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IS6110 Genomic Variability in the *Mycobacterium Tuberculosis* Complex Sputum Pulmonary TB and HIV TB Coinfection

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Abstract : *World's population is infected with MTB, a cause of death globally, with an increasing prevalence in several countries. To directly detect M.tuberculosis complex bacteria in non-cultured specimens, the target the IS6110 gene was used as a primer for in vitro amplification. Samples were analyzed by microscopy and molecular detection methods according to standard methods at the Clinical Microbiology Laboratory at Hasanuddin University. The 137 sputum samples of pulmonary TB patients. All samples were confirmed positive for TB and Rifampicin Resistance using the GeneXpert method. The analysis results showed that the sensitivity of the IS6110 PCR gene compared to TCM in pulmonary TB patients was 34%, while the specificity could not be assessed because there were no negative TCM results. In studying MTB infection, prompt diagnosis and identification are the main factors to control the spread of MTB bacilli and initiate appropriate tuberculosis management. The use of the IS6110 detection method is considered quite helpful because it can distinguish MTB from other Mycobacteria species. As a result, health facilities can provide a diagnosis fast and TB patients can immediately get appropriate TB drug therapy.*

Keywords - *GeneXpert, Polymerase Chain Reaction (PCR), acid-resistant bacilli*

I. INTRODUCTION

The increase in TB cases globally is of concern to every country in the world because TB is the second cause of death from all infectious diseases in the world [1]. Based on data for 2022, the Ministry of Health detected more than 700,000 new cases of TB. TB disease in India ranks third after India and China, with

824,000 cases and 93,000 deaths per year, or 11 per hour. Based on the 2022 Global TB Report, the highest TB cases are in the age group of 25 to 34 years. In Indonesia, the highest number of TB cases are aged 45-54 years [2]. Data on new case findings for 2023 shows a figure of 74% from the previous year and 86% for drug-sensitive TB and drug-resistant TB [3]. According to WHO, by 2021, around 470,000 people will have MDR (Multi Drug Resistant) Rifampicin Resistant TB, and around 180,000 will die from this form of TB each year [4]. The increase in TB cases globally is of concern to every country in the world because TB is the second cause recorded 214,000 people died from TB co-infected with HIV in 2020 [5]. In cases of severe immunodeficiency, the rate of extrapulmonary and disseminated TB is increased and, because it is difficult to diagnose, it can lead to mistaken hospital death [6]. People living with HIV have increased susceptibility to active tuberculosis and the largest HIV infection for tuberculosis worldwide [7]. None a very satisfactory approach and attention has recently turned to molecular diagnostic methods [8].

The PCR (Polymerase Chain Reaction) test is a fast and easy method for detecting *Mycobacterium tuberculosis* (MTB) using the principle of nested real-time PCR and molecular technology. The PCR (Polymerase Chain Reaction) method is more sensitive than microscopic examination. For sputum examination through a microscope, bacterial bacilli that have living cells in the form of rods or cylindrical must be visible. The duration of the sputum examination also affects the sensitivity of the examination results. Sputum must be sent to a hospital laboratory in less than two hours. For more than two hours, germs will die if not stored in the refrigerator. However, conventional culture techniques cannot provide fast diagnostic results and require BSL (Biosafety Level) II/III laboratory procedures and facilities which cannot be fulfilled by all health services [9]. The level of sensitivity and specificity was determined using the IS6110 primer of the *Mycobacterium tuberculosis* complex. Sensitivity measurement using primary dilution IS6110 can detect MTB up to a minimum of 10^{-1} CFU/mL, with a specificity of 100% (no amplification band is formed to identify other microorganisms). With this molecular detection, it can help identify MTB more quickly and accurately compared to the AFB method that has been used so far. AFB staining with the Ziehl-Neelsen method has a low sensitivity because it takes > 5,000 – 10,000 bacilli/mL sputum to get a positive result. In addition, the specificity value of AFB staining is low because this staining is very dependent on the quality of the slides made and the color contrast of AFB when observed through a microscope. In addition, the Ziehl-Neelsen method cannot differentiate MTB from other *Mycobacterium* species, so the specificity value is low. This is in line with the research conducted by Kohli and Osei which stated that IS6110 Gen PCR is a simple method and can be used routinely with practical use. The IS6110 Gen PCR test is no less sensitive than Ziehl Neelsen staining with a positive AFB result. In addition, the PCR method does not require a large number of samples in the process [10],[11].

