



HLA-DQB1*05:02 Allele Association with Anti-Tuberculosis Drug Induced Liver Injury: A Single-Hospital Based Study in Jakarta, Indonesian Population

Kinasih Prayuni¹, Hilyatuz Zahroh¹, Syafrizal², *Rika Yuliwulandari³

¹Genetic Research Center, YARSI Research Institute, YARSI University, Jakarta, Indonesia, ²Pasar Rebo General Hospital, Jakarta, Indonesia, ³Faculty of Medicine, University of Pembangunan Nasional Veteran Jawa Timur, Surabaya, Indonesia.

*Email: rika.yuliwulandari@yarsi.ac.id

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Abstract: Past studies have delved into the genetic factors underlying anti-tuberculosis drug-induced liver injury (AT-DILI), primarily concentrating on polymorphisms in genes responsible for drug-metabolizing enzymes. However, the immune system's potential impact on drug adverse effects, specifically through genes such as HLA, has received limited attention. Previous research has notably revealed an association between HLA-DQB1*05 and AT-DILI, specifically the prevalence of the HLA-DQB1*05:02 allele in AT-DILI patients. In light of this, our study aimed to investigate a potential link between HLA-DQB1*05:02 alleles and AT-DILI. In this study, we included 51 AT-DILI cases and 59 controls belonging to the Javanese ethnic group. The HLA-DQB1*05:02 genotypes were determined using a customized PCR-based typing method, and the results were further confirmed by analyzing five samples via the Luminex assay. Our findings revealed a significant association between HLA-DQA1*05:02 and the risk of AT-DILI ($P = 0.022$; OR (95% CI) = 6.11 (1.25-29.74)). Moreover, the consistent results obtained from the Luminex assay validated the reliability of the custom PCR-based genotyping approach. This preliminary study sheds light on the relationship between the HLA-DQB1*05:02 allele and AT-DILI within the Indonesian population. Furthermore, our study demonstrates the dependability of custom PCR-based genotyping in detecting HLA-DQB1*05:02 alleles. Nevertheless, further research is imperative to corroborate and expand upon our findings.

Keywords: Anti-tuberculosis-drug induced liver injury; human leucocyte antigen-DQB1*05:02; polymerase chain reaction; tuberculosis.

INTRODUCTION

Tuberculosis (TB) remains a significant global health concern, causing substantial morbidity and mortality. Although predominantly affecting the lungs, TB can manifest in various sites within the body, including bones and muscles. With over a quarter of the world's population impacted, TB primarily afflicts adults, with a higher incidence in males than females. In 2021, Indonesia contributed about 8% of cases and became the third largest country in the world for TB incidence after India and China (World Health Organization (WHO), 2021).

A combination of four anti-tuberculosis drugs, isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA) and ethambutol (EMB), was recommended as a first-line regimen for all populations (American Thoracic Society/Centers for Disease Control/Infectious Diseases Society of America, 2003). This first-line regimen is very effective but can be followed by a high risk of adverse effects. One of the most prevalent adverse

Corresponding Author: Rika Yuliwulandari

Faculty of Medicine, University of Pembangunan Nasional Veteran Jawa Timur, Surabaya, Indonesia

Email: rika.fk@upnjatim.ac.id

medication reactions in TB treatment is anti-tuberculosis drug-induced liver injury (AT-DILI) (Bao et al., 2018). AT-DILI incidence can impede the continuation of anti-tuberculosis treatment, resulting in treatment delays or even treatment failure (Shang et al., 2011).

AT-DILI susceptibility is influenced by various environmental factors, including age, gender, acetylation status, and genetic variants (Urban et al., 2014). Understanding AT-DILI's genetic predisposition is critical for avoiding or controlling the disease (Soedarsono & Riadi, 2020). Previous research on genetic risk factors for AT-DILI has mainly focused on genetic variations in genes encoding metabolic enzymes of TB drugs, especially isoniazid, including N-Acetyltransferase 2 (NAT2), glutathione S-transferase (GST) and Cytochromes P450 (CYP450) (Sahiratmadja et al., 2018; Singla et al., 2014; Suvichapanich et al., 2018; Wattanapokayakit et al., 2016; Yuliwulandari et al., 2019). However, several previous studies have shown that the immune system also has a high probability of contributing to the side effects of AT-DILI (Tostmann et al., 2008).

