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MOLECULAR GENETIC CHARACTERIZATION OF HEPATITIS B VIRUS IN BLOOD DONORS FROM SOUTH VIETNAM

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Abstract. The problem of transfusion safety preventing parenteral viral hepatitis transmission remains relevant. Viral hepatitis B (HB) is the most common viral infection transmitted through transfusion procedures. One of the natural phases of a chronic viral hepatitis B (CHB) course is occult hepatitis B infection (OBI) characterized by undetectable HBsAg level (regardless of other serological marker levels) along with detected hepatic HBV DNA as well as blood viral load ranging from extremely low to undetectable. In Vietnam, prevention of transfusion-based HBV transmission is focused on donor screening; it is still based solely on HBsAg serology. As such, OBI remains a potential threat to blood transfusion safety. Assessing hepatitis B virus (HBV) DNA is a reliable preventive measure against HBV transmission from HBsAg– donors, especially in highly endemic regions. The aim of our work was HBV identification and molecular genetic characterization in blood donors from South Vietnam. The study material was presented by 500 donor serum samples. Subjects were examined for HBV markers with qualitative detection of HBsAg, HBs IgG, and HBcore IgG. Amplification and subsequent HBV sequencing were performed using nested PCR with overlapping primer pairs jointly flanking the complete HBV genome (S, P, C, X genes). Full-size HBV genome nucleotide sequences were obtained for 58 samples. Among blood donors, taking into account HBsAg+ and HBsAg– samples, HBV DNA was detected in 11.6%, including 8.6% OBI. HBV phylogenetic analysis showed genotypes B and C. Vaccine escape mutations and mutations that contribute to disease progression were identified. Current screening in Vietnam is insufficient for eliminating the risk of transfusion-transmitted HBV infection. The major risk factor is OBI. PCR testing for HBV should be considered for blood donor screening.

Key words: *hepatitis B virus (HBV), hepatitis B viral markers, HBsAg-negative hepatitis B, genotypes, clinically significant mutations, blood donors, infection safety, South Vietnam.*

МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКАЯ ХАРАКТЕРИСТИКА ВИРУСА ГЕПАТИТА В У ДОНОРОВ КРОВИ ИЗ ЮЖНОГО ВЬЕТНАМА

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Резюме. Проблема инфекционной безопасности переливания крови с целью предупреждения передачи вирусов гепатитов является актуальной медицинской проблемой. Вирусный гепатит В (ГВ) остается наиболее

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распространенной вирусной инфекцией, передающейся при переливании крови. Одной из естественных фаз течения хронического вирусного гепатита В (ХГВ) является скрытый гепатит В (СкГВ), характеризующийся неопределяемым уровнем HBsAg (независимо от содержания других серологических маркеров) при наличии ДНК вируса в ткани печени, а также вирусной нагрузкой в крови, варьирующей от крайне низкой до необнаруживаемой. Вьетнаме профилактика трансфузионной передачи вируса гепатита В (ВГВ) сосредоточена на скрининге доноров, который по-прежнему основан только на выявлении HBsAg. Таким образом, СкГВ остается потенциальной угрозой безопасности переливания крови. Определение ДНК ВГВ является надежной профилактической мерой против передачи вируса от доноров с HBsAg– ГВ, особенно в высокоэндемичных регионах. Целью нашей работы была идентификация и молекулярно-генетическая характеристика ВГВ у доноров крови из Южного Вьетнама. Материалом для исследования послужили 500 образцов плазмы крови, полученных от доноров. Субъекты были обследованы на наличие серологических маркеров ГВ с качественным определением HBsAg, антител анти-HBs IgG и анти-HBcore IgG, а также молекулярно-биологического маркера — ДНК ВГВ. Амплификацию и последующее секвенирование генома ВГВ проводили с помощью гнездовой ПЦР с перекрывающимися парами праймеров, совместно фланкирующих полный геном HBV (гены S, P, C, X). Полные нуклеотидные последовательности генома HBV были получены для 58 образцов. Среди доноров крови, с учетом HBsAg+ и HBsAg– образцов, ДНК ВГВ была обнаружена в 11,6%, в том числе в 8,6% случаев СкГВ. Филогенетический анализ ВГВ показал наличие генотипов B и C. Были выявлены мутации, приводящие к иммунологическому ускользанию, и мутации, способствующие прогрессированию заболевания. Проводимый в настоящее время во Вьетнаме скрининг недостаточен для устранения риска трансфузионной передачи ВГВ-инфекции. Основным фактором риска является СкГВ, в связи с чем высокочувствительное ПЦР-тестирование на ВГВ следует рассматривать как дополнительный приоритетный метод для скрининга доноров крови.

