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Identification by enzyme immunoassay of escape mutants S143L and G145R of hepatitis B virus (*Hepadnaviridae: Orthohepadnavirus: Hepatitis B virus*)

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Introduction. The achievement of the goal of the World Health Organization to eliminate viral hepatitis B by 2030 seems to be problematic partly due to the presence of escape mutants of its etiological agent, hepatitis B virus (HBV) (*Hepadnaviridae: Orthohepadnavirus: Hepatitis B virus*), that are spreading mainly in the risk groups. Specific routine diagnostic assays aimed at identification of HBV escape mutants do not exist.
The study **aimed** the evaluation of the serological fingerprinting method adapted for routine detection of escape mutations in 143 and 145 aa positions of HBV surface antigen (HBsAg).
Material and methods. HBV DNA from 56 samples of HBsAg-positive blood sera obtained from donors, chronic HBsAg carriers and oncohematology patients has been sequenced. After the identification of mutations in HBsAg, the samples were tested in the enzyme-linked immunosorbent assay (ELISA) kit «Hepastrip-mutant-3K».
Results and discussion. Escape mutations were detected mainly in patients with hematologic malignancies. Substitutions in 143 and 145 aa were found in 10.81% and in 8.11% of such patients, respectively. The G145R mutation was recognized using ELISA kit in almost all cases. The kit specifically recognized the S143L substitution in contrast to the S143T variant. The presence of neighbor mutation D144E can be assumed due to it special serological fingerprint.
Conclusion. ELISA-based detection of escape mutations S143L, D144E and G145R can be used for routine diagnostics, especially in the risk groups. The diagnostic parameters of the kit can be refined in additional studies. This immunoassay and methodology are applicable for the development and quality control of vaccines against escape mutants.

Key words: *hepatitis B virus (HBV); viral hepatitis B; escape; mutant; HBsAg; G145R; S143L; S gene; enzyme-linked immunosorbent assay (ELISA); monoclonal antibody (AB); next generation sequencing (NGS); oncohematology; hematologic malignancies; serological fingerprint*

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Иммуноферментная идентификация escape-мутантов S143L и G145R вируса гепатита B (*Hepadnaviridae: Orthohepadnavirus: Hepatitis B virus*)

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Введение. Достижение цели Всемирной организации здравоохранения по ликвидации вирусного гепатита B к 2030 г. представляется проблематичным, отчасти из-за наличия мутантов ускользания (*англ.* escape) у возбудителя этого заболевания, вируса гепатита B (ВГВ) (*Hepadnaviridae: Orthohepadnavirus: Hepatitis B virus*), распространяющихся преимущественно в группах риска. Специфической рутинной диагностики, направленной на идентификацию escape-мутантов, не существует.
Цель исследования – оценка метода серологического портретирования, адаптированного для рутинного выявления escape-мутаций в 143 и 145 аминокислотных остатках (а.о.) поверхностного антигена (HBsAg) ВГВ.
Материал и методы. ДНК ВГВ из 56 образцов HBsAg-положительных сывороток крови, полученных от доноров, хронических носителей HBsAg, а также страдающих злокачественными заболеваниями крови лиц, секвенировали. После выявления мутаций в HBsAg образцы тестировали в иммуноферментной тест-системе «Гепастрип-мутант-3К».
Результаты и обсуждение. Escape-мутации выявлялись преимущественно у больных со злокачественными заболеваниями крови: замены в 143 и 145 а.о. обнаружены в 10,81 и 8,11% случаев соответственно. С помощью иммуноферментного анализа мутация G145R распознана почти во всех случаях. Тест-система специфично распознавала замену S143L в отличие от варианта S143T. Присутствие соседней мутации D144E может предполагаться благодаря ее особому серологическому портрету.
Заключение. Иммуноферментная детекция escape-мутаций S143L, D144E и G145R может применяться для рутинной диагностики, особенно в группах риска. Диагностические параметры тест-системы могут быть уточнены при дополнительных исследованиях. Данная иммуноферментная тест-система и методика применимы для разработки и контроля качества вакцин против escape-мутантов.

