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Molecular and genetic characterization of the hepatitis B virus full-length genome sequences identified in HBsAg-negative blood donors in Ural Federal District

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Abstract

Introduction. The World Health Organization estimates that as of 2019, more than 296 million people were living with chronic hepatitis B virus (HBV) infection. The prevalence of HBsAg-negative, occult form of the disease in blood donors varies depending on the region of the world and the sensitivity of the methods of analysis used. Considering that the genetic diversity of viruses demonstrates space and time variations and taking into account that the genetic profile of isolates in key groups, which may turn into a source of the pathogen spread, is important for forecasting of the epidemiological situation, the attention should be given to identification of HBV genotypes currently circulating among regular blood donors in regions of the Russian Federation.

The **aim** of this work was molecular and genetic characterization of HBV genomes identified in HBsAg-negative blood donors in the Ural Federal District.

Materials and methods. The study material was 1400 plasma samples obtained from HBsAg-negative blood donors in Ural Federal District. The study included the testing for HBsAg, anti-HBs IgG and anti-HBcore IgG antibodies, HBV DNA. For all identified HBV DNA containing samples, sequencing and analysis of the nucleotide sequences of the complete HBV genomes were performed.

Results. The prevalence of HBV DNA was 4.93%, including 4 (0.28%) cases of false occult hepatitis B. Among anti-HBcore IgG-positive samples, HBV DNA was found in 18.08% of cases, while in persons with detected HBV DNA the anti-HBcore IgG positivity rate was 46.38%. In 8.69% of the isolates, anti-HBs IgG antibodies and viral DNA were detected simultaneously in the absence of anti-HBcore IgG. Based on phylogenetic analysis, HBV subgenotypes distribution in HBsAg-negative blood donors was as follows: D3 — 53.62%, D2 — 21.74%, D1 — 18.84%, C2 — 5.8%. The high variability in the S, C, P regions of the virus genome in the examined group was shown. In all cases of HBsAg-negative chronic HBV infection identified in blood donors, viral sequences contained at least one amino acid substitution in positions, mutations in which are associated with immune escape. In 3 (4.35%) cases mutations in reverse transcriptase region of P gene that are associated with resistance to the following drugs were identified: lamivudine, telbivudine, entecavir. Mutations in the preCore/Core regions that contribute to the progression of liver disease were also identified.

Conclusion. Occult HBsAg-negative chronic HBV infection poses a threat of HBV transmission through transfusion of blood and its components due to the extremely low viral load, which does not allow the virus to be detected using routinely used diagnostic kits. The situation can be exacerbated by the abundance and diversity of virus amino acid substitutions that we have identified, including immune escape mutations, drug resistance mutations, and mutations that contribute to the progression of the disease.

Keywords: *hepatitis B virus, occult hepatitis B, serological markers, molecular biological markers, HBV variability, genotypes, clinically significant mutations, laboratory diagnostics*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the St. Petersburg Pasteur Institute (protocols No. 67, February 22, 2017 and No. 97, January 29, 2020).

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ORIGINAL RESEARCHES

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Молекулярно-генетическая характеристика полноразмерного генома вируса гепатита В у HBsAg-негативных доноров крови в Уральском федеральном округе

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Аннотация

Актуальность. По оценкам Всемирной организации здравоохранения, на конец 2019 г. более чем у 296 млн человек в мире зарегистрирован хронический вирусный гепатит В (ВГВ). Распространённость HBsAg-негативной, скрытой формы течения заболевания у доноров крови варьирует в зависимости от региона мира и чувствительности используемых методов анализа. Поскольку генетическое разнообразие вирусов демонстрирует пространственно-временные вариации, а генетический профиль изолятов в ключевых группах, потенциально способных становиться источником распространения патогена, важен для прогнозирования эпидемиологической ситуации, представляется значимым определить циркулирующие среди доноров крови в регионах России генотипы ВГВ.

Цель работы — молекулярно-генетическая характеристика геномов ВГВ, выявленных у HBsAg-негативных доноров крови в Уральском федеральном округе.

Материалы и методы. Материалом исследования служили 1400 образцов плазмы, полученных от HBsAg-негативных доноров крови Уральского федерального округа. Исследование включало определение HBsAg, антител анти-HBs IgG, анти-HBcore IgG, ДНК ВГВ. Для всех выявленных образцов проводили секвенирование и анализ нуклеотидных последовательностей полных геномов ВГВ.

Результаты. Распространённость ДНК ВГВ составила 4,93%, в том числе 4 (0,28%) случая ложного скрытого гепатита В. Среди анти-HBcore IgG-позитивных образцов ДНК ВГВ обнаружили в 18,08% случаев, в то время как у лиц с выявленной ДНК ВГВ анти-HBcore IgG — в 46,38%. У 8,69% изолятов обнаружены одновременно антитела анти-HBs IgG и ДНК вируса при отсутствии анти-HBcore IgG. На основании филогенетического анализа показано, что у HBsAg-негативных доноров крови представлены субгенотипы ВГВ в следующих соотношениях: D3 — 53,62%, D2 — 21,74%, D1 — 18,84%, C2 — 5,8%. Показана высокая вариабельность регионов S, C, P генома вируса в обследованной группе. Во всех выявленных нами случаях HBsAg-негативного хронического ВГВ у доноров крови были представлены вирусы, по крайней мере, с одной аминокислотной заменой в положениях, мутации в которых действуют как ускользающие от вакцины. В регионе обратной транскриптазы гена *P* в 3 (4,35%) случаях определены мутации устойчивости вируса к лекарственным препаратам: ламивудину, телбивудину, энтекавиру. В регионах preCore/Core выявлены мутации, способствующие прогрессированию заболевания печени.

