

The Pigeonhole of Occult Hepatitis B

Payam Dindoost¹, Narges Chimeh², Blain F. Hollinger³, Esmail Saberfar⁴,
Mehdi Norouzi⁵, and Seyed Mohammad Jazayeri⁵

¹ Department of Gastroenterology, Middle East Liver Diseases Center (MELD Centers), Tehran, Iran

² Department of Psychiatry, Family Research Institute, Shahid Beheshti University, Tehran, Iran

³ Department of Gastroenterology, Baylor College of Medicine One Baylor Plaza, Houston, USA

⁴ Department of Virology, Bayerpaul (BP) Vaccines and Pharmaceutical Company, Tehran, Iran

⁵ Department of Virology, Faculty of Public Health, Tehran University of Medical Sciences, Tehran, Iran

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Abstract- Occult Hepatitis B (OHB), or persistent hepatitis B virus (HBV) viremia in surface-antigen-HBsAg-negative patients, has been recognized as a medical concern during the last decade. The exact magnitude, pathogenesis and clinical relevance of OHB are unclear. This review organizes the published data on OHB and presents an overview of the current hypotheses on OHB's pathogenesis and clinical relevance. Many explanations have been offered for the pathogenesis of OHB, ranging from the inability of standard immunoassays to diagnose OHB to the involvement of the versatile virus-host factors. Also, special care should be taken regarding the diagnosis of OHB. It seems that both shared viral-host factors are involved in the pathogenesis of OHB. Further molecular studies on cohort patients group need to explore such association.

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Introduction

Hepatitis B virus (HBV) infection is usually diagnosed when circulating hepatitis B surface antigen (HBsAg) is detected. Evidence is accumulating that HBV is present in patients who are HBsAg negative but contain HBV DNA in serum and or liver tissue. The medical literature refers to this as Occult Hepatitis B infection, or OHB.

This type of infection is detectable by polymerase chain reaction (PCR) and has been classified into seropositive and seronegative infection depending on the positivity of anti-core antibody (HBc) and hepatitis B surface antigen (anti-HBs) (1-3). Occult HBV's real place in the clinical and biological spectra of HBV infection is not well known. Long-term studies of HBV chronic and recovered infections have shown that after HBsAg is no longer detectable, HBV DNA persists for years in serum or the liver (4).

Many issues are still open regarding occult HBV infection, beginning with the diagnostic criteria, which may have implications for liver transplantation.

OHB pathogenesis

The reasons for the lack of circulating HBsAg in OHB patients are unclear. The molecular basis of HBV persistence in HBsAg-negative subjects is believed to involve a combination of viral and host-dependent factors (Figure 1). Occult HBV infection is associated with ongoing virus replication in animal models (5), and the passaged particles might be able to infect virus-naïve animals (6,7), indicating long virus persistence. A number of explanations for the persistence of HBV DNA in HBs-negative samples have been proposed, including HBV DNA in low-copy numbers (8), altered host immune response (9), genetic variations of the S gene (10), viral DNA integration in the host genome (11), infection of peripheral blood mononuclear cells (12), immune complexes in which HBsAg is hidden (13,14), and interference of other viruses such as HCV (15-18) and HIV (Figure 1) (19-22).

Rearrangement in the HBV genome is one of the exceedingly proposed mechanisms for HBV DNA persistence (Figure 1). Mutation within the surface gene of the HBV genome is one of the factors contributing to the loss of HBsAg detection by immunoassay. HBV

Corresponding Author: S.M. Jazayeri

Department of Virology, Faculty of Public Health, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 88992660, Fax: +98 21 88992660, E-mail address: jazayerism@tums.ac.ir

surface gene mutants interfere with gene expression or produce an antigenically modified S protein (3). The “an” antigenic determinant is the primary determinant of HBsAg. Mutation in the “a” determinant may cause a conformational change in HBsAg, leading to undetectability of HBsAg as well as evading the host’s immune response (23). The most well known mutation within the “a” determinant is a glycine-to-arginine change at codon 145, created by a G-to-A substitution at

nucleotide 587 (3,23-25). Mutations at amino acid positions 120, 143, and 144 can result in poor reactivity with d/y subtyping monoclonal antibodies (26,27), and mutations in cysteine residues within the “a” determinant have been found to be essential for the antigenicity and presumably for the conformation of the protein (28).

