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Rapid Detection of Isoniazid Resistance in *Mycobacterium tuberculosis* Isolates by Multiplex Allelic Specific PCR

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Abstract

Mycobacterium tuberculosis is a type of bacteria causing infectious desease called tuberculosis. According to the Makassar City Health Office, total tuberculosis (TB) patients in 2019 were 5,412 with 83% cure rate. The number of TB cases had dropped to 3,250 in 2020 with 85% cure rate before it increased again to 3,911 in 2021. This study aimed at identifying Mycobacterium TB resistance to INH (isonicotinyl hydrazide) specifically in probable tuberculosis case in Makassar. Patients with suspected tuberculosis who have not taken medication were selected as study subject. They were examined through several stages including sample decontamination, microscopic examination (ZN Method), isolation on LJ media, culture on liquid medium MGIT (Mycobacteria Growth Indicator Tube), test on.DST (Drug Susceptible Test) MGIT, and DNA extraction using specific multiple allelic (MAS) techniques. According to the results of the culture, every sample (100%) was positive. With the use of the MGIT approach, we followed up on the encouraging culture results. In this instance, we used two MGIT tubes for each sample, one for the isoniazid antibiotic control and one for the subsequent sample. From the third day following vaccination through the twelfth, observations were made. On the third day, if the control tube is positive, then the second tube is observed until the fifth day (observation of the tube containing antibiotics is carried out two days after the control tube is positive. We also use isolates from positive culture results for Multiplex Allelic Specific PCR (MAS-PCR)).

Keywords: Resistance test, suspect, Tuberculosis, INH

Full length article **Corresponding Author*, e-mail: daengngerang73@gmail.com Doi # https://doi.org/10.62877/16-IJCBS-24-25-19-16

1. Introduction

The WHO Global Tuberculosis Report 2021 shows the number of TB patients in Indonesia has reached 824,000. This data contributes to global mortality rate increase due to TB from an estimated 1.2 million deaths among HIV negative people in 2019 to 1.3 million deaths in 2020. Yet, the Coronavirus pandemic has led to declines in TB diagnosis globally during 2019-2020. In this case, Indonesia becomes the second largest contributor to the decline (14 percent) after India (41 percent). This negative trend affected the fall of treatment coverage of TB patients to 59 percent in 2020 from 72 percent in 2019. Indonesia is placed as a country with low levels of TB patient care (below 50 percent). The WHO report also indicates a gap between the estimated incidence of TB and newly diagnosed people in 2020. Indonesia is the second country with the largest gap after Philippines [1-3]. One of the reasons for the failure of TB control programs in developing countries is inefficient diagnostic method to detect infection cases at early stage. In general, TB diagnostic methods are carried out conventionally through microscopic examination, culture and serology. However, these methods have many weaknesses. Microscopic examination requires germs/bacteria of at least 10,000 cells/ml. Besides, it cannot detect any mycobacterial species. Bacterial culture

examination has a high sensitivity and specificity, but requires longer time which ranges from 3-8 weeks. The sensitivity of this method is close to 100% and can be performed on clinical samples containing 10-100 cells of bacteria. The PCR method is considered faster, more sensitive and specific compared to cultivars for checking resistance [6-9]. According to a study conducted by

Nugrahaen (2013), from total 26 respondents being resistant to OAT, 10 (38.5%) had drug resistance to the combination of R, H (Rifampin and Isoniazid) and 4 (15.4%) had drug resistance to the combination of R, H, E, and S (Rifampin, Isoniazid, Erhambutanol, Streptomycin). Meanwhile, majority of anti-TB medications that are effective against Mycobacterium tub due to the impact of rising MDR-TB and the relatively small number of treatment drugs available. An effort was made to determine the molecular basis of Mycobacterium tuberculosis's resistance to OAT. Thus, it may be identified right away and the patient can receive prompt, precise therapy. Mycobacterium tuberculosis's primary resistance to INH in TB patients who have just started treatment has not yet been elucidated [10-14]. Despite significant progress over the previous ten years, tuberculosis (TB) continues to be one of the top four killers in Indonesia. Several challenges for the National TB Programs include the findings of recent survey which revealed higher prevalence of TB than previously believed, an increase in drug-resistant TB cases (MDR-TB or multidrug-resistant TB), findings of TB and MDR-TB cases in public and private hospitals with insufficient facilities, and the low contribution of private health service providers in case notification of TB [4, 16, 17]. Pulmonary TB cases are still rising from year to year (also known as re-emerging disease) in line with the increasing cases of HIV/AIDS. Ronal Bayer, a public health expert from the United States, stated that pulmonary TB cases were proof of the failure of public health experts, with the fact that growth in economic status could significantly escalate the cases [21-23]. *Mycobacterium tuberculosis* is known as acid-fast bacilli (BTA) because of their unique characteristics making them resistant to color washing with acids and alcohol. Besides, TB bacteria are also latent and aerobic. When heated to 100°C for 5-10 minutes and then exposed to 70-95 percent alcohol for 15-30 seconds, Mycobacterium tuberculosis perishes. The bacteria is neither light nor airflow resistant, although it can survive in air for 1-2 hours, especially in damp and gloomy environments (it can take months). When heated to 100°C for 5-10 minutes, 60°C for 10 minutes, and with 70-95% alcohol for 15-30 seconds, TB bacteria can be destroyed. Despite longer times they may take (months), the TB bacteria are resistant to damp, gloomy environments for 1-2 hours in the air, but not to light or air currents [21-24]. *M. tuberculosis* produces catalase, yet it stops producing when it is heated at 65°C for 20 minutes in a phosphate bath. *Mycobacterium tuberculosis* which is resistant to INH anti-tuberculosis drugs does not create catalase. Biochemical tests often used to differentiate M. tuberculosis from other species are niacin and nitrate tests. *Mycobacterium tuberculosis* gives positive niacin test results and it also reduces nitrate. Guinea pigs are known sensitive to *Mycobacterium tuberculosis*. Thus, it is frequently used as an experimental animal. If the guinea pig is injected with Mycobacterium tuberculosis, 10 days later you will see swelling at the injection site followed by swelling of the lymph glands and the spread of germs throughout the body [21-25]. There are several test methods applicable to obtain the results of the anti-tuberculosis drug sensitivity test (OAT). The most frequent method used today is the proportion method. The testing requires conducting culture on Lowenstein Jensen (LJ) medium given OAT in proportioned concentrations. In the process, this method takes longer time, about 4-6 weeks to have results [25-27].

