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Detection of *Aspergillus Sp* Fungus in Tuberculosis Patients with A Treatment Length of 2 Months using the *Polymerace Chain Reaction* (Pcr) Method

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Abstract

This study aims to detect the presence or absence of the Aspergillus sp fungus in Tuberculosis Patients within a 2-month treatment period. This research was carried out in the Microbiology Laboratory at Hasanuddin University Teaching Hospital. Laboratory methods. Samples were obtained from the Tabaringan and Pattingaloang Community Health Centers in Makassar City. Then testing was carried out using culture techniques and polymerace chain reaction techniques. The research results showed that by using the culture method from 30 samples, 9 samples obtained growing media, for 2 samples it was suspected that Aspergillus sp fungus was growing. Then for the polymerace chain reaction (PCR) method, from 30 samples the presence of Aspergillus sp fungus was detected, 4 samples using ASAP primers, then carried out again using ASPU primers (ASPU+Af3r, ASPU+Ni1r, ASPU+Fl2r) which resulted in 1 Aspergillus niger sample. From the results of this research, it can be concluded that the factor that causes the growth of fungus is the long-term administration of anti-tuberculosis drugs which only suppress bacteria from pulmonary tuberculosis.

Keywords: *Tuberculosis Treatment 2 Months,Aspergillus niger, Culture, PCR*

1. Introduction

Infectious diseases in various countries are a serious threat because the impact of these diseases can hinder the development of a country, even in rich and developed countries. Indonesia is a country that has a higher risk of infectious diseases than developed countries, one of which is tuberculosis [1]. Tuberculosis is an infectious disease caused by bacteria (bacilli) known as *Mycobacterium tuberculosis* [2].

Tuberculosis (TB) remains a major global health problem and is the second cause of death from infectious diseases worldwide, after the immunodeficiency virus (HIV). Data from the World Health Organization (WHO) states that there were 8.6 million new TB cases in 2012 and 1.3 million deaths due to TB disease. Even though the TB incidence rate in recent years can be reduced by the use of Anti-Tuberculosis Drugs (OAT), every year the estimate of new cases throughout the world remains high, reaching 9 million cases. Apart from that, pulmonary TB treatment can cause side effects such as progressive loss of lung function and Charonic Pulmonary Aspergillosis. If these tuberculosis bacteria enter and collect in the lungs, they multiply, especially in people with low immune systems and can spread through blood vessels or lymph nodes. Therefore, TB infection can infect body organs such as: lungs, brain, kidneys, digestive tract, bones, lymph nodes, etc., and can manifest in the oral cavity [3].

Pulmonary Tuberculosis, which can be cured with medication, often leaves residual lesions such as cavities, fibrosis, and so on, which are predisposing factors for pulmonary fungal infections. In patients

with pulmonary tuberculosis, the effects of lung anatomy accompanied by long-term administration of anti-tuberculosis drugs will suppress the normal flora so that the growth of opportunistic fungi is not inhibited [4].

The chronic nature of this disease gets worse if accompanied by opportunistic fungal infections. This opportunistic fungus is a potential pathogen in immunocompromised patients, namely patients with several pre- existing diseases and patients on long-term antibiotic therapy such as tuberculosis sufferers. This is caused by a decrease in the immune system which triggers fungal growth and worsens lung tissue [5]. Lung fungal infections often accompany other diseases and there are no typical symptoms so fungal infections are often undiagnosed and the presence of fungus in the lungs is unknown, therefore laboratory examination is very important to see whether the fungus is just normal flora or the cause of infection in the lungs [6].

The cause of the high rate of lung fungal infections in Indonesia is still relatively rare compared to bacterial or viral infections. The fungal group that often causes opportunistic infections in the lungs is *Aspergillus sp* [4].

Fungal lung infections often precede the emergence of other opportunistic infections, but often coexists with other pathogens. Most doctors often miss this fungal lung infection because it does not show specific clinical manifestations and is usually obstructed by other diseases such as tuberculosis and causes high morbidity and mortality rates. Therefore, it is very necessary to have an accurate diagnosis of opportunistic fungal pathogens, especially in Tuberculosis sufferers [7].

The high frequency of pulmonary tuberculosis in Indonesia is one of the causes of the high incidence of pulmonary fungal infections in Indonesia, although it is still relatively rare compared to bacterial or viral infections. The fungal group that often causes opportunistic infections in the lungs is *Aspergillus sp* [4].

Aspergillus sp can colonize the bronchi and lung cavities against the background of pulmonary tuberculosis. It turns out that the fungus *Aspergillus fumigatus* is the one that most often causes Aspergillosis in humans. Aspergillosis generally only develops in immunocompromised individuals. In immunocompromised patients, inhaled and colonized spores invade the lung tissue and grow until they cause lung tissue damage [8].

