the contamination on culture was significantly higher in samples treated with NALC-NaOH (21%) compared to Modified Petroff methods (13%) ( $p < 0.05$ ). In addition, the proportion of positive culture in NALC-NaOH was lower than Modified Petroff. In conclusion, our study proved that Modified Petroff methods are still more effective on sputum decontamination than NALC-NaOH based on the positivity rate of MTB culture. Though, not significantly different on AFB microscopic examination.

**Key words:** decontamination, Modified Petroff's methods, MTB, NALC-NaOH

Indonesia merupakan salah satu dari 22 negara dengan insiden TB tertinggi di dunia, khususnya yang berkaitan dengan kasus TB-HIV dan MDR-TB. Kontaminasi flora normal dari saluran nasofaring merupakan masalah utama dalam mengisolasi *Mycobacterium tuberculosis* (MTB) dari sputum. Oleh sebab itu diperlukan solusi yang aman untuk melakukan dekontaminasi sampel sputum tanpa membunuh bakteri MTB. Untuk memberikan hasil yang lebih baik dekontaminasi yang sering digunakan adalah metode berbasis N-asetil-L-sistein-natrium hidroksida (NALC-NaOH) dibandingkan dengan dari metode Petroff yang dimodifikasi dengan NaOH (4%). Sampel sputum yang digunakan untuk pengujian berasal dari 110 kasus suspek TB Paru dan dilakukan dekontaminasi menggunakan NALC-NaOH 2% atau (4% NaOH) yang sebelumnya sudah dilakukan pemeriksaan BTA. Selanjutnya, sputum tersebut dibiakkan pada media Lowensteins Jensen. Setiap kelompok dinilai berdasarkan tingkat kontaminasi dan hasil kultur positif. Kultur positif divalidasi menggunakan uji kromatografi untuk mendeteksi adanya antigen MPT-64 dan PNB. Hasil penelitian menunjukkan bahwa NALC-NaOH dan metode Petroff termodifikasi tidak berpengaruh nyata terhadap laju kepositifan BTA, dengan nilai masing-masing sebesar 71% dan 66%. Kontaminasi pada kultur secara signifikan ( $p=0,034$ ) lebih tinggi pada sampel yang diberi NALC-NaOH (21%) dibandingkan dengan metode Modified Petroff (13%). Proporsi kultur positif pada sampel yang diberi perlakuan NALC-NaOH memberikan hasil lebih rendah dari Modified Petroff, masing-masing 65% dan 70% dengan nilai  $p=1$ . Kesimpulan dari penelitian ini menunjukkan bahwa dekontaminasi sputum dengan metode Modified Petroff masih lebih efektif dibandingkan dengan NALC-NaOH untuk meningkatkan laju kepositifan kultur MTB. Namun kedua metode tersebut tidak berbeda nyata untuk mendapatkan hasil positif pada pemeriksaan mikroskopis BTA.

**Kata kunci:** dekontaminasi, metode *Modified Petroff's*, MTB, NALC-NaOH

Tuberculosis (TB) is one of the deadliest infectious disease which caused by *Mycobacterium tuberculosis*. TB is a major air-borne disease in human. It remains a major worldwide health problem with global mortality ranging from 1.6 to 2.2 million lives per year (WHO 2016). Direct smear microscopy for acid fast bacilli

(AFB) is rapid, inexpensive, highly specific, and capable of identifying the most infectious cases of TB. The only disadvantage of this method is low sensitivity (varying from 50 to 80%) relative to culture (Forbes *et al.* 2010).

The Gold standard for diagnosing pulmonary TB remains in culturing. Decontamination of clinical specimens such as sputum is an important and critical step in the isolation of mycobacteria, to obtain better

\*Corresponding author: Phone: +62-21-3160491; E-mail: [gahariyani@gmail.com](mailto:gahariyani@gmail.com)

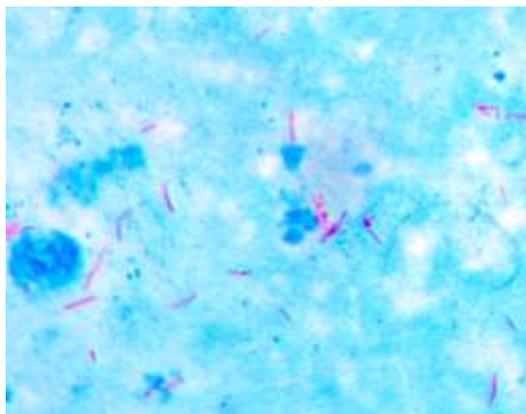


Fig 1 Microscopic examination of acid-fast bacilli with Ziehl Neelsen staining.

