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# The detection of occult HBV infection in patients with HBsAg negative pattern by real-time PCR method

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ABSTRACT

*Aim:* Diagnostic problems may be encountered in Hepatitis B virus (HBV) infections by serological tests and HBV DNA can be detectable in plasma and liver tissue while the HBsAg test is negative. This situation can be defined as occult or isolated Anti-HBc infections. Occult HBV infections may be divided into two categories by using hepatitis markers. One of them being that all hepatitis markers are negative and the other situation is having Anti-HBc +/- and Anti-HBs + patterns. These situations can be seen in isolated Anti-HBc cases.

*Method:* In this study, we aimed to detect the ratio of occult HBV infections by investigating HBV DNA in four different groups. These groups are: (1) 20 isolated Anti-HBc positive individuals, (2) 23 individuals naturally immune to HBV infection, (3) 20 individuals with seronegative hepatitis markers and high ALT levels, and (4) 23 vaccinated individuals against HBV. In order to detect HBV DNA the real-time PCR kit (QIAGEN, Artus HBV RG PCR Kit, Germany) with high analytical sensitivity ($\leqslant$3.8 IU/ml) was used.

*Results:* The reliability of the molecular methods was assessed by increasing the quantitation standards of internal, external and also positive controls. No HBV DNA was detected in any of the 86 individuals consisting of four study groups.

*Conclusion:* In conclusion, we did not detect occult HBV infection in our four study groups by using a high sensitivity real-time (RT) PCR method, while occult HBV infections with various frequencies were detected in other large, serial international studies in which highly sensitive analytical molecular methods were used. Although we also used a high standard molecular kit to detect occult HBV infections, we suggest that the reason for the absence of detection of occult HBV infections may be due to the small number of cases included in this study. However, it was assumed that the use of a nucleic acid amplification technology (NAT) with high analytical sensitivity in blood banks to prevent HBV transmission by blood transfusion is controversial due to both costs and diagnostic efficacy and for this reason we suggest that it will be useful to perform large serial studies regarding occult HBV infections in the future.

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## 1. Introduction

Hepatitis B virus (HBV) is the major cause of acute and chronical hepatitis and continues to be a serious public health threat in the world [1].

In the last decade after the introduction of new prophylactic treatments and therapies and highly sensitive molecular biology techniques for in vitro diagnosis of HBV infections, the presence of HBV in the liver and serum of HBsAg negative individuals has been demonstrated. The

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persistence of hepatitis B virus (HBV) in hepatitis B surface antigen (HBsAg) negative individuals is termed as occult HBV infection. Occult HBV infections may be divided into two categories by using hepatitis markers. One is a group which is negative for all hepatitis markers and the second one possesses Anti-HBc +/- and Anti HBs + patterns. Occult HBV infections were also reported in isolated Anti-HBc (Anti-HBc is the only positive marker found) cases [2,3].

Individuals with occult HBV infections are usually diagnosed as healthy after negative HBsAg results found in serology screens for organ transplantation and blood donations. Anti-HBc and HBV-DNA tests were not performed in these situations leading to an increase in HBV infections. In fact, the possible risk of HBV transmission was reported in HBsAg negative blood and transplantation donors with the transfusion of blood and blood products and organ transplantation because of the possibility of having occult HBV infection [4,5].

In this study, we aimed to detect the presence of occult HBV infections by monitoring HBV DNA by a real time PCR method in four different groups suitable to donate blood, blood products, tissue and organs but having a potential risk for HBV infection. These four groups included: (1) isolated Anti-HBc positive individuals, (2) naturally immune individuals to HBV infection, (3) individuals with seronegative hepatitis markers and higher ALT levels, and (4) vaccinated individuals to HBV (only Anti-HBs positive).

## 2. Material and method

### 2.1. Study centers and groups

This study was conducted as cross-sectional study between December 2010 and May 2011. Individuals who were applied to the serology laboratories of Medical Microbiology Departments of Cerrahpasa Medical and Istanbul Medical Faculties of Istanbul University with viral hepatitis pre-diagnosis or with any other reasons (for example, seroconversion after vaccination, post-hepatitis follow-up and blood donation) were included in this study. Routine hepatitis B indicators (HBsAg, Anti-HBs, HBeAg, Anti-HBe, Anti-HBc, and Anti-HBc IgM) of these individuals were investigated and the following groups of individuals were included in this study. These groups were: (1) 20 isolated Anti-HBc positive individuals, (2) 23 individuals naturally immune to HBV infection, (3) 20 individuals with seronegative hepatitis markers and high ALT levels, and (4) 23 individuals vaccinated to HBV (only Anti-HBs positive). All cases were approved by the ethics committee of Istanbul University.