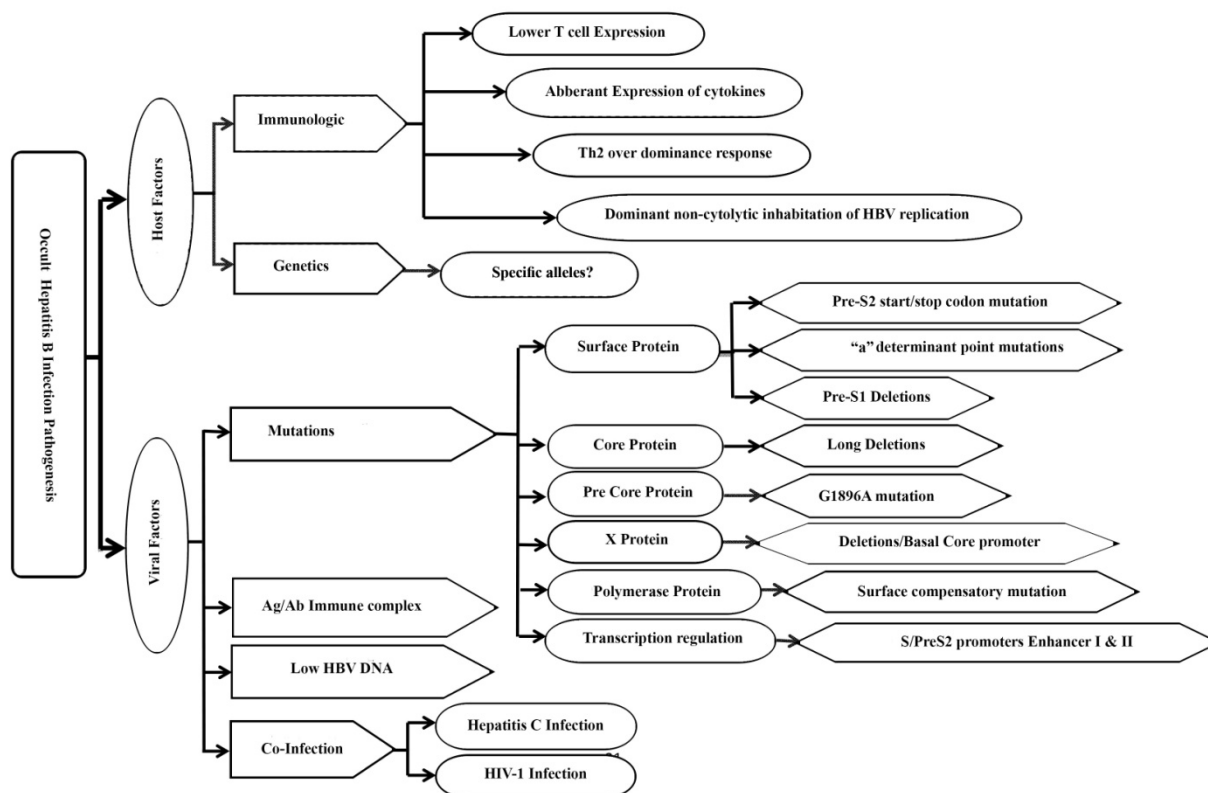


Figure 1. An algorithm showing the hypothesized pathogenesis of occult hepatitis B

Mutation outside the surface protein may also influence HBV replication capacity. According to earlier studies, these mutations have been reported to have a lower “replication fit” compared to the wild-type virus in vitro, providing a plausible explanation for the low HBV DNA levels (29,30).

