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Distribution of Hepatitis B Virus Genotypes Among Patients at Internal Medicine Unit, Dr. Soetomo General Hospital, Surabaya

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Abstract

Hepatitis B virus (HBV) infection is a major health problem worldwide, especially in developing countries. The study of HBV genotypes is important to find out the diversity of HBV genotypes related to the severity of the disease, response to therapy, and clinical symptoms.The aim of this study was to detect HBV genotypes among patients at Internal Medicine Unit, Dr. Soetomo Hospital Surabaya. This study was conducted to new patients at the Internal Medicine Unit of Dr. Soetomo General Hospital, Surabaya within a month. HBV surface genes were detected by Nested PCR. Sequencing and phylogenetic analysis to determine HBV genotype were performed on samples with positive HBV DNA. In this study, a total of 36 samples were obtained. The prevalence of HBV infection shown by positive HBsAg in patients with symptoms of liver disease was 55.55% (20/36 patients). Based on the results of electrophoresis from PCR products, positive HBV DNA was obtained 15 of these 20 patients (75%). After sequencing samples with positive HBV DNA, genotype B of Indonesian strain was found to be predominant genotype (100%). Subgenotype analysis showed that 7/15 samples had B3 subgenotype (46.67%). Patients at Internal Medicine Unit of Dr. Soetomo General Hospital, Surabaya, had a high prevalence of HBV infection (55.55%) with predominant genotype B. In Surabaya, HBV genotype infection still remained like the previous pattern, although in Indonesia there have been many inter-island and ethnic migration. Further similar studies are needed to obtain the diversity of other HBV genotypes.

Keywords: Hepatitis B virus, surface gene, genotype, subgenotype

Abstrak

[Distribusi Genotip Virus Hepatitis B pada Pasien di Unit Penyakit Dalam, Rumah Sakit Umum Dr. Soetomo, Surabaya]

Infeksi Virus Hepatitis B (VHB) merupakan masalah kesehatan di seluruh dunia, terutama di negara-negara berkembang. Studi tentang genotipe VHB penting untuk mengetahui keragaman genotipe yang dikemukakan berkaitan dengan keparahan dari penyakit, respon terhadap terapi, serta gejala klinis yang timbul. Mendeteksi genotipe VHB pada penderita gejala penyakit hati di Poli Hepatologi RSUD Dr.Soetomo Surabaya. Penelitian ini dilakukan pada penderita baru di Poli Hepatologi RSUD Dr.Soetomo Surabaya selama 1 bulan. Pada penelitian ini dilakukan nested PCR dengan menarget gen surface VHB sehingga dapat dilakukan penentuan genotipe. Pada penelitian ini didapatkan 36 sampel penderita dengan prevalensi infeksi VHB positif sebanyak 55,55% (20/36 pasien). Berdasarkan hasil elektroforesis produk PCR, didapatkan DNA VHB positif pada 15 dari 20 pasien tersebut (75%). Setelah dilakukan sequencing pada sampel dengan DNA VHB positif, didapatkan genotipe B galur Indonesia merupakan genotype dominan (100%). Pemeriksaan subgenotipe menunjukkan 7 dari 15 sampel memiliki subgenotipe B3 (46,67%). Pada penderita di Poli Hepatologi RSUD Dr.Soetomo Surabaya didapatkan prevalensi infeksi VHB yang tinggi (55,55%) dan dominasi genotipe VHB adalah genotipe B. Masih diperlukan penelitian sejenis lebih lanjut untuk mendapatkan keragaman genotipe VHB lainnya. Di Surabaya, pola genotipe VHB masih tetap seperti pola sebelumnya, walaupun di Indonesia telah banyak didapatkan migrasi antar pulau dan etnis.

