

Molecular Genotyping of HBV by using Nested PCR-RFLP among Hepatitis B Patients in Daerah Istimewa Yogyakarta Province and Surrounding Area

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Abstracts

Hepatitis B virus (HBV) can be classified into 8 genotypes, genotype A to H. Genotype of HBV is important for clinical and etiological investigations. Research for HBV genotyping, HBV transmission study using nested PCR and HBV genotyping based on RFLP using restriction enzymes have been reported. However, both of those methods have been not applied for HBV genotyping study among hepatitis B patients in endemic area, like Indonesia yet. Molecular genotyping of HBV will describe epidemiology, pathogenesis and clinical implication of HBV. Combination of nested PCR and RFLP (nested PCR-RFLP) method to determine HBV genotype in Indonesia is still less information. The objectives of study were to develop a system for HBV genotyping by nested PCR combined with RFLP (nested PCR-RFLP) method based on nucleotide sequence of surface protein encoding gene (S gene) in HBV genome and to confirm HBV genotypes which predominantly found among hepatitis B patients in Daerah Istimewa Yogyakarta Province and surrounding area. Total of 149 sera from chronic hepatitis B patients from Daerah Istimewa Yogyakarta and surrounding areas were collected for in this work. Viral DNA were extracted from sera of hepatitis B patients and used as template for first round nested PCR amplification using outer primers set. Amplicons of first round PCR were used as template for second round amplification using inner primers set. Then, amplicons of second round nested PCR were restriction digested by Sty I and Bsr I enzymes. For HBV genotyping then the restriction products were analyzed by RFLP based on restriction pattern. Results showed that the first round nested PCR amplification generated DNA fragments of whole S gene in length 1.233 bp, and in second round nested PCR amplification using inner primer set generated DNA fragments 585 bp in length. Genotype analysis for all samples using nested PCR-RFLP methods by restriction digested of Sty I and Bsr I enzymes found only 2 HBV genotypes among hepatitis B patients, namely genotype B and C. Quantification data showed that most of hepatitis B patients found infected by HBV genotype B (92,8%), genotype C (3,6%) and unidentified genotype (3,6%). Nested PCR-RFLP methods for HBV genotyping is simple and inexpensive for clinical diagnostic in large scale.

Key words : HBV, nested PCR, RFLP, genotyping, Sty I and Bsr I enzymes

Introduction

Hepatitis B virus (HBV) is an incompletely double-stranded (ds) DNA virus, which is worldwide a major causative agent of acute and chronic hepatitis, liver cirrho-

sis and hepatocellular carcinoma (HCC), especially in Asian, African and Southern European countries. The HBV genome consists of four open reading frames, Core (C), Surface (S), X and Polymerase (P) which covers more than 70% of complete genome and overlaps to the entire preS and S genes, X and C gene partially (Kann and Gerlich, 2005).

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HBV infection is showed some clinical manifestations from acute or fulminant hepatitis to diverse forms of chronic infection, including asymptomatic carrier state, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma or HCC (Chen, 1993). HBV genotypes have been partially clarified as influencing the clinical manifestation of chronic liver disease in hosts. A higher disease inducing capacity of HBV genotypes, moreover mutation has been reported to increase the risk of hepatocellular carcinoma (Kao *et al.*, 2003).

Recently, human HBV can be divide into eight genotypes, named genotype A, B, C, D, E, F,G and H, which diverge by 8–15 percent in the DNA sequence (Norder *et al.* 2004). The distribution of genotypes throughout the world has been confirmed. Genotype A is rather than ubiquitous and pandemic. It reported found prevalent among hepatitis B patients in Northern and Central Europe but is also present in North America and sub-Saharan Africa (Norder *et al.*, 1994.; Kramvis *et al.*, 2003; Bowyer *et al.*, 1997). Genotype A is also predominant isolate in Asian countries like India, Philippines and Nepal (Usuda *et al.*, 1999). Genotype B and C are confirmed to populations with origins in East Asia and the Far East (Kato *et al.*, 2002; Norder *et al.*, 1994). Genotype D is found worldwide but found most in the Mediterranean area, the Near and Middle east and South Asia (Usada *et al.*, 1999). Genotype E is confined to West Africa and is also endemic in that region (Odemuyiwa *et al.*, 2001). Genotype F in South America and genotype G in France, Germany, USA and Mexico (Magnius and Norder, 1995; Lindh *et al.*, 1997; Stuyver *et al.*, 2000). Genotype H was reported found in South and Central America (Arauz-Ruiz *et al.*, 2002).

