Comparison of chlorhexidine 0.7% and modified Petroff’s method on sputum decontamination for culture method to detect Mycobacterium tuberculosis

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ABSTRACT

Background: Tuberculosis (TB) is an infectious disease that is the leading cause of death from a single infectious agent worldwide. Culture is still the gold standard for the diagnosis of pulmonary TB. As an important specimen, sputum is often contaminated by normal flora at the time of collection, making the decontamination process an essential step of Mycobacterium tuberculosis culture. Decontamination with chlorhexidine 0.7% is expected to improve the detection of Mycobacterium tuberculosis in the culture method better than Modified Petroff’s method.

Methods: This research is an experimental study. Sputum was collected from pulmonary TB patients. Each sputum was decontaminated with chlorhexidine 0.7% and others with Modified Petroff’s. Decontaminated sputum inoculated on Lowenstein-Jensen media. Samples were incubated, and evaluated the recovery rate of Mycobacterium tuberculosis. Data were analyzed using SPSS version 25.0 for Windows.

Results: In total, we collected 16 sputum with 11 (68.75%) direct smear positive, 14 (87.5%) smear positive by chlorhexidine 0.7% method, and 13 (81.25%) by Modified Petroff’s method. The number of cultures positive for Mycobacterium tuberculosis by chlorhexidine 0.7% method was 14 (87.5%), of which 11 (68.75%) have specific colony characteristics of Mycobacterium tuberculosis, 3 (18.75%) non-tuberculous mycobacteria, and 1 (6.24%) mixed. In Modified Petroff’s, 6 (37.5%) were positive cultures and showed only growth of Mycobacterium tuberculosis. The contamination rate was 1 (6.25%) using chlorhexidine 0.7% and 8 (50%) using Modified Petroff’s method.

Conclusion: Chlorhexidine 0.7% has a higher recovery rate in the culture of Mycobacterium tuberculosis compared with Modified Petroff’s, showing recovery of non-tuberculous mycobacteria and a lower contamination rate.

Keywords: Mycobacterium tuberculosis, Sputum Decontamination, Modified Petroff’s, Recovery Rate.

INTRODUCTION

Tuberculosis (TB) is an infectious disease that is one of the top 10 causes of death worldwide, with the leading cause of death from a single infectious agent, above HIV/AIDS infection. WHO estimated that 5.8 million people were newly diagnosed with TB and reported in 2020.1 Indonesia has made great progress in expanding TB services over the past few years. However, the decline in TB incidence is still quite slow. Indonesia still ranks second for TB incidence globally, with estimated 258.355 cases found and cured per June 2022 based on Dashboard TB Indonesia.2 Tuberculosis is caused by Mycobacterium tuberculosis which most often attacks the lung.3 4 The high incidence makes detecting patients with active pulmonary TB disease an important component in TB control programs to reduce the chain of TB transmission.5 7 Sputum examination is important in diagnosing TB because it can provide an overview of lung conditions due to Mycobacterium tuberculosis infection. Currently, no accurate specimen besides a sputum biomarker-based test is available for TB.6 9 The process of taking sputum that passes through non-sterile areas of the upper respiratory tract makes excessive growth of contaminants such as Staphylococcus aureus, Pseudomonas aeruginosa, dan Candida albicans inhibits the efficiency of Mycobacterium tuberculosis isolation process which is the gold standard for the diagnosis of pulmonary TB.5 10–12 Sputum contamination makes the decontamination process crucial.13–15 Various decontamination methods have been carried out to minimize the growth of contaminations with minimum effect on Mycobacterium tuberculosis growth.14 15 Sodium hydroxide or NaOH-based decontamination protocol, including Modified Petroff’s method (NaOH 4%), is the most widely used method in laboratories worldwide.16–18 Sodium hydroxide 4% has been used as a standard...
agent to reduce contamination levels in specimens from non-sterile areas like the upper respiratory tract. However, this method still affects the viability of Mycobacterium tuberculosis in a large enough amount, namely 60-70%, so that it has the potential to reduce the recovery rate of Mycobacterium tuberculosis in culture due to false negative results, particularly in paucibacillary infection such as in patients with HIV co-infected TB. Based on those mentioned above, this study aimed to compare the performance of chlorhexidine 0.7% with modified Petroff’s method for sputum decontamination to increase the effectiveness of the Mycobacterium tuberculosis culture method from sputum specimens.

