

Prevalence of occult hepatitis B virus infection and characterisation of hepatitis B surface antigen mutants among adults in western Croatia

Bubonja-Šonje, Marina; Peruč, Dolores; Abram, Maja; Mohar-Vitezić, Bojana

Source / Izvornik: **Annals of Hepatology, 2024, 29**

Journal article, Published version

Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

<https://doi.org/10.1016/j.aohep.2023.101156>

Permanent link / Trajna poveznica: <https://urn.nsk.hr/urn:nbn:hr:184:219252>

Rights / Prava: [Attribution-NonCommercial-NoDerivatives 4.0 International](#) / [Imenovanje-Nekomercijalno-Bez prerada 4.0 međunarodna](#)

Download date / Datum preuzimanja: **2024-05-29**



Repository / Repozitorij:

[Repository of the University of Rijeka, Faculty of Medicine - FMRI Repository](#)





Original article

Prevalence of occult hepatitis B virus infection and characterisation of hepatitis B surface antigen mutants among adults in western Croatia

Marina Bubonja-Šonje^{a,b,*}, Dolores Peruć^{a,c}, Maja Abram^{a,b}, Bojana Mohar-Vitežić^{a,b}

^a Department of Microbiology and Parasitology, Faculty of Medicine, University of Rijeka, Braće Branchetta 20, Rijeka 51000, Croatia

^b Department of Clinical Microbiology, Clinical Hospital Centre Rijeka, Krešimirova 42, Rijeka 51000, Croatia

^c Department of Clinical Microbiology, Teaching Institute of Public Health of Primorsko-Goranska County, Krešimirova 52a, Rijeka, Croatia

ARTICLE INFO

Article History:

Received 4 June 2023

Accepted 11 September 2023

Available online 25 September 2023

Keywords:

anti-HBc

HBsAg mutants

HBV DNA

hepatitis B virus

occult HBV infection

ABSTRACT

Introduction and Objectives: Occult hepatitis B virus (HBV) infection (OBI) is characterised by low levels of hepatitis B virus (HBV) DNA in the blood/liver of patients with negative hepatitis B surface antigen (HBsAg). This study aimed to determine the OBI prevalence and virological characteristics (viral genotypes and HBsAg mutants) in patients with an "anti-HBc only" serological profile.

Materials and Methods: A total of 24 900 serum samples were routinely screened for hepatitis B markers over a five-year period. All anti-HBc-positive/HBsAg-negative/anti-HBs-negative sera were selected and analysed for the presence of HBV DNA. Mutational analyses of the HBs gene and polymerase gene sequences were performed.

Results: 1749 (7.02%) sera were anti-HBc positive, and 113 (0.45%) sera had an "anti-HBc only" serological profile (HBsAg/anti-HBs negative). HBV DNA was detected in 12/113 (10.61%) "anti-HBc only" positive sera, representing 0.048% of all routinely tested samples. Due to extremely low viremia, HBV genome was successfully sequenced in only two sera where subgenotype D3 was confirmed. Mutational analyses of the S gene revealed multiple missense mutations. In addition to the M133I, Y134F, and G145R mutations, already associated with diagnostic escape, we also found nine novel OBI-related S-gene mutations - S136Y, F158L, K160N, E164G, S167L, A168V, L175S, S210I and F212C.

Conclusions: We detected multiple known and novel S gene mutations in 2/12 (16.6%) OBI cases, nevertheless, further studies are required to determine their role in the pathogenesis of OBI. Understanding the frequencies of clinically relevant HBV mutations may contribute to improvement of diagnostic protocols.

© 2023 Fundación Clínica Médica Sur, A.C. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

1. Introduction

Occult hepatitis B virus (HBV) infection (OBI) is a complex clinical entity characterised by low levels of HBV DNA in the blood/ liver of HBV surface antigen (HBsAg) negative patients [1]. In practice, detection of OBI is usually based on finding antibodies to the HBV core protein (anti-HBc) as the only serological marker (referred to as "anti-HBc only", or "isolated anti-HBc"). It should be noted, however, that the "anti-HBc only" serologic pattern may be the result of: (1) Unresolved chronic infection with low-grade, intermittent virus production; (2) Resolved infection - a decline to undetectable anti-HBs titres

that occurs years later; (3) Chronic infection with a mutant HBsAg that cannot be detected by routinely used serologic assays, so-called "false OBI"; (4) The window phase of acute hepatitis B; (5) Passive transmission of anti-HBc; and (6) A false positive test.

