STUDY OF HEPATITIS B VIRUS (HBV) GENOTYPES IN KERMANSHAH PROVINCE, WEST OF IRAN

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Abstract
Hepatitis B virus (HBV), the prototype member of the family hepadnaviridae, is a human pathogen, causing the serious liver disease. The virus is the major cause of chronic hepatitis cirrhosis, and hepatocellular carcinoma worldwide. HBV has been classified into eight genotypes, which vary in geographic distribution. The aim of this study was to recognize the HBV genotypes in hepatitis B surface antigen (HBsAg)-positive carriers in west of Iran. In this study, one hundred HBsAg-positive carriers were used. Genotypes of HBV were recognized by nested-PCR that combined by restriction fragment length polymorphism (RFLP) technique. Eighty six out of hundred samples were positive-PCR and were used for HBV genotyping. Recognized genotypes were D and B, with frequency of eighty five and one cases (out of eighty six samples) respectively, while no result was obtained in other fourteen samples. This study indicated that predominant genotype of HBV in west of Iran is genotype D, while genotype B is very rare in this region.

Key words: Hepatitis B virus, Genotype, RFLP, Kermanshah, Iran

INTRODUCTION
The hepatitis B virus (HBV) is one of the most common chronic pathogens in the world, and high percent of the world's population have been exposed to this virus [1], and part of them have been died due to the consequences of this infection such as cirrhosis and hepatocellular carcinoma (HCC) [2]. HBV is the prototype member of hepadnaviridae family [3] and the Orthohepadnavirus genus [3,4]. The HBV genome is a partial, double-stranded DNA with four overlapping open reading frames [5].

In 1988, HBV was classified into four genotypes, and designated by capital letters of the alphabet from A to D [6]. In 1994 [7], an additional two HBV genotypes were found and named E and F, respectively. Genotype G was reported recently in 2000 [8] and genotype H, was proposed in 2002 [6]. Therefore HBV, the hepadnavirus infecting humans, is classified into eight genotypes, A-H, based on an intragroup nucleotide divergence of up to 4.2% of the S-gene sequences or in some cases up to 8.0% of complete genomes [9,10], until now. These genotypes arise during replication as a result of nucleotide misincorporations, in the absence of any proof-reading capacity by the viral polymerase [11].

HBV genotypes have distinct geographical distribution [12-14]. Genotype A is found in northwestern Europe, North America and Africa [15-17]. Genotypes B and C are characteristic of Asia and Oceania, whereas genotype D has a worldwide distribution but predominates in the Mediterranean area [14-16]. Genotype E is found in Africans on the West Coast of Africa [14,15,17] and Madagascar on the east [6], genotype F in the aboriginal populations of South America [18] and genotype H is limited to the Amerindian populations of Central America and has been detected in California and Mexico [19]. To date, the isolation of genotype G has been limited to HBV carriers in France [8], Germany [20], United Kingdom [16], Italy [16] and the United States of America (USA) [8,16].

The prevalence of HBV infection varies widely, with rates ranging from 0.1% to 20% in different parts of the world [21] and it has been reported that there are remarkable differences in the clinical and virologic characteristics between the patients that infected with different genotypes [22,23] therefore detection of HBV genotype is very important in every region of the world.

Overall, 45% of the world population lives in high prevalence regions (hepatitis B surface antigen (HBsAg) positivity rates > 8%), resulting in the massive global burden associated with the infection [24], and Iran is area of low endemicity [25]. The prevalence of HBsAg in Iran was reported in high range [26] but the prevalence rate is different in different parts of Iran [27]. There are evidence of high rate exposure to HBV among Iranian cirrhotics and patients with HCC [28]. These data show that HBV is the most common cause of cirrhosis and HCC in Iran.
There are several methods for genotyping of HBV. For this, in this study we used nested-PCR combined with restriction fragment length polymorphism (RFLP) strategy, that has been reported by Zeng et al [11] (by some modifications), because it could identify HBV genotypes A to H and would be applicable to large-scale studies. Data on genotype determination of HBV are very limited in west of Iran and not available data for this in kermanshah province. Thus the present study aims to determine the prevalence genotypes of HBV in Kermanshah province, that located in west of Iran.

MATERIALS AND METHODS

SERUM SAMPLES:

In this study, a number of blood donors who were from Kermanshah province and had come to Kermanshah Blood Transfusion Organization for blood donation, were studied in period during 2007-2008. Blood donors were screened for hepatitis B surface antigen (HBsAg) by ELISA method and retested again. One hundred HBsAg-positive serum samples, who had no HCV- and HIV-positive, were selected from carriers in different cities of Kermanshah province and used to determine the HBV genotypes. These serum samples were male/female = 88% /12% and mean age =34.6 years. These serum samples were stored at -70°C until using for DNA extraction.

DNA EXTRACTION:

Hepatitis B virus DNA was extracted from 200µl of each serum sample by using a nucleic acid extraction Kit (QIAamp MinElute Virus Spin Kit, QIAGEN, and Germany).

