

## Sequence-based genotyping of hepatitis B virus in general population

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### Abstract

**Background:** Hepatitis B Virus (HBV) causes acute and chronic liver disease worldwide. HBV has eight genotypes (A to H) which is the reflection of its genome with their characteristic geographical distribution. Each genotype could have different pathogenic and therapeutic characteristics. There have been few records on HBV genotyping in general population from our region. This study aimed to determine hepatitis B genotypes using sequencing in the general population of Shahrekord, a Southwestern region of Iran.

**Methods:** A total of 3000 serum samples (cluster sampling method) were enrolled from general population tested for HBsAg using ELISA. Using appropriate extraction kit, HBV DNA was extracted from HBsAg positive samples and each was subjected to nested PCR for detection of HBV DNA. Finally, using sequencing, the samples were used for HBV genotyping. Data were analyzed by SPSS 19 using descriptive statistics, chi square, and Fisher's exact test. P-value < 0.05 was considered as the level of significance.

**Results:** Out of 3000 serum samples, 40 (1.3%) were positive for HBsAg. HBV DNA was detected in 10 out of 40 (25%) of the samples studied. Genotype D was the predominant HBV type found in all of these 10 HBV positive samples.

**Conclusion:** Genotype D is probably the predominant HBV type in our region.

**Keywords:** Chronic hepatitis B, nested polymerase chain reaction, genotyping.

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### Introduction

One of the most important health problems worldwide is hepatitis B virus (HBV) infection. There are about 400 million persons who are chronically infected by this virus. Chronic carriers are exposed to the development of complications arising from hepatitis B infection, resulting in cirrhosis, hepatocellular carcinoma, liver failure or death (1, 2). HBV genotype is one of the most important factors that influence the outcome of infection (2, 3). Based on sequence divergence in the entire genome, HBV has been classified into at least 8 genotypes (A–H) that differ >8% from each other at the nucleotide level (2). Re-

cently, an additional provisional genotype (I) isolated from Southeast Asians (4) and a variant were also candidate as the tenth genotype J (5). Most genotypes can be split into subgenotypes that differ >4% from each other, such as HBV-A1 and HBV-A2 (6, 7).

As specific clinical associations with each genotype become increasingly apparent, HBV genotyping is becoming more and more important (6). It has been shown that HBV origin, course of infection, the severity of the disease (6-8), prognosis and response to antiviral treatment (7, 9), patterns of serological reactivity and replication of the virus (7) are mainly genotype

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dependent. Thus, understanding genotype distribution is important for epidemiologic characterization of HBV transmission and to control infection (10).

Geographic distribution of HBV genotypes regarding regional host population and endemicity have been widely considered. Accordingly, HBV/B and HBV/C are predominant in most parts of Asia, including China and Japan (11). Genotypes A, D and F are prevalent among the five geographic regions of Brazil. Genotype A was most prevalent in the United States (12) while the most common HBV genotype in Canada was B (13). Genotypes E and F are confined to Africa and the Americas (14). HBV/G has a global distribution, and HBV/H was first revealed in Central America (15). HBV genotypes I and J have been sporadically reported from Asia and Japan, respectively (4, 5). Genotype D is also predominant in the Middle East, including Iran (16).

Iran located in an intermediate endemic region for chronic HBV infection with prevalence rate of 2-8% (17). But, studies show improvement and prevalence transition from moderate to low (0.1-2%), due to vaccination in recent years (18,19). Genotype D has been reported as the predominant genotype in different regions of Iran (20-27). In our region, although there are some reports regarding HBV infection among chronically infected patients (27-29), there has been no report of HBV genotypes in general population. Therefore, this population-based study was aimed to determine HBV genotypes in general population of Chaharmahal va Bakhtiari, southwestern Iran.

## Methods

### *Serum samples*

This is a descriptive, cross-sectional and population-based study. The sample population consists of rural and urban adults over 15 years in Chaharmahal va Bakhtiari province. The sample size was decided to include 3000 individuals based on statistical advice, national prevalence of hepatitis

B and the province population, confidence interval 95%, and relative error 25%. Sampling method was clustering. The clusters were selected from seven counties of the province. Totally, 3000 serum samples were collected from healthy individuals in normal population.

