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

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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-Vitezić[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

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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.

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; EIA, 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; PGKC, Primorje Gorski 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).

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To date, multiple immune-associated escape HBsAg mutations have been identified which can evade neutralizing antibodies and allow persistent HBV infection [8]. Furthermore, escape mutations can affect HBsAg detection, posing a diagnostic challenge. Although OBI patients usually have low DNA levels (<200 IU/ml), there is also a risk of reactivation in immunocompromised patients or HBV transmission [9,10]. Correct diagnosis of OBI prevents reactivation, HBV transmission, and progression of liver fibrosis in patients.

The prevalence of HBV infection in Europe varies widely among different population groups. According to European Centre for Disease Prevention and Control estimates, Croatia is among the countries with a low HBsAg prevalence of about 1% [11]. The prevalence of OBI depends on HBV endemicity, study population and sensitivity of serological and molecular tests. OBI prevalence in the general population in different parts of the world is still largely undetermined and generally underestimated [1].

The aim of this study was to determine the prevalence and virological characteristics of OBI in the mixed population of Primorje Gorski Kotar County (PGKC) over a five-year period. Although OBI can occur sporadically in persons with detected anti-HBs, same as in persons without proven HBV markers (seronegative OBI), we limited our OBI estimate to individuals with "isolated anti-HBc" pattern, which is considered more common and clinically relevant [1]. The impact of HBV genomic variability on HBsAg undetectability was assessed.

2. Materials and Methods

2.1. Study population and serum samples

During the five-year period (from January 2014 to the end of 2018) overall 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 Centre Rijeka and at the Department of Clinical Microbiology, Teaching Institute of Public Health of PGKC. The study population included low-risk groups (general population, pregnant women) and high-risk groups (exposure risk, haemodialysis, or liver disease).

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 HBsAg and anti-HBs) were selected and stored at -80°C for subsequent HBV DNA testing and mutational analysis. HBsAg positive or anti-HBs positive sera were excluded from further study. Because of the expected intermittent low-level viremia, additional sera were collected from 113 "anti-HBc only" positive patients for DNA testing when available. In total, two to three serum samples were collected from most of the 113 selected patients during the study period. Previous HBV serological "markers" results (anti-HBc, HBsAg, anti-HBs, anti-HBe) were also considered when available.

2.2. Serological testing

Samples were tested for hepatitis B markers using Abbott Architect chemiluminescent microparticle immunoassays (CMIA): HBsAg Qual II (sensitivity 0.02 IU/ml), anti-HBc II, anti-HBs, and anti-HBe kits [specificity > 99.5%, sensitivity > 99.5% at the cut-off is < 0.45 Paul-Ehrlich-Institut (PEI) standard U/mL] on the Abbott Architect i1000SR automated analytical platform (Abbott, USA). Reactivity to "anti-HBc only" (HBsAg and anti-HBs negative) was confirmed with a second enzyme linked fluorescence assay (ELFA) VIDAS anti-HBc Total II (bioMerieux, France) or Enzyme Immunoassay (EIA) Siemens Enzygnost Anti-HBc monoclonal (Siemens, Germany). We consider a sample to be anti-HBc true-positive if the second anti-HBc test is reactive.

2.3. DNA extraction and HBV DNA testing

DNA was extracted from 500 μl of serum using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) with a final elution volume of 75 μl and analysed by polymerase chain reaction (PCR) using the COBAS TaqMan HBV test (Roche, Mannheim, Germany) on the COBAS TaqMan 48 instrument (Roche, Mannheim, Germany) according to the manufacturer's instructions. The COBAS TaqMan HBV test has an analytical sensitivity of 6 IU/ml and a specificity of 100% with a confidence limit of 99.54%. HBV DNA is searched in sequential (3-4) serum samples from the same patient, as HBV DNA is alternately detectable and undetectable during OBI.

2.4. HBV genome amplification, sequencing, and molecular analysis

DNA were extracted from 600 μl of HBV DNA positive sera and elution volume was reduced to 50 μl because all HBV DNA positive samples had very low viremia. The HBV genome was amplified in two fragments (A and B) using a modified seminested PCR protocol [12]. Amplification was performed using TaKaRa Taq™ DNA Polymerase Hot Start Version (Takara, France). Details of all primers used in this work are listed in Table 1.

