ImmunoAnalysis, 2022, 2, 7
doi:10.34172/ia.2022.07
https://ia.tbzmed.ac.ir/

Original Research

Phylogenetic and Immune Epitope Analysis of Hepatitis B Surface Antigen in Chronic Carriers from Tabriz, Northwest of Iran

Fatemeh Ebrahimi[1,2], Mahin Ahangar Oskoee[1,2], Hossein Bannazadeh Baghi[1,2], Javid Sadeghi[2], Mohammad Ahangarzadeh Rezaei[2], Omid Gholizadeh[1,2], Nader Mohammadzadeh[3], Ahmad Dashti[3], Vahdat Poortahmasebi[1,2*]

[1]Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Department of Bacteriology and Virology, School of Public Health, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Central Laboratory of East Azerbaijan Province, Tabriz University of Medical Sciences, Tabriz, Iran

ARTICLE INFO

Article History:
Received: October 13, 2022
Accepted: Decrember 13, 2022
ePublished: Decrember 25, 2022

Keywords:
HBV, Genotype, Phylogenetic analysis, Mutation

Abstract

Background: The purpose of this investigation was to specify the genotypes and subtypes of hepatitis B virus (HBV) by phylogenetic analysis and to investigate the mutations in the S gene region in chronic patients with hepatitis B surface antigen (HBsAg)-positive.

Methods: In the present study, Serum sample of 95 patients with chronic HBV infection were subjected. Nested polymerase chain reaction was used for detection of HBV DNA. The S gene region of the DNA isolates was subjected to direct sequencing and phylogenetic analysis.

Results: Genotype D was present in all isolates, and the predominant subtype was ayw2 (94.7%), followed by three isolates, ayw3 (3.1%), and two isolates, ayw4 (2.1%). There were totally 169 nucleotide changes. Eighty-one (47.92%) were missense, while 88 (52.07%) were silent. None of the nucleotide or amino acid changes were seen in 31 (32.6%) patients. Eight (8.4%) of the 95 samples had at least one mutation in the HBsAg "a" determinant region. Fifty-eight (71.6%) of the 81 amino acid changes in the surface protein occurred in immunological epitopes, with 16 (27.5%) occurring in B cell epitopes, 17 (29.3%) in T helper epitopes, and 25 (43.1%) in internal cytotoxic T lymphocyte epitopes.

Conclusion: This investigation demonstrated that the genotype D is the prevalent genotype in Eastern Azerbaijan province, similar to other Iranian areas and Mediterranean nations. Furthermore, the highest rate of mutation of antigenic epitopes occurred in the T lymphocyte epitopes of our patients.

Introduction

Hepatitis B virus (HBV) infection is the cause of one of the most severe liver diseases in the world and the most prevalent viral disease in humans.[1] Currently, HBV infection is considered as a health problem in the world, and the virus is the tenth most frequent cause of death globally and the primary cause of liver cancer.[2,3] The disease is highly contagious and is transmitted mainly through blood transfusions, unsafe injection methods, sexual contact, and mother-to-child transmission.[4-6] HBV can be associated with various clinical outcomes, from asymptomatic carriers to liver cirrhosis and hepatocellular carcinoma.[4,7] HBV is divided into ten genotypes, which differ by 8% intergenomic sequence divergence in the whole genome. With the identification of new HBV genotypes, they were gradually classified into subtypes using the 4-8% divergence and the four major subgroups, including ayw, ayr, adw, and adr, which have similar geographic distribution.[8-10]

Patients with HBV infection genotype A are predominately in North America, Northwestern Europe, and Africa, whereas genotypes B and C are more typical in China and Japan. The most common genotype, genotype D, is widespread, particularly in the Middle East, South Asia, and the Mediterranean.[11] Genotype C is common in cirrhotic patients. Genotype A frequently causes chronic disease, and genotype D is most frequently found among injecting drug users. Genotype C shows less response to antiviral drugs.[12] HBV genotype diversity appears to be effective in different clinical patterns of infection, disease severity, progression, and treatment response. Knowledge of HBV genotype distribution is critical in the vaccination, antiviral treatment, diagnosis, and prevention programs.[13,14]

High-endemic regions of HBV infection are known to exist in Southeast Asian and Middle Eastern nations.

