**ABSTRACT**

**Background:** The fundamental priority program for controlling Tuberculosis (TB) is accurate and rapid diagnosis. Variable Mycobacterium tuberculosis (MTB) phenotypes were discovered in sputum samples from pulmonary TB (PTB) patients. Active replicating bacteria and differentially culturable tubercle bacteria (DCTB) are present in the sputum. Differentially culturable tubercle bacilli did not grow on the standard medium. Culture filtrate (CF) H37Rv supplementation containing resuscitation-promoting factors (Rpfs) has the potential to increase sensitivity and reduce time to detection (TTD) on culture-based diagnostics.

**Methods:** This study was a true experimental laboratory. A clinical sample was taken from the sputum of 15 PTB patients, which was "MTB detected" by Xpert MTB/RIF assay. Sputum samples were divided into two groups and inoculated on MGIT 960 in one group with CF-H37Rv supplementation and without in the other as standard MGIT medium.

**Results:** On both standard MGIT medium and MGIT+CF H37Rv, the recovery rate was 100%. Seven samples (47%) show a growth promotion effect with CF H37Rv supplementation (ΔTTD > 10 hours), while eight samples (53%) show no change in TTD (ΔTTD < 10 hours). There is no evidence that CF H37Rv supplementation inhibits growth. Six of the seven samples with ΔTTD > 10 jam (85,7%) were dominated by samples with acid-fast bacilli (AFB) grading 1+, which could be an assumption related to re-treatment PTB cases, chronic cases, treatment stages, TB-HIV, or follow up of treatments.

**Conclusion:** There was no difference in recovery rate or TTD between standard MGIT medium and MGIT+CF H37Rv. The supplementation of culture filtrate H37Rv has a greater advantage on samples with low bacterial load (AFB 1+).

**Keywords:** Culture filtrate, MGIT 960 System, Mycobacterium tuberculosis, pulmonary tuberculosis, resuscitation-promoting factors, time to detection.

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**INTRODUCTION**

Tuberculosis (TB) is a contagious disease that is a major cause of illness and one of the leading causes of death globally. Indonesia is ranked second in the world regarding the number of TB patients in 2020, up from a third the previous year. Tuberculosis is caused by bacilli Mycobacterium tuberculosis (MTB) which is spread when people who are sick with TB expel bacteria into the air.

The main priority of the TB control program is the accurate and rapid diagnosis of active pulmonary TB (PTB) patients. This approach aims to prevent the transmission of MTB and provide appropriate therapy for TB patients. The microbiology laboratory is important in establishing a definitive diagnosis based on the detection, identification, and susceptibility testing of TB-causing agents through culture methods.

Several studies found various MTB phenotypes in sputum samples from patients with active PTB, including actively replicating and non-replicating or dormant bacteria. 80 to 99% of the MTB population in PTB patients’ sputum is dormant. Dormant MTB cannot grow on the standard medium used for the current diagnosis, resulting in a false negative diagnosis. This condition is reversible, and bacterial cells can resume metabolic activity when exposed to a rich medium or culture filtrate (CF) replicating mycobacteria. Differentially culturable tubercle bacteria (DCTB) are mycobacteria that can only grow when stimulated with CF.

The culture filtrate of growing MTB contains resuscitation-promoting factors (Rpfs). Resuscitation-promoting factors are a family of secreted proteins that can resuscitate non-replicating bacteria that were first discovered in Micrococcus luteus. MTB has five Rpf-like genes (Rpf A-E), which show biological activity similar to Rpfs in Micrococcus luteus.