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. Amplicons were 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 of amplicons A and B, as described by Gunther *et al* [13]. Amplicons were sequenced using the Big-Dye Termination v3.1 kit (Applied Biosystems AB, USA) and purified using the NucleoSeq kit (Macherey-Nagel MN, Germany) according to the manufacturer's recommendations. The purified sequencing reactions were dissolved in HiDi Formamide (Applied Biosystems AB, USA) and sequenced on the SeqStudio Genetic Analyzer ABI 310 (Applied Biosystems AB, USA). ABI PRISM Sequencing Analysis v 5.4. software was used in sequence analyses. Nucleotide sequences were aligned using MEGA (version 11) [14]. Genetic distances were obtained using the neighbour-joining statistical method, the Kimura 2 parameter model, and the bootstrap method with 1000 replicates. Sequences were submitted to the geno2pheno HBV database with the reference sequence NCBI accession number GQ922003.1 [15].

2.5. Statistical analysis

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

Table 1
The HBV primers used in nested PCR.

Frag	Primer	Sequences (5-3')	Position
A	P3	CTCGCTCGCCCAAATTTTTCACCTCTGCCTAATCA	1825-1841
	AR1	ACAGTGGGGGAAAGC	759-745
	R2	AGAAACGGRCTGAGGC	702-687
B	P4	CTGGTTCGGCCCAAAAAGTTGCATGGTGCTGG	1823-1806
	AF1	GTCTGCGGCGTTTTATC	419-435
	AF2	TGCCCGTTTGTCCTCTA	503-519

[1] Fragment A: primers P3 and AR1 are used in the first PCR, primers P3 and AR2 in the nested PCR. Fragment B: primers P4 and AF1 are used in the first PCR, primers P4 and AF2 in the nested PCR.

perform t-test, $\chi 2$ 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

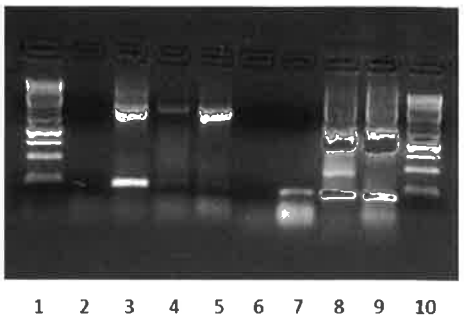

Fig. 1. Electrophoregram of both amplicons from HBV genome of three patients. (Lanes 2-5 P3 AR2 primers - 2092 bp; lanes 6-9 P4 AF2 primers - 1320 bp). Lane 1: DNA ladder; lane 2: negative control; lane 3: patient 1; lane 4: patient 2; lane 5: patient 3; lane 6: negative control; lane 7: patient 1; lane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

among Croatian blood donors was 1.5% in 2013-2016, while the prevalence among blood donors from endemic areas in the world can reach 75% [21–23]. The results of our study indicate that the prevalence of anti-HBc is 7.02%, the prevalence of "anti-HBc only" is 0.45%, and the rate of "anti-HBc only" pattern among all anti-HBc positive subjects is 6.46%. In 2017 the prevalence of anti-HBc among Croatian blood donors was 1.32%, while the rate of "anti-HBc only" among all anti-HBc positive blood donors varied between 9.6% and 16.4% [24]. In comparison to the reported prevalence of anti-HBc positivity among Croatian blood donors (1.32%), we found a higher prevalence (7.02%). Not surprisingly, the prevalence of anti-HBc positivity is higher in a heterogeneous population consisting of both low- and high-risk groups than in healthy blood donors. Lower anti-HBc positivity is expected in blood donors selected according to strict selection criteria and with no history of HBV exposure. This also explains why we found a much higher HBsAg positivity in the study population (1.04%) than was reported among blood donors during the same period (0.048%) [25].

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 Abbott 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 sera, which excluded three previously "anti-HBc only" positive individuals from further investigation. Thus, anti-HBc reactivity was confirmed in 113/116 (97.41%) "anti-HBc only" serum samples, with males (66.37%) more frequently affected than females (33.63%). We also performed an additional anti-HBe assay. Anti-HBe antibodies were found in 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 113 serum samples were true positive.