### 2.2. Sample collection and methods

#### 2.2.1. Sample collection

Ten milliliter samples of non-coagulated blood were collected from each donor to test for serological patterns of hepatitis. A second set of blood samples were collected from the same individuals into 10 ml sterile tubes containing anticoagulant and ethylenediamine tetra-acetic acid (EDTA) to obtain HBV-DNA. Alanine transaminase (ALT) and Aspartate transaminase (AST) levels of all participants were evaluated. Serum samples were obtained after centrifugation of blood at 3000 rpm. For the real-time PCR method, serum was similarly obtained from these blood samples by centrifugation at 3000 rpm and stored at −80 °C until the study.

#### 2.2.2. Serological study methods

The following test kits were used for the hepatitis studies; Surase B-96 (TMB)-GBC, Taiwan for HBsAg test; Anti corase B-96 General Biologicals, Taiwan for Anti-HBc (total) and Anti-HBc IgM tests; DiaPro, Diagnostic Bioprobes, Milano, Italy for Anti-HBs; HBeAg/HBeAb DiaPro, Diagnostic Bioprobes, Milano, Italy for HBeAg and Anti-HBe tests. These tests were carried on microELISA instrument (Trititrus, Grifols, Italy) by mentioned commercial test kits.

#### 2.2.3. Molecular study methods

*2.2.3.1. DNA extraction.* The HBV-DNA was extracted from the serum samples using QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's procedure.

*2.2.3.2. Quantitative real-time PCR.* Extracted HBV DNA was quantified by the real-time PCR method, using a Rotor-Gene Q instrument (Artus, Germany) and HBV RG PCR reagent kit (Artus, Germany). According to the manufacturer, the assay detection limit was 3.8 IU/mL (∼26.6 copies/ml). A 134 bp region of HBV genome was amplified in a total of 50 μl reaction mix containing the 30 μl HBV RG Master Mix and 20 μl of sample DNA. The real-time PCR was performed with negative and positive controls on the following program conditions: initial denaturation at 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 72 °C for 30 s. One independent real-time PCR reaction was performed for each sample. Quantification was performed using the software on Rotor-Gene Q instrument.

## 3. Results

Of the total 86 individuals belonging to the four study groups, 45 (52.3%) were female, 41 (48.1%) were male and the age distribution was between 3 and 69; the mean age was 43.07. In the HBV; Isolatad Anti-HBc group, 8 (40%) and 12 (60%) of the total 20 patients were female and male, respectively and age distribution was 3–69 and the mean age was 44.1. In naturally immune individuals to HBV infection group; 11 (47.8%) and 12 (52.2%) of the total 23 patients were female and male, respectively and age distribution was 20–61 and the mean age was 42.2. In group with seronegative hepatitis markers and higher ALT levels; 11 (55%) and 9 (45%) of the total 20 patients were female and male, respectively and age distribution was 23–62 and the mean age was 45.1. In vaccinated individuals to HBV (only Anti-HBS positive) group; 15 (62.23%) and 8 (34.8%) were female and male, respectively and the age distribution was between 21 and 69; the mean age was 40.9.

Real-time PCR kit (QIAGEN, Artus HBV RG PCR Kit, Germany) with high analytical sensitivity (⩽3,8 IU/ml) was used. The reliability of the molecular methods was applied

by increasing quantitation standards of internal and external and also positive controls. No HBV DNA was detected in any of the 86 individuals consisting of the four study groups.

