Study of Hepatitis B Prevalence in Parallel With the Most Frequent HBV Genotype in South Iranian Blood Donors

Mojgan Noroozi Karimabad,^{1,2} Gholamhossein Hassanshahi,¹ Ahmad Gharebaghiyan,² and Zohre Sharifi^{2*}

¹Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

²Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran

Background: Hepatitis B virus (HBV) is one of the leading causes of acute and chronic liver diseases and in turn responsible for a million of worldwide annual deaths. HBV has been classified into eight main groups, designated as A–H with different geographical distribution. Some genotypes are associated with different clinical outcomes and particular viral mutations. Current study was aimed to investigate the genotype and prevalence of HBV in blood donors of south of Iran. **Methods:** This experimental study investigated the prevalence of HBV positivity in 198,289 Iranian blood donors from south of Iran by both ELISA- and PCR-based methods. Within 198,289 donors, 120 HBsAg⁺ cases were selected, HBV-DNA was extracted, and the p gene sequences were amplified by nested-PCR. The HBV geno-

types were determined by direct sequencing of the polymerase gene of HBV. Phylogenetic trees also were constructed by the neighbor-joining (NJ) method. **Results:** Findings of this study indicated that 0.184%, 0.329%, and 0.215% of blood donors were HBsAg⁺ among Isfahan, Kerman, and Yazd provinces, respectively. Only 69 (57.5%) cases of 120 HBsAg⁺ donors were HBV-DNA⁺. Sequencing analysis revealed that all of HBV-infected donors had the D genotype of HBV. **Conclusions:** In conclusion, the prevalence of genotype D was 100% in Iranian HBV blood donors. These findings may have an impact on the immunological and genetic diagnosis of HBV, selection of diagnostic kits, and viral quality control panels to evaluate diagnostic methods. J. Clin. Lab. Anal. 26:407–411, 2012. © 2012 Wiley Periodicals, Inc.

Key words: HBV; blood donor; genotype; Iran

INTRODUCTION

It is very interesting to know that about 350 million of Hepatitis B Virus (HBV) contaminated individuals shift to chronic HBV infection and 4 million suffer from clinical onsets of the acute HBV infection yearly, or the mortality rate of HBV carriers is around 1 million annually (1–3), and finally WHO reports indicated an approximate HBV contact of 2 billion people per year (1–3).

HBV is among the most frequent causes of liver infection in human. The commonly known symptoms of HBV are jaundice, orangeade skin, and ophthalmic-protein associated with increased bilirubin due to the hepatocellular destruction (4).

The HBV genome contains a double-stranded DNA that is not completely cyclic and consist of four open reading frames, (ORF) as C, S, X, and P (5). The HBV

is transmitted by the following ways: (a) posttransfusion transmission by blood and blood components; (b) embryonic transmission; (c) sexual contact; and (d) via using common HBV-infected syringes by narcotic addicts. The chronic form of hepatitis B can lead to hepatocellular carcinoma (HCC) and in some cases joints inflammatory reactions with high mortality (6). According to the differences in complete genome, Okamoto et al. described A–D genotypes for HBV genome (7). Norder et al.

*Correspondence to: Zohre Sharifi, Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran. E-mail: Z.SHARIFI@IBTO.IB

Received 10 August 2011; Accepted 15 June 2012

DOI 10.1002/jcla.21534

Published online in Wiley Online Library (wileyonlinelibrary.com).

