ORIGINAL ARTICLE

doi: 10.5455/medarh.2014.68.376-380

Published online: 16/12/2014
Published print: 12/2014

Med Arh. 2014 Dec; 68(6): 376-380
Received: August 15th 2014 | Accepted: October 10th 2014
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Hepatitis B Virus Genotypes Distribution with HBsAg Positive in the North of Iran (Mazandaran) During 2011-2014

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ABSTRACT

Background: HBV infection is a major global health problem and ten genotypes (A to J) and multiples subtypes of HBV have been identified, and they show some distinct geographic distributions. The available data on HBV genotype in Iran are very heterogeneous and limited. Therefore in this study, we tried to identify the HBV genotypes by using polymerase chain reaction. **Methods:** In this cross-sectional study, HBV- positive serum samples of 122 patients with chronic hepatitis from 2011 to 2014 were studied. HBV-DNA was extracted from plasma samples using QIAamp® MiniElute® Virus Spin Kit (Qiagen). Plasma samples from HBsAg positive were confirmed the presence of HBV nucleic acid and determined the genotypes of HBV genome by PCR using the DNA PCR kit (Cinagene) with Taq-DNA polymerase enzyme and type of specific primers. All samples were examined in the virology laboratory of Sari Medical School. **Results:** The mean age of patients were 45 ± 25 (range, 20 to 70) year that 70 (57.37%) patients were male and 52 (42.62%) were female. The majority of HBV positive patients had a major surgery (44% patients) and then 32% patients followed by intra familial of hepatitis B virus infected and 11% of HBV positive patients had a history of blood transfusions. In this study, 91(74.59%) had genotype D, 7(5.73%) genotype B and 24(19.67%) genotype D and B. **Conclusion:** This study indicates that the genotype D is the most frequent followed by the mixed genotypes D and B and genotype B in our region. Prevalence and incidence of HBV genotypes are with distributed among of areas and different genotypes may show different responses with antiviral therapy.

Key words: Hepatitis B virus, Genotype of HBV, PCR.

1. BACKGROUND

Hepatitis B virus (HBV) infection can cause liver diseases including chronic hepatitis, cirrhosis and hepatocellular carcinoma (1, 2). Current estimates that there are nearly two billion people worldwide have been infected with this virus and more than 350 million of people live with chronic infection, of these; 0.5-1.2 million people die annually from complications of chronic hepatitis B (3-5) which is the 10th leading cause of death worldwide. Throughout the world, carrier variability rate for hepatitis B infection is estimated to be 0.1% to 20% (6), with regions classified as having low (<2%), intermediate (2-7%) and high (>8%) endemimicity. In Iran, it is estimated that near 35% of population have been exposed to HBV and the endemicity is intermediate, with a carrier rate 3% (7). It was reported that the HBV infection indicates an intermediate rate in this country and the distribution of carrier rate of HBV infection in the different provinces of Iran shows different (1.3% to 6.3%) (8). After HBV vaccination program, Iran can be considered one of the countries with low HBV infection endemicity (9).

The HBV belongs to hepadenaviridae family and is an enveloped, double-stranded DNA genome of approximately 3200 bass pairs. So far, ten HBV genotypes (A-J) and multiple subtypes have been identified (10-13) and HBV genotypes have distinct geographical distribution of the world (14-16). The study was shown that Turkish patients with chronic hepatitis B infection indicated very little genotypic heterogeneity. Genotype D of HBV represented almost the whole Turkish patient population infected with HBV (17, 18). In Pakistan, genotype D was the predominant type found in 128 (64%) patients followed by A in 47 (23%) and mixed A/D in 26 (13%) (19). A study was reported that 65.34% were classified into genotype D, 26.73% were of genotype B while 4.95% had genotype A. So in 2.98% samples, multiple genotypes were detected (genotype A+B; 1.98% and genotypes B+D; 1%) (20). In India, HBV genotype D was the most predominant

(56.0%) genotype followed by HBV genotype C (23.4%) and HBV genotype A (20.6%) (21). The study demonstrated that genotype D (35.67%) is the predominant genotype circulating in Afghani's population and followed by genotype C (32.16%), genotype A (19.30%), and genotype B (7.02%) (22). In Iran, Several studies were shown that the only genotype circulating in the some provinces of Iran found genotype D. in different clinical forms of HBV infections (23-28).