*Corresponding Author: Vahdat Poortahmasebi, Email: vahdat2011@gmail.com

However, there is little knowledge of the frequency of HBV infection in several of these places.[15] Nearly half a century after the development and implementation of an effective HBV vaccine worldwide, HBV infection is still considered a challenging program for the World Health Organization.[5] HBV spreads in different geographical and climatic regions, and analysis of different genotypes and sub-genotypes can give us a complete understanding of the original race of the virus and its origin. HBV genotypes and subtypes have drawn more attention recently because they influence the course and effects of chronic HBV infection. Patients with acute hepatitis are more likely to have genotype D, whereas chronic hepatitis B (CHB) patients are more likely to have genotype A.[16] Several investigations have been done on the distribution of HBV genotypes and subgroups in Iran.[1,17,18] The study on 19 patients with chronic HBV in Hormozgan showed that all patients had genotype D, sub genotype D1, and subtype ayw2.[19] Also, in another study conducted in Mazandaran on 100 patients with HBV infection, it was revealed that 69% of patients with genotype D, 7% were genotype B, and 24% were co-infections B and D.[20] The objective of this research was to offer a thorough understanding of how HBV changed over time in the Eastern Azerbaijan region and phylogenetic analysis based on hepatitis B surface antigen (HBsAg) sequence and genotype and subtype determination, which was influential in the public health system to improve prevention and treatment helps to identify strains that have evaded detection due to mutations in their HBsAg antigenic epitopes. Surface protein amplification and sequencing were done using sera from 95 patients.

Materials and Methods

Patients

Ninety-five patients with HBV infection who were HBsAg positive and had been sent to the Central Laboratory of Eastern Azerbaijan province during the year 2021 were enrolled in a cross-sectional study. HBsAg positivity for at least six months without any positive hepatitis D virus or hepatitis C virus results was required to participate in this study. Informed consent was given by each patient, and the ethics committee has approved this research design. Whole blood samples in 5 mL aliquots were taken from each patient. Serum separation was done by centrifuge in the virology laboratory of the Tabriz University of Medical Science (TBZMED) and then frozen at -70°C until evaluated. Before the time of specimen collection, all patients signed an informed consent provided by the local ethics committee.

Serological and biochemical markers

Following sample collection, hepatitis B serological tests were performed using the ELISA technique to check serum samples for the existence of HBsAg, HBeAg, anti-HBe, and anti-HBc (Acon, China). An automated analyzer was used to assess the alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

HBV DNA extraction and quantitative real-time PCR (qRT-PCR)

Under the directions provided by the manufacturer, HBV DNA was extracted using a viral nucleic acid extraction kit (Qiagen, Germany). 200 µL of serum was used to extract the DNA, as well as 50 µL of elution buffer, which was utilized to elute the DNA. At -70°C until required, the extracted DNA was stored. According to the manufacturer's recommendations, qRT PCR was carried out using the Altona kit (Hamburg, Germany) to ascertain the viral load (IU/mL) of HBV.

Nested polymerase chain reaction

To determine the existence of HBV-DNA in all samples, the nested PCR method was used due to its high specificity, and the standard gold method was used worldwide using the primers mentioned in Table 1. To identify mutations, genotypes, and subtypes, the full-length sequence (681 bp) of the HBsAg gene was amplified for all samples. To amplify the HBsAg region of the HBV DNA, two rounds of nested PCR were purposefully conducted. Taq activation at 95°C for 5 minutes was followed by 35 cycles of PCR amplification at the following temperatures: denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. Similar to the first round's amplification profile, the second round's cycling conditions called for initial denaturation at 95°C for 15 minutes and annealing at 58°C for 45 seconds.

HBsAg sequencing and sequence analysis

The products of the second round of nested-PCR were sequenced by using a DNA sequence analyzer and 0.5 µL of the relevant primers S6 and S7 (sequencing was done by Genetic Analyzer ABI-3130 DNA Sequencer). Chromas (version 2.1.1) and BioEdit (version 7.0.5.3) software were used to analyze the result. MEGA-X software (version 6.06), along with reference sequences of genotypes (A-I) and Woodchuck hepatitis virus (WHV), were used to

Table 1. The size and sequence of the primers used to amplify the full-length hepatitis B surface protein

Primers	PCR rounds	Sequence 5´ to 3´ of oligonucleotide	Tm (C°)
S1	First round	CCTGCTGGTGGCTCCAGTTC	63.4
S2		CCACAATTCKTTGACATACTTTCCA	58.9
S6	Second round	GCACACGGAATTCCGAGGACTGGGGACCCTG	74.8
S7		GACACCAAGCTTGGTTAGGGTTTAAATGTATACC	67.1

evaluate the phylogenetic tree analysis using the bootstrap resampling test with 1000 replicates.[21]

