Am. J. Trop. Med. Hyg., 106(1), 2022, pp. 174–181
doi:10.4269/ajtmh.21-0785

Occult Hepatitis B Virus Infection among β-Thalassemia Major Patients in Ahvaz City, Iran

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Abstract. Occult Hepatitis B Infection (OBI) is a critical risk factor for triggering post-transfusion hepatitis (PTH), cirrhosis, hepatocellular carcinoma, and hepatitis B virus (HBV) reactivation, which β-thalassemia major (BTM) patients are at risk of it due to multiple blood transfusions. This study was aimed at determining the prevalence of OBI among BTM patients from Khuzestan Province, Iran. In this cross-sectional study, 90 thalassemia patients, who have received blood 36 to 552 times, participated referred to the Shafa hospital of Ahvaz city from January 2018 to April 2019. ELISA for determining serological markers (HBsAg, anti-HBc, anti-HBs, and anti-HCV) and real-time PCR for detecting HBV-DNA were performed; Nested PCR was conducted for DNA sequencing and determining the genotype of OBI case. Phylogenetic and statistical analyses were done by R package. Of 90 subjects enrolled in this study; 95.5% (86/90) were HBsAg negative, and the frequency of OBI among them was 1.16% (1/86). The anti-HBs, anti-HBc, and anti-HCV were detected in 80.00%, 7.78%, and 12.2% of patients, respectively. HBV-DNA was assessed at four HBsAg-positive subjects as well, and all of them were negative. The phylogenetic analysis showed that the detected HBV DNA in the OBI case belongs to the genotype D. This research, for the first time, demonstrated that OBI is present among β-thalassemia patients in Iran. Also, further studies are necessary to determine the actual prevalence of OBI among BTM patients in Iran to decisions concerning OBI screening, especially in transfusion centers.

INTRODUCTION

Hepatitis B virus (HBV) is an enveloped, partly double-stranded DNA virus with approximately 3,200 base pairs belonging to the family Hepadnaviridae.[1] HBV infection is a severe problem for public health worldwide. It has been estimated that HBV has infected just over two billion people, of whom 257 million are chronically infected.[2] Chronic HBV infection is a significant cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), resulting in roughly 778,000 deaths per year.[3] Moreover, HBV is one of the most prevalent blood-borne infection agents, which has shown a more substantial risk of transmission by transfusion than Hepatitis C virus (HCV) and human immunodeficiency virus (HIV),[4,5] and therein lies the problem of post-transfusion HBV infections. Transfusion-transmitted infection (TTI) is a considerable challenge among β-thalassemia patients.[5]

β-thalassemia is one of the most common genetic diseases globally; whoever with this disorder is at significant risk of infection with blood-borne viruses due to receiving considerable amounts of transfusional blood components.[5] The likelihood of contracting HBV in these patients is dependent on the number of blood transfusions and the prevalence of HBV infection among blood donors.[6] HBsAg screening noticeably reduced the danger of transfusion-transmitted hepatitis B during the previous four decades; thus, most blood transfusion services use ELISA to detect it. Nevertheless, research has revealed that HBV spreading by negative-HBsAg blood components still occurs during the serologically negative window period and the late stage of infection.[7,8]

Studies have shown that a low level of HBV-DNA (HBV level is usually less than 104 copies/mL) remains detectable in hepatocytes and serum of some patients with negative HBsAg after acute self-limited HBV infection, chronic HBV infection, or even after successful anti-HBV therapy. This condition is termed occult HBV infection (OBI),[9] which has two forms: seropositive (anti-HBc and/or anti-hepatitis B surface [anti-HBs] positive) and seronegative (anti-HBc and anti-HBs negative).[10] Not only can OBI induce fibrosis, cirrhosis, and HCC, it is also a risk factor for disease transmission through blood transfusion or organ transplantation and acute exacerbation in immunosuppressive states.[11] Although the gold standard for OBI diagnosis in detecting HBV DNA in liver biopsy specimens, real-time PCR for HBV DNA detection in serum (or plasma) is used to detect OBI in many cases, difficulty in obtaining hepatic HBV DNA.[12] OBI's various features are still debatable, such as prevalence, pathobiology, and clinical implications.[10]

Reported from zero (Iran)[13,14] to 32.5% (Egypt)[5] in thalassemia patients, the prevalence of OBI is indeterminate because of depending on the sensitivity of HBsAg and DNA assays, and the prevalence of HBV infection in the target population.[15] Additionally, records on the prevalence of OBI among the Iranian population are inadequate. Hence, this study was planned to assess the frequency of OBI among thalassemia patients to identify the leading risk factors of HBV transmission and evaluate the necessity of OBI screening in blood transfusion centers.