Ключевые слова: *вирус гепатита B (ВГВ); вирусный гепатит B; ускользание; мутант; HBsAg; G145R; S143L; S-ген; иммуноферментный анализ (ИФА); моноклональное антитело (АТ); секвенирование нового поколения (NGS); онкогематология; злокачественные заболевания крови; серологический портрет*

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Introduction

Among numerous human diseases caused by viruses, only few are of the same global public health concern as viral hepatitis B. An estimated one third of the global population has been exposed to hepatitis B virus (*Hepadnaviridae; Orthohepadnavirus: Hepatitis B virus*) (HBV); approximately 360 million people have chronic infection. Such clinical complications as liver cirrhosis and hepatocellular carcinoma result in 1 million deaths worldwide annually [1]. In 2016, the 69th World Health Assembly endorsed the Global Health Sector Strategy for Viral Hepatitis, embracing a goal to eliminate hepatitis infection as a public health threat by 2030. The recommended targets are as follows: reduction in the HBV prevalence among 1-year-olds to ≤0.1%; reduction in the HBV-associated mortality to ≤5 per 100,000 population; a year-to year decrease in new cases of coinfection with HBV and HCV (*Flaviviridae; Hepacivirus: Hepatitis C virus*) [2].

In the meantime, HBV continues to spread despite the large-scale vaccination against hepatitis B and other epidemic control measures implemented by many countries. Among the factors responsible for this situation are various mechanisms adopted in the process of virus evolution and contributing to virus survival under immunological pressure [3]. A significant role is played by emerging diagnostic, immune and vaccine escape mutants that are being reported worldwide [4]. The vaccines based on the wild-type HBV virus surface antigen (HBsAg) do not protect against all escape mutants [5].

Apparently, such mutants do not spread uniformly; their distribution, first of all, depends on the genotype of the virus [4, 6]. In addition, escape mutations tend to accumulate in people belonging to special categories or, in other words, to special risk groups. For example, the highest prevalence of mutants has been demonstrated by oncohematological patients [7, 8], vaccinated children [7], and immunized infants born to HBV carrier mothers [10].

Penetration of escape mutants into populations (including those described above) is directly associated with errors in detection of these altered virus variants [11]. The analytical sensitivity of HBsAg assays depends on the HBV genotype or subtype; however, escape mutations make the situation much more complicated and ambiguous. Studies have shown that mutations in the HBV *S* gene, which are located in the a-determinant region of HBsAg or in regulatory elements, can have a significant effect on the antigenic profiles of HBsAg, sometimes leading to the situation when monoclonal antibodies (ABs) of diagnostic assays fail to recognize the above antigen [11, 12]. Even the most sensitive to date quantitative chemiluminescent Lumipulse HBsAg-HQ test (Fujirebio Inc., Japan) with a highly sensitive detection limit of 0.005 IU/ml (and the lower limit of detection of ~0.0011 IU/ml) for the wild-type HBV is significantly less efficient in detection of recombinant HBsAg with escape mutations than in detection of the wild type of this antigen [12]. At the same time, to assess adequately the sensitivity of tests in detection of escape mutations it is advisable to use serum panels with natural mutants, as the serological fingerprints of the naturally-occurred mutant HBsAg may be different from the fingerprints of its recombinant variant [13]. Furthermore, the sensitivity of the diagnostic assays can decrease, being affected by numerous escape mutations in HBsAg. Therefore, the insufficiently thorough assessment of the efficiency of the assays in detecting escape mutants can result in their underdetection.

Currently, there are no routine diagnostic tests for detecting such mutants. Next generation sequencing (NGS) is the most efficient and reliable technique [7]; however, this technique is quite labor-intensive and expensive. There have been solutions based on polymerase chain reaction (PCR) and gap ligase chain reaction (g-LCR), for example, in order to detect of escape mutants D144A [14] and G145R [15, 16]. There have been attempts to use limiting dilution cloning PCR (LDC-PCR) combined with sequencing to detect escape mutations at 120, 126, 128, 133, 141–145 aa [17, 18]. Microarrays have been offered for DNA identification of HBV genotypes and for detection of important mutations in *S*, *Pol*, *core,* and *X* genes [19]. However, all these innovations have not resulted in development of commonly used diagnostic tests. Previously, we offered the algorithm of serological search for mutations at positions 143 and 145 aa of HBsAg in the serum (S-HBsAg); it was based on serological fingerprinting – the enzyme immunoassay-based detection of any failures in the interaction between this antigen and monoclonal anti-HBsAg (ABs), using the criterion of the ≥10-fold decrease in sensitivity of the enzyme-linked immunosorbent assay (ELISA) for 10-fold serial dilutions of sera [20]. However, this solution was quite labor-intensive, involving 11 monoclonal conjugates.

The aim of this study was to assess the sensitivity and specificity of the serological fingerprinting technique adapted to routine detection of escape mutations at positions 143 and 145 aa of S-HBsAg.

Materials and methods

Samples. The study was performed on 56 blood serum samples collected from patients of different inpatient facilities and blood donor centers in Russia. Based on the past medical history or on the sample source, patients were divided into the following groups: donors (n = 11), chronic HBsAg carriers (the group of carriers) (n = 8), and patients with hematologic malignancies (the group of oncohematology patients) (n = 37).