Заключение. Скрытый, HBsAg-негативный хронический ВГВ представляет собой угрозу передачи ВГВ при переливании крови и её компонентов за счёт крайне низкой вирусной нагрузки, не позволяющей определить вирус с помощью рутинно используемых диагностических наборов. Ситуацию может усугубить выявленное нами обилие и разнообразие аминокислотных замен вируса, включающих мутации иммунологического избегания, мутации фармакорезистентности и мутации, способствующие прогрессированию развития заболевания.

Ключевые слова: *вирус гепатита В, скрытый гепатит В, серологические маркеры, молекулярно-биологические маркеры, вариабельность ВГВ, генотипы, клинически значимые мутации, лабораторная диагностика*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Этическим комитетом Санкт-Петербургского НИИ эпидемиологии и микробиологии им. Пастера (протоколы № 67 от 22.02.2017 и № 97 от 29.01.2020).

Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

The hepatitis B virus (HBV) is a DNA-containing blood-borne pathogen that can cause both acute and chronic liver infection progressing to cirrhosis and hepatocellular carcinoma (HCC). The World Health Organization estimates that as of 2019, more than 296 million people were living with chronic hepatitis B virus infection (CHB), over 820,000 people died from complications related to HBV infection, and only 10.5% of the infected people were aware of their condition [1].

The partially double-stranded circular virus genome approximately 3,200 base pairs (bp) in length is represented by *S*, *P*, *C,* and *X* genes with overlapping open reading frames encoding 7 proteins.

HBV owes its high genetic heterogeneity to the RNA-stage in its life cycle, to the lack of 3'-5' exonuclease activity in the reverse transcriptase, resulting in the virus failure to proofread the genome, and to the high rate of variability [2]. The HBV classification is based on the divergence of complete genomic nucleotide sequences. Today, 9 genotypes (A-I) have been identified by more than 8% sequence divergence, and nearly 50 subgenotypes that differ by 4.0–7.5% [3, 4]. Having high genetic variability, the virus can respond endogenous and exogenous selective pressure by active modification of its genome structure, thus triggering large numbers of naturally occurring mutations, some of which are of clinical significance. For example, the *S* gene region can frequently have amino acid substitutions at positions 123, 126, 129, 130, 133, 144, 145, and 181, which lead to evasion of the immune response and/or result in inefficiency of diagnostic tests for HBsAg detection, while mutations in the *P* gene, which partially overlaps the *S* gene, contribute to resistance of the virus to antiviral drug therapy [5, 6]. The *C* gene region is known for *T1753C*, *A1762T*/*G1764A* promoter mutations as well as *F24Y*, *E64D*, *E77Q*, *A80I/T/V*, *L116I*, *E180A* mutations associated with the progression of the disease and HCC [7].

The analysis and description of genotypic and mutation profiles HBV in the key population groups, which are potential contributors to the virus spread, have epidemiological significance for assessment of HBV models, transmission routes, and imported strains invading the virus population [8]. Blood donors represent one of these key groups. Transfusion of blood and blood products is of high importance for treatment of serious conditions of different origin, helping save millions of lives worldwide every year; in the meantime, without proper control over safety of the used materials, transfusion manipulations can turn into the infection source infecting recipients with blood-borne pathogens, including the human immunodeficiency virus (HIV), HBV, and the hepatitis C virus (HCV).

In Russia and in many other countries, the long-used primary diagnostic tool for detection of acute or chronic infection was serological detection of the HBV surface antigen (HBsAg); additional tests can be conducted to detect IgG antibodies to core antigen (anti-HBcore), if the test results for HBsAg are controversial. Currently, the donor screening protocol includes molecular and biological tests for the viral DNA detection, though the test can be performed using a minipool (not more than 6 samples) provided that the reagent kits have sensitivity of 100 IU/ml[1].

Despite the strict measures aimed at blood safety, the residual risk of HBV transfusion transmission is not eliminated completely [9]. In addition to the window period after the recent infection, another two characteristics of HBV contribute to this risk. Firstly, while high viral loads are required for infection with HIV and HCV, the HBV infectious dose is 16 copies (3 IU/ml) of HBV DNA, regardless of the volume of the transfused plasma/blood components [10]. Secondly, the natural course of CHB is the so-called occult HBV infection (OBI), which is detected by the presence of replication-capable viral DNA in liver tissues (episomal covalently closed circular DNA) and/or in blood when the test for HBsAg turned out negative. The seropositive OBIvariant is characterized by the presence of other CHB markers, primarily, anti-HBcore antibodies, while the seronegative variant can have no serological markers at all. In addition, as extremely low viral loads are typical of the above cases, the polymerase chain reaction (PCR) methods may not be sufficiently sensitive for detection of HBV DNA [11]. For blood transfusions, the most preferable options are PCR modifications with the sensitivity of 10 IU/ml and lower, and samples should be tested individually. Tests using minipools, i.e. a mix of 6-10 donations, which are commonly used to reduce the cost of screening, are characterized by lower sensitivity due to the dilution factor, thus leaving out cases with a low viral load. In mathematic modeling, the estimated residual risk of HBV ranges from < 1 to

[1] Decree No. 1166n of the Ministry of Health of the Russian Federation, 28/10/2020, "On Approval of the Procedure of Medical Examination of Donors and the List of Medical Contraindications (Temporary and Permanent) for Donation of Blood and (or) Its Components and Time Limits for Disqualification Based on Temporary Medical Indications Regarding Donation of Blood and (or) Its Components". URL: http://publication.pravo.gov.ru/Document/View/0001202011260032

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1.4 per 1 million donations in countries that are low endemic for HBV and from 16 to > 100 in countries that are highly endemic for HBV [12].