characterized S gene of HBV with a 4.1% difference among genotypes (8). To date, eight different genotypes were identified for HBV and association of HBV genotypes with cirrhosis is well documented (8). Several studies reported that some aspects of HBV infection such as clinical manifestation, prolonged diagnosis, and response to various treatment protocols are intensively related to its genotype (9, 10). It is now well documented that HBV genotypes are differently distributed in different geographical part of the world. For instance, type A genotype is epidemic in north of America, Europe, India, and Africa whereas B and C genotypes are more distributed in the Asian countries. The D genotype is distributed in south of Europe, Middle East, Mediterranean region, and India. The F genotype is frequent in the south of America but the G genotype is distributed in the west of America and European countries. The H genotype is quite prevalent in center of America and California (11, 12). The clinical course of HBV is correlated with age, immune response, and also the genetic variety of the virus. Less than 1% of acute infections can cause mortality (13). Most of the cirrhosis patients carrying C genotype and the A genotype of HBV may remain in carriers of chronic HBV infection for several years. In western countries, the D genotype carriers are often hepatitis B patients who used intravenous medications in western countries (14). It has been demonstrated that patients with C genotype exhibit a weaker response to interferons and antibody-dependent response to HBsAg and HBeAg, which leads to alteration in genotype (15). The pattern of geographical distribution of HBV genotype is remodeling due to the migration (16). Therefore, it is suggested that genotype should be considered as an important parameter in the assessment of primary serological patterns for HBV detection. Nowadays, several laboratory techniques such as PCR-RFLP or line-probe-assay are applied for determination of HBV genotype. At present, sequencing is the most precise method for HBV genotyping. Therefore, the aim of this study was to explore out the HBV positivity prevalence in parallel with HBV genotyping in south of Iran donors of blood transfusion services using sequencing methods.

MATERIAL AND METHODS

Subjects

In this experimental study, we investigated the prevalence of HBV positivity in 198,289 volunteer blood donors from south of Iran in Isfahan, Yazd, and Kerman provinces by both enzyme-linked immunosorbent assay (ELISA)- and PCR-based methods.

A total of 120 volunteer blood donors were randomly selected and peripheral blood samples were isolated from

donors of south of Iran blood transfusion services (IBTS) (Kerman, Isfahan, Yazd). The samples were centrifuged at $370 \times g$ for 4 min. All sera were separated and stored at -80°C for further use. The study protocol was approved by the ethical committee of IBTS.

All participants signed an informed consent form that was designed based on the aim and objectives of the study.

Detection of Serological HBV Markers

HBsAg screening and antibodies to hepatitis B core antigen (anti-HBc) tests were performed by ELISA (Maringburg, Germany). All of the samples were also screened by ELISA for possible HCV and HIV infections. Sera of 120 blood donors who were negative for fourth-generation HIV-combined antigen-antibody assays (Biomérieux, France) and third-generation HCV antibody (anti-HCV) (Ortho, Melville, NY) and also were positive for HBsAg were selected.

Serum HBV-DNA Extraction

The HBV-DNA was extracted from all serum samples with highly pure viral nucleic acid kit (Roche, Germany), according to the instructions of the manufacture.

Nested PCR and Gel Electrophoresis

Following HBV-DNA extraction, to amplify massive viral genomic DNA, a two-step nested-PCR was performed and accordingly a PCR reaction mixture was carried out by adding following reagents to a 0.2-ml microcentrifuge tube on ice: $2 \mu\text{l}$ $10\times$ PCR buffer, $0.4 \mu\text{l}$ of 10 mM dNTP, $1.6 \mu\text{l}$ of 25 mM MgCl_2 , $0.2 \mu\text{l}$ of each 10 pmol forward and reverse primers, $0.3 \mu\text{l}$ of 5 unit AmpliTaq Gold polymerase, $5 \mu\text{l}$ of prepared DNA, and sterile double-distilled water to a final volume of $25 \mu\text{l}$.

The sequences of forward and reverse primers are as shown in Table 1.

The amplification was performed with the following program: one cycle of initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 93°C for

TABLE 1. The Primers Used and Their Related Product Size

	Primers	Fragment size
STEP-1		
F	CCTGCTGGTGGCTCCAGTTC	1,360 bp
R	CGTCCCGCG(AC)AGGATCCAGTT	
STEP-2		
F	CYTGGCCWAAATTCGCAGTCCCT	721 bp
R	GCAAANCCCAAAGACCACAA	

45 sec, annealing at 63°C for 45 sec, extension at 72°C for 1 min, and final extension 72°C for 10 min. For second round of PCR amplification, the same temperature and cycles were used except annealing temperature that was 56°C.

PCR product was run on a 1.2% agarose gel, after adding loading buffer. In parallel with samples, a 100-bp DNA ladder was also run to estimate the molecular size of the DNA fragments.