Kata kunci: *Virus Hepatitis B, gen surface, genotipe, subgenotipe*

INTRODUCTION

HBV infection is a life-threatening infection worldwide. Despite its contribution to chronic liver disease, this virus can also cause cirrhosis and liver cancer that has the potential to cause death. WHO data shows that more than 257 million of the world's population suffer from HBV infections and 887,000 dies from complications of HBV infection.(1) Hepatitis B virus spreads through blood, sperm fluid, or other body fluids from patients infected with HBV. The transmission process includes mother to child transmission, sexual intercourse, alternating needles, and syringes, or direct contact with the patient's blood.(2)

Indonesia is a moderate to the high endemic country for HBV infection, with a number of HBV carriers 5-20 percent of the population.(3) To date, 10 HBV genotypes (A–J) have been identified based on the genetic differences in the genome sequence, especially in the surface gene. These ten HBV genotypes are differentiated based on differences in >8% nucleotide sequences. HBV genotypes are further subdivided into subgenotypes based on differences in 4-8% nucleotide sequences.(4) Genotype B HBV is the dominant HBV genotype in Indonesia, followed by genotypes C, D, and A.(5) Molecular data of HBV in Indonesia is still limited. There is still the possibility of finding new subgenotypes in various regions in Indonesia, such as B7, B8, and B9.(6) Shifting of HBV genotype from one population to another via human migration is a common phenomenon, as it has been done in several countries in the world. (7)

The study of HBV genotypes is important to find out the genotypic diversity expressed in relation to the severity of the disease, response to therapy, interferon, vaccine development, and clinical symptoms that arise.(8,9)

The aim of this study was to determine HBV genotype and subgenotype through nested PCR based on surface gene and sequencing in patients at the Internal Medicine Unit, Dr. Soetomo General Hospital, Surabaya.

MATERIAL AND METHOD

Sample collection.

This research was a cross-sectional descriptive study which took place at the Internal Medicine Unit, Dr. Soetomo General Hospital, Surabaya. Ethical approval of this study was obtained from the Ethics Committee of the Dr. Soetomo General Hospital, Surabaya (Ethical Clearance Certificate No. 396/Panke.KKE/X/2014). The inclusion criteria were: adult patients (>16 years old), had common liver disease symptoms (pain in the right quadrant over the abdomen, jaundice, hematemesis, melena, or tea-colored urine), and volunteered to participate in this study. A total of 27 blood samples were taken from patients in this study during October 2014. We measured serum HBsAg using Human Humareader kit (ELISA method). Then, in HBsAg positive samples, PCR was performed to detect HBV DNA, followed by sequencing and genotyping. Laboratory examination was performed at Laboratory of Hepatitis, Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya.

Viral DNA Extraction.

HBV DNA extraction from serum with positive HBsAg was carried out using Qiagen Cat QIAamp Viral DNA Mini Kit Kit. # 51104. Negative controls and positive controls were always included in every process. DNA extraction procedures were in accordance with the instruction manual from the manufacturer.(10)

Nested PCR.

Nested PCR based on surface gene overlapping with the polymerase region was performed using Qiagen Cat Top Taq Master Mix Kit. # 200403 according to the manufacturer's protocol. Primers used in the first round of PCR were P7: 5′ GTGGTGGACTTCTCTCAATTTTC3 ′ (256-278; sense) and P8: 5′ CGGTAAAAAGGGACTCACGAT3 ′ (796-776; anti-sense) (11). Primers for sec-

ond round were HBS1: 5′ CAAGGTATGTTGCCCGTT3 ′ (455-474; sense) and HBS2: 5′ AAAGCCCTACGAACCACT3 ′ (713-694; anti-sense).[12] Samples showing negative HBV DNA in the first round PCR were continued with second-round PCR. For each 4µL sample, 10 µL master mix was mixed with 1 µL of each primer and 4 µL distilled water. 35 cycles of PCR were carried out by Verity 96-well AB Applied Biosystem thermal cycler machine. Each cycle included: 25 seconds at 94 ° C for denaturation, 30 seconds at 53 ° C for annealing, and 50 seconds at 72 ° C for extensions. The final extension was carried out at 72 ° C for 10 minutes.

Gel Electrophoresis.

Electrophoresis of PCR products was carried out in 2% agarose gel containing ethidium bromide at 100 volts for approximately 30 minutes. Its results were then viewed under UV-light and documented with the digital camera.

Sequencing.

Sequencing was carried out by direct sequencing using the ABI 310 DNA Sequencer machine from Applied Biosystem, Inc.