MATERIALS AND METHODS

Ethics approval. The ethics committee approved this research of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1397.885) by with the ethical principles stated in the Declaration of Helsinki. Written informed consent was obtained from all patients enrolled in this study.

Study population. This cross-sectional study was performed in the Department of Virology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz,

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Iran, from January 2018 to April 2019 to determine the prevalence of OBI in β-thalassemia major (BTM) patients. It included 90 β-thalassemia patients admitted for blood transfusion to the thalassemia clinic of Shafa Hospital, Ahwaz, Iran, who has received blood 36 to 552 times. All patients received HBV vaccine (10 μg is the standard dose of vaccination should be administered to children are below $<$ 10 years of age and 20 μg injected for those $\geq$ 10 years of age; for three doses lasting up to six months).

Inclusion criteria included: 1) patients living in Khuzestan province, 2) patients with the mental capacity to give written informed consent, and 3) patients with β-thalassemia who regularly receive at least one unit of blood per month. The exclusion criteria included: 1) patients who disagreed to follow the study, 2) patients positive for HIV antibody, 3) hemophilic patients, and patients with other types of hemolytic anemia, such as α-thalassemia, sickle-cell anemia, and spherocytosis. After obtaining informed consent, a questionnaire was completed for each patient, including personal details (e.g., name, age, sex) and health data (e.g., history of the blood transfusion, history of other diseases, and the newest test result of liver function tests).

Collection and preparation of the samples. A blood sample (5 mL) was drawn from each participant; plasma was separated by centrifuging at 2,500 rpm, 10 minute, and stored at −70°C for further serological and molecular tests.

Serological tests. All samples were examined for HBsAg, anti-HBs, anti-HBc, and anti-HCV by ELISA (DIA.PRO Diagnostic Bioprobes Srl, Milano, Italy) based on the manufacturer's instructions.

Viral DNA extraction. Viral DNA was extracted from 200 μL of serum samples by using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instruction. The viral DNA was eluted in a volume of 50 μL of elution buffer used in molecular tests.

Quantitative real-time PCR. HBV DNA level was determined using the HBV RQ kit (Novin Gene, Karaj, Iran) on the QuantStudio3 real-time PCR System (Applied Biosystems Inc., Foster City, CA) according to the brochure; the analytical detection limit of the kit is 0.15 IU/μL.

Nested PCR and gel electrophoresis. The only OBI case was subjected to Nested PCR for amplifying a piece (402 bp) of the HBV genome, which enabled us to determine its sequence for designating HBV genotype and phylogenetic analysis. The HBV DNA was amplified by Nested PCR of the surface antigen gene using specific primers. The sequences of the outer primers were HBV179FS, CTAGGACCCCTGCTCGTGTT as the outer sense, and HBV704RS, CGAACCACTGAACAAATGGCACT as the outer anti-sense; 251f, GACTYGTGGTGGACTTCTC as the inner sense; 654r, GSCCCAMBCCCATAGG as the inner anti-sense primer[16] synthesized by Metabion Company, Germany. Concisely, Nested PCR amplification for the first round was carried out in a total volume of 25 μL, containing 2.5 μL PCR buffer, 0.5 μL dNTP, 1 μL $MgCl_2$, 1 μL of outer primers, 0.8 μL DNA Taq polymerase, 2.5 μL Template DNA, and 15.7 μL nuclease-free water In the second round, 1 μL product of the first round was used as a template, 2.5 μL PCR buffer, 0.5 μL dNTP, 1 μL $MgCl_2$, 1 μL of inner primers, 0.8 μL DNA Taq polymerase, and 17.2 μL nuclease-free water was mixed to reach the final 25 μL volume.

The first and second rounds of Nested PCR reaction mixture was subjected to a thermocycler (Peqlab, Germany) with the following thermal program: the amplification conditions for the first round of Nested PCR consisted of preincubation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 45 seconds.

For the second round of Nested PCR initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 49°C for 30 seconds, and extension at 72°C for 45 seconds. Finally, a volume of 5.0 μL of the final product was electrophoresed on 2% agarose gel to evaluate the suitability of DNA for sequencing. The expected PCR product for the first and second round was 526 bp and 402 bp, respectively.