Due to the absence of a crystallographic structural model, it is not possible to empirically predict the impact of a given mutation on the structure of HBsAg. Furthermore, multiple mutations and polymorphisms outside the surface protein could be found within the core, pre-S, X, and polymerase Xs, with known key regulatory functions, including enhancer regions (31,32), hyper editing APOBEC3G (31), altered methylation of HBV genome (31,32), altered RNA

splicing due to deletion in the core-surface interface (33), point mutations and deletions in the Pre-S1 and Pre-S2 promoters altering the ratio between small and large surface proteins (1,32,34), defects in polymerase protein due to point mutations in TP (35), and truncated precore/core-poly start codon (1). In these cases, a weak HBV replication, leading to levels of viral particles in the serum that are insufficient to allow HBsAg detection by diagnostic assays, could be the cause of HBsAg seronegativity.

Additionally, altered host immune factors, including lower T-cell expansion (CD4) (9), aberrant expression of cytokines (36,37), DNA damage of peripheral blood lymphocytes (38), and defects in the T-cell expansion and production of cytokines (9,36), might lead to

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cellular demise and immune hyporesponsiveness in occult hepatitis B infected patients (Figure 1). Involvement of CTL non-catalytic response (mediated by TNF- α and IFN-gamma cytokines) in the pathogenesis of blood-borne viruses (like HBV) plays a dominant role in the clearance of virus (39). However, this type of T cell response seems unable completely to eliminate viruses including HBV, leading to cryptic infection. In this scenario, the viral genetic changes might not be the cause of occult HBV infection but could instead be characteristic of less fit viral sequences that are not cleared as quickly from the liver by the immune system (31). Thus, the low HBV replication might also be due to the kind of balancing between a minute (and undetectable) production of antigen and an inefficient host immune system.

In the aspects of mutations involvement, revealed by sequencing application in most studies, the pattern of OHB could not be unraveled by the sequencing variations alone. Specifically, the current data suggest that the types and locations of mutations do not readily distinguish occult HBV from non-occult HBV at the level of an individual sequence, even if the entire genome is analyzed. Additionally, the differences in the geographical origins of the patients could, in part, account for sequence variations. Finally, infections of HBV mutants or simultaneous infections with wild-type and mutant viruses cannot be ruled out. It is possible that a minor amount of wild-type HBV in a population pool as a quasispecies coexists with the variant but cannot be detected by the use of a sequencing analysis. Therefore, the true proportion of patients carrying HBsAg variants should be higher than the rates found in previous studies.

Nonetheless, accumulating evidence indicates that the genomic heterogeneity of the virus does not account for its occult status in most cases (18, 40-42).

OHB epidemiology

The prevalence of occult HBV infection varies significantly between countries, geographic regions and patient populations (8,40,43). The discussion below provides several possible explanations for the differences in reported prevalence rates.

First, the technical differences might explain some of the discrepancies among these studies. Because the majority of individuals with occult HBV have viral loads under 10 viral copies/mL (typically in the range of 101 to 103 copies/mL), the sensitivity and specificity of the assays are important for its complete identification (44-46). Thus, the conflicting results may be explained

in part by the differences in the assay's sensitivity limits used to detect HBVDNA. For example, three studies examining OHB infection in South African AIDS patients found prevalence rates of 23%, 31%, and 88.4% (21,47,48). Also, the type and the number of HBV genomic domains examined might influence the results. In our results on the prevalence of OHB in cryptogenic cirrhosis, we found that the standard PCR-BASED based on the pre-S1 region was the most sensitive among the remaining parts of the HBV genome (42). In other studies, however, X (14,49,50), surface (49,51), poly (52), and core (14) (Karayiannis, personal communication) regions were the most sensitive parts.

