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Original article

OCCULT HEPATITIS B VIRUS INFECTION AMONG PATIENTS WITH LIVER DYSFUNCTION IN VARNA, BULGARIA

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ABSTRACT

Background: Occult hepatitis B infection (OBI) is a challenge in virology and a clinically relevant topic. The present study assessed the presence of HBV-DNA in serum samples of HBsAg negative, patients with data of liver dysfunction, positive for anti- HBc total Ab with or without anti-Hbs Ab.

Purpose: The goal of this study was to evaluate the prevalence of occult hepatitis B in Varna region, among patients with chronic liver dysfunction.

Materials and methods: The investigation was conducted among 79 people, predominantly patients at Gastroenterology Clinic in the University Hospital St. "Marina", Varna, Bulgaria. Quantitative determination of HBV DNA was performed in the National Reference Laboratory for Hepatitis viruses at the National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria.

Results: From 79 investigated patients with liver dysfunction16 (20. 25%), were considered as occult HBV carriers. Fourteen of them (17.72%) were positive for HBV DNA with very low values, below 200 IU/ml. Two of the cases (2.53%) were with serum levels comparable to those usually detected in the different phases of serologically evident (overt) infection and are considered as "false" OBI.

Conclusions: Our data showed that OBI is more widespread than expected and can be identified as a significant risk factor for the presence of more severe liver damages and an important oncogenic factor for developing cirrhosis and hepatocellular carcinoma.

Keywords: Hepatitis B, Occult hepatitis B infection (OBI), Anti-HBc total Ab, HBV DNA

INTRODUCTION:

Hepatitis B virus (HBV) infection is a considerable global health problem and approximately 2 billion of the world population have been infected, of which 250 million live with HBV infection [1]. In 2012, it ranked as the second leading cause of cancer death worldwide after lung cancer, causing about 600,000 deaths yearly. Among transmission routes, transfusion is the one that should be prevented, as HBV transmission by blood components negative for HBsAg can still occur [2]. Infection by HBV is divided into five clinical categories: asymptomatic, acute, chronic, fulminant and occult hepatitis B (OBI) or "silent" HBV infection [3]. In 2008, in Taormina (Italy), the European Association for the study of the liver (EASL) defined OBI as the presence of HBV DNA(< 200 IU/ml) in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing hepatitis B surface antigen (HBsAg) negative by currently available assays [4].

MATERIALS AND METHODS:

Study population

In our research, we have evaluated 79 patients with clinical data of liver dysfunction at the University Hospital St. "Marina", Varna, Bulgaria from 2015 to 2017. All of the patients were referred to the Laboratory of Clinical Microbiology and Virology at the University Hospital St. "Marina" Varna, Bulgaria. The specimens were stored at -70ºC until use. Seventy-nine patients, 29 women (36.7%, 95% CI: 26.1- 48.3) and 50 men (63.3%, 95% CI: 51.7-73.9), were enrolled in this study. All of them are Bulgarians, from 7 to 94 years old (mean age 54.8).

Methods

Serum samples were investigated for HBsAg, Anti-HBctotal Ab and anti-HCV Ab using commercially available ELISA test kits. EIAs based on the sandwich and competitive ELISA principle were performed, according to the manufactures' recommendations. The serological pattern of occult hepatitis B infection was revealed via following tests: HBsAg (Dia.PRO Milano Italy; ETI-MAK-4 DiaSorin Italy; SURASE B-96 Taiwan), anti HBc-totalAb (HBcAbDia. Pro Milano Italy; Anti-HBc total Elisa

BIOSOURCE Belgium) and for anti-HCV Ab (DIA.PRO Milano Italy; ETI-AB-HCV 3 DiaSorin Italy).

HBV DNA was extracted from 700 µL serum samples with Abbott sample preparation DNA kit according to manufacturer instructions. Quantitative determination of the viral load was performed by Abbott real time kit for HBV DNA with lower detection limit 10 IU/ml and a linear range from 10^1 to 10^9 IU/ml, at NRL"Hepatitis viruses", National Center of Infectious and Parasitic Diseases Sofia, Bulgaria.The assay is standardized against the World Health Organization (WHO) International Standard for Hepatitis B Virus DNA. The target sequence for the Abbott Real time consists of a highly conserved region in the surface gene of HBV DNA, which is essential for assembly and secretion of subviral particles and tolerates only minor structural changes. The choice of this target region means that all HBV genotypes (A to H) can be adequately detected and that the assay sensitivity is not compromised by YMDD mutations[tyrosine (Y)-methionine (M)-aspartic acid (D)-aspartic acid] conferring antiviral resistance and HBsAg escape mutations. The target sequence of the internal control (IC) is derived from the hydroxypyruvate reductase gene from the pumpkin plant *Cucurbita pepo* and is provided as a DNA plasmid in a buffer solution [5].