Based on the background, this research aims to detect the presence or absence of the *Aspergillus sp* fungus in Tuberculosis Patients within a 2-month treatment period. This research was carried out in the Microbiology Laboratory at Hasanuddin University Teaching Hospital.

2. Materials And Methods

The type of research used is laboratory observation. Researchers examined the fungus *Aspergillus sp* on sputum samples from patients with pulmonary tuberculosis using the culture method and *polymerase chain reaction* (PCR). The research design used is a descriptive design, where the research results are presented in the form of tables and narratives. This research has received approval from the Health Research Ethics Committee, Faculty of Medicine, Hasanuddin University, Makassar. The research was conducted at the Microbiology Laboratory at Hasanuddin University Makassar Teaching Hospital. The population in this study were pulmonary tuberculosis sufferers who were undergoing treatment for 2 months. Samples are taken *accidental sample* namely determining the sample by taking existing or available data on site as many as 30 samples.

The culture method used is to study fungal colonies that grow on the media *Sabouroud dextrose agar* (natural resources). Take 10µl of sputum using a loop. Shading on the media surface (SDA) *Sabroud dextrose agar*. Put it in the incubator at a temperature of 26-28^{o}C for 10 days. Interpretation of results colony *Aspergillus fumigatus* if white filament growth is found on the surface of the media which then produces velvet-like spores with a grayish white color. Sputum DNA extraction is carried out by preparing the sample, placing the sample in an Ependorf tube. Then centrifuge at a speed of 14.000–16.000 rpm for 3 minutes, remove the supernatant, add 200ul of GST Buffer solution and 20ul of proteinase K, vortex for 10 seconds, incubate at 60°C for 30 minutes, where every 5 minutes it is vortexed. (*Cell lysis*) Add 200ul GSB Buffer vortex, incubate at 60°C for 20 minutes where every 5 minutes vortex. (DNA Binding) Add 200ul Ethanol vortex for 10 seconds. Insert into the GS Column in a 2ml Collection tube. Centrifuge 14.000–16.000 rpm for 1 minute, discard the liquid in the collection tube. (Wash) Add 400ul W1 Buffer, Centrifuge 14.000–16.000rpm for 30 seconds. Discard the liquid in the collection tube, add 600ul Wash Buffer Centrifuge 14.000–16.000 rpm for 30 seconds. Replace the collection tube with a new one, centrifuge at a speed of 14.000–16.000 rpm for 3 minutes. (Elution) Transfer the GS Column to a sterile ependorf tube, add 100ul of Elution Buffer which has previously

been heated. Centrifuge with a speed of 14.000–16.000rpm for 30 seconds, removed from the GS Column.

DNA Isolate Extraction Make a suspension (Mac farland bacteria 0.5-1 fungus 1.8-2) in an ependorf tube, centrifuge for 5 minutes at a speed of 300x g. discard the supernatant and add 200µl PBS, add 20µl Proteinase K mix. (Cell Lysis) Add 200µl GBS Buffer vortex, incubate at 60oC for 20 minutes where every 5 minutes it is vortexed. (DNA Biding) Add 200µl Ethanol vortex for 10 seconds. Insert into the GS column in a 2ml collection tube. Centrifuge 14,000-16,000 rpm for 1 minute, discard the liquid in the collection tube. (Wash) Add 400µl W1 Buffer, centrifuge 14,000-16,000 rpm for 30 seconds. Dispose of liquid on *collection tube*, add 600µl Wash Buffer centrifuge 14,000- 16,000 rpm for 30 seconds. Replace the collection tube with a new one, centrifuge at a speed of 14,000-16,000 rpm for 3 minutes. (Elution) Transfer the GS Column to a sterile Ependorf tube, add 100µl of Elution Buffer which has previously been heated. Centrifuge at 14,000-16,000 rpm for 30 seconds, discard GS Column. The liquid contained in the Ependorf tube is a DNA product that is ready for PCR.

PCR was carried out in two stages with 4 different primers. The first PCR stage is intended to determine the type *Aspergillus sp.* The first PCR process uses primers ASAP1 and ASAP2 to amplify the 18S rDNA region. With a fragment size of 521 bp and a second PCR target to determine the specific type of fungus *Aspergillus.* The primers ASPU (Af3r), ASPU (Ni1r), ASPU (Fl2r) were used to produce DNA products with an internal fragment size of 310 bp.

