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ORIGINAL ARTICLE

Prevalence of Hepatitis B Virus Infection Among Voluntary Blood Donors from the Northeastern Region of Iran: Genotyping, Viral Load Characterization and Drug Resistance Prediction

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SUMMARY

Background: **Blood donor selection, along with laboratory screening of the HBV, plays a pivotal role in providing safe blood products. This study was aimed to evaluate the prevalence, genotype, and drug resistance prediction of HBV among Iranian blood donors.**

Methods: **This cross-sectional study was conducted on 47,506 blood donors referring to Golestan Blood Center from March 21, 2018, to March 20, 2019. Siemens Enzyngnost HBsAg6, INNO-LiPA Genotyping kits, and Nest-PCR were used for HBV screening, genotyping, and amplification of the polymerase gene, respectively. An online tool at hbv.geno2pheno.org and real-time PCR method were also utilized for drug resistance prediction and viral load measurement respectively.**

Results: **It was found that from among 47,506 donors, 47 (0.09%) were confirmed to be HBV positive subjects. About 0.94% of first-time blood donors (46 out of 4, 872) and 0.008% of repeated blood donors (1 out of 12,125) were found to be positive for HBV. First-time blood donors were also 8.6 times more likely to have a hepatitis B virus infection (odds ratio: 9.6; 95% confidence interval, 6.2 - 14.7). Seven donors had genotype D as predominant and one case had a mixed infection with genotypes A and D. Furthermore, the most predicted mutation in the polymerase gene was m204V, causing resistance to telbivudine and lamivudine.**

Conclusions: **The results showed that the risk of HBV transmission is higher among first-time blood donors. Therefore, it is recommended that pre-donation laboratory screening in first-time blood donors be conducted to improve the safety of the donated blood in the studied region.**

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KEY WORDS

hepatitis B virus, infection, blood donors, genotype, viral load, drug resistance

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LIST OF ABBREVIATIONS

TTI - Transfusion-transmitted infection
HBV - Hepatitis B virus
RT - Reverse transcriptase
IBTO - Iranian Blood Transfusion Organization
PCR - Polymerase chain reaction
AST - Aspartate aminotransferase
ALT - Alanine aminotransferase
IQR - Interquartile range
OR - Odds ratio
CI - Confidence interval
SHB - Small hepatitis B surface
MHR - Major hydrophilic region

INTRODUCTION

In recent years, the safety assessment of donated blood has become one of the most important tasks of blood services that can be achieved through strategies such as transfusion-transmitted infection (TTI) screening [1]. Furthermore, the type of donor is influential in blood safety, as the prevalence of TTI in regular voluntary donors is less than that in first-time donors [2]. One of the cases of TTI is the *Hepatitis B virus* (HBV). Approximately 250 million people are suffering from chronic HBV infection, about 40% of which progress to hepatic cirrhosis and hepatocarcinoma [3]. Screening methods including direct identification of the HBV (detection of HBsAg and nucleic acid test) and indirect methods (e.g., monitoring of the immune response by anti-HBc testing) have been useful in blood safety [4].

In epidemiological studies of HBV, alongside screening, genotyping is also of critical importance, because it provides information on how the virus is transmitted by migration or travel. Consequently, determining the virus genotype and its relationship to screening kits may also play an important role in the safety of the donated blood [5]. Ten genotypes of A to J and four major serotypes, including adr, adw, ayr, and ayw have been described for HBV [6]. The different geographical distribution of hepatitis B genotypes (genotype A in northern Europe, genotypes B and C in Asia, genotype D in the Middle East and genotype G in France and Germany) can explain the route of HBV transmission [7].

Furthermore, treatment of hepatitis is another effective factor in preventing the transmission of the HBV. Therefore, in addition to screening and genotyping, treatment of hepatitis can indirectly improve the health of the donated blood. The genotype of the infecting virus is one of the important factors in choosing the right treatment in hepatitis. For example, genotypes D and B have a weak response to treatment while genotypes A and C have a better response to treatment [8,9]. One of the viral components considered as a therapeutic target is the polymerase enzyme with reverse transcriptase (RT) activity. M240V mutation in the C-terminal domain of RT causes an alteration in the catalytic activity of the enzyme and resistance to drugs such as lamivudine and entecavir [10]. In the epidemiological classification of HBV, Iran is among regions with a low prevalence of HBV [11]. Nevertheless, the prevalence of HBV in the northeast of Iran among the general population is 5 - 7% (moderate prevalence) [12], and it is important to evaluate the prevalence of HBV in blood donors from this region. Therefore, this study was aimed to evaluate the prevalence of HBV and its genotypes in the northeast of Iran, to analyze the mutations in the gene of polymerase, and to predict drug resistance.