Nucleotide sequencing and phylogenetic analysis. The 402-bp PCR product of the partial S gene was purified with a QIAquick PCR Purification Kit (QIAGEN). The purified product was titrated by a nanodrop and then sequenced in both directions using an automated cycle sequencing method. The sequence was obtained with a DNA sequencer (ABI 3730XL DNA analyzer) and deposited in GenBank under the accession number MK791319. Multiple sequence alignment was performed between different HBV genotype (A–H) sequences (retrieved from GenBank), and the Ahvaz isolated the sequence obtained in this study by using ClustalW. A phylogenetic tree was constructed based on the maximum likelihood method and general time reversible model. The statistical authenticity of the phylogenetic tree was computed using the bootstrap method based on 1,000 replicates. The amino acid substitution was determined for Ahvaz to isolate by comparing the reference sequence of large S protein retrieved from NCBI.

Statistical analysis. Both sequence and statistical analyses were conducted in R (R Core Team). Fisher's exact test was carried out to evaluate the association between variables and groups. A comparison between means of groups was tested using the Mann–Whitney test. A P value $<$ 0.05 was considered statistically significant.

RESULTS

Patients' characteristics, serological marker, and molecular results. A total number of 90 thalassemia major patients with a median of 22.5 years (range 4 to 47 years), of whom 53.3% (48/90) were male, and 46.6% (42/90) were females, enrolled in this study. Of the enrolled cases 33.3% (30/90) received their first blood transfusion preceding HBV vaccination.

HBsAg, anti-HBs, anti-HBc, and anti-HCV were detected in 4.44%, 80.00%, 7.78%, and 12.2% of cases, respectively. Fifty-one percent (46/90) had an anti-HB titer greater than 100 WHO mIU/mL.

Anti-HBs response. Of the 90 children with thalassemia, 94.4% had titers exceeding 10 mIU/mL (responders), and 5.6% patients were nonresponders. All patients received three doses of vaccine, but some of them were nonresponders. A hepatitis B vaccine "nonresponder" refers to a person who does not develop protective surface antibodies after completing the full series of the hepatitis B vaccine.

HBV DNA positivity. HBV DNA was evaluated for each of the 90 patients, and it was detected in only one patient, who

was negative for all serological markers. One seronegative OBI case, whose viral load was 199 IU/ml, was detected among all the patients (1.1%). All serological and molecular results obtained in the current study have been shown in Figure 1.

OBI's risk factors. Statistical analysis of OBI's risk factors in this research includes sex, age, liver enzymes, anti-HBc, blood transfusion, and HBV vaccine status; no relation between these risk factors and OBI was observed (Table 1). In Table 2, the achieved results of serological tests versus parameters that may affect the outcome of these tests have been presented. There is an association between sex and HBsAg-seropositivity was found (*P* value 0.03). Similarly, a clear association between the age of BTM patients and both anti-HBs and anti-HCV tests because the mean age of patients who were positive for each of these tests was more than patients who were negative for anti-HBs or anti-HCV (*P* value 0.0003 and 0.00001, respectively). Aspartate transaminase (AST) and alanine transaminase (ALT) were significantly higher in BTM patients with positive HBsAg (*P* value 0.02 and 0.03, respectively). According to the statistical results, the more blood transfusion was correlated to the more possibility of positive anti-HBs and anti-HCV (*P* value 0.001 and 0.000009, respectively).

The *P* value (0.002) demonstrates a significant correlation between spleen removal and anti-HCV; therefore, splenectomy can predispose these patients to HCV infection.

The sequencing results. Evolutionary analysis was conducted to illustrate the molecular evolutionary relationship between the Ahvaz isolate, whose nucleotide sequence was obtained from the OBI case in this study, and 19 reference sequences of different HBV genotypes (A–H) reported in GenBank. The phylogenetic tree revealed that Ahvaz isolate was clustered with HBV genotype D isolates, as expected because this is the most prevalent HBV genotype in Iran (Figure 2). The amino acid sequence of the OBI case (accession: QCV56806. 1) revealed that amino acid substitutions occurred in the large S protein of HBV. The result of alignment with the consensus amino acid sequence of the large envelope protein (accession: YP_009173869.1, GI: 941241316) has been shown in Figure 3, in which asparagine and methionine were replaced by serine and threonine at positions 205 and 292, respectively.