PCR AMPLIFICATION:

In this study nested PCR assay that combined with restriction fragment length polymorphism (RFLP) was used, according to previously described method[11] (but by some modifications in profile of PCR, designed primers and restriction enzymes that were used in RFLP). The S gene sequences were amplified by two pairs of primers in nested PCR strategy that could amplify an amplicon of 585 bp (sequence between nt 203-787). The sequences of PCR primers were used in this study are shown in Table 1. PrsS3 and S1R primers as the first-round primers (outer primer pair) were used to amplify the sequence between nt 842 to nt 2820 (an amplicon of 1222 bp). YS1 and YS3 primers as the second-round primers (inner primer pair) were used to amplify the amplicon of 585 bp of S gene. PCR amplification was done for each two first- and second-round PCR under the following conditions: initial denaturation 3 min at 94°C, followed by 35 cycles of 94°C for 45 sec, 60 sec at 55°C and 72°C for 90 sec. Final extension was performed at 72°C for 6 min. A volume reaction was 25µl for first-round PCR and 50µl for second-round PCR. The first-round PCR was performed with the following amplification mixture: 2.5µl of extracted DNA, 0.5µl dNTP mix (10 mM), 2.5µl 10x Taq polymerase buffer, 0.75µl MgCl₂ (50 mM), 0.2µl Taq polymerase (5 unit/µl) and 1µl of each primers PrsS3 and S1R (10 pmol). A volume of 1µl of the first-round PCR products was then added to a second-round PCR mixture. 4µl of the second-round PCR products was visualized under UV light on a 1% agarose gel.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP):

The RFLP pattern that was used for HBV genotyping in this study, has been reported by Zeng et al [11] (by some modifications). The enzymes that were used in this study were MboI, StyI, HpaII, CfrI and BsrI. The enzymatic digestion was done on the second round PCR 585bp fragments. In this pattern, the genotype C is the only one of the genotypes that is cut by StyI. All of the genotypes B, A, E and G are cut by BsrI but the digestion pattern of the genotype B is different from the genotypes A, E and G. In this pattern genotype B has a cleavage site in nt position 126, 459bp but the cleavage site in the genotypes A, E and G is in nt position 300, 285bp. Genotypes D, F and H are not cut by BsrI and StyI therefore their second round PCR products remain intact. Thus the genotypes B and C are identified by doing a single step parallel enzymatic digestion. The genotypes E, F and H are cut by HpaII but the genotypes A, D and G have not HpaII cleavage site. Also CfrI has cleavage site in all of the genotypes except the genotype G. Otherwise MboI cuts genotypes H and D but its digestion pattern is different from genotype F digestion pattern (figure 1).

A volume of 4µl of the second-round PCR products was mixed with 4.5µl ddH₂O, 1µl of 10x buffer and 0.5µl of each restriction enzyme. The mixtures were incubated at 37°C for MboI, StyI, HpaII and CfrI but at 65°C for BsrI for 4 h. The restriction patterns were then electrophoresed on a 3% agarose gel, and visualized by ethidium bromide staining under ultraviolet light.
Table 1. primer's sequences that were used for HBV genotyping

<table>
<thead>
<tr>
<th>Primer’s name</th>
<th>Primer’s sequence</th>
<th>Binding position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrsS3</td>
<td>5'-GGGT[C]CACCATATTCTTGG</td>
<td>Sense, nt 2820-2837</td>
<td>Zeng et al [11]. By modification in fourth nucleotide, from A to T</td>
</tr>
<tr>
<td>S1R</td>
<td>5'-TTAGGGTTTAATGTATACCCA</td>
<td>Antisense, nt 842-821</td>
<td>Zeng et al [11].</td>
</tr>
<tr>
<td>YS1</td>
<td>5'-GCGGGGTTTTTCTTGTTGA</td>
<td>Sense, nt 203-221</td>
<td>Zeng et al [11].</td>
</tr>
<tr>
<td>YS3</td>
<td>5'-GGGACTCAAGATG[C]GTACAG</td>
<td>Antisense, nt 787-767</td>
<td>Zeng et al [11]. By modification in fourteenth nucleotide, from T to C</td>
</tr>
</tbody>
</table>

RESULTS

The 86 samples of 100 samples were PCR positive and in second-round PCR showed 585 bp bands and were used for HBV genotyping analysis, and in other 14 samples no result was obtained. Genotypes D and B were detected in this detection HBV genotypes study. Genotype D was detected in 85 of 86 samples (98.8%), while genotype B was recognized only in one sample of 86 samples (1.2%). In this study we used of MboI instead of DpnI, and revealed that DpnI cannot cleave unmethylated 5'-GATC-3' [29], that was reported by Zeng et al. Agarose gel electrophoresis of PCR- and RFLP- products of one sample are shown in Fig. 2.
Figure 1. Chart representation of the position of PCR primers and RFLP analyses. Digestion patterns for genotyping of HBV, that reported by Zeng et al and modified in this study, are shown in bold and colorful. BsrI, StyI, MboI, HpaII and CfrI are reported by 1, 2, 3, 4 and 5 numbers respectively, in this study. Enzymatic digestion by restriction enzymes was performed on second-round PCR products (585 bp length).
Figure 2. Second round PCR- and RFLP- products of S amplicon of HBV from some PCR-positive serum samples. (A): second round PCR-product. Lane 1: negative control, lane 2-9: positive PCR and M; 100bp DNA ladder. (B): lane 1 and 2; untreated PCR products (related to genotype D), lanes 3 and 4; RFLP products after digestion with BsrI, lanes 5 and 6; RFLP products after digestion with StyI and lanes 7 and 8; RFLP products after digestion with CfrI , lanes 9 and 16; RFLP products after digestion with MboI and M; 100bp DNA ladder plus. (C): lane 1; untreated PCR product (related to genotype B), lanes 2 and 3 are RFLP products after digestion with BsrI and StyI, respectively. In this study, 85 cases of genotype D and 1 case of genotype B were revealed.