The distribution of HBV genotypes may guide us in determining disease burden. HBV genotypes have been shown to differ with regard to prognosis, clinical outcomes and antiviral responses (29, 30). So, it is important to know the epidemiologically of HBV genotyping as well. The available data on HBV genotype in Iran are very heterogeneous. Therefore, this study was designed to determine and analyze the distribution of HBV genotypes among patients with HBsAg positive in Mazandaran province in the North of Iran by using polymerase chain reaction (PCR).

2. MATERIALS AND METHODS

2.1. Samples

During 2011- 2014, serum samples from 122 HBsAg positive patients with chronic hepatitis referred to Interior and Infections Disease Center in Sari Hospitals (Mazandaran province, north of Iran) were collected. Chronic hepatitis B was defined as infectious if the virus was persistent for more than six months. The blood samples were centrifuged and plasmas were separated and immediately stored at -70°C. All patients had elevated serum aminotransferases a positive test for HBsAg using enzyme linked immunosorbent assay (ELISA), and HBV genotypes were determined by DNA extraction kit using standard protocol. Factors such as, age, gender, suspected sources of infection (high-risk sexual relation, injective addiction and blood injection), last laboratory tests results (AST and ALT) were chosen through questionnaire for all patients. The data analyzed with SPSS 17 and Chi-square test.

2.2. DNA extraction

DNA extraction from plasma samples was extracted by QIAamp® MiniElute® Virus Spin Kit (Qiagen) using standard protocol. HBV was isolated from serum on following procedures; 25µl protease, 200µl plasma of patients with 200 µl AL Buffer (Lyses Buffer) mixed for 15 seconds by vortex and incubates at 65°C for 15 minutes, and centrifuges quickly. To add 250 µl Ethanol (96%-100%) and mixed for 15 seconds by vortex and incubates at room temperatures, after that all of them add to QIAamp Mini Elute columns. Samples were centrifuged for one minute at 8000 revolutions per minute (rpm) after finishing centrifuge, overlaid fluid separated and added in to the same volume. Next, tube content remained solution washed with 500µl of AW1 Buffer and centrifuged for one minute at 8000 rpm and discharged overlaid fluid, and washed with 500µl of AW2 Buffer and then washed with 500µl of Ethanol 96%-100%. Sample tubes centrifuge again for one minute at 8000 rpm and incubate for 3 minutes at 65°C for drying. Finally, 50 µl sterile distilled water or AVE Buffer added in the central above columns and incubated 1-2 minutes at room temperature, centrifuged at 14000 rpm for 1 minute. DNA quantification was determined using a spectrophotometer and resulted residue solved for next stages. All samples were examined in the virology laboratory of Sari Medical School.

2.3. PCR Test

Total DNA was isolated from serum samples and was done PCR-Test using DNA PCR kit (Cinagene) with Taq-DNA polymerase enzyme and according to special protocol with individual primers (31). Examination method summarized as follow: To make Master Mix of 1120 µl distil water, 30 µl dNTP, 150 µl 10x PCR Buffer and 90 µl $MgCl_2$. 11.5 µl of above reaction mix with 0.2 µl of Taq DNA polymerase, 40 pmol of each forward and reverse primers, 2 ng of DNA sample and up to 20 µl dH_2O. The above reaction placed into the Eppendorf Master Cycler PCR Machine and amplified. PCR program for amplification consisted of 95°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 58.5°C for 1 minute and 72°C for 1 minute and finally, 72°C for 10 minute.

2.4. Gel Electrophoresis

Agarose gel electrophoresis is an easy way to separate DNA fragments by their sizes and visualize them. We use 1.5% agarose gel to run PCR product (DNA fragment) from samples. This is a graphic representation of an agarose gel made by "running" DNA molecular weight markers. These gels are visualized on a UV analyzer by staining the DNA with a fluorescent dye (Ethidium bromide which is very carcinogenic). The DNA molecular weight marker is a set of DNA fragments of known molecular sizes that are used as a standard to determine the sizes of fragments.

3. RESULTS

During study period, 122 patients who had been infected to be HBsAg positive were enrolled in this study. It has been demonstrated that all of patients had chronic. The mean age of patients were 45 ± 25 (range, 20 to 70) year that 70 (57.37%) patients were male and 52 (42.62%) were female and all patients (100%) were married. The mean serum level of AST and ALT was 89.5 and 103.7 IU/lit respectively.