The inclusion criteria were being 15 years and over and consent to participate. For taking blood sample, written consent was obtained from the participants. The study holds ethics code of 90-2-6 obtained from the University Ethic Committee. For HBsAg test, Delaware Kit (Common Market) was used.

### *PCR amplification and detection of HBV DNA*

HBV DNA was extracted from the HBsAg positive serum samples using an extraction kit (QIAamp MinElute Virus Kits, Qiagen, Germany) and subjected to nested PCR according to the manufacturer's instructions using appropriate primers and positive control provided by kit (Plasma-Serum HBV PCR Detection Kit, Norgen-Canada). Strict measures were adopted to prevent any contamination. An aliquot of water was used as negative control. Samples were considered positive if they yielded at least two positive results in two different reactions and were considered negative when there were two negative results in two different reactions. To detect PCR product, the PCR products were analyzed by electrophoresis through a polyacrylamide gel, stained with silver nitrate and photographed.

### *HBV sequencing and phylogenetic analysis*

Detection of HBV genotypes was performed using sequencing. For sequencing, the PCR products were purified by Exo SAP-IT kit (USB Corporation, Ohio, USA) according to the manufacturer's instruction. Sequencing reactions were performed using 1-5µl purified PCR products, 1µl BigDye reaction mix (Life Technologies, Applied Biosystems, Darmstadt, Germany)

Table 1. Demographic characteristics of patients with positive PCR for DNA

		Number(%)
Gender	Male	5 (50%)
	Female	5 (50%)
Age (years)	15-24	1 (10%)
	25-34	3 (30%)
	35-44	1 (10%)
	45-54	2 (20%)
	55-64	3 (30%)
Marriage Status	married	6 (67%)
	single	2 (22%)
	widow	1 (11%)
Place of residence	Urban	7 (70%)
	Rural	3 (30%)
Education	Uneducated	1 (10%)
	Reading	5 (50%)
	Diploma and higher degree	4 (40%)
Vaccination status	vaccinated	3 (30%)
	Unvaccinated	7 (70%)

and 0.5 $\mu$ M of the HBV primers, with the same primers as those used for PCR amplification of pre-S region. The sequences obtained were compared with published sequences from the same genomic region of 8 HBV genotypes available in National Center for Biotechnology Information (NCBI) GenBank. Alignment was performed using CLUSTAL W in MEGA 5 software. The phylogenetic tree reconstruction was carried out using Maximum Composite Likelihood method and MEGA 5 software (Ibis therapeutics Carlsbad, Carlsbad, CA, USA). This method is a new genotyping strategy established by Ma et al in Shenyang, China (30). Data were analyzed by SPSS 19 using descriptive statistics, chi square, and Fisher's exact test.  $P < 0.05$  was considered as the level of significance.

### Results

Out of 3000 serum samples obtained from healthy individuals, 40 (1.3%) were positive for HBsAg using ELISA (data not shown). Ten out of 40 (25%) were positive for HBV DNA using nested PCR (Figure 1). Demographic characteristics are shown in Table 1. Chi square and Fisher's exact tests revealed no significant difference between these cases and HBV PCR negative individuals. The results of sequencing method showed that genotypes D was the predominant HBV type found in all of the

se 10 HBV positive samples (Figs. 2 and 3).

### Discussion

This is the first study aimed to evaluate the distribution of HBV genotypes in the general population of a Southwestern province of Iran, Charmahal va Bakhtiari. Based on a sequence divergence of 8% or greater of the entire genome sequences or a sequence divergence of 4.2% of the S genome sequences, HBV includes at least eight genotypes (A-H) (2) with two additional genotypes recently named I and J

