Hepatitis B virus genotypes in southwest Iran: Molecular, serological and clinical outcomes

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Abstract

AIM: To investigate the associations of hepatitis B virus (HBV) genotype with HBeAg and anti-HBe status, alanine aminotransferase (ALT) levels and HBV-DNA detection in different groups of HBV-infected patients in southwest Iran.

METHODS: A total of 89 HBsAg-positive serum samples were collected from the same number of patients. All sera were then investigated to determine HBV DNA and serological markers. For all the polymerase chain reaction (PCR)-positive samples, biochemical, histopathological assays and genotyping were also performed.

RESULTS: Genotype D was the only type of HBV found in different clinical forms of acute and chronic infections. There was a high prevalence of HBeAg-negative HBV-infected patients with chronic hepatitis (52.7%). Out of 55 patients with chronic hepatitis, seven (12.7%) were diagnosed with cirrhosis. A significant association between the presence of anti-HBe antibody and an increase in ALT level, among either HBeAg-negative (P = 0.01) or HBeAg-positive (P = 0.026) patients, was demonstrated. No significant differences were observed between the clinical outcomes of HBeAg-positive and -negative individuals (P = 0.24).

CONCLUSION: Genotype D has been recognized as the only type of HBV found in different clinical forms of HBV infections, including cirrhosis, among the residents of southwest Iran. Anti-HBe possibly plays a role in disease progression in some patients with chronic hepatitis, at least for a period of disease.

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Key words: Hepatitis B virus-D; Cirrhosis; Iran; Anti-HBe; Polymerase chain reaction

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INTRODUCTION

Infection with hepatitis B virus (HBV) is associated with a broad range of clinical infections, including acute hepatitis, asymptomatic carrier, chronic hepatitis, cirrhosis and hepatocellular carcinoma. The course of HBV infection depends on many factors, such as host immune status, age at infection, level of viral replication and probably the...
genetic variability of the virus influencing the expression of viral antigens [1]. Heterogeneity in the global distribution of HBV genotypes may account for differences in the clinical outcomes of patients infected with HBV, and their different responses to antiviral treatment [2,3].

DNA sequencing of HBV isolates has revealed the existence of 8 viral genotypes A-H, which vary in geographic distribution [4]. Genotypes B and C are dominant in the Far East and south-east Asia where HBV infection is highly endemic [5-8]. Some studies have found more severe liver disease to be associated with genotype C, as compared with genotype B [9]. Genotype C and reactivation of Hepatitis B is associated with increased risk of cirrhosis. By contrast, genotypes A and D are more common in Western Europe and North America [6,9]. A large cross-sectional study in Sweden showed that genotype D is associated with more active disease than genotype A [10]. Genotype D is also predominant in the Mediterranean area as well as in the Middle East, including India. It has been associated with anti-HBe-positive chronic Hepatitis B infection in the Mediterranean region [11,12]. However, the clinical relevance of HBV genotypes isolated from different geographical regions is poorly understood.

Based on HBsAg detection, Iran is located in an intermediate endemic region for chronic HBV infection in the Middle East, and patients with chronic HBV infection are presented with different clinical pictures. The first report of HBV genotyping of 26 HBV isolates from Iranian chronically HBV-infected individuals revealed that HBV genotype D is dominant in Iran [13-15]. Nevertheless, the clinical and serological statuses of patients infected with HBV-D in this geographic region need to be further investigated.

Our aim in this study was to investigate: (1) The prevalence of different HBV genotypes in HBsAg-positive individuals living in southwest Iran, and (2) the association of HBV genotype with HBeAg and anti-HBe status, ALT levels and HBV-DNA detection in different groups of HBV-infected patients.

MATERIALS AND METHODS

Subjects
A total of 89 HBsAg-positive serum samples were collected from the same number of Iranian patients (68 males and 21 females, aged between 15 and 77 years) attending the Gastroenterology and Hepatology Clinic at the Department of Internal Medicine, Shiraz University of Medical Sciences, Shiraz, southwest Iran. Patients were registered irrespective of HBeAg status, ALT level, HBV DNA level or antiviral treatment status. Patients were excluded for hepatitis C virus (HCV), hepatitis D virus (HDV) or HIV co-infection. All patients were then tested for the following: HBeAg, antibodies to HBeAg (anti-HBe), anti-HBc, HBV DNA by polymerase chain reaction (PCR) assay, liver panel [aspartate aminotransferase (AST), ALT upper limit of normal (ULN), 667 nkat/L], alkaline phosphatase (ALP), albumin and total bilirubin], complete blood count, international normalized ratio (INR), and \( \alpha \)-fetoprotein (AFP). An abdominal ultrasound was also performed to determine if there were features of cirrhosis. Liver biopsy was performed based on clinical indications. Liver damage was graded (0-8) according to the inflammatory components and staging (fibrosis; 0-6) was investigated using the modified histological activity indexing [16]. The definitions and diagnostic criteria for clinical terms were adopted from American Association for the Study of Liver Disease (AASLD) practice guidelines [17].