Ключевые слова: *вирус гепатита В (ВГВ), маркеры вирусного гепатита В, HBsAg-негативный гепатит В, генотипы, клинически значимые мутации, доноры крови, инфекционная безопасность, Южный Вьетнам.*

Introduction

One of the most common factors associated with liver disease is the hepatitis B virus (HBV). HBV transmission routes are associated with contact of mucous membranes with contaminated blood or other body fluids [38]. The incidence of chronic viral hepatitis B (CHB) is inversely proportional to the age of patients. Chronicity with infection under the age of 5 years exceeds 90%, whereas infection in adults leads to CHB in only 5% of cases. Approximately 20–30% of CHB patients develop cirrhosis and liver cancer. To date, about two billion people in the world have been infect-ed with HBV and, according to various sources, from 290 to 360 million of them are sick with CHB. Moreover, only about 30.4 million patients are aware of their disease [37]. One natural form of CHB is occult hepatitis B infection (OBI) in which HBsAg is not detected in patient peripheral blood. However, the virus persists as a covalently closed circular DNA in hepatocytes. Therefore, HBV DNA is detected in the liver tissues and/or in the blood. Detection of OBI in blood is complicated by the extremely low viral load and limited sensitivity of diagnostic kits [27]. With OBI, the level of HBsAg in patient blood plasma is insignificant, and the viral load does not exceed 200 IU/ml (in most cases ≤ 25 IU/ml). As such, OBI prevalence values determined by different research teams vary. They depend on HBV prevalence in the study population as a whole, the HBV vaccination program in the region, characteristics of the surveyed groups, risk factors, the sensitivity of the methods used, and single or multiple collection of samples [28].

The clinical significance of OBI remains under debate. On the one hand, certain conditions (a low viral load undetectable by routine diagnostic kits, absence of HBsAg in the blood) is precisely the outcome that is sought in the treatment of CHB. On the other hand, OBI is a risk factor for accelerated progression of cirrhosis and hepatocellular carcinoma (HCC) in chronic viral hepatitis C (CHC) and other liver diseases of various etiology [18]. An increased risk of developing HCC in patients with OBI without other concomitant liver diseases has also been shown [30]. In addition, HBsAg-negative CHB leads to a high likelihood of viral reactivation during immunosuppression. Thus, reactivation was shown in almost 40% of patients receiving immunosuppressive therapy and/or chemotherapy for oncology and other diseases [9]. In this light, blood donors deserve special attention since the transfusion of blood and its products is a significant therapeutic strategy in various severe conditions; a donor with OBI can become a source of infection for recipients [6].

Since the infectious dose of HBV is approximately 3.5 IU/ml, highly specific and sensitive tests (lower detection limit 2–4 IU/ml) must be used to detect viral DNA in blood donors. In addition, it is desirable not to use minipools in diagnostics as they significantly reduce analytic sensitivity [7]. However, standardized controlled diagnostic meth-

ods do not currently exist, and general recommendations imply the use of PCR variations (nested-PCR, droplet digital PCR) aimed at amplifying at least two different HBV genomic regions. It is important that the analysis be equally effective for different viral genotypes and subtypes [27]. Underdiagnosis of HBV in risk groups and groups potentially associated with the viral spread (primarily blood donors) remains a serious obstacle to the elimination of viral hepatitis B as a threat to public health [38]. It is extremely important to determine OBI prevalence in global regions among healthy blood donors in order to assess the likelihood of HBV transmission through transfusion and to assess the need to modify donor selection strategies in relation to risk reduction. Subsequent genotyping of detected strains and identification of clinically significant mutations can serve as an important epidemiological tool for studying the ways in which the virus spreads.