Resuscitation-promoting factors are potent in hydrolyzing peptidoglycan in the cell wall of MTB at picomolar concentrations to stimulate the bacteria out of the dormancy stage. A study showed that CF of growing MTB strain H37Rv increased the sensitivity of culture and accelerated the time for detecting...
Cell-free liquid culture in the exponential phase of MTB culture showed a resuscitation effect to substantially increase the growth capacity of non-culturable cells in liquid media.\textsuperscript{13,20,21} Time to detection (TTD) of MTB in commercial liquid media MGIT (Mycobacteria Growth Indicator Tube) 960 system ranged from 6 to 10 days.\textsuperscript{12} According to one study, the TTD of MTB in MGIT 960 for positive smears is 16 days, while the TTD for negative smear specimens is 20 days.\textsuperscript{23} The time required for culture is still quite long, which can result in patient management delays. Culture filtrate H37Rv supplementation containing Rpf s raises the prospect of improving the sensitivity and speeding up the time of MTB culture using currently available methods.\textsuperscript{13,17,24,25} This study aims to compare MTB's recovery rate and TTD in commercial liquid media MGIT 960 with and without CF H37Rv.

**METHODS**

**Study Design**

This study was a true experimental laboratory, comparing two groups of MGIT 960 (BD BACTEC\textsuperscript{TM} MGIT\textsuperscript{TM} 960 Mycobacteria Culture System, BD Biosciences, Sparks, USA) with and without CF H37Rv supplementation.

**Sample Collection**

The population in this study was 15 sputum samples of PTB patients, collected between October 20 and November 22, 2021, taken by consecutive sampling techniques. Sputum samples were collected from the dr. Soetomo hospital pulmonology clinic sent Xpert MTB/RIF (Xpert\textsuperscript{®} MTB/RIF, Cepheid\textsuperscript{®}, Sunnyvale, USA) examinations to the clinical microbiology laboratory. The inclusion criteria were sputum samples with the Xpert MTB/RIF assay result “MTB detected” Sputum samples that did not grow on both media, MGIT and MGIT+CF H37Rv, as well as samples that were contaminated during the culture process, were excluded from this study.

**H37v Culture Filtrate Preparation**

The colony stock of MTB H37Rv on Lowenstein Jensen (LJ) (Liofilchem, Rosetto d. Abruzzi, Italy) was homogenized with a glass bead and 7H9 broth (BD Bactec MGIT\textsuperscript{®} tube, Becton Dickinson, New Jersey, America) to make 1 Mcfarland suspension. A total of 100 μL of 1 Mcfarland suspension was inoculated to an MGIT tube supplemented with Oleic acid-Albumin-Dextrose-Catalase (OADC) (BD Bactec MGIT 960 supplemental kit, Becton Dickinson, New Jersey, America) and then incubated at 37°C for 12 days (mid-log phase) in the MGIT 960 system machine.\textsuperscript{26} The MGIT tube was removed from the MGIT 960 system machine after 12 days. Ziehl-Nelsen (ZN) staining (Becton Dickinson, New Jersey, America) was performed to ensure that acid-fast bacilli (AFB) were present in the MGIT-positive tube. Plating in Blood Agar (BBL Trypticase Soy Agar 2-100 μL of 1 Mcfarland suspension) was performed to ensure no contaminants grow.\textsuperscript{22} The contaminant-free MGIT tube was centrifuged for 20 minutes at 3000 g at 4°C. Minisart\textsuperscript{®} 0.2 μm filter (Minisart, Sartorius, Gottingen, Germany) was used to filter the supernatant. A second ZN staining was performed to ensure no AFB cells were filtered in the culture filtrate. The CF was tested for sterility by inoculating 500 μL of the filtered product into an MGIT tube and incubating it on the MGIT 960 system machine. The culture filtrate was kept at -80°C until it was time to use it.\textsuperscript{9,13,17} The effect of CF H37Rv on TTD can be classified into three categories: 1) TTD decrease >10 hours (growth promotion), 2) TTD prolongation >10 hours (growth inhibitor), and 3) TTD difference < 10 hours (no change in TTD).\textsuperscript{25}