Second, the nature of the specimen and the compartment explored (liver, serum, PBMC) may influence the results. Although HBVDNA detection in liver tissue is the standard for revealing OHB (53), HBV-DNA detection in serum or plasma using the correct biomolecular approach is easier to perform in large series studies because it can frequently be repeated and can identify individuals with active HBV replication. In a study by Knoll of 3,004 German donors, 552 (18.4%) had isolated anti-HBc, and among them, 44 (8%) had OHB in their serum, but in 39 liver biopsies of the same patients, 41% had OHB (54). Also, the method of extracting DNA from the specimen could have significantly affected the results.

Third, the geographical distribution of HBV infection is another potential explanation of the discrepancies among previous studies. Many studies have shown that the prevalence of occult HBV infection in HBV-endemic areas is higher than in regions with low HBV prevalence rates, and the differences in OHB prevalence might be closely related to the endemicity of HBV infection (8,55). In a Sicilian study, where the prevalence of HBV infection was quite high (15), the prevalence of OHB was reported to be 33%, whereas the distribution of 6.7% was reported in another study that used specimens from patients living in northern Italy, where the prevalence of HBV infection is lower (17).

These factors should be taken into consideration when determining the prevalence of occult HBV infection. Thus, knowledge of the prevalence of occult HBV infection is important for epidemiology and public health, especially in areas where HBV infection is prevalent.

OHB clinical relevance

OHB infection has been linked to several factors, but it is still unclear whether OHB is a result of a window period of seroconversion from acute HBV infection,

reinfection, immunological complexes, sequence divergence, poor laboratory detection (due to low-level HBs antigenemia), underlying HCV/HIV co-infection, immunosuppression or other host factors (2, 48, 56-58).

In general, the research on OHB research can be divided into two major perspectives. The first view includes researchers who believe that OHB is an important problem and that immune suppression poses a significant risk for HBV reactivation in patients receiving chemotherapy who require protection with an antiviral agent (59, 60). The second set of researchers, although recognizing the condition, consider that OHB is a diagnostic problem of concomitant HBV infection with minimal or no bearing on the severity of liver disease and that OHB is more a diagnostic problem of low HBV replication and not clinically relevant for the majority of patients. The latter view underscores that OHB may simply represent the late phase of HBV infection in which specific T-cell responses keep HBV replication under control (61). Despite the observation that occult HBV genomes may be infectious, it is not clear whether cryptic infection in the absence of detectable HBsAg is associated with increased morbidity and mortality from chronic liver disease and hepatocellular carcinoma (HCC) in patients with non-HBV cirrhosis or who have had a liver transplant. Furthermore, it is not established whether such patients require long-term protection against HBV reactivation with an anti-HBV agent.

Given that occult HBV infection has been found in advanced liver diseases and in liver cancer tissue, interest is increasing as to whether occult HBV infection may contribute to acute exacerbation and development of HBV-associated diseases such as progression of liver disease, cirrhosis, and hepatocellular carcinoma. In addition, strong evidence shows that the risk of cirrhosis and HCC development is higher in patients with occult HBV (15,58,62-64). Occult HBV infection has also been reported in populations without symptomatic liver diseases, such as blood donors, individuals with normal liver tests, and general populations (65,66). In contrast, other studies have failed to demonstrate any significant association between occult HBV infection and the severity of liver disease (67,68).

Another important debate surrounds occult HBV infection transmission to others. Occult HBV has been implicated in the transmission of the HBV via transfusion of blood and blood products and transplanted solid organs (58,68-70). Other studies have also documented in the transmission and development of OHB's via transfusion of blood or blood products or

organ transplantation, from mother to child, and from humans to chimpanzees (8,61,71-74). Additionally, studies have found that low levels of HBV DNA can be transmitted in humans (75), chimpanzees (76), and woodchuck animal models (77). Finally, occult HBV can be transmitted with few or no signs of ongoing viral replication (62,71,76,78).