Results

Demographic and clinical characteristics

Ninety-five patients with CHB participated in this study, 55 females (57.9%) and 40 males (42.1%), with 57.37 ± 8.55 (Mean ± SD) years old (range 20-71) (Table 2). The mean ALT levels were 40.21 ± 13.19 U/L, and AST levels were 38.50 ± 8.64 U/L. All the patients had HBsAg positivity for more than six months and were anti-HBc positive. 20% (n=19) and 80% (n=76) were HBeAg and anti-HBe positive, respectively. The median viral load of the patients was 9166 IU/mL. Four patients ranged in age from 20 to 28. In the "a" determinant region, two patients had mutations. All four patients have received the vaccine. 70 (73.7%) people have received antiviral drugs, and 25 (26.3%) people have not. Three people did not receive the vaccine, and seven did not know about receiving the vaccine.

Phylogenetic and mutational analysis

All HBsAg sequences were compared to the reference sequence of other HBV genotypes. The findings revealed that HBV genotype D was present in all samples (Supplementary file 1). Also, the predominant subtype was ayw2 (94.7%), followed by three subtypes, ayw3 (3.1%), and two subtypes, ayw4 (2.1%).

There were 40 (40.8%) mutant amino acids in total among the 95 patients, and silent nucleotide mutations were present in 24 (25.2%) patients. None of the nucleotide or amino acid changes were seen in 31 (32.6%) patients. There were 169 nucleotide changes in total, 81 (47.92%) of which were missense (changing amino acids), while 88 (52.07%) were silent (no amino acid change). Based on the average number of mutations per site, all sequences had an average nucleotide mutation frequency (*dN/dS*) of 0.92. The HBsAg area showed negative selection pressure when the ratio was less than "one." 1.7 occurrences per sample represented the average nucleotide mutations. 14 (14.7%) samples had at least one mutation in the HBsAg major hydrophilic region (MHR). Also, alteration of glycine (Gly) to arginine (Arg) at amino acid 145 (G145R) of HBsAg was found in one patient. The phylogeny tree (Figure 1) shows that our genotype is D and the hepatitis B genotype is the same as the reference we compared it to.

Analysis of mutations in immunological epitopes on surface proteins

Fifty-eight (71.6%) of the 81 amino acid changes in the surface protein occurred in immunological epitopes, with 16 (27.5%) occurring in B cell epitopes, 17 (29.3%) in T helper epitopes, and 25 (43.1%) in internal CTL epitopes. Eight (8.4%) of the 95 samples had at least one mutation in the HBsAg "a" determinant region (Supplementary file 1).

Discussion

Genotyping for HBV has been widely utilized globally. However, other areas, especially the Middle East, have had poor outcomes. Iran has limited knowledge about HBV genotypes, and this is the initial research to focus on the region of Tabriz. A phylogenetic study of the S region in 95 patients revealed that genotype D was the most common genotype of HBV in Tabriz. Subtype ayw2 strains predominated, while a minor number of ayw3 and ayw4 isolates were also found. These findings are in line with previous studies, which found that 24 of the 26 examined patients belonged to subtype awy2, whereas the remaining two isolates belonged to subtype ayw3.[22] Additionally, in prior research, it was determined that serotypes ayw3 and ayw4 were 0.2% and 0.4%, respectively.[23] Almost 250 million people worldwide have CHB.[24] Iran has a 2.2% prevalence of HBV infection, according to estimates.[22] HBV genotype D and subtype

Table 2. The pattern of nucleotide/amino acid distributions within the surface protein in correlation with the HBeAg status of the patients

HBsAg substitution levels	All patients (n= 95)	HBeAg positive (n= 19)	HBeAg negative (n= 76)	*P* value
Gender, n (%)				0.603
Male	40 (42.1)	7 (36.8)	33 (43.2)	
Female	55 (57.9)	12 (63.2)	43 (56.8)	
Age (Mean±SD)	57.37±8.55	54.50±12.61	58.77±7.46	0.174
ALT, (U/L)	40.21±13.19	41.11±11.14	36.79±8.06	0.127
AST, (U/L)	38.50±8.64	34.67±7.31	31.57±6.94	0.110
Viral load (IU/mL)	(Median: 9166, Range: 554–81437)	(Median: 5643, Range: 4406–81437)	(Median: 964, Range: 554–1379)	<0.001
Nucleotide mutations	64 (67.4)	12 (63.2)	52 (68.4)	0.661
Amino acid mutation	40 (42.1)	8 (42.1)	32 (42.1)	1
Nucleotide silent mutations	54 (56.8)	10 (52.6)	44 (57.9)	0.678
Immune mutation	48 (50.5)	6 (31.6)	42 (55.3)	0.064
B-cell epitopes	14 (14.7)	2 (10.5)	12 (15.8)	0.562
Th epitopes	15 (15.8)	2 (10.5)	13 (17.1)	0.481
CTL epitopes	19 (20)	3 (15.8)	16 (21.1)	0.608