MATERIALS AND METHODS

Study design

The present cross-sectional study was conducted in 2018 (from March 21, 2018, to March 20, 2019) to evaluate the status of blood donors in the northeast regions of Iran. To investigate the prevalence of HBV, 47,506 donors were enrolled in the study within one year. Determination of HBV genotype and viral load was done for cases that were found to be positive according to nested-PCR. All donors were included in the study with no gender restriction. However, HIV or HCV positive donors were excluded from the study.

Methodology

Golestan province is located in the northeast of Iran. Gorgan Blood Center is the main center in this province in addition to which Gonbad-e Qabus and Bandar Torkaman blood collection sites are active in the province. Based on the Iranian Blood Transfusion Organization (IBTO) standards, voluntary blood donors were enrolled in this study. Laboratory screening tests of HIV, HCV, HBV, and syphilis were done on all donated blood. HBsAg identification was performed for all voluntary blood donors as follows: venous blood samples were first collected in tubes without anticoagulants. The HBsAg (hepatitis B surface antigen) test was performed individually by the Da Vinci instrument, using the Enzygnost HBsAg 6.0 Kit (Siemens, Marburg, Germany) for this purpose.

Primary positive samples were retested in triplicate (one from the cord of blood bag and two from a serum sample). In case of a repeatable positive result, a confirmatory test was performed on the sample with an anti-HBc Monoclonal Siemens kit (Siemens, Marburg, Germany). Blood donors who had a positive confirmed result were permanently deferred from blood donation (Figure 1). Additionally, the results of screening tests and demographic data were extracted from a computerized donor database. Data of HBV positive blood donors were collected based on the approved questionnaire (http://www.aabb.org/tm/questionnaires/Pages/dhqaabb.aspx).

For molecular and genotyping tests, first-time voluntary blood donors with confirmed positive HBsAg were recalled to enroll into the study (telephone calls were

made to donors twice, and messages were sent if they did not respond). From the entire donors enrolled in the study, 5 mL whole blood in EDTA anticoagulant tubes was collected. Then, these samples were centrifuged at 3,800 RPM, and the isolated plasma was frozen at -30°C. Also, the HBV viral load was measured by real-time PCR techniques using a homemade kit (Iranian Blood Transfusion Organization). HBV genotyping was performed by the INNO-LiPA HBV Genotyping kit (Innogenetics; Ghent, Belgium) on the extracted DNA.

Nested-polymerase chain reaction (PCR)

HBV-DNA was extracted by the SinaPure DNA kit (SinaClon, Tehran, Iran) according to the manufacturer's instructions. HBV amplification of the polymerase gene was performed by nested PCR method using specific primers, as follows:

1-The outer primers were forward
(5'-CCTGCTGGTGGCTCCAGTTC-3')
and reverse
(5'-CGTCCCGCG (AC) AGGATCCAGTT-3'),
and length of the product was 1,360 bp.
2-The inner primers were forward
(5'-CYTGGCCWAAATTCGCAGTCCC-3')
and reverse
(5'GCAAANCCCAAAAGACCACAAT-3'),
and length of the product was 721 bp.

The PCR process was done using 2 x master mix (Amplicon-Bromol, Hamburg, Germany) in two steps to amplify the gene of polymerase as follows: The thermal profile used in one step (the first PCR) included 94°C for 5 minutes, followed by 30 cycles of 94°C for 45 seconds, 63°C for 45 seconds, and 72°C for 45 seconds, followed by a final extension of 72°C for 10 minutes, and the thermal profile used in the second PCR included 94°C for 5 minutes, 30 cycles of 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for 45 seconds, followed by a final extension of 72°C for 10 minutes [13]. Electrophoresis was carried out using 1% agarose gel with 0.5% Tris/Borate/EDTA buffer (TBE).