The studies of OBI prevalence among blood donors have been very few for a long time; however, in the recent years, there has been an increasing number of publications addressing the problem. The frequency of this type of CHB among blood donors varies across countries, depending on the representativeness of HBsAg in a geographic region. Among blood donors in Argentina, HBV was detected only in 0.06% of samples, and only 4 samples were HBsAg-negative. On the contrary, in Laos and Nigeria, the OBI prevalence was 10.9% and 17%, respectively [13, 14]. In the meantime, the reported prevalence of OBI among blood donors frequently varies in the same region and the findings of different research groups are prone to discrepancy. For example, in Iran, the frequency of HBsAg among the population and blood donors is 2.6% and 0.4%, respectively, while the prevalence of OBI among donors exceeds 4% [15–17]. Thus, the reported results vary, depending not only on the region, but also on the methods used for virus detection, including various commercial kits for detection of HBsAg and HBV DNA.

Considering that the genetic diversity of viruses demonstrates space and time variations and taking into account that the genetic profile of isolates in key groups, which may turn into a source of the pathogen spread, is important for forecasting of the epidemiological situation, the attention should be given to identification of HBV genotypes currently circulating among regular blood donors in regions of the Russian Federation.

The **aim** of our study was molecular and genetic characterization of HBV genomes identified in HBsAg-negative blood donors in the Ural Federal District.

Materials and methods

A total of 1,400 plasma samples collected from HBsAg-negative regular blood donors in the Ural Federal District in 2020 were used in the study. All participants signed the written informed consent for participation in the study. The study was approved by the Local Ethics Committee of the Saint-Petersburg Pasteur Research Institute of Epidemiology and Microbiology (minutes No. 67 of 22/2/2017 and No. 97 of 29/1/2020). At the healthcare centers responsible for collection and banking of blood and its components, samples from each donation were tested for markers of 4 blood-borne infections: HIV (HIV-1 p24 antigen, HIV-1/2 antibodies), HBV (HBsAg, HBV DNA in minipools), HCV (HCV antibodies, HCV RNA in minipools), syphilis (treponema pallidum IgM and IgG antibodies). At the Saint-Petersburg Pasteur Research Institute of Epidemiology and Microbiology, the received plasma was checked for presence of serological markers of CHB using an enzyme-linked immunosorbent assay-based test and qualitative detection of HBsAg, anti-HBs IgG antibodies, anti-HBcore IgG antibodies. The tests were repeated twice using DS-EIA-HBsAg, DS-EIA-ANTI-HBsAg, DS-EIA-ANTI-HBc (Diagnostic Systems RPC), and Vectohep B-HBs-antigen, VectoHBsAg-antibodies, HepaBest anti-HBc-IgG commercial kits (Vector-Best) in accordance with the manufacturer's instructions. Reagent kits with sensitivity of 0.05 and 0.01 IU/ml were used for detection of HBsAg.

HBV DNA was extracted using the RIBO-prep commercial kit (Central Research Institute of Epidemiology, Rospotrebnadzor). In all the samples, viral particles were concentrated by ultracentrifugation of plasma for 1 hour at 24,000*g* at 4°C.

The initial detection of HBV by real-time PCR with hybridization and fluorescence detection was performed using the commercial diagnostic AmpliSens® HBV-FL reagent kit (Central Research Institute of Epidemiology, Rospotrebnadzor) in accordance with the instruction. The sensitivity of the reagent kit was 50 IU/ml. The further detection of HBV DNA was performed using the method developed by the Saint-Petersburg Pasteur Research Institute of Epidemiology and Microbiology. The method is efficient for detection of HBV DNA in biological material at low viral loads (the sensitivity is 5 IU/ml), including HBsAg-negative CHB [18]. All the selected samples were sequenced and used for the further analysis of HBV complete genomic nucleotide sequences, as it was described earlier [19].

The primary analysis of the fragments received during the sequencing was performed using the NCBI Blast software to compare them with the nucleotide sequences available in the GenBank international database. The nucleotide sequences were aligned using the MEGA11 software and the ClustalW algorithm [20]. The phylogenetic trees were constructed, and the phylogenetic analysis was conducted by measuring distances between sequences using the neighbor-joining method for optimizing a tree following the balanced minimum evolution criterion; 1,000 bootstrap replications were done to assess the reliability of the constructed trees.

Serological subtypes of HBV were identified with the reference to the HBsAg amino acid sequence.

The deduced HBV sequences were also analyzed to identify genotypes and to assess any possible drug resistance and immune-escape mutations using 3 online tools from Geno2Pheno HBV web service[2], the HBVseq[3] resource from the Stanford HIV database, the HIV-GRADE HBV[4] tool.

The statistical analysis was performed using MS Excel, Prizm 5.0 programs (GraphPad Software Inc.). The Clopper-Pearson exact interval was used to mea-

[2] URL: https://hbv.geno2pheno.org
[3] URL: https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html
[4] URL: https://www.hiv-grade.de/cms/grade/explanations/hbv-tool

sure the statistical error. The results are presented using a 95% confidence interval (CI). Fisher's exact test or the χ^2 test with Yates' correction were used to measure the significance of differences between variables received by pairwise comparisons, depending on the characteristics of the samples. The significance threshold for differences was set at probability value $p < 0.05$.