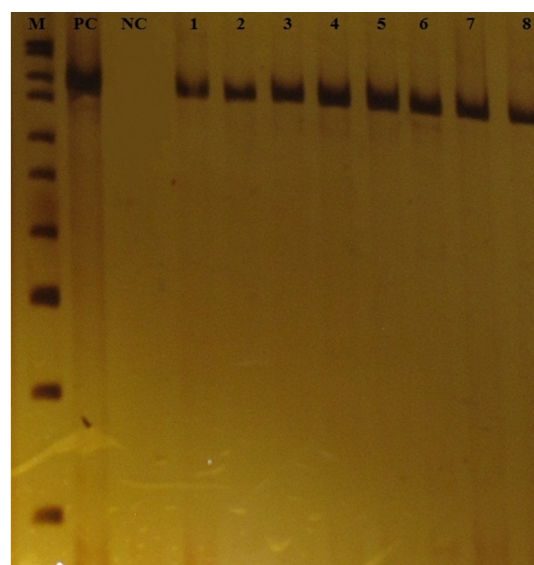


Fig. 1. Polyacrylamide gel electrophoresis of the reaction, products of nested PCR and a 50 KB DNA markers. The second round of amplification products (1-8), the positive (PC), the negative controls (NC), and molecular weight marker (M) are shown.

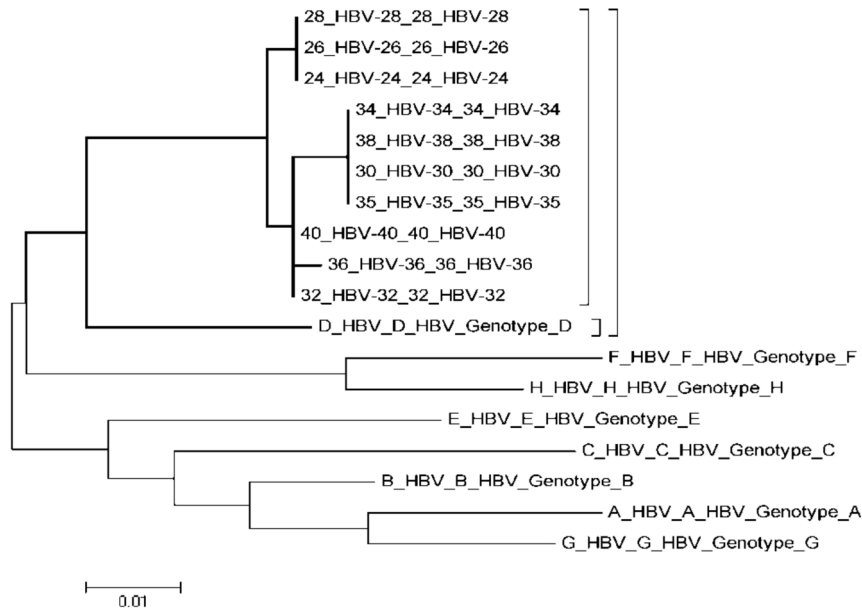


Fig. 2. Phylogenetic tree constructed with MEGA version5.05 software using the Maximum Likelihood method. Tree constructed based on 348 bp fragments from 10 HBV amplicon and 8 genotype of HBV obtained from NCBI database. Genotype of samples is closed with HBV genotype D.