HBV Genotyping, viral load, drug resistance prediction, and mutation analysis

The INNO-LiPA HBV Genotyping Kit (Innogenetics N.V. Belgium) was used for HBV genotype detection. The kit is based on probe hybridization of the PCR product by using biotin-mediated primers for the polymerase gene (domains B to C). The procedure was as follows: nested-PCR was first performed on the extracted DNA. Then, after hybridization, the product visualization steps were carried out in a staining process on probe-impregnated strips. Genotypes were determined according to the position and number of each band.

A homemade HBV real time-PCR (the IBTO kit, Iran) was used to measure the virus load. Based on AASLD 2018 Hepatitis B Guidance, the value less than or equal to 2,000 IU/mL was considered a low viral load, and values higher were considered high viral load [14].

Sequencing was also performed to confirm the results of genotyping. To this purpose, the result of the sequencing was aligned by https://blast.ncbi.nlm.nih.gov.

Additionally, the sequence analysis of PCR product of the polymerase gene was performed using the on-line tools on https://hbv.geno2pheno.org and www.ncbi.nlm.nih.gov/projects/genotyping in order to determine mutation and predict drug resistance.

ALT, AST, and Serology Tests

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) tests were assayed by kit (Pars Azmoon Company, Iran) manually according to the manufacturer's instructions.

Statistical analysis

Part of the data was collected from the donor's information software, including demographic information of age and gender. Data were analyzed using SPSS Version 16 (SPSS, Chicago, IL, USA) and STATA (StataCorp LP, USA) version 13. Chi-square was performed to compare the ratios. Significance level was considered less than 0.05. Furthermore, the odds ratio (OR) and 95% confidence intervals were calculated.

RESULTS

Prevalence of *Hepatitis B virus* in volunteer blood donors

The results revealed that of the 47,506 voluntary blood donors participating in this study, 45,077 were male (94.9%) and 2,429 female (5.1%), ranging in age from 18 to 65 years. Moreover, 4,872 (10.2%) were first-time donors, 12,125 (25.5%) were repeat blood donors, and 30,509 (64.2%) regular blood donors. Fifty-eight of the 47,506 blood donors were initially HBsAg positive, of whom 47 (0.09%) had a confirmatory test result. Furthermore, 0.94% (46 out of 4,872) of first-time blood donors and 0.008% (1 out of 12,125) of repeat blood donors had positive results. The difference in the prevalence rate of HBV between first-time and repeat donors was statistically significant ($p < 0.001$). First-time blood donors were also 8.6 times more likely to have a hepatitis B virus infection (OR: 9.6; 95% CI, 6.2 - 14.7, $p < 0.001$) (Table 1).

Characteristics of age, gender, and genotype

Of the 46 first-time blood donors with the confirmatory test result, 19 were available for re-sampling, of whom 11 (57.9%) were negative for nested-PCR and 8 (42.1%) were positive (Figure 2). The mean age of these 19 blood donors was 43 ± 5.3 years, including 17 males and two females. Genotyping was performed for eight donors who had a positive nested-PCR test. Seven blood donors had genotype D, and one blood donor had mixed genotypes A and D (Table 2).

Table 1. Prevalence of Hepatitis B virus in volunteer blood donors based on the type of donation.

Type of voluntary blood donors	Number (%)	Prevalence of positive confirmed HBV (95% CI)	p-value
First blood donors	4,872 (10.2)	0.94 (0.69 - 1.2)	< 0.001
Repeated blood donors	12,125 (25.5)	0.008 (-0.03 - 0.046)	
Regular blood donors	30,509 (64.3)		
Total	47,506 (100)	0.09 (0.07 - 0.13)	

Table 2. Characteristics of nested-PCR positive blood donors (HBV positive).

ID	Age (year)	Gender	ALT (IU/mL)	AST (IU/mL)	HBsAg	anti-HBc	Nested-PCR	Viral load (IU/mL)	Genotype	City
1	48	M *	12.5	7.54	positive	positive	positive	33	A, D	Gorgan
2	52	M	13.3	19.54	positive	positive	positive	4×10^4	D	Gorgan
3	49	M	24.41	16.65	positive	positive	positive	250	D	Gonbad
12	37	M	27.2	6.14	positive	positive	positive	265	D	Gorgan
13	38	M	31.4	12.4	positive	positive	positive	90	D	Gorgan
14	44	M	39.6	13.5	positive	positive	positive	98	D	Gonbad
18	36	M	37.5	15.6	positive	positive	positive	1.5×10^4	D	Gonbad
19	39	M	44	29.3	positive	positive	positive	50	D	Gorgan

* **M: - Male.**

Table 3. Drug resistance in genotype D of HBV.