Analysis of nucleotide sequences.

Molecular analysis was carried out using Genetyx for Windows version 10 software. Genotypes and subgenotypes were determined based on surface gene homology (> 8% and > 96%) by comparing HBV nucleotide sequences previously known. Phylogenetic trees were based on the unweighted-pair group method using arithmetic averages (UPGMA).

RESULTS AND DISCUSSION

In this study, 36 serum samples were collected from the patients at the Internal Medicine Unit, Dr. Soetomo General Hospital, Surabaya.

Table 1. Sex and age groups of patients in this study

Gender	Number of samples	Age (y.o)			
		≤20	21-40	41-60	>60
Male	19 (55.55%)	1 (2.78%)	7 (19.44%)	12 (33.33%)	0
Female	17 (44.44%)	0	4 (11.11%)	9 (25%)	3 (8.33%)
TOTAL	36 (100%)	1 (2.78%)	11 (30.56%)	21 (58.33%)	3 (8.33%)

The youngest patient in this study was 17 years old and the oldest was 80 years old, with an average age of 47.93 years. The number of male patients (55.55%) was more than female (44.44%). Middle age groups (41-60 years) were the most common group. The most common symptom of liver disease in this study was a pain in the right quadrant over the abdomen (58.33%).

Patients with HBV infection in this study indicated by positive HBsAg from serological results reached 55.55%. Based on previously published research, it is known that the prevalence of hepatitis B in Indonesia was as much as 9%, with the prevalence on Java Island around 5% (Mulyanto et al, 2009). While in data of Basic Health Research (Riskesdas), the prevalence of HBV infection was 9.4%.[13] This was because sampling was done in Internal Medicine Unit as high-risk groups so that the prevalence of HBV was more than 50 percent.

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Table 2. ALT values of patients with positive HBsAg

Gender	ALT			Total
	(0-1 x normal)	(1-2 x normal)	(>2 x normal)	
Male	5 (25%)	6 (30%)	2 (10%)	13 65%)
Female	4 (20%)	1 (5%)	2 (10%)	7 (35%)
TOTAL	9 (45%)	7 (35%)	4 (20%)	20 (100%)

Cutoff normal ALT values: In males: < 41 U/L, In females: < 31 U/L

Patients in this study have a wide range of ALT values (13-663 U / L). Only 4 out of 20 patients (20%) had elevated ALT exceeding 2 times the normal value limit. The increase in ALT indicates liver function disorders because ALT generally is a specific marker that can only be found in liver cells.[14] Disorders of liver function can be caused by hepatitis infection, other viral infections, toxic injury, alcoholic liver disease, ischemic injury, and autoimmune hepatitis.[15]

As much as 75% (15/20) samples with positive HBsAg showed positive HBV DNA in PCR examination. The cause of negative HBV DNA in PCR could be caused by a variation on primers attachment sites.[16] Another possibility is that HBV DNA titers are too low because the patients have taken anti-viral drugs. The use of anti-viral drugs as a therapy for HBV infection, both in the form of interferon injection and the use of oral nucleoside analogs can suppress HBV DNA replication so that undetectable HBV DNA titers can be considered markers of successful therapy even though HBsAg remains positive.[17]

From phylogenetic tree analysis as seen in Figure 1, it can be seen that 15 nucleotide sequences that have been obtained by researchers have one branch of the same phylogenetic tree with HBV sequences -B_D23678_204 which has B genotype of Indonesian strain. This shows that the samples obtained by researchers were dominated by B genotype of Indonesian strain.

HBV genotype plays an important role in the development of HBV infection. Genotype differences affect the tendency of HBV chronicity, response to therapy, tendency to mutations, seroconversions, cirrhosis, and hepatocellular carcinoma. Although not all mechanisms are known yet, differences in genotypes are thought to be related to immune responses.[4] A study in China showed longer immune clearance in patients with genotype C compared to patients with genotype B HBV. In addition, genotype C also showed higher viral replication, more histological changes in the liver, high ALT persistence, and a lower response to therapy. Genotype C has a lower helper T cell response, so it is not enough to maintain immune system activity longer.[18] Genotype C also has a higher risk of cirrhosis and hepatocellular carcinoma compared to genotype B.[19] In our study, no genotype C was found.