Several studies have shown that the Human leukocyte antigens (HLA) class II allele, namely HLA-DQ, plays a role in susceptibility to AT-DILI in tuberculosis patients. A previous study in Indonesia found that HLA-DQB*03:02 affects the concentration of isoniazid in the body, triggering an immune response, whereas the G allele in HLA-DQA*01:02 may confer protection against AT-DILI (Perwitasari et al., 2018). However, different results showed that HLA-DQB1*05:02 homozygotes had a higher risk of hepatotoxicity than heterozygotes and those without this allele (Chen et al., 2015a). A study in the Kazakhstan population found that the frequencies of the HLA-DQA1*03:02, HLA-DRB1*08:01, or HLA-DQB1*08:03 alleles were higher in AT-DILI patients than in controls (Kuranov et al., 2014).

Although the results from the previous studies are conflicting, our keen interest lies in investigating the association between HLA-DQ and AT-DILI, mainly focusing on HLA-DQB1*05:02. In this case-control study, we aim to explore the association between HLA-DQB1*05:02 and AT-DILI in Indonesian population, using an in-house PCR method. Polymerase chain reaction (PCR) is a simple and frequently used method for multiplying small amounts of DNA into many copies. It is also specific and sensitive to detect our interest gene target. Furthermore, we aim to evaluate the efficacy of the PCR method for detecting HLA alleles. The findings of this study are expected to contribute to the efficient and effective detection of the HLA-DQB1*05:02 allele in TB patients undergoing anti-TB drug treatment, thereby minimizing adverse treatment effects and optimizing TB treatment outcomes. Moreover, the outcomes of this research are anticipated to support the development of PCR-based kits, facilitating the implementation of personalized medicine approaches in Indonesia.

MATERIALS AND METHODS

Sample Collection

This study involved 110 tuberculosis (TB) patients from Pasar Rebo Hospital in Jakarta, Indonesia. The patients encompassed males and females, ranging in age from 17 to 70 years. The diagnosis of TB was established based on the standards set by the World Health Organization (WHO) (World Health Organization, 2010). All participants were receiving supervised treatment with anti-TB medications. Before their participation, patients provided informed consent by completing and submitting a consent form. The research protocol obtained approval from the Ethics Committee of YARSI University, number 096/KEP-UY/BIA/III/2021.

The eligible controls were patients who did not develop AT-DILI through 6 months of anti-TB therapy, were cured or had completed treatment according to the WHO criteria, and had liver function test results within the normal range during follow-up visits at months 2 and 6. The following were used to exclude the potential controls: Patients having a history of liver illnesses such as hepatitis A, B, and C, hepatoma, liver cirrhosis, and cholelithiasis, as well as those who had abnormal liver function test findings (alanine aminotransferase, aspartate aminotransferase, and total bilirubin) before starting anti-TB treatment (World Health Organization, 2010).

The criteria for selecting cases were as follows: patients over the age of 17 who were diagnosed with tuberculosis and received anti-TB treatment following the standard WHO regimen (2 months of INH, rifampicin, ethambutol, pyrazinamide/4 months of INH and rifampicin). These patients developed AT-DILI, as defined by the National Institute of Health's LiverTox (NIH) (<http://livertox.nlm.nih.gov/Severity.html>) (LiverTox, 2012), and exhibited at least one hepatitis symptom such as anorexia, fatigue, nausea and vomiting, mild fever, or a tender or enlarged liver.

Blood Collection and DNA Extraction

Peripheral blood samples were taken 3 mL from patients into EDTA-containing tubes. The Maxwell RSC Whole Blood DNA kit (Promega, Winconsin, USA) was used to extract genomic DNA from 3 mL EDTA-preserved blood samples using the Maxwell automatic machine (Promega, Winconsin, USA) following the instructions from the kit. DNA quality and quantity were measured using a spectrophotometer Tecan Pro 200 nanoquant (Tecan, Männedorf, Switzerland).