## 4. Discussion

Definition of occult HBV infections is established when HBV-DNA is present in plasma and liver tissue as detected by real time PCR methods with a high analytical sensitivity ($\leqslant$5 IU/ml or 30 copy/ml) and this poses a threat to human health for the transmission of HBV infections. HBsAg positive individuals with occult HBV infections may appear negative because of viral clearance by their immune system or after HBV infection therapy [6]. Occult HBV infections are generally asymptomatic and cannot be detected by routine serologic screening tests with the exception of special molecular methods. The reasons for the increasing clinical importance of occult HBV infections can be summarized as a sign of a new acquired HBV infection with symptoms appearing after blood and blood product transfusion or solid organ transplantation, the increasing possibility of cirrhosis and cancer development with a decreasing interferon response in patients with chronic hepatitis C infections and to be the cause of HBV infection reactivation with other various factors in patients under immunosuppressive therapy [3,4,7]. The number of studies investigating the cases with a negative HBsAg but with suspicion of an occult HBV infection has increased in recent years. These suspected cases may contribute to the increase of HBV prevalence and hepatitis incidences in society. As a matter of fact, the detection of a negative HBsAg in our four study groups; (a) isolated Anti-HBc positive individuals, (b) individuals naturally immune to HBV infection, (c) individuals with seronegative hepatitis markers and high ALT levels, and (d) Individuals vaccinated to HBV (only Anti-HBs positive) may be helpful to prevent new HBV infections if we can detect occult HBV infections in individuals of these groups. Because members of these groups are safe blood donors, blood products donors and organ donors..

Highly sensitive serologic tests are generally used in blood banks to monitor HBV but the risk of HBV transmission is still present with a low possibility and, in fact, it is nearly impossible to eliminate the risk of transmission completely.

In some studies, it was reported that samples of HBsAg negative blood by serologic screening still carried HBV which was responsible for the post-transfusion HBV infections in 5–10% of the total HBV infections. The probability of HBV transmission was reported as 1/63,000 due to transfusion [4,8]. It was thought that the screening for both HBsAg and Anti-HBc in blood and organ donors will most likely prevent occult HBV transmission whether via blood transfusion or organ transplantation. However, this situation may also cause donor loss in countries in which HBV infections are endemic [9,10]. Altunay et al. [11] reported that they detected 2748 (21.4%) Anti-HBc and 658 (2.4%) isolated Anti-HBc positivity in 12858 HBsAg negative serum samples from blood donors, respectively in the Istanbul Red Crescent Blood Center between June 2007 and October 2008. Nine (0.007) were detected as positive while 12,849 (99.93%) were detected as negative according to the NAT (Nucleic Acid Amplification Test) study. From these 9 NAT positive plasma samples, six (0.91%) were HBV-DNA positive. According to their data, taking into account the presence of higher HBV-DNA negativity (99.09%) in isolated Anti-HBc cases they concluded that there was no need to add the Anti-HBc test to routine serological screenings even it was shown that there may be a risk of about 1 out of 2142 transfusion in the light of the additional cost of the Anti-HBc test and possible donor loss.

Minuk et al. [12] reported 14 (18%) HBV-DNA positives of the 80 HBsAg negative, Anti-HBc positive individuals out of 487 individuals in Canada. Liang et al. [13] reported 30% HBV-DNA positives of the 3% isolated Anti-HBc group out of 1407 donors. Antar et al. [14] found 80 Anti-HBc positives out of 1026 HBsAg negative individuals and they also detected five (6.25%) HBV-DNA positives in Egypt. On the other hand Mohammed et al. [15] showed that there were 19 (12.4%) HBV-DNA positives of the 153 isolated Anti-HBc cases. Although different HBV-DNA positivity ratios were discovered in HBsAg negative and isolated Anti-HBc positive individuals in the studies performed in Turkey and other countries, similar results to our negative results regarding HBV-DNA positivity in isolated Anti-HBc groups were also reported in other international studies. However, Zervou et al. [16] found no HBV-DNA positivity in a 282 HBsAg negative isolated Anti-HBc group from Greece in 2001 and Daugles et al. [17] also showed no HBV-DNA positivity in 26,492 similar individuals. Although we did not detect occult HBV in the isolated Anti-HBc group, we suggest that this result may be a result of the scarce cases or due to other unknown reasons.

We found that HBV-DNA was absent in individuals naturally immune to HBV infection but the presence of HBV-DNA in naturally immune individuals and recovered HBV infected patients in other studies led to speculations about the sera obtained from these individuals as to whether it is contagious or not. As a matter of fact, Mohammed et al. [15] reported 12 HBV-DNA positives out of 260 HBsAg negative naturally immune individuals by an *in-house* PCR method in India and stated that highly sensitive analytical screening tests were needed to avoid potential risks with blood transfusions in blood banks. Similarly, in China, Ling-Na Shih et al. [18] demonstrated from 68 HBsAg negative naturally immune individuals, 5 (7.3%) HBV-DNA positives. In another study performed by Song et al. [19] in Korea, HBV-DNA was detected in 2 out of 365 naturally immune individuals and a mutation in the S gene was found in one of these two cases. In 2000 Kocazeybek et al. [20] reported that no HBV-DNA was present in 96 HBsAg negative naturally immune individuals from Turkey and they concluded that these results were in accordance with the classical literature suggesting that HBV-DNA cannot be detected in the serum samples or mononuclear cells obtained from individuals naturally immune to HBV.

