Original Article

Using Nucleotide Sequencing to Determine HBV Genotypes in Kerman Province

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Received: 19 December, 2017; Accepted: 04 April, 2018

Abstract

Background: Hepatitis viruses are one of the serious medical problems and Hepatitis B is one of the chief transferable disease via blood and its products. Nowadays, 11 genotypes of hepatitis B have known over the world by the genome sequencing. Hepatitis B viruses have special geographical distribution. The clinical importance of hepatitis B viruses and its relation with the mutations has recognized. The purpose of this study was to check the presence and prevalence of Hepatitis B virus genotypes among the referrals attended to the medical diagnostic laboratories in Kerman province.

Materials and Methods: In this cross-sectional study, twenty-one specimens were collected from blood samples available in the medical diagnostic laboratories of Kerman province during one year. After DNA extraction, PCR was carried on by specific primers, then they were sequenced. The obtained sequenced were compared with sequences in the NCBI gene bank and blasted for identification of their genotypes.

Results: Seven samples from twenty one samples (33.3%) had D genotype, 13 samples from 21 (62%) had D3 subgenotype and 1 sample from 21 (4.7%) had D4 subgenotype.

Conclusion: The prevalence of these genotypes in the Kermanian patients that recognized in this study can help to provide diagnostic kits for hepatitis B virus.

Keywords: Hepatitis B virus, genotype, PCR, Sequencing, NCBI.

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Please cite this article as: Monemi N, Hajirezaei M, Saleh-Gohari N. Using Nucleotide Sequencing to Determine HBV Genotypes in Kerman Province. Novel Biomed. 2018;6(3):147-51.

Introduction

Chronic hepatitis B is one of the biggest medical problems. It is reported annually more than 400 million cases in the world, 15-40% of these patients eventually develop liver cirrhosis, hepatic failure, and hepatocellular carcinoma[1,2]. Hepatitis B virus is a member of the Hepadenaviridea family. This small double strain DNA virus has four overlapping genes, including nucleocapsid, envelope, polymerase with transcriptase activity and X proteins[3].

Sometimes Chronic hepatitis B may overlap with several viral diseases. Chronic hepatitis B may be associated with the infection of important viruses like HIV, HTLV, HCV, HDV, HAV and HEV. In recent years, debate on chronic HBe-Ag negative hepatitis has been greatly increased. These patients are in two groups: patients with negative HBe-Ab and patients

with positive HBe-Ab[1].

Hepatitis B virus is transmitted through blood and other body fluids, including semen and saliva. The chance of this virus transmission is 100 times higher than the HIV virus and unlike the HIV; it can survive out of the body as well as in the dried blood for a couple of weeks[4]. This disease is often transmitted through embryonic or early childhood in Southeast of Asia, China, and Africa[5]. This is the reason of the high prevalence of this disease (5-20%) in these areas[6].

Lamivudine is a well-known drug used in chronic hepatitis B to stop the proliferation of B virus and improve liver lesions in 20-40% of European adult patients[6]. Lamivudine is an oral drug that is well tolerated and it is effective in HBe-Ag positive hepatitis and in the type of HBe-Ab positive. It acts as a nucleoside analog and acts as a DNA closure agent[7,8]. Mutation is possible in various sections of HBV genome. The most common mutation happens in precore region (G1896A), and in this case, HBe-Ag cannot be synthesized[9]. So far 11 genotypes A to J have been recognized by sequencing the complete genome of the hepatitis B virus which is isolated from patients over the world[10,11]. Hepatitis B virus genotypes have a specific geographic distribution and clinical importance of genotypes has been recently discussed in their pathogenesis[10,11]. The clinical pathology and the result of chronic hepatitis B are different in different genotypes[12]. HBV genotypes indicate their transmission as well as their geographic dispersion and in migrant populations show the geographical location of their migration[13]. It is reported that the type of genotypes are important in their clinical outcomes[14]. Some researchers believe that D genotype responds better to treatment of Lamivudine than genotype A[15]. In this study, the genotypes of Hepatitis B virus in patients from the Kerman province studied by molecular and sequencing methods.

Methods

Sample collection and DNA extraction: This study was a cross-sectional descriptive. Twenty-one specimens were collected from blood samples available in the medical diagnostic laboratories of Kerman province during one year (2016-2017). Including criteria was molecular and serologic positive samples. Samples were stored at -20°C before DNA extraction. For DNA extraction, the blood samples were centrifuged with 13000 RPM for 5 minutes then 200 µl of supernatant were used for DNA extraction. DNA extraction was performed using the Roch's High-pure viral nucleic acid kit. Then the DNA purity and concentration were determined by the NanoDrop and finally the appropriate samples were selected.

Polymerase chain reaction (PCR): To carry out PCR, the sequence of HBV primers was determined and synthesized in the SinaClon company in Iran (Table 1). Reaction of PCR was carried out using Taq DNA polymerase master mix (Ampliqon). The thermocycler was programmed at 95°C for 5 mins, followed by 95°C for 30s, 57°C for 30s, 72°C for 1 min and an additional 72°C for 5 mins. The PCR product was detected by gel electrophoresis on a 2% agarose gel. Samples with 541bps single band were sent to the Pouya Gostar Gene company in Iran for sequencing. Sequences were blasted with the genome of different genotypes of HBV virus in the NCBI gene bank and then the genotypes of the samples were determined.

Results

Result of PCR showed in Figure 1. All the 21 samples showed 541bps specific bands and then they were sequenced. Sequences were blasted in the NCBI gene bank and the genotypes of the samples were determined (Table 2). Based on the maximum nucleotide matching, these isolated sequences were similar (98-99%) to isolates in Belarus (named mn D-N15), Indonesia (named TRF68226) and Martinique (named mart B47, mart B58, and mart B36). Isolates in this study were assorted in D genotype, D3 and D4 subgenotpes.