Recent study has showed that genotype B and C are predominant genotypes in Japan (Kao *et al.*, 2002). It has been reported

that HBV genotype B may be associated with the development of HCC in young HBV carriers (Kao *et al.*, 2000). It is also associated with severe icteric flare-up of chronic HBV infection (Chan *et al.*, 2002). Genotype C is associated with more severe liver disease (Kao *et al.*, 2000; Kao *et al.*, 2002; Lindh *et al.*, 1999). Genotype C has a poor response to interferon therapy compared with genotype B (Kao *et al.*, 2000).

Genotyping of HBV is important for clinical and etiological investigations. There is less information about HBV genotype in Indonesia, especially found in Yogyakarta. The objectives of study were to develop a system for HBV genotyping by nested PCR combined with RFLP (nested PCR-RFLP) method based on nucleotide sequence of surface (S) protein encoding gene or S gene in HBV genome and to confirm HBV genotypes which predominantly found among hepatitis B patients in Daerah Istimewa Yogyakarta Province and surrounding area.

Materials and Methods

Samples Collecting

Blood samples were collected from Dr. Sardjito Hospital in Yogyakarta 45 samples, Dr. Karyadi Hospital in Semarang 47 samples, Red Cross Center in Salatiga 11 samples and Dr. Kustati Hospital in Surakarta 46 samples. Total of samples are 149 from HbsAg positive serologically patients.

DNA Extraction from Sera

Blood serum was separated from others blood cells by centrifugation in 6.000 rpm. Then, using micropipette 200 ml serum was collected into 1,5 ml Eppendorf tube. Viral DNA was extracted from the serum using High pure viral nucleic acid kit (Roche, Germany). Viral DNA extraction from serum was performed using procedures that recommended by Roche. Then the viral DNA from extraction product was used as template for first round of nested PCR.

Preparation of Nested PCR

Oligonucleotides primers set was purchased from the Genomic Company MWG (Ebersberg, Germany). Oligonucleotide primer sets for amplification of first round using outer primer set were designed to amplify conserved region into pre S gene with a genomic coordinate position 2820 to 2845 for primer sense and 191 to 168 for antisense. This outer primer set is able to amplify all of HBV genotypes, genotype A to H with amplification product 1.233 bp in length. For second round amplification was used inner primer set with a genomic coordinate position 203 to 221 for primer sense and 787 to 767 for primer antisense with amplification product 585 bp in length. Sequence of primers oligonucleotides for PCR amplification is shown in Table 1.

Table 1. Sequence of primers oligonucleotides for PCR amplification

Primers	Gene Target	Sequence	PCR product
Outer Primer Sense	S gene	5'- GGGACACCATATTCTTGG -3'	1.233 bp
Outer Primer Antisense	S gene	5'- TTAGGGTTTAAATGTATACC CA -3'	1.233 bp
Inner Primer Sense	S gene	5'- GCGGGGTTTTTCTTGTGA -3'	585 bp
Inner Primer Antisense	S gene	5'- GGGACTCAAGATGTTGTACA G -3'	585 bp

Nested PCR

First round amplification nested PCR was performed by thermocycler (Bio Rad) using Faststart PCR kit (Roche, Germany) with condition initial denaturation at 94°C for 3 min, followed by 35 cycles of amplification including denaturation for 45 sec at 94°C, annealing for 60 sec at 53°C and extension for 90 sec at 72°C. At the end of amplification was completed with final extension at 72°C for 10 min. For second round amplification, PCR product from first round amplification was used as template for amplification after diluted in dH₂O 1 to 10. Amplification cycle consist of initial dena-

turation at 94°C for 3 min, then denaturation for 45 sec at 94°C, annealing for 60 sec at 53°C and extension for 90 sec at 72°C. At the end of amplification was completed with final extension at 72°C for 10 min. second round amplification was performed in 25 cycles.