We think that our negative results with HBV-DNA in individuals naturally immune to HBV may be related to a mutation in the S gene and the reactivation of HBV

infections with the lymphoid system being a reservoir for HBV.

In individuals with seronegative hepatitis markers and higher ALT levels (>40 IU/ml), we also did not find HBV-DNA. Higher ALT levels and continuous viral replication were also reported in some cases with seronegative hepatitis markers which were preferable for the transfusion of blood and blood products and organ transplantation. These seronegative individuals are generally accepted as not contagious as the known transmission routes [3]. Song et al. [19] reported one HBV-DNA positive case out of 203 cases with seronegative hepatitis markers in Korea. In an occult HBV infection study from North America, 33 (8.1%) HBV-DNA positives were found out of 407 HBV seronegative cases and S gene variants were detected in 17 of the 33 HBV-DNA positive cases [12]. Although we could not detect any HBV-DNA positivity in the individuals with seronegative hepatitis markers and higher ALT levels, it was reported that occult HBV infections can be found in these individuals by utilizing highly sensitive molecular techniques to obtain HBV-DNA as IU/ml or copy number in recent years [21,22]. This situation may be related to a series of mutations [frequently in a determinant, arginine (Arg) was substituted with glycine (Gly) at the 145 position and localizes to 124–147 aa] in amino acid segments which determine a specificity in the major surface proteins (Surface – S region) of HBV. This mutation may prevent the neutralization of an Anti-HBs and lead to continuous replication of HBV. Classic diagnostic reactives could not detect this region. In individuals with seronegative hepatitis markers, high ALT levels may be caused from idiopathic factors other than HBV infections.

In the vaccinated individuals to HBV (only Anti-HBs positive), we could not observe HBV-DNA by RT-PCR method. Neutralizing Anti-HB antibodies are produced to the common determinant in S region of all subtypes of HBV after HBV infection or immunization and provide protection against the all subtypes of HBV. [22,23]. In cases known as Anti-HBs escape mutant, HBV-DNA positivity can be detected due to the unprotective antibodies formed against to differentiated region developed by mutations between 124 and 147 aa segment of the determinant. Therefore, it was suggested that HBV-DNA can be detected in Anti-HBs positive cases [24]. HBV replication with HBV-DNA positivity was reported in infants who were applied immunoprophylaxis, (Anti-HBs produced) and even no mutant HBV occured to their mothers from the first studies performed in Italy and Japan by vaccination. Arg substitution was found at 145 position in S protein from infants but Gly was present at the same position in the protein obtained from the mothers in Italian study. Threonine (Thr) at 126 position was also found substituted with Arg or isoleucine (Ile) in the infant S protein in a Japan study. When these first 2 reports and other results of the studies related with Anti-HBs escape mutants were evaluated together, it was suggested that unsufficient protection was given by vaccines which were prepared with the classical HBsAg sub-types (against a subtype which has the common determinant) [23,25].

In conclusion, we did not detect occult HBV infection in our four study groups by a high sensitivity real-time (RT) PCR method, while occult HBV infections with various frequencies were detected in other large, serial international studies in which highly sensitive analytical molecular methods were used. Although we also used a high standard molecular kit to detect occult HBV infections, we suggest that the reason for the absence of detection of occult HBV infections may be due to the small number of cases included in this study. However, it was assumed that the use of a nucleic acid amplification technology (NAT) with high analytical sensitivity in blood banks to prevent HBV transmission by blood transfusion is controversial both regarding cost and diagnostic efficacy and for this reason, we suggest that it will be useful to perform large serial studies on occult HBV infections in the future.