Statistical analyses

Statistical analyses were performed by SPSS ver. 23 software package. Results were expressed as mean (±) SD or median (range) as appropriate. Data were analysed by Pearson's χ^2test. Two-sided p-values < 0,05 were considered as statistically significant. Confidence intervals (95% CI) were determined using the formula P= p± 1,96 $(pq/n)^{1/2}$, where p is the frequency, q is 1-p, and n is the number of individuals tested in each group.

RESULTS:

Serum samples of 16 patients (20.25%, 95% CI:12.0-30.8) are presented in Figure 1. Five 5 women and nine men, presented in Figure 2 were tested HbsAg negative, anti HBc- total Ab and HBV DNA positive. They were defined as occult HBV carriers, like hepatitis B nucleic acid quantification via PCR with a lower limit of detection 10 IU/ml was the only reliable diagnostic marker.

Fig. 1. Occult HBV prevalence rate in the studied population (n=79)

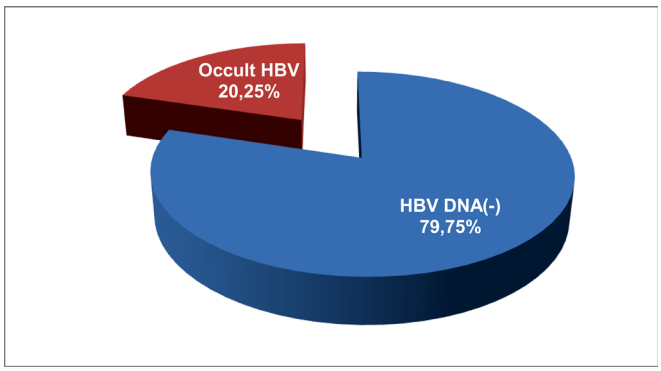
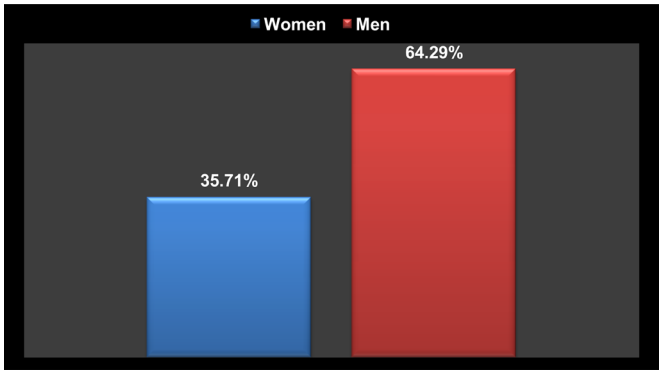
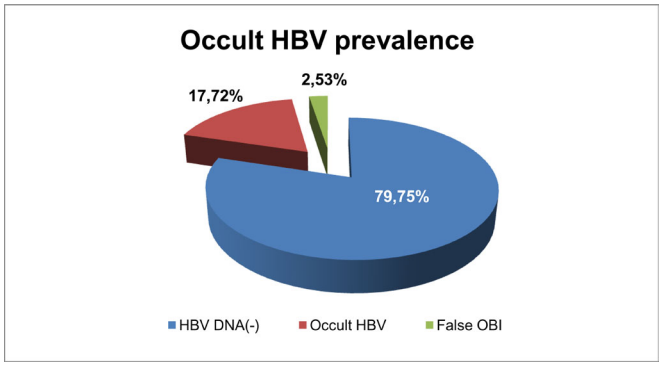


Fig. 2. Occult HBV state, according to the sex distribution.



Fourteen of the participants in the study (17.72%, 95% CI:10.0-27.9) were positive for HBV DNA with very low values below 200 IU/ml. Two of the cases (2.53 %, 95% CI: 0.3-8.8) were with serum levels comparable to those usually detected in the different phases of serologically evident (overt) infection and are considered as "false" OBI. Patients with OBI were from 25 to 94 years old (mean age 54.8), as shown in Figure 3.

Fig. 3. Occult hepatitis B prevalence among Bulgarian patients with liver impairment.