Results

The age of the blood donors in the group ranged from 18 years to 61 years, averaging 33.5 ± 8.7 years. The number of men in the studied group exceeded the number of women – 66.85 and 33.14%, respectively.

The prevalence of serological markers in the group was 72,71% (95% CI, 70.30–75.03%). No differences in the total prevalence of HBV markers between men (73.72%; 95% CI, 70.77–76.51%) and women (70,69% 95% CI 66.32–74.8%) were found. The results of the analysis of prevalence and distribution of the studied HBV markers in the group are presented in **Table 1**.

Thus, CHB serological markers are equally represented among men and women; however, anti-HBcore IgG antibodies were detected more frequently in men than in women (χ^2 = 10,166; p = 0.0014; df (degrees of freedom) = 1). During the analysis of the distribution of markers, the isolated anti-HBcore IgG antibodies were also more frequently detected in men than in women (χ^2 = 8.904; p = 0.0028; df = 1).

In the seropositive group, men (67.78%; 95% CI, 64.81–70.64%) outnumbered women (32.22%; 95% CI, 29.36–35.19%). The results of the analysis of prevalence and distribution of the studied HBV markers in the group of seropositive individuals are presented in **Table 2**.

The assessment of prevalence of serological markers in age groups showed that among HBV-seropositive individuals, 9.14% (95% CI, 7.44–11.07%) were people aged 18–22 years, 55.89% (95% CI, 52.78–58.97%) fell into the 23–40-year-old range, and 34.97% (95% CI, 32.04–37.99%) were older than 40 years.

Using molecular and biological methods, HBV DNA was detected in 4.93% (95% CI, 3.85–6.20%) of blood donors. Among anti-HBcore IgG-positive samples, HBV DNA was detected in 18.08% (95% CI, 12.71–24.55%) of samples, while among individuals with anti-HBcore IgG, it was detected in 46.38% (95% CI, 34.28–58.8%) of samples. A total of 8.69% (95% CI, 3.26–17.97%) of isolates had both anti-HBs IgG antibodies and viral DNA, while anti-HBcore IgG was absent. Note that although men significantly pre-

Table 1. Prevalence and distribution of the hepatitis B serological markers in the examined group, *n* (%; 95% CI)

Detected serological markers in blood plasma	Number in the group (*n* = 1400)	Number in the group among men (*n* = 936)	Number in the group among women (*n* = 464)
Prevalence of the hepatitis B serological markers			
HBs IgG+	947 (67,64; 65,12–70,09)	631 (67,41; 64,31–70,41)	316 (68,1; 63,65–72,32)
HBcore IgG+	172 (12,29; 10,61–14,12)	137 (14,64; 12,43–17,07)	40 (8,62; 6,23–11,55)
Seropositive	1018 (72,71; 70,3–75,03)	690 (73,72; 70,77–76,51)	328 (70,69; 66,32–74,8)
Seronegative	382 (27,29; 24,97–29,7)	246 (26,28; 23,49–29,23)	136 (29,31; 25,2–33,68)
Distribution of serological markers			
HBcore IgG+, HBs IgG+	101 (7,21; 5,91–8,79)	73 (7,8; 6,16–9,71)	28 (6,03; 4,05–8,6)
HBcore IgG+ isolated	71 (5,07; 3,98–6,35)	59 (6,3; 4,83–8,06)	12 (2,59; 1,34–4,47)
HBs IgG+ isolated	846 (60,43; 57,81–63,00)	558 (59,62; 56,39–62,78)	288 (62,07; 57,48–66,5)

Table 2. Prevalence and distribution of the hepatitis B serological markers among seropositive individuals, *n* (%; 95% CI)

Detected serological markers in blood plasma	Number in the seropositive group (*n* = 1018)	Number in the seropositive group among men (*n* = 690)	Number in the seropositive group among women (*n* = 328)
Prevalence of the hepatitis B serological markers			
HBs IgG+	947 (93,03; 91,28–94,51)	631 (91,45; 89,11–93,43)	316 (96,34; 93,7–98,1)
HBcore IgG+	172 (16,9; 14,64–19,34)	137 (19,86; 16,94–23,03)	40 (12,19; 8,86–16,23)
Distribution of serological markers			
HBcore IgG+, HBs IgG+	101 (9,92; 8,15–11,92)	73 (10,58; 8,38–13,12)	28 (8,54; 5,75–12,10)
HBcore IgG+ isolated	71 (6,97; 5,49–8,72)	59 (8,55; 6,57–10,89)	12 (3,66; 1,9–6,3)
HBs IgG+ isolated	846 (83,1; 80,66–85,36)	558 (80,87; 77,73–83,74)	288 (87,8; 83,77–91,14)