TABLE 1
Parameters affecting the presence of occult hepatitis B virus among beta-thalassemia patients

Parameter	OBI Pos	OBI Neg	*P* value	Stat. sig.
Sex				
Female	0	38	1	No
Male	1	47	–	–
Age				
< 23	2	42	1	No
≥ 23	0	43	–	–
ALT (IU/L)				
High	1	0	0.3	No
Normal	32	53	–	–
AST (IU/L)				
High	1	0	1	No
Normal	53	32	–	–
Anti-HBc				
Pos	0	7	1	No
Neg	1	78	–	–
Blood transfusion				
≤ 300	1	53	1	No
> 300	0	32	–	–
HBV vaccine			–	–
After	0	28	1	No
Before	1	57	–	–

ALT = alanine transaminase; AST = aspartate transaminase; HBV = hepatitis B virus; OBI = occult hepatitis B infection.

DISCUSSION

β-thalassemia major is a transfusion-dependent severe anemia caused by reduced (β+) or absent (β0) synthesis of

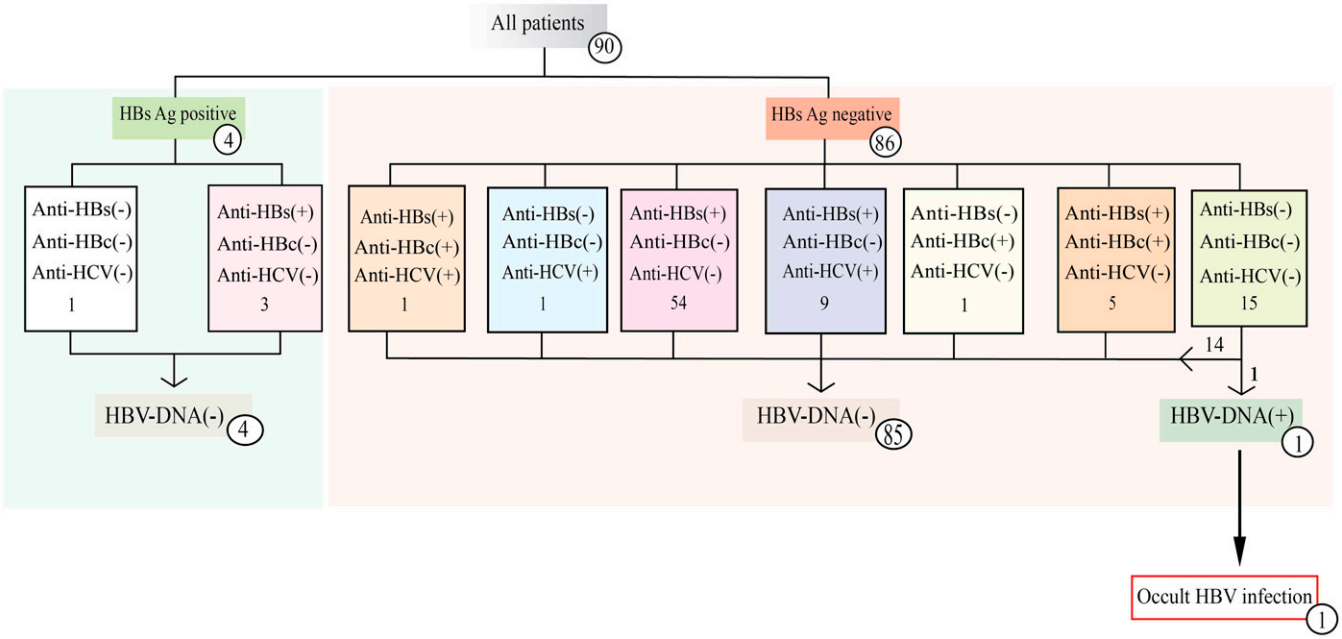

FIGURE 1. Flowchart of recruitment and testing of study participants. This figure appears in color at www.ajtmh.org.

