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Research

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Confirmation of *Mycobacterium tuberculosis* culture results with Ziehl-Neelsen staining and MPT64 antigen test

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Abstract

Background Culture of *Mycobacterium tuberculosis* (MTB) using egg-based solid media like Lowenstein Jensen (LJ) is the gold standard for tuberculosis diagnosis but requires extended incubation time. Rapid diagnostic tests, such as Ziehl-Neelsen (ZN) staining and the MPT64 antigen rapid test, are essential for early detection.

Objective This study aimed to evaluate the effectiveness of ZN staining and the MPT64 rapid test in detecting MTB and *Mycobacterium Other Than Tuberculosis* (MOTT) during different culture times.

Methods Using a cross-sectional design, 110 culture-positive samples were analyzed from Hasan Sadikin Hospital Bandung over two months. Specimens were cultured on LJ media for eight weeks, with weekly observation of colony growth. ZN staining and MPT64 tests were performed on growing colonies.

Results Less than four weeks culture, 61 samples (55.5%) were culture-negative, 45 (40.9%) were positive for MOTT, and the remainder were contaminated. In more than four weeks culture, 48 samples (43.6%) were positive for MTB, 45 samples (40.9%) were positive for MOTT, and 13 (11.8%) were culture-negative, and the remaining were contaminated. ZN-positive and MPT64-negative results indicated MOTT in less than four weeks culture, while ZN-positive and MPT64-positive results indicated MTB in more than four weeks culture.

Conclusion While ZN staining was positive for both MTB and MOTT colonies, the MPT64 rapid antigen test was specific for MTB, supporting its use in confirming MTB detection alongside culture methods.

Keywords: *Mycobacterium tuberculosis* | microbe | culture | MPT64 antigen | Ziehl-Neelsen staining

Introduction

Tuberculosis (TB) is caused by the bacterium *Mycobacterium tuberculosis* (MTB). This disease can affect any body part, but it most commonly targets the lungs (Boudville *et al.*, 2020). Symptoms of TB include chronic cough, fever, fatigue, weight loss, and night sweats. Antibiotics can be used for treatment, but the treatment duration is long, often up to 9 months. Inadequate treatment of TB can lead to the disease becoming more severe and spreading to other organs in the body (Alsayed & Gunosewoyo, 2023). TB prevention strategies include vaccination with the Bacillus Calmette-Guerin (BCG) vaccine and maintaining distance or avoiding close contact with infected individuals (Duarte *et al.*, 2018).

The diagnosis of tuberculosis can be confirmed through Ziehl-Neelsen (ZN) staining, Lowenstein-Jensen (LJ) culture, and the Mycobacterium protein tuberculosis (MPT)-64 antigen test. The MTB culture examination is

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the gold standard for confirming TB diagnosis. Culture media are broadly classified into two types: solid and liquid media. Egg-based solid media, such as LJ and Ogawa media, are today's most commonly used culture methods (Che-Engku-Chick *et al.*, 2016). Liquid media, like the *Mycobacterium* growth indicator tube (MGIT), are used to identify MTB growth in clinical samples. MGIT employs pressure and gas sensors to detect bacterial growth in culture samples (Ma *et al.*, 2020). Both techniques aid in detecting tuberculosis and other Mycobacterium infections.

The growth of MTB colonies on LJ media exhibits unique colony characteristics, such as granular, rough, warty, and dry cauliflower-like appearances (Kassaza *et al.*, 2014). For pulmonary TB, the MTB culture method using LJ media is considered the gold standard, but it has limitations, including a lengthy culture period of approximately six to eight weeks (Kassaza *et al.*, 2014). MTB positivity in LJ media cultures can be detected within three to four weeks; however, rapid colony growth in LJ media indicates saprophytic or non-pathogenic mycobacteria. This group of mycobacteria is recognized as *Mycobacterium* other than tuberculosis (MOTT). MOTT is also known as non-tuberculous *Mycobacterium*, atypical *Mycobacterium* (AM), opportunistic *Mycobacterium*, anonymous *Mycobacterium*, or environmental *Mycobacterium* (Sari *et al.*, 2023).

The longer time required to detect MTB growth in LJ media than MOTT is due to the unique characteristics and slower growth of MTB (Kassaza *et al.*, 2014). Therefore, faster methods are needed to confirm TB diagnosis, including rapid immunochromatography (ICT) (Ongut *et al.*, 2006), enzyme-linked immunosorbent assay (ELISA) (Yoo *et al.*, 2021), and real-time polymerase chain reaction (RT-PCR) (MacLean *et al.*, 2020).

