• VIRAL HEPATITIS •

A novel hepatitis B virus genotyping system by using restriction fragment length polymorphism patterns of S gene amplicons

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Abstract

AIM: Traditional hepatitis B virus (HBV) genotyping methods using restriction fragment length polymorphism (RFLP) can reliably identify genotypes A to F. As HBV genotypes G and H have been recently identified, this study was to establish an accurate and simple genotyping method for all eight HBV genotypes (A to H).

METHODS: Two hundred and forty HBV small S sequences obtained from GeneBank were analysed for restriction enzyme sites that would be genotype-specific. Restriction patterns following digestion with restriction enzymes BsrI, StyI, DpnI, HpaII, and EaeI, were determined to identify all eight HBV genotypes. Mixed genotype infections were confirmed by cloning and further RFLP analysis.

RESULTS: The new genotyping method could identify HBV genotypes A to H. Genotypes B and C could be determined by a single step digestion with BsrI and StyI in parallel. This was particularly useful in the Far East where genotypes B and C are predominant. Serum samples from 187 Chinese HBV carriers were analysed with this genotyping system, and the genotype distribution was 1.1% (2), 51.9% (97), 40.6% (76) and 4.8% (9) for genotypes A, B, C, and D, respectively. Mixed genotypes were found in only 3 patients (1.6%). Sequence data analysis confirmed the validity of this new method.

CONCLUSION: This HBV genotyping system can identify all eight HBV genotypes. It is accurate and simple, and can be widely used for studies on HBV genotyping.

INTRODUCTION

Hepatitis B virus (HBV) exhibits genetic variability which gives rise to the well recognized subtypes and genotypes of the virus. In addition, virus variants arise during replication as a result of nucleotide misincorporations, in the absence of any proof-reading capacity by the viral polymerase. Based on an inter-group divergence of 8% or more in the complete genome nucleotide sequence, HBV has been classified into at least 8 different genotypes[1-4]. Genotyping could also be accomplished based on partial sequences from the pre-S or S genes of the HBV genome[5-7].

HBV genotypes have distinct geographical distributions. Genotypes A and D occur frequently in Africa and Europe[5,8], while genotypes B and C are prevalent in Asia[1]. Genotype E is almost entirely restricted to Africa, and F is found preferentially in Central and South America[9]. Genotype G was reported in France and the United States[3]. Recently, the eighth genotype H has been described in Central America[4].

An increasing number of studies showed that HBV genotypes might influence HBeAg seroconversion rates[10], mutation patterns in precore and core promoter regions[11,12], severity of liver disease[13-15] and response to antiviral treatment[11,16]. In order to confirm and extend these observations, further studies are needed to be carried out, using reliable and simple methods for HBV genotyping. Though several HBV genotyping methods have been reported so far[5-7,17,18], these could identify genotypes A to F, but not G or H.

The aim of this study was therefore to establish a simple and accurate HBV genotyping system using restriction fragment length polymorphism (RFLP) of the small S gene region, which could identify HBV genotypes A to H and would be applicable to large-scale studies.

MATERIALS AND METHODS

HBV sequences and computerized analyses

Two hundred and thirty-two complete and 8 S sequences of HBV were obtained from the DNA database (GenBank), comprising 24 sequences of genotype A, 35 of B, 97 of C, 38 of D, 5 of E, 30 of F, 8 of G and 3 of H. The small S gene regions of all these sequences were aligned and analysed to identify conserved regions and restriction enzyme sites that were genotype-specific. Five restriction enzymes were deemed to be appropriate for this purpose, and restriction patterns were determined by computerized analysis of each of the above mentioned sequences. The DNASIS software package (Hitachi Software Engineering, 1991) was used in this study.

Patient sera and HBV genotypes

Serum samples from 190 Chinese patients with chronic hepatitis B were collected from 2 liver units in mainland of China, and stored at -70 ℃. All subjects (male/female = 161/29, mean age = 29.5±9.16 years) were HBsAg-positive by commercially available immunoassays (Abbort Laboratories). Of them, 116 (61%) were HBeAg positive, and the mean alanine transaminase value (ALT) was 248.5±342.8 IU/L. These samples were initially genotyped by RFLP analysis of pre-S amplicons as previously described[6].