Serum samples were tested for presence of serological markers of the hepatitis B virus (HBsAg, anti-HBs ABs, infectivity marker antigen HBeAg, anti-HBe IgG, anti-HBc IgM + IgG). In addition, the samples were examined for HBV viral load levels. The analysis of escape mutations at positions 143 and 145 aa was based on NGS and ELISA techniques with the latter employed with the help of the Hepastrip-mutant-3K enzyme-linked immunosorbent assay kit. The sera were also tested for ABs to hepatitis C and D viruses.

Diagnostic kits. The sera were tested for HBsAg, using the Hepastrip B enzyme-linked immunosorbent assay kit (Nearmedic Plus LLC, Russia). In some samples, HBsAg levels were quantified using the industry-specific standard HBsAg 42-28-311-00 (Diagnostic Systems Research and Production Company, Russia). Testing for other markers of HBV infection and for the total ABs to hepatitis C and D viruses was performed using ELISA kits manufactured by Vector-Best CJSC (Russia): VectoHBe-IgG (cat. No. D-0578), Vecto-HBe antigen (cat. No. D-0576), Vecto-HBc antibodies (cat. No.D-0566), Vecto-HBsAg antibodies (cat. No. D-0562), Best anti-HCV (set 3) (cat. No. D-0773), and Vectohep D-antibodies (cat. No. D-0954). ELISA testing was performed in accordance with the manufacturer's instructions.

The isolation and quantification of HBV DNA was performed using real-time PCR and the RealBest HBV DNA reagent kit (the quantitative version) (cat. No. D-0599, Vector-Best CJSC, Russia) in accordance with the manufacturer's instruction.

Monoclonal conjugates. Mouse monoclonal ABs to HBsAg (11F3, H2) as well as their horseradish peroxidase conjugates prepared using the technique offered by Tijssen P. et al. [21], were produced at the Immunity Mediators and Effectors Laboratory of the Gamaleya National Research Center of Epidemiology and Microbiology (NRCEM) of the Ministry of Health of Russia. We also used commercially available conjugate NF5 (Sorbent LLC, Russia).

Whole-genome deep sequencing of isolates of the hepatitis B virus. The NGS-based genome-wide study was performed for all 56 isolates. Primers located in conserved genomic regions were used for amplification of DNA samples, taking into account overlapping amplified loci. Each amplification reaction was performed separately, and included the following primers [22, 23]:

pair 1: 1-TCACCATATTCTTGGGAACAAGA,
2-CGAACCACTGAACAAATGGC;
pair 2: 1-GCCATTTGTTCAGTGGTTCG,
2-TGGGCGTTCACGGTGGT;
pair 3: 1-ACCACCGTGAACGCCCA,
2-TCTTGTTCCCAAGAATATGGTGA.

The length of the first PCR product was 1103 bp; the second product was 946 bp long, and the third one was 1226 bp in length. The amplification was followed by agarose gel electrophoresis of the PCR products. The resulting amplicons were further used to measure the DNA concentration with the Qubit 2.0 fluorometer (Invitrogen, United States). Then, all the 3 amplification products were mixed in equimolar amounts in one sample; the amount of nucleic acid in the total amplified samples was measure with the Qubit 2 fluorometer. The DNA concentration in the samples was normalized to 15 ng/μl. A total of 100 ng of each sample was used in the reaction to prepare indexed libraries; the libraries were prepared in accordance with the manufacturer's standard protocol.

The NGS was performed using the Ion PMG platform (Life Technologies, United States) and 316-type arrays, following the standard protocol. Each array included 16 prepared indexed genomic libraries at the theoretical design capacity ranging from 300 Mbp to 1 Gbp, i.e. from 300 million to 1 billion nucleotides. The practical capacity of 500 Mbp was accepted as the design value, based on the HBV genome length equal to ~3200 bp. The design sequencing depth for the concurrent analysis of 16 indexed genomic libraries was ~9,700 reads per sample. The results of the sequencing showed that the average reading depth for different samples was ~1,000–10,000.

The alignment of *S* gene nucleotide sequences and the comparative analysis of the primary nucleotide sequence were performed using the Vector NTI 9.0 program (ThermoFisher Scientific Inc. (Invitrogen), United States). Sequences of HBV genomes (genotypes A–H) from the GenBank database were used as reference sequences. HBV genotypes, subtypes, and mutations were identified, taking into consideration the data from the studies published previously [11, 25, 26]. The sequences were aligned using the following reference data from GenBank: JX096956 (Latvia, sub-genotype D2), and X98077, subtype adw [27]. This article describes the analysis of the part of *S* gene, which corresponds to HBV S-HBsAg.