Serological markers
All serological tests were performed as instructed by the manufacturers. HBsAg, anti-HBc-IgG and IgM antibodies, anti-HDV antibody, HBeAg and anti-HBe antibody were measured using commercially available standard one-step enzyme immunoassay kits (MonoLISA, Bio-Rad, France). Anti-HCV (third generation assay) was measured by enzyme immunoassay (EIA) according to the manufacturer’s instructions (Innogenetics, Belgium).

PCR assay
Preparation of DNA samples from the sera: Strict measures were adopted to prevent any contamination. Purification of DNA samples was performed using a previously described method [18].

PCR amplification and detection of HBV genotypes: DNA amplification and detection of HBV genotypes were performed based on a nested PCR assay, using the type-specific primers previously described [19]. Six genotypes (A-F) of HBV were identified by the assay system. To test the validity of the results, detection of HBV genotypes was also performed by quantitative real time PCR using a previously described method [20]. Quantitative real time PCR assay was carried out using SYBER-Green signal detection. A standard curve was constructed using ten-fold serial dilutions (10\(^{-8} - 10^{-5}\) copies/L) of plasmid DNA including the complete clinically isolated HBV-genome.

Detection of PCR product: Ten milliliters of reaction product was electrophoresed in a 1.5% agarose gel made in Tris-acetated-EDTA (TAE) buffer, pH = 8-8.5, and visualized by UV illumination after ethidium bromide staining.

Statistical analysis
Fisher’s exact test, chi-square test with Yate’s correction and the Student’s t test were used where appropriate. \( P < 0.05 \) was considered statistically significant.

RESULTS
All 89 patients were found to be infected with HBV of genotype D. Genotype D was the only detected type found in different clinical forms of acute and chronic infections, in all HBeAg-positive and -negative patients, in all patients who had elevated or normal ALT levels and at all ages. Thirty-two (36%) out of the 89 patients were categorized as inactive HBsAg carriers. Two patients (2.2%) were diagnosed to have acute hepatitis. The remaining 55 (61.8%) were classified as having chronic hepatitis. Based on histological, clinical and laboratory findings, seven
(12.7%) patients out of 55 were diagnosed as having cirrhosis. The cirrhotic patients consisted of 6 males (85.7%), aged 23 to 77 (average; 49.6 years) and one female (14.3%) aged 52 years. All cirrhotic patients had ALT levels that were at the upper limit for the normal level, but the ALT level was lower than the AST level in all patients. Four (57.1%) of the 7 cirrhotic patients were HBcAg negative. Based on HBcAg serology results, the 55 patients with chronic hepatitis were subdivided into two groups: (1) 26 patients (47.3%) positive for HBcAg, and (2) 29 patients (52.7%) negative for HBcAg. In the latter group, 24 (82.8%) patients had an ALT level that was higher than the normal value. Twenty-three of these were positive for anti-HBe antibody, indicating a possible genetic mutation in the precore/core region of the HBV-DNA genome. However, no significant correlation between the presence or absence of HBcAg and an increase in the level of ALT was observed in patients with chronic hepatitis (P = 0.13).

Thirty-two patients with chronic hepatitis (58.2%) were positive for anti-HBe. Significant associations between the presence of anti-HBe antibody and an increased ALT level among both HBcAg negative (P = 0.01) and HBcAg positive (P = 0.026) individuals were observed. The number of individuals who had HBV DNA levels > 10^6 copies/L was higher among HBcAg-positive patients (11/26) than among HBcAg-negative subjects (4/29) (P = 0.01).

None of the 32 inactive HBsAg carriers demonstrated ALT levels higher than the normal value. They all were negative for HBcAg, but positive for anti-HBe antibody.

Based on histopathological status, more damage to hepatocytes was demonstrated in patients with chronic active hepatitis who were positive for anti-HBe compared with patients who were anti-HBe negative (P = 0.001). The laboratory results for the patients are presented in Table 1.

### Table 1. Laboratory results for patients infected with hepatitis B, virus genotype D n (%)  

| Test                      | CH HBeAg+ (n = 26) | CH HBeAg (n = 29) | Inactive HBeAg carriers (n = 32) | Cirrhosis (n = 7) |
|---------------------------|--------------------|-------------------|----------------------------------|------------------|
| Anti-HBe                  | 16 (61.5)          | 23 (79.3)         | 30 (93.8)                        | 3 (43.0)         |
| Anti-HBe                  | 10 (38.5)          | 6 (20.7)          | 2 (6.2)                          | 4 (57.0)         |
| ALT > 667 nkat/L          | 16 (61.5)          | 20 (69.0)         | 0 (0)                            | 7 (100.0)        |
| ALT > 667 nkat/L          | 10 (38.5)          | 9 (31.0)          | 32 (100.0)                       | 0 (0)            |
| DNA molecules < 10^3/L    | 2 (7.7)            | 2 (6.9)           | 17 (53.1)                        | 3 (43.0)         |
| DNA molecules 10^3-10^6/L | 7 (26.9)           | 14 (48.3)         | 13 (40.6)                        | 1 (14.3)         |
| DNA molecules 10^6-10^9/L | 6 (23.1)           | 9 (31.0)          | 2 (6.2)                          | 2 (28.6)         |
| DNA molecules > 10^9/L    | 11 (42.3)          | 4 (13.8)          | 0 (0)                            | 1 (14.3)         |

CH: Chronic hepatitis.