**Sputum Culture**

TB sputum culture is started with a decontamination process using 4% NaOH (Emsure, MerckKGaA, Darmstadt, Germany). After adding the NaOH in a 1:1 ratio with the amount of specimen and vortexed, the tube is allowed to stand for about 15-20 minutes. After 15-20 minutes, phosphate buffer saline (PBS) (pH 6.8) (Emsure, MerckKGaA, Darmstadt, Germany) was added until the tube reached 50 mL. Specimens were centrifuged for 15-20 minutes at 3000 g. The supernatant was disposed of in a disinfectant-containing container. The sediment was resuspended in 1-2 mL PBS (pH 6.8). The 500 μL pellet/sediment suspension is used to inoculate the MGIT tube, with one drop used for the smear.\textsuperscript{22,27} Each specimen was inoculated into two different MGIT tubes. One control tube has a composition similar to the standard MGIT medium used in the culture of MTB. One experimental tube was added with culture filtrate H37Rv with a volume of 0.7 mL (1:10) (15), and 500 μL of decontaminated sputum was inoculated into the control and experiment tube and incubated in MGIT 960 TB System machine.

The MGIT tube showing a positive signal is removed from the MGIT 960 TB system machine. Positive timing or TTD was recorded. Positive MGIT tubes < 7 days are considered early positive MGIT, which means positive MGIT tubes with negative confirmatory test results.\textsuperscript{22} Make a smear of 1-2 drops of positive liquid MGIT on a clean glass object. The ZN staining was performed to see the presence or absence of AFB. The positive MGIT tube is also inoculated on Blood Agar to see whether contaminant bacteria or fungi grow. Blood Agar is incubated in a CO\textsubscript{2} incubator for 48-72 hours.\textsuperscript{22,27} Tubes that found contaminants were excluded from the analysis. Tubes with positive smears and no contaminants were declared as positive cultures. Re-treatment cases include failed therapy, drug withdrawal, and relapse.\textsuperscript{28}

**Data Analysis**

A comparative test was used to analyze the data. Mann-Whitney was used to compare recovery rates. The TTD comparison was tested using the T-test, and SPSS version 25.0 for Windows was used for data processing.

**RESULTS**

**Sample Characteristic**

The Xpert MTB/RIF assay examination revealed that all sputum samples tested positive for MTB or “MTB Detected,” with 11 people (73%) sensitive to rifampicin (RIF), 3 people (20%) resistant to RIF, and one person (7%) indeterminate to RIF. According to the treatment history, 12 patients (80%) were new cases, and three were re-treatment cases (20%). Table 1 shows the patient's RIF sensitivity pattern and treatment status.
Table 1. Susceptibility pattern to rifampicin and treatment status.

<table>
<thead>
<tr>
<th>Treatment status</th>
<th>Rifampicin Sensitive (N=11)</th>
<th>Rifampicin Resistant (N=3)</th>
<th>Rifampicin Indeterminate (N=1)</th>
<th>Total (N=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New case, n (%)</td>
<td>9 (60.0)</td>
<td>2 (13.0)</td>
<td>1 (7.0)</td>
<td>12 (80.0)</td>
</tr>
<tr>
<td>Re-treatment case, n (%)</td>
<td>2 (13.0)</td>
<td>1 (7.0)</td>
<td>0 (0.0)</td>
<td>3 (20.0)</td>
</tr>
</tbody>
</table>

Table 2. Sample consistency and AFB grading.

<table>
<thead>
<tr>
<th>Macroscopic Appearance</th>
<th>AFB Grading</th>
<th>Total (N=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1+ (N=7)</td>
<td>2+ (N=3)</td>
</tr>
<tr>
<td>Purulent, n (%)</td>
<td>3 (20.0)</td>
<td>1 (7.0)</td>
</tr>
<tr>
<td>Mucus, n (%)</td>
<td>3 (20.0)</td>
<td>1 (7.0)</td>
</tr>
<tr>
<td>Bloody Sputum, n (%)</td>
<td>1 (7.0)</td>
<td>1 (7.0)</td>
</tr>
</tbody>
</table>

AFB: Acid Fast Bacilli

Table 3. TTD on standard MGIT group.