results. However, bacterial and fungal contamination of the culture frequently may interfere with the interpretation data result. This condition can result the need to repeat the culture and lead to reduce the effectiveness of culture as a method of diagnosis of tuberculosis, because it will take more time for a colony to appear (Bruchfeld *et al.* 2000; Sjahrurachman *et al.* 2012; Korean CDC 2014; Gopi *et al.* 2018). Contamination of culture and its harmful effect can be prevented by decontamination of sputum sample. Decontamination and concentration of sputum samples by Modified Petroff's method is one of the most commonly used methods for *M. tuberculosis* culture. However, N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) solution may recommended as a gentle but effective digesting and decontaminating agent. Addition of a large volume of phosphate buffer (PB) with pH 6.8 makes strong shift in pH, washes the specimen, dilutes toxic substances and decreases the specific gravity of the specimen so that centrifugation is more effective. Therefore, it is generally accepted that the NALC-NaOH method (Kent and Kubica 1985) should be given preference over the modified Petroff's methods. But, the technical and procedural factors may influence the sensitivity of each method (Peres *et al.* 2009). Studies in different laboratory may give different results since the result of decontamination may be influenced by various technical factors (Forbes *et al.* 2010; Peres *et al.* 2009; Sjahrurachman *et al.* 2012). The aim of this study is to compare the NACL-NaOH-based and Modified Petroff methods with 4% NaOH to obtain better result on microscopic examination of AFB and MTB culture.

## MATERIALS AND METHODS

**Sputum Samples.** The cross-sectional study was

conducted in the tuberculosis laboratory, Microbiology Departement, Faculty of Medicine, Universitas Indonesia, from December 2016 to July 2017. One hundred ten sputum samples were isolated from suspected case of pulmonary tuberculosis. The samples then be decontaminated with NALC-NaOH-based method or Modified Petroff's methods.

**Microscopic Examination.** Microscopic examination was performed before and after decontamination. AFB smear was conducted using Ziehl-Neelsen technique recommended by World Health Organization (WHO 2016; Tripathi *et al.* 2014; Burdz *et al.* 2003). Specimens for digestion and decontamination were mixed well using vortex and equally divided into two parts, and each treated by NALC-NaOH and modified Petroff's method before inoculated it directly on Lowenstein-Jensen (LJ) medium (Peres *et al.* 2009; Tripathi *et al.* 2014).

**NALC-NaOH-based Method.** NALC-2% sodium hydroxide-sodium citrate solution was prepared as described by Kent and Kubica (Tripathi *et al.* 2014). An equal volume of NALC-NaOH citrate reagent was added into 3-5 mL of seeded sputum sample, and vortexed briefly for 30 sec. The samples then were incubated for 15 min at room temperature and added by 0.067 M of phosphate buffer (pH 6.8) until the volume reach 50 mL. After homogenization, the sputum samples were pelleted by centrifugate it at 3,000g for 15-20 min. The supernatant was discarded, and the pellet was resuspended with 1 mL of PBS. The smear was made and a 0.5 mL of cell suspension was inoculated on LJ slopes. The culture slants were incubated at 35-37°C.

**Modified Petroff's Method.** In brief, 3-5 mL of sputum was homogenized for 15 min in a shaker using an equal volume of 4% NaOH. After centrifugation at 3.000g for 15-20 min, the seeds were neutralized with

20 mL of sterile distilled water or aqua pro injection. The samples were again centrifuged at 3.000g for 15-20 min. From the sediment, LJ medium was inoculated and smear was made. The culture slants were incubated at 35-37 °C (Pathak *et al.* 1973; Tripathi *et al.* 2014; Costa *et al.* 2018). All slopes were observed daily for first week and weekly for 8 weeks. Some parameters such as, growth rate, optimum temperature, colony morphology and pigmentation were also observed as growth parameters. The chromatography test was using to detect the antigen of MPT-64 and PNB tests. The absence of growth at the end of 8<sup>th</sup> weeks was regarded as negative culture. Contamination, if any, was recorded separately. The number of culture failures for a certain decontamination method, included the number of specimens with negative culture as well as number of contaminated cultures. The data acquired from the study is processed using SPSS software version 20. Bivariate analysis was done using Chi-square test.

## RESULT

Based on direct microscopy, out of 110 samples, 88 (80%) were smear positive and 22 (20%) were negative. The smear was carried out again after decontamination, and the result showed that 71 samples (71%) were positive by NALC-NaOH and 73 samples (66%) were positive by modified Petroff's methods (Table 1).

The total number of culture failures (which includes both contamination and negative cultures) were 33 (30%) in NALC-NaOH as against 38 (35%) in modified Petroff's methods (Table 2). The contamination rate was higher NALC-NaOH 23 (21%), whereas it was lowest in modified Petroff's methods 14 (13%) (Table 2).