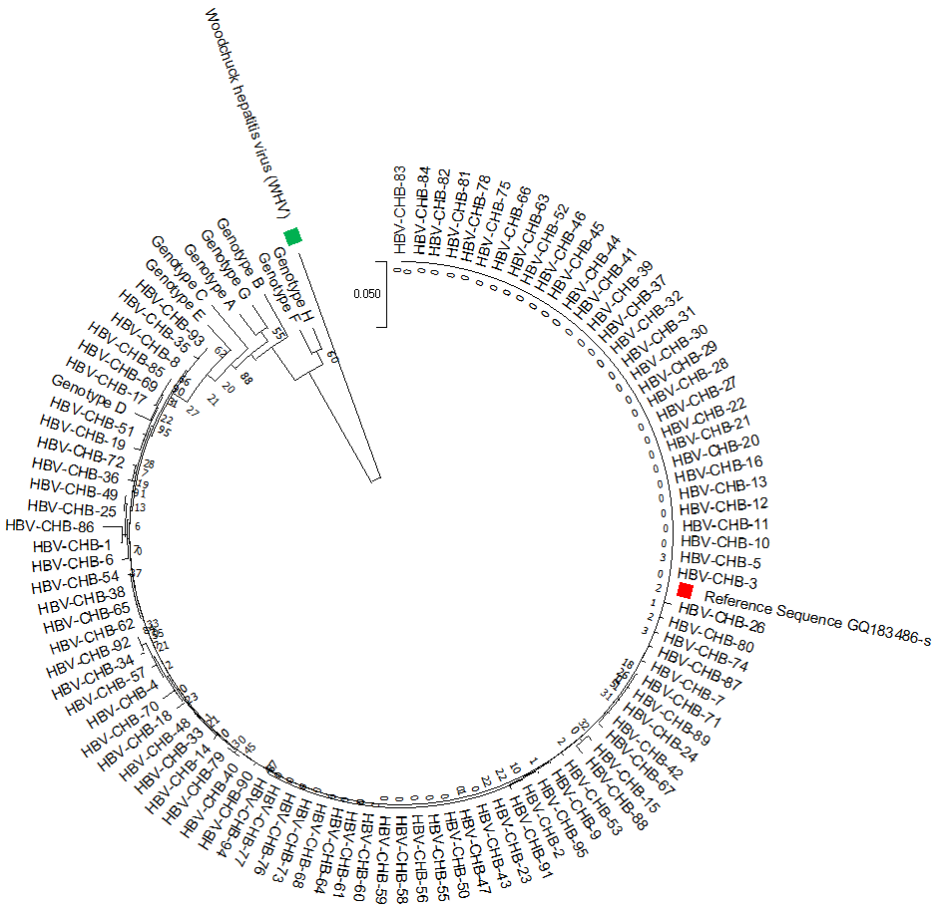

Figure 1. Maximum likelihood phylogenetic tree construction based on Clustal W alignment of HBsAg sequences HBV isolates from patients.