The assessment of HBsAg escape mutants using the adapted serological fingerprinting technique and ELISA Hepastrip-mutant-3K kit. The technique employing the Hepastrip-mutant-3K enzyme-linked immunosorbent assay kit is based on the study by Bazhenov A.I. et al. [20]. It is a sandwich ELISA variant developed to search for HBsAg mutants in the positive for this marker samples selected by screening of human sera with any routine diagnostic test. During the analysis the polyclonal anti-HBs ABs sorbed on the surface of the plate wells bind HBsAg in the human serum or plasma; the resulting antigen-antibody complex is detected with the help of mouse monoclonal peroxidase conjugated ABs by the chromogenic assay. The technique requires 3 conjugates characterized by different specificity toward HBsAg of the wild and mutant types. Conjugate 11F3 is highly inefficient in detecting HBsAg variants with mutations S143L and G145R, while conjugate H2 detects wild-type HBsAg and the above variants. The third conjugate (NF5) interacts with the mutant in region 143 (but not 145) aa.

For each studied HBsAg-containing serum, we prepared a series of 10-fold dilutions (from 1/10 to 1/1,000,000) with the buffer containing 125 mM HEPES ((4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid)), 438 mM sucrose, 192 mM sodium chloride, 1.25% (v/v) casein, 3.3 mM p-hydroxyphenylacetic acid, 5% (w/v) BSA (bovine serum albumin), 1% (w/v) human γ-globulin, 12.2 μM methyl orange, 1.95 μM bromophenol red, 0.1% (v/v) ProClin-300, 10% (w/v) merthiolate, 21.6 μM Amphotericin B, 0.01% (v/v) gentamicin, and 1% (v/v) Tween 20. Each serum dilution was tested with conjugates in 4 repeats. The ability of conjugates to detect HBsAg in serum samples was assessed using wild-type HBsAg (ImBio, Microgen, Russia) in the concentration of 2 μg/ml.

Working solutions of conjugates 11F3 (0.8–1.0 μg/ml), H2 (2 μg/ml), and NF5 (2 μg/ml) were prepared with the buffer containing 0.01M EDTA (ethylene-di-

amine-tetraacetic acid), 0.5% (w/o) milk powder, 12.5% (v/v) FBS (fetal bovine serum) (inactivated for 30 minutes at 56 °C), 12.5% (v/v) normal rabbit serum (inactivated in the similar way), 0.05% (w/o) saponin, 0.0125% (v/v) Triton X-405, 0.00625% (v/v) Tween 80, 15 mM potassium iodide, 0,1% (v/v) n-propyl gallate, 0.15 M sodium chloride, 0.5 M urea, 0.005% (w/o) bromocresol purple, 0.35% (w/o) potassium thiocyanate, 0.1% (w/v) Zwittergent, 10% (w/v) merthiolate (sodium ethyl mercuri thiosalicylate), 21.6 μM Amphotericin B, 0.01% (v/v) gentamicin in Versene solution.

To perform the reaction in wells with immobilized goat polyclonal anti-HBs ABs of the plate from the Hepastrip-mutant-3K kit, we added 50 μl of the solution of conjugates 11F3, H2 or NF5. Each of them was tested separately, including all serum dilutions (or the control wild-type antigen). Then, we added 100 μl of wild-type HBsAg or the buffer used for sera dilutions (negative test control) to the control wells of the plate; the remaining wells were used for adding dilutions of the tested sera. The plate was incubated in the wet chamber at +37 °C for 2 hrs and then was washed 8 times with PBST (Phosphate Buffered Saline Tween 20) solution (0.15 M sodium chloride, 2.67 mM sodium phosphate dihydrate, 0.01% sodium azide, 0.1% (v/v) Tween 20; pH 7.2–7.5). Then, we added 100 μl of newly prepared standard solution of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) with substrate buffer containing hydrogen peroxide to all wells and incubated for 30 min at +37 ºC. To discontinue the reaction, we added 50 μl of 2M sulfuric acid, to each well. The results were checked immediately using the Sunrise microplate reader (Tecan, Switzerland) for measuring absorbance at 450 nm against the reference wavelength of 620 nm.

The comparative analysis of activity of conjugates was performed for dilutions of HBsAg-containing serum with the optical density values that would make it possible to compare the activity of 2 conjugates, i.e. ≤3.0 (plateau).

The sera characterized by similar optical density values with conjugates H2 and 11F3 belong to the wild type. If samples with conjugate H2 demonstrate the optical density values exceeding >10 times the values recorded for 11F3, they may contain a mutation at positions 143 or 145 aa and require further testing. Therefore, we use the third conjugate, NF5, which interacts with the mutant at position 143 aa (but not 145 aa). If the reactivity of the serum sample with conjugate H2 does not exceed >10 times that of NF5, it means that this sample has HBsAg with a mutation at position 143 aa. If the reactivity of the serum with conjugate H2 exceeds >10 times the reactivity with 11F3 and with NF5, the conclusion is that HBsAg has a mutation at position 145 aa.

