

Prevalence of viral hepatitis and molecular analysis of HBV among voluntary blood donors in west Iran

Abbas Doosti¹, Samad Amini-Bavil-Olyaei², Elaheh Tajbakhsh¹, Ahmad Adeli², Fereidoun Mahboudi²

¹Biotechnology Research Centre, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran;

²Biotechnology Department, Pasteur Institute of Iran, 13164 Tehran, Iran

SUMMARY

To determine the prevalence of viral hepatitis infection and hepatitis B virus (HBV) molecular characterization, 11,200 blood donors from citizens of Shahrekord (a city located in west of Iran) were investigated. Results showed HBsAg-positive in 1.78% of persons (n=200), anti-HDV-positive in 3% of HBsAg-positive cases (n=6) and anti-HCV-positive in 0.67% of donors (n=76). HBV phylogenetic analysis disclosed HBV genotype D, sub-genotype D1, and subtype *ayw2*. Amino acid mapping of the HBV *pol* region revealed various HBV drug-resistance mutations though donors had no antiviral therapy. In conclusion, this study demonstrated notable viral hepatitis seroprevalence rate in blood donors in west of Iran.

KEY WORDS: HBV, HCV, HDV, Seromolecular survey, Blood donors, Iran

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Around 400 million people are chronically infected with hepatitis B virus (HBV) worldwide. Over 15 million people are infected with hepatitis D virus (HDV) and about 170 million of the world population are infected with hepatitis C virus (HCV) (Dehesa-Violante & Nunez-Nateras 2007; Farci 2006). In the Middle East, HBV infection shows an intermediate rate, varying between 2% and 7% in different countries (Qirbi & Hall 2001). In Iran, it is estimated that over 35% of population have been exposed to the virus and about 2.5% of population are hepatitis B chronic carriers ranging between 1.3 and 8.7% in different provinces of Iran (Merat *et al.*, 2000). After the national HBV vaccination programme for neonates in 1993, Iran can be considered one of the countries with low HBV infection endemicity (Alavian 2007b). HCV and HDV infection are also endemic in the Middle East countries. In Iran, about 0.5% of population

are affected with HCV. In addition, delta virus infection is reported in 5.6% of patients with chronic hepatitis B infection (Alavian & Alavian 2005; Alavian 2007a).

Hepatitis B, C and D viruses are potentially dangerous complications of transfusion therapy. Although effective serologic and molecular screening tools are employed to detect transfusion transmitted agents among healthy blood donors, the transmission risk remains (Liu *et al.*, 2006). Seroepidemiological studies revealed different rates of HBV, HCV and HDV infection in healthy blood donors in different provinces of Iran (Table 1). In addition, several surveys were conducted to show viral hepatitis molecular epidemiology in Iran.

Results showed circulating HBV genotype D (Amini-Bavil-Olyaei *et al.*, 2008; Amini-Bavil-Olyaei *et al.*, 2005), HDV genotype I (Mohebbi *et al.*, 2008), and HCV genotypes 1a/1b/3a (Samimi-Rad *et al.*, 2004) in Iranian infected patients. Although there are several reports of viral hepatitis prevalence among healthy blood donors in Iran, there is no report of seroprevalence of viral hepatitis infections rate in Shahrekord, the capital of Chahar-Mahaal-Bakhtiari province in west

Corresponding author

Abbas Doosti

Biotechnology Research Centre

Islamic Azad University, Shahrekord Branch

Shahrekord, Iran

E-mail: samini@ukaachen.de

TABLE 1 - Sero-epidemiological data of hepatitis infection (HBV, HCV and HDV) among blood donors in different provinces of Iran.

Province	Location	Year of study	Sample size	HBsAg	Anti-HCV	Anti-HDV	Reference
Kashan	Centre	2001-2002	600	0.5%	0.5%	ND	(Moniri <i>et al.</i> , 2004)
Yasouj	Southwest	2000	4,980	0.96%	0.02%	ND	(Nabavizadeh & Haghbeen 2000)
Urmia	Northwest	1998	2,000	ND	0.57%	ND	(Sadeghi <i>et al.</i> , 1998)
Tehran	Centre	1986-1988	120	ND	ND	2.5%	(Rezvan <i>et al.</i> , 1990)
Kashan	Centre	1996-2001	43,731	0.62%	1.1%	ND	(Afzali <i>et al.</i> , 2001)
Ghazvin	West-central	2000-2002	39,595	1.08%	0.25%	ND	(Vahid <i>et al.</i> , 2005)
Shiraz	Southwest	1998	7,879	1.07%	0.59%	ND	(Ghavanini & Sabri 2000)
Tehran	North-central	1979	168,890	3.4%	ND	ND	(Farzadegan <i>et al.</i> , 1979)
Shahrekord	West	2003-2004	11,200	1.78%	0.67%	3%*	This study

*In HBsAg-positive cases. (ND: Not Determined).