Genotyping PCR and restriction enzyme treatment

The S gene sequences were amplified by nested PCR. Based

on the most conserved regions, we designed PCR primers to amplify the sequence between nt 203 to nt 787, yielding an amplicon of 585 bp. The outer primers were PrsS2 (sense, nt 2 820-2 837, 5'-GGGACACCATATTCTTGG) and S1R (antisense, nt 842-821, 5'- TTAGGGTTTAAATGTATACCCA). The inner primers were YS1 (sense, nt 203-221, 5'-GCGGGGTTTTTCTTGT TGA) and YS2 (antisense, nt 787-767, 5'-GGGACTCAAGATG TTGTACAG). DNA was extracted from the serum as previously described[19]. A 5 μL of the resuspended DNA was added to an amplification mixture containing 5 μL of 10× Taq polymerase buffer, 5 μL of 25 mmol/L deoxyribonucleotide triphosphates, 1 μL (2U) of Taq polymerase (Promega, Beijing, China) and 10 pmoL each of primers PrsS2 and S1R (total volume of 50 μL). The PCR profile was an initial 3 min denaturation at 94 ℃, followed by 35 cycles of amplification including denaturation for 45 s at 94 ℃, annealing for 60 s at 53 ℃, and extension for 90 s at 72 ℃. Strand synthesis was completed at 72 ℃ for 6 min. A 1 μL of the first-round PCR products was then used for the second-round PCR under the same conditions but with the primers YS1 and YS2.

A 10 μL of the second-round PCR products was mixed with 0.5 μL (5U) of the chosen restriction enzyme (New England Biolabs, Hong Kong, China), 1.5 μL of 10× buffer and 3.0 μL of water. After incubation at 37 ℃ for 4 h, the samples were electophoresed on a 30 g/L agarose gel. A 10 μL of undigested second-round PCR products was run in parallel with the enzyme-digested samples. The restriction patterns were read visually under ultraviolet light.

Identification of mixed genotypes
When non-specific, atypical or mixed RFLP patterns were found, and the small S gene region was amplified with primers BS1 (sense, nt 56-76, 5'-CCTGCTGGTGGCGCCAGTTCC) and S1R, yielding an amplicon of 797-bp in length. These PCR products were purified and ligated into the pGEM-T vector using a commercial kit (Promega, Beijing, China). Ten positive clones were selected for further analysis. Extracted plasmid DNA was amplified with primers YS1 and YS2. PCR products were digested with restriction enzymes and analysed by electrophoresis. Samples that did not give clear results were then sequenced directly.

Sequencing and sequence data analysis
Three serum samples each of genotypes B, C and D, and 2

serum samples of genotype A by our method were randomly selected for sequencing. The small S gene region was amplified with primers BS1 and S1R and ligated into the pGEM-T vector as described above. Plasmid DNA was extracted from positive clones and sequenced using an ABI automatic DNA sequencer. Sequences were then edited, aligned and compared with reference sequences using the DNASIS software.

RESULTS
Predicted RFLP patterns
Following alignment of S gene sequences, five restriction enzymes, StyI, BsrI, DpnI, HpaII and EaeI were deemed to be suitable for yielding restriction patterns that would identify all eight HBV genotypes.

Genotype C had a StyI site at nt position 455, cutting the S gene into two fragments of 253- and 332-bp in length. This restriction pattern was found in 95 of 97 genotype C sequences examined. This restriction site was absent in all other genotypes. All genotype B sequences could be distinguished by the fact that the S gene had a unique BsrI site at nt position 328, which gave two characteristic bands of 126- and 459-bp in length. A BsrI site at nt position 502 was observed in 22 of 24 genotype A and all genotype E and G sequences. Moreover, genotype E PCR products could be digested at position 706 by HpaII, while genotype G had no EaeI site, which was present in all other genotypes. The BsrI site at nt position 502 was also found in 1 of 38 genotype D sequences, which could be mistaken as genotype A. Thus the sequences which still left unresolved were those of genotypes D, F and H. For genotype F, a DpnI site was found at nt positions 491 and 747, while genotypes D and H were cut at nt position 491 but not at 747. Finally, a HpaII site at nt position 292 in genotype H could be differentiated from genotype D. The sequences recognized by these enzymes are shown in Figure 1. The patterns created by these enzymes from the small S gene region are shown in Table 1.