Drug	Prediction	Scored mutations
Lamivudine	resistant	M204V
Adefovir	susceptible	none
Entecavir	partly resistant	M204V
Tenofovir	susceptible	none
Telbivudine	resistant	M204V

Table 4. Insertion mutations predicted for mixed genotype A and D.

Predicted genotype1:	Identical 96% D (D1)
Mutations RT domain:	H124N, Y135S, S176R, A194N/D, M204V, S213T, T222S, F227G, L228Q, L247G, N248H, V253G/V, G255D, C256M, Y257S, G258R, S259K, L260I, P261G, Q262A, H264G, I265 G
Mutations SHB protein:	T127P, A168G, R169P, G185[n.d.], I195M, S204R, P214A, I218M, F219D, F220S
Predicted genotype 2:	Identical 94% A (A2)
Mutations SHB protein	T133S, T114S, K122R, N131T, F134Y, A159G, Y161F, V168G, R169P, G185 N/D, A194V, I195M, S204R, V209M,1213L, P214A,1218M, F219D, F220S

Abbreviation: SHB - small hepatitis B Surface protein, RT - reverse transcriptase.

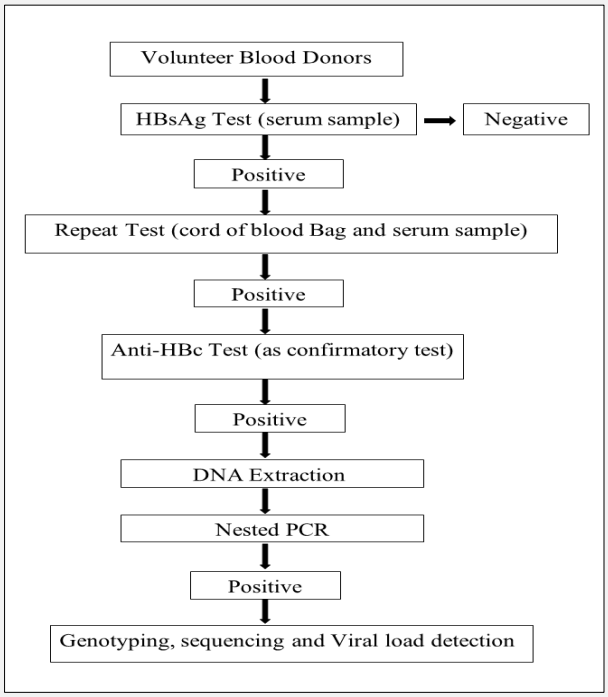

Figure 1. HBV screening algorithm in blood donors.

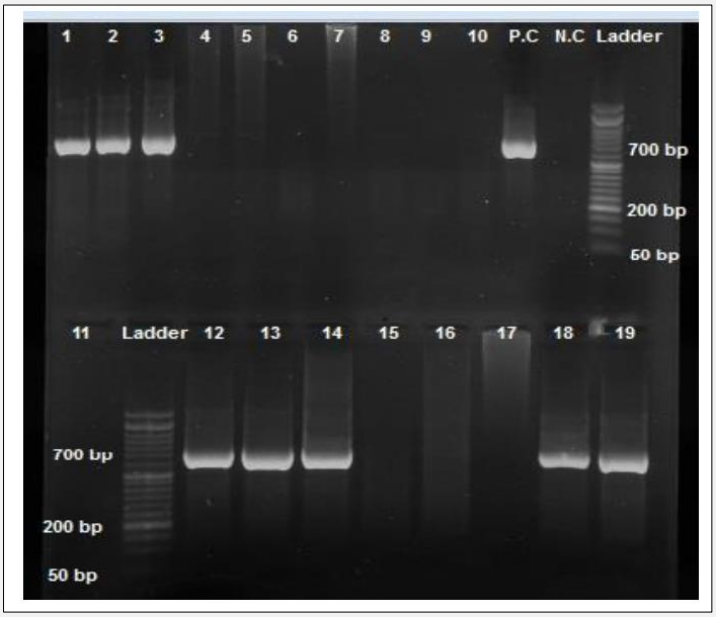

Figure 2. Nested PCR Test for HBV-confirmed serologic cases, 8 of the 19 first-time donors had positive Nested PCR (N.C: Negative control, P.C: Positive control).

