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The Frequency of Genotype D of Hepatitis B Virus in Isfahan, Iran

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Abstract

Background: Approximately 600,000 deaths occur every year as a result of the acute and chronic consequences of hepatitis B virus infection. Ten different hepatitis B virus genotypes have been identified with distinct geographical distributions. Different clinical outcomes, including the rate of mutations, development of hepatocellular carcinoma, chronicity, response to treatment, transplantation rejection and occult infections, are affected by specific genotypes. The aim of the present study was to determine the frequency of genotype D of the virus in Isfahan, Iran.

Patients and Methods: In this study primarily hepatitis B virus positive patients were identified by the detection of HBs antigen using ELISA test and then PCR was used as a confirmatory test. Fifty five patients that were identified as hepatitis B positive were tested for hepatitis D genotype using type - specific PCR.

Results: The patients included 30 (54.5%) females and 25 (45.5%) males. In total, frequency of genotype D was 29 out of 55 cases (52.7%). Genotype D was detected in 19 (63.3%) females and 10 (40.0%) males indicating no statistically significant difference. The difference in the level of liver enzymes in patients infected with genotype D and non-genotype D hepatitis B virus were not significant.

Conclusion: In the present study the frequency of genotype D among patients with hepatitis B virus infection in Isfahan, Iran, was 52.7%. No significant relation was observed between the level of liver enzymes and infection with the genotype D.

Keywords: Hepatitis B virus, genotype D, PCR, Isfahan, Iran.

Introduction

Nearly 2 billion people worldwide are infected with Hepatitis B virus (HBV) with a multitude of different genotypes with different geographical distributions [1-13]. Acute and chronic forms of this disease, cause 600 thousands deaths per year [1]. Chronic hepatitis B is a major cause of liver cirrhosis and in the most cases the disease develops to hepatocellular carcinoma (HCC). [1,7,9,10,13]. At present 10 different genotypes of the virus have been identified with different geographical distributions and have been designated as A to J. A-D genotypes were discovered in 1988, E-F in 1994, G -H in 2001 and finally I-J in 2008. As well HBV is consistent of 9 serological subtypes of adw2, adw4, ayw1, ayw2, ayw3, ayw4, adrq+, adrq- and adyr according to the antigenic determinants of HBs Ag [2,3,14]. Diversity among genotypes is 8% or more and for the sub-genotypes it is between 4-8% [11]. There is evidence that different clinical outcomes, such as development of HCC, chronicity, response to treatment, transplantation rejection and occult infections are affected by specific genotypes [9].

These genotypes have different geographical distributions. Predominant genotypes in central and east Asia are B and C, south and west Asia D, Europe A and D, Australia A, E and G, North America A, B, C, D and F , central and south America H and F and finally two new genotypes I and J have been reported from Vietnam and Japan respectively [9,10,15-21].

Regarding the prevalence of Hepatitis B virus, Iran is in the middle range. A limited number of

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hepatitis B virus genotypes have been identified in Iran. Among which, the genotype D sub-genotype D1, subtype ayw2 is reported as dominant genotype, while small numbers of D2/ayw3 and D3/ayw2 have also been reported [1-13-17].

As only a few studies regarding the hepatitis B virus genotypes have been conducted in Iran, the aim of this study was to determine the frequency of genotype D of hepatitis B virus in Isfahan, Iran.

Patients and Methods

In a descriptive study, patients with clinical diagnosis of hepatitis were tested for HBV infection using HBsAg screening test ELISA kit (RADIM, Italy) according to manufacturer's guideline [8]. Then a PCR detection kit (Cinagen, IRAN) for HBV was used as a confirmatory test according to guidelines of the manufacturer [18].