2. Materials and Methods

2.1. Study Subject

The population in this study were patients with pulmonary tuberculosis who had checked themselves at the Center for Community Lung Health (BBKPM) Makassar. The samples collected were 100 sputum samples which had been confirmed positive for BTA. Sample processing was carried out at the BBKPM Microbiology Laboratory, while molecular examination was conducted at the Hasanuddin Medical Research Center (HUMRC) laboratory, Faculty of Medicine, Hasanuddin University.

2.2. Sputum Decontamination

Sputum was placed into a sterile 50 ml Falcon tube. Then, a decontamination solution (4% NaOH + 2.9% Sodium Citrate + N-Acetyl L-Cystein) was added in the same volume ratio (1: 1). Next, it was vortexed for 10 seconds and left for 15 minutes at room temperature. Afterwards, it was added with Phosphate Buffer Saline (PBS) pH 6.8 until the volume reached 50 ml, then it was centrifuged at 3000 g for 15 minutes. The supernatant was removed slowly and 1-2 ml of PBS was added to the precipitate/pellet. Furthermore, the decontaminant results were used for smear microscopy with ZN staining and culture and DNA extraction.

2.3. Culture on LJ Medium (Lowenstein Jensen) and Inoculum Preparation

A total of 20µl of the decontaminated specimen was put into the LJ tube. Then it was incubated at 370C for ± 48 days. A total of 1 Ose colony growing on LJ medium was positioned into a sterile tube containing 4 ml of PBS 1x, then it was vortexed and left for 15 minutes. A total of 1 ml of the sample suspension was set into a new sterile tube contained 4 ml of PBS once and then it was vortexed and left for 15 minutes. Prepare for an 0.5 Mcfarland of the suspension.

2.4. DST (Drug Susceptible Test) by MGIT **(***Mycobacteria Growth Indicator Tube)*

Before adding the specimen to the MGIT tube, put 100 µl isoniazid antibiotic and 800 µl OADC (Oleic Acid Dexrose Catalase) first. In this case, 2 MGIT tubes were used for each sample, 1 tube for control (without adding drugs) and 1 tube for the next additional antibiotic isoniazid. Then 500 µl of inoculum which had been diluted before (at the inoculum preparation stage) was added. Furthermore, it was incubated at 370C for ± 42 days. Observations began on the third day after inoculation until the twelfth day. If the control tube is positive on the third day, then the second tube is observed until the fifth day (observation of a tube containing antibiotics is carried out two days after the positive control tube. Readings are conducted by using the MGIT READER.

2.5. DNA Extraction

The DNA extraction method used was sonication and boiling. A total of 1 ml of decontamination results was placed into the Eppendorf tube. Then it was centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded and 100 ul sterile water (ddH2O) was added. The Eppendorf tube containing isolates was put into a water bath (temperature 950C) for 25 minutes, then it was sonicated for 20 minutes and centrifuged at 13,000 rpm for 15 minutes. After that, the supernatant containing DNA was transferred to a new tube. After DNA extraction completed, it can be stored at -200C (if not directly in PCR).