DNA Sequencing

HBV genotypes were determined by direct sequencing of the polymerase gene of HBV. The p gene sequences were amplified by nested-PCR (Figure 1) and then all PCR products were purified with highly pure PCR product purification Kit (Roche). The purified PCR products were then sequenced using the PCR primers with dideoxy method (TakapooZist, Iran).

The obtained sequences and the reference sequences were aligned with Clustal W software. Phylogenetic trees were constructed by the neighbor-joining (NJ) method (Figure 2).

RESULTS

In this study, we screened all of volunteer blood donors of three big provinces in south of Iran (Isfahan, Kerman, and Yazd). Our serological (using ELISA) results demonstrated that in Isfahan, 224 (0.224%) of 99,603 donors, in Kerman 227 (0.255%) of 63,940, and in Yazd 83 (0.225%) of 36,743 donors were HBsAg⁺ (Table 2). To confirm the HBV positivity by PCR, DNA was extracted from 120 volunteer blood donors who were serologically positive for HBsAg. A PCR was performed using specific primers to the polymerase regions of HBV. Our results showed that 184 (0.184%), 211 (0.329%), and 79 (0.215%) donors were positive for HBV-DNA in Isfahan, Kerman, and Yazd, respectively. Total number of collected volunteer donors was 198,296 (Table 2).

We selected 17 samples from each province for sequencing analysis and subsequent to the optioning of the chromatograms, overall, the most visible and well-sequenced

30 samples (ten from each province) were phylogenetically analyzed. Our phylogenetical analysis demonstrated that all of donors had the D genotype of HBV. Moreover, searching by BLAST software among gene bank data indicated that all of the 30 studied samples showed the highest level of homology with isolated sequences from Uzbekistan, Kazakhstan, India, China, and Belorussia. Finally, all of our isolated HBV-DNA sequences could be classified in D genotype and D¹ subgenotype.

DISCUSSION

Hepatitis B is one of the major posttransfusion transmitted infections. Iran is located in Middle East with a median range of HBV prevalence. In the past decades, Farzadegan et al. reported an HBV prevalence of 3.4% in whole population and 0.5% in blood donors of Iran (17). More recently, studies reported some cases of occult hepatitis B infection in Isfahan and Kerman, the two big central and southeastern provinces of Iran (2, 18). In contrast to the USA where HBV is responsible for chronic viral hepatitis, in Iran HBV causes 70–80% of viral hepatitis and is the main cause of liver malignancies (19). In the current study, 120 HBsAg⁺ blood donors were recruited. Of the donors, 69 subjects were HBV-DNA⁺ while 51% were HBV-DNA⁻. This could probably be due to the low rate of sensitivity and specificity of applied techniques for detection of HBV-DNA in sera samples or even decreased viral load. There are also some cases of HBsAg⁺ in which viral duplication is ceased while patient is in the phase of anti-HBs appearance and HBsAg is still present in patient's serum (20). The studies that were performed in Iran most often focused on S and C genes of HBV. Consistent with our results, there are two reports indicating that D genotype is frequent in Iran, but their studied population was limited to Tehran (the capital of Iran). Goodarzi et al. indicated that in Tehran, D genotype is frequent in HCC caused by chronic HBV infection (21). With a wider sample size, Milani et al. showed that the most frequent genotype in the Iranian HBsAg⁺ blood donors is the D genotype (22). In agreement with our finding, in addition to Iran accumulating data from different parts of neighboring countries also confirmed that the D genotype of HBV is frequent in these countries, including Afghanistan, Turkey, Pakistan, and Russia (6, 23, 24). As we searched by BLAST, our isolated sequence had the maximum level of homology with Belarus, Kazakhstan, Uzbekistan, India, and China. Goodarzi et al. showed that HBV sequences isolated from Iranian HCC patients have the maximum level of homology with Swedish type (AF121240-SW) (21). Again, in agreement with our findings, Amini-Bavil reported that HBV isolated from Iranian patients had a high level of homology with Saudi Arabia and Turkey type of HBV

TABLE 2. The Number of Donors With Number and Percentage of HBsAg Positive Donors by ELISA- and PCR-Based Methods in Each Province

Province	Number of donors	ELISA-based positivity (%)	PCR-based positivity
Isfahan	99,603	224 (0.224%)	184 (0.184%)
Kerman	63,940	227 (0.355%)	211 (0.329%)
Yazd	36,743	83 (0.225%)	79 (0.215%)

Fig. 1. Detection of HBV-DNA by nested PCR demonstrating a 721-bp band amplified in positive samples followed by 1% agarose gel electrophoresis. M = 100-bp DNA ladder molecular size marker, bp = base pair.

