

# Hepatitis B virus Genotypes in West Azarbayjan Province, Northwest Iran

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## Abstract

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**BACKGROUND:** Infections caused by Hepatitis B are one of the world health's most serious problems. According to assessments, nearly 500,000 to 1.2 million people die each year due to chronic hepatitis, cirrhosis of the liver and hepatocellular carcinoma. Hepatitis B is one of the diseases which can be transferred through blood and its products. Clinical importance of genotypes of hepatitis B virus and their relations with mutations are well known.

**AIM:** Since epidemiological data resulting from determining genotypes and sub-genotypes of hepatitis B can help a lot in defining a vaccination plan, antiretroviral therapy, detection and prevention of diseases, genotypes of this virus in hepatitis B patients were evaluated in West Azarbayjan province.

**MATERIALS AND METHODS:** In this cross-sectional study, serum samples of 100 hepatitis B patients (70 male/30 female) were taken randomly from Urmia University of Medical Sciences (UMSU) referrals, Urmia, Iran; and were tested positive for the presence of surface antigens of hepatitis B virus (HBsAg) using ELISA method. In the first method, after extracting the DNA of the virus, sequencing of S genes was carried out using Sanger method, and the sequences were aligned and edited using Bioedit software. In the next step, phylogenic analysis of the sequences was done in comparison with the reference sequences which were extracted from a gene bank, utilising Neighbour-joining assay method with CLUSTAL W software. To ensure genotyping accuracy, the samples were tested once more, using Nested PCR method.

**RESULTS:** The results were consistent with the sequence method and the dominant genotype in patients suffering hepatitis was type D. In other words, Iranian's HBV genotypic types are homogeneous and in close coordination with each other.

**CONCLUSIONS:** The results reveal that D genotype is the main genotype of HBV in West Azarbayjan province, northwest Iran. Presence of this genotype was in conformity conformed with the low rate of acute liver diseases caused by hepatitis B chronic infection, cirrhosis of the liver and hepatocellular carcinoma.

## Introduction

Hepatitis B is one of the global health problems. According to the statistics of world World Health Organization, it's it is the third most prevailing infectious and contagious disease after Tuberculosis and Malaria and is the fifth cause of death from infectious disease in the world. There are 2 billion people infected with hepatitis B and more than 350 million people (5% of total population

of the world) are reported to be chronic carriers of hepatitis B [1].

There are four million new clinical hepatitis B patients each year and one million of hepatitis B carriers die from active chronic hepatitis and cirrhosis of liver annually. Hepatitis B virus (HBV) is one the causes of acute and chronic hepatitis and liver cancer in Asia and especially in China, Taiwan, Africa and South America [2]. Hepatitis B genome is a double-stranded DNA which is not completely circular and has has 3200 base pairs. Hepatitis B genome have

four open reading frames called S, C, P and X. Using advanced evolutionary analysis and based on HBV similarity of similarity HBV between full genome of virus, 8 genotypes are identified which are based on more than 8% complete A-H genome sequence variation [3].

DNA of the virus consists of 4 groups of genes: *S gene* (surface shield of antigen or s protein codes HBsAg), *C gene* (Hepatitis B Nucleocapsid proteins, viral capsid or nuclear antigen or HbcAg and codes e antigen), *P gene* (DNA polymerase) and *X gene* (which codes transactivating protein) which increases hepatitis B virus duplication. *P gene* covers almost 180 percent of genome of virus, fully covers *S gene* and a part of it overlaps with *C* and *X genes*. In addition, pre-core and core promotore shares are a significant area of genome sequence with *X gene* [3, 4].

Studies show that clinical outbreak, prognosis and response to treatment of the disease depends on genotype of virus. Hepatitis B genotype has a certain type of geographical distribution. In North America, Northern Europe, India and Africa, A genotype, in Asia, B and C genotypes, in Middle East, Mediterranean area and India, D genotype, in west and south Africa, E genotype, in Southern and central America, F genotype and in America and Europe, G and H genotype are dominant [5-8].