Both host and viral factors are involved in the induction of OBI, although recent studies have more strongly emphasized the role of host immunological and epigenetic control mechanisms in this context [2–5]. The low fidelity of HBV polymerase and the high mutation rate allows the virus to modify its genome structure. HBV genome contains four partially overlapping open reading frames (ORF). The HBsAg pres/S ORF is a highly heterogenic part of the HBV genome. HBsAg contains the major hydrophilic region (MHR) located between codon positions 99 and 169. Within the MHR the "a" determinant is located at codon positions 124–147. This determinant is an immunodominant epitope critical for recognition of HBsAg by anti-HBs and immune cells [6]. Mutations that cause a conformational change within the "a" determinant may affect the ability of serological assays for the detection of HBsAg [7].

Abbreviations: CMIA, chemiluminescent microparticle immunoassays; EIAs, enzyme immunoassay; ELFA, enzyme linked fluorescence assay; HBc, HBV core protein; HBsAg, HBV surface antigen; HBV, hepatitis B virus; MHR, major hydrophilic region; OBI, occult hepatitis B virus infection; ORF, open reading frame; PGK, Primorje Goški kotar County; RT-PCR, real-time polymerase chain reaction; S/CO, sample/cutoff

* Corresponding author at:

E-mail address: marina.bubonja@uniri.hr (M. Bubonja-Šonje).

Table 1		The HBV primers used in nested PCR.		
	Primer	Sequences (5'-3')	A	B
F	P3	CCTGCTCCCA	AR1	AGAACACCCRCTC
A	P4	GTCCTCCGCCCA	R2	AGAACACCCRCTC
F	AF1	CTCTCCGCCCA	P4	GTCCTCCGCCCA
A	AF2	TCCCGCTTGC	AR1	AGAACACCCRCTC
F	Fragments A: Primers P3 and AR1		B: Primers P4 and R2	
A	and AF2 in the nested PCR.		Primers P3 and AR1	

The results were analyzed using the Microsoft Excel program 2021. The SPSS program (version 21) (SPSS Inc., Chicago, IL) was utilized to

2.5. Statistical analysis

First amplifications were performed under the following conditions: 95 °C/5 min, 35 cycles of 95 °C/30 s, 58 °C/45 s, 72 °C/2.5 min and final extension 72 °C/7 min. Nested PCR conditions were 95 °C/5 min, 25 cycles of 95 °C/30 s, 58 °C/45 s, 72 °C/2.5 min and final extension 72 °C/7 min. Nested PCR conditions were 95 °C/2.5 min, 35 cycles of 95 °C/30 s, 58 °C/45 s, 72 °C/2.5 min and final extension 72 °C/7 min. Amplification products were gel visualised by gel electrophoresis and purified from the gel using the Nucleospin Gel and PCR Clean up kit (Macherey-Nagel MN, Germany) according to the manufacturer's recommendations. Sequencing primers were used for partial sequencing reactions. Sequencing of amplicons A and B, as described by Günther et al [13], amplicons were sequenced using the Big-Dye Terminator cycle sequencing kit (Applied Biosystems AB, USA). ABI Prism Sequencing Analysis v.5.4. Software was used in sequence analysis. ABI 310 (Applied Bio-sequencer) was used on the Sequitudo Genetic Analyzer ABI 310 (Applied Biosystems AB, USA) and ABI 310 (Applied Bio-sequencer) in Hdi format sequencing reactions were performed according to the manufacturer's recommendations. The purified sequencing reactions were desorbed in Hdi formamide (Applied Biosystems AB, USA) and sequenced in Hdi formamide (Applied Biosystems AB, USA) and sequenced in Hdi formamide (Applied Biosystems AB, USA) and sequenced in Hdi formamide (Applied Biosystems AB, USA).

2.4. HBV genome amplification, sequencing, and molecular analysis

DNA was extracted from 500 μ l of serum using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) with a final elution volume of 75 μ l and analysed by polymerase chain reaction (PCR) using the COBAS TaqMan HBV test (Roche, Mannheim, Germany) on the COBAS TaqMan HBV test (Roche, Mannheim, Germany) according to the manufacturer's instructions. The COBAS TaqMan 48 instrument (Roche, Mannheim, Germany) was used to analyse the samples. An initial sensitivity of 6 IU/ml and a specificity of 100% with a confidence limit of 99.54% for HBV DNA is reported. In addition, the COBAS test has an analytical sensitivity of 6 IU/ml and a detection limit of 3-4 ng serum samples from the same patient, as HBV DNA is almost entirely detectable and undetectable during OBI.