One of the countries with the highest mortality from liver disease is the Socialist Re-public of Vietnam (Vietnam). In this case, the main disease is hepatocellular carcinoma, caused by viral hepatitis [12]. The prevalence of CHB in patients with liver disease reaches 47% [16]. Prevention of HBV infection in Vietnam is based on the Newborn Universal Vaccination Program, as well as screening of blood donors for the presence of HBsAg. However, vaccination coverage rates have fluctuated in recent years, and detection of HBsAg is not sufficient to detect all cases of infection in blood donors [14]. The estimated CHB prevalence in Vietnam in 2019, depending on region, ranged from 8% to 25%, but these calculations are based on a limited number of studies. In addition, most studies assessing CHB prevalence in specific groups were based on detection of HBsAg and/or anti-HBcore IgG. The use of molecular genetic methods is limited not only in research, but also in routine laboratory diagnostics. The ability to detect infections with low viral loads is available only in central laboratories of large cities, or not available at all. Few works devoted to assessing OBI prevalence in the region use anti-HBcore IgG antibodies as a disease marker. Moreover, an analysis of cases with anti-HBcore antibodies only (which can be considered a surrogate marker of OBI in high-risk groups) is presented only in one study; it reported an extremely high (39.7%) level of this indicator [14]. Another research team, finding antibodies to HBcore in 50% of 110 HBsAg– samples in the absence HBV DNA, considered this to be evidence of a significant number of resolved HBV infections. This, however, may be explained by the relatively high HBV DNA detection limit of the method used (> 300 IU/ml) [13].

Thus, information on HBV prevalence among blood donors in Vietnam is limited in the literature, and information on the prevalence of HBsAg– disease in this group is completely absent.

The aim of this work was identification and molecular genetic characterization of HBV among blood donors in the South Vietnam.

Materials and methods

The study was approved by the Ethics Committee of the Saint Petersburg Pasteur Institute. The study material was 500 blood plasma samples collected from blood donors in South Vietnam. All those examined denied an anamnesis of HBV infection. As part of the study, serological and molecular biological markers of CHB (HBsAg, anti-HBs IgG antibodies, anti-HBcore IgG, HBV DNA) were determined as described earlier [2]. Detection of HBV DNA in HBsAg– individuals was carried out using a technique that allows detection of the virus at a load of 3 IU/ml with a 500 µl volume of extracted material [3]. As recommended by Taormina Workshop on Occult HBV Infection Faculty Members, when the virus was detected, nested PCR was applied using a set of primers co-flanking the full viral genome, as shown previously [27, 1].

The primary analysis of the obtained fragments was performed using the BLAST algorithm (http://www.ncbi.nlm.nih.qov/BLAST) on the nucleotide sequences provided in the GenBank sequence database. The resulting sequences were aligned in the MEGAv.11 program using the ClustalW algorithm. The phylogenetic tree was constructed using the neighbor-joining method; the significance of the tree was assessed using bootstrap analysis with 1000 replicates. The nucleotide sequences obtained were submitted to the HBVseq (https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html), HBVdb (https://hbvdb.ibcp.fr/HBVdb/) and Genafor (https://hbv.geno2pheno.org) databases to search for possible mutations. The amino acid sequence of the proteins was determined by translating the corresponding nucleotide sequence according to the open reading frame. The serotypes of the identified isolates, which characterized their antigenic specificity, were identified using the analysis of the nucleotide sequence of the conserved region of HBsAg α-determinant.

Statistical data processing was carried out using the Excel (Microsoft Corp.) and Prizm 5.0 (GraphPad Software, Inc.) software packages. The "exact" Clopper–Pearson interval was used to estimate statistical uncertainty. Results are represented as a median (Me) indicating 95% confidence interval (95% CI). Depending on sample characteristics, the Fisher exact test or Yates-corrected Chi-Squared test was used to evaluate the statistical significance of numeric data obtained during paired comparison. A probability value of $p < 0.05$ was taken as the statistical significance threshold.

Results

The ages of the examined blood donors ranged from 18 to 64 years; the median age was 36.8 years. The number of men in the group exceeded the number of women: 59.2% and 40.8%, respectively. The mean age among women was 36.3 years. For men, it was 37.1 years. The sample distribution by age group and sex is shown in Fig. 1.

When evaluating HBV serological markers, prevalence among blood donors was deter-mined to be 65.0% (95% CI: 60.64–69.18%). However, HBsAg was detected in only 3.00% (95% CI: 1.69–4.9%) of individuals. The HBV serological marker prevalence and distribution in blood donors are shown in Table 1.

Women represented 20% of HBsAg+ in the study group; the remaining 80% were men. HBsAg prevalence in men (4.05%; 95% CI: 2.11–6.97%) exceeded that in women (1.47%; 95% CI: 0.03–4.24%). However, there were no significant differences.