HLA-DQB1*05:02 Allele Amplification

Amplification of the HLA-DQB1*05:02 allele using forward and reverse primers in the following order: FQB05:5-ACG TGG GGG TGT ACC GGG CG-3' and RQB05:5'-GTCCACCGACGCCCGGGCCCC-3' (synthesized by Macrogen Inc, BioPolis, Synapse, Singapore). The primary design used Primer3 targeting exon 2 of the HLA-DQB1 gene with the allele sequence HLA-DQB1*05:02 as a reference. (Untergasser et al., 2012) OligoCalc was used to calculate the physical properties of our primer set, including melting temperature, molecular weight, and %GC content. It also calculates self-complementarity between two identical oligonucleotide molecules and calculates potential intra-molecular hairpin loop formation (Kibbe, 2007).

The PCR reaction mixture is consisted of 12.5 µl of KAPA Multiplex Mix (KAPA Biosystem, Massachusetts, USA), 20 ng of genomic DNA, 10 µM of forward and reverse primers, and nucleus-free water (NFW) (Promega, Wisconsin, USA) up to a total volume of 25 µl then placed in the T-100 thermal cycler machine (BioRad, California, USA). PCR reaction profile was as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, 30 s of annealing at 64°C, and 3 min of extension at 72°C, and the final extension at 72 °C for 1 min. Agarose gel electrophoresis was used to separate PCR products in 2% agarose gel concentration at 100 V for 30 minutes in 1x TAE buffer (Promega, Wisconsin, USA). An 81 bp band of PCR products was visualized by UV gel Documentation (GelDoc) ((BioRad, California, USA). A total of 5 samples were randomly selected for the Luminex Assay test for the HLA-DQ allele to confirm the quality and accuracy of the data from the PCR results. The samples were sent to Laboratorium Terpadu, Faculty of Medicine, University of Indonesia, for Luminex Assay testing. The assay utilized combined Luminex xMAP technology (Thermo Fisher Scientific, Massachusetts, USA) along with the OneLambda LABType (OneLambda, West Hills, CA, USA) reverse sequence-specific oligonucleotide (SSO) method. This was conducted on a suspension array platform using colour-coded microspheres as a solid support to

immobilize oligonucleotide probes. The target DNA is amplified by polymerase chain reaction (PCR) and then hybridized with the bead probe array.

Data analysis

The data of samples possessing the HLA-DQB1*05:02 allele and those lacking this allele were compiled into an Excel spreadsheet. Subsequently, we calculated the frequency of the HLA-DQB1*05:02 allele. The Chi-Square test was employed to evaluate the correlation between HLA-DQB1*05:02 alleles and AT-DILI in both the case and control samples. P-values less than 0.05 were considered statistically significant. Additionally, the odds ratio (OR) and 95%CI were used to express the statistical strength of the link between sample populations and genetic markers. The Mann-Whitney U-Test was utilized for all comparisons in demographic data. Excel spreadsheet software (Microsoft, Wisconsin, USA) was used to complete all statistical calculations.

RESULTS AND DISCUSSION

Our study investigated the relationship between HLA-DQB1*05:02 and AT-DILI with the PCR method using our in-house primer design. We collected TB patients with and without AT-DILI. The demographic data of patients is shown in Table 1. There was no statistical difference in sex between case and control patients, while there was a significant difference in age. There was no statistical difference between AT-DILI patients and controls in baseline liver function tests (data not shown). However, after anti-TB treatment, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin levels were significantly higher in case patients than in control patients. (Table 1).

Table 1. Demographic Data of Case Patients with AT-DILI and Control Patients without AT-DILI

Demographic	Case (n=51)	Control (n=59)	p-value
Sex - No. (%)			
Female	27 (52.9)	31 (52.5)	NS
Male	24 (47.1)	28 (47.5)	
Mean age - Year (range)	44.73 (17-70)	38.25 (18-68)	0.01*
Liver test Function (Mean (range))			
AST	186.57 (29-527)	46 (30-65)	<0.0001*
ALT	180.25 (50-497)	34.67 (18-65)	<0.0001*
Bilirubin	1.85 (1.12 - 6.76)	0.62 (0.3- 0.72)	<0.0001*

ALT: alanine transaminase; AST: aspartate transaminase; NS: not significant
 *Significant differences (p <0.05) were determined using the Mann-Whitney U-Test

