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TOPIC HIGHLIGHT

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Diagnostic strategy for occult hepatitis B virus infection

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Abstract

In 2008, the European Association for the study of the liver (EASL) defined occult hepatitis B virus infection (OBI) as the "presence of hepatitis B virus (HBV) DNA 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". Several aspects of occult HBV infection are still poorly understood, including the definition itself and a standardized approach for laboratory-based detection, which is the purpose of this review. The clinical significance of OBI has not yet been established; however, in terms of public health, the clinical importance arises from the risk of HBV transmission. Consequently, it is important to detect high-risk groups for occult HBV infection to prevent transmission. The main issue is, perhaps, to identify the target population for screening OBI. Viremia is very low or undetectable in occult HBV infection, even when the most sensitive methods are used, and the detection of the viral DNA reservoir in hepatocytes would provide the best evaluation of occult HBV prevalence in a defined set of patients. However, this diagnostic approach is obviously unsuitable: blood detection of occult hepatitis B requires assays of the highest sensitivity and specificity with a lower limit of detection < 10 IU/mL for HBV DNA and < 0.1 ng/mL for HBsAg.

Key words: Occult hepatitis B virus infection; Hepatitis B surface antigen; Hepatitis B virus DNA; Anti-HBc

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INTRODUCTION

According to European Association for the study of the liver (EASL), about one third of the world's population have serological evidence of past or present hepatitis B virus (HBV) infection, and more than 350 million people may be affected by chronic HBV infection[1]. In addition, chronic HBV infection is the worldwide primary cause of cirrhosis and hepatic cellular carcinoma, and it is among

the top ten causes of death[2]. The clinical evolution of HBV is variable, ranging from mild liver disease to fulminate hepatitis, cirrhosis, or hepatic cellular carcinoma (HCC). In some individuals, in whom the HBV infection persists, serological markers can identify different clinical states of viral persistence[2,3].

Chronic hepatitis B

Patients with hepatitis B surface antigen (HBsAg) detectable for six months or more are defined as having chronic hepatitis B. Usually these patients have elevated serum liver enzymes, high levels of HBV DNA, and high risk of transmission, both related to the positivity of the hepatitis B e antigen (HBeAg). They also have the highest risk of cirrhosis and HCC[2]. In some patients, HBeAg is undetectable, in spite of persistent replication of the virus.

In these patients, the virus has mutations that prevent expression of the "e" protein. The mutations are located in the basal core promoter (BCP) region (A1762T and G1764A) and in the precore (PC) region (G1896A) of HBV genome[4]. These variants are more common in Mediterranean countries and Asia. The appearance of hepatitis B e antibody (anti-HBe) does not necessarily indicate clinical improvement. The HBV DNA levels in these subjects tend to be lower[5].

"Healthy" carrier

These patients are characterised by a positive HBsAg that persists more than six months, but with normal liver enzymes values. They are negative for HBeAg, and are associated with low or undetectable serum HBV DNA and low risk for progression to cirrhosis or HCC[2].

Occult hepatitis B infection

Owing to modern molecular analysis we know the viral genome of HBV can persist indefinitely in previously infected HBsAg-negative subjects[5]. This persistence occurs by conversion to a covalently closed circular HBV DNA (ccc) DNA in the hepatocyte, which then binds to proteins, forming a mini chromosome. This cccDNA is the molecular basis of occult hepatitis B infection because to its stability and long-lasting persistence in the nuclei of hepatocytes[6].

SEROLOGICAL PATTERN OF OCCULT HEPATITIS B INFECTION

The antibodies produced by the host and proteins released from the virus provide us with valuable information. Within the group of occult hepatitis B infection (OBI) patients, it possible to observe differences based on the results from serological markers.

Seropositive subjects

These are OBI subjects with anti-HBc and/or positive anti-HBs in which serum HBsAg is not detected because of the resolution of acute hepatitis B (after a few months of HBsAg carriage) or after years of chronic HBsAg positive infection[1]. Thirty-five percent of patients with OBI have positive anti-HBs and forty-two percent of have positive anti-HBc[2]. The HBV DNA detection rate is higher in individuals who are positive anti-HBc but negative for anti-HBs. When patients give a positive result for both antibodies, they have intermediate HBV DNA levels[3]. One explanation for this serological pattern is that positive anti-HBc patients with chronic HBV infection clear HBsAg to an undetectable level, with or without anti-HBs: this pattern is associated with older age and anti-HBe[5].

