

Polymerase gene mutation pattern in patients of chronic Hepatitis B treatment in Prof. Dr. W.Z. Johannes Hospital, Kupang, Indonesia

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Abstract. Djuma AW, Dewu S, Kambuno NT, Banunu A, Silaen OSM. 2024. Polymerase gene mutation pattern in patients of chronic Hepatitis B treatment in Prof. Dr. W.Z. Johannes Hospital, Kupang, Indonesia. *Biodiversitas* 25: 136-141. Hepatitis B virus (HBV) infection is a significant global health concern, impacting over two billion people worldwide. Among them, more than 240 million individuals develop chronic HBV infection, placing them at a high risk of developing severe liver conditions such as cirrhosis, liver failure, and Hepatocellular Carcinoma (HCC). In 2013, Nusa Tenggara Timur (NTT) emerged as one of the Indonesian provinces with a high incidence of hepatitis cases. Long-term therapy has proven effective in halting disease progression but may also lead to the emergence of resistant mutations. This study aims to analyze mutation patterns in the Polymerase (P) gene as indicators of viral resistance to nucleotide analogs (NUCs) in HBV patients at RSUD Prof. Dr. W.Z. Johannes Kupang, a previously unreported case. The research was conducted at the Hematology-Oncology Medical Laboratory Division, Faculty of Medicine, Universitas Indonesia, RS. Dr. Cipto Mangunkusumo, Department of Internal Medicine, Jakarta. A total of 28 individual samples with positive Hepatitis B surface Antigen (HBsAg) were subjected to PCR sequencing, resulting in 23 positive samples. Mutation profiles were analyzed by comparing them with the reference sequence M54923. Sequencing of the Polymerase (P) gene region was successfully carried out for 14 samples. Among these, 12 (85.71%) exhibited nucleotide substitutions, while the remaining two (9.14%) did not show any mutations. The most prevalent substitution patterns in the P gene included rtQ228H, rtS127R, rtM135L, rtV243I, rtL257A, rtS127A, rtK323N, rtL326R, rtV214I, rtT312S, rtV281E, rtF290Y, rtM299I, rtQ306H, and rtK308N. No mutations associated with antiviral resistance were detected. The use of NUCs in chronic hepatitis B treatment is considered safe for patients at Prof. Dr. W.Z. Johannes Kupang Hospital.

Keywords: Antiviral resistance, global health, HBV, liver diseases

INTRODUCTION

Hepatitis B disease is a liver condition caused by Hepatitis B Virus (HBV) infection and is a global health issue, especially in developing countries. Approximately 257 million people worldwide have chronic HBV infection based on HBsAg status, with 887,000 deaths in 2015, primarily due to complications like cirrhosis and liver cancer (Borgia et al. 2012; Kim and Kim 2015; Zheng et al. 2015; Muljono 2017). HBV belongs to the Hepadnaviridae family, and its genome is composed of circular DNA, approximately 3.2 kb in length (Philips et al. 2021). Within the genome, there is an overlapping region that contains four distinct genes, namely S, C, P, and X. The S gene primarily encodes a surface protein located in the HBV envelope, all within a lengthy open reading frame. This gene contains start codons that divide it into smaller, medium-sized, and larger polypeptides. These polypeptides are often used individually or in combination with other HBV genes to detect HBV DNA (Pollicino et al. 2014). The P gene encodes the Polymerase protein, a crucial component of the reverse transcription process during HBV replication. The HBV replication cycle lacks a proofreading mechanism, leading to

the production of a highly genetically diverse progeny (Abdukadirova et al. 2020; Bello et al. 2023).

Due to this significant genetic diversity, HBV is classified into ten genotypes, denoted as A through J, with approximately 7.5 percent genetic variance between them. With the exception of E and G, all genotypes are further divided into 25 sub-genotypes, each exhibiting approximately 4 percent variability in amino acids (Munn et al. 2015; Shamseer et al. 2015). The distribution of HBV genotypes varies depending on geographical regions. The prevalence of HBsAg in Indonesia, with a population of over 250 million, was 9.4% in 2007 (Balitbangkes 2007; Wallace et al. 2012), dropping to 7.1% in 2013 (Balitbangkes 2013), shifting the country from highly to moderately endemic (Ie et al. 2015; Zampino et al. 2015; Wijayadi et al. 2018). Previous study reported that the prevalence of hepatitis B and hepatitis C among blood donors in NTT, based on data from the Indonesian Red Cross Society, was 3.5% and 0.5%, respectively (Kambuno et al. 2018). Other studies reported the prevalence of hepatitis B among students in the city of Kupang (Kambuno et al. 2019, 2020), cases of hepatitis B transmission in orphanages in Kupang (Kambuno et al. 2021a), and the prevalence of HBsAg, anti-HBc, and

anti-HBs at 13.2%, 39.8%, and 28.7%, respectively, among high school students in the city of Kupang (Kambuno et al. 2021b).