The ICT method has been widely used to identify MTB antibodies in serological examinations. Improved accuracy and speed of diagnosis are necessary to enhance TB control efficiency. An example of a rapid, accurate, and precise ICT diagnostic method for TB detection is the MPT64 antigen test (Jørstad *et al.*, 2018). MPT64 is a 24 kD protein antigen produced by MTB and is extensively used for diagnostic purposes and as a vaccine component (Cao *et al.*, 2021). When the bacteria are alive, MTB releases this antigen into the patient's sputum. The presence of this antigen in a sputum specimen indicates an active pulmonary tuberculosis infection (Cao *et al.*, 2021).

The more extended culture period necessitates combining it with faster diagnostic methods for confirmation. This study aimed to evaluate the positivity rate of MTB colony growth before and after four weeks of culture using ZN staining and the MPT64 antigen rapid test, utilizing specimens from suspected TB cases examined at Hasan Sadikin Hospital, Bandung, West Java.

Methods

Study design

The Health Research Ethics Committee of Politeknik Kesehatan Kementerian Kesehatan Bandung approved this study with ethics approval number No.77/KEPK/EC/XII/2023. A descriptive-analytic method was employed with a cross-sectional approach. The study subjects comprised all specimens from suspected TB patients cultured daily at Hasan Sadikin Hospital, Bandung, West Java. The sample in this study included all MTB-positive culture specimens collected over two month period, specifically from September to October 2023. Sampling was conducted in the Microbiology Laboratory of Hasan Sadikin Hospital, Bandung, West Java.

Culture

The samples used included sputum and non-sputum samples, excluding cerebrospinal fluid. Decontamination was performed using 4% NaOH for sputum samples and 2% NaOH for non-sputum samples, followed by a 15-minute incubation. The samples were placed in Falcon tubes or screw-capped centrifuge tubes and centrifuged for 15 minutes at 3000 rpm (equivalent to 537 g). The supernatant was discarded, and the sediment was resuspended in sterile distilled water and centrifuged again for 15 minutes at 3000 rpm. The supernatant was discarded, leaving approximately 1 mL of sediment, and 0.2 mL of the sediment was inoculated onto LJ media (Becton Dickinson, USA). The cultures were incubated in an incubator (Memmert Thermostat, Germany) at 37°C for eight weeks. Colony development was observed weekly. An example of a positive MTB culture can be seen in **Figure 1A–B**.

ZN staining

ZN staining was performed using Ziehl-Neelsen reagents (Indo Reagen, Indonesia) following the method described previously (Van Deun *et al.*, 2006). A colony was taken with a sterile loop and spread on a glass slide, adding one drop of 0.9% NaCl, then fixed using a Bunsen burner. In the initial staining step, 0.3% carbol fuchsin was applied to the smear, heated until steaming but not boiling, and allowed to sit for 5 minutes. After cooling, the slide was rinsed with running water to remove excess carbol fuchsin. Decolorization was done by applying 3% acid alcohol until the smear appeared clean (pale). The slide was rinsed again with running water. Then, 0.1% methylene blue was applied, allowed to sit for 10–20 seconds, rinsed with running water, and air-dried. The dried slides were ready for microscopic examination at 100× objective lens magnification. A sample was considered ZN-positive if acid-fast bacilli were observed as red-stained bacteria (**Figure 1C**).

