

Determination of HBV Genotypes among Hbs Ag Positive Blood Donors in Tehran, Iran Using PCR-RFLP

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(Received 7 May 2008; accepted 11 Jan 2009)

Abstract

Background: Hepatitis B virus (HBV) is one of the major causative agents of acute and chronic liver disease worldwide and is believed to be responsible for a million deaths annually. On the basis of a comparison of complete genomic sequences, HBV has been classified into eight genotypes A-H which show a geographical distribution. Some genotypes are associated with different clinical outcomes. Identification of HBV genotypes is important to begin and follow up the treatment.

Methods: In this cross-sectional study, the serum samples of blood donors were collected from Tehran Blood Transfusion Centers in period during "2005-2006". Sera of 55 blood donors who were positive for hepatitis B surface antigen were selected. DNA was extracted using commercial kit and the S gene sequence was amplified by nested-PCR. PCR products were then analyzed for restriction enzymes that would be genotype specific.

Results: Genotype D was found the only type in all HBV DNA positive serum samples, in Tehran.

Conclusion: Genotype D is dominant among Tehran's blood donors, which is consistent with Iran and the Middle East dominant genotype.

Keywords: *HBV, Hbs Ag, PCR-RFLP, Blood Donors, Iran*

Introduction

Hepatitis B virus infection (HBV) is an important public health problem worldwide and leading to significant morbidity and mortality especially in the developing countries (1, 2). The clinical manifestation of HBV infection range in severity from asymptomatic sub clinical infection (70%), symptomatic hepatitis (30%), fulminate severe disease with liver failure (0.1-0.5%), following to exposure to HBV, up to about 10% of the patient will progress to hepatitis B, which is defined as persistent of the infection for more than a month (3). Infection with HBV is preventable by vaccines, but as yet, there is no long-standing efficient treatment for chronic carriers of the virus (4). The chronic hepatitis B then progress to liver cirrhosis and hepatocellular carcinoma in about 15-40% of the patients (3).

Approximately 2 billion people in the world have been infected by HBV, 400 million have chronic infection and 600,000 die each year from HBV

related liver disease (1, 5). At least 35% of Iranian has been exposed to HBV and 3% are chronic carriers (6). The prevalence of HBs Ag in general population is 1.7% and among blood donors is 0.5% (6, 7). Compared to the United States where HBV infection is responsible for 25% of chronic hepatitis, HBV accounts for up to 70-80% of chronic hepatitis in Iran, indicating that HBV alone is the leading cause of chronic liver disease in Iran (8).

The primary risk categories for infection by HBV are blood transfusion, blood derivatives, dialysis, needle accident among health-care professionals, intra venous drug abuse and unprotected sexual contact. Vertical transmission is common in endemic regions such as Africa and Southeast Asia (9). The most common routes of transmission in Iran are prenatal and intravenous drug abuse (10). HBV has been classified into eight genotypes (A-H) (1, 11). Okamoto et al, first defined four genotypes (A-D) based on a divergence of 8%

or more of the complete HBV genomes (12). Norder et al, compared S gene sequence of the HBV genomes formerly classified by Okamoto. They showed that the smallest differences of S gene sequences between genomes were 4.1% (13). Thereafter, two new genotypes designated as E and F followed by G and H recently reported from Belgium and Central America (12). Genotype A prevails mainly in North America, Northern Europe, India and Africa, and Genotype B and C in Asia, Genotype D in southern Europe, the Middle East and India, Genotype E in West and South Africa, Genotype F in South and Central America, Genotype G in the United States of America and Europe and genotype H in Central America and California (14).

HBV belongs to the *Hepadnaviridae* family and has some unique properties. It is highly species specific, infecting only humans, chimpanzees and some other primates (4). The intact and infectious virus is 42-47 nm in diameter and circulates in the blood in concentration as high as 10^8 virion per ml (5). HBV is a circular, partially double-stranded DNA with approximately 3200 nt (9). This highly compact genome contain four major open reading frame encoding the envelope (pre S1, pre S2, and surface antigen HBs Ag), the pre C-C (pre core-core) region encodes hepatitis B core antigen (HBc Ag) and hepatitis B e antigen (HBe Ag), the P coding region for the viral polymerase and X encodes the viral X protein which modulates host-cell signal transduction and can affect host and viral gene expression (8, 15).