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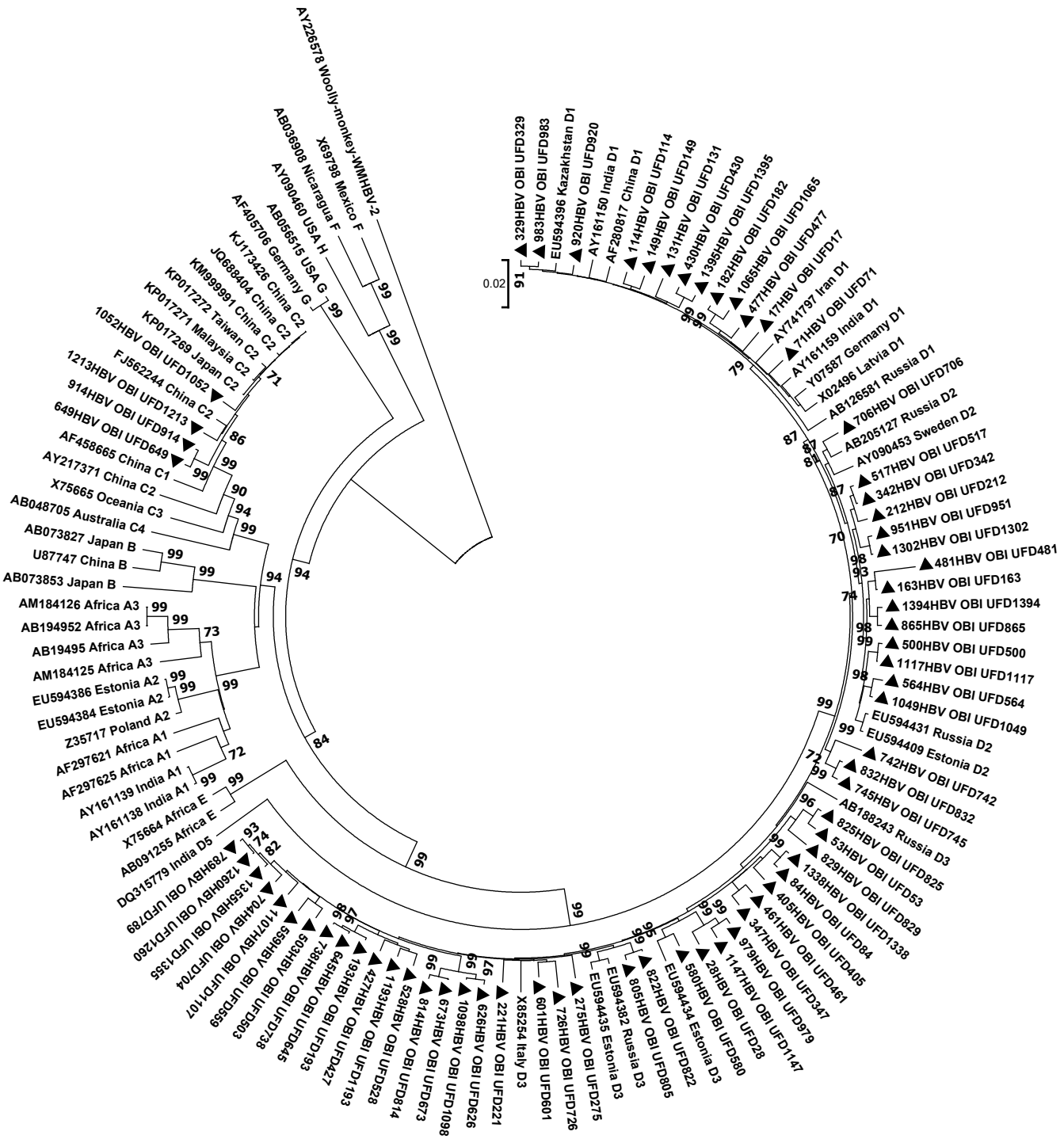
vailed in the group of blood donors and among individuals with the detected HBV DNA (75.36%; 95% CI, 63.51–84.94%), the frequency of OBI among men (5.56%; 95% CI, 4.18–7.22%) was only insignificantly higher than among women (3.66%; 95% CI, 2.15–5.8%); no significant differences were found ($p = 0.1488$).

In most of the samples (94.2%), the viral load was less than 50 IU/ml, though in 4 samples it reached 10^5 IU/ml.



Phylogenetic analysis of the HBV complete genomic nucleotide sequences from blood donors compared to reference sequences from the GenBank international database.

The reference sequences have the assigned GenBank codes showing the genotype and the region of the sample. Nucleotide sequence AY226578 of woolly monkey HBV was used as an out-group. The studied samples are marked with black triangles. The phylogenetic tree was constructed using the neighbor-joining method. Bootstrap values ≥ 60 are used.

The complete genomic nucleotide sequences in the HBV isolates were deposited to the GenBank international database with accession numbers OP153940–OP154008.

The phylogenetic analysis of 69 isolates showed that HBV genotype D (94.2%; 95% CI, 85.82–98.4%) prevailed in the studied group; genotype C was identified in 4 cases (5.8%; 95% CI, 1.60–14.18%). Among the patients with HBV genotype D, the highest frequency was demonstrated by subgenotype D3 (56.92%) compared to subgenotypes D2 (23.08%) and D1 (20%). Thus, among 69 samples from HBsAg-negative blood donors, HBV subgenotypes were distributed as follows: D3 — 53.62%, D2 — 21.74%, D1 — 18.84%, C2 — 5.8% (**Figure**).

The identification of serological subtypes in the detected isolates showed the predominance of serotype ayw2 (73.91%; 95% CI, 61.94–83.75%); serotypes ayw3 (14.49%; 95% CI, 7.18–25.14%), adw3 and adr — 5.8% (95% CI, 1.60–14.18%) demonstrated rarer occurrence. At the same time, serotype ayw2 was identified in all isolates with subgenotype D1 as well as in most isolates with subgenotypes D2 and D3; ayw3 was also identified in isolates with subgenotypes D2 and D3; adw3 was found only in isolates with HBV subgenotype D2; adr was identified in isolates with subgenotype C2.