In this study, the presence and diversity of *Mycobacterium tuberculosis* isolates in the IS6110 insertion sequence were analyzed using the PCR method. This method is most widely used to differentiate strains based on the variability of the number and position of the chromosomes IS6110 [12],[13]. IS6110 detection by PCR method can be used for diagnosis of *Mycobacterium tuberculosis* and this gene can cause antibiotic resistance in isolates. For this reason, this study aims to reveal whether there is specificity in identifying the IS6110 gene in *Mycobacterium tuberculosis* isolated from the sputum of pulmonary TB patients in the HUMRC and HIV TB laboratories at Dr. Wahidin Sudirohusodo Hospital and BBKPM Makassar. In particular, there are three main concerns of this study: 1) the degree of positive AFB in the sputum examined; 2) the sensitivity and specificity of the results of the Molecular Rapid Test for sputum; and 3) the IS6110 gene in *Mycobacterium tuberculosis* isolated from the sputum of pulmonary TB patients at the Research Center Laboratory Hasanuddin University Medicine, Makassar, South Sulawesi, Indonesia.

II. MATERIAL AND METHODS

The study for pulmonary TB samples was carried out from September 2022 to February 2023 at the Hasanuddin University Medical Research Center (HUMRC) laboratory, Hasanuddin University Medical Faculty, Makassar, South Sulawesi. Patients enrolled in the Pulmonary TB sample study were Multi-Drug Resistant Tuberculosis (MDR-TB) who participated in the LPA (Line Probe Assay) program from the Ministry of Health of the Republic of Indonesia. In addition, this study also conducted HIV TB sampling which was carried out from December 2022 to May 2023 at the Makassar Community Lung Health Center and Dr. Wahidin Sudirohusodo Hospital Makassar. This study also identified the IS6110 gene through sputum samples taken directly from HIV TB patients treated at the Makassar Community Pulmonary Health Center and Dr. Wahidin Sudirohusodo Hospital as many as 23 samples.

A total of 137 sputum samples from pulmonary TB patients and 23 HIV TB samples underwent GeneXpert examination and smear examination for initial TB screening. DNA extraction was carried out from processed decontaminated sputum specimens using the spin column method (Geneid "gSYNC™ DNA Extraction Kit) and continued with PCR examination to detect *Mycobacterium tuberculosis* complex and *Mycobacterium tuberculosis* by identifying the IS6110 gene region of the bacterium. In this test, the H37Rv-1

strain was used as a positive control whereas *Mycobacterium tuberculosis* H37Rv-1 isolate was the ATCC reference gene. Identification and differentiation of MTBC and MTB were carried out using a pair of primers in the IS6110 and MTP40 regions (Table 1) [14].

III. RESULT AND DISCUSSION

Characteristics of Study Samples in Pulmonary TB and HIV TB Patients

Most of the pulmonary TB respondents ranged from 31-50 years (40.9%), the smallest proportion was 17-30 years or (27.0%), and the rest >50 years (32.1%). Based on gender, most were male (56.9%) and the rest were female (43.1%). Positive smear examination found 71 (51.8%). This shows a positive result for the presence of *Mycobacterium tuberculosis* based on Ziehl Neelsen staining. Meanwhile, there were 66 (48.2%) negative BTA. The results of the Molecular Rapid Test (GeneXpert) examination of 137 samples of pulmonary TB isolates were all Rifampicin Resistance (Table 2).

23 research samples confirmed TB-HIV. The results of the BTA sputum examination obtained 56.5% negative smear results and 43.5% positive smear results. The age of the sample that was taken was mostly 31-50 years old at 47.8% and a small portion was aged > 50% as much as 13.1% while the rest were aged 18-30 years as much as 39.1%.

Distribution of IS6110 Gene PCR Examination Results and GeneXpert TCM Examination Results Pulmonary TB patient

The IS6110 gene PCR examination results obtained 46 (33.6%). This showed a positive result for the presence of *Mycobacterium tuberculosis* complex based on the IS6110 gene PCR examination, while a negative result for the IS6110 PCR gene was obtained by 91 (66.4%). The results of the analysis showed the sensitivity of the IS6110 Gen PCR compared GeneXpert TCM in Pulmonary TB patients is 34%, while its specificity cannot be assessed because there are no negative TCM results (Figure 1).

Examination with negative TCM resulted in negative IS6110 Gen PCR 0 (0%), while the positive TCM test resulted in 46 (33.6%) positive IS6110 Gen PCR (Table 3). Table 3, it cannot be analyzed using continuity correction because no negative TCM was found.