The prevalence of HBV DNA with a viral load above 200 IU/mL was 3.4% (95% CI: 1.99–5.39%), broadly consistent with the prevalence of HBsAg. An additional 43 HBsAg– blood donors were found to have viral DNA with a viral load of less than 25 IU/mL. Thus, OBI prevalence was 8.6% (95% CI: 6.29–11.41%). The prevalence of HBV DNA in the study group was 11.6% (95% CI: 8.93–14.74%). Most of the HBV samples were obtained from men, 81.03% (95% CI: 68.6–90.13%). The prevalence of viral DNA among men (15.88%; 95% CI: 11.91–20.55%) significantly exceeded that in women (5.39%; 95% CI: 2.72–9.44%): $\chi^2 = 11.948$, RR = 2.945, p = 0.0005, df = 1.

Phylogenetic analysis of HBV obtained from HBsAg+ individuals revealed a pre-dominance of the B genotype (80%; 95% CI: 51.91–95.67%) over the C genotype (20%; 95% CI: 4.33–48.09%). However, analysis of all HBV samples showed a smaller difference in the group between the B genotype (63.79%; 95% CI: 50.12–76.01%) and the C genotype (36.21%; 95% CI: 23.99–49.88%). With regard to subtype prevalence, the results were as follows: 56.9% B4; 18.97% C1; 6.9% B2; 8.62% C2; 5.17% C3; and 3.45% C5. The nucleotide sequences of the complete HBV genomes (genotype B) examined in this work were submitted in the GenBank database (OP796800 — OP796836). The HBV subtype distribution depending on HBsAg detection is shown in Fig. 2.

Based on analysis of the conserved nucleotide sequence region of the HBsAg "a" determinant the viral serotypes characterizing antigenic specificity were determined (Table 2).

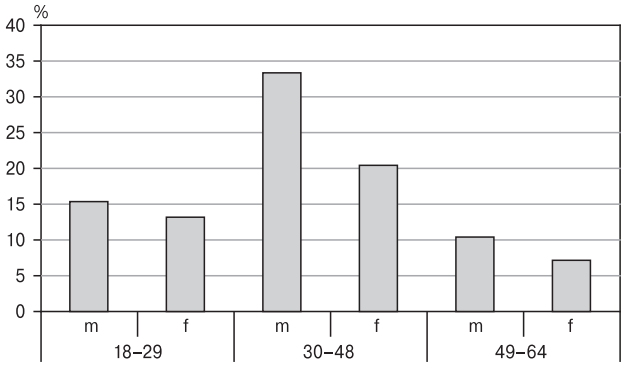

Figure 1. Age group and sex sample distribution
Note. M — male; F — female.

Table 1. HBV serological markers prevalence and distribution in blood donors (HBsAg, HBcore IgG, HBs IgG)

HBV serological marker prevalence	Number in the overall group (n = 500), share of the group, 95% Confidence Interval
HBsAg+	15 (3.00%, CI: 1.69–4.9%)
HBs IgG+	284 (56.8%, CI: 52.33–61.19%)
HBcore IgG+	149 (29.8%, CI: 25.82–34.02%)
Seronegative	175 (35.0%, CI: 30.82–39.36%)
HBV serological profile distribution	
HBsAg+	7 (1.4%, CI: 0.56–2.86%)
HBsAg+, HBcore IgG+	8 (1.6%, CI: 0.69–3.13%)
HBcore IgG+, HBs IgG+	115 (23.00%, CI: 19.38–26.94%)
HBcore IgG+ isolated	26 (5.2%, CI: 3.42–7.53%)
HBs IgG+ isolated	169 (33.8%, CI: 29.66–38.13%)

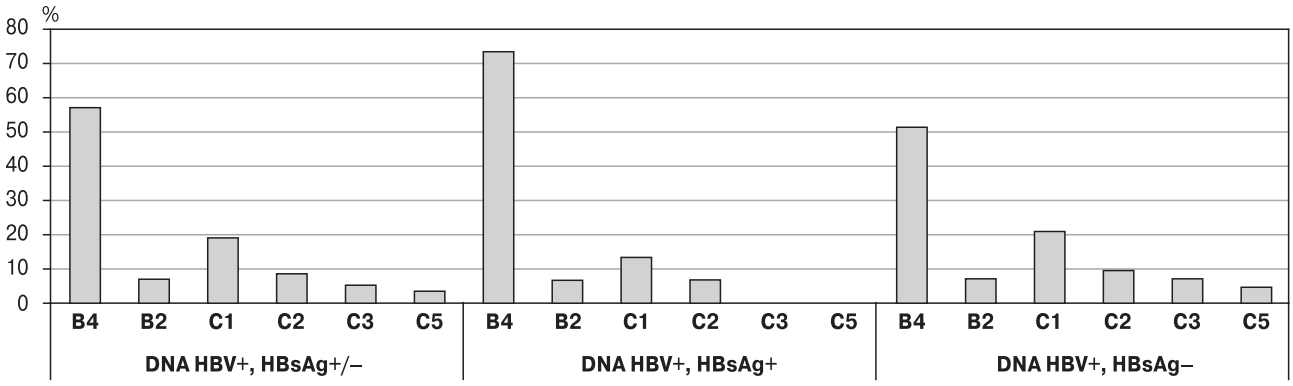

Figure 2. Distribution of HBV genotypes among HBsAg-positive and negative blood donor samples