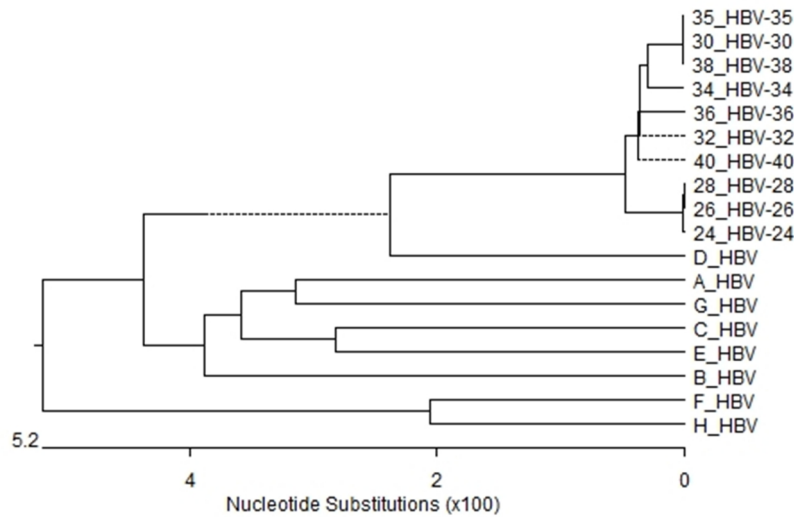


Fig. 3. Phylogenetic tree of 348 bp fragments from 10 HBV isolated in the studied population and 8 standard genotypes from NCBI database. Tree constructed with DNASTAR megalign software. Genotype of samples is closed with HBV genotype D.

(4,5,31). There have been a number of reports indicating that most HBV genotypes are restricted to geographical regions and associated with a host population around the world, while others tend to have a worldwide distribution, or still remain unknown (3,4). Just as with the genotypes, the distribution of HBV subgenotypes is also distinctly geographic (8,9). It has been suggested that polymorphism, transmission, mutation and the evolutionary history of HBV have led to the above outcomes.

Different biological and epidemiologic behaviors have been attributed to HBV genotypes (6). Accordingly, they are closely associated with outcome of chronic liver disease, antiviral response, severity of the diseases, and disease time course. Therefore, detection and monitoring of HBV genotypes are very important to clarify the pathogenesis, route of infection and virulence of the virus and also in epidemiologic setting (7-9).

Studies of the distribution of HBV geno-

types have largely been conducted among patients with chronic hepatitis B (CHB) from Iran (26). However, limited studies carried out to determine genotypes of this virus in Iranian general population (24). Accordingly, this is the first study aimed to understand the overall distribution of HBV genotypes in general population from a Southwestern province of Iran. Our results identified genotype D as the predominant genotype in this region. These findings are concordant with those of the previous studies conducted in the different regions of the country where only genotype D was detected in CHB patients (26). Although in this study, HBV genotype D was detected in apparently healthy individuals in the general population, all these results, together, from different regions, confirm that genotype D may be the only genotype circulating in this country both in CHB patients and most probably in the general population. This result is also consistent with other published reports from other countries of the Middle East (16,25) indicating predominance of this genotype in this region of the world.

It has been suggested that HBV genotypes have a characteristic geographic distribution and are closely related to the process of human evolution and migration (16,25). It was found that genotype D is the predominant HBV genotype in South Asia and the Middle East including India, Afghanistan and Iran (16) and is dominant in most parts of Europe (25). Historically, Aryans who firstly colonized to the North of the Caspian Sea migrated to Iran, India, and Europe. Therefore, there is a possibility that the population of this colony has been infected with genotype D before their migration and then transmitted the virus to the following generations after their migration. Thus, this may be the best explanation for the prevalence of genotype D in India, Iran and most parts of the Europe. In countries with high levels of immigration in the Europe and North America a variety of genotypes are being reported (24) which may depict the impact of immigration on

HBV genotype distribution. Also, the dominance of this genotype in Iran and the neighboring countries (25) may indicate immigration among these countries which results in circulation of this genotype among these populations.

The clinical outcomes of the individuals infected with HBV of genotype D are still controversial. It has been suggested that it causes severe chronic liver diseases more frequently than other genotypes (1,3,16); it causes high risk of provoking fulminant hepatitis as well. However, there are some reports indicating HBV genotype D is related to acute self-limited hepatitis (9). Furthermore, HBV genotype D has been found in the majority of asymptomatic carriers (84.2%) and has not been found in patients with liver cirrhosis; hepatocellular carcinoma is lower in genotype D (17). No association between HBV of genotype D and distinct clinical phenotypes has been found in the Turkish population infected with HBV (16). Based on our findings, apparently no clinical symptoms were seen in the infected healthy individuals. From these results, together, it may be concluded that the infection caused by genotype D mainly is mild and/or asymptomatic.

### Conclusion

The results of this study may provide useful regional epidemiologic information. However, as these results cannot be generalized for all Iranian population, a large scale, nationwide study should be done to determine HBV genotypes in general population of Iran.

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