There were 4 samples of IS6110 gene PCR results which were confirmed by sequencing and analyzed with the NCBI Blast tool. Blast results show that four samples have 99% identity of *Mycobacterium tuberculosis* strain H37Rv-1 (Figure 2).

Sensitivity and Specificity Between GeneXpert TCM Results and IS6110 Gene PCR Results In TB-HIV Patients

Based on the results of the analysis, the sensitivity and specificity cross tables obtained from the TCM GeneXpert results and the IS6110 gene PCR results were obtained. Based on the results of the TCM examination, as many as 8 patients (57.1%) were confirmed positive for the IS6110 gene. Based on these results, a sensitivity value of 57.1% was obtained. Based on the results of the TCM examination, 3 patients (33.3%) who were negative for TB-HIV were confirmed negative for the IS6110 gene with a specificity value of 33.3%. Based on the results of the Fisher exact test, $p = 0.312$ ($p > 0.05$) it can be concluded that there is not much difference in sensitivity and specificity between the TCM GeneXpert results and the results of the IS6110 gene PCR examination in TB-HIV patients. Fourteen HIV TB samples that were positive for PCR Gen IS6110 were followed by sequencing and blast results showed 99% identity of Mycobacterium tuberculosis strain H37Rv-1.

Sensitivity and Specificity Between MTP40 Gene PCR Results and IS6110 Gene PCR Results In TB-HIV Patients

Based on the results of the MTP40 gene PCR examination, as many as 12 patients (85.7%) were confirmed positive for the IS6110 gene. Based on the results, a sensitivity value of 100%. Based on the results of the PCR examination, as many as 9 patients (100%) who were negative for TB-HIV, were confirmed

negative for the IS6110 gene with a specificity value of 81.8%. Based on the results of the Fisher exact test, p = 1.000 (p <0.05) it can be concluded that there is a difference between the PCR results of the MTP40 gene and the PCR results of the IS6110 gene in TB-HIV patients.

The research subjects obtained were 23 research samples who were confirmed TB-HIV and met the inclusion criteria. The research characteristics consist of the demographic characteristics of the research subjects and the clinical characteristics of the research subjects. The demographic characteristics of the research subjects are age, gender, occupation, education level, and marital status. The clinical characteristics of the research subjects, namely the results of BTA. Based on the age of the sample, most of the samples were aged 31-50 years by 47.8% and a small proportion were aged >50% as much as 13.1% while the rest are aged 18-30 years as much as 39.1%. Based on gender, some of the research samples were male 87.0% while women are 13.0%. Based on the work environment, most of the samples had worked as much as 60.9% while not working as much as 39.1%. Based on the level of education, most of them are basic education, 78.3%, while higher education is 21.7%. Based on marital status, 52.2% were not married, and not much different from married status, 47.8%. Based on the results of the BTA sputum examination, 56.5% of BTA negative results were obtained and 43.5% of BTA results were positive.

The frequency distribution of IS6110 gene identification results in the sputum of TB-HIV patients. Based on the results of these examinations, out of 23 TB-HIV sufferers, 14 patients (60.9%) confirmed positive results for the IS6110 gene and as many as 9 patients (39.1%) confirmed negative for the IS6110 gene. . Based on the results of the TCM examination, as many as 8 patients (57.1%) were confirmed positive for the IS6110 gene based on these results, a sensitivity value of 57.1% was obtained. Based on the results of the TCM examination, as many as 3 patients (33.3%) were negative, confirmed negative for the IS6110 gene with a specificity value obtained of 33.3%. After conducting the FisheFishert test, a value of p = 0.312 (p> 0.05) was obtained, which means that there is not much difference in sensitivity and specificity between the TCM GeneXpert results and the IS6110 gene PCR examination in HIV TB patients.

In the context of TB-HIV co-infection, IS6110 has been used to investigate epidemiological aspects of TB among HIV-infected persons. For example, in a study conducted in Australia, analyzing TB clinical isolates used IS6110-based restriction fragment length polymorphism analysis (RFLP) to determine the proportion of extrapulmonary TB cases caused by new infections and to identify risk factors for extrapulmonary TB [15]. Espirito Santo State, Brazil characterized TB strains isolated from patients with extrapulmonary and pulmonary TB using the IS6110 fingerprint to explore possible associations between genotype and clinical TB. Several of these research studies show that the IS6110 gene has been used to assess genotypic diversity and the dynamics of TB transmission in HIV-infected persons [16].