For performing genotype specific PCR, DNA was extracted by a phenol-chloroform method [18]. A primer set of D-S (Forward) (5'-GCCTCCAAGCTGTGCCTTG-3') and D-AS (Reverse) (5'-TGTGATCTTGTGGCAATGACCCAT-3')[12] (each 1.2 pmol/µl) were used in a PCR reaction with a total volume of 25µl including: 20 ng DNA, 2.5 micro liters of 10X buffer (potassium chloride 500mM, Tris HCl 500mM, PH = 8.4), 1mM magnesium chloride, 30µM of each dNTPs, 1.5U Smar Taq DNA polymerase (Cinagen, IRAN). PCR consisted of a denaturation at 94º C for 5 min, 35 cycles of amplification consisting of denaturation, annealing and elongation at 94 º C for 30 seconds, 55 º C for 30 seconds and 72 º C for 30 seconds, respectively and a final elongation at 72 º C for 5 min. A 2% agarose gel was used to observe the PCR product in gel electrophoresis. PCR product band was extracted from the gel by Fermentas extraction kit (Fermentas, Germany) according to the manufacturer's guidelines and was sequenced. Liver enzymes were measured using PARS LAB kit (PARS LAB, Iran) following the manufacturer's guidelines. The DNA extraction accuracy was analyzed by 1% agarose gel electrophoresis (Figure 1). In PCR expected 867 bp bands were observed in 2% agarose gel after gel electrophoresis (Figure 2). The sequence of PCR product was submitted in Gene bank with accession number of KM199285.

A nucleotide BLAST search of the sequence against the NCBI (National Center of Biotechnology Information) nucleotide sequence database was performed. Using neighbor-joining method a phylogenetic tree was constructed in MEGA5 (Molecular Evolutionary Genetic Analysis Software version 5.1) [20].

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad USA).

Results

The frequency of infection with the genotype D of HBV in different age groups and sexes are shown

Table 1: The frequency of individuals with genotype D of HBV infection in different sexes and age groups.

Age groups	Males			Females			P value
	Number of patients with HBV	Number of Patients with genotype D	Percentage	Number of patients with HBV	Number of Patients with genotype D	Percentage	
20-30	3	1	33.3%	16	11	68.7%	0.243*
31-40	9	7	77.7%	7	5	71.4%	>0.99**
41-50	3	1	33.3%	4	2	50%	>0.99**
> 50	10	1	10%	3	3	100%	0.014**

* Based on Chi-square test.
** Based on Fisher exact test.

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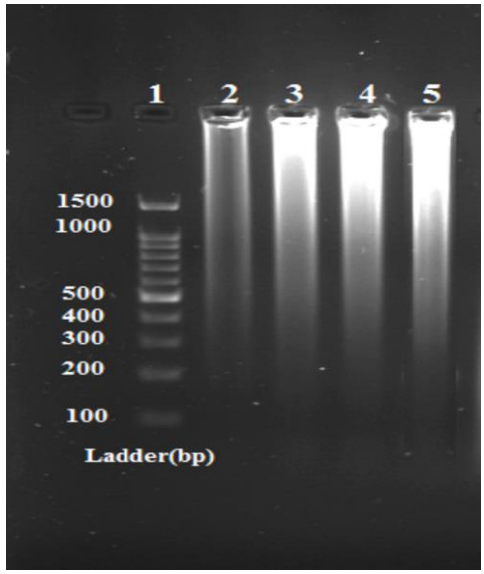

Figure 1: Electrophoresis results testing the accuracy of DNA extraction. Line 1: 100 bp DNA Ladder, Lines 2, 3, 4 and 5 extracted DNAs.

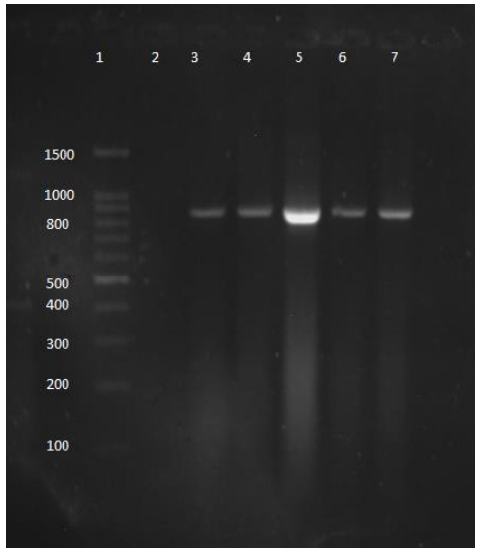

Figure 2: Electrophoresis results of type-specific PCR. Line 1: 100 bp DNA Ladder, Line 2: negative control, Lines 3-7: PCR products of different individuals with genotype D.

in table 1. In total, the frequency of genotype D was 29 out of 55 cases (52.7%). It was detected in 19 (63.3%) females and 10 (40%) males indicating no statistically significant difference.