Although in most of the cases, HBV nucleotide sequences in OBI do not differ from those in HBsAg-positive infection and are defined as the "wild type", the studied group of donors had some natural polymorphic variants of the virus in the genomic regions of all isolates, including amino acid substitutions that were found in 100% of HBV samples in RT, SHB, MHB, LHB, and Core regions as well as in 71.01% of samples in the preCore region.

The analysis of mutations affecting the HBsAg antigenicity included the major hydrophilic region (MHR) and the "a" determinant region representing a cluster of major B-cell epitopes located between amino acids 124 and 147 (or 149). The analysis revealed amino acid substitutions at 22 positions of 144 positions, accounting for 15.28% (95% CI, 9.83–22.21%). The following polymorphic variants were identified: *Y100S/L, Q101R/H, G102R, L109Q/I, I110L, G112E, T113S, T114S, T118V/A, P120S, K122R, T125M, T127P, A128V, N131T, S132Y, F134Y, A159G, Y161F, V168A*. In all isolates of HBV genotype C, the above region had substitutions that were not detected among isolates with genotype D: *T126I, K160R*.

The reverse transcriptase region of the *P* gene also had polymorphic variants: *A7V, E11G, H12L, I16T, A21S, A38G/E, R41G, Q48E, Y54H/N/D, V63I/C/G, N71K, L72R, N76T/G, S85C/L, F88S, Y89*/L, H90S, L91I, H94D, A97G, H100D, S105C, Y111N, S117Y, N118T, F122L, H124Y/N/H, H126R, T128I/N/T/A, M129L, Q130P, D134N, Y135S, N139D, L145M, K154Q, L164M, R167G, L180M, T184A, I187L, M204V, S213T, V214A, H216Q, L229F, A223S, N248H, C256S, Y257H, D263E/A, I266V/R, Q267H/L, E271D, V278I, I282V, L293H, F300L, M309K, S317A/F/S, K318R, A329T, N337T*. Most of the studied samples (21.74%; 95% CI, 23.71–33.31%) had 7 mutations in the RT region; 15.94% (95% CI, 8.24–26.74%) had 6 mutations; 11.59% (95% CI, 5.14–21.57%) had 10 and more mutations; 11.59% (95% CI, 5.14–21.57%) had 8 mutations; 10.14% (95% CI, 4.18–19.79%) had 3 and 4 mutations; 8.7% (95% CI, 3.26–17.97%) had 5 mutations; 7.25% (95% CI, 2.39–16.11%) had 9 mutations. Drug-resistance mutations were detected in 3 samples (4.35%; 95% CI, 0.91–12.18%) (**Table 3**).

The analysis of the preCore region revealed polymorphic variants *W28L/S, G29D/A*. The *W28L/S* frequency was 13.04% (95% CI, 6.14–23.32%), *G29D/A* — 23.19% (95% CI, 13.87–34.91%), each of 2 mutations – 5.8% (95% CI, 1.60–14.18%).

In the Core region, there were 39 positions that had amino acid substitutions: *T12S, S21T/H/A/Q, F24Y, V27I, D29Q, A34T, E40D/Q, A41P, P45H, L55I, E64D, M66L/R, T67N/S, A69V/S, N74G/V, E77D, P79Q, A80I/T, N87S, N92H, I97F/L, I105V, T109S, E113Q/D, L116V/I, P130A, A131T, P135Q, N136D, T142L, L143I, T147A, V149I, R151P, D153*, R154*, S157T, Q179K, S183P*.

Discussion

In 2016–2017, a total of 2,643 cases of acute hepatitis B (AHB), 0.915 per 100,000 population, were reported in Russia, demonstrating a 1.6-fold decrease in the incidence over the last 5 years. The distribution of

Table 3. Drug resistance mutations identified in the study group

Isolate	Subgenotype	Mutation	Description
HBV_OBI_UFD53	D1	*M204V*	Resistance to lamivudine, telbivudine, partial resistance to entecavir
HBV_OBI_UFD1147	D2	*L180M*	Limited sensitivity to lamivudine
		T184A, L180M	Compensatory mutation in entecavir resistance
HBV_OBI_UFD829	D2	*L180M, M204V*	Resistance to lamivudine
		M204V	Resistance to telbivudine
		T184A, L180M, M204V	Resistance to entecavir

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incidence rates across regions lacks uniformity, ranging from 0-0.54 to 0.86-3.79 per 100,000 population. In the meantime, high incidence rates of newly diagnosed CHB are still reported — approximately 21,045 cases per 100,000 population, including patients with CHB and patients who were previously referred to as HBV carriers. The discrepancy between the AHB and CHB incidence rate is most likely associated with several factors. First of all, most of the patients infected with HBV are asymptomatic initially, meaning that the detected acute infection cases represent only a certain percentage of the actual rate of infection frequency. Secondly, the awareness of the population is still relatively low, and the availability of advanced molecular and genetic diagnostic methods is still limited; therefore, new cases of CHB are detected either accidentally, for example, during routine medical check-ups, or when patients seek medical attention, having pronounced clinical symptoms typical of advanced stages of the disease. In fact, such detected new cases are not new and represent only a certain percentage of actual patients with CHB. The Ural Federal District falls into the group of regions with the highest AHB incidence – 0.86–3.79 per 100,000 population and highly variable CHB incidence: ranging from 0.26 to 7.66 in Kurgan, Sverdlovsk, and Tyumen regions, and from 11.63 to 61.72 in the Yamal-Nenets Autonomous District [21]. Therefore, the Ural Federal District was selected as one of the first regions for the pilot study of prevalence of OBI among blood donors in Russia.