Iran, with an estimated population of 200,000. The present study investigated the seroprevalence of HBV, HCV and HDV infections among 11,200 voluntary healthy blood donors. In addition, HBV molecular epidemiology and its genetic variability were explored in HBV-positive cases.

11,200 healthy blood donors who voluntarily referred to the Shahrekord Blood Transfusion Organization Centre, during 2003-2004, participated in the present cross-sectional study. All donated blood was checked for blood borne viruses as HBV, HCV, and HDV (anti-HDV was just done on HBsAg-positive samples). Serological markers including HBsAg, anti-HBc, anti-HDV and anti-HCV were checked by RADIM ELISA kit (Diagnostic Bioprobes Srl, Milano, Italy). HBsAg-positive samples were rechecked by HBsAg Confirmatory Kit (Dia Sorin S.p.A, Saluggia, Italy). HCV-positive sera were confirmed by two different methods: Genelabs Diagnostics HCV BLOT kit (MP Biomedicals, Solon, OH, USA) and a RT-PCR based method (STRP™-Hepatitis C virus Detection Kit, CinnaGen, Tehran, Iran). Liver enzymes (ALT and AST) were measured by an autoanalyzer. In addition, around one-fourth of HBsAg-positive

donors were randomly selected for HBV molecular analysis using direct sequencing followed by phylogenetic analysis. HBV DNA was extracted from HBsAg-positive sera using DNP™ DNA extraction kit (CinnaGen). A part of the HBV S gene including 'a determinant' domain was amplified as previously described (Amini-Bavil-Olyae *et al.*, 2006). Amplicons were cleaned up using a gel extraction kit (Qiagen, Halden, Germany) and were subjected to bi-directional sequencing by BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) at the Sequence Laboratories Göttingen GmbH (SE-QLAB) in Germany. To generate a phylogenetic tree, thirty-eight Iranian HBV sequences were compared to A-H defined HBV strains, retrieved from GenBank as reference genes. Sequences were aligned by the CLUSTAL X program; the Kimura two-parameter algorithm was used for genetic distance calculation and a phylogenetic tree was constructed by the neighbour-joining method. Bootstrap re-sampling and reconstruction were carried out 1,000 times to confirm the reliability of the phylogenetic tree. MEGA3 software was utilized for phylogenetic and evolutionary analysis (Kumar *et al.*, 2004).

Out of 11,200 blood donors, 276 showed positive results for HBV, HCV and HDV markers (80% male and 20% female; age range: 18-64 years; with a mean age of 27 years). Out of 276 infected persons, 1.78% (n=200) of donors showed positive HBsAg, from whom six individuals (0.05%) showed positive serological markers for HDV indicating HBV/HDV coinfection (3% of HBsAg-positive cases) and 0.67% of donors (n=76) showed positive anti-HCV. Liver enzymes like AST and ALT for all positive cases (n=276) showed normal values, less than 45 IU/L. Ninety percent of HBV-positive cases were HBsAg/HBcAb-positive suggesting asymptomatic hepatitis B virus carriers. All HBsAg-positive cases were retested using HBsAg confirmatory kit based on binding inhibition or neutralization of binding activity principle. The results of the HBV confirmatory assay were in agreement with previous HBV ELISA results. HCV infection in HCV-positive cases (n=76) were also confirmed by two different methods, HCV BLOT kit and RT-PCR technique. Using these two methods, HCV infection could be detected in 87% and 62% of anti-HCV-positive cases respectively. Detection of the HCV genome by RT-PCR method was less, which may be due to unsuitable sample processing and storage. In this survey, none of the patients was HCV/HBV coinfecting.

Sixty HBsAg-positive sera from 200 HBV-positive blood donors were randomly selected for HBV molecular characterization. Among 60 selected HBV-positive cases, HBV DNA could be successfully amplified in 45 cases (perhaps because of low viral load) and ultimately 38 satisfactory HBV sequences with unambiguous sequencing chromatograph were subjected to phylogenetic analysis. Phylogenetic results revealed genotype D and subgenotype D1 in all Shahrekord HBV isolates with a 99% bootstrap value, 1,000 replicates (Figure 1). HBV subtype was deduced from amino acid of the HBV S-gene, and all isolates were *ayw2* subtype.

No evidence of vaccine escape mutant was found during amino acid mapping of HBsAg gene, in particular at the highly immunologic 'a determinant' domain in the isolated HBV. In this study, among 38 sequenced isolates, 18.4% (n=7) showed different HBV drug resistance mutations though no antiviral therapy had been performed in the donors' lives. In the *Pol* region of the iso-

lated HBV, drug-resistance mutations for adefovir dipivoxil (ADF) were determined in 15.8% of isolates (n=6). The rtA181T substitution (known ADF-drug resistance mutation) was detected in 4 cases; whereas, two isolates showed rtQ215H/P substitutions (likely ADF-drug resistance mutation). One isolate (2.6%) showed rtT184S substitutions indicating entecavir (ENT) drug-resistance mutation. It is noted that lamivudine drug resistance mutation was not observed in isolated HBV.

