Original article

A study on genotypes of hepatitis B virus among hemodialysis patients in Khuzestan province

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How to cite this article:
Neisi N, Makvandi M, Samarbaf-Zadeh AR. A study on genotypes of hepatitis B virus among hemodialysis patients in Khuzestan province. Jundishapur J Microbiol. 2011; 4(2): 65-70.

Received: February 2010 **Accepted:** October 2010

Abstract

Introduction and objective: Hepatitis B virus (HBV) is a member of *Hepadnaviridae* and a major causative agent of chronic and acute hepatitis, liver cirrhosis and hepatocellular carcinoma. Genotype determination of HBV is based on PCR-RFLP and sequencing of genome of the virus. The genotype formation is mainly due to mutations of HBV precore, S, and YMDD (tyrosine-methionine-aspartate-aspartate motif in the C domain of the HBV DNA polymerase gene) genome area. Moreover, some of the mutant HBV remains undetectable by serological tests (occult hepatitis). Since the genotypes of HBV and occult hepatitis B has not been studied in our area, this study was conducted to determine both occult hepatitis B infection and genotypes among hemodialysis patients.

Materials and methods: Two hundred and fifty hemodialysis patients were selected in this study. The sera of the patients were collected and the extracted DNA was used as template of PCR to amplify a 479bp fragment of the viral genome. The PCR products were digested by Ava2 and Mbo1 restriction enzymes. Based on RFLP patterns, the genotypes were determined. The HBV markers including; HBV surface antigen (HBsAg), hepatitis Bc antibody (HBcIgG), hepatitis B virus e antigen (HBeAg) and hepatitis B virus e antibody (HBeAb) were carried out for all the patients by ELISA test.

Results: Fifty (20%) out of 250 sera showed positive HBV by PCR. Out of the 50 positive cases for HBV, 46(92%) belonged to genotype D2 and 2(4%) cases of them were B6 genotype. Ten cases were positive for HBV by PCR test but negative by ELISA test (4% occult hepatitis).

Conclusion: Prevalence of HBV infection was high among the dialysis patients (20%), and occult hepatitis B was 4% in these patients. The dominant genotype of HBV was D2 (92%) followed by genotypes B6 (4%) in hemodialysis patients.

Keywords: Hepatitis B virus (HBV); Hemodialysis; Genotyping; RFLP

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Introduction

Hepatitis B virus (HBV) is a partially double stranded DNA, and highly prevalent in hemodialysis patients. The high levels of prevalence of HBV infection is mainly due to impact of dialysis and transfused blood units to these patients [1]. An investigation conducted in Iran has revealed that 2.6% of hemodialysis patients are suffering from HBV infection [2]. Some of HBV infections are not detected in routine serological tests, because mutation has changed some of the important Ag of this virus. Among the many mutants of the HBV, the most important are the precore mutant, the S mutant, and the YMDD variants [3].

The existence of HBV genome in HBV surface antigen (HBsAg) negative individuals is called occult HBV infection. Occult HBV status is associated in some cases with mutant viruses undetectable by HBsAg assays, but more frequently it is due to a strong suppression of viral replication and gene expression. Clinically, occult HBV has direct impact on transmission of the infection by blood transfusion or organ transplantation, especially if the immunity of the patients is compromised. The outcome of antiviral therapy depends on the genotypes of HBV infection.

The aim of this study was to investigate the prevalence of HBV, epidemiological distribution of HBV genotypes (RFLP) and occult Hepatitis among hemodialysis patients in Khuzestan province (south west of Iran).

Materials and methods

Population study and sample collection

This is a prospective cross sectional descriptive study. The sera of 250 dialysis patients [(169, 67.6%) male and 81(32.4%) female] were collected, who underwent dialysis at least six months, from winter 2008 to spring 2009. Patients suffering from hepatitis C virus (HCV), hepatitis D virus (HDV) or HIV co-infection were excluded from the study. Serum samples were kept at -70°C until PCR and genotyping. Age of the patients was between 10-85 years (mean 47 years).