Strategy for HBV genotyping using the new method
The optimal strategy, using the new method, which could be applied to a particular geographical region according to the most prevalent HBV genotype in that region, is summarized in Figure 2. For example, genotypes B and C were the most prevalent in the Far East. After parallel digestion by BsrI and

Table 1 Restriction digestion patterns that could identify HBV genotypes

Genotypes	Pattern	No. of sequences	Fragments obtained with				
			BsrI	StyI	DpnI	HpaII	EaeI
A	A1	12	300 285	585	85 204 296	585	100 485
	A2	10	300 285	585	289 296	585	100 485
	A3	1	585	585	85 204 296	585	100 485
	A4	1	585	585	289 296	585	100 485
B	B1	33	126 459	585	289 296	585	100 485
	B2	2	126 184 275	585	289 296	585	100 485
C	C1	95	585	253 332	585	585	100 485
	C2	2	585	585	585	585	100 485
D	D1	36	585	585	289 296	585	100 485
	D2	1	585	585	585	585	100 485
	D3	1	300 285	585	585	585	100 485
E	E1	4	300 285	585	289 296	504 81	100 485
	E2	1	126 174 285	585	289 296	504 81	100 485
F	F1	29	585	585	289 256 40	90 495	100 485
	F2	1	585	585	289 296	90 495	100 485
G	G1	8	300 285	585	289 296	585	585
H	H1	3	585	585	289 296	90 495	100 485

StyI, more than 90% of the serum samples from Chinese patients could be genotyped.

Comparison with genotyping by RFLP of PreS1 regions

Of the 190 serum samples, 3 were HBV DNA negative by nested PCR. Thus, 187 serum samples were analysed in this study, and compared to the results obtained by RFLP analysis of the pre-S1 region. One hundred and eight-one of the 187 serum samples were classified as genotypes A (2), B (95), C (75) and D (9). These results were in full agreement with those obtained with our new method. Because of nonspecific amplification and atypical restriction patterns, 6 samples could not be classified by RFLP analysis of pre-S1 amplicons. However, these were resolved into either genotypes B (2), C (1) or mixed B+C (3) by