TABLE 2
Statistical comparison between parameters affecting in beta-thalassemia patients tested by HBsAg, anti-HBs, anti-HBc, and anti-HCV

	HBs Ag		Anti-HBs		Anti-HBc		Anti-HCV	
Parameter	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Age (years)	24.5 ± 6.8	23 ± 8.4	24.6 ± 7.8	16.8 ± 7.6	22.4 ± 6.9	23.1 ± 8.4	32.7 ± 2.7	21.7 ± 7.9
P value	0.68		0.0003*		0.71		0.00001*	
Sex								
Male	0	48	39	9	4	44	8	40
Female	4	38	33	9	3	39	3	39
P value	0.03*		0.75		0.84		0.17	
No. blood transfusion	282 ± 87.2	265 ± 102	283 ± 91.3	196 ± 112	279 ± 76.1	264 ± 103	376 ± 26	250 ± 99
P value	0.76		0.001*		0.7		0.000009*	
AST (IU/L)	74 ± 29.3	52 ± 33.8	53 ± 35.7	52 ± 25.4	48 ± 16.9	53 ± 34.8	60 ± 41.3	48 ± 25
P value	0.06		0.7		0.8		0.3	
ALT (IU/L)	76 ± 30.2	57 ± 48.1	58 ± 49.8	57 ± 38.5	52 ± 13.1	58 ± 49.4	65 ± 34.7	51 ± 31,1
P value	0.09		0.8		0.6		0.1	
Lymphocyte %	38 ± 7.5	43.9 ± 9.6	43.4 ± 9.7	44.3 ± 9.4	42.9 ± 6.4	43.7 ± 9.8	40.5 ± 7.2	44 ± 9.8
P value	0.2		0.5		0.9		0.3	
Splenectomy								
Yes	0	19	18	1	2	17	6	12
No	4	67	54	17	5	66	5	67
P value	0.2		0.07		0.6		0.002*	

ALT = alanine transaminase; AST = aspartate transaminase.
* *P* value 0.05 was considered statistically significant.

the β-globin chains of hemoglobin.[17] β-thalassemia major patients need a lifetime blood transfusion, which is associated with the risk of acquiring TTIs.[18] Hepatitis B virus is one of the most common causes of liver diseases in thalassemia patients, which has five clinical forms: acute, chronic, fulminate, asymptomatic, and occult HBV infection.[13,19] The present work assessed the prevalence of OBI among the Iranian subjects with BTM referred to the Shafa Hospital of Ahvaz city who had negative results for HBV DNA in their serum samples. In an international workshop in Taormina (Italy) in October 2018, OBI was described as a presence of replication-competent HBV DNA in the liver (with or without HBV DNA in serum) and the absence of serum HBV surface antigen (HBsAg).[10] This study was primarily designed to assess OBI's prevalence among BTM patients from Khuzestan Province, Iran.

The current estimated prevalence of HBsAg is 1.79% among the general Iranian population.[20] In this research, the prevalence of HBsAg in thalassemia major patients was 2.4 times greater than the general population (4.4% versus 1.79%). It is interesting to note that all four HBsAg-positive patients were HBV DNA negative, which supports the idea that there is no correlation between HBsAg positivity and HBV DNA.[21–23]

The prevalence of occult HBV infection has been studied in different parts of the world and among various groups; however, there are conflicting with results even in a similar group. As an illustration, OBI prevalence among hemodialysis patients was 1.5% (Turkey),[24] 0.5% (Iran),[25] 0% (Germany),[26] 1.3% (Japan),[27] and 0% (Italy)[28]; in hemophilia patients was 1.73% in Pakistan,[29] 9.3% in Iran,[30] and 0% in Polish[31]; among thalassemia patients was 31.4% (India),[6] 32.5% (Egypt),[5] and 0% (Iran)[13,14]; in individuals with HCC was 30% (Japan),[32] 38.5% (Korea),[33] 50% (Egypt),[34] and 70.4% (China)[35]; among blood donors in different parts of the world was 3.7% (Egypt),[36] 4.71% (India),[37] 1.98% (Colombia),[38] 0.3% (Lebanon),[39] 0% (Iran),[8] and 7.4% (Libya).[40] OBI also has been found in patients with leprosy in Brazil (5.3%),[41] individuals suffering from hyperlipidemia in China (9.5%),[42] patients with primary glomerulopathy (3%) and lupus nephritis (13.3%) in Iran,[43] and among ART (antiretroviral therapy) naïve HIV-seropositive individuals in Mozambique (8.3%).[44]

Despite other studies in which the frequency of OBI among Iranian BTM patients was0%,[13,14] data obtained in this study showed that one of 86 BTM patients, who were negative for HBsAg, had OBI (1.16%). This prevalence is far fewer than those reported in India (31.4%)[6] or Egypt (32.4%).[5] This broad-ranging OBI prevalence in different studies could be related to the sensitivity of HBV DNA detection assays, studied population size and characteristics, the endemicity of HBV in different regions, and vaccination status.[28,45]