3. DNA extraction and HRV-DNA testing

example to be anti-HBc IgM-positive in the second anti-HBc test is negative.

Samples were tested for hepatitis B markers using Abbott Architect hemiluminescent microparticle immunoassays (CMIA). HBsAg total II (sensitivity 0.02 IU/ml), anti-HBc II, anti-HBs, and anti-HBe total II (bioluminescence assay (ELFA) VIDAS anti-HBc second enzyme linked fluorescence assay (ELFA)). HBsAg "anti-HBc only" (HBsAg and anti-HBs negative) was confirmed with a "anti-HBc only". Enzyme linked immunosorbent assay (ELISA) (Siemens Total II (bioluminescence assay (ELFA)) or Enzyme linked immunosorbent assay (ELFA) (Siemens (Germany)). We consider a diagnosis of anti-HBc monoclonal (Siemens, Germany).

2.2. Serological testing

All samples used in the study were collected and delivered to diagnostic laboratories for routine screening, and not particularly collected or serologically tested for this study. Only sera with a specific serological pattern confirmed by repeated testing (defined by positive anti-HBC and negative HBS) were selected and stored at -80°C for subsequent HBV DNA testing and multiregional analysis. HBSAg positive or anti-HBs positive sera were excluded from further study. Because of the expected intermediate low-level yields, HBSAg negative sera were also excluded from the study.

During the five-year period from January 2014 to the end of 2018, over all 24 900 serum samples, all collected from different individuals were tested for hepatitis B markers as a part of the routine serological testing at the Department of Clinical Microbiology, Clinical Hospital Central Rijeka and at the Department of Clinical Microbiology, Teaching Institute of Public Health of PCKC. The study population included low-risk groups (general population, pregravid women) and high-risk groups (exposure risk, haemodialysis, or liver

2.1. Study population and serum samples

2. Materials and Methods

The aim of this study was to determine the prevalence and virulence of oral opportunistic pathogens in the mixed population of Primitive Gorillas. The results of this study will be used to determine the impact of Hb on the pathogenicity of these organisms.

generally underestimated [1].

tion in different parts of the world is still largely undetermined and

serological and molecular tests. OBL prevalence in the general popula-

OBI depends on HBV endemicity, study population and sensitivity of

tries with a low HbsAg prevalence of about 1% [11]. The prevalence of

americum population groups according to language, culture, and ethnicity.

The Picardianne of the Picardie in the European Union among different populations according to European Centre for International Affairs (Paris)

Education, and Professionalism in Practice among

Mission [9,10]. Offered dialogues of UBI Previews features & Benefits, HRV

risk of reactivation in immunocompromised patients of HBV trans-

OBL patients usually have low DNA levels ($<200 \text{ ng/ml}$), there is also a

can affect HBsAg detection, posing a diagnostic challenge. Although

allow persistent HBV infection [8]. Furthermore, escape mutations

have been identified which can evade neutralizing antibodies and

To date, multiple immune-associated escape HBsAg mutations

perform t-test, χ^2 or Fisher's exact test. The significance level was set at 0.05.

2.6. Ethical statement

The study was conducted in accordance with the current Helsinki Declaration and approved by the ethics committees of Clinical Hospital Centre Rijeka (Approval Number: 2170-29-02/15-18-2) and Teaching Institute of Public Health of PGKC (Approval Number: 08-820-62/247-18) with a waiver of informed consent because this was a study of de-identified, routinely collected data and clinical samples.

3. Results

3.1. HBV serology and HBV DNA PCR

The overall prevalence of HBsAg positive sera among all routinely tested samples was 261/24 900 (1.04%). Of all 24 900 sera, 1 749 were anti-HBc positive (7.02%), whereas 116 samples had the "anti-HBc only" pattern (0.47%). In 116 "anti-HBc only" sera an additional anti-HBc confirmatory assay was performed which excluded three anti-HBc negative sera from further investigation. One hundred and thirteen patients (0.45%) with an "anti-HBc only" pattern represented 6.46% of all anti-HBc positive individuals; with males (66.37%) more frequently affected than females (33.63%); $P < 0.001$. Thirty of 113 "anti-HBc only" sera showed anti-HBe positivity (26.55%).

We further analysed all sera collected from 113 "anti-HBc only" positive patients for the presence of viral DNA. HBV DNA was detected in 12 of 113 patients (10.6%), representing 0.048% of all 24 900 patients. In nine HBV DNA positive sera, the viral load was < 6 IU/ml (lower limit of detection), whereas in the remaining three positive sera, the viral load was 10 IU/ml, 50 IU/ml, and 141 IU/ml. The data are summarized in Table 2.