ayw2 predominate in the Iranian population, based on prior research of Iranian HBV chronic patients. Moreover, these results matched up with a previous study done in Golestan using 100 HBV-infected patients.[25] The survey of 163 samples in Sistan and Baluchistan also showed that genotype D in Iran is dominant.[26] Another study of 70 patients with Urmia confirmed the relevant findings.[27] But different genotypes were reported in specific researches. In 2012, a study on the serum of 160 people who tested positive for HBsAg showed that in 93.8% of the cases, these individuals' dominant genotype was the D genotype. While 6.2% of the samples had a mixture of genotypes, including D (2.9%), F (2.5%), B (0.4%), and A (0.4%).[28] Moreover, another investigation identified genotype B, which may be about adjacent countries.[29] Additionally, HBV genotype D is widespread in nearby countries, including Pakistan,[30] Afghanistan,[31] and Turkey.[32] Despite being relatively uncommon in northern Europe and the Americas, HBV genotype D is present worldwide.[33] In our cases, the proportion of missense versus silent nucleotide variants was approximately 0.92, which means that the protein sequence has maintained its stability over time. On the external surface of the MHR, the "a" determinant domain of the HBsAg, which is a widely conserved area of the protein, where antibodies (anti-HBs) against the HBsAg attach. That is responsible for producing defense-related antibodies in an infected host. This region is found between amino acid positions 124 and 147.[34,35] Virion secretion can be affected by mutations in the S-gene, which codes for HBsAg, particularly those that affect the "a" determinant in the main hydrophilic loop. Conformational alterations may result from amino acid substitutions in the "a" determinant domain. These mutations can also interfere with the ability to neutralize antibodies to attach to the viral surface.[36,22] As a result, they may have significant clinical implications, such as evasion of the immunological response induced by vaccination, evasion of immunoglobulin therapy, babies born to mothers who are carriers, and clinical failure of standard serological testing. Nine (11.11%) of the 81 amino acid alterations were found in the "a" determinant region of HBsAg. G145R (replacing Gly with Arg) is the highest significant mutation in the "a" determinant of HBsAg. Also, genotypes B, C, and D are the most common ones to exhibit G145R mutations.[37,38] Even with significant quantities of anti-HBs, this mutation is persistent over time and can be horizontally transmitted.[39] Previous research discovered that the secondary structure of the G145R mutation reveals that this change adds a new β-strand at the 121-124 region. As a result, the HBsAg's "a" determinant can become stiffer and more aggregation-prone due to the G145R mutation, which changes its immunogenic function and secretion.[40] In this study, G145R mutation was found in only one patient, and our findings are consistent with previous studies.[41,25] Contrary to our results, another study found no G145R mutation in

Ahvaz.[42] Considering that since 1993, the hepatitis vaccine has been officially included in Iran's children's vaccination program,[43] one of our patients is infected with hepatitis B despite being under 30 years of age and receiving the vaccine, which can be due to having a mutation in the "a" determinant domain and G145R mutation. Our investigation revealed that, from 81 amino acid changes, 58 (71.60%) eventuate in the antigenic epitopes of the S gene, as follows: 25 (43.1%) in CTL epitopes, 17 (29.3%) in T helper epitopes, 16 (27.5%) in B cell epitopes, and 9 (9.8%) in the "a" determinant region. The latter conclusion agreed with those of other investigators, particularly in patients who were infected with genotype D,[25,44,45] the highest rate of mutation of antigenic epitopes occurred in the CTL epitopes of our patients. These findings are consistent with previously reported results that found that the majority of mutations were concentrated in the CTL immune epitope.[46,47] On the contrary, Shokatpour et al[44] and Karami et al[45] found that the most mutations occurred (72.0%) in T helper epitope and the B cell epitope (47.82%), respectively. Escape mutations in HBV immunological epitopes are an important issue since they can play a role in how chronic HBV pathogenesis. According to previous studies, the worldwide prevalence of MHR variants was 21.73%, with the most frequent substitution occurring at position P127T. This is comparable with our results showing the overall frequency of 19.75% MHR mutations with the most frequent one of P127T.[45] Sayan et al[48] identified many HBV vaccine-escape mutations (S143T, D144E, G145R, E164D, and I195M), of which three (G145R, E164D, and I195M; one case each for G145R and E164D, but two instances for I195M) are shown in the current investigation.

Conclusion

According to this study, Iranian patients primarily had HBV genotype D and subtype ayw2 infections. The genotypes' frequency and distribution matched those observed in Iran's various geographical areas. This is fascinating and requires additional research with a bigger sample size. The findings imply that a local modification in the "a" determinant region of the HBsAg allows it to evade detection by the host immune system, vaccination, and diagnostic tests.

Acknowledgments

This study is supported by the Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran (Project code: 67508).

Author Contributions

Conceptualization: Vahdat Poortahmasebi.
Data curation: Vahdat Poortahmasebi, Mahin Ahangar Oskoee.
Formal Analysis: Vahdat Poortahmasebi.
Funding acquisition: Vahdat Poortahmasebi.
Investigation: Vahdat Poortahmasebi, Fatemeh Ebrahimi, Mahin Ahangar Oskoee.
Methodology: Fatemeh Ebrahimi, Vahdat Poortahmasebi, Omid Gholizadeh, Nader Mohammadzadeh, Ahmad Dashti.
Project administration: Vahdat Poortahmasebi.
Supervision: Vahdat Poortahmasebi, Mahin Ahangar Oskoee, Hossein Bannazadeh Baghi, Javid Sadeghi, Mohammad Ahangarzadeh Rezaei.
Validation: Vahdat Poortahmasebi, Fatemeh Ebrahimi,Mahin Ahangar Oskoee.
Visualization: Vahdat Poortahmasebi, Mahin Ahangar Oskoee.
Writing – original draft: Vahdat Poortahmasebi, Fatemeh Ebrahimi.