RFLP by *Sty I* and *Bsr I* enzymes

RFLP was performed by restriction digestion of second round PCR product by *Sty I* and *Bsr I* enzymes. A 1 ml of the second round PCR product was mixed with 1ml *Sty I* or *Bsr I* (Fermentas), 1 ml of 10x buffer and 7 ml H₂O in total volume of 10 ml. After incubation at 37°C for 3 hours, samples were electrophoresed on 1,5% agarose gel (GIBCO-BRL). Then agarose gel was stained by sybersafe (Invitrogene) and the restriction patterns were read visually under ultraviolet light using Transilluminator UV in dark room.

Results and Discussion

Nested PCR

Amplification of S gene sequence was performed by nested PCR methods using outer primer set for first round amplification and using inner primer set for second round amplification. The outer primer set was designed based on the most conserved region to yield amplification product 1.233 bp in length (Zeng *et al.*, 2004). Electrophoresis of PCR products using outer primers set is shown in Figure 1.

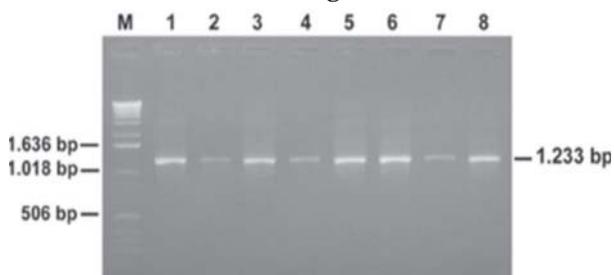


Figure 1. Electrophoresis of PCR products using outer primers . Lane 1-8 are PCR products, M indicate the molecular weight marker 1 kb plus DNA ladder

In nested PCR, the PCR product of first round amplification was used as template for second round amplification using inner primer set after diluted in dH₂O 1 to 10. The inner primer set was designed also based on the most conserved region in S gene sequence with an amplicon 585 bp in length (Zeng *et al.*, 2004). Electrophoresis of PCR products using inner primers set is shown in Figure 2.

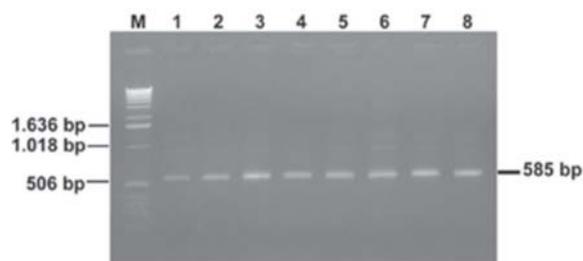


Figure 2. Electrophoresis of PCR products using inner primers. Lane 1-8 are PCR products.

M indicate the molecular weight marker 1 kb plus DNA ladder

RFLP

Nested PCR with genotype specific primer and RFLP are common methods which have been used for HBV genotyping (Naito *et al.*, 2001). This study is using nested PCR combined with RFLP using restriction enzymes for HBV genotyping. This combination genotyping method is convenient because it is simple to do and not expensive for large-scale studies in HBV genotyping. As reported by Norder *et al.* (1994) that genotyping of HBV could be accomplished based on the sequence of S gene. In this study RFLP was performed by restriction digestion by Sty I and Bsr I enzymes. For HBV genotyping the second round PCR product, which were 585 bp in length were digested by Bsr I. HBV genotype B and C could be typed in one step using parallel digestion with Sty I and Bsr I enzymes. Patterns of restriction digestion by Sty I could be seen in Figure 3.

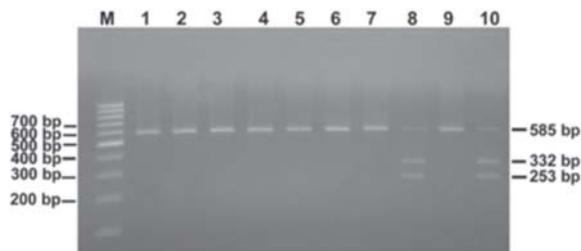


Figure 3. Restriction Patterns of RFLP after digested with Sty I enzyme. M indicate the molecular weight marker 100 bp DNA ladder. Lane 1-10 are restriction products.