Characteristics of ALT, AST, and viral load

For ALT, AST, and viral load, median and interquartile range (IQR), (IQR1 - IQR3) were as follows: ALT, 29.3 IU/mL (IQR, 16.07 - 39.07), AST, 14.5 IU/mL (IQR, 8.7 - 18.7) and viral load 174 IU/mL (IQR, 60 - 11,316). Viral load measurement in the nested-PCR positive group showed that six blood donors had less than 2,000 IU/mL, and two donors had more than 2 x 10^3 IU/mL. The lowest value was 33 IU/mL in mixed genotypes A and D. Also, the highest value was 4 x 10^4 IU/mL in genotype D. Overall, low, and high viral loads, along with normal ALT and AST enzymes were seen in 6 and 2 of blood donors, respectively (Table 2).

Genotype characteristics based on drug resistance and mutation

Due to M204V mutation in the HBV genotype D, drug resistance to lamivudine and telbivudine and partly to entecavir was seen in one HBV infected blood donor. However, it was susceptible to adefovir and tenofovir (Table 3).

Individual characteristics of mixed genotypes A and D

This mixed genotype A and D was detected in a 48-year-old man who was a farmer with a 2-year history of residence in Turkey. Genotype characteristics are as follows: genotype D (D1) subtype ayw, (Identical = 96%) and genotype A (A2) (Identical = 94%). Moreover, mutation of R169P, G185[n.d.], I195M, S204R, P214A, I218M, F219D, and F220S was common in both small hepatitis B surface (SHB) proteins of genotypes A and D (Table 4).

DISCUSSION

In this study, the prevalence of HBV among the studied blood donors was 0.09%. Various studies have investigated the prevalence of HBV among blood donors, including a comprehensive systematic review in the Middle East region. According to this study, the overall prevalence of HBV was 1.62%. In particular, it was 5.05%, 3.02%, 2.84%, 1.68%, and 1.58% in Yemen, Saudi Arabia, Pakistan, Turkey, and Egypt, respectively [15]. The prevalence of HBV among the general population was 1.8% in Iran and 5% in Golestan province [16]. Compared with previous studies, the prevalence of HBV among blood donors in this study is lower in the general population of Golestan and lesser than countries like Turkey and Egypt. Perhaps the more regular and repeat blood donors, as well as the orderly consultations and greater awareness of blood donors about TTIs, contributed to this difference. Also, in the present study, the prevalence of HBV was higher among first-time blood donors (0.94%) than regular and repeat blood donors (OR = 9.6, 95% CI = 6.2 - 14.7). Also, based on data extracted from a questionnaire given to HBV positive blood donors, they had received no vaccination against HBV and had no migration history (The only exception was a blood donor with mixed genotypes A and D who had a migration history to Turkey.) Various factors such as epidemiological changes, migration, and travel may contribute to this difference [17-19]. Additionally, awareness about TTIs, regular counseling for TTI prevention, and HBV vaccination resulted in a lower prevalence of HBV among regular and repeat blood donors compared to first-time blood donors [20]. The effectiveness of the HBV vaccine was 80% in the Iranian population and 89% in the north-eastern part of Iran [21]. As a result, factors such as high prevalence of HBV in the general population, donor awareness of HBV transmission routes, continuous consultation with blood donors, and implementation of HBV vaccine program may affect the prevalence of HBV in different regions and among blood donors in this study.

In this study, genotyping was performed by two methods, namely the Inno-Lipa kit and sequencing. The results showed that genotype D was the dominant genotype among blood donors. However, a mixed genotype A and D was also detected for the first time in this study, which was confirmed by sequencing. The sequence analysis performed using an online tool accessible at https://hbv.geno2pheno.org predicted two genotypes (Table 4). The major genotype in Iran is genotype D. One of the causes of different genotype reporting (mixed A and D) in this study may be traveling to areas where genotype A is more prevalent. Furthermore, different reports of mixed genotypes may be affected by the type of the method used, including hybridization, multiplex-nested PCR, whole-genome sequencing, and ultra-deep pyrosequencing [22,23]. The hybridization-based Innolipa kit is one of the most common kits for genotyping. The sensitivity of this kit is greater than 10 IU/mL [24]. Consequently, the use of methods that differ in the detection of the mixed genotype may result in different reports. In Iran, various studies have investigated the prevalence of HBV genotypes among blood donors based on genes of the virus, and the methods include genotype D (gene of S and RFLP method) in Tehran province, genotype D, sub-genotype D1 (gene: polymerase, method; nested-PCR) in South of Iran, and genotype D-sub genotype D1 (gene: S and C, method: sequencing) in the Persian Gulf region [25,26]. A comprehensive study in 29 provinces of Iran reported genotype D, sub-genotypes D1 (93.7%), D2 (2.8%), and D3 (3.5%), and the most prevalent serotypes in this study included ayw2 (93.3%), ayw3 (2.9%), and ayw4 (0.3%) [27]. Despite different methods for genotyping in Iran, genotype D is the prevailing genotype.