Currently, chronic hepatitis B treatment options include Nucleos(T)ide Analogs (NAs) and pegylated-interferon alpha (peg-IFN), which can be used alone or together. NAs inhibit the reverse transcriptase enzyme, effectively reducing HBV replication and circulating HBV levels in the host. Several NAs are available, such as Lamivudine (LAM), Adefovir Dipivoxil (ADV), Entecavir Monohydrate (ETV), Telbivudine (LdT), Tenofovir Disoproxil Fumarate (TDF), and emtricitabine. Long-term NA treatment can lower the viral load, slow disease progression, achieve HBeAg seroconversion in some patients, lead to HBsAg loss in a few cases, and lower ALT levels (Wibowo et al. 2020). NAs are preferred due to their oral administration, minimal side effects, high response rates, and relatively low costs. However, prolonged use, especially of NAs with a low genetic barrier or as monotherapy, may result in NA resistance, long-term safety concerns, and the need for indefinite treatment to suppress virus replication (Nguyen et al. 2020).

Long-term NAs treatment can suppress disease progression in patients with high viral loads and reverse fibrosis and cirrhosis. The combination of nucleoside and nucleotide analogs is recommended to overcome drug resistance. Genetic variations in the polymerase gene, especially in the reverse transcriptase domain, can pose challenges to NAs treatment (Wibowo et al. 2020; Pley et al. 2022). There has been no previous research on the mutation patterns of hepatitis B patients undergoing NAs treatment at Prof. Dr. W.Z. Johannes Kupang Hospital. This study represents the inaugural effort to report on the mutation patterns of the polymerase gene at RSUD Prof. Dr. W.Z. Johannes Kupang. Understanding mutation patterns is crucial for clinicians in selecting the most appropriate therapy, which can reduce the viral load and suppress disease development.

MATERIALS AND METHODS

Study subjects

The study subjects consisted of a convenience sample of 28 inpatients and outpatients at Prof. Dr. W.Z. Johannes Kupang Hospital who were undergoing therapy with positive HBsAg. The subjects voluntarily agreed to participate in this research and signed informed consent forms after receiving explanations. This research obtained ethical approval from the Research Ethics Commission of Polytechnic of Health Ministry, Kupang with certificate number LB.02.03/1/0112/2022.

DNA extraction

DNA extraction was performed according to the manufacture procedure using the DNATM Miniprep Kit (ZYMO RESEARCH). Amplification of the HBV Polymerase (P) gene region was conducted using the Polymerase Chain Reaction (PCR) technique with specific primers. DNA amplification of the P region was carried out using primer pairs S2-1 (CAAGGTATGTTGCCCGTTTG

and HS6-2 (GCCAAGTGTGGCTGACGCA) in the first stage and S088 (TGTTGCCCGTTTGCCTCTA) and HS4-2 (CCTATTGATTGGAAGGTGTG) for the second stage. The PCR products were 740 and 514 base pairs (bp). The PCR process utilized the GeneAmp PCR System 9700 (Applied Biosystems, USA) with a total reaction volume of 25 µL, consisting of My Taq Has Ready Mix (BIOLINE) 12.5 µL, forward and reverse primers (400 nM) 1 µL at each, 5 µL of extracted DNA, and 5.5 µL of Diwater. The PCR cycling conditions, including denaturation, annealing, and extension, were as follows: 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min and 30 sec for both stages (35 cycles for the first stage and 30 cycles for the second stage) (Silaen et al. 2020). PCR cycling began with a pre-denaturation step at 94°C for 5 min and concluded with a final extension step at 72°C for 5 min. Positive PCR products, with sizes of 249 bp for the S gene and 740 bp for the P gene, were purified using a PCR purification column (QIAGEN, USA) for sequencing with a DNA sequence analyzer ABI 3130xl (Applied Biosystems, USA).

Analysis of HBV polymerase gene characteristics

The DNA sequences of the polymerase gene were aligned with the HBV sequences obtained from GenBank (Acc. number M54923) as a normal reference, and base numbering in the HBV genome sequences began from the EcoRI restriction enzyme cutting site. Amino acid sequences were obtained by translating DNA sequences after alignment, starting from the ORF P, with amino acid numbering beginning from amino acid 349 of protein P as rt1.22.

