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Evaluation of the Use of Distilled Water as a Sodium Hydroxide Wash in Antimicrobial Cultures

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Abstract

Background: Respiratory specimens subjected to mycobacterial detection were initially pretreated with N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) to remove the mucus and normal flora. Next, they were washed and neutralized with a phosphate-buffered solution (PBS). The effectiveness of distilled water (DW) compared to PBS as a washing neutralizer during the identification of mycobacteria was evaluated in this study. *Methods:* We analyzed the results of the mycobacterial test conducted at a general hospital in Gwangju from October 2016 to September 2018. PBS and DW were used as a respiratory sample-washing agent for one year each. *Results:* The positive culture rate for the culture of mycobacteria was 12.7% (1,843/14,532) and 14.7% (2,095/14,291), when PBS and DW were used, respectively. The recovery rate of the mycobacteria growth indicator tubes (MGIT) and the separation rates of the *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria (NTM) showed no significant change. However, in the 2% Ogawa medium, as the NTM culture increased from 47.4% (399/841) to 56.1% (630/1,122), the recovery rate increased from 45.6% (841/1,843) to 53.6% (1,122/2,095). The MGIT contamination rate decreased from 6.5% to 4.1%. *Conclusion:* DW as a washing agent for NALC-NaOH increased the recovery rate of Ogawa medium and reduced the contamination rate of MGIT. Therefore, the use of DW instead of PBS as a washing neutralizer during the identification of mycobacteria might be useful.

Keywords

Mycobacterial culture
Mycobacterium spp.
Nontuberculous mycobacteria
Sodium hydroxide
Sputum

1. Introduction

Antimycobacterial culture tests for the diagnosis of non-tuberculous mycobacterial (NTM) infections, including *Mycobacterium tuberculosis* complex (MTBC), suffer from a significant time-consuming difficulty due to their slow growth rate compared to normal bacteria [1]. For this reason, the method that relies only on solid culture has been replaced by simultaneous liquid culture, which shortens the time required for culture testing and increases the detection rate [2]. In recent years, the incidence of tuberculosis has decreased and the isolation rate of NTM from clinical specimens has increased [3], and more than 90% of the diseases caused by NTM are lung diseases [4,5], so there is a need for further research on sample preparation methods and culture methods to improve the detection rate of antimicrobials in respiratory specimens. Due to the potential contamination of sputum with mucus and commensals, N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH), which is used as a pretreatment agent to dissolve mucus and remove contaminating bacteria, can be used to increase detection rates [6]. During this process, the presence of lipids in the *M. tuberculosis* cell wall provides resistance to acidic and alkaline environments [7], making *M. tuberculosis* resistant and allowing for the removal of contaminating bacteria. Liquid cultures are more culturable than solid cultures but require the washing of alkalized specimens with distilled water (DW) or phosphate-buffered solution (PBS, pH 6.8) before media inoculation [8-11]. In previous studies [5-8], PBS was used to neutralize the wash and then incubated, while in some studies DW was used to neutralize the wash and then incubated in PBS [2]. However, although studies have compared antimicrobial detection rates and liquid culture mycobacterium growth indicator tube (MGIT; Becton Dickinson, Franklin Lakes, NJ, USA) contamination rates according to pretreatment methods [2,8], no comparisons of antimicrobial detection rates or contamination rates according to wash neutralization methods of pretreatment agents have been reported.

In this study, we analyzed the results of antimicrobial cultures and polymerase chain reaction (PCR) tests performed after wash neutralization with PBS from October 2016 to September 2017 and after wash with DW and without neutralization from October 2017 to September 2018 to determine the usefulness of using DW instead.

2. Materials and methods

2.1. Subjects

We analyzed 28,823 clinical specimens referred for antimicrobial testing to the Microbiology Laboratory of Gwangju Christian Hospital during the 2-year period from 1 October 2016 to 30 September 2018, of which 6,211 cases were referred for antimicrobial culture and PCR testing. Of the 14,532 cases from 1 October 2016 to 30 September 2017, respiratory specimens (sputum, bronchial lavage fluid) were washed and neutralized with PBS after pretreatment, and 14,291 cases from 1 October 2017 to 30 September 2018 were washed with sterile distilled water for perfusion (JW pharmaceutical, Seoul, Korea). A retrospective investigation was performed on the results of antibacterial cultures and PCR tests using both liquid and solid cultures. This study was approved by the Institutional Review Board of Gwangju Christian Hospital (IRB No. KCHIRB-RE-2020-006).