There is some evidence that the long-term prognosis, the initial clinical pictures and the response to treatment may differ depending on which genotype has infected the patient (4, 12). For instance, genotype D of HBV is associated with more sever liver disease than genotype A and may lead to HCC in young patients. It has also been reported that genotype D of HBV has a lower response rate to lamivudine in comparison with genotype A of HBV (16). Lamivudine inhibits viral DNA polymerase in HBV and can be effective for rapidly reducing the serum HBV

DNA titer and affect the outcome of disease. Lamivudine resistance among HBV-infected patients during therapy has been reported. It has been demonstrated that the emergence of lamivudine-resistant HBV is associated with mutations of the YMDD motif within the conserved sub-domain C in the polymerase gene. Interestingly, lamivudine-resistant mutants have been reported among patients not treated with lamivudine. It has been shown that there are a relationship between HBV genotypes and YMDD mutant profile in untreated patients. For example, in a study in Iran no YMDD mutation was found in patients with genotype D who not treated with lamivudine (17).

Several methods have been used for HBV genotyping including sequencing, PCR-RFLP, Line-probe Assay, primer specific PCR and Enzyme-linked Immuno-sorbant Assay (18, 19). Currently, PCR-RFLP is the most commonly used method for HBV genotyping because it is simple and inexpensive (19).

The aim of this study was to investigate the prevalence of HBV genotypes using PCR-RFLP method in HBs Ag positive blood donors in Tehran Province.

Material and Methods

HBV DNA extraction

In a cross-sectional study, a number of blood donors who were from Tehran province and had attended to Tehran Blood Transfusion Center for blood donation were studied in period between "2005-2006". Blood donors were screened for hepatitis B surface antigen (HBsAg) by ELISA method and neutralization test was carried out for confirmation the results. Blood donors, who were positive for HBsAg, again were recalled and standard questionnaires were completed by each of them. Fifty five serum samples were selected from these study population and stored at -70°C . The study population included 46 males (83%) and 9 females (17%), aged (22 to 66) yr (mean \pm SD, 37.5 ± 11.14). All tested serum were HBs Ag positive by ELISA method (10). HBV

DNA was extracted from 250 µl serum with the high pure viral nucleic acid kit (Roche, Germany) according to the instruction of the manufacturer. For suspension of HBV DNA, 30 µl elution buffer was used. Among samples obtained from 55 HBs Ag positive blood donors, HBV DNA was detectable in 31(56.3%). Thus, a total of 31 samples positive for HBV DNA were proceeding for the further test.

Primers

PCR primers were amplified the sequence between nt 203 to nt 787, yielding an amplicon of 585 bp. The outer primers were PrsS2 (sense, nt 2820-2837, 5'-GGGACACCATATTCTTGG) and S1R (antisense, nt 842-821, 5'-TTAGGGTTT-AAATGTATACCCA). The inner primers were YS1 (sense, nt 203-221, 5'-GCGGGTTTTTCT-TGTTGA) and YS2 (antisense, nt 787-767, 5'-GGGACTCAAGATGTTGTACAG) (17).

PCR

HBV genotyping was performed using amplification of a segment of the HBV surface gene by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism. The S gene was amplified by nested-PCR on the most conserved regions. In briefly, 5µl of the resuspended DNA was added to an amplification mixture containing 2µl of 10x PCR buffer for each samples, 0.5 µl dNTP, 0.2 µl each of outer primers (PrsS2 and S1R), 0.3 µl (1.5U) of taq polymerase (Roche, USA) and 11.8 µl water (Total volume of 20 µl).

The PCR profile was an initial 3 min. denaturation at 94° C, followed by 35 cycle of amplification including denaturation for 45s at 94° C, annealing for 60 s at 53° C, and extension for 90 s at 72° C. Strand synthesis was completed at 72° C for 6 min. A 1 µl of the first round PCR products was then used for the second round PCR under the same condition but with the primers YS1 and YS2 and water was 15.8 µl. PCR products were analyzed by horizontal gel electrophoresis on 2% agarose gel in TAE 1x buffer at 85 V/1h. A single 585 bp band was preserve in all cases after the second PCR (Fig. 1).