Figure 1 Genotype-specific sites recognized by restriction enzymes. GeneBank accession numbers and consensus sequences from HBV genotypes A to H are listed. The shaded letters indicate the sequences recognized by the relevant enzyme. StyI recognizes sequence C | C (A/T) (A/T) GG; BsrI, C | CAGT; DpnI, GA | TC; HpaII, C | CGG; EaeI, (C/T) | GGCC (A/G). *nt position number is from the unique *Eco*RI site according to the reference sequence ×001587 (genotype C).
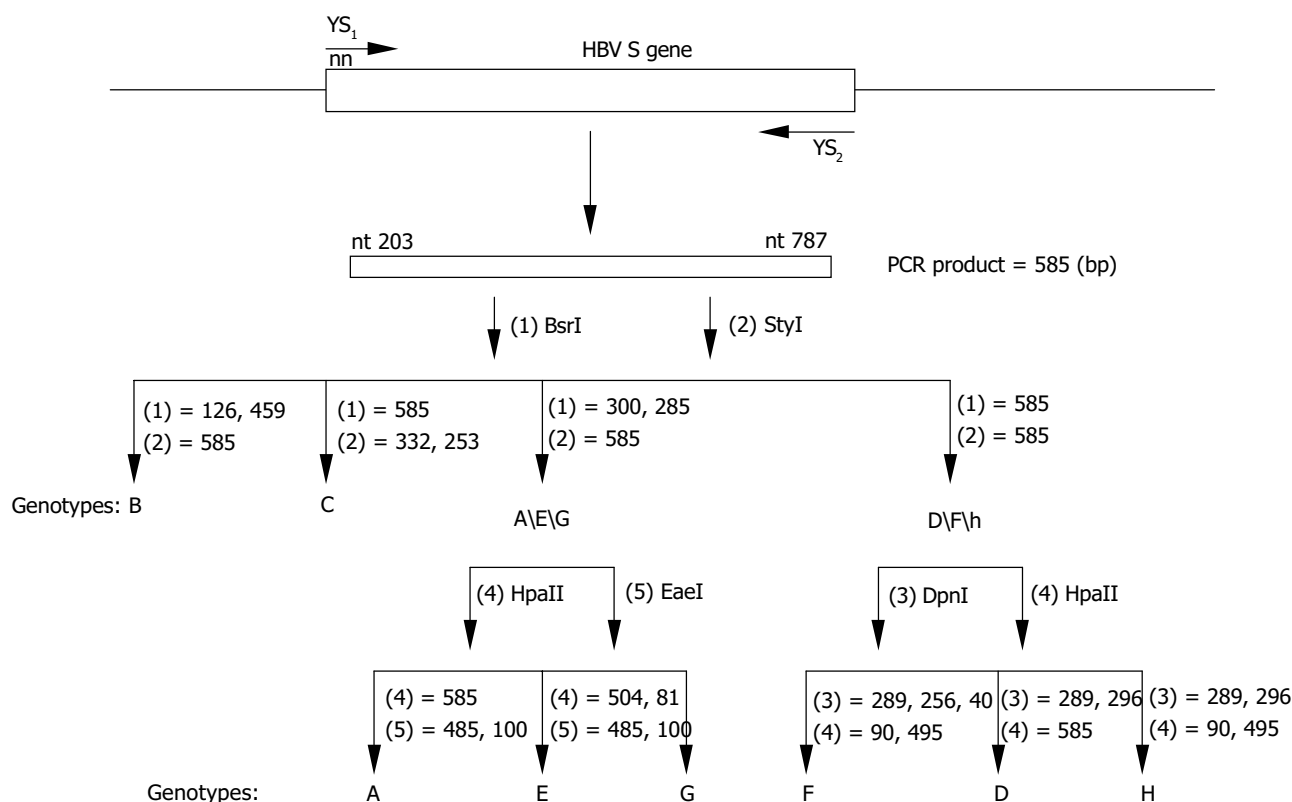
Figure 2 Diagrammatic representation of the position of PCR primers and RFLP analyses for HBV genotyping. The second round PCR products, which were 585 bp, were digested by (1) BsrI, (2) StyI, (3) DpnI, (4) HpaII and (5) EaeI. Genotypes B and C could be typed in one step using parallel digestion with BsrI and StyI.
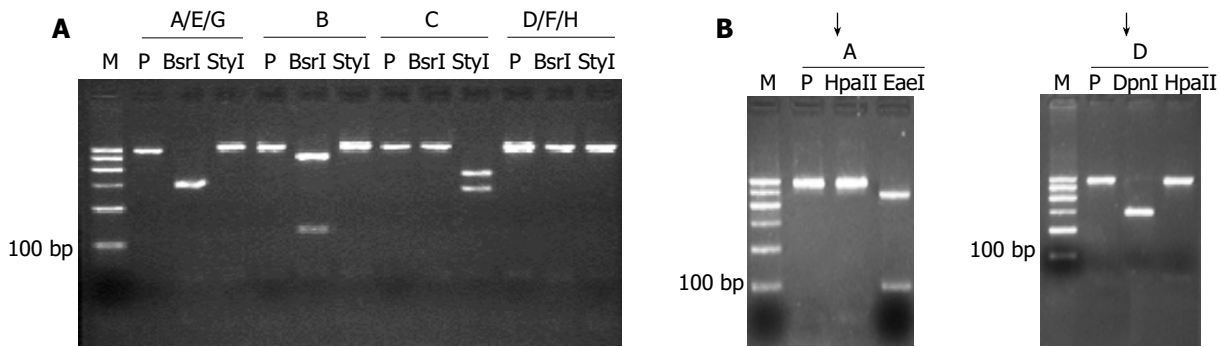
Figure 3 RFLP of S amplicon of HBV from 187 nested PCR-positive serum samples from Chinese carriers. Lane M: 100 bp ladder from 100-600 bp fragments, P: undigested PCR product. A: After the first step of parallel digestion with BsrI and StyI, the samples were divided into four groups: genotypes B, C, A/E/G and D/F/H; B: Groups A/E/G and D/F/H could be further resolved by the second parallel digestion. In this study, genotypes E, F, G and H were not observed, while genotypes B and C were the major ones.

our method after cloning and repeat RFLP analysis. These results were verified subsequently by direct sequencing of the S gene. Thus the final genotype distribution of the samples was A, 2 (1.1%); B, 97 (51.9%); C, 76 (40.6%); D, 9 (4.8%) and mixed (genotypes B and C), 3 (1.6%). Examples of RFLP analysis using the new method are shown in Figure 3.