Seronegative OBI

Patients who are not positive for anti-HBc and anti-HBs represent twenty-two percent of OBI patients[2]. They have very low levels of HBV DNA[7]. This pattern of antibodies may appear from the beginning of the infection when patients have not yet developed positive hepatitis B specific antibody ("primary OBI") or because of clearance of the hepatitis B specific antibodies[1]. Therefore, this pattern should always be kept in mind, because almost anybody can be a potential carrier of occult B hepatitis.

Moreover, there are cases termed as "False" OBI. They are carriers of mutations in HBsAg (in the S gene) that are not recognized by some routine detection assays. In these cases, the DNA result resembles other cases of HBV, because they are usually positive for HBsAg[1].

MARKERS FOR SCREEN OBI PATIENTS

HBsAg

OBI diagnosis is based upon detection of HBV DNA when HBsAg is absent. It is very important to define the optimal methodology to test this marker to prevent false positive results, depending on HBsAg assay sensitivity.

The quantification of HBsAg presents several problems associated with the virus, the host, or the test kits. To correctly test HBsAg, International Standard samples with known quantities of HBsAg are required. Thus, using World Health Organization (WHO) International Standard for HBsAg (00/588) with a potency of 33 IU/vial, it is possible to know that 1 international unit (IU) is equivalent to 5.6 Abbott ng, 1.9 French ng, and 0.43 PEI units or ng (confirming that ng values applied to other standards are not equivalent and some of the values have changed over time)[7].

The correlation between the HBsAg and the number of HBV particles is a key point, because this marker is used with a wide range of HBV particles in blood, depending on the infection state. One ng of HBsAg protein is equivalent to approximately 2×10^8 22 nm subviral particles and to approximately 5×10^7 HBV particles (assuming the virus particles have a four times larger surface). Different assays for testing HBsAg have variable detection limits, from 0.04 to 0.62 ng/mL. At a detection limit of 0.04 ng/mL, there must be around 2 million particles in the blood for a positive result. The presence of excess

Table 1 Serological markers and hepatitis B virus DNA in different states of persistence of hepatitis B virus[2,4]

	HBV DNA	HBsAg	Total anti-HBc	anti-HBs	HBeAg	anti-Hbe
Chronic hepatitis B	+++	+	+	-	+	-
Chronic hepatitis B with variants pre-core	++	+	+	-	-	+/-
Healthy carrier	$< 10^6$ IU/mL	+	+	-	-	+
OBI seropositive	< 1000 IU/mL	-	+	+/-	+/-	+/-
OBI seronegative	< 1000 IU/mL	-	-	-	-	-

HBV DNA data are copies/mL. -: Negative, the marker is not present in the serum of the patient; +/-: The marker can be present in the serum of the patient; +: Positive, the marker is present in the serum of the patient; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; anti-HBs: Hepatitis B surface antibody; HBeAg: Hepatitis B e antigen; anti-Hbe: Hepatitis B e antibody; OBI: Occult hepatitis B virus infection; IU: International unit.

subviral particles can improve detection.

A level of 2 million HBV genomes/mL (quantity estimated at the time of HBsAg seroconversion) is considered to exist in a ratio of 1:1000 for the amount of HBsAg in the virus to that in subviral particles[8]. However, this is not always true. Minegishi *et al*[9] found six HBsAg seroconverters among 76 prism-negative blood donations with $> 10^4$ genomes/mL, generating a ratio of 1: < 200. Gerlich *et al*[8] found a blood donor in the incubation phase with a ratio of 1:100, which became 1:500 during the chronic phase.

Accordingly, the ratio is variable, from 1:100 in patients with OBI (HBsAg negative with high levels of HBV DNA) to 1:100 000 or more when HBsAg is detected in association with low concentrations of HBV DNA. Most HBsAg commercial assays are able to detect all genotypes and subtypes of the wild-type virus, but some of them may miss mutations in the S region[9,10]. Usually, wild-type virus is the dominant species detected at the beginning of the infection; however, mutations can increasingly appear because of the lack of viral proofreading exonuclease activity.

Mutations in the S gene cause changes in the amino acids of the "a" region, which is very important for inducing immunity, being a target of anti-HBs[7,10]. Immunological pressure may cause a decrease of HBsAg, but might favour the selection of HBsAg mutants. Thus, the presence of anti-HBs and the clearance of AgHBs do not necessarily reflect viral clearance.