Genotyping by RFLP

A 5 µl of the second round PCR products was mixed with 1µl of the chosen restriction enzymes, HpaII (Roche 5000 u, 10 U/µL), DpnI (Biolab 20000 U/mL), BsrI (Biolab 5000 U/mL), StyI (Roche diagnostic USA) and EaeI (Roche 200U, 10U/µL), 2µL of each 10x buffers (L, 4, 3, H) and 12.8 µl of PCR grade water (Roche). After incubation at 37 °C (except BsrI which is at 65 °C) for 16 h, the samples were electrophoresed on a 2.7% agarose gel and the restriction patterns were read under ultraviolet light after staining the gel with ethidium bromide. Molecular weight 100 bp marker (Roche) was induced in each analyzes (Fig. 2).

Results

A total of 55 blood donors with mean age 37.5±11.14 yr including 46 men and 9 women age ranged (22 to 66) were investigated in this HBV genotyping study (Table 1). No significant association between RFLP patterns and age or sex was shown. Although the HBV transmission route remained unidentified in subjects, all subjects had two or even more of the criteria: 22 (40%) had a history of surgery, 22(40%) had history of hospitalization, 45(81.8%) had history of dentistry, 3(5.45%) had history of blood transfusion, 7(12.72%) had history of contact with needle stick, 7(12.72%) followed by intra familial and 3(5.45%) had history of tattooing (Table 2). Of 55 serum samples, 24 were HBV DNA negative by nested-PCR, thus 31 serum samples were analyzed further to compare the results obtained by RFLP analysis of the S gene region (Fig. 1). In this study, all samples were cut by EaeI enzyme, which has site in all genotypes (except genotype G) for cut at nt position 100,485bp. All HBV genotypes could be identified by four chosen enzymes (BsrI, StyI, DpnI and HpaII, (Fig. 2). No samples were cut by these enzymes which were classified as genotype D.

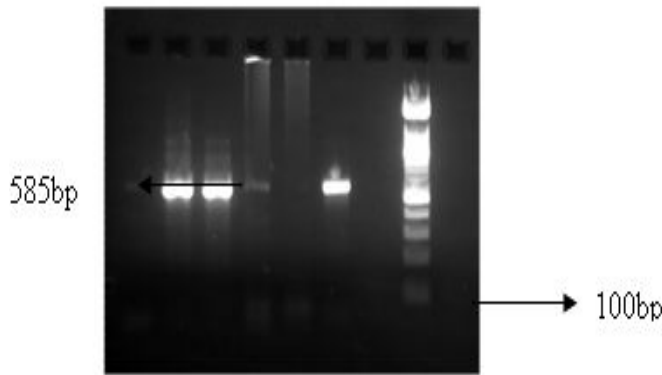


Fig. 1: DNA amplification by nested-PCR using conserved nature of nucleotide sequences in the S region followed by gel electrophoresis, after ethidium bromide staining

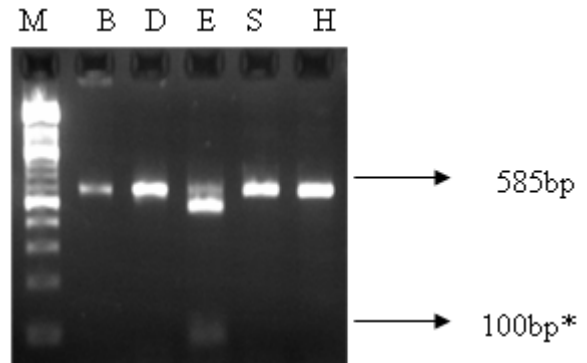


Fig. 2: RFLP of S amplicon of HBV from 31 nested-PCR-positive serum samples from HBs Ag positive Blood donors. Lane M: 100 bp ladder from 100-600 bp fragments, H (HpaII), S (StyI), D (DpnI), E (EaeI), B (BsrI) enzymes. * At nt position 100 bp the S gene sequence cut by EaeI.

Table 1: Demographic characteristics of volunteer blood donors in Tehran, Iran

AGE	HBV DNA Positive		Total
	(n)	(%)	
20-30	10	71.42	14
31-40	10	66.66	15
41-50	7	41.17	17
51-60	3	37.5	8
≥60	1	100	1
SEX			
Male	28	60.86	46
Female	3	33.33	9

Table 2: HBV genotype distribution among Tehran’s blood donors as determined by RFLP method

Risk factors	Level(n)	(%)	HBV Genotype							
			A	B	C	D	E	F	G	H
Blood injection	3	5.45	-	-	-	+	-	-	-	-
Surgery	22	40	-	-	-	+	-	-	-	-
Hospitalization	22	40	-	-	-	+	-	-	-	-
Familiar history	7	12.72	-	-	-	+	-	-	-	-
Dentistry	45	81.8	-	-	-	+	-	-	-	-
Needle stick accident	7	12.72	-	-	-	+	-	-	-	-
Tattooing	3	5.45	-	-	-	+	-	-	-	-

Discussion

HBV infection is a well recognized major health problem especially in the developing countries. It causes acute hepatitis of varying severity and

persist in 95% of children and 2-10% of adult patient, leading to chronic liver disease, cirrhosis, hepatocellular carcinoma and even fulminate hepatitis (1). The prevalence of HBV infection

virus varies with rates ranging from 0.1% to 20% in different parts of the world (10).