D was also found in three patients with a definite diagnosis of hepatocellular carcinoma. Nevertheless, these patients were excluded from the study, because their complete clinical and serological data were not available.

The appearance of anti-HBe usually marks non-replicative viral infection and inactive disease or response to treatment. However, 58.2% of the samples collected from patients with chronic hepatitis containing genotype D were anti-HBe positive. It should be noted that, in Iran, about 58% of HBV-infected individuals are infected with precore mutants, and may have anti-HBe antibodies in spite of actively replicating the virus. The findings of this study confirm the presence of a significant association between the presence of anti-HBe antibodies and increased ALT levels among both HBcAg-negative (P = 0.01) and HBcAg-positive (P = 0.026) individuals. These results suggest that anti-HBe may play a role in the progression of the disease.

Apart from HBcAg status, 65.5% of our patients with chronic hepatitis showed ALT levels that were higher than the normal value. In contrast to a study by Yalcin et al., diagnosis of cirrhosis, as well as hepatocellular carcinoma, among our patients with chronic hepatitis indicated that this may be associated with ethnic background. Ethnic background might also be an influencing factor on disease progression in patients infected with HBV-D.

Although the number of HBcAg-positive patients with HBV DNA levels higher than 10^6 molecules/L was less than that of HBcAg-negative individuals (23% vs 31%), no statistically significant differences were demonstrated between the clinical outcomes of the two groups (P = 0.24).

In conclusion, this study suggests the unique characteristic of HBV-D infection is related to geographical location as well as ethnicity. Based on ALT levels and histopathological outcomes, we assume that anti-HBe plays a role in disease progression in some patients with chronic hepatitis, which consequently might lead to cirrhosis and hepatocellular carcinoma. The obtained evidence suggests HBV genotype D is associated with reactivation of chronic disease.

### DISCUSSION

HBV has eight genotypes, which have distinct geographical distributions. There is some evidence the long-term prognosis and the initial clinical picture and response to treatment may differ depending on the genotype of the HBV having infected the patient. The viral genome controls antigen expression, leading to different genotypes and a disease spectrum after infection. Genotype D is dominant in the Mediterranean region, the Middle East and Central Asia. However, the clinical outcomes of the individuals infected with HBV of genotype D are still controversial. HBV genotype D is reported to be related to acute self-limited hepatitis. Furthermore, HBV genotype D has been found in the majority of asymptomatic carriers (84.2%) and it is not found in patients with liver cirrhosis and hepatocellular carcinoma. These findings are in contrast with other studies. No association between HBV of genotype D and distinct clinical phenotypes has been found in the Turkish population infected with HBV.

In our study, HBV-D was the only detectable genotype in different clinical forms of HBV infections, in patients with acute (2.2%), inactive HBsAg (36%) or chronic hepatitis (61.8%). Seven (12.7%) out of 55 patients with chronic hepatitis were diagnosed with cirrhosis. Genotype D was also found in three patients with a definite diagnosis of hepatocellular carcinoma. Nevertheless, these patients were excluded from the study, because their complete clinical and serological data were not available.

The appearance of anti-HBe usually marks non-replicative viral infection and inactive disease or response to treatment. However, 58.2% of the samples collected from patients with chronic hepatitis containing genotype D were anti-HBe positive. It should be noted that, in Iran, about 58% of HBV-infected individuals are infected with precore mutants, and may have anti-HBe antibodies in spite of actively replicating the virus. The findings of this study confirm the presence of a significant association between the presence of anti-HBe antibodies and increased ALT levels among both HBcAg-negative (P = 0.01) and HBcAg-positive (P = 0.026) individuals. These results suggest that anti-HBe may play a role in the progression of the disease.

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### COMMENTS

#### Background

Heterogeneity in the global distribution of hepatitis B virus (HBV) genotypes may account for differences in the clinical outcomes of HBV-infected patients and their...
responses to antiviral treatment. The clinical and serological statuses of patients infected with HBV of a specific genotype in this geographic region (Iran) need to be further investigated.

**Research frontiers**

Genotype D was the only type found in different clinical forms of acute and chronic infections. A significant association between the presence of anti-HBe antibody and increased alanine aminotransferase (ALT) levels among HBeAg-negative and HBeAg-positive individuals was demonstrated.

**Innovations and breakthroughs**

This study suggests the unique characteristics of HBV-D infection are related to geographical location as well as ethnicity. The obtained evidence suggests that HBV genotype D is associated with reactivation of chronic disease.

**Applications**

Further research should explain the mechanism of pathogenesis of different HBV genotypes and its relation to special geographical region.

**Peer review**

This is an interesting and well-written clinical epidemiology study on Hepatitis B genotypes in Iran with disease correlation. The paper contributes to the viral hepatitis literature as there is very little from the Middle East in the area of Hepatitis B.

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