The study was performed with the informed consent of the patients. The research protocol was approved by the Local Ethics Committee of the National Medical Research Center for Hematology of the Ministry of Health of Russia (Protocol No. 104 dated January 28, 2015).

Results and discussion

The NGS results showed that only 5 (8.93%) of 56 samples had a mutation at 143 aa; 4 of them belonged to the group of oncohematology patients. Thus, the prevalence of the mutation at position 143 aa was 10.81% (4 of 37) (see **Table 1**). Note that 3 of 5 samples had homogeneous mutation S143T, while the other 2 had mutation S143L. All samples with variant S143T, which belonged to subtype adw1 of genotypes A and D, were identified by the Hepastrip-mutant-3K enzyme-linked immunosorbent assay as samples containing the wild type, though they had fairly high levels of viral DNA (10^5–10^8 copies/ml). The low-titer serum with heterogeneous mutation S143L accounting for 31% of viral population was also identified as the serum containing the wild-type virus. Only 1 of 5 serum samples with a HBV mutation at position 143 aa of S-HBsAg was correctly identified by the ELISA test. It was a high-titer serum collected from the donor and containing HBV of genotype D and subtype ayw3 (see **Table 1**).

Compared to substitutions at 143 aa, the mutation at position 145 aa of S-HBsAg was detected more rarely and similarly prevailed in the samples from the group of oncohematology patients. It was detected more effectively by ELISA (see **Table 2**). All of the four substitutions detected at position 145 aa represented mutation G145R. The detection rate for this mutation in oncohematology patients was 8.11% (3 of 37), while among all of the tested samples, its prevalence was 7.14% (4 of 56). In 2 cases, the mutation was homogeneous; in the other 2 cases, it was represented by minor populations (22–25%). Both samples with 100% mutation G145R were identified by the enzyme-linked immunosorbent testing system. Nevertheless, the sensitivity of the assay was not sufficient to detect minor G145R-containing populations: in one case, the sample was identified as the sample containing a wild type; in the second case, it was identified as the sample containing a substitution at position 143 aa. In the latter case, the sample (No. 66) contained the HBV genotype, which can be assigned to recombinant form D/E. At the same time, in S-HBsAg, it had minor mutation L216Opal (21%) together with substitutions V118A and V128A belonging to diagnostic escape mutations, which could affect the detection of G145R mutation.

Among the other 47 samples, 8 ones were sera with mild defects relating to monoclonal conjugates (≤10 times) (see **Table 3**). No common mutations which would help clearly identify specific serological fingerprints were found (see **Tables 3** and **4**). Interestingly, escape mutation D144E, which was detected as homogeneous in samples 65 and 10, demonstrated the serological response different from the mutation-associated alterations at positions 143 and 145 aa, though it was located in close proximity. For example, both samples with substitution D144E had a mild defect regarding conjugate NF5. The same mutation was detected in one more sample from the group of oncohematology patients, though its content did not exceed 72%; this may be good explanation why the Hepastrip-mutant-3K test did not detect even a mild defect in recognition. The serological fingerprints of the

other 38 samples do not have any difference from the fingerprints of the wild-type virus.

HBsAg is a primary serological marker for detection of acute HBV infection and for monitoring of chronic HBV infection. The viral DNA level in virus carriers, who undergo treatment with nucleoside/nucleotide analogs, can decrease below the detection limit. Therefore, the monitoring of chronic HBV infection includes HBsAg quantification, which can be used during phases of immune tolerance, immune clearance, and immune response control (the non-active phase) as well as during re-activation of the HBeAg-negative form of the disease [12]. However, even when the most sensitive HBsAg detection methods are used, there is still a chance for diagnostic errors. Such errors may occur when a sample has escape mutations, especially in case of their multiple occurrences in the same patient. As a result, the epidemiological threat is increasing, espe-

Table 1. Detection of mutation in 143 amino acid position of S-HBsAg hepatitis B virus in sera using the enzyme-linked immunosorbent assay kit «Hepastrip-mutant-3K»

Таблица 1. Выявление мутации в позиции 143 аминокислотного остатка S-HBsAg вируса гепатита В в сыворотках с помощью иммуноферментной тест-системы «Гепастрип-мутант-3К»