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-HBeAb positivity can be explained by the fact that anti-HBeAb may appear several months or years after the anti-HBc detection, or may fall to undetectable levels over time. Besides false 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 patient was in the window phase of acute HBV infection leading to persistent infection.

In persistently infected patients HBsAg can sometimes become undetectable years after the resolution of acute hepatitis. Spontaneous HBsAg seroclearance occurs infrequently (~1% per year) in treatment-naive adults with chronic infection [26]. Transition of low-viraemic HBsAg carriers to OBI 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.

In the present study, HBV DNA was detected in 12/113 (10.61%) "anti-HBc only" positive cases, corresponding to an overall OBI prevalence of 0.048% (12/24 900). It is higher than the OBI 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" positive, HBV DNA negative patients from this study (101/113) cleared infection with loss of anti-HBs, and in most cases anti-HBe antibodies, over the years. However, since undetectable serum HBV DNA does not exclude OBI, there is a possibility that viral levels were below the analytical sensitivity of the commercial PCR method used. Although detection of replication-competent HBV DNA in the liver is the gold standard for OBI diagnosis, liver biopsy is an invasive test that is less often performed in clinical practice and was not used in this study [1].

Using the commercial COBAS TaqMan HBV assay, which amplifies the conserved pre-core/core region of the HBV genome as the target DNA sequence, intermittent low-level viremia was detected in repeated serum samples collected during the five-year period from twelve "anti-HBc only" positive persons. HBV DNA positive samples were retested using the nested PCR assay. However, the nested PCR assay failed to amplify the HBV genome in the nine sera with extremely low viral loads (<6 IU/ml) close to the COBAS TaqMan HBV RT-PCR assay detection limit, probably because only residual nucleic fragments were detected by the commercial RT-PCR test. Amplification of HBV genome segments with nested PCR was possible in three of twelve HBV DNA positive sera with DNA level greater than 10 IU/ml. However, sequencing results revealed usable sequences for only two HBV DNA positive serum samples with the highest viral loads (50 and 141 IU/ml) confirming that the higher the viral load, the better the amplification products.

Finally, we wanted to detect HBsAg mutations that could be associated with the development of OBI. Because of the overlapping reading frame of the polymerase gene and HBsAg, mutations in the polymerase domain may lead to mutations in the preS/S ORF resulting in a modified S protein that is not detected in serological screening tests. Mutations in the S gene are known as "escape variants" and can lead to vaccine escape, and detection failure. It has been shown that mutations responsible for a conformational change within the "a" determinant of HBsAg can lead to failure of serological detection [7]. In the present study, four mutations were detected in the "a" determinant: M133I, Y134F, S136Y, G145R. Three of the mutations (M133I, Y134F, and G145R) have already been associated with diagnostic-escape [29]. The G145R mutation is the most common and best-defined HBsAg mutation that also leads to vaccine-escape [30]. It has been demonstrated that HBsAg serological assay used in this study is highly sensitive for the detection of G145R [31], so multiple mutations interacting with each other may be responsible for diagnostic failure. In addition to the three previously described mutations in the S region [32], we found nine novel mutations that, to our knowledge, have not been previously reported. The following mutations were found outside the "α" determinant of the S gene: F158L, K160N, E164G, S167L, A168V, L175S, S210I and F212C. Most of these mutations that we have found can lead to changes in the amino acids' polarity, from hydrophilic to hydrophobic or *vice versa*. A change in the biochemical properties of amino acids, such as hydrophilicity/hydrophobicity, can change the shape of the protein molecule. Some of the MHR mutations upstream or downstream of the "a" determinant sequence are also known to alter the hydrophilicity of the HBsAg protein; affecting HBV antigenicity and leading to immune escape, or false-negative detection of HBsAg by commercial tests (8). However, further bioinformatic and functional analyses are needed to verify whether the novel amino-acid substitutions we found, alone or in combination, can lead to a conformational change of the S protein and a failure of HBsAg detection. Both, the impact of the individual mutations and the complementary interactions between individual mutants on diagnostic assays, require further study, e.g., cloning of S-gene mutants from serum samples to expression plasmids and their transfection into cells.

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 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: D144E, M133I, M133L, P120S, Q101H, R122K [29]. The immune escape mutation M133I detected by Grgić *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 subgenotype, which is consistent with the distribution of

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.

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Conflicts of interest

None.

CRediT authorship contribution statement

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

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