Table 2. Distribution of hepatitis B subtypes and serotypes, according to the determinant "a" nucleotide sequence

Isolate	Subtype	Nucleotide sequence	Serotype
Viet_ocHBV_bd9	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd25	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd49	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd103	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd122	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd135	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd141	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd159	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd178	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd181	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd187	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd198	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd237	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd242	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd248	B4	AGAAAAACTGCAACA	ayw3
Viet_HBV_bd253	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd266	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd268	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd277	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd285	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd294	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd310	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd324	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd350	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd353	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd383	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd385	B4	AGAAAACCTGCATCA	ayw1
Viet_HBV_bd426	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd456	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd471	B4	AGAAAACCTGCATCA	ayw1
Viet_ocHBV_bd481	B4	AGAAAACCTGCAACA	ayw1
Viet_HBV_bd491	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd498	B4	AGAAAACCTGCAACA	ayw1
Viet_ocHBV_bd154	B2	AAAAAACCTGCAACA	adw2
Viet_ocHBV_bd259	B2	AAAAAACCTGCAACA	adw2
Viet_HBV_bd327	B2	AAAAAACCTGCAACA	adw2
Viet_ocHBV_bd495	B2	AAAAAACCTGCAACA	adw2
Viet_ocHBV_bd3	C1	AAGAAACCTGCAACA	adw2
Viet_HBV_bd27	C1	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd90	C1	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd117	C1	AAGAAACCTGCAACA	adw2
Viet_HBV_bd201	C1	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd282	C1	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd287	C1	AAGAAACCTGCAACA	adw2
Viet_ocHBV_bd407	C1	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd418	C1	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd475	C1	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd493	C1	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd28	C2	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd87	C2	AAGAGACCTGCAACA	adr
Viet_HBV_bd129	C2	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd256	C2	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd322	C2	AAGAGACCTGCAACA	adr

Isolate	Subtype	Nucleotide sequence	Serotype
Viet_ocHBV_bd167	C3	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd371	C3	AAGAGACCTGTAACA	adr
Viet_ocHBV_bd427	C3	AAGAGACCTGCAACA	adr
Viet_ocHBV_bd60	C5	AAGAAACCTGCAACA	adw2
Viet_ocHBV_bd299	C5	AAGAAACCTGCAACA	adw2

Table 3. The most common clinically significant mutations identified in the examined blood donor group