<table>
<thead>
<tr>
<th>AFB grading</th>
<th>&lt; 7 days (N=6)</th>
<th>≥ 7 days (N=9)</th>
<th>Total (N=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+, n (%)</td>
<td>0 (0)</td>
<td>7 (47)</td>
<td>7 (47)</td>
</tr>
<tr>
<td>2+, n (%)</td>
<td>3 (20)</td>
<td>0 (0)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>3+, n (%)</td>
<td>3 (20)</td>
<td>2 (13)</td>
<td>5 (33)</td>
</tr>
</tbody>
</table>

AFB: Acid Fast Bacilli; TTD: Time to Detection; MGIT: Mycobacterium Growth Indicator Tube

Table 4. TTD on MGIT + CF H37Rv group.

<table>
<thead>
<tr>
<th>AFB grading</th>
<th>&lt; 7 days (N=6)</th>
<th>≥ 7 days (N=9)</th>
<th>Total (N=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+, n (%)</td>
<td>2 (13)</td>
<td>5 (33)</td>
<td>7 (46)</td>
</tr>
<tr>
<td>2+, n (%)</td>
<td>3 (20)</td>
<td>0 (0)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>3+, n (%)</td>
<td>3 (20)</td>
<td>2 (13)</td>
<td>5 (34)</td>
</tr>
</tbody>
</table>

AFB: Acid Fast Bacilli; TTD: Time to Detection; MGIT: Mycobacterium Growth Indicator Tube; CF: Culture Filtrate

Difference of Time to Detection (ΔTTD)
The growth of MTB in liquid media generally takes 6-10 days. In the manual of MTB culture, TTD in liquid media (MGIT) was divided into < 7 days (< 168 hours) and ≥ 7 days (≥ 168 hours). In this study, there were six samples with TTD < 7 days (< 168 hours) and nine samples with TTD ≥ 7 days (≥ 168 hours) from the standard MGIT group. Table 3 displays the TTD in the standard MGIT group.

Meanwhile, for the MGIT + CF H37Rv group, there were eight samples with TTD < 7 days (< 168 hours) and seven samples with TTD ≥ 7 days (≥ 168 hours). Samples with TTD <7 days also showed the growth of MTB. Table 4 presents the TTD in the MGIT + CF H37Rv group.

The following diagram in Figure 1 shows that sputum samples cultured on MGIT medium with CF H37Rv supplementation demonstrated faster growth and TTD. Figure 2 shows the TTD using standard MGIT and MGIT + CF H37Rv correlated with AFB grading. On MGIT+CF H37Rv medium, samples that took longer than seven days to become positive on standard MGIT medium had a ΔTTD of more than 10 hours, as indicated in Figure 3.

From the results of the T-test, it was found that Sig. Lavender’s test for equality of variance is 0.905 >0.05, which means that the data variance between the MGIT and MGIT+CFH37Rv groups is homogeneous or the same. The results of Sig. (2–tailed) obtained 0.68 >0.05, so the administration of CF H37Rv in the experimental group did not cause any difference in TTD with the control group.

DISCUSSION
Macroscopically, sputum with a 53% purulent consistency made up most of the sample. The typical sample size is around 3 cc. According to the ZN examination...
As a result, the treatment process may cause the emergence of CF-dependent MTB.

The time required for MTB growth in the experiment and control groups, or TTD, shows that the TTD in the experiment group is generally faster than the TTD in the control group. Fourteen of the fifteen samples (93%) inoculated into the MGIT+CF H37Rv tube had a faster TTD than the control group. A previous study found that Rpfs can shorten the lag phase time and cause mycobacteria to enter the log phase immediately. The exponential growth of mycobacteria in the log phase consumes a large amount of oxygen. It causes fluorescence to accumulate in the MGIT tube, allowing a positive signal to be detected faster. In the control group, 60% of the samples had a TTD of ≥ 7 days, whereas only 46% of the samples in the experiment group had a TTD of ≥ 7 days.