In this research, only one marker (HBsAg) was correlated to sex. To this extent Baruch and their collaborators indicated in most human populations, there is a higher prevalence of chronic carriers of HBV (persistently HBsAg+) among males than females.[52] Arababadi et al.[13] did not find any relationship between sex and HCV infection similar to our study. Furthermore, they observed a significant difference between the age of HCV infection, positive patients, and HCV infection negative patients. In this research, a connection between the age of beta-thalassemia patients and anti-HCV was found; moreover, their finding on the correlation between blood transfusion frequency and HCV infection agreed with our result. Contrary to a study conducted in Egypt,[7] reporting a significant association between the number of blood transfusions and anti-HBc positivity, in our study, only anti-HBs and anti-HCV were related to blood transfusion frequency.

The rate of anti-HCV positivity among BTM patients in the present study is 12.2%, which can be compared with results obtained from other Iran regions. This prevalence is lower than that reported in Mazandaran (16.9%)[46] and Mashhad (16%),[47] but higher than Kurdistan (5.6%)[48] and Lorestan (4.2%).[49]

The finding of a correlation between levels of liver enzymes (AST and ALT), and HBsAg-seropositivity obtained in the present study is consistent with another study conducted in Egypt.[50] A statistically significant difference in the

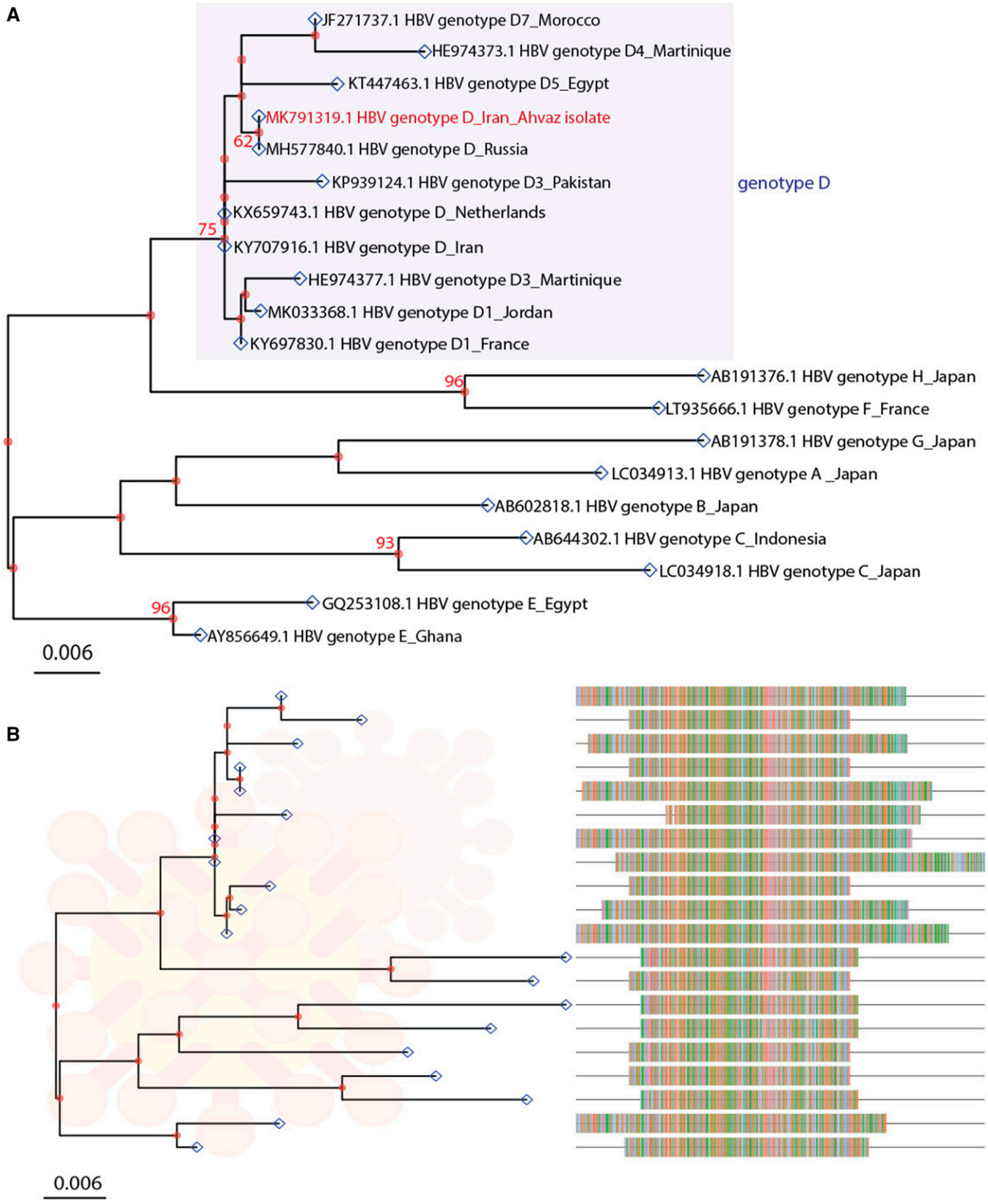

FIGURE 2. Phylogenetic tree constructed using the Maximum likelihood method. (**A**) It includes 19 reference sequences from the GenBank Database representing HBV genotypes (A–H), which have been indicated with their accession numbers, as well as Ahvaz isolate shown by a red tip label whose nucleotide sequence was determined in this study. Branch lengths were estimated with the best fitting nucleotide substitution model (GTR) selected with model test and drawn to scale with the bar denoted 0.006 nucleotide substitutions per site. Bootstrap resampling was done to determine the robustness of branches, and only values of 60% (from 1,000 replicates) have been shown. (**B**) Visualization of the phylogenetic tree with multiple sequence alignment (ClustalW aligned the sequences). This figure appears in color at www.ajtmh.org.

FIGURE 3. A comparison of consensus amino acid sequence of large envelope protein (accession: YP_009173869.1, GI: 941241316) (top) with the amino acid sequence of Ahvaz strain (accession: QCV56806.1). Replacement of amino acids at positions 205 and 292 of L-HBsAg has been shown within red ovals. This figure appears in color at www.ajtmh.org.