It is unknown whether detection of very low levels of HBV DNA has any relevance in clinical practice. However, a major question about occult HBV is whether such small amounts of HBV DNA are associated with progressive liver damage. In the vast majority of patients with OHB infection, low threshold level of HBV DNA (<1,000 copies/mL) may not be sufficient to cause progressive liver disease in immunocompetent chronic HCV patients, as demonstrated in previous studies (79). In a recent survey, 49 recipients of blood transfusions traced from 10 donors with OHB in a retrospective study. Only one recipient had a sequence homology of 95% with her donor. Furthermore, after inoculation of 2 donor's serum samples to 4 chimeric mice, only one mouse contained HBV DNA (80). On the contrary, a low-level HBV infection can reactivate in the immunocompromised individual, resulting in profound liver injury and liver failure (60,81).

OHB diagnosis

Although numerous studies have examined occult HBV infection in immunocompetent patients, the precise prevalence of this clinical entity remains difficult to define. The frequency of diagnosis depends on the relative sensitivity of both HBsAg and HBV DNA assays as well as on the prevalence of HBV infection in the study population. Also, the literature has clearly demonstrated that the prevalence of occult HBV infection is dependent on the time interval from HBsAg clearance to HBV DNA assessment, with longer time intervals being associated with lower prevalence rates of occult HBV infection (15, 82).

Specimen

Considering that viremia is very low or undetectable in occult HBV infection (even when sensitive methods are used), and assuming that the viral cccDNA reservoir is located in hepatocytes, liver-tissue extracts provide the best evaluation of occult HBV prevalence in a defined set of patients (43,61). The detection of HBV DNA in the liver could simply represent integration of HBV DNA into the host genome rather than an intact virus (15). Thus, the diagnosis of occult HBV infection might be better reserved for those

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in whom HBV DNA is detected by two independent sets of primers in the sera or in those with cccDNA detected in the liver (72).

HBsAg Serology. HBsAg detection is the mainstay for the diagnosis of infection and the routine screening of blood donors. A wide variety of commercial assays is available for the detection of HBsAg. Clearly, the sensitivity of an assay to detect a variant is dependent on the anti-HBs used. Thus, it is not surprising that there are examples of variants that cannot be detected by all assays. Many test manufacturers have improved their HBsAg screening assays by replacing the monoclonal tracer antibody with polyclonal antibody (ies) against both wild-type and mutant HBV strains. The polyclonal capture, antibody-based assays showed a significantly better recognition of HBsAg mutants. A large number of HBsAg mutants are thought to be able to influence the performance of commercial assays (83). Although currently available HBsAg assays are superior to their predecessors, more developments are needed to improve the ongoing assessment of the ability of assays to detect a wide range of HBsAg variants. Several studies compare the sensitivity of modern assays for the detection of HBsAg variants in different panels (84-87). Regardless, if the level of antigenemia is low, as is generally the case in healthy blood donors with inactive hepatitis B, low levels of circulating HBsAg mutants may not be detected, even if the immunoassay is capable of detecting the recombinant form of the corresponding mutation (88).

In regard to OHB, the cutoff for HBsAg detection based on viral load is reported to range from 720 to 1,1431 copies/mL (180 to 2,858 IU/mL assuming 4 copies or geq/mL). Below these values, the HBsAg assay is usually nonreactive, whereas the reverse occurs at higher levels (89).

Molecular tests

In patients with OHB, serum DNA levels are frequently below the limits of detection for many of the quantitative assays. To date, there is no standardized method of viral quantification for low DNA levels in patients with occult HBV infection (Carman, personal communication) (61). Serum HBV level is usually less than 104 copies/ml in these patients (45,61). Because the majority of individuals with occult HBV infection have very low viral loads, more sensitive methods should be applied to identify occult HBV infection. A variety of testing methodologies are available to quantify HBV DNA, and it is virtually impossible to make direct comparisons due to the absence of standardization values.