2.2. Reagents

A solution of 6.0% NaOH (Ducksan pure chemical, Ansan, Korea) and 2.9% sodium citrate tribasic dehydrate (Ducksan pure chemical) was prepared by autoclaving at 121°C, 15 lb psi for 15 min, and then the NaOH concentration was adjusted to 3% by vortex mixing, and NALC (Sigma-Aldrich, Darmstadt, Germany) was added to a concentration of 0.5% by mixing well to dissolve completely. PBS (pH 6.8) was prepared by dissolving 4.74 g sodium phosphate dibasic anhydrous (Na_2HPO_4) (Ducksan pure chemical) and 4.54 g potassium phosphate monobasic

(KH_2PO_4) (Ducksan pure chemical) in 1 L of DW and autoclaving.

2.3. Respiratory specimen pretreatment

A 50 mL screw cap centrifuge tube containing respiratory specimens including sputum was treated with 2–3 times the volume of the specimen with NALC-3% NaOH and vortexed vigorously 3–4 times at 5 min intervals for 20 min at room temperature. After sputum digestion, PBS or sterile distilled water was mixed up to 50 mL for washing and neutralization and then centrifuged at 4°C, 3,600 rpm for 20 minutes using a Continent 512R (Hanil, Gimpo, Korea) to remove the supernatant, which was used for antibacterial staining, and the precipitate was suspended in 5 mL of PBS or DW and used for culture.

2.4. Antibacterial culture test

After the pretreatment, the sediment samples suspended in PBS or DW sterile were allowed to stand for 5 min to allow debris such as epithelial cells to settle, and then 100 μL were inoculated into 2% Ogawa medium (Union Lab, Seoul, Korea), a solid medium, and placed in a 37°C incubator for 1 day to dry the moisture on the surface of the medium, and then incubated upright for 8 weeks to check for growth. Liquid culture was performed by inoculating 0.5 mL of the sample in 800 μL aliquots of well-mixed growth supplement (Becton Dickinson, Franklin Lakes, NJ, USA) and PANTA (Becton Dickinson, Franklin Lakes, NJ, USA) on MGIT before testing. The MGIT was mounted on a BACTEC MGIT 960 system (Becton Dickinson, Franklin Lakes, NY, USA) and incubated for up to 6 weeks to determine the presence of bacterial growth by measuring fluorescence, which is activated as bacterial growth consumes oxygen.

2.5. Treatment of MGIT liquid culture-positive specimens

After mixing the MGIT with a positive signal on the BACTEC MGIT 960 system, 1 mL was taken after centrifugation, and slides were prepared to check the presence of antioxidant bacteria by fluorescent staining (auramine-rhodamine), followed by PCR.

2.6. Calculation of respiratory specimen contamination rate

There were 14,023 respiratory samples in PBS and 13,794 respiratory samples in DW, and the contamination rate was calculated when the MGIT960 system gave a positive signal but no actual antibacterial bacteria were detected,

2.7. Detection of MTBC and NTM by PCR

The specimens were pretreated in the same way as the culture and then subjected to the SLAN-96P Real-Time PCR System (LG chem, Seoul, Korea) using the test kit AdvanSure TB/NTM real-time PCR Kit (LG chem, Seoul, Korea) according to the manufacturer's instructions.

2.8. pH measurement

The pH of pretreatment solution NALC-3% NaOH and PBS, samples after pretreatment, and MGIT after inoculation were measured using an HM Digital PH-200 pH meter (HM Digital, Inc. Culver City, CA, USA).

2.9. Statistical analysis

Differences in test results between PBS and DW were tested for significance using the χ^2 test using the statistical program Medcalc (version 19.5.3, MedCalc Software Ltd., Ostend, Belgium). Significant differences were interpreted as $P < 0.05$.