The results of this study showed 7 samples of 21 (33.3%) had nucleotide similarity to D genotype isolates (mnD-N15 isolate from Belarus and TRFO8286 isolate from Indonesia), 13 samples of 21 (62%) were similar to D3 subgenotype isolates (mart B47 and mart B58 isolates from Martinique) and 1 of 21 (4.7%) were similar to D4 isolate (mart B36 isolate from Martinique).

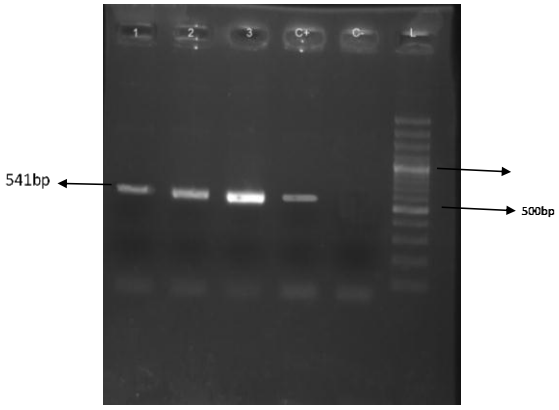

Figure 1. Specific PCR from HBV. The 5µl PCR product was loaded and 541bp band was seen. Columns 1 to 3 are positive samples, Column C-: negative control, Column C+: positive control, Column L: 100bp ladder.

Table 1: HBV specific primers for PCR[27].

Forward	5'-CGGTAAAAAGGGACTCAAGAT-3
Reverse	5'-GTGGTGGACTTCTCTCAATTTTC-3'

Discussion

Chronic liver inflammation changes to liver fibrosis enhances the risk of hepatocellular carcinoma[16]. Therefore, accurate diagnosis of this disease is important in the early stages. Different studies results indicated the high accuracy of molecular methods in detecting of HBV in patients. In many studies, along with serological methods, molecular methods are used for confirmation[17, 18] and some studies confirmed the accuracy of molecular methods[17, 18, 19]. Molecular methods have also been used to identify and determine the genotypes of this virus in various studies[20, 21]. In this study, due to the precision of molecular methods, PCR technique was used to identify patients.

Several studies are proving the prevalence of one or more specific genotype among people in a population. The genotype infecting people in Spain and Sweden was reported genotype D, genotype H was in California and Mexico[22]. In other studies, 121 patients in France and the United States were investigated and genotypes A and G were the dominant genotypes of the disease[23]. In Bangladesh, HBV genotyping studies on 39 patients with hepatitis B indicated the genotype C is dominant compared to D and A genotypes in this region[10]. A South Korean study showed that all of 6 people with hepatocellular carcinoma that were investigated, had genotype C of HBV[24]. HBV Genotype determining studies in Iran indicated the prevalence of genotype D in Golestan provinces[19], Yazd[25], Esfahan[26] and Mashhad[27]. In addition, a study on blood donors in Kerman province confirms the prevalence of genotype D in donors[26]. Also, based on a study performed on 23 patients in Shahrekord city, D and C genotypes were found[28]. In Tehran, D genotype and D1 subgenotype were reported[29]. The results of most studies in Iran indicated that D genotype is the most dominant genotype in Iran[30, 31].

In this study, HBV genotypes were determined by the NCBI genotyping tools. This study showed that D genotype is prevalent genotype in Kerman province. The present study results has confirmed the result of Sharifi et al, study[26]. In addition, some Iranian studies showed the high prevalence of D genotypes of HBV in different regions of Iran, and these data confirm the results obtained in this study. In the neighboring countries such as Turkey, Pakistan, Afghanistan, and Russia, the most common genotype has also been reported D. Also in other countries like India, Saudi Arabia and, Egypt genotype D were reported[19, 21, 31]. Thus, the most common genotype reported in the Eastern Mediterranean region is the genotype D that is consistent with the present study.

Recent studies in Iran has shown the similarity of isolates of Iran with isolates from Turkey, Saudi Arabia, Lithuania, Element, France, Kazakhstan, and Belarus[26]. In the present study, HBV isolates show nucleotide similarity to the HBV isolates of Belarus, Indonesia, and Martinique.

Conclusion

Different HBV genotypes are varied in pathogenicity and response to treatment so HBV genotypes investigation make facilitate severity of disease, complications, response to treatment, vaccine response studies, purchase of diagnostic kits, immunological and genetic diagnosis, and the completion and improvement of screening program in Iran. The analysis of DNA extracted from HBV patients in Kerman province showed that the isolates of this study

Table 2: Analyzing information: 33.3% of samples (7 of 21) had D genotype, 62% (13 of 21) had D3subgenotype and 4.7% (1 of 21) had D4 subgenotype. Six isolates with D genotype were similar to mnD-N15 isolate from Belarus; one was similar to TRF08226 isolate from Indonesia. One isolate with D3 subgenotype was similar to martB47 isolate and three were similar to martB58 isolate from Martinique .Only one isolate with D4 subgenotype was similar to mart B36 isolate from Martinique.

Number	Isolates	%	Number	Genotype
				Sub genotype
6	mnD-N15			
		33.3%	7	D
1	TRF08226			
10	martB47			
		62%	13	D3
3	martB58			
1	Mart B36	4.7%	1	D4
21		100%	21	Total

have D genotype and D3, D4 subgenotypes. These results can also be used to provide quality control panels for evaluating the diagnostic methods for the virus.

Acknowledgment

Especial thanks to the medical diagnostic laboratory of Dr Salehgohari for providing the required equipments and stuffs for doing the experiments of this study.

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