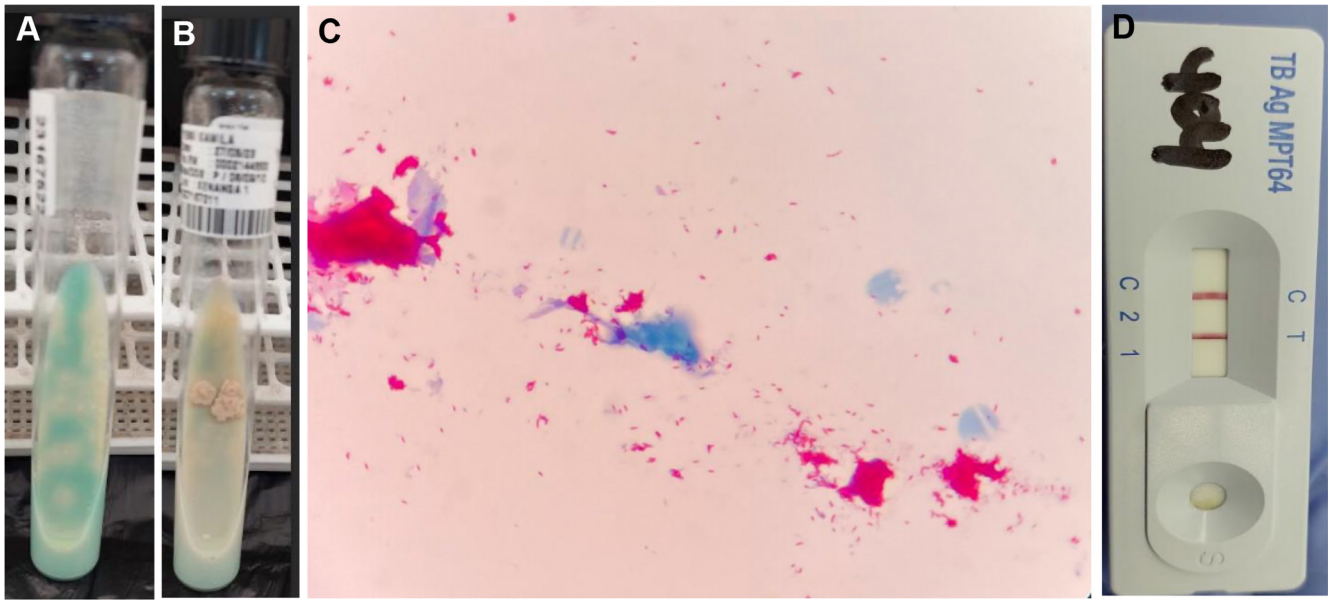

Figure 1 A-B. Culture of M. tuberculosis colonies on Lowenstein Jensen media, with colonies showing a cauliflower-like appearance (B). C. Acid-fast bacilli positive colonies stained with Ziehl-Neelsen, viewed at 100× lens objective magnification. D. Positive result of MPT64 antigen rapid test.

MPT64 antigen rapid test

A total of 200 µL of buffer, provided in the kit (Standard Diagnostics Inc., South Korea), was added to a tube. Approximately 3–4 colonies were picked and suspended in the buffer solution, then homogenized. Two drops (100 µL) of the sample suspension were taken using the pipette provided in the kit and added to the test cassette's S area. Result interpretation was conducted precisely at the 15-minute mark, as reading the results beyond 15 minutes may yield incorrect results. A sample was considered MPT64-positive if two red lines appeared on the kit, one in the test area (T) and one in the control area (C) (**Figure 1D**).

Data analysis

The primary data, consisting of the number of M. tuberculosis culture colonies that tested positive with Ziehl-Neelsen staining and the MPT64 antigen rapid test, were analyzed using the chi-square test with the Statistical Product and Service Solution (SPSS, IBM, version 27) software. The analysis of variables aimed to determine whether the influence of the two variables was statistically significant at $p<0.05$.

Results

The characteristics of the study subjects are presented in **Table 1**. The data showed that the study subjects were evenly distributed by gender, with 56 females (50.9%) and 54 males (49.1%). Furthermore, the data reveal that the majority of study subjects were aged between 12 months and two years (49.1%), followed by those aged 5–11 months (39.1%), with smaller proportions in the 24–26 years and 46–65 years age groups, each comprising 6.4% and 5.5% of the sample, respectively. Regarding ward classification, the data indicate that most study subjects were in the Class 3 ward (81.8%).

Table 1 Characteristics of study subjects by gender, age, and ward classification

Characteristics	Subjects	Number	%
Gender	Male	54	49,1
	Female	56	50,9
Age	5–11 mo	43	39,1
	12 mo–2 yr	54	49,1
	24–26 yr	7	6,4
	46–65 yr	6	5,5
Ward classification	Class 1 & 2	20	18,2
	Class 3	90	81,8
Total subjects		110	100,0

mo: months, yr: years

The culture results for sample colony growth at culture times of less than four weeks and more than four weeks are presented in **Table 2**. Culture results for less than four weeks showed that the growing colonies were MOTT bacteria (45 samples, 40.9%), while the culture with no colony growth (negative, 61 samples, 55.5%) indicated that MTB bacteria could not yet be identified because MTB colony growth typically occurs after four weeks (Baker *et al.*, 2014). Four samples were contaminated during culture within less than four weeks. These contaminated samples were discarded and not further cultured. Culture results for more than four weeks revealed that 48 samples (43.6%) showed positive colony growth, indicating the possibility of MTB, while 13 samples (11.8%) remained negative with no colony growth.

The results of colony examination with Ziehl-Neelsen staining and the MPT64 antigen rapid test are presented in **Table 3**. The findings showed that all MOTT and MTB-positive culture samples, at less and more than four weeks, were positive with Ziehl-Neelsen staining. However, the

Table 2 Culture results for sample colony growth

Culture periods	Colony growth	Number of samples	%
Less than 4 weeks	Negative	61	55,5
	Positive MOTT	45	40,9
	Contaminated	4	3,6
More than 4 weeks	Negative	13	11,8
	Positive MTB	48	43,6
	Positive MOTT	45	40,9
	Contaminated	-	-

Negative: no bacterial colony growth; Positive MOTT: growth of colonies suspected to be *Mycobacterium* other than tuberculosis; Positive MTB: growth of colonies suspected to be *Mycobacterium tuberculosis*.