3. Results

3.1. Antimicrobial culture results and contamination rates

Table 1 showed that the positive rate of antibacterial

culture was 12.7% (1,843/14,532) when PBS was used as a washing neutralizer for the pretreatment digestate and 14.7% (2,095/14,291) when DW was used, which was significantly different ($P < 0.01$). The contamination rate was 6.5% (907/14,023) with PBS, while it decreased to 4.1% (563/13,794) with DW ($P < 0.01$).

3.2. Results of solid and liquid cultures

After using DW instead of PBS, the positive rate of solid culture increased from 45.6% (841/1,843) to 53.6% (1,122/2,095) ($P < 0.01$, **Table 2**), and the culture performance of NTM improved from 47.4% (399/841) to 56.2% (630/1,122) ($P < 0.01$; **Figure 1**). The positive rate of liquid-cultured MGIT as a percentage of the overall positive rate was 98.1% and 98.0% in PBS and DW, respectively, with no significant difference (**Table 2**).

3.3. MTBC and NTM detection ratio

The MTBC and NTM detection rates were 37.1% (684/1,843) and 62.9% (1,159/1,843) when PBS was used (October 2016 to September 2017) and 36.0% (754/2,095) and 64.0% (1,341/2,095) when DW was used (October 2017 to September 2018), respectively, with no significant difference. MTBC continues to show a downward trend since before this study period (**Figure 2**). The isolation rates by media were 37.7% (681/1,808) and 62.3% (1,127/1,808) for MTBC and NTM in MGIT and 36.6% (751/2,054) for MTBC and 63.4% (1,303/2,054) for NTM in DW. The overall positivity rate of Ogawa cultures increased as the number of NTM isolates increased ($P < 0.01$), from 52.6% (442/841) and 47.4% (399/841) in Ogawa media to 43.9% (492/1,122) and 56.1% (630/1,122) in the DW period.

Table 1. Mycobacteria culture results of specimen processed by PBS and DW

Washing solution	No. of specimen	No. (%) of positive culture for			No. (%) of contamination*
		All	MTBC	NTM	
PBS	14,532	1,843 (12.7)	684 (4.7)	1,159 (8.0)	907/14,023 (6.5)
DW	14,291	2,095 (14.7)	754 (5.3)	1,341 (9.4)	563/13,794 (4.1)

*Respiratory specimen. Abbreviations: MTBC, *Mycobacterium tuberculosis* complex; NTM, nontuberculous mycobacteria; PBS, phosphate buffered solution; DW, distilled water.

Table 2. Comparison of MGIT 960 system and Ogawa medium results by PBS and DW

Washing solution	No. (%) of positive culture for				
	Total	MGIT	MGIT only	Ogawa	Ogawa only
PBS	1,843 (100)	1,808 (98.1)	1,002 (54.4)	841 (45.6)	35 (1.9)
DW	2,095 (100)	2,054 (98.0)	973 (46.4)	1,122 (53.6)	41 (2.0)

Abbreviations: MGIT, mycobacterium growth indicator tube; PBS, phosphate buffered solution; DW, distilled water.

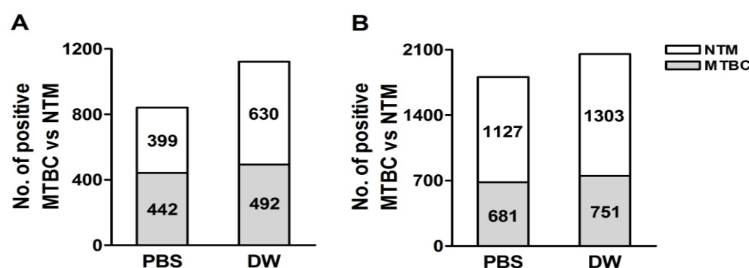

Figure 1. The number of *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacterium (NTM) cultured in Ogawa (A) and MGIT (B) medium. Abbreviation: MGIT, mycobacterium growth indicator tube; PBS, phosphate buffered solution; DW, distilled water.