METHODS

This study was an experimental study that was carried out at the Microbiology Clinic Department, Dr. Soetomo Hospital, Surabaya, Indonesia, from December 2021 to March 2022 after receiving ethical approval from the Health Research Ethics Committee of Dr. Soetomo Hospital on December 3rd, 2021. The sampling technique used in this study is consecutive sampling. The inclusion criteria were sputum collected from patients that confirmed pulmonary TB by Xpert MTB/RIF with sensitivity to rifampicin in 15 (93.75%) specimens and 1 (6.25%) rifampicin indeterminate. From treatment history, 14 (87.5%) were new cases, 1 (6.25%) were relapsed cases, and 1 (6.25%) were lost follow-up cases, as seen in Table 1.

Specimens collected have a variation of storage time, from 1 to 11 days, with a mean of 5 days. This variation did not affect the viability of Mycobacterium tuberculosis. Out of 16 sputum samples, 11 (68.75%) were smear positive by the direct method, 14 (87.5%) were smear positive by the chlorhexidine 0.7% method, and 13 (81.25%) were smear positive by Modified Petroff’s method. Decontamination increased the positivity of acid-fast staining can be seen in Figure 1.

Sputum Culture

The sputum culture was transferred 3.4 drops of resuspended pellet to the Lowenstein-Jensen medium (Liofilchem, osetto d. Abruzzi, Italy) with a sterile pipette and spread evenly over the entire surface of the Lowenstein-Jensen medium. Positioned the bottle at an inclination of 30° on a shelf for 24 hours and incubated at 37°C. Tightened the bottle after 24 hours, then continued the incubation for eight weeks. Direct observation of the recovery rate and colony characteristics of Mycobacterium tuberculosis was carried out in the eighth week from incubation time. The number of colonies that grew was then counted and recorded. The contamination rate was evaluated using a blood agar medium (Becton Dickinson, New Jersey, USA). Resuspended pellet 100µl were transferred onto the center surface of the blood agar medium. Spread evenly over the entire media surface with a sterile spreading spatula moved back and forth while the Petri dish was turned. They were incubated at 37°C for 48 hours. Direct observation of the blood agar medium was carried out every 24 hours, semiquantitative by counting and recording the presence or absence of colony growth during incubation. Sputum was also stained with acid-fast staining Ziehl-Neelsen (Becton Dickinson, New Jersey, USA) before and after each decontamination procedure and examined under light microscopy.

Results

The Man-Whitney test was used to compare the recovery rate, colony characteristics of Mycobacterium tuberculosis, and the contamination rate, a p-value <0.05 was considered statistically significant. SPSS version 25.0 for Windows was used for data processing.

Sample characteristic

All 16 sputum in this study were Mycobacterium tuberculosis confirmed by Xpert MTB/RIF with sensitivity to rifampicin in 15 (93.75%) specimens and 1 (6.25%) rifampicin indeterminate. From treatment history, 14 (87.5%) were new cases, 1 (6.25%) were relapsed cases, and 1 (6.25%) were lost follow-up cases, as seen in Table 1.

Recovery rate

A higher recovery rate was obtained from the chlorhexidine 0.7% method with 14 (87.5%) positive cultures, whereas 11 (68.75%) have a specific colony of Mycobacterium tuberculosis, 3 (18.75%) have non-tuberculous mycobacteria colony, 1 (6.25%) has a mixed colony, and 1 (6.25%) showed no growth. In comparison, the recovery rate from Modified Petroff’s method has 6 (37.5%) positive cultures with specific Mycobacterium tuberculosis colonies, 9 (62.5%) showed no growth, and 1 (6.25%) was contaminated.

Contamination rate

The growth of non-acid fast bacteria colonies was significantly higher with chlorhexidine 0.7% method 1 (6.25%) than with Modified Petroff’s method 8 (50%) (p <0.05, Man-Whitney test), as shown in Table 2.
**Table 1.** Patient’s Rifampicin susceptibility result and treatment status.