Extraction of viral DNA

HBV DNA was extracted from 100µl of serum by using High yield DNA Purification Kit (CinnaGen Inc. Iran). Briefly, after adding 5µl of protease to 100µl of serum, HBV DNA was extracted according the manufacturer instruction. Finally the DNA was dissolved in 30µl distilled water.

PCR of HBV-DNA

HBV-DNA was amplified in an automated thermocycler (Bio-Rad iCycler model, USA), using the following primer (nt 2833-2845:5' TCACCATATTCTTTGGGAACA-AGA), and reverse primer (nt 80-61:5' TTCCTGAACTGGAGCCACCA) that amplify a conserved region of HBV DNA genome [4]. In order to do PCR, 20µl of the extracted DNA was used as template in a 50µl reaction.

The reaction mixture was 1µl dNTP (10mmol/L), 5µl 10x PCR buffer, 50pmol of each primer (0.8 and 0.7µl), and 0.3µl Taq DNA polymerase (5u/mL). The volume was adjusted to 50µl by distilled water. All PCR components were purchased from CinnaGen Inc. PCR condition was as follows: After an initial 3mins denaturation step at 94°C, 40 cycles of amplification were performed, each including 45S denaturation at 94°C, 60S annealing at 53°C and 90S extension at 72°C, followed by a final 7mins extension at 72°C. 10µl of PCR product was loaded on a 2% agarose gel (Amersham Pharmacia Biotech AB, Swiden) and electrophoresed at 100V for 60mins. After ethidium bromide staining, the gel was visualized on a UV

transilluminator (vilber lourmat, France). The size of the amplicon was 479bp.

Genotyping (RFLP)

A volume of 10µl of the PCR product were mixed with 1.5µl of 10X buffer, 3µl of water and an 0.5µl (5u) of *AvaII* (Fermentas Inc. Ukraine) which recognized GGWCC,W=A or T and *mboI* (Fermentas, Inc. Ukraine) which recognized GATC sites respectively in separate reactions and incubated at 37°C for 3h. A 3% agarose gel was prepared by mixing 2% low melting paint agarose (Sigma Chemical Co., USA) and 1% standard agarose. 10µl of restriction enzyme digestion products were loaded on the gel. After electrophoresis and staining, the gel was visualized. The RFLP patterns obtained were compared with the restriction endonuclease analysis reference profiles for HBV genotype classification as described [4].

Serological assay

To detect the HBV, the 50 positive samples of PCR, were tested, using the serological HBV markers including HBsAg, HBeAg, HBcAb, HBeAb (DiALAB, Austria). All the serological tests were performed according to manufacture instructions.

Results

Out of 250 collected samples from hemodialysis patients, 50(20%) were HBV positive (Fig. 1), including; 46(92%) of D2, 2(4%) of B6 and 2(4%) of unknown genotypes (Figs. 2, 3). Among the positive PCR samples, 34(68%) were male and 16(32%) were female. 10(4%) PCR positive sera patients showed negative for HBsAg; while they were positive for HBcIgG test. Out of 10 negative HBsAg, 4(1.6%) patients showed positive reaction to HBeAb and 6(2.4%) HBeAg markers (Table 1).

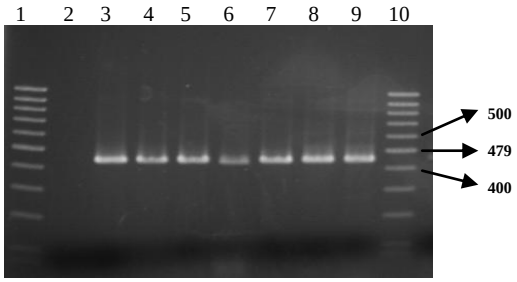

Fig. 1. PCR pattern of HBV positive patients; 1 and 10 Ladder; 2. Negative control; 3. Positive control; 4-9. Patient samples