DISCUSSION

In this study, we focused on studying of HBV genotypes. According to obtained results of our research, genotype D were detected as the dominant genotype in our region (west of Iran). HBV has been classified into 8 genotypes (A-H), that show a distinctive geographical distribution [14]. Because HBV is an etiologic agent of acute and chronic disease throughout the world [30], and also its genotypes might influence mutation patterns in precore and core promoter regions, severity and activity of liver disease, patterns of serological reactivity, replication of the virus, prognosis and response to antiviral treatment [11], detection of HBV genotypes is very important to clarify the pathogenesis, route of infection and virulence of the virus. In our country data about HBV genotypes are very limited, and because in some cases, differences in the distribution of the genotypes can be found within a single country [14], HBV genotypes should be determined in different district of each country. There is no information for this in Kermanshah province. Thus in presented study, we focused on detection of HBV genotypes in Kermanshah province, that located in west of Iran.

In our study obtained results for genotype D are similar to other reports from Iran and other countries of Middle East area. For examples, in Iran genotype D was the only detected HBV genotype in 109 patients that were used for genotyping by INNO-LiPA methodology [31]. Also in other study by using type- specific primers method, done in Shiraz province (in southwest Iran) revealed HBV genotype D was the only detectable genotype in different clinical forms of HBV infections in patients with acute, inactive HBsAg or chronic hepatitis [32]. There is also other study, that carried out by Amini et al, revealed that all 26 chronic hepatitis B Iranian patients were HBV genotype D carriers [33]. In study was done in Saudi Arabia, by nested PCR-
mediated amplification of the target sequence and hybridization with sequence-specific oligonucleotides method, showed that 57 patients of 70 were genotype D [34]. There is also a study in Egypt showed HBV-D genotype was dominant in this region [35]. Also in another study in this country showed genotypes D and B were related to pediatric cancer patients [36]. In addition one study by phylogenetic analysis method (of surface region sequence) in Yemen revealed that genotype D was predominant genotype [37]. Also similar study in India determined genotype D as the predominant genotype of HBV. According this study among patients with chronic HBV infection, genotypes D, A and C were detected in 57.3%, 18% and 11.5% respectively [38]. In one study in Pakistan, genotype A, B and C were reported as the predominant genotypes and genotype D was rare [39]. In Turkey, a study showed all studied patients were infected by genotype D [40]. In addition in another study in this country, genotype D was detected in 78 of 88 patients [41].

In our study, genotype B was revealed as a finding only in one sample. The result of presentation of genotype B in this study, conform with previous studies, including those were done in Pakistan [39], Egypte [36] and Taiwan [42]. There is no report about present of genotype B in Iran so far. Therefore present of this genotype in this region is remarkable. Because the distribution of HBV genotypes can be influenced with time and with population migration [43], observation this genotype in this region, Kermanshah province, can be concluded population migration from other region of Asia to the area. Also distribution of HBV genotypes can be influenced by the ethnic background and the country of origin of the individual carriers of the virus [14]. Otherwise this genotype is very rare in our position. This finding can clarify that predominant genotype of HBV in Kermanshah province similar to other region of Middle East area is genotype D, regardless to ethnic.

Otherwise did not obtain result in 14% samples. Because the serum of an infected patient may contain 1000 to 10,000 empty particles for every one that contains the viral nucleocapsid [44], these negative samples have low titer of complete virion.

In this study we used of MboI restriction enzyme instead of DpnI restriction enzyme that was reported previously by Zeng et al [11], because DpnI cannot cut unmethylated GATC. DpnI and MboI recognize the same sequences, 5'-GATC-3', but show differential cleavage specificity based on methylation status of the adenine residue. DpnI cleaves 5'-GATC-3' when the adenine is methylated, but MboI is inhibited by methylation, and only cleaves 5'-GATC-3' when the adenine is unmethylated [29]. Therefore with regard to say above, we used of MboI, because PCR product cannot methylated.

In conclusion, we found that genpotype D is main and frequent HBV genotype in Kermanshah province. This study can open the way for doing other studies such as understanding of antiviral drugs effects on the patients affected with HBV. Otherwise, in another researchs, we can study and detect the HBV subgenotypes and serotypes, and by this we can help to survey main and effective antiviral drugs to cure the affected patients.

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