The absence of HBsAg in the studied group is consistent with the information received from the healthcare centers that provided the biological material. The high level of anti-HBs IgG is most likely associated with vaccination against the virus. The detected cases with the combination of anti-HBs IgG and anti-HBcore IgG indicate that 7.21% of donors retained certain levels of neutralizing antibodies after infection acquisition and natural recovery. The predominance of men among the seropositive individuals can be apparently explained by the prevailing number of men in the studied group. This is supported by similar prevalence rates of CHB markers among the men and women. The lower detection rate of anti-HBcore IgG in the women compared to the men can imply the comparatively less risk-related behavior of representatives of this cohort and, consequently, a lower risk of infection.

It is commonly known that the HBV prevalence is measured by the frequency of HBsAg and, consequently, there are geographic regions with high (> 8%), moderate (2–7%) and low (< 2%) rates of infection prevalence among the population. Russia belongs to countries characterized by moderate prevalence. However, this method cannot be used for estimation of the prevalence of the occult, HBsAg-negative infection. The fact that we detected 4.93% of donors with HBV DNA during our study proves that the currently used methods for screening of blood donors for HBV markers are not sufficient. As there are no significant differences in HBV DNA frequencies between men and women, the male sex cannot be seen as the risk factor for OBI development, though uncontrolled sexual behavior and other types of risk-related behavior are more typical of the male sex. Previously, analyzing the prevalence of HBsAg-negative CHB among blood donors in Kazakhstan, we also pointed out the absence of significant differences, along with a slight tendency toward more frequent infection rates among men (10.1%) compared to women (6.9%) [22]. On the other hand, among blood donors in Guinea, the HBV DNA was detected significantly more frequently in men (18.61%) than in women — 10.5% ($\chi^2 = 27.285$; $p < 0.0001$; df = 1) [23]. It can be assumed that the presence or the absence of differences can be associated with the prevalence of the pathogen in the region as well as with the difference in behavior patterns of men and women in Russia, Kazakhstan, and Guinea.

On the one hand, the detection of only 18.08% of HBV DNA samples among all donors' samples positive for anti-HBcore IgG implies the redundancy of this serological marker for diagnostic tests. On the other hand, anti-HBcore IgG was detected only in 46.38% of HBV-positive individuals, thus suggesting that anti-HBcore IgG tests are not sufficient for detection of all donors with OBI. Special attention should be given to donors who were tested positive for HBV DNA, while having the combination of anti-HBs IgG and anti-HBcore IgG antibodies as well as to individuals having only anti-HBs IgG. Previously, this phenomenon was observed primarily among patients with immunosuppression of different origin, though it was also found in immunocompetent blood donors. For example, among HBsAg-negative blood donors with HBV DNA in China, 85% were anti-HBcore-reactive, 36.2% — anti-HBc and anti-HBs-reactive; 11.3% did not have any serological markers [24]. Considering that this serological profile in individuals with HBsAg-negative CHB is more common in countries of Asia (13%) than in Europe (2%), it can be assumed that it is associated with the insufficient vaccination coverage [12].

The improving sensitivity of PCR methods used for HBV detection at extremely low viral loads can result in false-positive results. The divergence of results obtained from serological and highly sensitive molecular OBI tests makes it difficult to differentiate between true and false-positive cases of detection of viral DNA. Thus, sequencing of nucleotide sequences of all isolates is a reliable method for confirming HBsAg-negative CHB.

The genotypic profile HBV in the studied group has several distinctive features. Previously, the Ural Federal District was characterized by presence of only genotype D, being consistent with our findings, while Russia, on the whole, was characterized not only by

prevalence of genotype D, but also by prevalence of genotype A subgenotype A2 and genotype C subgenotype C1 [25]. Although HBV subgenotype D2 is common in the central and northwestern parts of the country, in this study, the leading role is played by subgenotype D3, while subgenotype D2 has insignificantly higher frequency of occurrence compared to subgenotype D1. We think that such distribution of subgenotypes of genotype D can be explained by the close proximity to Kazakhstan and a large number of migrants from that region with high frequencies of HBV D3 and D1 [26]. In addition, it is known that the predominance of HBV D3 is typically observed in groups of patients with risk-involving sexual behavior, while multiple virus variants are reported in cohorts represented by people who believe that the infection was acquired during medical manipulations [27]. The results of the study prove that the epidemiological HBV profile in Russia has changed due to immigrant workforce from countries of Central Asia. The variety of genetic variants of the virus within subgenotypes implies multiple independent infection sources of detected cases with genotype D in the studied group. Special attention should be given to 4 isolates with subgenotype C2, which is hardly represented in our country; they were isolated from men aged 25–30 years, living in the same city and not related. To improve the differentiation of the received isolates, we selected 8 full-length HBV genomes with subgenotype C2 from the GenBank international database, as our samples have the highest similarity with them. However, during the phylogenetic analysis, the isolates that we received did not form any monophyletic clade related to any reference sequence. Based on the aforesaid, despite the anamnestic data of blood donors, we assume that there were at least 2 independent cases of importation of the above subgenotype to the region.