According to the report of the Iranian Blood Transfusion Organization (IBTO), from March 2003 to March 2004, a total of 1,489,935 blood units were donated throughout the country, i.e. 28 provinces, which had 0.8% and 0.065% positivity for HBsAg and anti-HCV, respectively (Mahmoodian-Shooshtari & Pourfathollah 2006). Shahrekord was ranked as 21st province in the number of blood donations in 2004 with 11,200 donors. However, the prevalence of HBsAg and anti-HCV in this city was found to be higher than the overall infection rate in the whole country. Seroprevalence of HBsAg among 11,200 voluntary healthy blood donors was assessed to be 1.78%, which was relatively similar to other studies conducted in different provinces of Iran (see Table 1). HBsAg positivity among voluntary blood donors in Iran's neighbours was also reported. In Pakistan, the HBsAg positivity rate was 4.8% among 1,474 Pakistani donors [17]; in Kuwait, HBsAg was measured among 3,314 donors and 1.9% of participants were HBsAg-positive (Ameen *et al.*, 2005); in Turkey, 6,240,130 donated bloods were examined for HBsAg and the frequency was 4.19% (Gurol *et al.*, 2006); and in Saudi Arabia, 1.9% of 26,606 blood donors was HBsAg-positive (Panhotra *et al.*, 2005). It can be concluded that, HBsAg rate among blood donors in Iran and also in Shahrekord is still less in comparison with other neighbouring countries of Iran.

This research also revealed that the only HBV genotype circulating in infected patients in Shahrekord citizens is D, subgenotype D1 with subtype *ayw2*, in agreement with previous surveys carried out in Iran (Amini-Bavil-Olyae *et al.*, 2008). This genotype is more or less pandemic, and it is a predominant genotype in the Mediterranean area and the Middle East (Qirbi & Hall 2001; Sertoz *et al.*, 2008). Currently, the relation between HBV genotype and outcome of