Serum number* № сыворотки*	Patients' group Группа пациентов	Markers of viral hepatitis in the serum Маркёры вирусных гепатитов в сыворотке								HBV properties according to NGS Свойства ВГВ по результатам NGS			Hepastrip-mutant-3K Гепастрип-мутант-3К			
		HBV ВГВ						Others Другие					Optimal dilution of serum (1 : x) for detecting by conjugate‡ Оптимальное разведение сыворотки (1 : x) для определения конъюгатом‡			S-HBsAg assessment Оценка S-HBsAg
		HBsAg§	anti-HBsAg§ анти-HBsAg§	HBeAg	anti-HBe IgG анти-HBe IgG	anti-HBc IgM + IgG анти-HBc IgM + IgG	DNA, copies/ml ДНК, копий/мл	anti-HCV анти-HCV	anti-HDV анти-HDV	Genotype Генотип	Subtype Субтип	Mutation in 143 aa† Мутация в 143 а.о.†	11F3	H2	NF5	
18	Hematologic malignancies Онкогематология	+	–	+	–	–	$3{,}0 \times 10^8$	–	–	A	adw1	S143T 99%	10^4	10^4	10^4	wild type дикий тип
29		+	–	–	+	+	$2{,}9 \times 10^5$	–	–	D	adw1	S143T 100%	10^4	10^4	10^4	wild type дикий тип
33		+	–	–	+	+	$4{,}5 \times 10^8$	–	–	D	adw1	S143T 100%	10^4	10^4	10^4	wild type дикий тип
35		+	–	–	+	+	$3{,}7 \times 10^3$	–	–	D	ayw3	S143L 31%	10^3	10^3	10^3	wild type дикий тип
77	Donors Доноры	95	<10	–	+	+	$7{,}4 \times 10^8$	+	+	D	ayw3	S143L 100%	original исходный	10^4	10^5	substitution in 143 aa position замена в позиции 143 а.о.

Note. *the numbers of sera from 1 to 40 from the group «Hematologic malignancies» correspond to the numbering according [7], which contains the details of the diagnosis and molecular biological features; §the value indicates the measured concentration of the analyte (IU/ml); †the value indicates the content of the HBV with such mutation relative to the total viral pool; ‡the recognition defect that is not less than 100 times is highlighted in dark gray, and the recognition defect that is not less than 10 times is highlighted in light gray; «+», positive result; «–», negative result; NGS, next generation sequencing.

Примечание. *номера сывороток 1–40 из группы «Онкогематология» соответствуют нумерации из работы [7], содержащей детали диагноза и молекулярно-биологические характеристики; §величина обозначает установленную концентрацию аналита (МЕ/мл); †величина обозначает содержание ВГВ с указанной мутацией относительно общего пула вируса; ‡темно-серая заливка обозначает дефект распознавания в ≥100 раз, светло-серая – в ≥10 раз; «+» – положительный результат, «–» – отрицательный результат; NGS – секвенирование нового поколения.

cially considering the accumulation of mutation-associated alterations in risk groups [7].

The obtained results show that routine search of escape mutants is justified in the above sub-populations, one of which is the group of patients with hematologic malignancies. Escape mutations S143L/T, D144E, and G145R were detected mainly in this category of patients, demonstrating frequencies of 10.81%, 5.41%, and 8.11%, respectively. The sensitivity of the enzyme immunoassay-based detection of these mutations was fairly low. The best result was reached at 100% homogeneity of the mutation and the high HBV DNA concentration. Nevertheless, it was found that the enzyme-based immunodetection was specific for substitution S143L compared to S143T. In addition, the reliable detection of mutations S143L and G145R depends on the depth of the detected serological defects. In case of mutation S143L, the ≤10-fold decrease in the depth of defect of the recognition of mutant HBsAg with monoclonal conjugate 11F3 resulted in the detection error, identifying samples with HBV as suspicious, though they did not have any substitution. Therefore,

Table 2. Detection of mutation in 145 amino acid position of S-HBsAg hepatitis B virus in sera using the enzyme-linked immunosorbent assay kit «Hepastrip-mutant-3K»

Таблица 2. Выявление мутации в позиции 145 аминокислотного остатка S-HBsAg вируса гепатита В в сыворотках с помощью иммуноферментной тест-системы «Гепастрип-мутант-3К»