Table 1. *Sequencing* Nucleotide Primer Pairs used in PCR

Primary	**DNA *Sequencing* (5'-3')**
ASAP1	CAGCGAGTACAT-CACCTTGG
ASAP2	CCATTGTTGAAA-GTTTTAACTGATT
ASPU	ACTACCGATTGAATGGCTC-G
Af3r	CATACTTTCAGAACAGCG-TTCA
Ni1r	ACGCTTTCAGACAGT-GTTCG
Fl2r	TTCACTAGATCAGACAGAGT

For every 25µl of PCR mixture, approximately 50-150 ng of total DNA is used *templates*. The first step of PCR uses primers ASAP1 and ASAP2, the second step uses primers ASPU (Af3r), ASPU (Ni1r), ASPU (Fl2r). PCR using the method *Thermal Cycler* (Perkin-Elmer Catus), in order: first PCR, process 4 minutes at temperature 94ºC, then carried out 30 cycles for 1 minute at a temperature of 94ºC, 2 minutes at 55ºC, 90 seconds at 72ºC and the thermal cycle was terminated with polymerase for 10 minutes at 72ºC. Second PCR with the same mixture 25µl. The process is 4 minutes at 94ºC, then carried out 30 cycles for 1 minute at a temperature of 94ºC, 15 seconds at 60ºC, 15 seconds at 72ºC and the thermal cycle was terminated with polymerase for 10 minutes at 72ºC.

PCR results are separated using 2% *agarose gel electrophoresis* and stained with ethidium bromide. The gel is cooked and cooled at a temperature of 50-60ºC after adding ethidium bromide with a concentration of 2g in 100ml TAE Buffer, the gel was allowed to harden for approximately 15 minutes. Then put it on *electrophoresis tanks* with 1x TAE buffer. Electrophoresis was carried out at 100V for 60 minutes. The amplified DNA that has been electrophoresed is visualized using *gel documentation.* The DNA band will be visible and its size will be known based on the molecular size marker which is expressed by *base pair.*

3. Results and Discussion

This research was conducted at the Tabaringan Health Center and the Pattingaloang Health Center in Makassar City with a total sample of 30. The PCR technique was carried out at the Microbiology Laboratory, Hasanuddin University Teaching Hospital.

Table 2. Demographic Data by Gender

Gender	Amount	%
Man	16	54 %
Woman	14	46 %
Total	30	100%

Table 2. It can be seen that out of 30 samples, 16 patients (54%) had TB suffered by men, while 14 had women (46%).

Table 3. Demographic Data by Age

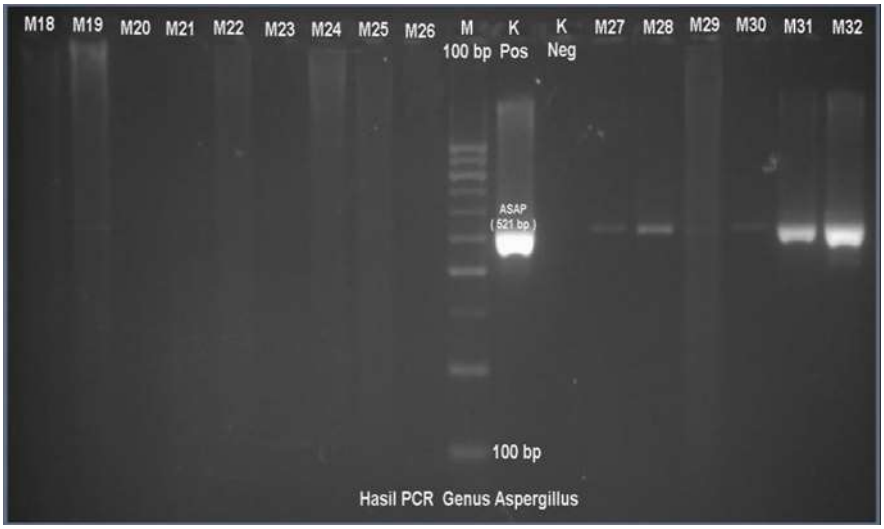
Age	Amount	%
0-20 years	0	0%
21-45 years	16	54%
> 45	14	46%
Total	30	100%

Table 3. it can be seen that our of 30 samples, 16 patients (54%) had TB aged 21-45 years, while aged >45, 14 patients (46%) had TB.

PCR result: Sputum samples of 30 that met the inclusion and exclusion criteria were cultured using SDA media. Further extraction and DNA amplification was carried out using the PCR method using 2 primers (ASAP) and (ASPU+Af3r, ASPU+Ni1r, ASPU+F12r).