All genotypes of HBV will cause liver disease, but their clinical signs are different. C genotype is prevalent in cirrhotic patients. A genotype often lead to chronicity of illness and D genotype is typically observed in patients who are intravenous drug users. But some researches demonstrate that B genotype was more frequently observed in Taiwan youngsters who didn't have cirrhosis of liver rather than D genotype. D genotype was also less connected to acute liver diseases and cancer compared to A genotype [5, 9].

Studies conducted on a limited number of patients show that chronicity ratio in individuals with A and D genotypes is higher than B and C genotypes. The ratio of development of persistent HBV infection after severing hepatitis B infection is higher in A patients rather than B and C genotype patients. They also reported a higher chronicity after HBV infection in D genotype patients [10].

The only host to HBV is a human being, and chronic carriers are the only source of virus in nature. It is estimated that more than 35% of Iranians had contact with HBV and 3% are chronic carriers which 1.7% of them are in Fars province and more than 5% are in Sistan-Va-Balouchestan province. In a study carried out on 250,000 of healthy volunteers of blood donation, 3.6% of men and 1.6% of women were carriers of HBsAg [11, 12].

Anti-HBV antibody was identified in 37% of this population. Thus, it seems that 8% of Iranians

which are suffering hepatitis B will be chronic carriers. Studies conducted in the last decade demonstrate that in patients with cirrhosis of the liver, 70% to 84% have evidence of contacting HBV and 51% to 56% are vectors. Also, in Iranian patients with hepatocellular carcinoma, HBsAg was positive in 72% of virus encounters, and carriage rate is reported to be 46% [2, 11, 12].

Since epidemiologic data resulting from determining genotypes and sub-genotypes of hepatitis B virus help a lot in defining a vaccination program, antiretroviral therapy, diagnosing and prevention of the disease, this research was conducted to study hepatitis B virus genotypes in West Azarbaijan, northwest Iran; province which was not studied previously.

## Material and Methods

Cross sectional study were carried out on 100 patients (70 male/30 female) in 2014-2015 year. hepatitis B patients were chosen randomly from UMSU hospitals. Five milliliters of blood were collected from each patient. After 15 minutes incubation at room temperature samples were centrifuged at 1000 g. Serum were separated and kept at -40 degree centigrade until the experiments were preformed. Sample were Samples were assayed for presence of HBsAg by using the ELISA method (PishTaz Teb, Tehran, Iran).

In order to determine hepatitis B genotypeing, positive 100 serum samples which are positive in the ELISA method, DNA purification carried out by using Nucleic acid extract kit(QIA amp DNA miniKit, Germany) which was based on affinity chromatography utilizing silica columns, and DNA extracted samples divided in two parts.

### **HBV genotyping by sequencing**

The first part of the extracted DNA from hepatitis B virus was sent to South Korea with the aid of Iran Pasteur Institute. Sequencing of *S genes* were carried out using Sanger method, and the sequences were aligned and edited using Bioedit software. In the next step, phylogenic analysis of the sequences were done in comparison with the reference sequences which were extracted from a gene bank, utilizing Neighbor joining assay method with CLUSTRAL W software.

### **NESTED PCR method**

Also nested Nested PCR method was carried

out by hepatitis B virus kit (Genekam Biotechnology AG, Germany) by second part of extracted DNA, extracted materials as described before.

Primers were used as are shown in table 1, [13]. Electrophoresis were carried out by PCR products in 60 volts in TAE buffer (2 M Tris-Acetate, 0.05 M EDTA-K2) and 2% agarose gel containing Syber Green dye. DNA bands in compare with molecular size marker with maximum 500 bp in visible zero UV rays to the gel.

