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Hepatitis B virus genotype frequency in patients of northern counties from Iran by Multiplex allele-specific PCR

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ABSTRACT

Hepatitis B is of the main causes of liver cell carcinoma (HCC) and Cirrhosis. It has been shown that HBV genome nucleotide diversity affects the disease progression. Therefore, genotyping of hepatitis B virus can be crucial step in clinical and epidemiological diagnosis. This study aimed to determine the prevalence of HBV genotypes among patients from two Iranian cities, Sari and Rasht, by using a molecular technique, Multiplex allele-specific PCR. This study was done on 160 patients with hepatitis B (HBsAg positive) during year 2010-2011. Samples were collected from Rasht and Sari counties in northern Iran. After extraction of HBV DNA, their genotypes were determined by Multiplex allele-specific PCR method. The data obtained were analyzed through electrophoresis of amplified fragments on Agarose gel and compared with size marker. 93.8% of patients (150 samples) were positive only for genotypes D, while 6.2% showed a mix of different genotypes including D(2.9%), F (2.5%), B (0.4%), and A (0.4%). The result obtained by this study showed that Multiplex allele-specific PCR can be used as a precise technique for identification of different genotypes of hepatitis B virus in both large scale epidemiological investigations and clinical studies.

Keywords: Hepatitis B virus, genotyping, Multiplex allele-specific PCR.

INTRODUCTION

Hepatitis B virus (HBV) is a member of the Hepadnavirus family with a genome size 2.3 kbp. HBV is of an important health problem throughout the world with more than 300 million chronic infections and about one million deaths per year due to Hepatocellular Carcinoma (HCC) and Cirrhosis [1-3]. Chronic carriers of HBV are at high risk of HCC development with the highest global prevalence of mortality in Asia. On the other hand, 53% of the HCC causes are attributed to the HBV [4]. According to reports, around 15-40 % of HBV infected people and are under risk of liver failure, HCC and Cirrhosis [2-3]. In Iran more than 35% of individuals are exposed to HBV, and HBV is considered as the most common cause of HCC [5].Different studies done on HBV have showed that HBV genotypes affect the disease progression [6-8]. HBV genome nucleotide diversity causes a difference in response to antivirus treatment [9-10]. Hepatitis B virus is classified to 8 genotypes (A to H) based on the difference in more than 8% of complete genome sequence [11-12]. These genotypes have different geographical distribution. Genotypes A and D are more common in Europe, Russia, India, and North of Asia; genotypes B and C are more common in East Asia and Australia; and genotypes H, F, and G are more distributed in Central and South America. During the past decade, PCR based techniques have extensively been used in molecular diagnosis of diseases [13-15]. The ability of Multiplex allele-specific PCR technique allows us to simultaneously determine several genotypes of a virus in one PCR reaction. In this study we assessed the HBV genome diversity among HBsAg positive carriers from two northern regions of Iran, Sari and Rasht, by using Multiplex allele-specific PCR technique.

MATERIALS AND METHODS

Blood samples were collected from 160 people with hepatitis B positive sera (HBsAg positive) who were referred to Fajr and Dr. Ashtiani medical detection laboratories in Sari and Rasht, respectively during year 2010-2011. DNA of all samples was extracted by High Pure Viral Nucleic acid kit (Roche,Germany). The quality and quantity of extracted DNAs were analyzed by Biophotometer (Eppendorf, Germany). Primers used in this study were those used by Chen et al. [16] (Table 1).

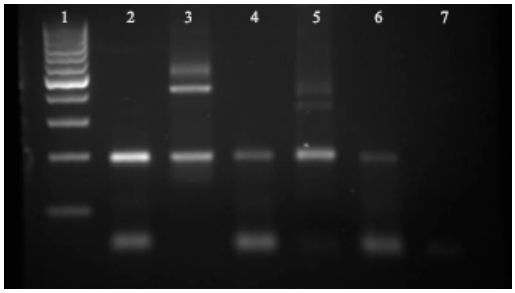
Table 1- Primer Sequence and product size for each HBV genotype

