

Original Article

Sequence Analysis of the Polymerase Gene in Hepatitis B Virus Infected Blood Donors in Iran

Zohreh Sharifi PhD¹, Fatemeh Yari PhD¹, Ahmad Gharebaghiyan PhD¹

Abstract

Background: Hepatitis B is one of the most common infectious diseases worldwide that can be transmitted by blood transfusion. The hepatitis B virus (HBV) has eight different genotypes that show different geographical distributions and clinical manifestations. This study aims to investigate the sequence of the HBV polymerase gene and the frequency of HBV genotypes among Iranian blood donors.

Methods: The sera of 223 blood donors who were positive for hepatitis B surface antigen (HBsAg) as determined by the ELISA method were selected. HBV DNA was extracted from the sera of 134 blood donors by a commercial kit, and the entire polymerase gene was amplified by nested-PCR. HBV genotypes were determined by direct sequencing of the HBV polymerase gene. Phylogenetic trees were constructed by the neighbor-joining (NJ) method.

Results: No known base mutations were found in the entire HBV polymerase gene of infected blood donors, and only genotype D was detected among HBV-infected blood donors. The sub-genotype D1 of HBV was dominant in the subjects.

Conclusions: This study shows that antiviral-resistant mutations, such as lamivudine-resistant HBV strains, do not exist naturally among Iranian blood donors. More studies on the full-length HBV genomes are required to determine genome evolution of HBV among infected Iranian blood donors.

Keywords: Blood donor, genotyping, hepatitis B virus, sequencing

Cite the article as: Sharifi Z, Yari F, Gharebaghiyan A. Sequence Analysis of the Polymerase Gene in Hepatitis B Virus Infected Blood Donors in Iran. *Arch Iran Med.* 2012; **15**(2): 88 - 90.

Introduction

Hepatitis B virus (HBV) infection is a major health problem worldwide, with 2 billion infected people each year.¹ HBV belongs to the Hepadnaviridae family and is an enveloped virus with a double-strand DNA genome.

The HBV circular genome is nearly 3200 bp long, with four partially overlapping open reading frames (ORF); named surface (S), core (C), polymerase (P), and X. HBV is classified into eight genotypes (A-H) based on divergence of 8% or more of the complete HBV genomes. The HBV genotypes are geographically distributed. Genotype A is predominant in North America, Northern Europe, India, and Africa. Genotypes B and C are predominant in Asia. The most prevalent genotype in the Mediterranean region is genotype D. Genotype E is observed in parts of East, Central, and West Africa. Genotype F is found in South and Central America, genotype G in the USA and France, and genotype H is observed in Central America.^{2,3}

Lamivudine is the first nucleoside analogue for the treatment of chronic hepatitis B.⁴ A Lamivudine resistance mutant can develop among HBV-infected patients who undergo treatment.⁵ Mutation of tyrosine-methionine-aspartate-aspartate (YMDD) motif of HBV to tyrosine-valine-aspartate-aspartate (YVDD) or tyrosine-isoleucine-aspartate-aspartate (YIDD) in the P gene of HBV can

occur during treatment with Lamivudine, and also some studies showed that HBV-drug resistance can occur in the absence of antiviral therapy (Lamivudine) in HBV-infected patients.⁶

The surface gene is completely overlapped by the P gene.⁷ Because of the overlap between the S and P genes, induced changes that relate to antiviral resistance in the P gene may also show changes in the S gene as well. A few mutations in the P gene could also affect hepatitis B surface antigen (HBsAg) production.

There are some reports about HBV genotyping and sequence analysis from Iran that have been based on C and S genes in chronic patients,⁸ but there are no reports on the P gene of HBV-infected blood donors.

The aim of this study was to investigate the polymorphism of the P gene of HBV and the frequency of HBV genotypes among Iranian blood donors.

Subjects and Methods

This was a cross-sectional study on a number of volunteer blood donors from all the blood transfusion centers in Iran between 2007–2009. The sera of 223 volunteer blood donors were collected. Donors were negative for fourth generation HIV-combined antigen-antibody assays (BioMerieux), third generation HCV antibody (anti-HCV; Ortho) and positive for HBsAg (Dade Behring). Blood donors were screened for HBsAg by the ELISA method and a neutralization test was carried out to confirm the results. Blood donors who were positive for HBsAg were recalled, of which all completed questionnaires. All serum samples were selected and stored at -70°C. The study population included 185 males and 38

Authors' affiliation: ¹Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran.

Corresponding author and reprints: Zohreh Sharifi PhD, Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran. Tel: +98-218-860-1501-30, Fax: +98-218-860-1555, E-mail: z.sharifi@ibto.ir.

Accepted for publication: 29 June 2011

females, aged 20 to 65 years. HBV DNA was extracted from 250 μ L serum using the High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturer's instructions.