Moreover, in this study, the results of drug resistance and mutation prediction were as follows: The M204V mutation in the P gene was one of the most important predicted mutations. This mutation causes drug resistance to lamivudine, telbivudine, and entecavir. Furthermore, mutations of R169P, I195M G185, S204R, P214A, 1218M, F219D, F220S, were common in both genotypes A and D. Three mutations, L80V, L180M,

and M204V, were identified in the polymerase gene in patients with genotype D treated with lamivudine, which caused resistance to this drug [28]. Other drugs, such as entecavir has less drug resistance [29]. Consequently, the drug resistance prediction based on genotype may help select a suitable drug for the treatment of donors.

Retroviral-RT is an enzyme derived from the polymerase gene that is not capable of proofreading. Therefore, this property plays a role in the induction of different HBV strains. Also, because of the overlap between S and P genes, the mutation in the p gene may alter the S gene and thus induce the failure of HBsAg detection [30]. In this study, mutation of M240V in the P gene did not play a role in HBsAg detection. In Iranian blood donors, P gene mutations, including N248H, S106N, D131N, D111N, P1095, and T118N have been reported [13]. In our study, the N248H mutation was observed. Mutations in the SHB region cause the virus to escape from the immune system, and no response to the HBV vaccine is generated [31]. The major hydrophilic region (MHR) is a part of HBsAg that is identified by B lymphocyte cells. The second dominant domain of MHR is located between amino acids 124 and 147. This region is important in identifying the virus by diagnostic kits and the immune system. Thus, mutations in this region alter amino acids, leading to false negative results of virus identification tests and virus escape from the immune system [32,33]. In a comprehensive study on HBV positive blood donors in Iranian blood transfusion centers, the important mutations identified in MHR were as follows: G130S, T131N, P120T, 129P, S144T, M133L, D144E S143P [27]. In another study among the general population of Golestan province, mutations P120S, P120T, R122K, M133I, T140I, S143L, and G145R were associated with immunotherapy escape, failure in HBsAg detection, and vaccine escape [34]. In this study, the mutations predicted in MHR (amino acids 124 - 147) included the following: T127P, T133S, N131T, and F134Y, and these mutations were not related to three escape mutants.

The final finding of this study was to determine the viral load as well as ALT and AST enzymes. Eleven HBsAg/anti-HBc positive and nested-PCR negative blood donors were considered as inactive carriers. In six donors, normal ALT and AST tests with low viral load (< 2,000 IU/mL) may indicate chronic hepatitis. In two (18.2%) blood donors, viral load was greater than 2,000 IU/mL. Consequently, the measurement of viral load in asymptomatic blood donors with normal ALT and AST enzymes showed that donors with high viral load may have more of a role in HBV transmission to others.

There were some limitations in this study. The first is related to the type of study which was cross-sectional, and this was because it was impossible to design a prospective cohort study. No access to all HBV positive blood donors was the second limitation of the present study.

CONCLUSION

The results showed that the risk of HBV transmission is higher among first-time blood donors. Also, the overall prevalence of HBV among blood donors in northeastern Iran is lower than that in the general population of Iran. It is recommended that the use of pre-donation laboratory screening in first-time blood donors be used in order to improve the safety of the donated blood in this region. Also, the use of genotyping and, subsequently, drug resistance prediction for all HBV positive blood donors may play a role in improving and preventing the transmission of the virus to others.

Ethics Approval:

The study was approved by the Ethics Committee of the High Institute for Research and Education in Transfusion Medicine, Iranian Blood Transfusion Organization, and informed consent was obtained from the enrolled blood donors.

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Declaration of Interest:

The authors declare no conflicts of interest.

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