Recently, digital PCR (dPCR) has been used for the diagnosis of TB, using IS6110 and other targets to detect *M. tuberculosis* DNA. A study assessed dPCR accuracy using IS6110 and IS1081 as amplification targets and found that IS6110-dPCR was more sensitive than IS1081, with a sensitivity of 40.6% and a specificity of 93.4% [17].

IS6110 has been used in various molecular techniques for the identification and detection of TB. Two methods commonly used are IS6110 restriction fragment length polymorphism (RFLP) and spoligotyping. IS6110 RFLP involves the digestion of MTBC DNA with restriction enzymes and visualization of the resulting fragments by gel electrophoresis. Spoligotyping is a PCR-based method that identifies the presence or absence of certain spacer sequences at the CRISPR locus. Both methods rely on the presence of IS6110 in the MTBC genome for the identification of strains and genotypes [18].

The use of IS6110 in the diagnosis of TB has also been explored. Studies have evaluated the amplification of the IS6110 sequence, along with other genetic markers, in sputum samples to evaluate the efficacy of a specific PCR-based test for the direct detection of M. tuberculosis in patients with suspected TB. The sensitivity and specificity of this nucleic acid amplification test were found to be higher than the bacteriological detection [19].

A digital PCR test incorporating the IS6110 has been developed to detect Mycobacterium tuberculosis DNA in the plasma of TB patients to help diagnose TB smear-negative, which can be a challenge in the management of TB. The IS6110 insertion sequence is a valuable tool for molecular epidemiology, diagnosis, and characterization of Mycobacterium tuberculosis strains, especially in the context of TB and HIV co-infection. However, due to time limitations in research, it is necessary to carry out genotype analysis to determine patterns of transmission, explore risk factors, and investigate the relationship between TB/HIV genotypes. In the study of pulmonary TB patient specimens, we used stored sputum samples (in the form of isolates) which were used for LPA 1st-line examination. a decrease in the number of bacteria so that the PCR-positive detection rate becomes low.

IV. FIGURES AND TABLES

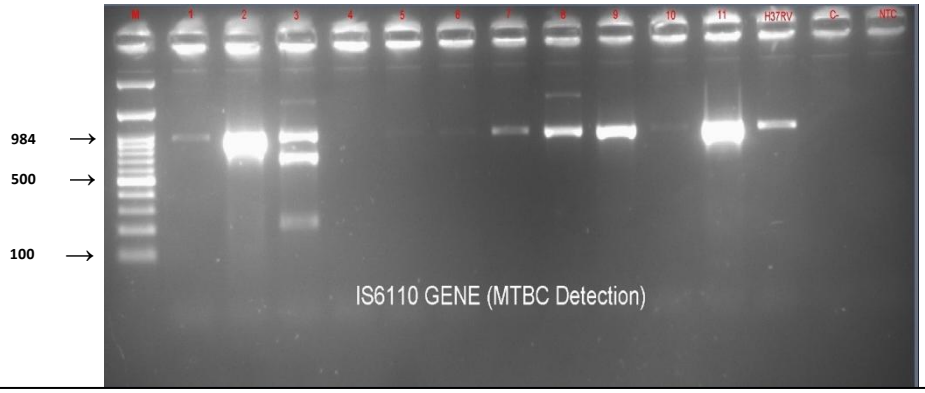

Figure 1. Agarose gel electrophoresis of multiplex PCR for MTBC info. Lanes-1-3, 6-11 represent clinical samples positive for IS6110; lanes 4 and 10 represent negative clinical samples; lane 12: H37RV as a positive control; lane 13: negative control and lane 14: Non template control