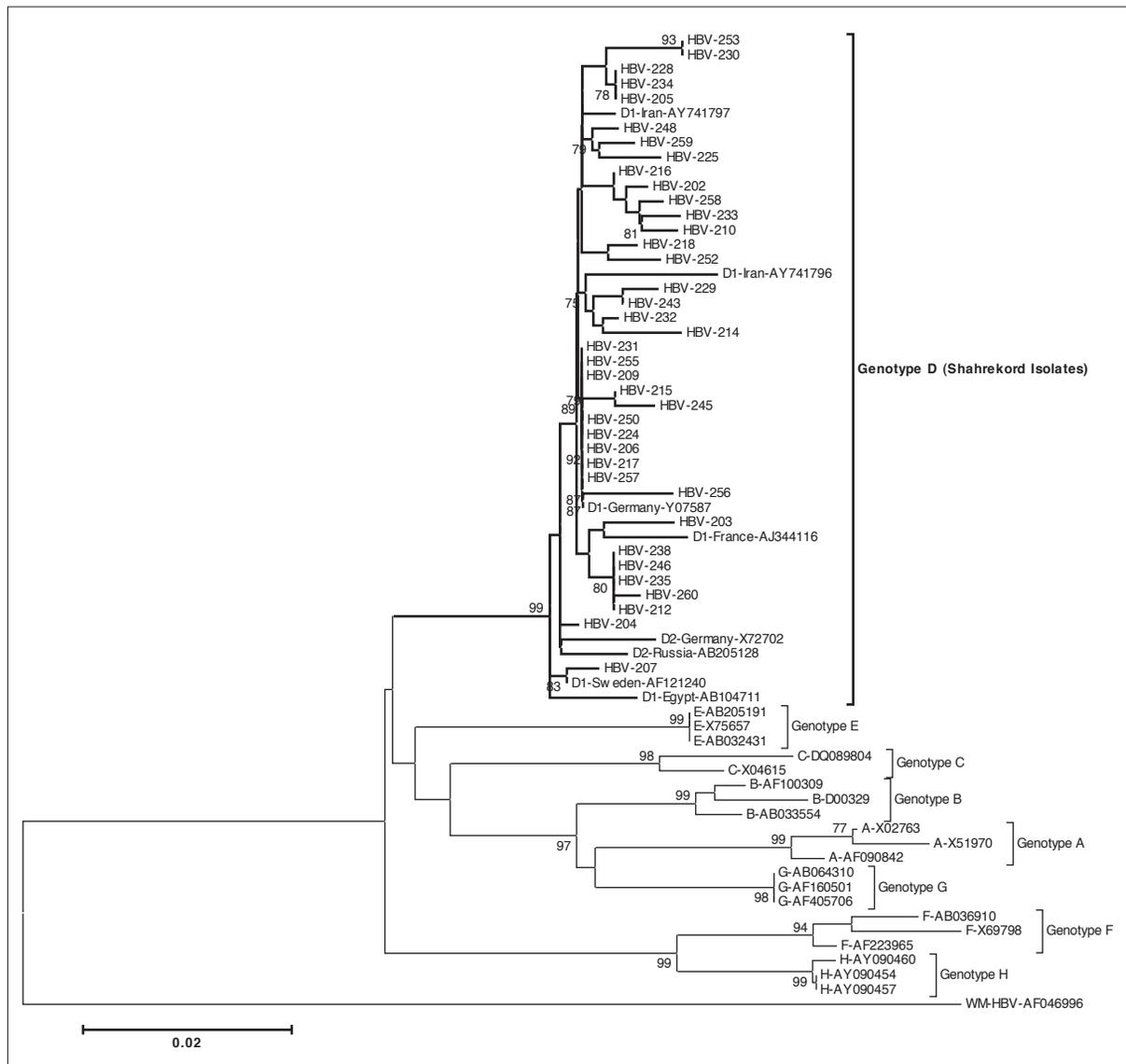


FIGURE 1 - A phylogenetic tree was generated by the neighbour joining method based on the partial HBs nucleotide sequences of the 38 HBV isolates from HBV-positive blood donors living in Shahrekord (West Iran). The 38 HBV isolates obtained in this study are marked by HBV-numbers. Twenty HBV reference gene sequences with different A to H genotypes were retrieved from GenBank, which was shown by their accession number. Woolly monkey hepatitis B virus (WM-HBV) was utilized as the out-group. Bootstrap values are indicated for each node as a percentage of the data obtained from 1,000 re-samplings.

disease and also treatment are well-documented (Verschuere *et al.*, 2005; Enomoto *et al.*, 2006). Thus, understanding the HBV genotype is considerably important for public health. For instance, in one study found evidence of a direct association between genotype D of HBV and severe hepatitis active disease and an elevated ALT level (Kidd-Ljunggren *et al.*, 2004).

Surprisingly, HBV polymerase amino acid sequence mapping revealed naturally ADF and ENT drug resistance mutations in 18.4% of HBV isolates from donors who had never received antiviral medication. Lamivudine is the most common antiviral medication for treatment of HBV infection in Iran and it was supposed that circulation of this type of HBV drug resistance mutation

should be frequent in Iran. However this survey found ADF and ENT drug resistance mutation in untreated infected individuals. Other studies have also reported HBV drug-resistance isolates among untreated infected patients (Medici *et al.*, 2006; Thibault *et al.*, 2002). This could either be due to an immune selection in chronic infected people or to the vaccination program on the circulating virus (Kobayashi *et al.*, 2001). Consequently, the mutant variants can potentially circulate in the population. Prescription of ADF and ENT medicines has been recently started in Iran. Therefore, monitoring of natural HBV drug resistance mutations (in particular at baseline) before starting treatment should be wisely considered.

HDV infection can be found worldwide and in Iran the prevalence of HDV infection was reported around 2.5% among Iranian blood donors (Rezvan *et al.*, 1990). The present study found 3% (among HBsAg-positive samples) in Shahrekord' citizens. In Saudi Arabia, the rate of hepatitis D infection was detected around 3.3% of 3,147 blood donors (Al Traif *et al.*, 2004) which shows a higher rate of HDV infection compared to our result. Anti-HDV screening of donated blood is essential since it is known that patients with HDV infection will more likely develop severe and fulminant hepatitis, and those with chronic HDV infection are significantly at risk of liver cirrhosis. Interestingly, AST and ALT levels were normal in HBV/HDV coinfecting donors in this study. The rate of anti-HCV seropositivity was reported 0.02-1.1% among Iranian blood donors in different provinces. In this study, HCV infection prevalence was found to be 0.67%, a relatively similar rate to previous surveys (see Table 1). Anti-HCV positivity among voluntary blood donors in Iran's neighbouring countries like Pakistan and Kuwait was higher, reported as 1.8% and 1.5% of blood donors, respectively (Khan *et al.*, 2007; Ameen *et al.*, 2005).

In conclusion, this survey showed a noticeable seroprevalence of HBV, HCV and HDV infection among voluntary blood donors in Shahrekord, west Iran. In addition, the molecular epidemiology of HBV revealed genotype D1 of HBV in infected blood donors residing in Shahrekord. We also detected naturally ADF and ENT drug resistance mutations in isolated HBV despite no antiviral therapy suggesting HBV drug resistance circulating in untreated infected persons.

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