In this apparent "resolved" infection, cytotoxic T cells are responsible for controlling the replication (but absolutely eliminating it). The role of Anti-HBs would be in controlling traces of circulating virus, although there is a risk of selecting mutants. This process may underlie seropositive OBI, and it is very important in patients under immunosuppressive therapy, in patients with liver disease, or in cases of blood donors, because of the risk for transmitting the virus[8]. Mutants have also been detected in vaccinated patients, in patients who have been treated with hepatitis B immunoglobulin, and in patients with chronic infection[11]. As a consequence, all patients with a serological pattern consistent with possible OBI should be investigated to rule out HBsAg mutants. HBsAg should be tested with an alternative method that can detect the most common mutants. Quantitative HBV DNA testing should also be considered[11].

HBV DNA

The gold standard for OBI diagnosis is the study of extracted DNA (from liver or blood). For this purpose, a very sensitive and specific assay is required. The experts meeting in Taormina[1] recommended assays with detection limits of less than 10 copies of HBV DNA per reaction. Current technologies used for DNA detection are: nested-PCR, real-time PCR, and transcription based mediated amplification (TMA). Using these assays, it is possible to decrease the lower detection limit (< 5 IU/mL of HBV DNA). This is particularly important in OBI, because the HBV DNA levels vary from < 10 to 425 copies/mL. However, the false negative and positive rates are around the cut-off level due to the Poisson distribution of the virions and blank specimens[7].

According to Taormina Group recommendations, primers must be specific for different HBV genomic regions and be complementary to highly conserved (genotype shared) nucleotide sequences[1]. Usually, the genes amplified by PCR are S and X; the former has been found to be the most sensitive in serum, and the second has been described as the most sensitive in the liver[2]. The use of very sensitive methods, such as PCR, increases the risk of false positive results, due to contamination or to different amplicons within the target. To resolve these problems, inclusions of appropriate controls in each assay run, as well as sequencing the amplicons, are recommended. In the case of serum samples, it is recommended DNA from is extracted from at least 1 mL of serum[1,2].

Differences can be observed because of the different amounts of material used in the assay. To reduce this problem, it is possible to quantify the DNA in comparison with, or normalized to, a host cell gene (such as beta-globin)[1]. DNA detection from a liver biopsy would be the best option because of the persistence of viral genomes in hepatocytes. For this procedure, frozen samples are preferred to formalin fixed tissues[1,7].

Usually, when a blood sample is positive for HBV DNA, the liver sample is too; however, HBV DNA from the liver can be detected even when HBV cannot be detected in serum. Patients with undetectable HBV DNA in the liver have also undetectable levels of HBV DNA in peripheral blood[2]. However, there are no standardized assays and liver specimens are not routinely obtained[7]. Commercial assays use serum or plasma to de-

termine the presence and the amount of HBV DNA. In some studies using the woodchuck model, occult WHV was shown to persist in peripheral blood mononuclear cells (PBMC)[13,14]. Samples for studying HBV DNA should be collected and stored in appropriate conditions, and the risk of cross-contamination should be avoided[1]. However, which is the best DNA extraction method to apply in the OBI diagnosis remains unclear[2].

To reduce variability and risk of contamination, reagents that are ready to use or that use automatic systems to extract DNA have been proposed. Nine years ago twenty-two laboratories participated in an international collaborative study to establish a WHO international standard (97/746) for HBV DNA nucleic acid amplification techniques (NAT)[15]. A subtype adw2 genotype A isolate was used. Based on this study, one IU of the standard is equivalent to 6.31-6.42 genomic equivalents (geq) if a PCR assay is used. Therefore, the results of the DNA for HBV must be expressed as IU/mL, but accurate conversion factors depend on the chemistry used for HBV DNA quantification, and range from 5.26 to 7.3 copies/IU. Despite these attempts, there is still variability in the results; therefore, using one assay should be used to monitor any particular patients or group of patients[7]. In case of blood banks the NAT is used to screen for Hepatitis C virus (HCV), Human Immunodeficiency Virus 1 (HIV-1), and HBV. Plasma pooling is often used because of high cost issues, which introduces a dilution factor and decreases the sensitivity of the assay. This is critical in the case of OBI, because the levels of HBV DNA are very low. Moreover, the use of plasma pooling can be aggravated by using triplex assays to detect the three viral genomes at the same time. González *et al*[16], using blood donors in Madrid, found that donors in the window period and donors with OBI were not uncommon. Furthermore, they were detected at a higher frequency using individual nucleic acid testing (NAT) than with minipool NAT blood.