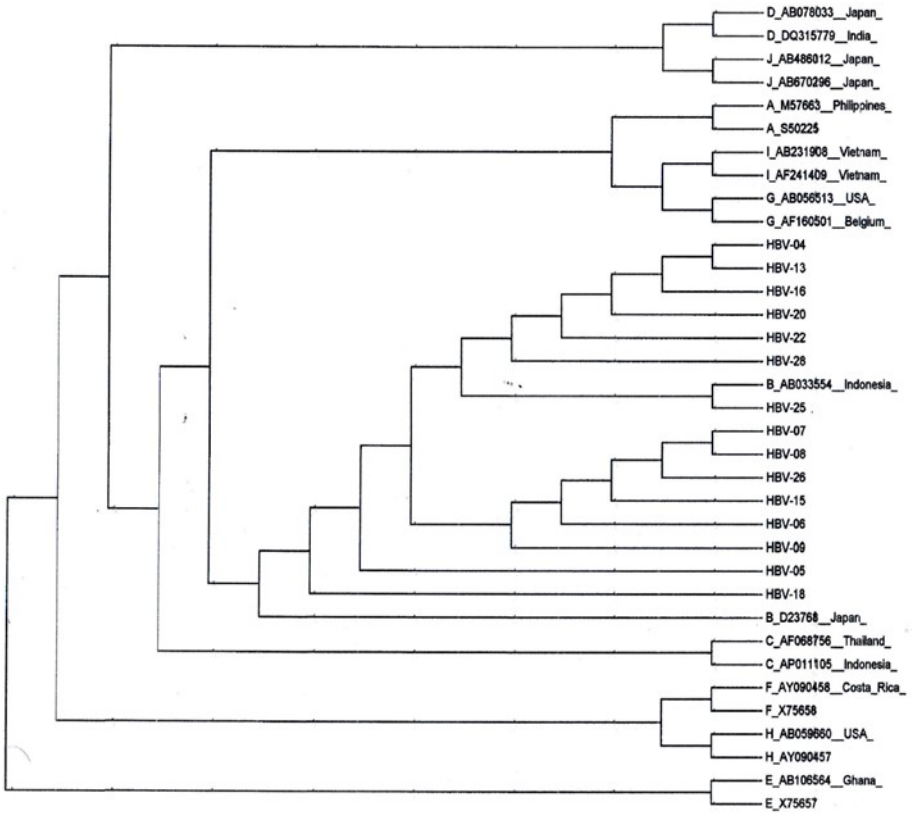

Figure 1. Phylogenetic tree genotype of 15 HBV samples in this study

HBV consists of 10 genotypes, from A to J, where genotypes B and C are more prevalence in Asia.[4] Countries in Asia have a wide diversity of HBV genotypes, especially in Southeast Asian countries, including Indonesia. Indonesia is an archipelago with a diversity of ethnic populations, and each ethnicity based on its geographical location has its own characteristics. Genotype diversity is very possible to see the number of ethnicities and the existence of migrations from various islands.[20,21] Research by Thedja (2012) shows that the distribution of genotypes and subgenotypes in Indonesia is very complex and different in each ethnicity. Although dominated by B3 subgenotypes, many new subgenotypes are found in Indonesia, for example, subgenotypes B7, B8, and B9.[6] The results of this study indicated that all samples with positive HBsAg and positive HBV DNA had genotype B. Moreover, it can be seen from the phylogenetic tree in Figure 1 that all samples had homologous genotype B by reference to genotype B Indonesia sequence (B_AB033554) but not in one branch with genotype B originating from Japan (B_D23768). This is in accordance with the results of several previous studies which showed that genotype B is the most common HBV genotype found in Indonesia, followed by genotypes C, D, and A. This study was also in line with another study in Surabaya, which took samples from chronic HBV blood donor, chronic liver disease patient, and hemodialysis patients.[22] Surabaya is the second largest city of Indonesia which has long been an arrival city of traders, migrants, and travelers. Thus, the urbanization rate is high in Surabaya.[23] Population migration can be the cause of change of HBV genotype and subgenotype distribution.[24] However, in this study, it reveals that no HBV genotype change had occurred.