Table 3 Results of Ziehl-Neelsen (ZN) staining on colonies grown in culture

Culture periods	Culture results	ZN staining		MPT64 antigen test		p-value
		Positive	Negative	Positive	Negative	
Less than 4 weeks	Negative	0	61	0	61	0,001
	Positive MOTT	45	0	0	45	
More than 4 weeks	Negative	0	13	0	13	
	Positive MTB	48	0	48	0	
	Positive MOTT	45	0	0	45	

Negative: no bacterial colony growth; Positive MOTT: growth of colonies suspected to be *Mycobacterium* other than tuberculosis; Positive MTB: growth of colonies suspected to be *Mycobacterium tuberculosis*. ZN: Ziehl-Neelsen staining shows positivity for all *Mycobacterium* spp.; MPT64 antigen rapid test shows positivity for *Mycobacterium tuberculosis*.

MPT64 antigen rapid test yielded positive results only for samples with MTB-positive colonies in cultures more than four weeks.

The chi-square test in **Table 3** assessed the relationship between culture time and the type of examination method. The chi-square test results showed a probability value of 0.001, which is lower than the statistical significance threshold of $\alpha = 0.05$, indicating that culture times more than four weeks were more likely to detect TB positivity with MPT64 than with ZN staining.

Discussion

This study achieved a balanced distribution of patient gender, which helps reduce bias or confounding factors in comparing TB diagnostic methods. The most frequent age group of TB patients in this study were children under two years old, consisting of the 5–11 months age group (43/110, 39.1%) and the 12 months–2 years age group (54/110, 49.1%). When combined, TB cases in children under two years old accounted for 97/110 (88.2%). TB in children is often determined by risk factors such as a history of contact with TB patients (Nandariesta *et al.*, 2019). The majority of TB patients (81.8%) were treated in Class 3 wards, representing lower socioeconomic groups with financial constraints. This finding is consistent with previous research (Saputra & Herlina, 2021), which indicated that TB incidence is more common among low-income groups.

MTB colonies exhibit slow growth at temperatures of 35–37°C, with in vivo division occurring every 23–32 hours. In vitro culture on solid media typically takes 3–6 weeks, while in liquid media, MTB colonies can grow faster, between 1–3 weeks (Chai *et al.*, 2018). In contrast, MOTT colonies thrive within less than seven days (rapid growth) on solid and liquid media (Sharma & Upadhyay, 2020). This difference in growth rates between MTB and MOTT is used to differentiate colonies of the two types of bacteria.

The results of MTB and MOTT cultures show that both MTB and MOTT thrive on LJ media. Bacterial growth in culture, considered positive when colonies appear on LJ medium, is subsequently confirmed with ZN staining. Between 40% and 70% of patients with tuberculosis demonstrate positive culture results with positive ZN smear staining (Essawy *et al.*, 2014). MTB are classified as acid-fast bacilli, characterized by their resistance to acid decolorization during the ZN staining procedure (Reynolds *et al.*, 2009). Once stained, the color cannot be removed using the acids typically employed. This essential and unique feature allows for classifying and detecting MTB bacteria using microscopic procedures such as ZN staining.

Table 3 shows positive ZN staining in both MTB and MOTT colonies. Both MTB and MOTT belong to the same genus, *Mycobacterium*. Culture results that were positive in less than four weeks showed negative MPT64 antigen test results. This outcome corroborates the positive MOTT culture. Conversely, positive culture results after more than four weeks showed positive MPT64 antigen test results, which confirm positive MTB cultures, as MPT64 is a protein secreted by MTB complex species. Therefore, only MTB is detected by MPT64, while MOTT is not (Cao *et al.*, 2021). MTB produced MPT64 protein, a significant filtrate protein from culture encoded by the RD2 gene, and is specific enough to differentiate MTB from MOTT (Cao *et al.*, 2021). Thus, the MPT64 antigen rapid test is very useful in confirming culture colonies that are positive for MTB.

Conclusion

Positive MOTT colony cultures, amounting to 45 samples (40.9%), were obtained from cultures less than four weeks old. In contrast, MTB colonies, amounting to 48 samples (43.6%), were obtained after cultures of more than four weeks. All positive MOTT and MTB colony cultures showed positive ZN staining, while the MPT64 antigen rapid test was only positive for MTB colonies. After four weeks of culture, the MPT64 antigen rapid test confirmed MTB-positive colony cultures.

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Author Contributions WI, IK, AD, and HIS: conceptualization, literature review, and editing; WI: investigation, data analysis, and initial manuscript drafting, review, and editing.

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