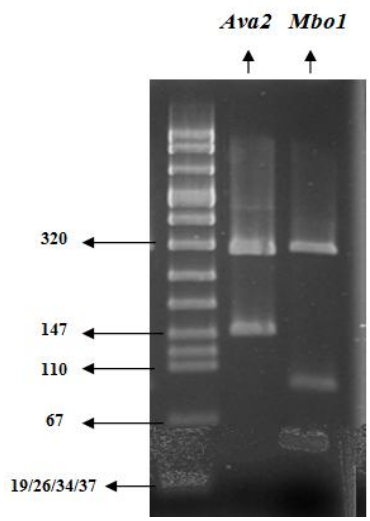

Fig. 2: Genotype B pattern B

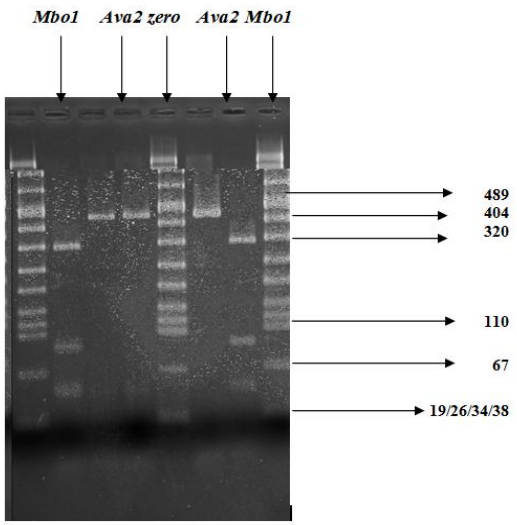

Fig. 3: Genotype D pattern D2

Table 1: The result of serology of PCR patient's samples

Serological markers		$HBsAg^+$	$HBeAg^+$	$HBeAb^+$	$HBcAb^+$	$HBsAg^-$	$HBeAg^-$
Genotype	D	37(14.8%)	40(16%)	4(1.6%)	46(90%)	9(3.6%)	7(2.8%)
	B	1(0.4%)	1(0.4%)	1(0.4%)	2(4%)	1(0.4%)	0
	Unknown	2(0.8%)	2(0.8%)	2(0.8%)	2(4%)	0	0
Total	250	40(16%)	43(17.2%)	7(2.8%)	50(20%)	10(4%)	7(2.8%)

The relation between age and prevalence with respect to genotype was analyzed. Prevalence of HBV genotypes D, B and the unknown were 8(80%), 1(10%) and 1(10%) in <30 years olds of patients, respectively. The genotype of one of the 30-50 year old patients was not detected, but the 17(94.4%) and one (5.5%) of them were of genotypes D, and B, respectively. Among the more than 50 year old patients, 21(95.45%) of them were of genotype D and one of them (4.5%) was of unknown genotype. The genotype B_6 was not seen. Prevalence of HBV in males was 31(91.18%) with genotype D, 1(2.95%) with genotype B and 2(5.9%) with unknown genotype. Prevalence of HBV in females with genotype D was 15(93.75%) and with genotype B was 1(6.25%) and the unknown genotypes were not detected.

Discussion

The hepatitis B virus is highly prevalent in hemodialysis patients. Hemodialysis patients are counted to have increased risk of HBV infection because of the opportunity of exposure to HBV associated with the dialysis procedure. The reservoir of infection for potential transmission of HBV is greater in hemodialysis patients. It is known that different methods of control, cleaning and disinfection of the hemodialysis membranes, machines, instruments and environmental surfaces may interfere with determined prevalence [1].

In this study, the distribution of HBV genotypes and occult hepatitis among hemodialysis patients in the Khuzestan province was analyzed by RFLP method. Ten patients showed negative HBsAg, but PCR in these samples was positive, indicating surface mutation of HBV infection. It may be associated with mutations in the S gene that make HBsAg undetectable by serological assays or with low-level infection below the limit of detection of the HBsAg detection test kits [3]. This is called occult hepatitis.