According to the SPSS test results, the minimum TTD difference between the two groups was only 1 hour, with the experiment group 1 hour earlier. Meanwhile, when the two groups’ maximum TTD time was compared, the MGIT + CF H37Rv experiment group was 20 hours faster. The difference in mean time between the control and experiment groups was 14.6 hours. The SPSS test results using a t-test of two different samples revealed a p-value of 0.68 > 0.05, indicating no significant difference between the two groups. In general, CF H37Rv supplementation accelerated culture TTD, but this was not statistically significant. As a result, the researchers hypothesized that CF supplementation might not provide broad-spectrum benefits for all specimen types.

In response to the addition of CF H37Rv, growth was promoted in 7 samples (47%), with AFB 1+, three samples with AFB 2+ (21%), and five samples with AFB 3+ (33%), respectively. Most of the sputum used to produce the BTA 3+ result had a purulent nature. The findings of this study support previous findings that the gross appearance of purulent and blood-tinged sputum samples was associated with smear positivity when compared to mucoid or salivary consistency. Sample volume greater than 4 mL was also associated with smear positivity compared to sample volume less than 4 mL.

The results of decontaminated sputum inoculation in MGIT and MGIT + CF H37Rv tubes revealed no difference in recovery rate between the experiment and control groups. With Xpert/MTB RIF assay, all samples were confirmed to contain MTB, and 100% could grow on MGIT medium alone or MGIT + CF H37Rv medium. This contradicts previous findings that supplementing CF can increase the sensitivity of cultures for DCTB detection by 75% compared to media lacking CF. Another study discovered three negative specimens in culture under standard conditions but grew with CF H37Rv supplementation. Both groups recovered completely in this study was most likely because the sample was dominated by patients with new cases who had not received treatment. Hence, MTB active replicating bacteria still dominated the MTB population. Although several studies have shown that pre-treatment patient samples contain a high concentration of DCTB, which can only grow with CF H37Rv supplementation, the proportion of DCTB increases with treatment duration.
more pronounced in samples with low bacterial load, such as smear-negative, Xpert MTB/RIF positive with a high cycle threshold (Ct) value, or Xpert MTB/RIF negative samples. Another study found that supplementing CF H37Rv decreased TTD time from 9 days to 6 days (p ≤ 0.001) and increased the baricillary load on the sputum culture of pre-and post-treatment patients by 30%, 13, 35

In terms of treatment status, 7 of 12 new patients (58.3%) had a shortened TTD<10 hours, while the other 5 (41.6%) had a shortened TTD ≥ 10 hours. The percentage between the groups with ΔTTD <10 hours and ≥10 hours did not differ significantly. Three individuals with re-treatment patients were studied, and it was discovered that two of them (67%) had a TTD reduction of≥10 hours, while just one sample (33%) had a decrease of TTD of less than 10 hours. The TTD for one patient from the failed therapy case was shortened by 36 hours despite having a smear grade of 2+ that revealed a substantial bacterial load. This could be because patients who have failed therapy have a high concentration of dormant bacteria or DCTB. 9, 33 As a result, CF H37Rv supplementation can reduce TTD by up to 10 hours. This is consistent with previous research, which found that treatment caused MTB to enter a dormant state, and the presence of dormant bacteria was linked to therapeutic failure in TB patients. 9, 13, 32, 34