A significant difference ( $p < 0.05$ ) in the proportion of contaminated and uncontaminated culture was shown between those treated with NALC-NaOH and Modified Petroff. Higher proportion of contaminated culture is observed from group treated with NALC-NaOH in comparison to Modified Petroff (Table 3). However, the proportions of uncontaminated culture with positive and negative results show no significant difference between group treated with NALC-NaOH and Modified Petroff (Table 4).

## DISCUSSION

Sputum culture method is an important tool for TB

control programs—because it is more sensitive than smear microscopy in diagnosing TB. The culture also facilitates the drug susceptibility testing, but since sputum samples pass through the oropharynx tract during collection, culture contamination limits the diagnostic yield of sputum culture for TB (WHO 2016; Forbes *et al.* 2010).

Decontamination process for removing bacteria and yeast in order to isolate *Mycobacteria* in the sputum, unfortunately may also kill *Mycobacteria*. The percentage of killed organisms will vary according to the method used and also the population of mycobacteria in the specimen. In this study the use of NALC-NaOH for sputum digestion, as in Kent and Kubita method, will have final concentration about 2% NaOH. Such concentration will only give less destructive to *Mycobacteria* compare to the used of 4% NaOH in Petroff's method (GLI 2014; Tripathi *et al.* 2014; Burdz *et al.* 2003).

In the analysis of 110 samples, the decontaminated by the NALC-NaOH method provided isolation of *M. tuberculosis* in a lowest percentage (65%) compared to modified Petroff's method (70%). This is in accordance with the research of Sharma *et al.* in 2012 showed that the proportion of positive culture results was greater in samples processed with NALC-NaOH 63.7% than samples processed with Modified Petroff 46.7%. Meanwhile, from the research of Chatterjee *et al.* (2013) showed that the proportion of positive culture results was greater either in samples processed with NALC-NaOH was 62.7% than samples processed with Modified Petroff 58.5% (Chaudhary *et al.* 2013). Meanwhile, the research results of Pathak SK *et al.* (1973) showed that the proportion of positive culture results was 79.4% for NALC-NaOH. This figure is much greater than the proportion of positive culture results for NALC-NaOH which was shown well by the study of Sharma *et al.* 2012 and Chatterjee *et al.* 2013.

The probable reasons maybe are, 4% NaOH is used for modified Petroff's method as compared NALC-NaOH method which uses 2% NaOH while concentrating the sputum may kill or seriously injure few *Mycobacteria* (Korean CDC 2014). Hence, recovery by NALC-NaOH was faster and better than modified Petroff's method. In our study we found smear positivity higher than the culture positivity. The reason might be that microscopy sometimes gives false positive results and in our condition, it cannot distinguish between dead and live bacteria. In such cases, the patients might be treated with antitubercular drugs and in the microscopy of these samples, the AFB

Table 1 Percentage from microscopy using Ziehl Neelsen Staining

Smear	No. positive microscopy	No. negative microscopy
Direct	88 (80%)	22 (20%)
NALC- NaOH	78 (71%)	32 (29%)
Modified Petroff's	73 (66%)	37 (34%)

Table 2 Comparison of the NALC-NaOH and Modified Petroff's methods (in regards rate of contamination, negative cultures and culture failures)

Methods	No of Contaminated Slopes	Negative cultures (No. of slopes with no growth up-to 8 weeks)	Total culture failures/ negative
NALC- NaOH	23	10	33
Modified Petroff's	14	24	38

Table 3 Chi-square test result showing a significantly higher proportion of contaminated culture in group treated with NALC-NaOH compared to the treated with Modified Petroff's

Decontamination Method	Contamination n (%)	Uncontaminated n (%)	P*
NALC- NaOH	23 (41.8%)	32 (58.2%)	0.034
Modified Petroff's	14 (25.5%)	41 (74.5%)	

\*statistically significant for  $p < 0.05$

Table 4 Chi-square test result showing no statistically significant difference in the proportion of positive and negative results in uncontaminated culture previously treated using NALC-NaOH and Modified Petroff

Decontamination Method	Negative culture n (%)	Positive culture n (%)	P*
NALC- NaOH	38 (34.5%)	72 (65%)	1,000
Modified Petroff's	33 (30%)	77 (70%)	

\*statistically significant for  $p < 0.05$

might be dead. For these reason, the dead isolates did not grow in the L-J culture media. This also reveals that AFB microscopy does not always give accurate results for the diagnosis of TB (Sharma *et al.* 2012; GLI 2014).