First panel shows the anti-Hbc total Ab (+), HBV DNA (-) participants.Second panel represent the seropositive occult, HbsAg(-), anti- HbcAb (+) and HBV DNA carriers(<200 IU/ml). The third panel is for "false" OBI cases with HbsAg(-), anti- HbcAb (+) HBV DNA (>327 IU/ml).

Pursuant to the HBV DNA levels 5 main groups of patients were identified: 6 patients were with viral load between 10IU/ml and 30 IU/ml (42.9%, 95% CI: 17.7-71.1); 3 between 30IU/ml and 50 IU/ml (21.4%, 95% CI: 4.7-50.8%); 2 between 50IU/ml and 70IU/ml (14.3%, 95% CI: 1.8-42.8); 1 between 70IU/ml and 90IU/ml (7.1%, 95% CI: 0.2-33.9) and 2 patients with viral load up to 200IU/ml (14.3%, 95% CI: 1.8-42.8).HBV viremia ranged from 1×10^1 to $1,3\times10^2$ IU/ml. Ten participants from OBI group (71.4%, 95% CI: 41.9-91.6) were patients at Gastroenterology Department at University Hospital St. "Marina", Varna, Bulgaria. Cirrhosis was evident in seven patients (50.0%, 95% CI: 23.0-77.0), as presented in Table 1.

Table 1. Demographic, clinical and laboratory characteristics of the tested patients.

	Sex	Age	HBV DNA IU/ml	Clinic	Diagnose
1	Female	35	104	Hematology	Acute lymphoblastic leukemia.Liver steatosis.
2	Female	63	82	Internal diseases	Liver cirrhosis
3	Male	54	56	Hepatogastroenterology	Liver carcinoma
4	Male	72	21	Hepatogastroenterology	Liver cirrhosis
5	Male	40	10	Hepatogastroenterology	Toxic liver failure
6	Male	54	63	Hepatogastroenterology	Liver steatosis.Ascites
7	Female	57	130	Hepatogastroenterology	Liver steatosis
8	Male	38	11	Hematology	Essential thrombocytopenia. Liver steatosis.
9	Male	50	23,2	Hepatogastroenterology	Liver cirrhosis
10	Male	56	23,2	Hepatogastroenterology	Liver cirrhosis
11	Male	64	16	Internal diseases	Liver steatosis
12	Female	25	28	Hepatogastroenterology	Liver cirrhosis
13	Male	66	10	Hepatogastroenterology	Liver cirrhosis
14	Female	94	10	Hepatogastroenterology	Liver cirrhosis

Serological marker anti-HBs was not excluded from discussion although the patients hadn't been tested for it.

HBV DNA levels observed in two patients with "false" occult OBI, were higher than 200 IU/ml, introduced as a cut-off value for OBI and were similar to those with evident overt infection, as it's shown in Table 2.

Table 2. Demographic, clinical and laboratory characteristic of the "false" OBI patients.

	Sex	Age	HBV DNA IU/ml	Clinic	Diagnose
1	**Male**	**60**	**327**	**Hepatogastroenterology**	**Liver cirrhosis**
2	**Male**	**35**	**394**	**Internal diseases**	**Liver steatosis**

DISCUSSION:

Occult hepatitis B is a complex clinical entity. In the last decade, there have been significant advances in understanding the molecular mechanisms underlying OBI [6]. In this research, we analyzed a well-characterized group of 79 patients with liver function abnormalities, with the aim to evaluate the prevalence of occult hepatitis B in Varna and the region. The prevalence of OBI varies from 1% to 87% in different regions of the world [7]. During the last 10 years, the application of highly sensitive molecular techniques began expounding occult hepatitis B virological features and possible clinical inferences. The variability relies upon the sensitivity of HBV DNA detection assays, the sample size, and the detection of HBV DNA in liver tissue and serum by nested PCR or real-time PCR [1,8]. As the specific primers used in the real-time PCR cover, all HBV genotypes its applications possesses better outcomes**.**

HBV remains the most frequent transfusion-transmitted viral infection [6, 9]. Regards to eastern countries occult hepatitis B had been reported in 0.1%-2.4% of blood donors [2, 3]. The similar results were established and for the United States, where only 5% of the population has been exposed to HBV. The prevalence of OBI in the Asian population, however, is much higher and ranges from 7.5%-16 %**.** In Korea, HBV DNA was detected in 31/195 (15.9%) healthy Korean subjects without detectable HbsAg [10, 11].The infection was more prevalent in male participants, as compared with female and this socio-demographic result was confirmed with hereby survey.