Determination of mutations associated with NAs

The determination of mutations related to NAs followed guidelines developed for the Asia-Pacific region, including mutations rtM204V/I, rtL180M, and/or rtA181V/T associated with LAM resistance; mutations rtM204I and/or rtA181V/T associated with LdT resistance; mutations rtA181V/T and/or rtN236T associated with ADV resistance; mutations rtL180M, rtT184S/A/I/L/G/C/M, rtM204V, and/or rtM250V associated with ETV resistance; and mutation rtA181T/V for TDF resistance.

Determination of HBV subtypes

The determination of HBV subtypes was based on phylogenetic analysis of the nucleotide sequences of the polymerase gene, which spans 797 bases, overlapping with the base sequences of the HBsAg gene. HBV subtypes were determined based on amino acid types at positions 122 and 160 of the HBsAg protein.

RESULTS AND DISCUSSION

Demographic and viral characteristics

The material for this study consisted of serum samples from 28 chronic hepatitis B patients undergoing treatment who visited the Department of Internal Medicine at Prof. Dr. W.Z. Johannes Kupang Hospital. Out of the 28 samples, positive HBV DNA was detected in 23 samples. Among these, DNA amplification of the HBV P gene was successfully

performed in 14 samples. HBV diversity can be distinguished based on HBsAg serological reactions and genetic characteristics. There are four known serotypes of HBsAg determined by the antigenicity of immunological epitopes at amino acids 124-147 in the 'a' determinant of HBsAg. Subtypes d/y are determined by the presence of lysine/arginine at amino acid position 122, and subdeterminants w/r are determined by position 160 (Reuter et al. 2022). Based on these positions, four main HBsAg serotypes are recognized: adw, adr, ayw, and ayr. There were 10 individuals with adw subtype, 2 with adr subtype, and 2 with ayw subtype. Furthermore, in terms of genetic characteristics, based on nucleotide sequence similarity, HBV genomes are classified into eight genotypes (A-H) distributed geographically. The age range of the subjects in this study was 22 to 57 years, with an average age of 40.71 years. The distribution of age, gender, genotype, and subtype is depicted in Table 1.

Polymerase gene analysis

The region of the P gene analyzed in this study is the reverse transcriptase region, which is the target for Nucleoside Analogue (NAs) antiviral agents. Patterns of mutations encoding resistance to several NA antivirals that have been widely reported in previous studies (Zoulim 2004; Liaw et al. 2012; Liu et al. 2015) were examined in this study.

This study did not find any mutation patterns related to antiviral resistance. Some new variants were also identified, and these variants are shown in Table 2. The analysis of nucleotide variations was conducted by comparing the P gene sequences of each sample with the wild-type HBV nucleotide sequences from GenBank (Acc. number M54923), which served as the consensus.

Amino acid substitution patterns Q228H, S127A, V243I, and L257A were the most frequently observed. Figure 1 shows the positions in the P gene related to NA antiviral resistance and the forms of new variants identified in this study.

Discussion

Mutations in Gene P

Mutations commonly found in the P gene, particularly in the reverse transcriptase region, have been reported to be associated with resistance to Nucleoside Analogue (NA) antiviral agents (Papatheodoridis 2013). Several antivirals, such as lamivudin, entecavir, telbivudine, and adefovir, have been reported to exhibit specific patterns of amino acid substitutions, which are typically observed in patients who have received treatment for more than 6 months (Kang et al. 2014; Shedain et al. 2017). However, it has been reported that substitutions associated with NA resistance can also be found in untreated subjects (Zhang et al. 2016; Coffin et al. 2018; Qian et al. 2020). This can be explained by the fact that mutations in the HBV genome occur randomly, and variants carrying specific mutations can survive during treatment and evolve into dominant populations (de Almeida Ribeiro et al. 2022).

In this study, no amino acid substitution patterns associated with antiviral resistance were detected. There were no mutation patterns related to resistance to LAM,

Ldt, ADV, EFV, and TDF (Figure 1). Recent mutation patterns in the P gene that have been reported to be associated with reduced treatment efficacy include rtT128N, rtD134E, rtS202G (Belaiba et al. 2022), rtL180M, A181V, T184I, M204V (Tokgöz et al. 2018; Liu et al. 2021), rtT38A, T54Y, I122F, T128A, M129L, Q130P, D131N, S135T, R138K, V191I, T225S, and N238H (Tang et al. 2021), rtQ215S, rtF221Y, rtQ149K, rtL122F, rtN118D/T, rtL157M, rtH124Y (Ziaee et al. 2016).