The contamination rate by NALC-NaOH method was 21% in our study which is higher than that reported by other workers. Sharma *et al.* (2012) reported 13.2% while Chatterjee *et al.* (2013) reported 4.98%. Several studies show the contamination rate by modified Petroff's was 13% in our study which is lower than that reported by other workers. Sharma *et al.* 2012 reported 23.1% while Chaudary *et al.* in 2013 reported 8% and TripathiK *et al.* in 2014 12%. Our study showed, first the NALC-NaOH method for AFB smear and culture improves the sensitivity when compared to modified Petroff's method. Second, Modified Petroff method has lower contamination rate than NALC-NaOH, and culture using LJ medium NALC-NaOH method is not suitable for routine use. Third, the modified Petroff's method for AFB smear and culture improves the sensitivity when compared with the NALC-NaOH method.

In conclusion, the Modified Petroff methods is more effective than NALC-NaOH to prevent

contamination from highly contaminated sputum. However, the positivity result of microscopic examination of AFB was not significantly different between this two methods.

## REFERENCES

- Bruchfeld J, Aderaye G, Palme IB, Bjorvatn B, Källenius G, Lindquist L. 2000. Sputum concentration improves diagnosis of tuberculosis in a setting with a high prevalence of HIV. *Trans R Soc Trop Med Hyg.* 94: 677-80. doi: 10.1016/s0035-9203(00)90230-x.
- Burdz TVN, Wolfe J, Kabani A. 2003. Evaluation of sputum decontamination methods for *Mycobacterium tuberculosis* using viable colony counts and flow cytometry. *Diagn Microbiol Infect Dis.* 47: 503 - 9. doi: 10.1016/s0732-8893(03)00138-x.
- Costa RR, Silva SF, Fochat RC, Macedo RL, Pereira TV, Silva MR, Pinto CP, Leite IC. 2018. Comparison between Ogawa-Kudoh and modified Petroff techniques for mycobacteria cultivation in the diagnosis of pulmonary tuberculosis. *Einstein. São Paulo.* 16(2):1-5. doi: 10.1590/S1679-45082018AO4214.

- Chatterjee M, Bhattacharya S, Karak K, Dastidar SG. 2013. Effects of different methods of decontamination for successful cultivation of *Mycobacterium tuberculosis*. Indian J Med Res. 138(4): 541 - 8.
- Chaudhary SK, Mishra B. 2013. Comparison of hypertonic saline-sodium hydroxide method with modified Petroff's method for the decontamination and concentration of sputum samples. Int J Infect Microbiol. 2(3): 78 - 81. doi: 10.3126/ijim.v2i3.8664.
- Forbes BA, Sahm DF, Weissfeld DS. 2010. Infection of lower respiratory tract: Bailey and Scott's Diagnostic Microbiology. Mosby Elsevier. USA. 12:805-6.
- Gopi A, Samreen F, Madhulata C. 2018. A comparative study between microscopy and culture in detection of M.tb among smear negative pulmonary and extra pulmonary tuberculosis. Indian Journal of Microbiology Research, July-September, 5(3):313-317 doi:10.18231/2394-5478.2018.0066.
- GLI, Mycobacteriology Laboratory Manual. 2014.
- Kent PT, Kubica GP. 1985. Public health microbiology: A guide for the level III laboratory. Centers for Disease Control, Atlanta.
- Korea Centers for Disease Control and Prevention. 2014. Korean guidelines for tuberculosis. Joint Committee for the Revision of Korean Guidelines for Tuberculosis. Seoul and Cheongwon. : p. 2. doi: 10.4046/trd.2016.79.1.1
- Pathak SK, Deshmukh PA, Menon CRN. 1973. A comparison of different culture techniques. Ind J Tuberc. 20: 85.
- Peres RL, Maciel EL, Morais CG, Ribeiro FCK, Vinhas SA. 2009. Comparison of two concentrations of NALC-NaOH for decontamination of sputum for mycobacterial culture. Int J Tuberc Lung Dis. 13(12): 1572 - 5.
- Sharma M, Misra RN, Gandham NR, Jadhav SV, Angadi K, Wilson V. 2012. Comparison of modified Petroff's and N-Acetyl-L-cysteine-sodium hydroxide methods for sputum decontamination in tertiary care hospital in India. Med J Dr. D. Y. Patil University. 5(2): 97 - 100. doi: 10.4103/0975-2870.103323.
- Sjahrurachman A, Rintiswati N, Gartinah T, Solihin I, Woro E, Panjaitan R, et al. 2012. Petunjuk teknis pemeriksaan biakan, identifikasi, dan uji kepekaan *Mycobacterium tuberculosis* pada media padat. Jakarta: Kementerian Kesehatan RI; 60 - 73.
- Tripathi K, Tripathi PC, Nema S, Shrivastava AK, Dwidewi K, Dhanvijay AK, et al. 2014. Modified Petroff's method: an excellent simplified decontamination technique in comparison with Petroff's method. Int J Recent Trends in Science and Technology. 10(3): 461 - 64.
- World Health Organization. Global tuberculosis report. 2016. Geneva: World Health Organization. P. 24.