In addition to the serological classification of HBV isolates according to the antigenic determinants of their HBsAg, a genetic classification based on the comparison of complete genomes or S gene sequence has defined eight genotypes of HBV (A-H) (20).

When genotypic classification of HBV came into use, the first reports were based on complete genome sequence (12). Gradually the sequence of single genes or parts of genes was used in order to facilitate the comparison of a larger number of strains (12). Genotyping based on complete genome sequence is in ideal method, but sequencing is costly and can not be easily carried out in clinical diagnostic laboratories for large scale (19). Currently, PCR-RFLP is the most widely used method because it is less complicated compared to direct sequencing and sequence analysis (15). We studied the prevalence of HBV genotypes among HBsAg positive blood donors in Tehran. Many blood donors were unaware that they were infected with HBV until they were found positive for HBsAg during routine screening. A finding indicates that frequent monitoring of these blood donors is warranted as they may subsequently develop HBV-associated chronic liver disease (21).

All genotypes except genotype G could be cut with EaeI at nt position 100, 485 bp (Fig. 2). Genotype B and C could be typed in one step using parallel digestion with BsrI and StyI. A/E and G genotypes could be identified with BsrI. In our study, no samples were cut by these two enzymes (BsrI, StyI). Thus the sequence which still left unresolved were those of genotype D, F and H. HpaII has no site in genotype D while it has site for cut in F and H genotypes. Thus, genotype D is differentiated from genotype H and F. All serum samples were not cut by HpaII and typed as genotype D. Our finding genotype (genotype D) is consistent with previous studies. Also in another study of 109 Iranian HBsAg positive patients, HBV genotype D was the only detected type (10). Another study (22) also revealed that the

HBV genotype D with sub genotype D1 dominates in the Iranian infected patients. In our neighboring countries such as Turkey, Pakistan and Afghanistan there are similar results. Genotype D is predominant in Pakistan (1). In Turkey, genotype D was dominant and among three different resulted sub genotypes in the entire study population (D1, D2 and D6), D2 was the most frequent restriction fragment length polymorphism pattern (13). In Afghanistan, results revealed genotype D (95% bootstrap value) and sub genotype D1 (98% bootstrap value) in all Afghan isolates (23). Relationship between the four major HBs Ag subtypes (adw, adr, ayw and ayr) and the 8 genotypes have been determined (24). Also, some of difference in the proportion of anti-HBe-positive patients seen was found to be genotype related (4). In a study, association between genotype and level of ALT was shown (25). Genotype D infected patients were found to be anti-HBe-positive significantly more often, and among the anti-HBe-positive patients, those infected with genotype D had significantly higher ALT level. Also most of isolates from genotype D-infected patients had mutation at the end of the gene for the Pre Core region (4). The appearance of anti-HBe follows the disappearance of HBe Ag usually marks non-replicative viral infection and inactive disease. It should be noted however that in Iran about 58% of HBV infections are Pre Core mutants and may have anti-HBe in spite of being actively replicating, thus an undetectable HBe Ag or the presence of anti-HBe should never be assumed to indicate non-replicative infection and HBV DNA testing or another serologic testing seems to be necessary (26,27). These findings detect the importance of HBV genotyping and are effective in selection, buying and designing kits to complete and improve screening's programs in our country.

It is recommended that to determine the genotypes of HBV among HBs Ag positive blood donors in other provinces and cities. Such studies also shed lighter on the molecular, virological and host mechanisms underlying the pathogenesis of HBV related disease.

Acknowledgments

The authors wish to acknowledge the staff of blood donation unit, Tehran Blood Transfusion Center for their help in the sample collection. We also wish to express our thanks to all the members of virology lab, at research center, Iranian Blood Transfusion Organization, who have been a great source of inspiration and technical expertise. The authors declare that they have no conflict of interests.

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