We compared anti-HBc sample/cutoff (S/CO) values between HBV DNA positive and HBV DNA negative "anti-HBc only" positive sera. Although anti-HBc values in HBV DNA positive sera were higher than anti-HBc values in HBV DNA negative sera, the mean difference was not significant: (S/CO 9.62 versus 7.73; $p = 0.14$) (results not shown).

3.2. Seminested-PCR and genome sequencing

Due to the low viral load, we used seminested-PCR to sequence HBV genome in 12 "anti-HBc only" positive HBV DNA positive serum samples. Amplification of both HBV genome fragments (2092 bp and 1320 bp) was successful in 3/12 sera. The nested PCR amplicons of both fragments from patients labelled 1, 2, and 3 are shown in Fig. 1.

However, the sequencing results yielded usable sequences for only patient 2 (viral load 50 IU/ml) and patient 3 (viral load 141 IU/ml). The partial sequences were aligned with another 383 whole genome HBV sequences including the reference sequence as an anchor to obtain a reliable alignment. The sequence labeled patient 2 was aligned to the reference HBV genome from position 576 to 1784.

Table 2
Serological and molecular test results.

Category	Number (%)	95% CI
Total serum samples	24 900 (100)	
HBsAg positive sera	261/24 900 (1.04)	0.93-1.18
all anti-HBc positive sera	1749 (7.02)	6.71-7.35
"anti-HBc only" positive sera	113/24 900 (0.45)	0.37-0.55
"anti-HBc only"/ all anti-HBc positive	113/1749 (6.46)	5.35-7.72
HBV DNA positive/all tested sera	12/24 900 (0.048)	0.03-0.08
HBV DNA positive/ "anti-HBc only"	12/113 (10.6)	5.61-17.82
anti-HBe positive/ "anti-HBc only"	30/113 (26.55)	18.68-35.68
HBsAg mutation/HBV DNA positive	2/12 (16.6%)	2.09-48.41

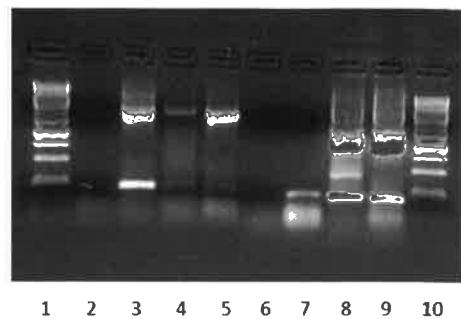


Fig. 1. Electrophoregram of both amplicons from HBV genome of three patients. (Lanes 2-5 P3 AR2 primers - 2092 bp; lanes 6-9 P4 AF2 primers - 1320 bp). Lane 1: DNA ladder; lane 2: negative control; lane 3: patient 1; lane 4: patient 2; lane 5: patient 3; lane 6: negative control; lane 7: patient 1; lane 8: patient 2; lane 9: patient 3; lane 10: DNA ladder-Molzym 1kb.

and the sequence labeled patient 3 was aligned to the reference HBV genome from position 574 to 1786.

Two patients 2 and 3, were genotyped by submitting 4 sequences (2 partial HBV sequences from the patients and the closest relatives identified by phylogenetic analysis) to the Geno2Pheno HBV genotyping database. The samples were classified as genotype D, subgenotype D3 (data not shown). Because of the overlap of the HBsAg and HBV RT polymerase genes, the amino acid sequences of both coding regions were determined. Escape mutant analysis and drug resistance mutations were performed. The amino-acid substitution (mutations) profiles in the HBs protein and polymerase domain are listed in Table 3.

HBV sequences obtained from patients 2 and 3 were identical in analysed S and polymerase regions. A dozen mutations were detected in the S region of HBV DNA. Three of the observed mutations (M133I, Y134F and G145R) have already been associated with the escape of anti-HBs. Mutations in 5 different amino acids encoding the polymerase genomic region were detected in both samples.

4. Discussion

According to recent data, HBsAg prevalence among voluntary blood donors in Croatia is 0.05% and has been steadily decreasing over the years [16]. Seroprevalence studies conducted in 2010-2011 in different subpopulations estimate that the prevalence of HBsAg carriers in the general Croatian population is between 0.5% and 0.7% [17,18]. In the present study that covered a heterogeneous population comprising both, low-risk groups, and high-risk groups an overall HBsAg prevalence of 1.04% was found. The slightly higher HBsAg prevalence found in our study can be explained by the difference in the population studied, previous Croatian studies included only routinely screened patients and healthy blood donors.