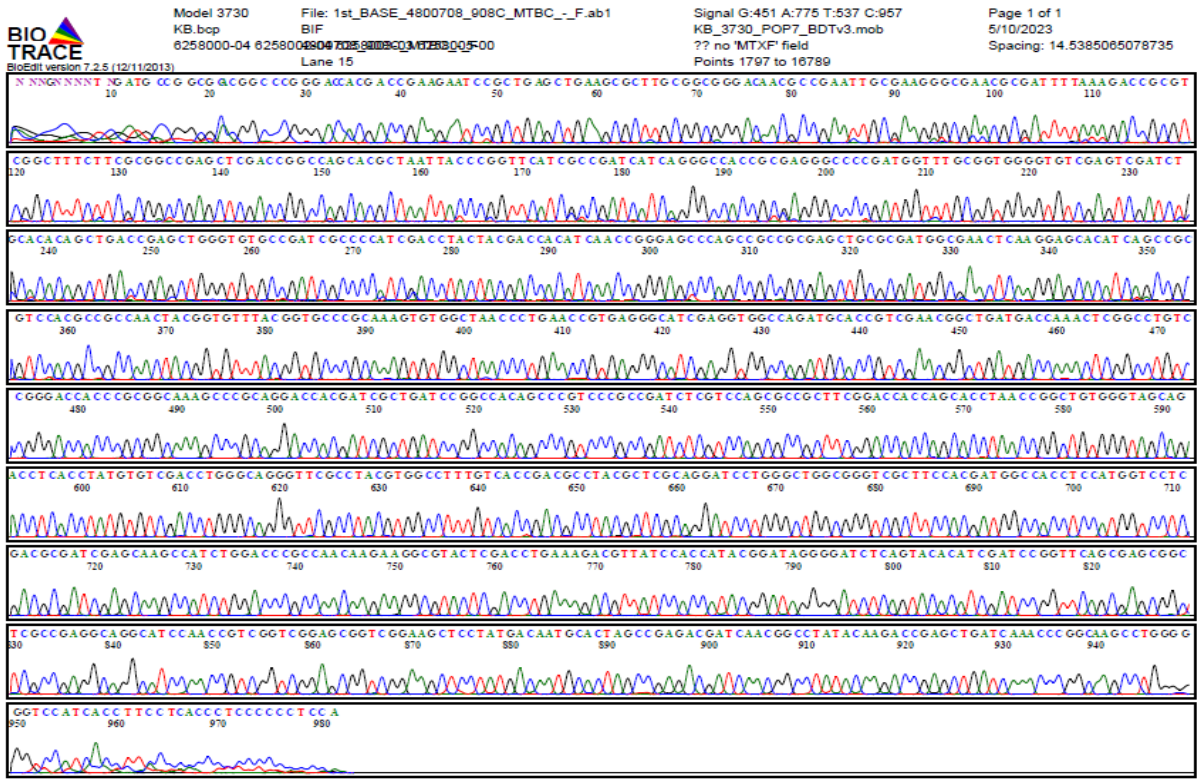

Figure 2. The sequencing result of IS6110 gene

Table 1. Sequences Of Primers Used In Nested Multiplex PCR For Detecting *Mycobacterium spp*

Genes	Primers	Sequence (5'-3')	Size (bp)	Reference
MTP40[a]	MTB F MTB R	CGGCAACGCGCCGTCGGTGG CCCCCACGGCACCGCCGGG	396	Herrera E.A. 1996
IS6110[b]	MTBC F MTBC R	CGGAGACGGTGCGTAAGTGG GATGGACCGCCAGGGCTTGC	984	Wojciech, 1992

[a]specific for *Mycobacterium tuberculosis*

[b]specific for *Mycobacterium tuberculosis complex*

Table 2. Characteristics of the study sample (n=137) in pulmonary TB patients

Variable	n	%
Age		
17-30	37	27.0
31-50	56	40.9
> 50	44	32.1
Gender		
Male	78	56.9
Female	59	43.1

Results acid-resistant bacilli		
Positive (+)	71	51,8
Negative (-)	66	48.2
GeneXpert		
Rifampicin Resistance Positive	137	100
Negative	0	0
PCR IS6110 Gene		
Positive (+)	46	33.6
Negative (-)	91	66,4
Total	**137**	**100**

Distribution of study sample frequencies

Table 3. Distribution of IS6110 Gene PCR Examination Results and GeneXpert TCM Examination in Patients Pulmonary TB

PCR IS6110 Gene Result	GeneXpert TCM Examination Results				Total	p
	Positive		Negative			
	n	%	n	%	n	%
Positive	46	33,6	0	0	46	33,6
Negative	91	66,4	0	0	91	66,4
Total	137	100	0	0	137	100

Sensitivity : 34%
Specificity : Can not be assessed

V. CONCLUSION

The development of the IS6110 detection method is very helpful in making a diagnosis quickly so that therapy can be given earlier to prevent the risk of bacterial transmission. In addition, the therapy given is considered more adequate because this method can differentiate MTB from other Mycobacteria species. It was found that 14 TB-HIV patients (60.9%) confirmed positive results for the IS6110 gene and as many as 9 patients (39.1%) confirmed negative for the IS6110 gene with a sensitivity value of 57.1% and a specificity of 33.3%. Based on the results of the MTB PCR examination, a sensitivity value of 100% and the specificity obtained was 81.8%.

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