2.6. Multiple Allelic Spesific PCR (MAS-PCR)

This procedure was carried out on isolated DNA samples. M tuberculosis DNA extract as a positive control and aquadest as a negative control. "PCR mix" was put into a PCR tube consisting of 5 µL 10x PCR buffer, 1 µL 10 mM dNTP mix, 2 µL 25 mM MgCl2, 10 µM katGO-F 1 µl, 10 µM katG315-R 1 µl, 0, 25 µL Hostar Taq DNA polymerase 35.75 µL Nuclease free water and 5 µL DNA template/sample. Amplification was conducted by using a PCR machine (DNA thermal cycler). The initial stage was denaturation at 95 ºC for 15 minutes, then 95 ºC for 50 seconds, annealing at 60 ºC for 40 seconds, extension 72 ºC for 1 minute for 35 cycles followed by final extension at 72 ºC for 7 minutes and 4 ºC, 30 minutes for storage.

3. Results and discussion

In this investigation, sputum samples from patients with TB suspicion underwent direct smear testing first. Then, it continued with a decontamination procedure to eradicate all other microorganisms than mycobacteria, including gram-negative bacteria and fungi. It was then followed by a culture procedure using LJ medium after decontamination. According to the results of the culture, all samples (100%) were positive. MGIT approach was applied to follow up encouraging culture results. In this instance, two MGIT tubes were used for each sample, one for the isoniazid antibiotic control and one for the subsequent sample. From the third to the twelfth day after vaccination, observations were made. On the third day, if the control tube is positive, the second tube will be observed until the fifth day (observation of the tube containing antibiotics was carried out two days after the control tube was positive. Isolates from positive culture results was also used for Multiplex Allelic Specific PCR (MAS-PCR). From the results of the DST-MGIT test on 100 positive smear samples, 74 samples (74%) were still sensitive to isoniazid while 26 samples were resistant (26%) as indicated by the presence of colony growth on MGIT media. The objective of the study was examining molecular analysis of isoniazid resistance in pulmonary tuberculosis patients in Makassar. Sample collection was started from patients with suspected TB. Then, they were tested for resistance to isoniazid antibiotics using the MGIT and MAS-PCR methods. These methods were applied to see if there were any samples resistant to isoniazid in Makassar and to discover correlation of resistance occurrence and mutations in katG, inhA and ahpC genes [28-31]. Specimens of sputum or sputum from patients who are suspected of having TB used in this study were first subjected to direct smear examination. Then, it was followed by a decontamination process to kill germs other than mycobacteria, especially gram-negative bacteria and fungi. The decontamination results were used for culture, DST and PCR (MAS-PCR). Based on the direct BTA smear examination results conducted at the Makassar Pulmonary Health Center, 100 samples with positive smears and positive culture result were collected for overall samples. The DST test of all samples obtained 26 samples resistant and 74 samples sensitive to isoniazid antibiotics [32-34]. Ziehl Nelsen staining was used to determine if the colonies emerged on the MGIT media were M. tuberculosis or contaminants for all positive DST results. The appearance of cord development on the AFB smear suggested the existence of M. tuberculosis colony growth. In order to identify mutations in the katG gene at position 315, the ahpC gene, and the inhA gene (markers of isoniazid resistance indicated by the presence or absence of a band formed after electrophoresis), isolates from positive culture results were subjected to M. tuberculosis DNA analysis using the MAS-PCR method [4, 5, 15]. PCR amplification results showed 79 samples were sensitive or susceptible as indicated by the presence of bands in the region of the katG315 gene (293bp), ahpC gene (383bp) and inhA gene (193bp) and 21 remaining samples were resistant to isoniazid. The absence of amplified bands in the katG gene with a target band of 293 bp in 16 samples and the inhA gene with a target band of 193 bp in 2 samples suggested the presence of isoniazid resistance in the 21 samples. Two genes, katG and ahpC, were discovered to be mutated in the other 3 resistant samples [4, 5, 32]. This study examined the effectiveness of molecular methods in rapid resistance identification through mutation profiles of resistance-related genes, specifically the katG, ahpC, and inhA genes which are linked to isoniazid resistance and used as first-line treatments for TB cases. We discovered 81% of INH resistance, as assessed by the gold standard MGIT DST (culture technique), was acquired via MAS-PCR. Meanwhile, no mutations were found in three target genes. Five samples noticed resistant to the DST test indicated only 19% different findings. Perhaps, it was caused by the presence of other mutation hotspots as markers of INHA resistance besides the 3 target genes being tested [5, 15, 25, 32].

Table 1. Distribution Result of DST MGIT and MAS-PCR The samples collected were 100 sputum samples which had been confirmed positive for BTA

DST-MGIT	MAS-PCR		Total
	Resistance	Susceptible	
Resistance		6 (6%)	26 (26%)
Susceptible		74 (74%)	58 (74%)
Total	20 (20%)	80 (80%)	**100**

4. Conclusions

Compared to the culture approach which takes additional two weeks to get results, the PCR method considered produce more quick results. We advise all MAS-PCR results should be verified by sequencing techniques in order to verify the precise DNA sequence. The findings of this study suggest MAS-PCR can be used as a quick test to predict DST yet it can only be used as addition to the gold standard MGIT DST.

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