Data Availability Statement

The data that support the findings of this study are available in Supplementary Data.

Ethical Issues

All experiments and procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical research (Ethics code: IR.TBZMED.REC.1400.873).

Conflict of Interest

The authors declare that they have no competing interests.

Supplementary Files

Supplementary file 1 contains Tables S1-S4.

References

1. Stanaway JD, Flaxman AD, Naghavi M, Fitzmaurice C, Vos T, Abubakar I, et al. The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study 2013. Lancet. 2016;388(10049):1081-8. doi: 10.1016/s0140-6736(16)30579-7.
2. Hofmann F, Kralj N. Criteria for successful hepatitis B vaccination in adults: results of a case study. Infection. 2009;37(3):266-9. doi: 10.1007/s15010-008-7410-y.
3. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet. 2015;386(10003):1546-55. doi: 10.1016/s0140-6736(15)61412-x.
4. Tian T, Song C, Jiang L, Dai J, Lin Y, Xu X, et al. Hepatitis B virus infection and the risk of cancer among the Chinese population. Int J Cancer. 2020;147(11):3075-84. doi: 10.1002/ijc.33130.
5. Liu Z, Zhang Y, Xu M, Li X, Zhang Z. Distribution of hepatitis B virus genotypes and subgenotypes: a meta-analysis. Medicine (Baltimore). 2021;100(50):e27941. doi: 10.1097/md.0000000000027941.
6. Seto WK, Lo YR, Pawlotsky JM, Yuen MF. Chronic hepatitis B virus infection. Lancet. 2018;392(10161):2313-24. doi: 10.1016/s0140-6736(18)31865-8.
7. Bréchot C, Degos F, Lugassy C, Thiers V, Zafrani S, Franco D, et al. Hepatitis B virus DNA in patients with chronic liver disease and negative tests for hepatitis B surface antigen. N Engl J Med. 1985;312(5):270-6. doi: 10.1056/nejm198501313120503.
8. Kramvis A. Genotypes and genetic variability of hepatitis B virus. Intervirology. 2014;57(3-4):141-50. doi: 10.1159/000360947.
9. Schaefer S. Hepatitis B virus taxonomy and hepatitis B virus genotypes. World J Gastroenterol. 2007;13(1):14-21. doi: 10.3748/wjg.v13.i1.14.
10. Pourkarim MR, Amini-Bavil-Olyaee S, Kurbanov F, Van Ranst M, Tacke F. Molecular identification of hepatitis B virus genotypes/subgenotypes: revised classification hurdles and updated resolutions. World J Gastroenterol. 2014;20(23):7152-68. doi: 10.3748/wjg.v20.i23.7152.
11. Pettersson JH, Myking S, Elshaug H, Bygdås KIE, Stene-Johansen K. Molecular epidemiology of hepatitis B virus infection in Norway. BMC Infect Dis. 2019;19(1):236. doi: 10.1186/s12879-019-3868-8.