The reported prevalence of OBI is much lower than the prevalence of the serological pattern "anti-HBc only" [19,20]. This is not surprising, as the "anti-HBc only" pattern does not provide an accurate diagnosis. It may be the only detectable serological marker of OBI, "false OBI" (HBV infection with mutant HBsAg strains), resolved HBV infection, or a false positive result.

Until today, data on the prevalence of anti-HBc and OBI in Croatia are available only for blood donors. The prevalence of anti-HBc

Table 3
Missense mutations in the S and polymerase domains of HBV genome.

HBV domain	Mutations detected
S protein	M133I, Y134F, S136Y, G145R, F158L, K160N, E164G, S167L, A168V, L175S, S210I, F212C
Polymerase	D263E, I266V, Q267L, S317T, L336M

Patients [33]. There are very little data on the frequency of HBV immune escape mutations in Croatia. Recently, six immune escape mutations in genotype D virus have been reported; D14E, M13I, M13L, P12S, Q10I, R12K [29]. The immune escape mutation M13I detected by Grigic et al was also detected in our two patients. Although we focused our study on "anti-HBc only" positive OBI patients, we confirmed that both successfully sequenced HBV DNA samples belong to the D3 subtype, which is consistent with the distribution of the D3 subtype [34].

Thus, HBV S gene sequences of 2/12 HBV DNA positive patients from this study (16.6%) were found to have multiple mutations that could be associated with the decreased HbsAg expression and low immune recognition of the virus. "False OBI" cases, The prevalence of "false OBI" has previously been reported to be as high as 40% in OBI

DNA sequence, intermediate low-level viremia was detected in repeated serum samples collected during the five-year period from 1980 to 1985. Positive persons, however, were all "anti-HBc only," whereas only two positive samples with detectable HBV DNA were found among 50 and 141 viral loads.

Using the COMMERCIAL COBAS TaqMan HBV assay, which amplifies the conserved pre-core/core region of the HBV genome as the target gene in clinical practice and was not used in this study [1].

In the present study, HBV DNA was detected in 12/113 (10.61%) "anti-HBc only" positive cases, corresponding to an overall QBI prevalence of 0.048% (12/24 900). It is higher than the QBI prevalence among Croatian VBD, which has been reported to be 0.001-0.002% in recent studies [16,28]. It can be assumed that the rest of the "anti-HBc only" patients (16/113) were asymptomatic carriers of chronic hepatitis B. The sensitivity of the commercial PCR method used, although not explicitly mentioned, is less often performed at the level of individual laboratories.

In persistentily infected patients HbsAg can sometimes become undetectable years after the resolution of acute hepatitis. Spontaneous HbsAg seroconversion occurs in frequently ($\sim 1\%$ per year) in treatment-naïve adults with chronic infection [26]. Transition of low-mitamic HbsAg carriers to DBL was reported in 20% of inactive HbsAg carriers within 5-years [27]. This phenomenon could also be one of the reasons for the "anti-Hbc only" positivity analyzed in our study.

Anti-HBe is usually indicative of completed infection but may also be detectable in chronic hepatitis B. Given the high-performance characteristics of the anti-HBe assay used in this study, the relatively low proportion of anti-HBe positive patients in this study suggests that anti-HBc may appear several months or years after the acute hepatitis B infection, or may fail to undetectable levels over time. Besides HBC positivity, other reasons for the "anti-HBc only" serological pattern could be the window phase of acute HBV infection, cleared infection with loss of anti-HBs, or OBI. In reviewing the patients' medical records, we found that one, "anti-HBc only" HBV DNA positive patient was HBSAg positive several months before the start of the study. This was probably due to the window phase of acute HBV infection. This patient was found to be "anti-HBc only" during the study.

One of the reasons for the "anti-HBc only" serological pattern could be a false positive test due to the imperfect specificity of anti-HBc assays. The specificity of the Abbot Architect anti-HBc II assay used in this study is reported to be 98.0%. To minimize false positive anti-HBc results, we performed a second assay on all "anti-HBc only" positive individuals, which included three assays on all "anti-HBc only" positive individuals. The specificity of the Abbot Architect anti-HBc II assay used in these assays was found to be only a quarter of the "anti-HBc only" positive sera. However, based on the anti-HBc positivity in two different serological tests, we can assume that these 13 serum samples were true positive.

genotype D in Europe as well as the predominance of the D3 subtype in Croatia (42%) [29].