**Table 1: Designed primers for identification of hepatitis B genes using nested PCR method**

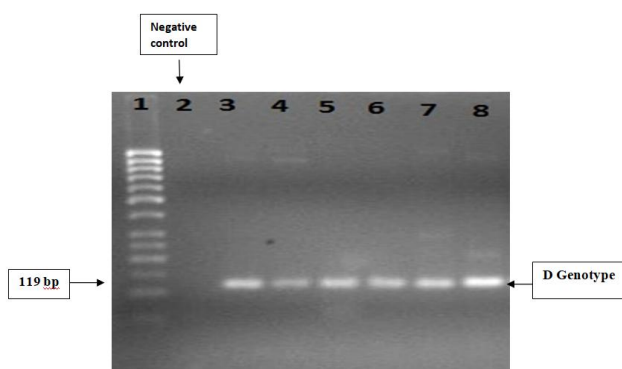
Primer	Sequence a (position, specificity, and polarity)
<b>first PCR</b>	
P1b	5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (nt 2823-2845, universal, sense)
S1-2	5'-CGA ACC ACT GAA CAA ATG GC-3' (nt 685-704, universal, antisense)
<b>2nd PCR</b>	
<b>Mix A</b>	
B2	5'-GGC TCM AGT TCM GGA ACA GT-3' (nt 67-86 types A to E specific, sense)
BA1R	5'-CTC GCG GAG ATT GAC GAG ATG T-3' (nt 113-134, type A specific, antisense)
BC1R	5'-CAG CCT AGG AAT CCT GAT GTT G-3' (nt 165-186, type C specific, antisense)
<b>Mix B</b>	
BD1	5'-GCC AAC AAG GTA GGA GCT-3' (nt 2979-2996, type D specific, sense)
BE1	5'-CAC CAG AAA TCC AGA TTG GGA CCA -3' (nt 2955-2978, type E specific, sense)
BF1	5'-GYT ACG GTC CAG GGT TAC CA-3' (nt 3032-3051, type F specific, sense)
B2R	5'-GGA GGC GGA TYT GCT GGC AA-3' (nt 3078-3097, types D to F specific, antisense)

## Results

After genotyping HBV with S gene sequence utilizing Sanger sequencing method, all samples shows hepatitis B virus D genotypes.

Also carrying out Nested PCR by nested method genotyping and compare with DNA molecular size with maximum of 500 bp in agarose gel all samples were revealed in 119 bp witch which is the sign of type D genotype.

There are no other genotypes were observed in our experiments, figure Figure 1.



**Figure 1:** According to the marker, all bands belong to 119 bp and identified D genotype which for type A in 68 bp, for type B in 281 bp, for type C in 122 bp, for type E in 167 bp and for type F in 97 bp no bands were visible

## Discussion

Hepatitis B chronic infection is a widespread disease which infected more than 5% of the world's population and is one of the biggest health problems of Iran and the world. The prevalence of hepatitis B is endemic in the world. 90% of the world's population are living in areas with high prevalence of 8% of positive HBsAg or with medium prevalence of 82-28% of HBsAg [11]. Iran is located in the middle east Middle East and according to Center for Disease Control, have has medium prevalence of the infection. Prevalence of positive HBsAg in 1357 1982 in Iran was reported to be 2.5%-7.2% [12, 14]. Even though hepatitis B has low prevalence in Bahrain and Kuwait, medium in Iran, USA and United Arab Emirates and high in Oman, Palestine, Yemen and Sweden, positive HBsAg prevalence in ordinary people was reportedly 1.7% and in blood donors was reported to be 0.5% [12, 15, 16].

This study was first carried out in West Azarbayjan province, north west Iran; in order to determine hepatitis B genotypes among hepatitis B patients utilizing sequencing method and Nested PCR method on polymerase gene of hepatitis B virus. In this method, blood samples of one hundred patients which were randomly chosen and tested positive for the presence of surface antigens of Hepatitis hepatitis B virus (HBsAg) using ELISA method and HBV with PCR method, were taken and the experiment were carried out on these samples.

Studies show that there is a meaningful relation between HBV genotypes with illness severity, Cirrhosis cirrhosis of the liver and Hepatocellular hepatocellular carcinoma and response to treatment [17]. Recent data suggests that patients with C genotype would probably have a greater chance of being subject to severe liver diseases while those who have B genotypes have a greater chance of hepatocellular carcinoma [18, 19]. According to the latest studies, 8%-20% of individuals with hepatitis B are its carriers and prevalence of hepatitis B in Middle East and Iran is reported to be 2%-7% [12, 20]

One of the discussions made in hepatitis B researches, is the geographical distribution of this virus which has different patterns in different regions of the world. D genotype is dominant in Middle East and Mediterranean countries which are confirmed through multiple studies [21].