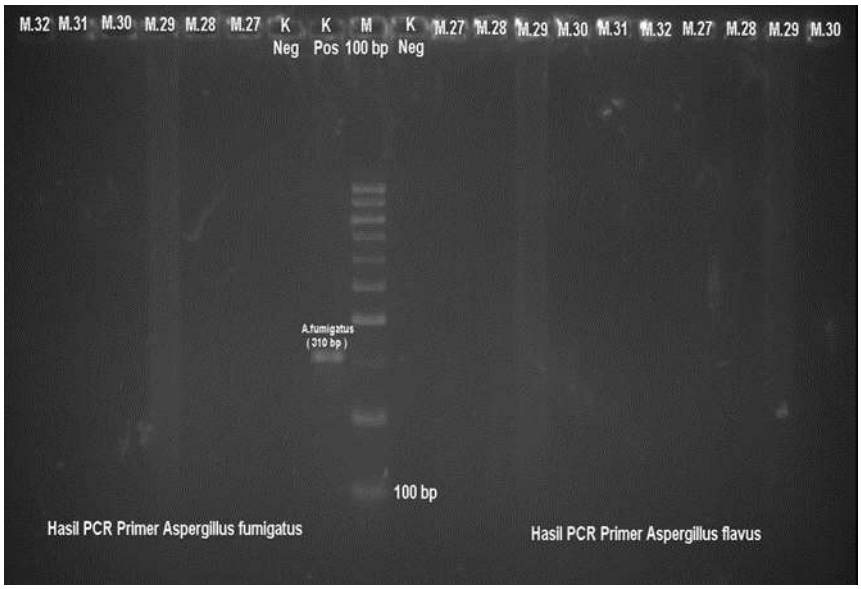
Mushroom PCR Examination Results *Aspergillus* (Direct sputum samples) can be seen for fungal detection *Aspergillus* Of the 30 direct sputum samples that had been extracted, no fungus was found *Aspergillus* is phenotypically but DNA from fungi was found *Aspergillus Sp* based on the ASAP primer sequence from the PCR journal reference *Identification system for the genus Aspergillus and three major pathogenic species: Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger* [9].

In Picture.1 below are the results of DNA amplification for several samples using ASAP primers. It can be seen that in samples M27, M28, M29, M30, M31, and M32 a band was formed at 521bp which means it was found *Aspergillus sp.*



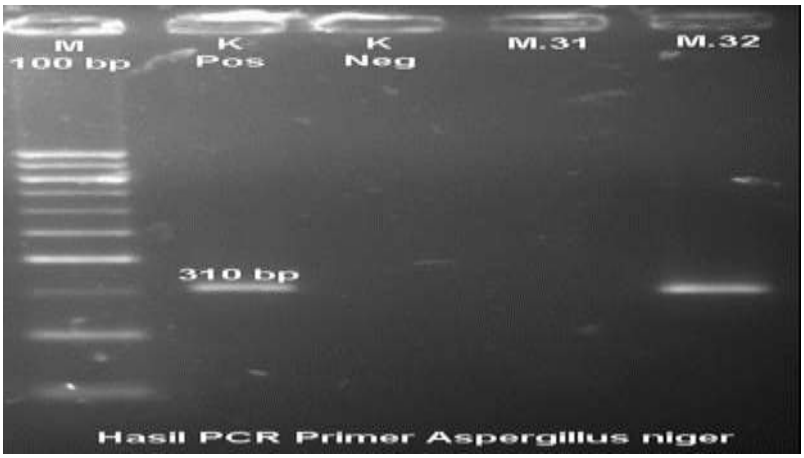
Picture. 1 ASAP Primer
(M18-M26 sample code), M (marker) K+ (Positive control), K- (negative control),
(M27-M32 sample code).

Then the results of the Mushroom detection *Aspergillus* Of the 30 samples, 2 of them, namely numbers M31 and M32, sputum results from SDA culture media were found to be fungus. *Aspergillus* by phenotype and identified by genotype *Aspergillus sp* based on ASPU primer sequences (Af3r, Ni1r and Flr2) used from PCR journal references *Identification system for the genus Aspergillus and three major pathogenic species: Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger* [9].



Picture 2. ASPU+Af3r and ASPU+Fl2r primers
(M32-M27 Sample code), K-(Negative control), K+(Positive control), M (Marker), K- (Negative control), (M27-M30 Sample code).

Picture 2. Above is the result of DNA amplification using primers ASPU+Af3r and ASPU+Fl2r. It can be seen that some samples did not form bands but in the positive control *Aspergillus fumigatus* a 310bp band is formed.