The P gene was amplified by Nest-PCR in a 50 μ L reaction that consisted of 5 μ L 10 \times PCR buffer with 1.5 mM MgCl₂, 1 μ L 10 mM dNTPs, 1 μ L of outer primer (10 pmol/ μ L), 0.5 μ L AmpliTaq gold DNA polymerase (5 U/ μ L), 36.5 μ L nuclease-free water and 5 μ L of the extracted nucleic acid. The thermal profile used on the Techne thermal cycler consisted of 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 63°C for 45 s, and 72°C for 45 s; followed by a final extension of 72°C for 10 min, and a final hold program of 4°C. The outer primers were F1 (sense, nt 56-76, 5'-CCTGCTGGTGGCTCCAGTTC-3') and R1 (antisense, nt 1395-1416, 5'-CGTCCCGCG (AC) AGGATCCAGTT-3'). PCR primers were amplified at the sequences between nt 56 to nt 1416, that yielded an amplicon of 1360 bp.

For nested-PCR amplification, 48 μ L PCR master mix that contained the inner primers F2 (sense, nt 298-320, 5'-CYTGGC-CWAAATTCGCAGTCCC-3') and R2 (antisense, nt 997-1019, 5'-GCAAANCCCCAAAAGACCACAAT-3') and 2 μ L of the first round PCR products were used for the second round PCR under the same conditions. To produce the amplicon, the cycling profile for the second round PCR consisted of 94°C for 5 min, 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s, followed by a final extension of 72°C for 10 min, and a final hold program of 4°C.

PCR products were analyzed by horizontal gel electrophoresis on 1% agarose gel in TAE 1 \times buffer at 85 V/1 hr. A single 721 bp band was shown after the second PCR.

The P gene was amplified by PCR in a 100 μ L volume and then purified with the High Pure PCR Product Purification Kit (Roche, Germany). All PCR products were sequenced with the dideoxy method (Takapozist Company) using the PCR primer.

Using the CLUSTAL W 1.8 software package, the nucleotide-sequences of HBV strains were aligned with a reference panel of sequences representative of each subtype retrieved from the GenBank database. Phylogenetic analysis was performed using the neighbor-joining method (NJ plot software) for tree drawing. The reliability of the phylogenetic classification was evaluated by a 1000-cycle bootstrap test. The amino acid sequence obtained from the nucleotide sequence was also analyzed to assess possible drug resistance mutations in the HBV sequence.

Results

Of the 223 HBV positive blood donors who were studied, 79% were male and 21% were female. Mean age of the blood donors was 35.5 \pm 9.11 years. There were 60% (134 of 223) of the blood donors who were positive for HBV-DNA by the nested-PCR assay. The entire P gene was amplified by PCR. The PCR products that were sequenced in the region of the HBV genome contained the main domains (A-E) of P that encoded the YMDD motif. The P gene in the HBV-infected blood donors was found intact and there was no insertion or deletion detected in this gene. Phylogenetic analysis showed that all Iranian HBV isolates were genotype D.

A comparison of the sequences to the available sequences in the Genbank showed that these isolates had more than 95% similarity with the D1 genotype. Some sequences have been submitted to the GenBank database under accession no. FJ 606839 -FJ 606875

Discussion

Despite the HBV vaccination, HBV infection remains an important public health problem worldwide. In Iran, the prevalence of HBsAg has been reported as 1.7% in the general population and 0.4 % in blood donors. Over 35% of the Iranian population have been exposed to HBV.⁹

Genetic evolution occurs in all viruses, but at different rates. Retroviral RT is an enzyme without proof-reading ability; thus reverse transcription is itself error-prone.¹⁰

The replication of HBV DNA occurs through an RNA reverse transcriptase intermediary step. HBV variants are generated due to random misincorporation of bases into the replicating DNA strand. The generation of multiple variant transcripts from a single template and the formation of a quasi-species pool provide the source material for the emergence of a mutant when selection pressure is applied.¹¹

A mutation selected in one gene can potentially lead to an amino acid change in the overlapping reading frame. Since the S gene completely overlaps with the P gene, pre-S, and S mutations affect the structure of the P.

On the other hand, because of the overlap between the HBV P and S genes, P mutations (associated with drug resistance) may alter antigenic sites in the HBsAg. A triple mutational pattern (rt-V173L + rtL180M + rtM204V/I) reduces binding of the antibody to HBsAg and this mutant virus could escape vaccine-induced protection.⁷ The V555I and V542I mutations in the C domain cause premature termination of the HBs proteins.

Some reports have indicated that HBV-drug resistant mutants (YIDD and YVDD) can be detected in infected patients that none of them took lamivudine therapy.^{6,12} These mutants would potentially produce false-negative test results in susceptible HBsAg immunoassays that are used for screening blood donors. HBsAg is one of the most important serological markers used to screen HBV-infected blood donors. Therefore, it is important that studies on HBV isolates should be performed for detecting these mutants.

In this research, we collected samples from HBV-infected blood donors from all blood transfusion centers in Iran, and HBV P gene were amplified by nested-PCR with specific primers. All PCR positive samples were analyzed by sequencing and phylogenetic analysis. The results showed that the LLAQ motif, from amino acids 179 to 182 as well as YMDD-motif, from amino acids 203-206 [which is placed in the reverse transcriptase (RT) region of the P gene], was conserved in all Iranian isolates. In the P region and in comparison with the reference gene, 19 amino acid substitutions (I91L, P1095, T118N, N121I, I122F, Y1244H, D131N, K149Q, S223A, I224V, N248H, S256C, W257Y, T259S, E263D, V266I, L267H, L269I, Q271E) were detected in all Iranian isolates found to be dominant in genotype D.