Verification of reliability of the new method

Nucleotide sequences of 11 samples randomly selected were determined and then compared to reference sequences for genotypes A (X02763), B (D00329), C (X01587) and D (X59795). The homologies of the S gene region under investigation were 97.9-98.3%, 99.3-99.9%, 97.5-98.8% and 98.2-98.5%, respectively, which confirmed the validity of the new RFLP technique.

DISCUSSION

Great importance has been attached to the differences between HBV genotypes. In earlier studies[20,21], a higher frequency of liver dysfunction was observed in patients with subtype adr (most often genotype C) compared to those with subtype adw (mainly genotype B). These findings concurred with those reported by Lindh *et al.*[13] and Orito *et al.*[15]. Mayerat *et al.*[14] found that genotype A was more frequent in chronic hepatitis B patients than genotype D, while the opposite situation was true in acute hepatitis B patients. A study from Taiwan reported that genotype C was associated with more active liver disease compared to genotype B[22], and this was supported by a more recent study[23]. A retrospective study of 332 cases[10] showed that patients with HBV genotype B had a lower prevalence and earlier seroconversion to anti-HBe than those with genotype C, which could explain the less active liver disease seen in patients with genotype B. Another study[24] suggested that HBV genotype might influence HBV recurrence after liver transplantation as patients with genotype D appeared to have a higher risk for HBV recurrence and mortality. The correlation between HBV genotype and response to interferon therapy has also been reported. HBV genotype C was associated with a higher frequency of core promoter mutation and a lower response rate to interferon alfa therapy[16]. Hou *et al.*[11] studied 103 chronic hepatitis B patients from 16 European centers and found that HBV genotype A responded better to standard interferon treatment than other genotypes. This appeared to be related to its molecular characteristics, having a greater tendency to develop core promoter mutations and less variation in the nucleocapsid protein. A study reported that patients carrying the adw subtype were associated with a higher risk of lamivudine resistance than those with ayw subtype[25], although Chan *et al.* found that HBeAg seroconversion after treatment by lamivudine was not influenced by the HBV genotype[26]. To

interpret these differences between HBV genotypes, larger-scale investigations are needed. So, development of accurate, simple and inexpensive HBV genotyping methods would be very useful in this respect.

Several methods have been used for HBV genotyping including direct sequencing, RFLP, PCR with genotype-specific primers[17], line probe assay[18] and enzyme-linked immunoassay[27]. Genotyping based on complete genome sequences is an ideal method, but sequencing is costly and cannot be easily carried out in clinical diagnostic laboratories for large-scale studies. Currently, PCR-RFLP is the most widely used method for HBV genotyping because it is simple and inexpensive. Norder *et al.*[2] found that HBV genotyping could be accomplished based on the sequence of the S gene. After analyzing 73 HBV sequences from GeneBank, Lindh *et al.*[5] developed a genotyping method based on RFLP patterns of a S gene amplicon, which first identified HBV genotypes A-F. They also reported another genotyping technique based on RFLP analysis of the pre-S region[6]. However, pre-S gene was less suitable for genotyping than S gene because it was not so conserved as S gene[28]. Mizokami *et al.*[7] compared 68 complete and 106 small S HBV sequences and confirmed that S gene could be used to accurately identify the six HBV genotypes A to F. They also described a RFLP genotyping system using five enzymes, HphI, NciI, AlwI, EarI, and NlaIV. Because genotypes G and H were not discovered then, they could not be assessed by these methods.

Compared with previous methods, our new method has several relative advantages. Firstly, it can identify all eight HBV genotypes. Secondly, it is more accurate because it was based on analysing many of the sequences deposited in GeneBank. Thirdly, a simple and inexpensive strategy can be adopted according to the most prevalent HBV genotypes in a particular geographical region. For example, the strategy shown in Figure 2 was especially suitable for the Far East where genotypes B and C are mostly found (85-98.6%)[21,29,30]. The RFLP patterns are simple, with one or two bands, and therefore easy to be recognized.

In this study, a new method for HBV genotyping based on RFLP analysis of S gene amplicons was established, and could identify all eight HBV genotypes. This HBV genotyping system is accurate, simple and can be expected to be widely used in studies of HBV genotyping.

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