levels of ALT and AST between HBsAg-positive and HBsAg-negative β-thalassemia patients was found; moreover, in that study, there was no significant relation between splenectomy and anti-HCV, in contrast to the current study.

Several mechanisms are responsible for OBI, including deficiency of the host immune system, multiple amino acid substitutions in the S protein affecting HBsAg detection by current immunoassays, mutations in regulatory factors that negatively affect virus replication mutations affecting post-transcriptional mechanisms controlling S protein expression.[51]

HBV genotypes show distinct geographical distribution, genotype D is predominant in Iran. Despite the different geographical areas in different parts of Iran, genotype D is considered as the predominant genotype.[4] In this study, sequence analysis of a 402-bp fragment of the HBV S gene revealed that HBV genotype D had infected the OBI case; in addition, two amino acid substitutions were found at positions of N205S and M292T in large S protein, which may have affected HBsAg detection by the commercial ELISA kits. Based on substitution scores in BLOSUM 62, threonine rarely substitutes for methionine in homologous proteins, but serine frequently substitutes for asparagine. These substitutions—"M → T" and "N → S"—decrease the mass of a protein by 57 Da, and reduce the stability index of the protein as well.

The presence of HBV DNA in HBsAg negative blood units have been reported from many parts of the world, including from our center.[6] HBsAg screening reduces but does not abolish, the occurrence of post-transfusion hepatitis B, and HBsAg-negative HBV DNA-positive units can be infective.

CONCLUSION

In conclusion, until now, all studies conducted in Iran have reported OBI's rate among BTM patients as 0%; thus, this is the first time that an OBI case has been observed in Iranian BTM patients. The present study showed that HBsAg is not a useful marker for the diagnosis of HBV infection among

BTM patients, and it is essential that these patients be checked for HBV DNA by molecular assays. Since the number of published studies related to OBI in thalassemia patients is quite low, therefore, to determine a more accurate prevalence of OBI, especially in at-risk groups, more studies on more cases with more sensitive diagnostic methods should be conducted.

Received July 20, 2021. Accepted for publication August 23, 2021.

Published online October 4, 2021.

Acknowledgments: This research was part of a thesis written by Fatemeh Amirhashchi, MSc student of Medical Virology (Thesis number: OG-9745). It was financially supported by the Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The American Society of Tropical Medicine and Hygiene (ASTMH) assisted with publication expenses.

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