Based on the characteristics of MTB susceptibility to RIF, 11 samples were sensitive, with 5 (45.5%) experiencing a TTD shortening of > 10 hours and 6 (54.5%) experiencing a TTD shortening of <10 hours. In four samples, consisting of 3 samples with resistant RIF and 1 sample with RIF indeterminate, two of them (50%) experienced a shortening of TTD >10 hours, and the other two samples (50%) experienced a shortening of the TTD < 10 hours. It was concluded that the shortening of the TTD with the addition of the CF H37Rv was not associated with the susceptibility pattern of MTB to RIF. This finding is consistent with studies showing that CF is dispensable for detecting DCTB from drug-resistant strains. 36, 37

CF-H37Rv supplementation was also found to be more beneficial in samples with a TTD of ≥ 168 hours (≥7 days) on a standard MGIT medium. Six of the nine samples (66.7%) with a TTD of ≥ 168 hours on standard MGIT medium had their TTD reduced by > 10 hours on MGIT+CF H37Rv medium. As a result, CF-H37Rv supplementation demonstrated more significant growth promotion effects in samples that took longer to become positive. Previous research using recombinant RpfB and RpfE in sputum samples confirmed to contain MTB by fluorescence microscopy found that the reduction in TTD occurred only in samples with TTD greater than 20 days. 15, 30

At the beginning of this study, the researchers hypothesized that the decrease in TTD in MGIT liquid cultures was partly due to DCTB reactivation and replication. However, in the absence of a difference in recovery rate between the two groups, the sample is most likely still dominated by active replicating bacteria. A study that used the MPN assay to calculate the number of DCTBs discovered that samples with reduced TTD > 10 hours contained less DCTB than samples that did not change TTD or experienced an inhibitory effect from CF H37Rv supplementation. This finding suggests that CF may increase the metabolic activity of actively dividing MTB cells rather than resuscitating dormant cells. 25, 38

This study’s source of culture filtrate is a virulent strain of MTB H37Rv, which necessitates cautious handling and high-level biosafety. A study that looks into the sources of culture filtrate to resuscitate DCTB found that CF from H37Ra, Mycobacterium smegmatis, W-Beijing, and Erdmann strains had a significant resuscitation effect in increasing the number of MTB CFUs in the sample. Regardless of virulence, various mycobacteria can resuscitate dormant MTB cells in vitro. 13 Another study found that CF from Mycobacterium smegmatis had no resuscitation effect on dormant MTB. Adding Rpf ABMTB and Rpf ABCDEMTB to Mycobacterium smegmatis CF resulted in a slight but statistically significant increase in the DCTB recovery rate. 24

The study is limited by the small sample size and sputum sample selection, which only included samples with “MTB detected” on the Xpert MTB/RIF assay. Based on the findings of this study, which demonstrated that the effect of CF H37Rv was more beneficial in samples with a low bacterial load, future research could focus on patients with paucibacillary tuberculosis, such as in cases of extrapulmonary TB (EPTB) and TB-HIV, re-treatment cases, chronic cases, other follow up treatment stages, and patients with clinically TB symptoms but culture shows negative result. Another weakness in this study is the use of CF H37Rv, a virulent strain of MTB that requires extreme caution and takes a long time to grow. Sources of CF from other mycobacteria that are rapidly growing and less virulent can be considered in future studies to reduce preparation time and increase safety.

CONCLUSION

The recovery rate and TTD of the MGIT+CF H37Rv group and the standard MGIT medium groups were not statistically different. Culture filtrate supplementation did not benefit all types of specimens equally. The supplementation of culture filtrate H37Rv has a more significant advantage on samples with low bacterial load (AFB 1+) and samples with a longer time to positivity [TTD ≥7 days (≥ 168 hours)].

CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

ETHICAL CONSIDERATION

The Health Research Ethics Committee at RSUD Dr. Soetomo Hospital approved this study based on a certificate of ethical conduct No.0659/LOE/301.4.2/X/2021 dated October 19, 2021.

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AUTHOR CONTRIBUTION

All authors contributed by preparing the proposal, data collection, data analysis, writing and revising the manuscript, and approving the final version for publication.
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