Genotype C had a Sty I site at position 455 into S gene. Restriction digestion by this enzyme generated two DNA fragments 253 bp and 332 bp in length respectively. This restriction site was not found in other HBV genotypes, including genotype B (Zeng *et al.*, 2004). HBV genotype B could be distinguished from other genotypes by a unique Bsr I site at nucleotide position 328 in S gene. Restriction digest by Bsr I enzyme (Figure 4) generated two DNA fragments 126 and 459 bp in length (Zeng *et al.*, 2004).

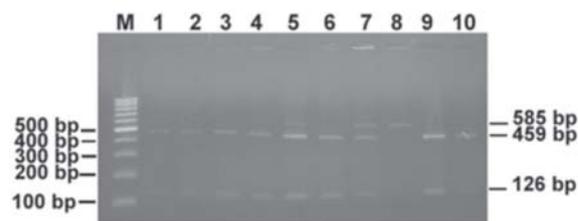


Figure 4. Restriction Patterns of RFLP after digested with Bsr I enzyme. M indicate the molecular weight marker 100 bp DNA ladder. Lane 1-10 are restriction products.

Our HBV genotyping using nested PCR combined with RFLP (nested PCR-RFLP) method showed that in Daerah Istimewa Yogyakarta Province and surrounding area most of hepatitis B patients infected with HBV genotype B than C. Genotype B and C of HBV are characteristic found in Asia countries. Genotype B has been found to cause hepatitis B early (HBe) seroconversion more frequently than genotype B, so patients infected with HBV genotypes B have

better prognoses than genotype C (Sunitha *et al.*, 2000).

If compared to sequencing analysis, HBV genotyping using nested PCR combined with RFLP method is less complicated. This genotyping method it may be important to evaluate the etiological or clinical relevance of HBV genotypes to predict the progression of liver disease or to investigate routes of infection (Mizokami *et al.*, 1999). Study in Taiwan reported that HBV genotype C was associated with more active liver disease compared to genotype B (Kao *et al.*, 2000).

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Table 2. Quantification data of HBV genotyping among hepatitis B patients

No	Sample Origin	Sample Number	RFLP Genotyping		
			B	C	unidentified
1	RS. Sardjito, Yogyakarta	45	44	0	1
2	RS. Karyadi, Semarang	47	46	0	1
3	RS. Kustati, Surakarta Red Cross Center,	46	46	0	0
4	Salatiga	11	9	2	0
Total		149	147	2	2
Percentage		100%	92,8%	3,6%	3,6%

Quantification data (Table 2) showed that majority of hepatitis patients infected by HBV genotype B 145 samples (92,8 %) and genotype C are 2 sample (3,6%). Quantification also recorded 2 sample (3,6 %) are unidentified genotype. This data is in line to Kato *et al.*, (2002) who reported that genotypes B and C are confined to populations with origin in East Asia. However

some researchers also reported that genotype B and C are predominant in Japan (Orito *et al.*, 2001; Kato *et al.*, 2002). HBV genotype B has been reported associated with the development of hepatocellular carcinoma (HCC) in young HBV carriers (Kao, 2000), and also associated with severe icteric flare-up of chronic HBV infection (Chan *et al.*, 2002). However genotype C has a poor response to interferon therapy compared to genotype B (Kao *et al.*, 2000).

Several genotyping methods have been reported used for HBV, such as direct sequencing, line probe assay and enzyme-linked immunoassay (Grandjacques *et al.*, 2000; Usuda *et al.*, 1999). HBV genotyping based on complete genome sequences is an ideal methods, but sequencing is still expensive and not easy to carried out for large scale study. This nested PCR-RFLP methods for HBV genotyping is simple and inexpensive for clinical diagnostic in large scale.

Amplification products using outer primer set generated DNA fragment 1.233 bp in length. This DNA fragment is sequence of surface or S gene. Amplification product using inner primer set generated an amplicon in size of 585 bp. Genotype analysis using nested PCR-RFLP method showed that in Daerah Istimewa Yogyakarta Province and Central Java Province among hepatitis B patients found 2 genotypes, namely genotype B and C. Quantification data showed that the most of hepatitis B patients found infected by HBV genotype B (92,8%), genotype C (3,6%) and unidentified genotype (3,6%). Nested PCR-RFLP methods for HBV genotyping is simple and inexpensive for clinical diagnostic in large scale.

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