<table>
<thead>
<tr>
<th>Treatment status</th>
<th>Rifampicin Sensitive (N=15)</th>
<th>Rifampicin Resistant (N=0)</th>
<th>Rifampicin Indeterminate (N=1)</th>
<th>Total (N=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New, n (%)</td>
<td>13 (81.25)</td>
<td>0 (0.00)</td>
<td>1 (6.25)</td>
<td>14 (87.50)</td>
</tr>
<tr>
<td>Relapse, n (%)</td>
<td>1 (6.25)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>1 (6.25)</td>
</tr>
<tr>
<td>Lost follow up, n (%)</td>
<td>1 (6.25)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>1 (6.25)</td>
</tr>
<tr>
<td>Total, n (%)</td>
<td>15 (93.75)</td>
<td>0 (0.00)</td>
<td>1 (6.25)</td>
<td>16 (100.00)</td>
</tr>
</tbody>
</table>

*Statistically significant if p-value less than 0.05*

**Table 2.** Comparison of chlorhexidine 0.7% and Modified Petroff’s method for contamination rate in Blood Agar medium.

<table>
<thead>
<tr>
<th>Growth Status</th>
<th>Blood Agar Media (N=16)</th>
<th>Chlorhexidine 0.7% (N=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth, n (%)</td>
<td>8 (50.00)</td>
<td>1 (6.25)</td>
<td>0.007*</td>
</tr>
<tr>
<td>No Growth, n (%)</td>
<td>8 (50.00)</td>
<td>15 (93.75)</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

*Statistically significant if p-value less than 0.05*

**Table 3.** Analyses of presentable colony characteristics of Mycobacterium tuberculosis in Modified Petroff’s method and chlorhexidine 0.7% method.

<table>
<thead>
<tr>
<th>Colony Characteristic</th>
<th>Decontamination</th>
<th>NaOH 4% (N=8)</th>
<th>Chlorhexidine 0.7% (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cauliflower, n (%)</td>
<td></td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Cream buff/yellowish, n (%)</td>
<td></td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Rough and dry surface, n (%)</td>
<td></td>
<td>6 (37.50)</td>
<td>11 (73.33)</td>
</tr>
</tbody>
</table>

**Table 4.** Comparison of Mycobacterium tuberculosis colony characteristics in chlorhexidine 0.7% method and Modified Petroff’s method.

<table>
<thead>
<tr>
<th>Colony Growth</th>
<th>Decontamination</th>
<th>NaOH 4% (N=16)</th>
<th>Chlorhexidine 0.7% (N=16)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td></td>
<td>6 (37.50)</td>
<td>11 (68.75)</td>
<td>0.005*</td>
</tr>
<tr>
<td>Non-characteristic</td>
<td></td>
<td>0 (0.00)</td>
<td>4 (25.00)</td>
<td></td>
</tr>
<tr>
<td>No Growth, n (%)</td>
<td></td>
<td>10 (62.50)</td>
<td>1 (6.25)</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant if p-value less than 0.05*

**DISCUSSION**

The result of acid-fast staining from both decontamination methods in this study showed an increase in sensitivity to the decontaminated specimen. This is in accordance with a systematic review by Steingart KR et al. if the decontamination process increases microscopic sensitivity. The importance of proper decontamination greatly affects the success of Mycobacterium tuberculosis culture. In this study, sputum inoculation with chlorhexidine 0.7% showed significantly higher results than Modified Petroff’s method. The increasing concentration of NaOH (3% or 4%) in Modified Petroff’s method can reduce the contamination rate but is toxic to Mycobacteria, which gives a risk of a false negative result.

Positive cultures from specimens decontaminated with chlorhexidine 0.7% gave a higher number of colonies in the Lowenstein-Jensen medium, which indicated that this method gave more viable Mycobacterium tuberculosis. This would be in accordance with research conducted by Rikimaru T et al. if chlorhexidine had no bactericidal effects on three different Mycobacteria species (Mycobacterium avium, Mycobacterium kansasii, and Mycobacterium tuberculosis) after exposure for 120 minutes with chlorhexidine 0.5%. This positive culture was due to the Mycobacteria’s nature, which is known to be resistant to many chemical agents and antiseptics, such as chlorhexidine, compared to other bacteria. This high resistance ability is primarily due to the unique arrangement of the mycobacteria cell wall, which contains a thick layer of lipids. This thick layer of lipids inhibits the penetration of chemical agents, especially chlorhexidine, which is also an antiseptic to enter the target bacteria membrane, resulting in cell death.