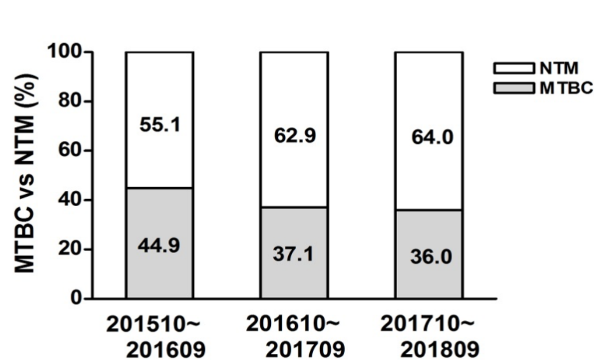

Figure 2. Annual change in the proportion of *Mycobacterium tuberculosis* complex (MTBC) vs nontuberculous mycobacteria (NTM) isolated in patients.

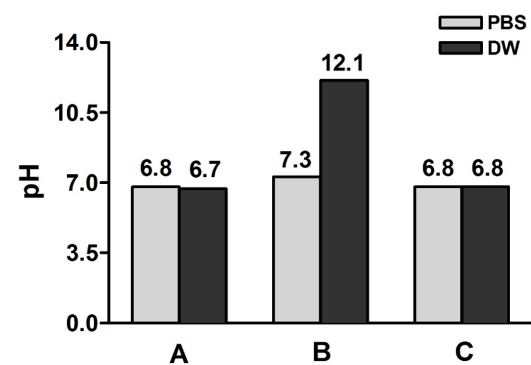

Figure 3. Changes of pH after washing pretreatment agent with PBS or DW and after inoculation of the specimen in MGIT. (A) pH of PBS and DW, (B) pH of the specimen after pretreatment and washing, and (C) pH of the MGIT after inoculation of the specimen. Abbreviation: MGIT, mycobacterium growth indicator tube; PBS, phosphate buffered solution; DW, distilled water.

Table 3. PCR results of specimen processed by PBS and DW

Washing solution	No. of specimen	No. (%) of positive PCR for		
		MTBC	NTM	Mycobacteria
PBS	3,080	130 (4.2)	38 (1.2)	168 (5.4)
DW	3,131	162 (5.2)	60 (1.9)	222 (7.1)

Abbreviations: PCR, polymerase chain reaction; MTBC, *Mycobacterium tuberculosis* complex; NTM, nontuberculous mycobacteria; PBS, phosphate buffered solution; DW, distilled water.

3.4. PCR test results

Table 3 showed the PCR positivity rate increased from 5.4% (168/3,080) in PBS to 7.1% (222/3,131) in DW ($P < 0.01$). MTBC and NTM were identified in 4.2% (130/3,080) and 1.2% (38/3,080) of PBS and 5.2% (162/3,131) and 1.9% (60/3,131) of DW.

3.5. pH change between PBS and DW

After washing and neutralizing with PBS, the pH of the samples averaged 7.3 and after inoculation with MGIT, the pH was 6.8. Samples washed with DW had an average pH of 12.1 and a pH of 6.8 after inoculation with MGIT (**Figure 3**).

4. Discussion

In this study, the results of mycobacteria testing over a two-year period were analyzed to determine the usefulness of using DW instead of PBS as a cleaning agent for respiratory specimen preparation. Although it is appropriate to use the same specimen over the same period of time to provide an accurate comparison of the use of the two cleaning agents, due to the nature of sputum, it is not easy to divide the specimen exactly in half, so the results of one year of PBS and one year of DW were analyzed, and direct comparison of results is limited. Pretreatment of respiratory specimens is very important to increase the detection rate of mycobacteria by dissolving the mucus and collecting antioxidant bacteria in between [6]. In this process, the use of DW instead of PBS, which was used as a washing agent for the pretreatment agent NALC-3% NaOH, simplifies the preparation process of reagents for testing, saving time and effort for testing. The results analyzed in this study showed that the positive rate of antioxidants increased from 12.7% (1,843/14,532) to 14.7% (2,095/14,291) when DW was used instead of PBS, while the contamination rate decreased from 6.5% (907/14,023)

to 4.1% (563/13,794). There are reports that the concentration of the pretreatment NALC-NaOH affects the contamination rate in antimicrobial cultures, with a decrease in the contamination rate and an increase in the detection rate when the NaOH concentration was increased to 6% [8], and a study using 1.5% NaOH reported a contamination rate of 13.4% [9]. However, in this study, we found that using DW as a cleaning agent while keeping the NaOH concentration at 3% reduced the contamination rate and increased the positivity rate. However, because we did not have accurate information on the prevalence of patients referred for testing when DW was used, we cannot conclusively attribute the increased detection rate to an increase in culture positivity, but we do know that the use of DW did not affect antimicrobial cultures.