Commercial molecular assays are routinely used in diagnostic laboratories, which are reported to vary significantly in sensitivity and specificity, linear range, genotype inclusivity, and sample volume; they may be insufficient for detecting very low HBV-DNA levels typical in patients with occult HBV infection. These empirical discrepancies appear to be mainly dependent on the different sensitivities and specificities of the assays used in the various studies, all of which limited testing for occult HBV to patient serum. Thus, assays of the highest sensitivity and specificity must be used for the diagnosis of OHB. As of this writing, the optimal standard for diagnosis is the analysis of HBV-DNA extracts from plasma performed by real-time, polymerase chain reaction (PCR) techniques. Quantitative real-time PCR is an effective method to detect occult HBV infection in blood centers. When the procedures are simplified, and the problem of contamination and high cost is overcome, quantitative real-time PCR should be the preferred method to detect occult HBV infection. The standard lower limit of detection (LLOD) for HBV DNA is 5 IU/mL or 30 copies/mL (89). Target amplification assays, such as the real-time PCR assay or the transcription-mediated amplification (TMA) assay, make it possible to detect quantities of <5 IU/mL of HBV DNA and are rapidly approaching the level of a single HBV genome (89).

For research purposes and to avoid false-negative and false-positive results, PCR primers that span at least three genomic regions of the HBV genome such as the S, X, and core gene should be employed. Validation should require detection from at least two regions of the genome (53). Full-genome sequencing is the method of choice, although it is expensive and time consuming. In diagnostics, however, nested PCR would be time-consuming and might increase the risk of PCR product carryover contamination.

OHB and HBV serologic markers

The diagnosis of OHB depends on the HBV serological marker profiles. This relationship has been studied before, and although the meaning of markers of previous HBV infection has no universal interpretation, studies have shown that the prevalence of occult HBV infection has usually been higher in subjects positive for both anti-HBs and anti-HBc than in those negative for all serologic markers (46-80% versus 20-50%) and high frequencies of HBV-DNA positivity have been observed among anti-HBc only individuals (10% to 80%) (8,15,49,67), suggesting that this antibody does not result in complete HBV elimination (90). Furthermore, most

studies have suggested that detection of “anti-HBc alone” could reflect unrecognized occult HBV infection. The term anti-HBc only has frequently been used to describe individuals infected with the human immunodeficiency virus (HIV) (56,91-94) or hepatitis C virus (HCV) (15,17,49,95-99). However, occult HBV infection has also been detected in patients who tested negative for all HBV serum markers, and prior studies have not always clearly explained their diagnostic approaches and the mechanisms of anti-HBc-negative and anti-HBs-negative serology in individuals with occult HBV infection. A possible explanation for apparent cases of “anti-HBc only” is a false negative HBsAg readings in several studies, which were reported to be a result of mutations in the “a” determinant (1,41,42).

Regardless of mutations in the surface or other HBV proteins that might influence the expression of HBsAg, such OHB infections may be detected in (a) individuals with resolving HBV infection who test positive for both anti-HBc and anti-HBs, (b) anti-HBc-only carriers who are seronegative for HBsAg in a window period of infection (15,61,63,100), and (c) false positive anti-HBc results due to the variable sensitivity and specificity of commercial assays (84-87). The possibility of false negative results for IgG anti-HBc should also be considered. Specifically, a portion of subjects with occult HBV infection who tested negative for IgG anti-HBc might not have truly been negative; rather, they might have falsely tested negative due to low-antibody titer (Hollinger, personal communication).

Concluding remarks

The pathogenesis of occult hepatitis B is not yet clear, and it seems that both shared viral-host factors are involved. Further molecular studies on cohort patients group need to explore such association. HBsAg may not be an effective tool for differentiating HBV and OHB infection. Specifically, in light of this review, routinely used serological markers of HBV infection do not rule out occult and ongoing hepatitis B virus infection. This emphasizes that molecular methods should be used to detect occult HBV infection in these patients.

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