The amino acid positions most frequently undergoing substitution and resulting in mutations were positions 228, 127, 243, 257, and 323 (Table 2). The mutation patterns observed in untreated subjects can be explained by the random emergence of variants due to the lack of a proofreading system during HBV replication. Another determining factor is the availability of replication space that supports the development and survival of these variants. For instance, during the immune-tolerant phase, viral levels are high, and all available space is occupied by the wild type, leaving no opportunity for variants to develop (Seto 2019).

Table 1. Demographic characteristics

Characteristics	n=14
Age (median,min;max) (+SD)	42 (22;57)
Men (n)	8
Woman (n)	6
Subtype	
<i>adw</i> , (n)	10
<i>adr</i> , (n)	2
<i>ayw</i> , (n)	2

Table 2. Amino acid substitution patterns in the Polymerase (P) Gene

Samples code	Gen P and Polymerase variant	
	Substitution Gen P	Polymerase variant
S03	G843T	Q228H
S04	Ins 538G539	S127R
	A562C	M135L
	A894G	V243I
	T928G	L257A
S07	Del 541AG542	S127A
	A894G	V243I
	T928G	L257A
S08	G843T	Q228H
S09	G843T	Q228H
S11	G843T	Q228H
S13	Ins 538G539	S127A
	G843T	Q228H
	A1128C	K323N
S14	A1128C	K323N
	G936C	L326R
S19	T1081A	V214I
	A894G	T312S
S28	T1001A	V281E
	T1028A	F290Y
S33	G1056T	M299I
	A1077T	Q306H
	Ins 1083T1084	K308N
S36	T538G	S127A

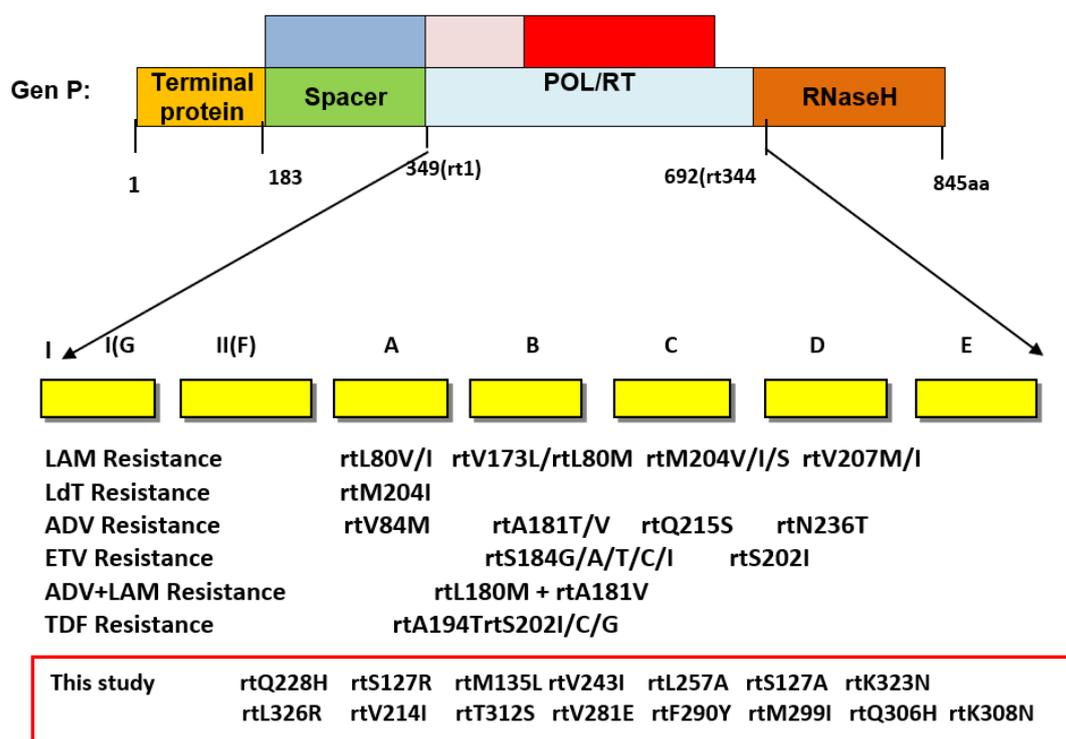


Figure 1. Mutations in the P Gene associated with antiviral resistance. In this study, mutations in the P Gene include rtQ228H, rtS127R, rtM135L, rtV243I, rtL257A, rtS127A, rtK323N, rtL326R, rtV214I, rtT312S, rtV281E, rtF290Y, rtM299I, rtQ306H, and rtK308N

Conversely, when the host's immunity is strong or antiviral drugs are administered, all viruses, including variants, are eliminated. Variants have an opportunity to develop in the presence of replication space, which occurs when the host's immune response is weak or when treatment is administered with antivirals that cannot suppress the replication of all viruses. Under such conditions, variants can thrive and form their own populations (Terrault et al. 2016).