The liquid medium MGIT contains 7 mL of a modified Middle brook 7H9 broth base, and a large amount of dissolved oxygen is present in the liquid medium, which inhibits fluorescence, but when antimicrobial bacteria or other bacteria grow and consume oxygen, they exhibit orange fluorescence under 365-nm UV light, which is detected by a fluorescence detector, and antimicrobial bacteria including *M. tuberculosis* can be detected [8]. At this time, if the contaminating bacteria grows first, the MGIT is removed from the device and treated as contamination during the positive confirmation process, and even if antibacterial bacteria are present, they cannot be detected. In this study, samples pretreated with NALC-3% NaOH were washed with DW and resuspended to maintain an alkaline condition (pH 12.1) until MGIT inoculation, which is thought to have less effect on the survival of antioxidant bacteria due to their lipid cell wall characteristics [2], while more resident contaminants were killed and removed. After inoculation, the disodium phosphate and monopotassium phosphate in MGIT acted as buffers to maintain the proper pH for the culture, so the culture environment was not affected (**Figure 3**). However, the MGIT medium requires careful cleaning due to the possibility of false positives if the NaOH concentration is high, and the detection rate may be affected if the sample is left in a strong alkaline state for a long time, so in this study, the centrifuged samples were inoculated into MGIT without delaying for more than 5 minutes after suspension in DW; however, no experiments were conducted on the contamination rate and positive rate changes according to the delay time after suspension in DW.

In domestic reports, the contamination rate of MGIT varies depending on the concentration of NaOH and pretreatment conditions, and contamination rates of 5%–10% or more than 10% have been reported [10,12]. However, the TB guidelines issued by the National Institutes of Health of the Korea Centers for Disease Control and Prevention suggest that contamination by normal commensal bacteria usually occurs at 5%–10%, and if the contamination rate is lower than 5%, the antibacterial bacteria themselves may be affected, and if the contamination rate exceeds 10% for a period of time, it is necessary to check the decontamination process. In this study, the MGIT contamination rate was 6.5% when PBS was used, but it was reduced to 4.1% when sterile DW was used, indicating that the contamination of commensal bacteria was further eliminated. It should be noted that it was not possible to calculate the solid culture contamination rate due to the lack of Ogawa media culture contamination records during this study.

The increase in solid media culture positivity as a percentage of total culture positivity from 45.6% to 53.6% when DW was used indicates an improvement in solid media culture positivity regardless of the prevalence of patients referred for specimen culture. However, due to the acidity of Ogawa's medium, by washing with DW and inoculating the sample in an alkaline condition, neutralization occurs in the medium, which improves the culture environment and leads to an increase in the positive rate, suggesting that the use of DW may be more suitable. Increased positivity in solid media has the advantage of replacing

the need for additional tests, such as antituberculosis drug susceptibility testing, when liquid media is contaminated. In addition, quantitative tests required to evaluate the treatment progress of TB patients and follow-up of lung diseases caused by NTM are possible, and the morphology of colonies can be directly observed, and antimycobacterial bacteria that grow only in solid media can be detected, solving the problem that not all antimycobacterial bacteria can be detected by liquid culture alone [12,13]. On the other hand, MGIT liquid culture did not affect the performance of liquid culture, unlike solid culture, as the overall positive rate was 98.1% when using PBS and 98.0% when using DW. As for the cultivation rate of NTM, it was reported that MGIT liquid culture had a significantly higher NTM cultivation rate than solid culture [12], but the use of DW resulted in an increase in NTM from 47.4% to 56.2% without decreasing the isolation of MTBC in Ogawa media (**Figure 1**). However, in this study, even after using DW, the culture performance of Ogawa media was still 53.6% of the total positive rate (**Table 2**), which is lower than other reports of more than 60% and 80% [8,13,14], so further studies are needed to increase the positive rate of solid culture.