The sequences from this study were compared to GenBank sequences in a BLAST search. The best matches and the high-scoring matches were from Kazakhstan (EU595396), Belarus (EU414136, EU414137), Uzbekistan (AB222713), India (EF103279), and China (FJ386590). Afterwards, the sequences from Iranian isolates with isolates from these countries were aligned and a phylogenetic tree was drawn. High similarity between these sequences was shown and the results of the BLAST search confirmed.

The results of this study showed that HBV genotype D with sub-genotype D1 is dominant in Iranian infected blood donors. Our findings (genotype D) are consistent with previous studies. In a

study, the sera of 55 blood donors positive for HBsAg have been selected and HBV genotyping was done on the S gene sequence. Genotype D was found to be the only type in all HBV DNA positive serum samples.¹³

Our finding is consistent with the predominance of genotype D in the Mediterranean basin and the Middle East. The predominant genotype of HBV in our neighboring country (Turkey) is genotype D (98.3%–100%).¹⁴ Genotype D is also predominant in other neighboring countries such as Russia, Pakistan, and Afghanistan.^{15,16}

Sequence analysis showed no specific mutation (rtM204V/I, rtL180M) on the P gene in HBV-infected blood donors and HBV lamivudine-resistant strains did not exist naturally among these donors either.

This study has also shown that genotype D, sub-genotype D1 of HBV was dominant in Iranian HBV-infected blood donors. There was high similarity between the P gene sequence in Iranian isolates and isolates from other countries such as Kazakhstan, Uzbekistan, Belarus, India, and China.

References

1. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat.* 2004; **11**: 97–110.
2. Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology.* 2003; **46**: 329–338.
3. Echevarria JM, Avellón A, Magnius LO. Molecular epidemiology of hepatitis B virus in Spain: identification of viral genotypes and prediction of antigenic subtypes by limited sequencing. *J Med Virol.* 2005; **76**: 176–184.
4. Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus *in vitro* by 2',3'-dideoxy-3'-thiacytidine and related analogues. *Proc Natl Acad Sci USA.* 1991; **88**: 8495–8499.
5. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DLJ. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine *in vivo*. *Hepatology.* 1996; **24**: 714–717.
6. Kobayashi S, Ide T, Sata M. Detection of YMDD motif mutants in some lamivudine-untreated asymptomatic hepatitis B virus carriers. *J Hepatol.* 2001; **34**: 584–586.
7. Sheldon J, Soriano V. Hepatitis B virus escape mutants induced by antiviral therapy. *J Antimicrob Chemother.* 2008; **61**: 766–768.
8. Amini-Bavil-Olyae S, Sarrami-Forooshani R, Mahboudi F, Sabahi F, Adeli A, Noorinayer B, et al. Genotype characterization and phylogenetic analysis of hepatitis B virus isolates from Iranian patients. *J Med Virol.* 2005; **75**: 227–234.
9. Alavian SM. Ministry of Health in Iran is serious about controlling hepatitis B. *Hepat Mon.* 2007; **7**: 3–5.
10. Ngui SL, Hallet R, Teo CG. Natural and iatrogenic variation in hepatitis B virus. *Rev Med Virol.* 1999; **9**: 183–209.
11. Weinberger K, Bauer T, Bohm S, Jilg W. High genetic variability of the group-specific a-determinant of hepatitis B virus surface antigen (HBsAg) and the corresponding fragment of the viral polymerase in chronic virus carriers lacking detectable HBsAg in serum. *J Gen Virol.* 2000; **81**: 1165–1174.
12. Tuncbilek S, Kose S, Elald AI, Akman S. Lamivudine resistance in untreated chronic hepatitis B patients in Turkey. *Turk J Gastroenterol.* 2008; **19**: 99–103.
13. Milani S, Sharifi Z, Hosseini M. Determination of HBV genotypes among HBsAg positive blood donors in Tehran, Iran, using PCR-RFLP. *Iran J Public Health.* 2009; **38**: 41–47.
14. Bozdayi AM, Bozkaya H, Turkyilmaz AR, Sarioglu M, Cetinkaya H, Karayalcin S, et al. Nucleotide divergences in the core promoter and precore region of genotype D hepatitis B virus in patients with persistently elevated or normal ALT levels. *J Clin Virol.* 2001; **21**: 91–101.
15. Baig S, Siddiqui A, Chakravarty R, Moatter T. Hepatitis B virus sub-genotypes D1 and D3 are prevalent in Pakistan. *BMC Research Notes.* 2009; **2**: 1–7.
16. Amini-Bavil-Olyae S, Alavian SM, Adeli A, Sarrami-Forooshani R, Sabahi F, Sabouri E, 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**: 358–364.