In addition to several mutations found in the S region of HBV, we also identified substitutions in five different amino acids encoding the genomic polymerase region (D263E, I266V, Q267L, S317T, and L336M), none of which were shown previously to result in antiviral drug resistance [6,7,15,29]. It is worth noting that both patients with sequenced HBV DNA did not previously receive antiviral therapy. Therefore, the mutations arose from host factors alone, without selection by anti-HBV therapy.

Because we selected only sera with isolated anti-HBc pattern for OBI testing, we may have missed some occult HBV infections in unusual HBV antibody profiles (anti-HBs positive or seronegative OBI cases) and underestimated the prevalence of OBI. However, the "anti-HBc only" pattern is considered more common and clinically relevant than other serological profiles. Due to the extremely low viral load in OBI patients, it was difficult to amplify the HBV genome and therefore we had a limited number of characterised OBI strains. This study has three main limitations. First, although the accumulation of several mutations found outside the "a" determinant of the S gene likely alters the immunogenicity of HBsAg, evasion of detection by diagnostic tools has yet to be demonstrated. Therefore, further bioinformatic and functional analyses are needed to determine whether these novel mutations could affect secondary structure and antigenicity of HBsAg. Second, we selected only sera with an "isolated anti-HBc" pattern for the study, which means that we may have failed to detect seronegative, or anti-HBs positive OBI cases. Third, the small number of patients whose HBV DNA was sequenced reduces the power of the study.

5. Conclusions

The findings of this study provide insight into the OBI prevalence and HBsAg mutations in our patients with an "anti-HBc only" serological profile. Our study is one of the few studies conducted in Croatia that includes a heterogeneous population of adults (including those at low and high risk). Investigations of HBV genetic variability and knowledge of the prevalence of clinically relevant viral mutations may contribute to improvement of diagnostic protocols.

Data availability statement

All data generated or analysed during this study are included in this article.

Funding

This work was supported by the University of Rijeka (MBS, grant number uniri-biomed-18-215) and (MA, grant number uniri-biomed-18-277).

Conflicts of interest

None.

CRediT authorship contribution statement

Marina Bubonja-Sonje: Conceptualization, Methodology, Writing – original draft, Data curation, Writing – review & editing. **Dolores Peruć:** Validation, Data curation, Writing – review & editing. **Maja Abram:** Supervision, Data curation, Writing – review & editing. **Bojana Mohar-Vitežić:** Conceptualization, Writing – original draft, Data curation, Writing – review & editing.

Acknowledgments

We would like to thank our laboratory technicians for their assistance in performing the serological tests.