Genotype		DNA sequence (from 5′ to 3′)	Position	Size (bp) of product
A	A–S	AAA CTA CTG TTG TTA GAC GAC GAG ACC	2334–2360	644
	A–AS	CTG GAT TGT TTG AAT TGG CTC CG	2955–2977	
B	B–S	CCA AAC TCT TCA AGA TCC CAG AGT CA	16–41	331
	B–AS	ACA AGT TGG TGA GTG ACT GGA GAT TT	321–346	
C	C–S	CTC CCA TCT CTC CAC CTC TAA GAG ACA GT	3164–3192	242
	C–AS	CAG GGG TCC TAG GAA TCC TGA TGT TG	165–190	
D	D–S	CAG ACG CCA ACA AGG TAG GAG CT	2972–2994	189
	D–AS	GAG TGT TTC TCA AAG GTG GAG ACA GA	3135–3160	
E	E–S	ATA CCC TAT GGA AGG CGG GCA TCT	2752–2775	130
	E–AS	CCC ATT CGA GAG GGA CCG TCC A	2860–2881	
F	F–S	TAT CTG TGG GTA TCC ATT TGA ATA CTT C	815–842	487
	F–AS	CGA GCG AAA CAA GCT GCT AG	1282–1301	

The PCR reaction was performed in a total volume of 50 µl including 2.5U Taq DNA polymerase, 5 µl buffer 10X (potassium chloride 500 mM, Tris-cl 500 mM, PH = 8.4), 10 pmole primers, 1 mM magnesium chloride, 0.2 mM dNTPS, and 20 ng Template DNA. The PCR cycling was carried out on Eppendorf Master Cycler (Germany). Cycling condition was performed as follows: 95 °c for 5 minutes as initial Denaturation, 94 °c for 1 minute, 59 °c for 1 minute, and 72 °c for 1 minute for 35 cycles, and 72 °c for 5 minutes as final extension. The PCR products were run on 2% agarose gel for electrophoresis. The types of all genotypes were determined base on their PCR product size through their comparison with 100bp size marker.

RESULTS

In this study, 160 patients with hepatitis B (HBsAg positive) including 118 females and 42 males were studied. Genotyping by Multiplex allele-specific PCR technique indicated that genotype D is the dominant genotype in all patients. The results obtained from 10 patients also showed a mix of several genotypes. Two patients showed genotypes A, F and D; two patient showed genotypes B, F, and D; and six patients showed genotypes F and D.



Genotype A: 644 bp Genotype D: 189bp
Genotype E: 130bp Genotype B: 331bp
Genotype C: 242bp Genotype F: 487bp

Line-1 :DNA Ladder 100 bp, Line 2-6 DNA: PCR products of different individuals, Line- 7: negatve control

DISCUSSION

The different genotypes of HBV have different serological conversion rate for HBeAg, Precore/core mutation patterns, liver disease, and response to antivirus treatment. For example, in a comparison between genotypes B and C, both commonly found in Asia, genotype B has a faster and higher rate for HBeAg serologic conversion, causes less of cirrhosis, and is better in response to interferon treatment. Although both genotypes have a role in development of HCC, but studies in Taiwan and Japan showed that they play role in different people, genotype B in people below 50 years old while genotype C in those who are over 50 years old [17].

Mutations in Precore/core decreases HBeAg expression and increases viral DNA replication. It has been shown that Mutations in Precore is more common in genotype D and cause more severe liver disease. However, mutations in the core area occur in all genotypes with a higher frequency in patients with genotypes F. In a study done in 2003 in southern Taiwan, Lee and his colleagues determined that genotypes C is associated with more severe forms of disease [18]. Another study performed in Pakistan showed that the most common genotypes in patients with HCC were D and then the combination of A, and A/D [19].

In studies carried out on Pres1/Pres2 sequence and Precore/core area in Iranian HBV patients determined that the most common genotype among Iranians is D. A study done in 2005 on 26 Iranian patients with chronic hepatitis reported only genotype D infection for all patients [20]. Genotype D has also been the only genotype reported by some other studies done on Iranian population [21-23]. Some different methods have been developed for HBV genotyping including PCR-Sequencing, Line probe assay, PCR-RFLP, Genotype-Specific PCR. These methods vary in sensitivity, specificity, duration, hardness testing, and costs. Multiplex allele specific PCR is a molecular technique that is able to detect all HBV genotypes only by a pair of specific primers in a reaction mix. In Nested Multiplex PCR method used for HBV genotyping Naito and his colleagues [24] primers were selected according to the nucleotide diversity in PreS1 or S area. Also, Krischber and his colleagues [25] developed a Multiplex PCR technique to determine genotypes A-F by using six pairs of specific primers. The Method used in this study in a comparison with others such as Naito and Krischber methods has some more advantages such as higher-speed, lower cost, and more sensitivity. The results obtained by this study showed that genotype D is the dominant genotype in patients with hepatitis B virus in both studied cities in northern Iran, and also found some mix of different genotypes including D (2.9%), F (2.5%), B (0.4%), and A (0.4%). Furthermore, this study shows that Multiplex allele-specific PCR technique can be used as a powerful tool in large-scale epidemiological investigations and clinical studies related to HBV genotyping.

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