Picture 3. ASPU+Ni1r primer
M (Marker), K+(Positive), K-(Negative), (M31, M32 sample code)

Picture 3. Above is the result of DNA amplification using the ASPU+Ni1r primer. It can be seen that the M32 sample was 310bp, which means the presence of fungus was detected *Aspergillus niger*

Based on research conducted at Hasanuddin University Teaching Hospital with the title Detection of *Aspergillus sp* Fungus in Tuberculosis Patients with a Treatment Time of 2 Months Using 2 methods, namely the culture method and the PCR method. The culture method is the gold standard method for identifying types of fungi and bacteria which is then followed by staining. Standard sputum culture has low yields in detecting fungi. Conventionally only a small proportion of cultured specimens. Therefore the performance of High Volume Culture (HVC) in where all specimens were plated on Sabouraud Dextrose Agar (SDA) [10]. Then, from the culture results on 30 samples that grew, namely 7 samples, it was continued with gram positive staining. For the PCR method, different primers are used with the aim of detecting positive Aspergillus sp fungi and detecting the type of fungus of the *Aspergillus sp*.

Seen in Table. 3 of 30 samples detected *Aspergillus sp*. There are 4 samples using Primer (ASAP). Then continue with the Primary (ASPU+Af3r, ASPU+Fl2r, ASPU+N1rl) that is detected is *Aspergillus niger* on the sample with code M32. *Aspergillus sp* is a type of opportunistic fungus that is commensal in immunocompetent people, but has the potential to cause disease in people who are immunocompromised.

In this study, there were several factors that caused the presence of *Aspergillus sp* fungus in tuberculosis sufferers, namely gender, age and length of treatment. The results obtained are in line with Kawanura's

previous research which stated that the sex with the most *Aspergillus sp* fungus is men compared to women [11]. This is because women are thought to be due to the hormone Estradiol in women which functions to increase the cellular immune response through macrophage activity by IFN-gamma which causes women have more resistance against Tuberculosis than men. Other impacts are also lifestyle exposure such as smoking and work. Then another factor is age which is in line with research from Putu Agus's that almost 70% of pulmonary tuberculosis patients are in the productive age group between 15-64 years due to changes in the anatomy and physiology of the body's organs [3].

In this study, PCR results showed that *Aspergillus niger* fungus was detected in pulmonary tuberculosis patients who were given anti-tuberculosis drug treatment. Patients with pulmonary tuberculosis infection are very susceptible to suffering from opportunistic mycosis infections as a result of which the body's defenses are compromised.Pulmonary tuberculosis accompanied by opportunistic fungal infections tends to be virulent and more fatal. This is also in line with previous research from India. In 2002 to 2003, with sputum cultures of patients who were positive for chronic pulmonary tuberculosis and had received treatment, the most common patient suffering from fungal infections (46%) was *Aspergillus niger*. *Aspergillus niger* is a filamentous fungus that grows everywhere.

The discovery of fungi in sputum can also occur due to contamination of the normal flora of the oral cavity during sputum collection. Candida albicans is a normal flora of the oral cavity, this fungus can be found in the sputum of patients who do not have pulmonary mycosis because the patient does not collect sputum aseptically [12].

Long-term use of antibiotics is also a factor that causes these fungi to grow, where long-term use of antibiotics can cause resistance and increase colonization, because there is suppression of endogenous flora, antibiotics cause the body to lack vitamin B12 and vitamin K. Increased colonization causes function from phagocytosis which can then disrupt the body's defense process to fight infections that enter the body, long-term use of antibiotics causes the immune system to decrease and fungi easily grow and become pathogens, in the scope of Ahmad's research [13].

Contamination can occur at various stages of the diagnostic process (a) During sampling, handling or storage of clinical specimens, (b) During DNA extraction, either from exogenous environmental contamination or endogenously from the extraction reagent itself, (c) During PCR setup, again from environmental contamination or from PCR reagents At any point in the test implementation chain due to inherent contamination from previously generated PCR, (d) Products present in high concentrations in the laboratory, (e) During DNA extraction or PCR setup due to cross sample contamination when samples with very high levels of template DNA cause template migration into adjacent reactions through aerosol production [14].

The advantage of the PCR method is that it has high specificity, it can provide the same results very quickly on the same day, it can differentiate between microorganism variants and the microorganisms detected do not have to be living [15].

4. Conclusion

Based on research conducted at the Unhas Teaching Hospital. Mushroom results were obtained *Aspergillus sp* on samples M27, M28, M29, M30, M31 and M32. Then continued running the second PCR was obtained *Aspergillus niger* on sample M32. The factor that causes the growth of fungus is the long-term administration of anti-tuberculosis drugs which only suppress bacteria from pulmonary tuberculosis.

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