In BLAST search in NCBI, sequence identity of up to 99% was observed with sequences of genotype D of HBV reported from other countries. In the phylogenetic construct (Figure 3) our sequence was placed in the same cluster with the already reported sequences from Sudan, Egypt, Mongolia and Pakistan which differed from the sequences reported from far east (Indonesia, Japan and India), Europe (Belarus and Spain) and Turkey that were placed in distinct clusters.

The mean level of liver enzymes in patients infected with genotype D of hepatitis B virus was 36.333±10.786 and 20.667±6.408 in males and females respectively for ALT. In the case of AST

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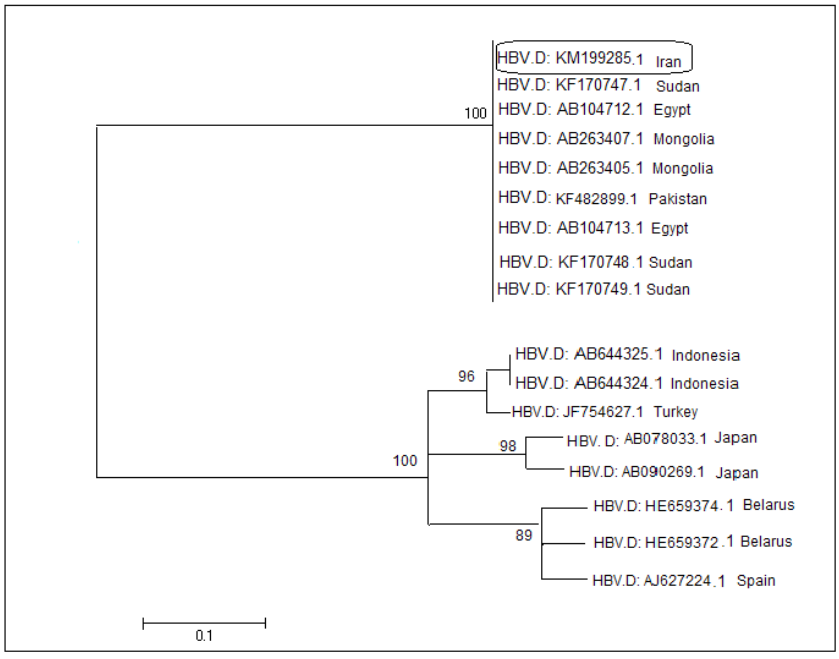

Figure 3: Phylogenetic tree of the genotype D in different parts of the world was constructed using neighbor-joining method implemented in MEGA 5.1 with 1000 bootstrap replicates. The numbers displayed at each branch separation are bootstrap values in percent.

this was 214±299.23 and 33±20.833 in males and females respectively. Level of liver enzymes in patients infected with non-genotype D hepatitis B virus were 25.714±9.552 and 22.750±12.842 in males and females respectively for ALT. In the case of AST they were 39.571±23.950 and 45.250±33.059 in males and females respectively. The difference in the level of liver enzymes in males and females infected with genotype D and non-genotype D hepatitis B virus were not significant.

Discussion

As pathogenesis and also response to immunotherapy and antiviral drugs for different genotypes of HBV varies, identification of HBV genotypes in each region is essential. In the present study, genotype D of the virus was detected in 52.7% of the cases tested in Isfahan. The frequency of genotype D of HBV reported in this study is much less than frequencies of 95% and 97% reported by Sharifi et al. [17], (2012) and Mohebbi et al. [13], (2008) from all parts of Iran but higher than frequency of 2.9% from north of Iran reported by Naderi et al. (2012) [15]. It seems that the frequencies of infection in different parts of Iran are not the same.

In studies in India, Taiwan and Pakistan, among the genotypes detected, the highest liver enzymes were observed in cases infected with genotype A of the virus [3-10-12], but in Japan genotype D of the virus was the case [19]. In this study only one genotype of the virus was detected in which no significant relation between the level of liver enzymes and infection with genotype D was observed.

In the phylogenetic construct (Figure 3) our sequence was placed in the same cluster with sequences reported from North Africa, Pakistan, Egypt and Mongolia [5,16,22]. As these countries are placed in nearly the same region, it seems that distinct viruses are circulating in closed geographical regions. On the other hand, the same viruses are circulating in Europe and for East Asia [4,6,14].

Conclusion

In the present study the frequency of genotype D among patients with hepatitis B virus infection in Isfahan, Iran, was 52.7%. No significant relation was observed between the level of liver enzymes and infection with the genotype D.

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