HBV genome region	Mutation	Prevalence in overall group	Genotype; prevalence in genotype	Description
RT	S204L	1.72%	C1 (9.09%)	The mutation is not described. However, aa change at the position can be considered a possible mutation conferring resistance to lamivudine, telbivudine, and entecavir.
RT	V207M	12.07%	B4 (21.21%)	AA change at given position is a mutation conferring resistance to lamivudine.
MHR	Y100F	1.72%	C3 (33.33%)	Associated with HBsAg-negative CHB.
MHR	Q101K/H	6.9%	C1 (18.18%), C3 (66.67%)	Associated with HBsAg-negative CHB or genotype-specific polymorphism.
MHR	L109F	1.72%	B2 (25%)	Associated with HBsAg-negative CHB.
MHR	I110L	10.34%	B2 (25%), C1 (27.27%), C5 (100%)	Associated with HBsAg-negative CHB.
MHR	S114P	5.17%	B4 (9.09%)	Associated with HBsAg-negative CHB.
MHR	K122R	56.9%	B4 (100%)	Associated with HBsAg-negative CHB or genotype-specific polymorphism.
MHR	C124S/W	6.9%	B4 (6.06%), C1 (9.09%), C2 (20%)	Associated with HBsAg-negative CHB. Escape mutant (immune escape, vaccine escape, diagnostic escape). MHR is the most important antigenic determinant in envelope proteins and is composed of two loops bounded by disulfide bridges that are broken due to mutations in this genomic region. Substitutions in these regions can lead to a change in the three-dimensional conformation of the extravirion loop compared to wild-type strains. Some amino acid substitutions lead to surface antigen production differences.
MHR	I126T/N	18.97%	C1 (63.64%), C2 (40%), C5 (100%)	
MHR	P127T	1.72%	B4 (3.03%)	
MHR	Q129P	3.45%	B4 (6.06%)	
MHR	M133I/L	5.17%	B4 (9.09%)	
MHR	C139S	1.72%	B2 (25%)	
MHR	T140S	3.45%	B4 (6.06%)	
MHR	K141R	3.45%	B2 (50%)	
MHR	T143M	15.52%	B4 (27.27%)	
MHR	V184A	3.45%	B4 (100%)	Associated with increased risk of HCC.
PreCore	T16I	3.45%	B4 (6.06%)	Suspected of being associated with severe disease in HBsAg-negative patients.
PreCore	L27P	13.79%	B4 (24.24%)	Suspected of being associated with severe disease in HBsAg-negative patients.
PreCore	W28* (G1896A)	15.52%	B4 (15.15%), B2 (25%), C2 (60%)	Negatively affects HBeAg production. Creates a stop codon (preventing HBeAg synthesis). Responsible for more than 90% of defective HBeAg secretion, affecting HBeAg serostatus.
PreCore	W28S/C	12.07%	B4 (12.12%), C1 (27.27%)	May be a transitional mutation to W28*.
Core	E113D	1.72%	C2 (20%)	Changes between aa 113 and 143 influence the antigenicity and stability of the particle. May create immune escape mutants leading to chronic viral persistence and severe liver disease. Located within B-cell epitopes, and is associated with disease progression, cirrhosis, and hepatocellular carcinoma development.
Core	T128A	3.45%	B4 (6.06%)	
Core	P130T/L/S	27.59%	B4 (24.24%), B2 (25%), C1 (45.45%), C2 (40%)	
Core	P134Q	1.72%	C1 (9.09%)	
Core	P135Q/A	17.24%	B4 (24.24%), B2 (50%)	
Core	A137G	1.72%	B2 (25%)	

The donor group had high HBV amino acid variability in the PreCore, Core, and SHB regions. Mutation V207M, which is the cause of resistance to lamivudine, was identified in 12.07% (95% CI: 4.99–23.3%) of donors. Another amino acid substitution detected (S204L) may also be associated with resistance (lamivudine, entecavir, telbivudine). The most common clinically significant mutations present in the examined blood donor group are shown in Table 3.

Discussion

Studies on the prevalence of OBI among blood donors have long been scarce, but in recent years there have been more and more publications on the problem. The prevalence of this CHB form among blood donors in different countries varies depending on the HBsAg prevalence in the geographic region. Thus, among blood donors in Argentina, HBV was detected in only 0.06% samples, of which only four were HBsAg–. In contrast, OBI prevalence was 10.90% and 17.00% in the Lao People's Democratic Republic and Nigeria, respectively [26, 19]. Furthermore, reported OBI prevalence among donors often fluctuates within the same region, and the results of different research teams are contradictory. For example, in Iran, the prevalence values of HBsAg in the population and in blood donors are 2.60% and 0.40%, respectively, while OBI prevalence among donors exceeds 4.0% [4, 31, 35]. Thus, the data generally vary not only by region, but also by virus detection methods, which include a variety of commercial diagnostic kits for HBsAg and HBV DNA.

The prevalence values for HBsAg, anti-HBs IgG, and anti-HBcore IgG in the study group were 3.0%, 56.8%, and 29.8%, respectively. Thus, the detection of IgG antibodies to HBcore and HBs indicates contact with the virus in at least 29.8% of the examined blood donors, with markers present in 65%. Interestingly, the serological marker prevalence among blood donors that we found differs from that among adults in Vietnam generally. Thus, in Binh Thuan province, with a similar level of anti-HBs IgG prevalence (60.3%) to our data, the prevalence of anti-HBcore IgG was 71.7%; this is more than twice as high as the results obtained [12]. The same high prevalence of anti-HBcore IgG (68.2%) was shown earlier in Thai Binh province [24]. The reason for the differences with our results may be both a greater prevalence and risk of HBV infection in rural areas, alongside different factors in the blood donors we examined (lower risk of infection, higher standard of living and health). An indirect confirmation of this may be the significantly lower prevalence of HBsAg among blood donors compared with adult populations studied in Binh Thuan (15.3%) and in Thai Binh (19.0%) provinces [12, 24].