Genotype B is most commonly found in western Indonesia, while genotype C is more commonly found in Eastern Indonesia.[19] Most of the patients in the sam-

ple came from Java, which is a part of Western Indonesia, with the dominance of the East Java region. Only 1 patient was from outside Java (Palu-Eastern Indonesia), but this patient also had B genotype. This shows the pattern of genotype distribution in Indonesia, especially in Java, which is still dominated by genotype B with the same strains, although inter-island, ethnic and state migration has been found in Indonesia. [25,26]

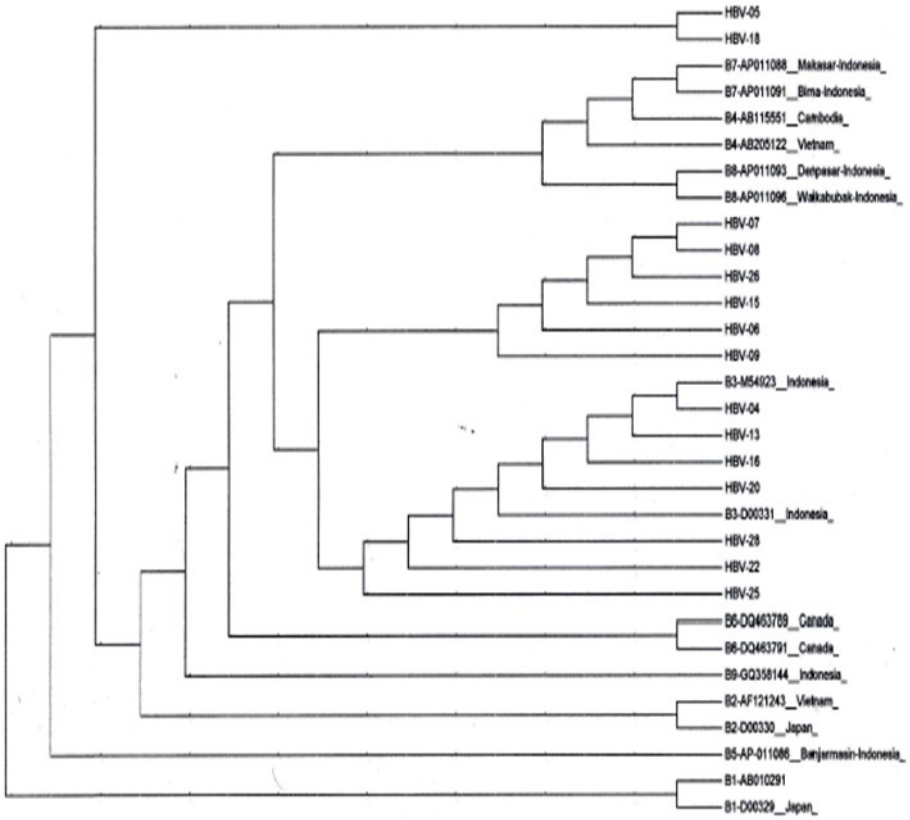

Figure 2. Phylogenetic trees subgenotype of 15 HBV samples in the study

The results of this study showed that 7 of 15 positive HBV samples (46.67%) had B3 subgenotypes. Six other samples showed a close relationship with the B3 subgenotype, which can be seen in the phylogenetic tree as in the same branch with B3 subgenotype. In a previous study by Shi et al (2012), it was also shown that B3 subgenotype is not monophyletic (only forms one branch of the phylogenetic tree), but is diffuse. This allows the existence of a B3 quasi-subgenotype.[27] The other two subgenotypes could not be identified. Accurate determination of subgenotypes can be done using the entire genome length as the gold standard, although this method is more expensive and take more time.[9]

CONCLUSION

The prevalence of HBV infection in patients at the Internal Medicine Unit was high (55,55%). Nested PCR based on surface gene was able to detect 75% (15/20) positive HBV DNA from positive HBsAg patients. Genotype distribution of these patients was dominated by B3 genotype, as in previous studies, and it seemed was not influenced by migration.

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