Fig. 2. The neighbor-joining phylogenetic analysis of the p gene of HBV isolates from Iranian blood donors. Note that samples are shown in this figure and other results are same as presented in Figure 2 of [13].

(25). One of the background reasons that could be addressed for this homology probably is Hadj ceremony. During the period of Hadj and Umra, several Iranian pilgrims travel to Saudi Arabia every year (since emerging Islam) and may receive transfusion for any clinical reason, which further increase transmission of HBV. This scenario can also occur in relation to the other neighboring countries by tourism including Turkey. Based on the homology of C and S genes of HBV, a closed relation between Iran, Litany, Germany, and France was observed (Amini-

Bavil). There was also an association between HBV genotype in Iran and Uzbekistan and Kazakhstan, India, and China as well. Also, this could probably be explained by the fact that the HBV is not only the virus of 20th and 21st centuries, but was also obviously present in parallel with the history. Therefore, it might be possible that due to the population intermix in this part of the world, HBV transmitted from one generation to another. Basically, south of Iran is the industrial part of the country and most of big industries such as copper and steel industries as well

as big mines are located there, hence, staff, engineers, and managers of these economic sectors most often used to travel to the industrial countries. It is also likely that due to separation of Eurasian countries from Soviet Union, traveling of Iranian industrial managers and staff to these countries has increased. So, this could possibly be one of the main causes of HBV transmission, thus, Iranian HBV genotype is homolog to these countries. Finally, posttransfusion transmission of HBV might not just follow the pattern of HBV transmission and sexual manner should also be considered. Even marriage is popular between the people of these neighboring countries with Iranians. To confirm the above-discussed reasons of genotype homology in HBV, among these neighboring countries we could refer to research performed by Kumar who well described the shifting pattern of HBV genotype in north of India from B to A, following migration from Europe during historical era (26). In contrast to other studies that used C and S genes of HBV we used polymerase region of the gene for genotyping, so this region might show similarity with present genotypes commonly found in different countries. This problem could be solved via analysis of sufficient sample size and also sequencing of complete HBV gene in further studies.

ACKNOWLEDGMENTS

Authors of this article take this opportunity to appreciate all the donors who attended this research program.