The PCR test positivity rate increased from 5.4% (168/3,080) to 7.1% (222/3,131) when DW was used (**Table 3**). The increase in the positive rate of the PCR test, which is thought to be less affected by pH than culture, cannot exclude the possibility that the positive rate increased when DW was used, but the number of patients referred for PCR testing was only 22% of those referred for culture, and the detection rates of MTBC and NTM were different from those of culture, so it is not possible to correlate the increase in the positive rate with the actual increase in prevalence. However, the recent increase in NTM has not reduced the overall antimicrobial culture positivity rate, so continued surveillance and detailed analysis are needed. In addition, although not shown here, antibacterial staining results also increased from 7.2% (1,311/18,189) to 9.1% (1,577/17,371) during the same period, indicating that DW use did not affect antibacterial detection in antibacterial staining tests.

In conclusion, the use of DW instead of PBS as a washing neutralizer for respiratory specimen pretreatment reduces the contamination rate of MGIT liquid culture and does not affect the detection of antimicrobials in culture, PCR, and antimicrobial staining, and increases the culture rate of antimicrobials, especially in solid media, so it is useful to use DW as a washing agent for pretreatment.

Disclosure statement

The authors declare no conflict of interest.

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References

[1] Hwang SS, Oh KJ, Jang IH, et al., 2011, Evaluation of the Diagnostic Performance of the AdvanSure TB/NTM Real-Time PCR Kit for Detection of Mycobacteria. Korean J Clin Microbiol, 14: 55–59.

[2] Kim YS, Jo YH, Lee HJ, et al., 2001, Comparison of the MGIT (Mycobacteria Growth Indicator Tube) with Ogawa Media for Recovery of Mycobacteria. Korean J Clin Microbiol, 4: 58–61.

[3] Go UY, Park MS, Kim UN, et al., 2018, Tuberculosis Prevention and Care in Korea: Evolution of Policy and Practice. J Clin Tuberc Other Mycobact Dis, 11: 28–36.

[4] Schlossberg D. Tuberculosis and nontuberculous mycobacterial infections. 6th ed. 2011, ASM Press, Washington, DC.

[5] Griffith DE, Aksamit T, Brown-Elliott BA, et al., 2007, An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases. Am J Respir Crit Care Med, 175: 367–416.

[6] Kang H, Sung N, Lee S, et al., 2008, Comparison of Smear and Culture Positivity Using NaOH Method and NALC-NaOH Method for Sputum Treatment. Tuberc Respir Dis, 65: 379–384.

[7] Beran V, Havelkova M, Kaustova J, et al., 2006, Cell Wall Deficient Forms of Mycobacteria: A Review. Vet Med, 7: 365–389.

[8] Yi JY, Kim JP, Shin JH, et al., 2000, Detection of *Mycobacterium tuberculosis* using BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 System: Comparison with BACTEC 460 TB System and Ogawa Media. Korean J Clin Pathol, 20: 384–391.

[9] Subramanyam B, Sivaramakrishnan G, Sangamithrai D, et al., 2020, Reprocessing of Contaminated MGIT 960 Cultures to Improve Availability of Valid Results for Mycobacteria. Int J Microbiol, 1721020: 1–3.

[10] Jung H, Bang HI, Choi TY, 2019, Evaluation of the Effectiveness of a Re-Decontaminating Process with Bacterial Contaminated Specimens Showing a Positive MGIT Signal for the Detection of Mycobacteria. Korean J Clin Lab Sci, 51: 171–176.

[11] Krasnow I, Wayne LG, 1966, Sputum Digestion. I. The Mortality Rate of Tubercle Bacilli in Various Digestion Systems. Am J Clin Pathol, 45: 352–355.

[12] Bae E, Im JH, Kim SW, et al., 2008, Evaluation of Combination of BACTEC Mycobacteria Growth Indicator Tube 960 System and Ogawa Media for Mycobacterial Culture. Korean J Lab Med, 28: 299–306.

[13] Joung US, Jeong J, Lee SH, et al., 2004, Comparison of Mycobacterial Culture by Mycobacterium Growth Indicator Tube and Ogawa Media. Korean J Clin Microbiol, 7: 135–138.

[14] Kim YS, Jo YH, Lee HJ, et al., 2001, Comparison of the MGIT (Mycobacteria Growth Indicator Tube) with Ogawa Media for Recovery of Mycobacteria. Korean J Clin Microbiol, 4: 58–61.

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