Another crucial factor influencing the survival of a variant is its position in the HBV gene. In genes that do not overlap, such as the pre-core region, the chance of mutations occurring is higher because only one ORF is affected. However, if mutations occur in critical gene positions essential for survival, such as the enhancer or promoter, the resulting mutants will not survive (Nagahashi et al. 2016).

In this study, no patterns related to resistance to antivirals were found in the 14 subjects examined. This suggests that all subjects in this study remain sensitive to NA antiviral treatment (Figure 1). Based on this study, NA antiviral therapy remains promising for use in Indonesia for patients who have not yet received treatment, with the caveat that a consensus algorithm for NA use should be applied (Izzaty et al. 2016; Sarin et al. 2016).

Considering that resistance tends to develop after some time on antiviral treatment, it is crucial for patients to undergo regular monitoring to detect resistance and characterize the types of mutations that emerge. To identify mutations with substitution patterns associated with resistance, it is advisable to examine samples from a large number of subjects, including those from different geographical

locations, to determine the dominant mutant types.

Amino acid substitution patterns typically reported to lead to mutations causing resistance to NA antivirals involve changes from methionine to valine, isoleucine, or from valine to leucine, leucine to methionine, etc. The patterns found in this study are similar to those previously reported, including the change of glutamine to histidine (rtQ228H) (Turyadi et al. 2018), serine to alanine (rtS127A), serine to arginine (rtS127R), valine to isoleucine (rtV243I), leucine to alanine (rtL257A), and lysine to asparagine (rtK323N) (Table 2). The impact of these amino acid changes is not yet clear, although they involve a shift from polar to non-polar groups and a loss of hydrophilic properties, becoming hydrophobic. Changes in amino acid properties in the P gene can result in a decrease in the virus's ability to replicate, leading to low viral levels in the serum, making them undetectable (Thedja et al. 2011). To confirm this, a separate in vitro molecular study is required.

Amino acid changes in the P gene can also have clinical implications. The development of drug resistance begins with mutations in the P gene, followed by an increase in viral load (viral breakthrough), an increase in ALT levels (biochemical breakthrough) several weeks or months after treatment, and eventually the development of liver disease (clinical breakthrough) (Lok et al. 2007).

Measurement of viral load is essential for monitoring and confirming resistance to antivirals because nearly all cases of NA resistance are initially identified by a continuous increase in viral load during treatment. However, an increase in viral load is not solely related to NA resistance, as it can also be caused by patient non-

compliance with antiviral drugs and/or pharmacogenomic factors. Therefore, indications of NA resistance should be confirmed through genotypic and phenotypic testing (Durantel et al. 2005; Lok et al. 2007).

To identify genes with high resistance properties, the amino acid sequences of HBV polymerase in patients experiencing virologic breakthrough should be compared with sequences isolated from the pre-treatment period of the same patients. Direct sequencing can detect mutants if the number of mutants present exceeds 20% of the total quasispecies. Cloning methods can address this issue but require the analysis of a large number of clones (Lok et al. 2007; Liu et al. 2015).

In this study, cloning was not performed, which could have confirmed the presence of resistant mutants within the viral population. The characterization of mutations in this study was carried out by analyzing the sequence of PCR products, which represents the amplification results of all viruses present in an individual. This study has limitation of the small sample size (n=14), and we did not include other clinical data (viral load, HBeAg status, treatment history). As a continuation of this study, it is recommended to clone the identified variants to ensure that the mutations originate from a single virus strain.

In conclusion, out of the 14 samples analyzed, 12 (85.71%) exhibited nucleotide substitutions, while 2 (9.14%) samples did not undergo mutations. The most frequently observed substitution pattern in the P gene included rtQ228H, rtS127R, rtM135L, rtV243I, rtL257A, rtS127A, rtK323N, rtL326R, rtV214I, rtT312S, rtV281E, rtF290Y, rtM299I, rtQ306H, and rtK308N. No mutations related to antiviral resistance were detected. The absence of mutations associated with antiviral resistance suggests that the patients in this study are still sensitive to Nucleos(t)ide Analog antiviral (NAs) treatments. However, it is essential to emphasize the need for regular monitoring during treatment to detect and manage any potential development of resistance in the future. Further research and larger-scale studies involving subjects from various geographic locations are recommended to better understand the dominant mutant types and their clinical implications.

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