Our PCR assay demonstrates its capability to detect the target HLA alleles, specifically in screening patients for HLA- DQB1*05:02. PCR, a widely utilized molecular biology technique, holds immense value in clinical applications. It offers simplicity, ease of understanding and use, and rapid results. Moreover, this technique is relatively cost-effective while displaying high sensitivity in generating specific target gene copies for various clinical applications (Khalil, 2021). The amplification product size, obtained using FQB05 and RQB05, was determined to be 81 bp, as shown in Figure 1. The figure illustrates that sample 1 and sample 9 exhibit the presence of the

HLA-DQB1*05:02 allele, while samples 2-8 and 10 do not possess this allele. However, although PCR is a valuable technology, it also has limitations. Any contamination in the PCR reaction can produce a misleading interpretation. Another issue is that the PCR primers can anneal non-specifically to similar sequences but not identical to the target DNA. Furthermore, even at a shallow rate, DNA polymerase might incorporate the wrong nucleotides into the PCR sequence (Garibyan & Avashia, 2013).

The genotype frequencies in the case and control groups are presented in Table 2. Allele HLA-DQB1*05:02 are more frequent in case samples (17.56%) than in controls (3.39%). Our findings showed that there is a significant association between HLA-DQB1*05:02 and AT-DILI (P = 0.022; OR (95% CI) = 6.11 (1.25-29.74)). Genetic variants in the Human Leucocyte Antigen (HLA) gene have been linked to vulnerability to T-cell-mediated adverse effects from various medications, making it a candidate gene for pharmacogenetic investigations.(Barbarino et al., 2015) Several previous studies showed the association of HLA with increased risk of idiosyncratic DILI, including HLA-DQB1*02:01 and HLA-DQB1*05 to anti-TB treatments (Chen et al., 2015b; Sharma et al., 2002). HLA-B*58:01 and DRB1*01:02 to nevirapine-containing anti-retrovirus (ARV) regimens (Phillips et al., 2013). HLA-B*57:01 and HLA-A*33:03 variant alleles to flucloxacillin and ticlopidine (Daly et al., 2009; Hirata et al., 2008). A comprehensive genome-wide association analysis discovered a substantial link between amoxicillin-clavulanate-induced liver injury and variant alleles of HLA-A*02:01, HLA-DQB1*06:02, and DRB1*15:01 (Lucena et al., 2011). However, a study in the Spanish population could not find an association between HLA alleles and AT-DILI (Leiro-Fernández et al., 2016). Those studies demonstrated that the connection of HLA alleles varies by ethnic group of population (Maróstica et al., 2022).

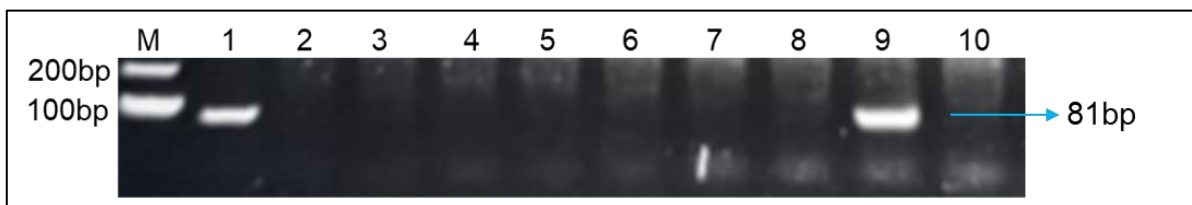


Figure 1. Agarose Visualization of HLA-DQB1*05:02 Allele. M, 100 bp Ladder; Lanes 1 and 9: Contain HLA-DQB1*05:02; Lanes 2-8 and Lanes 10: no HLA-DQB1*05:02 Allele.

Table 2. Frequency of HLA-DQB1*05:02 Allele

HLA Allele	Case (51)	Control (59)	p-value	Odd Ratio (95% CI)
HLA-DQB1*05:02	9 (17.65%)	2 (3.39%)	0.022*	6.11 (1.25-29.74)
Non HLA-DQB1*05:02	42 (82.35%)	57 (96.61%)		

*Significant difference (p <0.05) was determined using the Chi-square test

Our result found that the presence of HLA-DQB1*05:02 increases the risk of susceptibility to AT-DILI among TB patients. Notably, HLA-DQB1*05:02 was found to be more frequent in patients with AT-DILI compared to patients without AT-DILI. Our findings align with the study in the Chinese population (Chen et al., 2015a).