The detection of HBsAg in 3.0% of blood donors is significantly lower than the prevalence of this marker among apparently healthy people (12.3%) in Ho Chi Minh City [2]. However, it is approximately twice the prevalence this marker in blood donor cohorts in Vietnam. Despite the fact that until 2011 the prevalence of HBV among donors was practically the same as the prevalence in the general population, in the last decade it fell to 1.44% [15]. We assume that such a contradiction may be due to the high sensitivity (0.01 IU/mL) of the diagnostic kit used in this work. Note that HBsAg prevalence in certain risk groups, for example blood recipients, was 8–10% [15]. Apparently, HBV-infected blood donors with low blood levels of HBsAg, as well as HBsAg–, can serve as a source of infection. An indirect confirmation of this assumption is the high OBI prevalence (8.6%) in the study group.

Among the blood donors examined in this study, HBV DNA prevalence was 11.6%, which exceeds the figures presented by other research groups. We suggest that the most likely reason for this is the use a method in our study that allows the determination of viral DNA at low viral loads. The revealed higher prevalence of HBV DNA in men (15.88%) compared to women (5.39%) may be due to cultural features and freer conduct of men. OBI prevalence in different countries and global regions varies, but generally correlates with the prevalence of HBsAg+ CHB. In addition, in endemic countries, CHB (including OBI) is most common among low-income populations [32]. The high OBI prevalence revealed in this study is typical for regions highly endemic for HBV. This study included residents of the capital with a predominantly favorable socio-economic situation. As such, it can be assumed that the OBI prevalence among blood donors in rural areas will be higher.

As is known, the high mutation rate of HBV has led to the fact that the virus is currently divided into ten genotypes. One of them (genotype J) is phylogenetically positioned between the hepatitis B viruses of humans and monkeys. Nine others (genotypes A-I) differ from each other in nucleotide sequence by more than 8.0%. In addition, within a number of genotypes, subtypes have been described that differ by 4–7.5%. Each geographic region has its own HBV genotypic/subgenotypic profile. In most regions, one or two main genotypes and a limited number of subtypes circulate. This makes it possible to extract additional epidemiological information from data on prevalence and changes in viral genetic variants in a particular region and group [29]. At the same time, the genotypic/subgenotypic viral profile of a population can gradually change due to labor and tourist migration, as well as due to the use of prophylactic agents in some countries (or absence in others). For example, HBV vaccination is not a universal preventive measure, and screening

of blood donors in countries with different income levels differs in the laboratory diagnostic methods used [29].

According to a number of studies, genotypes and subtypes in HBsAg– CHB in a particular region, in most cases, correlate with those in the HBsAg+ form of the disease [15, 20]. It was shown that genotype B (71.43%) prevailed among patients with CHB in Vietnam compared to genotype C (27.55%) [33]. A similar prevalence of HBV genotypes was shown by us for HBsAg+ individuals: 80% B and 20% C. However, in a recent study of HBV genotypes in HCC patients in Vietnam, genotype B was detected in only 57.9% of patients and genotype C in 42.1% [17]. These results are close to those obtained by us for the entire cohort of blood donors (63.79% B, 36.21% C) and are almost identical to the results we obtained for HBsAg– individuals (58.14% B, 41.86% C).

Interestingly, even more differences have been noted in the distribution of HBV subtypes. Among patients with CHB, HBV subtype B4 prevailed (66.33%), followed by subtype C1 (26.53%), B2 (5.1%), C2 (1.02%), and C3 (1.02%) [33]. At the same time, only two subtypes were present in HCC patients. The frequencies in the HCC group were: B4 in 57.9% of patients; and C1 in 42.1% of patients [17]. Our results are similar regarding B4 frequency in HCC patients. Among the examined blood donors, B4 was present in 56.9% of cases. However, the B2 subtype was also detect-ed (6.9%). This is similar to the frequency of this subtype in the population [33]. The C genotype subtypes we have identified differ in diversity compared to the previously presented data. The C1 subtype predominates (18.97% of patients), but the subtypes C2 (8.62%), C3 (5.17%), and C5 (3.45%) are also present.

When comparing the distribution of viral subtypes among HBsAg+ and HBsAg– samples from blood donors, there was no difference between groups. However, among individuals with OBI, a greater diversity of viral subtypes was seen. The greater diversity in our group of genotype C subtypes among OBIs (those with low viral load), is particularly interesting because genotype C in Vietnam is characterized by higher viral loads and more severe liver disease than genotype B [34]. However, the aforementioned predominance of genotype B in HCC patients compared to genotype C [17] casts doubt on these data. It is likely that insufficient methods (which do not permit detection of HBsAg– CHB) have limited the possibility of detecting cases of HBV genotype C when viral loads are not high. An indirect confirmation of this assumption is the higher frequency of the C genotype and diversity of subtype C genotypes in neighboring countries.