12. Mayerat C, Mantegani A, Frei PC. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? J Viral Hepat. 1999;6(4):299-304. doi: 10.1046/j.1365-2893.1999.00174.x.
13. Toyé RM, Cohen D, Pujol FH, Sow-Sall A, Lô G, Hoshino K, et al. Hepatitis B virus genotype study in West Africa reveals an expanding clade of subgenotype A4. Microorganisms. 2021;9(3):623. doi: 10.3390/microorganisms9030623.
14. Hou J, Liu Z, Gu F. Epidemiology and prevention of hepatitis B virus infection. Int J Med Sci. 2005;2(1):50-7. doi: 10.7150/ijms.2.50.
15. Nodeh MM, Mosavat A, Valizadeh N, Zadeh AM, Boskabadi A, Mashkani B, et al. Genotype characteristic and phylogenetic analysis of hepatitis B virus in northeast-Iran. Infect Genet Evol. 2018;59:148-54. doi: 10.1016/j.meegid.2018.02.004.
16. Zhong YW, Li J, Song HB, Duan ZP, Dong Y, Xing XY, et al. Virologic and clinical characteristics of HBV genotypes/subgenotypes in 487 Chinese pediatric patients with CHB. BMC Infect Dis. 2011;11:262. doi: 10.1186/1471-2334-11-262.
17. Hutin Y, Nasrullah M, Easterbrook P, Dongmo Nguimfack B, Burrone E, Averhoff F, et al. Access to treatment for hepatitis B virus infection - worldwide, 2016. Am J Transplant. 2018;18(10):2595-8. doi: 10.1111/ajt.15093.
18. Lin CL, Kao JH. Natural history of acute and chronic hepatitis B: the role of HBV genotypes and mutants. Best Pract Res Clin Gastroenterol. 2017;31(3):249-55. doi: 10.1016/j.bpg.2017.04.010.
19. Norouzi M, Ghorashi SA, Ataei B, Yaran M, Malekzadeh R, Alavian SM, et al. Hepatitis B virus surface antigen variants clustered within immune epitopes in chronic hepatitis B carriers from Hormozgan province, south of Iran. Iran J Basic Med Sci. 2010;13(4):213-24. doi: 10.22038/ijbms.2010.5117.
20. Haghshenas MR, Mousavi T, Rafiei A, Hosseini V, Hosseini-Khah Z. Prevalence of hepatitis B virus genotypes with HBsAg positive patients in the northern of Iran (Mazandaran) during 2010-2011. HealthMED. 2012;6(5):1568-73.
21. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985;39(4):783-91. doi: 10.1111/j.1558-5646.1985.tb00420.x.
22. Sali S, Azarmmanesh S, Ghalikhani H, Vaezjalali M. Phylogenetic analysis of hepatitis B virus among household members with HBV chronic infection. Avicenna J Med Biotechnol. 2019;11(3):221-8 .
23. Mohebbi SR, Amini-Bavil-Olyaee S, Zali N, Noorinayer B, Derakhshan F, Chiani M, et al. Molecular epidemiology of hepatitis B virus in Iran. Clin Microbiol Infect. 2008;14(9):858-66. doi: 10.1111/j.1469-0691.2008.02053.x.
24. Taherkhani R, Farshadpour F. Prevalence, genotype distribution and mutations of hepatitis B virus and the associated risk factors among pregnant women residing in the northern shores of Persian Gulf, Iran. PLoS One. 2022;17(3):e0265063. doi: 10.1371/journal.pone.0265063.
25. Moradi A, Zhand S, Ghaemi A, Javid N, Tabarraei A. Mutations in the S gene region of hepatitis B virus genotype D in Golestan province-Iran. Virus Genes. 2012;44(3):382-7. doi: 10.1007/s11262-012-0715-z .
26. Yoosefi K, Taheri M, Khosravi S, Saneie-Moghadam E, Mohagheghi-Fard AH. Genotyping of hepatitis B virus by multiplex PCR in Sistan and Baluchestan province. Zahedan J Res Med Sci. 2016;18(2):e5986. doi: 10.17795/zjrms-5986.
27. Ansari MHK, Rasmi Y, Abbasi L. Hepatitis B virus genotypes in West Azarbayjan province, Northwest Iran. Open Access Maced J Med Sci. 2017;5(7):875-9. doi: 10.3889/oamjms.2017.206.
28. Naderi S, Nazemi A, Jafarpour M, Tohidi F, Yaghoubian SS, Safarnejad Tameshkel F. Hepatitis B virus genotype frequency in patients of northern counties from Iran by multiplex allele-specific PCR. Ann Biol Res. 2012;3(8):3842-5.
29. Neisi N, Makvandi M, Samarbaf-Zadeh AR. A study on genotypes of hepatitis B virus among hemodialysis patients in Khuzestan province. Jundishapur J Microbiol. 2011;4(2):65-70.
30. Masood I, Waheed U, Arshad M, Saeed M, Farooq A, Moneeba S, et al. Molecular epidemiology of hepatitis B virus genotypes in blood donors in Islamabad, Pakistan. J Lab Physicians. 2019;11(3):240-3. doi: 10.4103/jlp.jlp_150_18.
31. Husseini AA, Islam Saeed KM, Yurdcu E, Bozdayı AM. Molecular epidemiology of hepatitis B virus, hepatitis C virus, and hepatitis D virus in general population of Afghanistan. Turk J Gastroenterol. 2020;31(9):658-66. doi: 10.5152/tjg.2020.19169.
32. Bozdayi G, Türkyilmaz AR, Idilman R, Karatayli E, Rota S, Yurdaydin C, et al. Complete genome sequence and phylogenetic analysis of hepatitis B virus isolated from Turkish patients with chronic HBV infection. J Med Virol. 2005;76(4):476-81. doi: 10.1002/jmv.20386.
33. Norder H, Couroucé AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology. 2004;47(6):289-309. doi: 10.1159/000080872.
34. Alavian SM, Carman WF, Jazayeri SM. HBsAg variants: diagnostic-escape and diagnostic dilemma. J Clin Virol. 2013;57(3):201-8. doi: 10.1016/j.jcv.2012.04.027.
35. Weber B. Diagnostic impact of the genetic variability of the hepatitis B virus surface antigen gene. J Med Virol. 2006;78 Suppl 1:S59-65. doi: 10.1002/jmv.20610.
36. Simon B, Kundi M, Puchhammer E. Analysis of mutations in the S gene of hepatitis B virus strains in patients with chronic infection by online bioinformatics tools. J Clin Microbiol. 2013;51(1):163-8. doi: 10.1128/jcm.01630-12.
37. Yano Y, Azuma T, Hayashi Y. Variations and mutations in the hepatitis B virus genome and their associations with clinical characteristics. World J Hepatol. 2015;7(3):583-92. doi: 10.4254/wjh.v7.i3.583.
38. Konopleva MV, Borisova VN, Sokolova MV, Semenenko TA, Suslov AP. Recombinant HBsAg of the wild-type and the G145R escape mutant, included in the new multivalent vaccine against hepatitis B virus, dramatically differ in their effects on leukocytes from healthy donors in vitro. Vaccines (Basel). 2022;10(2):235. doi: 10.3390/vaccines10020235.
39. Kanji JN, Penner RED, Giles E, Goodison K, Martin SR, Marinier E, et al. Horizontal transmission of hepatitis B virus from mother to child due to immune escape despite immunoprophylaxis. J Pediatr Gastroenterol Nutr. 2019;68(5):e81-e4. doi: 10.1097/mpg.0000000000002318.
40. Rezaee R, Poorebrahim M, Najafi S, Sadeghi S, Pourdast A, Alavian SM, et al. Impacts of the G145R mutation on the structure and immunogenic activity of the hepatitis B surface antigen: a computational analysis. Hepat Mon. 2016;16(7):e39097. doi: 10.5812/hepatmon.39097.
41. Vaezjalali M, Goudarzi H, Rashidpour S, Davoodbeglou F, Thijssen M, Pourkarim MR. Persons with intellectual disability: a potential reservoir of invasive strains of hepatitis B virus. Hepat Mon. 2019;19(9):e85661. doi: 10.5812/hepatmon.85661.
42. Khodadad N, Seyedian SS, Moattari A, Biparva Haghighi S, Pirmoradi R, Abbasi S, et al. In silico functional and structural characterization of hepatitis B virus PreS/S-gene in Iranian patients infected with chronic hepatitis B virus genotype D. Heliyon. 2020;6(7):e04332. doi: 10.1016/j.heliyon.2020.e04332.
43. Moghadami M, Dadashpour N, Mokhtari AM, Ebrahimi M, Mirahmadizadeh A. The effectiveness of the national hepatitis B vaccination program 25 years after its introduction in Iran:

a historical cohort study. Braz J Infect Dis. 2019;23(6):419-26. doi: 10.1016/j.bjid.2019.10.001.
44. Shokatpour N, Vaezjalali M, Foster GR, Sali S. Nucleotide substitutions in hepatitis B viruses derived from chronic HBV patients. Mediterr J Hematol Infect Dis. 2019;11(1):e2019046. doi: 10.4084/mjhid.2019.046 .
45. Karami C, Adli AH, Zhand S, Tabarraei A, Talei R, Saeidi M, et al. Study of genotype, subtype and mutation in the S gene in hepatitis B patients co-infected with HIV in Iran. Jundishapur J Microbiol. 2016;9(12):e34009. doi: 10.5812/jjm.34009.
46. Ramezani F, Alavian SM, Sadeghi A, Khedive A, Ghalichi L, Norouzi M, et al. Evolution of hepatitis B virus surface gene and protein among Iranian chronic carriers from different provinces. Iran J Microbiol. 2015;7(4):214-20.
47. Khedive A, Norouzi M, Ramezani F, Karimzadeh H, Alavian SM, Malekzadeh R, et al. Hepatitis B virus surface protein mutations clustered mainly in CTL immune epitopes in chronic carriers: results of an Iranian nationwide study. J Viral Hepat. 2013;20(7):494-501. doi: 10.1111/jvh.12045.
48. Sayan M, Sentürk O, Akhan S, Hülagü S, Cekmen MB. Monitoring of hepatitis B virus surface antigen escape mutations and concomitantly nucleos(t)ide analog resistance mutations in Turkish patients with chronic hepatitis B. Int J Infect Dis. 2010;14 Suppl 3:e136-41. doi: 10.1016/j.ijid.2009.11.039.