References

- [1] Raimondo G, Locarnini S, Pollicino T, Leviero M, Zoulim F, Lok AS, Taormina Workshop on Occult HBV Infection Faculty Members. Update of the statements on biology and clinical impact of occult hepatitis B virus infection. *J Hepatol* 2019;71(2):397–408. <https://doi.org/10.1016/j.jhep.2019.03.034>.
- [2] Koumbi L, Karayannidis P. The epigenetic control of hepatitis B virus modulates the outcome of infection. *Front Microbiol* 2016;6:1491. <https://doi.org/10.3389/fmicb.2015.01491>.
- [3] Yuan Y, Yuan H, Yang G, Yun H, Zhao M, Liu Z, et al. IFN- α confers epigenetic regulation of HBV cccDNA minichromosome by modulating GCN5-mediated succinylation of histone H3K79 to clear HBV cccDNA. *Clin Epigenetics* 2020;12(1):135. <https://doi.org/10.1186/s13148-020-00928-z>.
- [4] Qin YP, Yu HB, Yuan SY, Yang Z, Ren F, Wang Q, et al. KAT2A promotes hepatitis B virus transcription and replication through epigenetic regulation of cccDNA minichromosome. *Front Microbiol* 2022;12:795388. <https://doi.org/10.3389/fmicb.2021.795388>.
- [5] Zhang W, Luo S, Li T, Wang M, Huang J, Liao Q, et al. Hepatitis B virus-specific cellular immunity contributes to the outcome of occult hepatitis B virus infection. *Front Microbiol* 2022;13:850665. <https://doi.org/10.3389/fmicb.2022.850665>.
- [6] Shen T, Yan XM. Hepatitis B virus genetic mutations and evolution in liver diseases. *World J Gastroenterol* 2014;20(18):5435–41. <https://doi.org/10.3748/wjg.v20.i18.5435>.
- [7] Caligariu P, Cerruti R, Icardi G, Bruzzone B. Overview of hepatitis B virus mutations and their implications in the management of infection. *World J Gastroenterol* 2016;22(1):145–54. <https://doi.org/10.3748/wjg.v22.i1.145>.
- [8] Lazarević I, Banko A, Miljanović D, Cupic M. Immune-escape hepatitis B virus mutations associated with viral reactivation upon immunosuppression. *Viruses* 2019;11(9):778. <https://doi.org/10.3390/v11090778>.
- [9] Wang Y, Han SB. Hepatitis B reactivation: a review of clinical guidelines. *J Clin Gastroenterol* 2021;55(5):393–9. <https://doi.org/10.1097/MCG.0000000000001520>.
- [10] Chang Y, Jeong SW, Jang JY. Hepatitis B virus reactivation associated with therapeutic interventions. *Front Med* 2022;8:770124 (Lausanne). <https://doi.org/10.3389/fmed.2021.770124>.
- [11] European Centre for Disease Prevention and Control. Systematic review on hepatitis B and C prevalence in the EU/EEA. Stockholm: ECDC; 2016. Available at: <https://www.ecdc.europa.eu/en/publications-data/systematic-review-hepatitis-b-and-c-prevalence-euea> [Online, 29.05.2022].
- [12] Wang X, Xu L, Chen Y, Liu A, Wang L, Xu P, et al. Integrating nested PCR with high-throughput sequencing to characterize mutations of HBV genome in low viral load samples. *Medicine* 2017;96(30):e7588 (Baltimore). <https://doi.org/10.1097/MD.00000000000007588>.
- [13] Günther S, Li BC, Miska S, Krüger DH, Meisel H, Will H. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 1995;69(9):5437–44. <https://doi.org/10.1128/JVI.69.9.5437-5444.1995>.
- [14] Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. *Mol Biol Evol* 2021;38(7):3022–7. <https://doi.org/10.1093/molbev/msab120>.
- [15] <https://hbv.genopheno.org/> [Online, 20.05.2022].
- [16] Strauss Patko M., Oćtić T., Grubešić D., Babić I. Izvješće o rezultatima rada transfuzijske službe u 2020. godini. In: Transfuziološki vjesnik br.65/2021. Available at: <https://hzum.hr/wp-content/uploads/2018/09/transfuziolski-vjesnik-bilog-65.pdf> (in Croatian)
- [17] Kaic B, Vilibic-Cavlek T, Kurecic Filipovic S, Nemethi Blazic T, Pemi Novosel I, visekruna Vucina V. Epidemiology of viral hepatitis. *Acta Med Croatica* 2013;67:273–9.
- [18] Vilibic-Cavlek T, Kucinac J, Ljubin-Sternak S, Kaic B, Lazaric-Stefanovic I, Kolarić B. Prevalence of viral hepatitis in Croatian adult population undergoing routine check-up, 2010–2011. *Cent Eur J Public Health* 2014;22(1):29–33. <https://doi.org/10.21101/ceph.a3844>.
- [19] Pondé RAA, Cardoso DDP, Ferro MO. The underlying mechanisms for the 'anti-HBc alone' serological profile. *Arch Virol* 2010;155:149–58. <https://doi.org/10.1007/s00705-009-0559-6>.
- [20] Gutiérrez-García ML, Fernandez-Rodriguez CM, Lledo-Navarro JL, Buhigas-Garcia I. Prevalence of occult hepatitis B virus infection. *World J Gastroenterol* 2011;17(12):1538–42. <https://doi.org/10.3748/wjg.v17.i12.1538>.
- [21] Samardžija M, Drenjančević D, Miletic M, Slavulj B, Jukić I, Zibar L, et al. The impact of positive anti-HBc marker on permanent deferral of voluntary blood donors in eastern Croatia and estimation of occult hepatitis B virus infection rate. *Acta Clin Croat* 2020;59(1):126–34. <https://doi.org/10.20471/acc.2020.59.01.15>.
- [22] Olotu AA, Oyelese AO, Salawu L, Audu RA, Okwuraiwe AP, Aboderin AO. Occult Hepatitis B virus infection in previously screened, blood donors in Ile-Ife, Nigeria: implications for blood transfusion and stem cell transplantation. *Virology* 2016;13:76. <https://doi.org/10.1186/s12985-016-0533-3>.
- [23] Candotti D, Laperche S. Hepatitis B virus blood screening: need for reappraisal of blood safety measures? *Front Med (Lausanne)* 2018;5:29. <https://doi.org/10.3389/fmed.2018.00029>.
- [24] Miletic M, Bingulac-Popovic J, Stojic Vidović M, Hećimović A, Berendika M, Babić I, et al. Anti-HBc prevalence among Croatian blood donors in a 14-year period

