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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

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

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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