Occult HBV infection is a world-wide diffused entity although its distribution may reflect the general prevalence of the HBV in the various geographical areas and in the various populations [5]. By PCR method, occult hepatitis can is detectable. Prevalence of occult HBV is 0% to 36% in the most recent reports (Table 2) [6-12].

Table 2: Occult hepatitis and genotypes in other countries

Country	Prevalence of occult HBV in hemodialysis patients	Genotype	No of patients
Greece	0.9%	A	366
Turkey	9.8%	D	71
Italy	26.6%	A,C	128
Korea	0%	C	83
North America	3.8%	A,B,C	241

As mentioned above, however, these discrepancies appear to be mainly dependent on the different sensitivity and specificity of the assays utilized in the various studies. In this context, it is of note that several authors consider occult HBV as a possible source of virus spread in

hemodialysis units (thus representing a risk of infection for both patients and staff), and suggest some precautions including HBVDNA screening for all hemodialysis patients [11].

Out of the 10 patients negative for HBsAg, 4(1.6%) were negative for HBeAg and positive for HBeAb which it may have a precore mutation, because frequently the mutation occurs after seroconversion to Anti-HBe. These mutations are commonly found in patients from Southern Europe, Asia, Africa, and the Middle East who are HBV chronic carriers or exhibit HBV-induced chronic active hepatitis [13]. HBV is classified into eight genotypes by a sequence divergence in the entire genome exceeding 8% and designated by capital letters of the alphabet from A to H.

Genotypes of HBV shows different response to chemotherapy of virus, for example core promoter and lamivudine resistance mutations were found to be more common in genotype C and A, while pre-core stop mutations have been observed more frequently in genotypes B and D [14]. Genotype D is the most widely distributed genotype and has been found universally, with its highest prevalence in Southern Europe and North Africa to India, in West and South Africa [1]. Genotype D was classified into three groups, D_1, D_2, and D_{del}. Genotype B was also classified into six pattern, B_1, B_2, B_3, B_4, B_5, and B_6 [4].

We found a predominant frequency of HBV genotype D pattern D_2 46(92%) and genotype B pattern B_6 2(4%) in hemodialysis patients and 2(4%) genotype were untypeable by this method (Table 1, Figs. 1-3). Some previous studies in Iran have shown that all patients in different type of HBV infection have genotype D [15,16]. According to recent studies, genotype D in Asia is associated with more severe disease and may predict occurrence of hepatocelluar carcinoma especially in younger patients [17]. Thus, knowledge of the genotype infecting hemodialysis patients can assist a physician in making clinical and therapeutic decisions.

The prevalence of different HBV genotypes reflects the origin of immigrants and other patterns of migration [18]. Existence of genotype B in Iran correlates with migration from southern east of Asia. Patens of two samples could not be classified because of the mixed type of genotypes. The RFLP method cannot type mixed genotype infections [19]. Therefore, some advanced methods are needed. In this study the correlation of genotypes and gender was considered. In our study 31(91.18%) of male patients have genotype D, 1(2.95%) genotype B and untypeable genotype were 2(5.9%). In females, genotype D was found in 15(93.75%) patients and genotype B was found in 1(6.25%) patients.

Previous studies have shown that in chronic HBV, female patients response to treatment is more successful than males because the level of DNA virus is lower and the level of transeaminase is higher in these [20]. Similarly, males response to treatment may be low our study.

Conclusion

In conclusion, HBV circulates in two different genotypes in hemodialysis patients in Khuzestan province. HBV genotype D is highly predominant in these patients with respect to the serological tests that cannot detect the mutant samples, so it is necessary to carry out the PCR as a routine test for these patients.

Acknowledgment

The authors would like to thank vice chancellor for research affairs of Ahvaz Jundishapur university of Medical Sciences.

This study is based on an MSc thesis which was granted by infectious and tropical diseases research center (Grant No. 86114).

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