- [29] Gragic I, Hercog L, Goranec L, Kurecic I, Vincic A, Zidovacic Lepic S. Hepatitis B virus genotypes, detection of reverse transcriptase resistance and immune escape mutations in persons with chronic hepatitis B from Croatia Mol Exp Biol Med 2020;3(1):23-8.
- [30] Camman WF, Zanetti AR, Karayannidis P, Waters J, Manzillo G, Tanezi E, et al. Vacione (HbsAg), Precore SK, Lukaszewski TX, Tyroor RE, Williams CT, Leary TP. An improved Abbott ARCHITECT assay for hepatitis B virus surface antigen (HbsAg). J Clin Virol 2011;53(1):59-63. <https://doi.org/10.1016/j.jcv.2010.09.019>.
- [31] Lou SC, Precore SK, Lukaszewski TX, Tyroor RE, Williams CT, Leary TP. An improved Abbott ARCHITECT assay for the detection of hepatitis B virus surface antigen (HbsAg). J Clin Virol 2011;53(1):59-63. <https://doi.org/10.1016/j.jcv.2010.09.019>.
- [32] Coppola N, Onorato L, Minichini C, Di Caprio G, Starace M, Sagripanti C, et al. Clinical significance of hepatitis B surface antigen mutations. World J Hepatol 2015;7(27):2729-39. <https://doi.org/10.4234/wjh.v7i272729>.
- [33] Ahn SH, Park YN, Park YC, Chang HY, Lee JW, Shin JE, et al. Long-term clinical and virological outcomes in patients with spontaneous hepatitis B surface antigen seroconversion. J Hepatol 2005;42(1):88-94. <https://doi.org/10.1016/j.jhep.2004.10.026>.
- [28] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-67.pdf (in Croatian).
- [27] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-67.pdf (in Croatian).
- [26] Zhou K, Cottagé C, Whitalak E, Terrault N. Spontaneous loss of surface antigens among adults living with chronic hepatitis B virus infection: a systematic review and pooled meta-analyses. Lancet Gastroenterol Hepatol 2019;4(3):227-38. <https://doi.org/10.1016/j.lgh.2018.12.002>.
- [25] Stausas Pako M, Ogič T, Mitević M, Balaič I, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-60.pdf (in Croatian).
- [24] Stausas Pako M, Ogič T, Mitević M, Balaič I, Ivjeković D, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-60.pdf (in Croatian).
- [23] Oliver F, Sturzbecher L, Cavallo D, Colombari P, Ricci G, Salvati N, et al. Long-term outcome of inactive and chronic low viraemic HBeAg-negative-hepatitis B virus infection: benign course towards HbsAg clearance. Liver Int 2017;37(1):1622-31. <https://doi.org/10.1111/liv.13416>.
- [22] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-67.pdf (in Croatian).
- [21] Bubalo-Jasojiević D, Perinic M, Abraminić M, Babić I, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-68.pdf (in Croatian).
- [20] Stausas Pako M, Ogič T, Mitević M, Balaič I, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-69.pdf (in Croatian).
- [19] Stausas Pako M, Ogič T, Mitević M, Balaič I, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-70.pdf (in Croatian).
- [18] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-71.pdf (in Croatian).
- [17] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-72.pdf (in Croatian).
- [16] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-73.pdf (in Croatian).
- [15] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-74.pdf (in Croatian).
- [14] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-75.pdf (in Croatian).
- [13] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-76.pdf (in Croatian).
- [12] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-77.pdf (in Croatian).
- [11] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-78.pdf (in Croatian).
- [10] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-79.pdf (in Croatian).
- [9] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-80.pdf (in Croatian).
- [8] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-81.pdf (in Croatian).
- [7] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-82.pdf (in Croatian).
- [6] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-83.pdf (in Croatian).
- [5] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-84.pdf (in Croatian).
- [4] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-85.pdf (in Croatian).
- [3] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-86.pdf (in Croatian).
- [2] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-87.pdf (in Croatian).
- [1] Stausas Pako M, Ogič T, Balaič I, Grubasic D, Ivjeković D, Živković O, Špoljarić Špoljarić-Vještak Špoljarić-Vještak-88.pdf (in Croatian).