Serum Number* № сыворотки*	Patients' group Группа пациентов	Markers of viral hepatitis in the serum Маркёры вирусных гепатитов в сыворотке								HBV properties according to NGS Свойства ВГВ по результатам NGS			Hepastrip-mutant-3K Гепастрип-мутант-3К			
		HBV ВГВ						Others Другие					Optimal dilution of serum (1 : x) for detecting by conjugate‡ Оптимальное разведение сыворотки (1 : x) для определения конъюгатом‡			S-HBsAg assessment Оценка S-HBsAg
		HBsAg§	anti-HBsAg§ анти-HBsAg§	HBeAg	anti-HBe IgG анти-HBe IgG	анти-anti-HBc IgM + IgG HBc IgM + IgG	DNA, copies/ml ДНК, копий/мл	anti-HCV анти-HCV	anti-HDV анти-HDV	Genotype Генотип	Subtype Субтип	Mutation in 145 aa† Мутация в 145 а.о.†	11F3	H2	NF5	
1	Hematologic malignancies Онкогематология	60	30	–	+	+	$1{,}0 \times 10^8$	–	–	D/E?	ayw2/ ay?	G145R 100%	10^2	10^5	10^4	substitution in 145 aa position замена в позиции 145 а.о.
2		95	–	+	±	+	$9{,}4 \times 10^7$	–	–	D	adw3	G145R 100%	10^2	10^5	10^3	substitution in 145 aa position замена в позиции 145 а.о.
15		+	–	–	+	+	$1{,}4 \times 10^8$	н/т# n/t#	–	D	ayw?	G145R 25%	10^4	10^4	10^4	wild type дикий тип
66	HBsAg carriers HBsAg-носители	>1	<10	+	+	+	$3{,}7 \times 10^8$	–	–	D/E?	ayw3	G145R 22%	10^4	10^5	10^5	substitution in 143 aa position замена в позиции 143 а.о.

Note. *the numbers of sera from 1 to 40 from the group «Hematologic malignancies» correspond to the numbering according [7], which contains the details of the diagnosis and molecular biological features; §the value indicates the measured concentration of the analyte (IU/ml); †the value indicates the content of the HBV with such mutation relative to the total viral pool; ‡the recognition defect that is not less than 100 times is highlighted in dark gray, and the recognition defect that is not less than 10 times is highlighted in light gray; «+», positive result; «–», negative result; #n/t, not tested.

Примечание. *номера сывороток 1–40 из группы «Онкогематология» соответствуют нумерации из работы [7], содержащей детали диагноза и молекулярно-биологические характеристики; §величина обозначает установленную концентрацию аналита (МЕ/мл); †величина обозначает содержание ВГВ с указанной мутацией относительно общего пула вируса; ‡тёмно-серая заливка обозначает дефект распознавания в ≥100 раз, светло-серая – в ≥10 раз; #н/т – не тестировано; «+» – положительный результат, «–» – отрицательный результат.

Table 3. Features of sera with mild defects of hepatitis B virus recognition by monoclonal conjugates with the enzyme-linked immunosorbent assay kit «Hepastrip-mutant-3K»

Таблица 3. Характеристика сывороток со слабовыраженными дефектами распознавания вируса гепатита В моноклональными конъюгатами из иммуноферментной тест-системы «Гепастрип-мутант-3К»

Serum number* № сыворотки*	Patients' group Группа пациентов	Маркёры вирусных гепатитов в сыворотке Markers of viral hepatitis in serum								HBV properties according to NGS Свойства ВГВ по результатам NGS			Hepastrip-mutant-3K Гепастрип-мутант-3К			
		HBV ВГВ						Others Другие					Optimal dilution of serum (1 : x) for detecting by conjugate‡ Оптимальное разведение сыворотки (1 : x) для определения конъюгатом‡			S-HBsAg assessment Оценка S-HBsAg
		HBsAg§	anti-HBsAg§ анти-HBsAg§	HBeAg	anti-HBe IgG анти-HBe IgG	anti-HBc IgM + IgG анти-HBc IgM + IgG	DNA, copies/ml ДНК, копий/мл	anti-HCV анти-HCV	anti-HDV анти-HDV	Genotype Генотип	Subtype Субтип	Mutual mutations Общие мутации	11F3	H2	NF5	
14	Hematologic malignancies Онкогематология	+	–	–	+	+	$6{,}0 \times 10^7$	n/t† н/д†	–	D	ayw2	none нет	10^3	10^3	10^4	substitution in 143 a.o. position? замена в позиции 143 а.о.?
38	Hematologic malignancies Онкогематология	+	–	+	+	+	$5{,}0 \times 10^8$	n/t н/д	–	D2	ayw3	none нет	10^5	10^5	10^6	substitution in 143 a.o. position? замена в позиции 143 а.о.?
40	Hematologic malignancies Онкогематология	+	–	–	+	+	$1{,}1 \times 10^5$	–	–	D2	ayw3	none нет	10^3	10^3	10^4	substitution in 143 a.o. position? замена в позиции 143 а.о.?
63	HBsAg carriers HBsAg-носители	>1	–	+	+	+	$4{,}4 \times 10^5$	–	–	D	ayw2	none нет	10^5	10^5	10^4	?
64	HBsAg carriers HBsAg-носители	>1	–	+	+	+	$1{,}4 \times 10^8$	–	–	D	ayw2	none нет	10^5	10^5	10^4	?
65	HBsAg carriers HBsAg-носители	>1	–	+	+	+	$2{,}5 \times 10^7$	–	–	D/E?	adw2	none нет	10^6	10^6	10^5	?
9	Hematologic malignancies Онкогематология	+	n/t н/т	–	n/t н/т	+	n/t н/т	n/t н/т	n/t н/т	D	ayw3	none нет	10	10	original исходный	?
10	Hematologic malignancies Онкогематология	+	n/t н/т	n/t н/т	n/t н/т	n/t н/т	$4{,}7 \times 10^6$	n/t н/т	n/t н/т	D	adw3	none нет	10^5	10^4	10^4	?