Chlorhexidine is a biguanide compound widely used in various applications and most active against Gram-positive bacteria, but it also has activity against Gram-negative bacteria, fungi and some enveloped viruses. The antimicrobial activity of chlorhexidine is dose-dependent, with the most commonly used concentration of 0.5-4 % in the form of gluconate. Chlorhexidine has bacteriostatic activity at low concentrations (0.02-0.06%), causes damage to bacterial membranes, and changes the osmotic balance of bacterial cell walls, causing leakage of potassium ions and other low-weight molecules. A higher concentration (>0.12%) has
However, one specimen had a false positive result (Xpert MTB/RIF assay detected with negative culture). A study conducted by Theron G et al. showed that the occurrence of false positives was associated with recent previous tuberculosis due to the presence of residual Mycobacteria DNA in respiratory tract and detection of non-viable bacilli; low Mycobacterium DNA load as measured by high cycle threshold (CT) (>30) and no sign of pulmonary inflammation in radiological images. In this study, samples with false positive results came from new TB cases with CT 30.06 with no data of radiological examination to trace. Besides the thick lipid composition of the cell wall that caused Mycobacteria to be more resistant to antiseptics, such as chlorhexidine, there was varying tolerance level of Mycobacteria species to NaOH, causing species that have a low tolerance to NaOH will die in the decontamination procedure. Several studies have also found that chlorhexidine is effective in recovering non-tuberculous mycobacteria in culture after decontamination.

The contamination rate was higher in Modified Petroff’s method, as seen in Table 2, in line with the previous study by Asmar S and Dracourt M that compared chlorhexidine at different concentrations, one of which was 0.7% with a standard protocol based on NaOH, the contamination rate was carried out in Columbia agar media with a total of 75 sputum specimens, giving the best result in chlorhexidine 0.7% concentration. Some previous studies also found a lower contamination rate with the chlorhexidine 0.7% method.

In this study, chlorhexidine 0.7% is assumed to give a bactericidal effect. In positive cultures from specimens with decontaminated chlorhexidine 0.7%, non-tuberculous mycobacteria colony growth was also found, as seen in Figure 2 and Table 3. All inclusion specimens were sputum from Xpert MTB/RIF assay-confirmed pulmonary TB patients.

In several other studies, it has also been described that chlorhexidine has a broad spectrum ability on non-acid-fast bacteria but minimal effect on Mycobacteria. In this study, non-tuberculous mycobacteria colonies grew on culture from the chlorhexidine method in line with a study conducted by Ferroni A et al., stating that the chlorhexidine method provides bactericidal activity, causing increasing cell membrane permeability leading to cytoplasmic leakage and, ultimately, coagulation and deposition of intracellular constituents, including nucleic acid. In this study, chlorhexidine 0.7% is assumed to give a bactericidal effect.
better recovery for non-tuberculous mycobacteria than the Modified Petroff’s method. An increase in recovery rate could be because chlorhexidine is better at preserving the viability of Mycobacteria, resulting in a higher positive culture. In other studies, it was stated that non-tuberculous mycobacteria were more sensitive to NaOH.\(^{13,40,41}\) Observation of the morphological characteristic of non-tuberculous mycobacteria colonies on solid media can be distinguished from Mycobacterium tuberculosis. However, it cannot determine non-tuberculous mycobacteria species, so further confirmation is needed by molecular examination.\(^{48,49}\)

All inclusion specimens were sputum from Xpert MTB/RIF assay-confirmed pulmonary TB patients. However, in this present study, one specimen with a false positive result (Xpert MTB/RIF assay detected with negative culture) was found in the Lowenstein-Jensen medium inoculated with sputum from the chlorhexidine decontamination method. Further study with sputum from Xpert MTB/RIF assay not detected is necessary to evaluate chlorhexidine effectiveness to grow non-tuberculous mycobacteria.

CONCLUSION

Chlorhexidine 0.7% has a lower contamination rate and higher yield in culture positivity of Mycobacterium tuberculosis with more colony recovered and more specific characteristics of Mycobacterium tuberculosis compared with Modified Petroff’s method. Chlorhexidine 0.7% also showed an effect on the recovery of non-tuberculous mycobacteria, making it a better method for decontamination.

CONFLICT OF INTEREST

The author reports no conflict of interest in this work.

ETHICAL CONSIDERATION

The subject was fully informed and explained the protocol, and informed consent was obtained before testing. The protocol of this study was approved by the Health Research Ethics Committee of Dr. Soetomo Hospital on December 3rd, 2021, with ethical number 0323/KEPK/XII/2021.

FUNDING

None.

AUTHOR CONTRIBUTION

RHL, NMM, LA, and SS have equally contributed to the designing, data analysis, interpretation of data, drafting or revision of critically important intellectual content, given final approval of the version to be published.

REFERENCES