REFERENCES

1. Arababadi MK, Pourfathollah AA, Jafarzadeh A, Hassanshahi G, Rezvani ME. Association of exon 9 but not intron 8 VDR polymorphisms with occult HBV infection in south-eastern Iranian patients. *J Gastroenterol Hepatol* 2009;25(1):90–93.
2. Arababadi MK, Hassanshahi G, Yousefi H. HBV-DNA in hemodialysis patients infected by HCV. *Saudi J Kidney Dis Transpl* 2009;20(3):398–401.
3. Kuhns MC, Busch MP. New strategies for blood donor screening for hepatitis B virus: Nucleic acid testing versus immunoassay methods. *Mol Diagn Ther* 2006;10(2):77–91.
4. Kennedy M, Alexopoulos SP. Hepatitis B virus infection and liver transplantation. *Curr Opin Organ Transplant* 15(3):310–315.
5. Chu CJ, Keeffe EB, Han SH, et al. Hepatitis B virus genotypes in the United States: Results of a nationwide study. *Gastroenterology* 2003;125(2):444–451.
6. Alam MM, Zaidi SZ, Malik SA, et al. Molecular epidemiology of hepatitis B virus genotypes in Pakistan. *BMC Infect Dis* 2007;7(115):115–121.
7. Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. *J Gen Virol* 1988;69(Pt 10):2575–2583.
8. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnus LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 1992;73(Pt 5):1201–1208.
9. Niel C, Moraes MT, Gaspar AM, Yoshida CF, Gomes SA. Genetic diversity of hepatitis B virus strains isolated in Rio de Janeiro, Brazil. *J Med Virol* 1994;44(2):180–186.
10. Chevaliez S, Bouvier-Alias M, Laperche S, et al. Performance of the Cobas AmpliPrep/Cobas TaqMan real-time PCR assay for hepatitis B virus DNA quantification: A polymerase chain reaction-based assay to identify genotype F of hepatitis B virus. *J Clin Microbiol* 2008;46(5):1716–1723.
11. Kidd-Ljunggren K, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. *J Gen Virol* 2002;83(Pt 6):1267–1280.
12. Ozdemir FT, Duman D, Ertem D, et al. Determination of hepatitis B genotypes in patients with chronic hepatitis B virus infection in Turkey. *Turk J Gastroenterol* 2005;16(4):183–187.
13. Kato H, Orito E, Sugauchi F, et al. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. *J Virol Methods* 2001;98(2):153–159.
14. Blackberg J, Kidd-Ljunggren K. Occult hepatitis B virus after acute self-limited infection persisting for 30 years without sequence variation. *J Hepatol* 2000;33(6):992–997.
15. Kao JH, Wu NH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes and the response to interferon therapy. *J Hepatol* 2000;33(6):998–1002.
16. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group: A genotype identified in isolates from South Africa. *J Gen Virol* 1997;78(Pt 7):1719–1729.
17. Farzadegan H, Harbour C, Ala F. The prevalence of hepatitis B surface antigen and its antibody in blood donors and high risk groups in Iran. *Vox Sang* 1979;37(3):182–186.
18. Alavian SM. Ministry of health in Iran is serious about controlling hepatitis B. *Hepatitis Monthly* 2007;7(1):3–5.
19. Alavian SM, Keyvani H, Rezaei M, Ashayeri N, Sadeghi HM. Preliminary report of hepatitis B virus genotype prevalence in Iran. *World J Gastroenterol* 2006;12(32):5211–5213.
20. Robinson SW. Hepatitis B virus and hepatitis D virus. In: Mandell GL, Bennet JE, Dolin R, editors. *Principle of Infection Disease*. New York: Churchill Living Stone; 2000. p 1406–1439.
21. Goodarzi Z, Malekzadeh R, Montazeri G, et al. Phylogenetic analysis of HBV based on PreS region in Iranian hepatocellular carcinoma patients. *Hepatitis Monthly* 2007;7(4):201–205.
22. Milani S, Sharifi Z, Hosseini M, Mahmoodian Shooshtari M. Determination of HBV genotypes among Hbs Ag positive blood donors in Tehran, Iran. Using PCR-RFLP. *Iranian J Publ Health* 2009;38:41–47.
23. Amini-Bavil-Olyaei S, Sarraimi-Forooshani R, Adeli A, et al. Complete genomic sequence and phylogenetic relatedness of hepatitis B virus isolates from Iran. *J Med Virol* 2005;76(3):318–326.
24. Amini-Bavil-Olyaei S, Alavian SM, Adeli A, et al. Hepatitis B virus genotyping, core promoter, and precore/core mutations among Afghan patients infected with hepatitis B: A preliminary report. *J Med Virol* 2006;78(3):358–364.
25. Alavian SM, Fallahian F, Lankarani KB. The changing epidemiology of viral hepatitis B in Iran. *J Gastrointest Liver Dis* 2007;16(4):403–406.
26. Kumar K, Kumar M, Rahaman SH, et al. Distribution of Hepatitis B virus genotypes among healthy blood donors in eastern part of North India. *Asian J Transfus Sci*. 2011;5(2):144–149.
27. Hassanshahi G, Arababadi MK, Khoramdelazad H, Yaghini N, Zarandi ER. Assessment of CXCL12 (SDF-1 α) polymorphisms and its serum level in posttransfusion occult HBV-infected patients in Southeastern Iran. *Arch Med Res* 2010;41(5):338–342.