As a general rule, patients with OBI show anti-HBc total Ab positive and /or anti-HBs Ab positive result, when

serologically tested in the absence of HBsAg-seropositive OBI. About 20 % of the patients with OBI are with seronegative-OBI-anti HBc total Ab and anti-HBs Ab negative [12]. All of the included individuals in the present trial were anti- HBc total Ab (+), without anti- HBs Ab marker tested. Studies have shown that the prevalence of OBI infection is closely related to the endemicity of HBV infection [13].

The prevalence of OBI is estimated to be 4% –25% in anti-HBc Ab positive patients [2]. The majority of individuals with OBI have viral loads less than 10^5 IU/ml, typically 10^1-10^3 IU/ml, interestingly, in a plurality of OBI patients viral load of 20 IU/ml was reported, no matter the age, as per our data [14,15]. Our results confirmed that there were no indicative differences as per HBV DNA levels and age (χ^2= 47,7, p=0,19). Six patients had a viral load between 10 and 30 IU/ml, predominantly men (4.3%), without statistical significance (χ^2=10.09, p=0.73).

WHO reported Bulgaria in the zone of the intermediate prevalence of HBsAg.Varna and the region was in the high intermediate zone– 5.2%, as per aggregated data from our previous survey [12].

We made a seroepidemiological screening survey for HbsAg (-), anti-HBc total Ab (+) people, based on ELISA and CLIA commercially available tests, conducted from 2010 to 2015 [16]. In a general population (n = 2326) we found positive results in 8.25 %. In the patients group (n=1596) with various chronic diseases, 23.6% were HbsAg (-), anti-HBc total Ab (+).The upshot from our studies showed that anti-Hbc total Ab is not the ideal marker for OBI determination, but it can be used as a screening marker and a substitute in the absence of HBV DNA molecular techniques. In addition, anti-Hbctotal Ab is useful in OBI diagnosis, even when HBV DNA is available, because of the possibility of intermittent viremia [15].

Numerous studies have shown that the molecular basis of HBV persistence in HbsAg-negative subjects involves a combination of viral and host-dependent factors [17].The molecular basis of persistent OBI is related with genome integration as viral genome can persist indefinitely by conversion to a covalently circular HBV DNA (ccc) DNA in the hepatocyte, binding to proteins and forming a chromosome. A small number of cases of OBI are due to infection with HBV mutants [4,8,13]. Genetic mutations and deletions in HBV such as mutations in the "a" determinant of HbsAg (sG145R mutation) can bring about the structural arrangement of the protein and lead to undetectable HbsAg. Mutations in the pre S1 and pre S2 region also can lead to lack of detectable HbsAg in serum [7,18]. Other possible mutations are treatment-associated mutations (lamivudine treatment) and splicing (nonfunctional DNA). HBV immune complexes with anti-HBs can impair HBsAg detection by conventional serological assays and leads to OBI. We have not studied the genetic causes in our patients' cohort with OBI. Our data showed predominantly low levels of viral load in 16 patients (20.25%). In 2 of them (2.53%), we can assume undetectable escape mutants.

Half of our participants were with cirrhosis, and one was with liver cancer. Results were demonstrative when comparing diagnosis and HBV DNA levels (χ^2= 57.62, p=0,009). Historically, patients with chronic hepatitis B and cirrhosis have a poorer prognosis, including greater risk for development of HCC, than their chronic hepatitis counterparts without cirrhosis [19].

Coinfection with hepatitis C virus (HCV) is well documented. In the recent survey, we found 4 patients (5.1 %, 95% CI: 1.4-12.5) anti-HCV Ab (+), anti HBc total Ab (-). One of them - a woman with liver cirrhosis was with viral load 82 IU/ml and was registered with OBI. Results showed no statistical significance between men and women (χ^2=0.37, p=0.54). It has been described that about one-third of patients with chronic HCV infection had detectable serum HBV DNA but undetectable HBsAg. The presence of OBI in chronic HCV infected patients increases the risk of hepatocellular carcinoma (HCC) [20].

CONCLUSIONS:

The study of occult HBV infection should involve routine, screening, serological methods and molecular assays techniques of DNA extracts. The infection is often the result of multiple mechanisms and prevalence seem to be higher among subjects at high risk of HBV infection and with liver disease than among individuals at low risk of infection and without liver disease [18]. Our data showed that OBI is more widespread than expected and can be identified as a significant risk factor for the presence of more severe liver damages and an important oncogenic factor for developing cirrhosis and hepatocellular carcinoma.

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