Furthermore, our study revealed that the HLA-DQB1*05 allele can be an essential marker for AT-DILI in TB patients, in addition to previous markers, namely NAT2, CYP2E1 and GST genes (Sun et al., 2008; Wang et al., 2012). However, a large-scale study should investigate the role of other HLA alleles in AT-DILI within the Indonesian population.

The HLA-DQB1*05:02 allele is also a risk factor for flupirtine-induced liver injury in the German population (Nicoletti et al., 2016). Another study reported that HLA-DQB1*05:02 increase the risk of Myasthenia Gravis, an autoimmune disease that affects neuromuscular transmission, in both the Italian and Spanish population (Salvado et al., 2022; Testi et al., 2012). A study in Malaysia also revealed that HLA DQB1*05:02:01 alleles may be associated with an increased risk of colorectal cancer (CRC) (Johdi et al., 2016). The present study showed that HLA-DQB1*05:02 is associated with a higher risk of liver cirrhosis observed in the Iranian population in three-generation, two-generation, and intra-familial groups (Naderi et al., 2023).

Table 3. Luminex Assay Results for Random Selection of 5 Case Samples

No	Sample	HLA Class II (HLA-DQ)		
			Allele 1	Allele 2
1	Sample 1*	HLA-DQA HLA DQB	DQA1*01 DQB1*03	DQA1*06:01 DQB1*05
2	Sample 2	HLA-DQA HLA DQB	DQA1*01 DQB1*03	DQA1*05 DQB1*06
3	Sample 9*	HLA-DQA HLA DQB	DQA1*01 DQB1*03	DQA1*03 DQB1*05
4	Sample 10	HLA-DQA HLA DQB	DQA1*01 DQB1*03	DQA1*06:01 DQB1*06
5	Sample 13*	HLA-DQA HLA DQB	DQA1*01 DQB1*05	DQA1*01 DQB1*06

*Contain HLA-DQB1*05 Allele

The Luminex assay was performed on a subset of 5 case samples, yielding the following outcomes: 3 samples exhibited the presence of the HLA-DQB105 allele, while 2 did not possess these alleles. The results of the Luminex assay are shown in Table 3. Notably, the bold entries in samples 1, 9, and 13 signify the presence of the HLA-DQB1*05 allele, aligning with the PCR visualization results depicted in Figure 1, where samples 1 and sample 9 displayed amplification for HLA-DQB1*05:02. Sample 13 also displayed an 81 bp band in PCR result visualization; however, these results were not depicted in Figure 1. In contrast, the Luminex results for samples 2 and 10 indicated the absence of the HLA-DQB1*05 allele, which coincided with the visualization results showing no amplification in these specific samples.

Over the last decade, the bead-based assay using the Luminex® equipment has become the gold standard for HLA testing. Luminex is the primary technique for regular HLA typing and antibody screening, with little competition from new technologies (Dunn, 2011; Tait, 2016). Generally, the resolution of the Luminex assay is medium but may be high when a rare allele is detected (Dunn, 2011). Our Luminex results showed only two digits of HLA-DQB1, emphasizing the need for confirmation from other equipment capable of detecting at least four HLA alleles, such as next-generation sequencing (NGS). Several high-throughput HLA-typing approaches based on NGS have been developed, offering both high-throughput and high-resolution capabilities (Hosomichi et al., 2015). However, despite the NGS's potential benefits, the cost of sequencing remains relatively high, which could limit its broad adoption. These factors highlight the challenge of balancing accuracy and cost-effectiveness when choosing the best HLA typing approach for specific research situations. In addition, our PCR result could lead to false positive results because PCR is a susceptible technology and any form of contamination of the sample by even trace amounts of DNA can produce misleading results. Furthermore, our PCR method only detects the presence or absence of HLA-DQB1*05:02; it is not helpful if patients need to know the complete allele of HLA-DQB1, especially for organ transplantation.

CONCLUSION

In conclusion, we have successfully developed a PCR assay for detecting HLA-DQB1*05:02 allele, which holds significance in pharmacogenetic screening for AT-DILI among tuberculosis patients. However, it is essential to validate this developed assay using larger sample sizes for further study. Our study reveals a significant association between HLA-DQB1*05:02 and AT-DILI within the Indonesian population. These findings serve as preliminary data, highlighting the need for further research involving larger patient cohorts.

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CONFLICT OF INTEREST

There are no conflict of interest.

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