In Cambodia and Thailand, genotype C prevailed: 80.49% and 81.3%, respectively. Genotype B has been found in regions bordering Vietnam [21, 8]. The diversity of subtypes and recombinant forms of HBV in Laos, including such rare variants as B1, C5, I1, and I2 [5], may indicate a potential diversity HBV in regions of Vietnam, as evidenced by the high nucleotide sequence identity between some of the OBI cases we identified and samples from neighboring countries. However, we did not find B1, I1, or I2 genotypes among blood donors. Attention is drawn to HBV genotype C5, which is characteristic of Southeast Asia, but previously noted in Vietnam in only isolated cases [13]. The identification this subtype in such a limited OBI sample suggests a higher prevalence of HBV C5 in Vietnam than previously thought. Further OBI research in this geographic region is needed to confirm or refute this assumption. However, indirect confirmation is the fact that we previously detected HBV C5 among OBIs in HIV-infected individuals [2].

A consequence of high HBV variability is a variety of clinically significant viral mutations, including amino acid substitutions in the reverse transcriptase domain, which are defined as drug resistance mutations. Among the examined blood donors, mutations were found at two positions (204, 207) associated with resistance to antiretroviral drugs. The high prevalence of the V207M mutation (associated with lamivudine resistance) may be-come a public health problem in the future [10]. It should be noted that the V207M mutation was the most common (38%) among children with CHB and HBV genotype B, including those who had not previously received treatment [25]. Apparently, this mutation is characteristic of genotype B and is currently widespread in Vietnam.

Of particular interest are mutations in the MHR of HBsAg associated with the ability of the virus to elude neutralizing antibodies. Several dozen such mutations have been described so far [11]. The most common amino acid substitutions associated with vaccine/diagnostic escape and OBI include mutations at positions 123, 126, 129, 130, 133, 144, 145, and 181 [22, 36]. In this study, ten immune-associated escape mutations were identified among blood donors, with most of them leading to vaccine escape (Table 3). Some of these mutations are associated with failed diagnostics, and some with vaccine escape, leading to the possibility of infection of vaccinated people. Given the identification of such strains among blood donors, the problem of HBV spread in the region may be more global than it appears.

Indirect confirmation of this is the prevalence of escape mutations (8.1%) among patients with HCC [17]. Presumably, this may be due to high variability of determinant “a” in CHB patients with simultaneously detected HBsAg+ and anti-HBs IgG+, while the detection of anti-HBs IgG+ without HBsAg in most cases indicates the development of immunity against HBV [23]. The mechanism of this seems to be connected with the fact that amino acid substitutions

at positions 124–137 are able to influence natural and vaccine-induced antibodies to HBsAg.

Another widespread mutation in the blood donor group was a substitution in the PreCore region at position 1896. The prevalence of the W28* (G1896A) mutation, contributing to disease progression and HCC development, was 15.52%. In addition, W28S/C amino acid substitutions were found in 12.07% of individuals. We hypothesize that the W28S/C mutations are transitional mutations which may later become a stop codon W28*.

In blood donor samples, it is a serious challenge to identify HBV strains with: undetectable HBsAg; extremely low viral load; mutations that hinder vaccine-induced anti-bodies; drug resistance mutations; and/or mutations associated with disease development/progression. Such complex mutants presumably have an increased ability to over-come genetic barriers, thereby leading to the spread of drug-resistant viral variants in the population, despite universal vaccination.

Conclusion

Our work shows a high prevalence of CHB markers among blood donors in South Vietnam. The high incidence of OBI among blood donors indicates not only widespread HBsAg– forms of the disease in the population, but also surveillance shortcomings. The insufficiency of generally accepted analytic methods and/or inadequate sensitivity of di-agnostic tests to detect CHB, requiring further attention and effective measures to ensure safe blood transfusion. The revealed hypervariability of the viral genome, multiple escape mutations, and mutations that contribute to disease progression, make it necessary to study distinguishing features of the pathogen and the host immune response in OBI. Highly sensitive PCR testing for CHB should be considered for blood donor screening in Vietnam.

Conflict of interest

All authors declare no conflict of interest.

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