In this study, majority (44% patients) of HBV positive patients had a history of surgery, and 32% patients followed by intra familial of hepatitis B virus infected and 11% of HBV positive patients had a history of blood transfusions. Our result also showed that some patients had cirrhosis.

		Type			Total (%)	p-value
		B (%)	D (%)	B+D (%)		
Gender	Female	7 (5.73)	35 (28.68)	10 (8.19)	52(42.62)	0.38
	Male	3 (2.45)	48 (39.34)	19 (15.57)	70(57.37)	
Type of infection	chronic	10 (8.19)	85 (69.67)	27 (22.13)	122 (100)	0.353
Age	<25	2 (1.63)	7 (5.73)	2 (1.63)	11 (9.01)	0.106
	25-45	3 (2.45)	48(39.34)	11 (9.01)	62(50.81)	
	>45	3 (2.45)	26 (21.31)	20 (16.39)	49(40.16)	

Table 1. Genotypes distribution according to gender, disease and age group

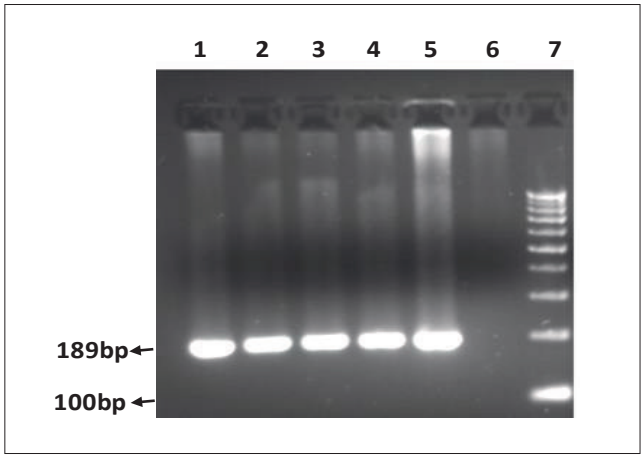

Figure 1. The electrophoresis pattern of HBV genotypes by genotype specific primers amplification (31). 1-4 are HBV-D positive Samples (189 bp), 5 is a control positive, 6 is a control negative and 7 is 100 bp DNA marker.

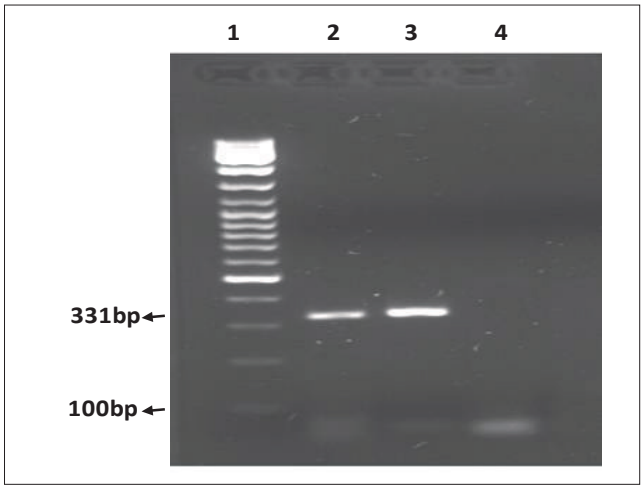

Figure 2. The electrophoresis pattern of HBV genotypes by PCR using genotype specific primers (31). 1 is 100 bp DNA marker, 2 is a HBV-B positive sample (331 bp), 3 is a control positive and 4 is a control negative.

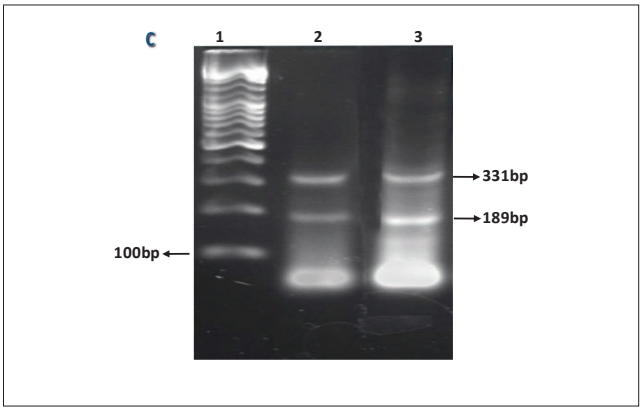

Figure 3. The electrophoresis pattern of HBV genotypes by PCR using genotype specific primers (31). 1 is 100 bp DNA marker, 2 is a HBV-B , D positive sample (331-189bp), 3 is a control positive.