Note. *the numbers of sera from 1 to 40 from the group «Hematologic malignancies» correspond to the numbering according [7], which contains the details of the diagnosis and molecular biological features; §the value indicates the measured concentration of the analyte (IU/ml); †n/t, not tested; ‡the recognition defect that is not less than 10 times is highlighted in gray; «+», positive result; «–», negative result; «?», the details of the changes are unclear.

Примечание. *номера сывороток 1–40 из группы «Онкогематология» соответствуют нумерации из работы [7], содержащей детали диагноза и молекулярно-биологические характеристики; §величина обозначает установленную концентрацию аналита (МЕ/мл); †н/т – не тестировано; ‡серая заливка обозначает дефект распознавания в ≥10 раз; «+» – положительный результат, «–» – отрицательный результат; «?» – характер изменений неясен.

Table 4. Mutations in S-HBsAg in samples with mild defects of hepatitis B virus recognition by monoclonal conjugates of enzyme-linked immunosorbent assay kit «Hepastrip-mutant-3K»

Таблица 4. Мутации в S-HBsAg в образцах со слабовыраженными дефектами распознавания вируса гепатита В моноклональными конъюгатами иммуноферментной тест-системы «Гепастрип-мутант-3К»

Serum number* № сыворотки*	Patients' group Группа пациентов	Conjugate defect in ELISA Дефект по конъюгатам в ИФА	HBV genotype Генотип ВГВ	HBV subtype Субтип ВГВ	Amino acid substitutions in S-HBsAg, and their homogeneity Аминокислотные замены в S-HBsAg и степень их гомогенности
14	Hematologic malignancies Онкогематология	11F3, H2	D	ayw2	V118T (100%); V128A (100%); E164A (40%); T189I (21%); S207N/H/R (39%); L222I (21%)
38	Hematologic malignancies Онкогематология		D2	ayw3	none нет
40	Hematologic malignancies Онкогематология		D2	ayw3	S114P (<100%)
63	HBsAg carriers HBsAg-носители	NF 5	D	ayw2	V118T (100%); V128A (100%); T189I (68%); L216Opal (21%)
64	HBsAg carriers HBsAg-носители		D	ayw2	R24K (27%); V118T (100%); V128A (100%)
65	HBsAg carriers HBsAg-носители		D/E?	adw2	P11L (99%); V118T (100%); V128A (100%); Y134H (94%); D144E (100%); V177A (98%)
9	Hematologic malignancies Онкогематология		D	ayw3	S114A (97%)
10	Hematologic malignancies Онкогематология	H2, NF5	D	adw3	V118T (100%); V128A (100%); D144E (100%); S174N (100%); L222R (66%); I226S (99%)

Note. *the numbers of sera from 1 to 40 from the group «Hematologic malignancies» correspond to the numbering according [7], which contains the details of the diagnosis and molecular biological features; ELISA, enzyme-linked immunosorbent assay.

Примечание. *номера сывороток 1–40 из группы «Онкогематология» соответствуют нумерации из работы [7], содержащей детали диагноза и молекулярно-биологические характеристики; ИФА – иммуноферментный анализ.

further tests are needed to specify quantitative criteria of the assay.

Another important aspect of the approach described in this article can be its prospective use for development and quality control of vaccines against escape mutants. Then, recombinant HBsAg with a mutation can be seen as a homogeneous protein, and the Hepastrip-mutant-3K test can be efficiently used for identification and quantification of the folding of this mutant protein.

Conclusion

The enzyme-linked immunosorbent detection of escape mutations S143L, D144E, and G145R can be used in routine laboratory diagnostic testing, especially in risk groups. Nevertheless, the parameters of diagnostic sensitivity and specificity of the assay as well as the criteria of the presence of mutation-associated alterations can be precised by additional studies performed on the larger number of samples described by using molecular and biological methods. In addition, this assay and technique can be used for development and quality control of vaccines against escape mutants.

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