In a research carried out by Eftekhari et al., in 2010 on positive HbsAg patients, GAP-PCR method was utilized in order to determine the genotype. Results showed that in 60% of the individuals, HBc-Ab antibody was tested positive and the dominant genotype in these patients was type D [22].

In a similar study by Goodarzi et al. in 2007 [23] which was conducted to determine the genotype

of HBV in Iran, S and C genes of the virus were studied using sequencing and phylogenetic analysis method in preS area. The results of their investigation demonstrated that in HCC patients all extracted genotypes were typed D. Albeit in positive HBsAg patients, utilising reverse hybridisation method demonstrated that in 86% of chronic hepatitis patients, 10% of Cirrhosis patients and 2.7% of carriers, the only identified genotype is type D [24].

In researches done in different areas of Iran, the dominant genotype is D genotype, but some studies reported B and C genotypes [11]. The research was conducted by Karimi et al. in 2013 [25] on serums of 116 patients with the positive surface antigen of HBV. The results showed that 19.8% were positive concerning hepatitis B nucleic acid and the dominant genotype in these individuals was D genotype in 73.9% of the cases. 26.1% of patients were found to have C genotype.

In a similar study in India, A and D genotypes were dominant. According to this study, D, A and C genotypes were observed in 57.3%, 18% and 11.5% of patients with chronic HBV infection respectively, respectively. D genotype was more prevalent in chronic liver patients in New Delhi [26]. In north east areas genotype C is dominant in high risk individuals and is more connected with liver disease progression [27]. Other researches of the same kind performed in middle eastern Middle Eastern countries like Saudi Arabia, lead to similar results which shows D genotype in more than 80% of hepatitis B (HBV) patients [28].

In other areas such as Mediterranean area, Middle East and South Eastern Asia, D genotype is prevalent and similar results in Turkey demonstrated that all 44 patients which were tested, had D genotypes [29].

In this study, D genotype was found to be dominant in infected patients. In other words, HBV genotypic types are in close connection with each other and are homogeneous and there is no connection between the genotype of virus and the investigated population (epidemiologically). This fully complies with the results previously obtained in Iran, Middle East, Afghanistan, Central Asia, Turkey, Egypt, New Delhi and British Colombia [30].

Despite all the investigations carried out in Iran, data on HBV genotypes are very limited because of the fact that in some cases, differences in genotypes can be found within a country and thus the necessity of regional studies in order to determine HBV genotypes in West Azarbayjan province is justified [31].

The results of this study demonstrate that the dominant genotype in West Azarbayjan is similar to other regions of Iran. D genotype is the dominant genotype in Iran and other Middle Eastern countries. In a research conducted by Alavian et al., the only

genotype found in HBV patients in Iran was type D [9]. Utilizing different methods in the studies lead to similar results. Therefore it can be conducted that the dominant genotype in Iran was type D. Analogous results despite different methods utilized, are an evidence of gene dominance [32].

In a study performed by Yalchin et al., liver enzymes level were investigated and were found to be normal. In this research, ALT and AST liver enzymes were measured and were found to be normal. It seems like enzyme variation doesn't have a meaningful connection with HBV virus and does not change significantly [29] and was in conformity with the present research.

In conclusion, the results show that D genotype is the main genotype of HBV in West Azarbayjan province. This study opens the door to perform further studies such as understanding the effects of antiviral drugs on HBV infected patients. Researches on subgenotypes of HBV and its serotypes can be conducted, and the original and effective anti-viral drugs may be used to treat infected patients.

These results can be utilised in the immunological and genetic diagnosis of HBV to make hepatitis B diagnostic kits and quality control panels for evaluation of diagnostic methods of the virus. Genotyping can be done in bigger cities of West Azarbayjan and may be compared to the present results.

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