Genotyping was using polymerase chain reaction of HBV DNA positive serum samples. Of these, 91 (74.59%) of cases were infected with genotype HBV-D, 7 (5.73%) were infected with genotype HBV-B, 24 (19.67%) were infected with genotypes HBV-D and HBV-B.

The most prevalent genotype in patients was genotype HBV-D. There was no significant relation between HBV genotypes and according to gender, disease and age group (Table 1).

PCR for identification of HBV genotypes by gel electrophoresis:

4. DISCUSSION

The carrier variability rate for hepatitis B infection is estimated from 0.1% to 20% throughout the world (6). In the Middle East, it has been reported that HBV infection is an intermediate rate, varying between 2% and 7% in different countries (32). In blood donors, this rate was 0.8 % in Iran (33), 4 % in Pakistan (34), 1.1-3.5 % in Kuwait (35), 4.19 % in Turkey (36) and 1.9 % in Saudi Arabia (37). It can be concluded that HBsAg rate among blood donors in Iran is still less in comparison with other neighboring countries of Iran. At present, HBV has been classified into ten different genotypes (A-J) by genome sequencing of HBV strains (10-13). Genotypes of HBV have different geographic distribution in the world (38, 39). The aim of this survey was to determine HBV genotype among of HBsAg positive patients who referred to Sari hospitals in the Northern of Iran. In this study, the majority of HBV positive patients had a surgery (44% patients), and 32% patients had a family of HBV infected, and 11% of patients had a history of blood transfusions. The results showed that among the 122 samples, the proportions of genotype D, B and mixed genotype (D+B) were 91%, 7% and 24% respectively. Same to our study, it has been shown the HBV genotype D is distributed worldwide, and reported frequently (20-23, 40). It has been reported that the distribution of HBV genotypes was not just one genotype but some samples were mixed. It has been reported that 62.2% were found genotype D, 13% were found genotype A and 12%. Mixed genotype D+A was found in 12% of acute patients, 5.6% of chronic patients and 5.6% of carriers (40), 85.1% were genotyped as type D/E, 4.4% were genotyped as type A, 1.4% were genotyped as type C, and 0.7% were genotyped as type F (41) and other study was indicated the HBV genotype frequencies were: B, 57.9%; C, 16.0%; and BC, 26.1% (42). A study conducted among injecting drug users showed that the presence of genotype D in 62%, genotype A in 9% while 29% individuals were found to be infected with both of genotype A and D (43). But some studies from different part of Iran have reported that the genotype D is the only detectable genotype in the different clinical forms of HBV infection, including carriers HBV, chronic liver disease and cirrhosis (23-28) that is not the same as our results because we found HBV genotype D, B and mixed D+B. Genotypes A and D were most prevalent in co-infected patients with HIV and so, HBV subtype A was present among three-fourth of patients infected through sexual contact, whereas the same percentage of subtype D was isolated among injection drug users (44).

HBV genotyping may guide us in selection of the duration and type of antiviral therapy and to predict the likelihood of sustained HBV clearance after therapy. It seems that there are different types of HBV genotypes in different parts of countries, due to wide range of geographical distribution and influence of the neighbors in the abundance of different types of HBV genotypes. Furthermore, factors

such as repeated blood transfusion and treatment of the patients also can be some of the most important criteria which can cause this wide range of different genotypes. In conclusion, the present study describes HBV genotyping in Mazandaranian (in the north of Iran) patients infected with HBV. This preliminary report describes that the genotype D is not the only genotype in the patients with HBsAg, which is different as other studies. Thus, further studies are needed to achieve the confirmation and so to determine the distribution of genotype of HBV subtype in patients with HBV positive.

Acknowledgments

This study was granted (grant number: 89-45) by Vice-Chancellor for Research of Mazandaran University of Medical Sciences. I would like to thank from them, medical college and all coworkers of Sari hospitals.

CONFLICT OF INTEREST: NONE DECLARED.

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