Other assays to detect a genome use a probe labelled with a different dye to permit the identification of the different viruses. However, a positive result from this type of screen needs to be confirmed by other tests[17].

Anti-core antibody

This is the first antibody to appear, even preceding HBsAg, and targets the nucleocapsid of HBV. The anti-core antibody can induce anti-HBc responses without T-cell activation. This antibody can be found in almost every patient with a previous contact with HBV, even in HBV carriers without other responses. This serological pattern is called "anti-HBc alone", and might reflect an occult HBV infection. Anti-HBc is present in the different phases of hepatitis, including recovery, and may persists longer than anti-HBs or anti-HBe; however, it is not protective. Anti-HBc IgM may help in the diagnosis of the acute phase. Moreover, this IgM can be positive during flares[3].

In some patients, anti-HBc cannot be detected at any phase of HBV infection because of a defective host immunological response (such as in HIV coinfection or organ transplantation) or virus infection by variants of HBV[3,8]. Although anti-HBc is not an ideal marker, the Taormina group recommended its use as a surrogate marker whenever an HBV DNA test is not available to identify potential seropositive OBI individuals such as in cases of blood, tissue or organ donation, or in cases of patients undergoing immunosuppressive therapy[1].

In addition, anti-HBc determination is useful in OBI diagnosis, even when HBV DNA is available, because of the possibility of intermittent viremia[3]. In such cases, not all anti-HBc positive individuals are positive for HBV DNA, and anti-HBc tests might provide false-positive results[1]. Furthermore, the absence of this antibody does not exclude OBI (seronegative OBI). If this marker is used in combination with HBV DNA, the prevalence of HBV infection in the area should be considered, because when prevalence of anti-HBc is higher than a 50% of the donor population, a positive result is unhelpful[17].

Anti-surface antibody

This is the last antibody to appear (about three months after acute phase), and it is able to neutralize the virus. In vaccinated subjects it is the only positive marker.

This antibody can be used with anti-HBc to study the serological status of patients with a probable OBI.

TARGET POPULATIONS FOR INVESTIGATING THE PRESENCE OF OBI

Investigation of possible OBI should be done in case of blood and solid organ donors, because of the risk of transmission to others[7]. The study and monitoring of reactivation in case of serologic markers of past HBV infection must also be done in patients undergoing immunosuppressant therapy, because of the risk of reactivation after the therapy.

Reactivation risk can be explained if immunosuppression permits viral replication, represses the function of immune cells, and after the treatment, the response of the immune system is exaggerated, leading to cellular injury. The main factors for reactivation are positive HBsAg, grade of immunosuppressant, liver disease, primary malignancy, and toxicity of these drugs. Among the group of patients treated with immune suppressors, there are patients with autoimmune liver diseases. In this group Georgiadou *et al*[18] studied the prevalence of OBI in patients treated with immunosuppressants, and they concluded that there was a significantly higher proportion of OBI cases among these patients compared to blood donors. Interestingly, under immunosuppression, these patients did not seem to deteriorate during the follow-up[18,19].

Patients undergoing haemodialysis must also be studied for OBI because of the risk of reactivation due to immunosuppression and the risk of infection[7].

On the other hand, patients with chronic hepatitis C, patients affected by a Hepatic cellular Carcinoma, and patients with cryptogenetic liver disease, must be investigated for OBI because of its possible influence on the development of these diseases[7].

In case of pregnant women, Kwon *et al*[20] studied the prevalence of HBV DNA in 202 healthy pregnant women. They concluded that the vertical transmission of OBI through the cord blood does not represent a clinical problem because of the low HBV DNA level in the mother's blood, although they acknowledged that more studies are needed.

CONCLUSION

The study of occult HBV infection involves serological and molecular assays. The serology should include, firstly, AgHBs. This test must be done using the most sensitive method, because, depending on this result, the hepatitis B infection can be further classified. With a negative HBsAg result, HBV DNA should be studied only in cases previously exposed, to rule out OBI. Moreover, serological tests should include anti-HBc and anti-HBs.

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