As is known, antigenic specificity is characterized by serotypes, which are often identified concurrently with genotyping, as, based on some data, the clinical course of CHB can depend on the genotype and serotype of the virus [28]. In Russia, serotypes ayw2 and ayw3 are typically detected in isolates with genotype D, thus demonstrating consistency with our findings. However, 4 cases of isolates D2 with serotype adw3 can be associated with mutations of the "a" determinant, the tertiary structure of which can recognize antigenic specificity [29]. In most of the CHB cases detected in the group of donors, the viral load was extremely low; only in the above samples it reached 10^5 IU/ml, thus suggesting the existence of false OBI, which is characterized by fairly high viral loads and modified HBsAg that was not detected by the tests due to the inefficiency of diagnostic kits.

HBV infection can be prevented by safe and effective vaccines providing 98–100% protection against the virus and preventing the development of complications, including chronic disease and primary liver cancer [1]. However, some mutations that develop in the virus genome both naturally and due to the selective effect of external factors can lead to evasion of therapeutic, preventive, and diagnostic measures. The major HBV envelope protein consists of the N-terminus (amino acid positions 1–99), MHR (amino acid positions 100–169) and C-terminus (amino acid positions 170–226). The major hydrophilic region is composed of two loops linked by disulfide bridges between cys124-cys137 and cys139-cys147 and is the target for antibodies induced by immunization and used in diagnostic tests [30]. The destruction of the bridges, which is caused by mutations, results in changes in the protein conformation and, consequently; it affects the ability of the vaccine-induced antibodies to neutralize the virus and contributes to the above-mentioned inefficiency of diagnostic kits due to the inability of antibodies to recognize a modified epitope [6]. Thus, preventive vaccination or prophylaxis with immunoglobulins can be inefficient in some cases. All the isolates used in this study carried amino acid substitutions in MHR, including mutations associated with HBsAg-negative CHB, previously described as escape-mutations or vaccine-escape mutations [31]. Furthermore, some of the detected mutations in the S region are associated with progressive liver disease. For example, according to Thi Cam Huong et al., the risk of HCC development was 3.38 in patients infected with the virus having the *P120S* polymorphism [32]. It should be noted that all the detected OBI cases in blood donors were associated with the viruses that had at least 1 amino acid substitution at positions 120, 126, and 131, in which mutations act as vaccine-evading mutations. This circumstance may pose a threat, as the pathogen can be transmitted to vaccinated individuals. In addition, isolates with escape mutations, which circulate in the group of blood donors, increase the risk of HBV reactivation HBV in immunocompromised patients; this problem cannot be neglected when transfusion of blood and its components is involved [33]. Based on the aforesaid, the variability of the reverse transcriptase region in the *P* gene comes as no surprise; on the other hand, amino acid substitutions in this domain can also lead to low levels of HBV DNA and HBsAg. The detected drug resistance mutations (*L180M*, *M204V*) and compensatory mutation *T184A* are observed in patients with HIV + HBV coinfection and are most commonly detected in patients receiving antiretroviral therapy during the European multicenter study [34]. The detection of 3 OBI cases combining escape mutations and drug resistance mutations pose an apparent risk of infection to recipients with the therapy-resistant virus, the detection of which can be difficult without advanced, highly sensitive molecular and biological methods.

In our study, the mutations in the preCore region were detected in 71% of cases; any changes in the sequence of this fragment can be seen as markers of the progressive disease. For example, the detected poly-

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morphism at positions 28 and 29 is clearly associated with development of liver cirrhosis and HCC. As is known, the stop codon at position 28 (*W28**) can affect the HBeAg synthesis, being responsible for more than 90% of cases with defective secretion of the above protein [7]. In this study, we did not identify stop codons and can assume that *W28L* and *W28S* represent a transient stage to mutation *W28**.

Mutations associated with liver disease development (for example, *F24Y, E40D/Q, E77D, A80I/T*), which are located in the region of T-cell epitopes (*F24Y, E40D/Q*) and B cells (*E77D, A80I/T, L116V/I*), were detected among the variety of polymorphic variants in the Core region [35]. Modifications of the amino acid sequence between positions 113 and 143 affect the antigenicity and stability of the virus. Theoretically, mutations in major immune epitopes can interfere with the immune response, causing persistent infection and most likely contributing to OBI development among the blood donors as well as to the variety of detected polymorphic variants in all regions of the virus genome [36]. Our findings correlate with the studies of Wang et al. who found significantly higher variability of *S* gene regions of the HBV genome, including MHR, and *P* gene regions, including the reverse transcriptase region, in OBI cases compared to HBsAg-positive infection [37].

OBI is associated with the risk of HBV transmission during transfusion of blood and its components; the situation can be aggravated by the detected multiple and various amino acid substitutions including immune escape mutations, drug resistance mutations and mutations contributing to the progression of the disease, cirrhosis, and HCC. Note that infants and children account for a large percentage of recipients and approximately 90% of them can develop chronic infection when infected with HBV before they reach the age of 5. The insight into characteristics of the full-length HBV genome in blood donors from different regions will help understand the frequency of OBI among donors and the role of HBsAg-negative CHB in the spread of the pathogen in the population.

Conclusion

High frequency of OBI among blood donors implies not only high prevalence of HBsAg-negative cases in the population, but also insufficiency of the standard test methods and/or sensitivity of diagnostic tests for CHB detection, calling for attention and effective measures focused on safety of blood transfusion. The detected hypervariability of the virus genome proves the significance of further studies of specific characteristics of the pathogen and host immune response in cases of latent HBV infection. Molecular phylogenetics can provide an insight into the epidemiology of the infection process, helping understand the spread specifics and the role of "imported" HBV genotypes in the virus circulation in the region.

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