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## References

[1] Maddrey WC. Hepatitis B: an important public health issue. J Med Virol 2000;61:326–62.
[2] Hu K-Q. Occult hepatitis B virus infection and its clinical implications. J Viral Hepat 2002;9:243–57.
[3] Torbenson M, Thomas DL. Occult HBV. Lancet Infect Dis 2002;2:479–86.
[4] Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ. FTR-EDS. The risk of transfusion transmitted viral infections. N Engl J Med 1996;334:1685–90.
[5] Dickson RC, Everhart JC, Lake JR, Wei Y, Seaberg EC, Wiesner RH, et al. Transmission of hepatitis B by transplantation of livers from donors positive for antibody to hepatitis B core antigen. Gastroenterology 1997;113:1668–74.
[6] Hollinger FB, Sood G. Occult hepatitis B virus infection: a covert operation. J Viral Hepat 2010;17:1–15.
[7] Marusawa H, Uemoto S, Hijikata M, Ueda Y, Tanaka K, Shimotohno K, et al. Latent hepatitis B virus infection in healthy individuals with antibodies to hepatitis B core antigen. Hepatology 2000;31:488–95.
[8] Badur S. Non-A, non-B hepatitis viruses. J Klimik 1988;1:20–4.
[9] Chazouilleres O, Mamish D, Kim M, Carey K, Ferrell L, Roberts JP, et al. 'Occult hepatitis' B virus as source of infection in liver transplant recipients. Lancet 1994;343:1685–90.
[10] Bal SH, Heper Y, Kumaş LT, Mıstık R, Töre O. Investigation of the presence of HBV-DNA in isolated Anti-HBc positive cases and their importance in blood banking. Bul Microbiol 2009;43:243–50.
[11] Altunay H, Kosan E, Birinci I, Aksoy A, Kirali K, Saribas S, et al. Are isolated anti-HBc blood donors in high risk group? The detection of HBV DNA in isolated anti-HBc cases with nucleic acid amplification test (NAT) based on transcription-mediated amplification (TMA) and HBV discrimination. Transfus Apher Sci 2010;43:265–8.
[12] Minuk GY, Sun D, Uhanova J, Zhang M, Caouette S, Nicolle LE, et al. Occult hepatitis B virus infection in a North American community-based population. J Hepatol 2005;42:480–5.
[13] Liang TJ, Bodenheimer HC, Yankee Ronald Brown NV, Chang K, Huang J, Wands JR. Presence of hepatitis B and C viral genomes in US blood donors as detected by polymerase chain reaction amplification. J Med Virol 1994;42:151–7.
[14] Antar W, El-Shokry MH, Abd El Hamid1 WA, Helmy MF. Significance of detecting anti-HBc among Egyptian male blood donors negative for HBsAg. Transfus Med 2010;20:409–13.
[15] Ali R, Khan LA, Husain SA, Singla R, Kar P. Significance of anti-HBc screening of blood donors & its association with occult hepatitis B virus infection: implications for blood transfusion. Indian J Med Res 2010;132:312–7.
[16] Zervou EK, Dalekos GN, Boumba DS, Tsianos EV. Value of anti-HBc screening of blood donors for prevention of HBV infection: results of a 3-year prospective study in Northwestern Greece. Transfusion 2001;41:652–8.

[17] Douglas DD, Taswell HF, Rakela J, Rabe D. Absence of hepatitis B virus DNA detected by polymerase chain reaction in blood donors who are hepatitis B surface antigen negative and antibody to hepatitis B core antigen positive from a United States population with a low prevalence of hepatitis B serologic markers. Transfusion 1993;33:212–6.
[18] Shih LN, Sheu JC, Wang JT, Huang GT, Yang PM, Lee HS, et al. Serum hepatitis B virus DNA in healthy HBsAg-negative Chinese adults evaluated by polymerase chain reaction. J Med Virol 2000;32:257–80.
[19] Song EY, Yun YM, Park MH, Seo DH. Prevalence of occult hepatitis B virus infection in a general adult population in Korea. Intervirology 2009;52:57–62.
[20] Kocazeybek B, Ordu A, Ayyıldız A, Aslan M. The investigation of HBV-DNA in naturally immunized cases with hepatitis B infection. J Viral Hepat 2000;1:30–2.
[21] Aspinall S, Steele AD, Peenze I. Detection and quantitation of hepatitis B virus DNA: comparison of two commercial hybridization assays with polymerase chain reaction. J Viral Hepat 1995;2:107–11.
[22] Kosan E, Kocazeybek B, Altunay H, Aymelek M, Alan E, et al. Can the nucleic acid amplification test (NAT) be an alternative to the serologic tests? A prospective study, the results of 18200 blood donors from the Turkish Red Crescent. Transfus Apher Sci 2010 Dec;43:269–72.
[23] Thomas HC, Carman WF. Envelope and precore/core variants of hepatitis B virus. Gastroenterol Clin North Am 1994;23:499–514.
[24] Carman WF, Zanetti AR, Karayiannis P, et al. Vaccine-induced escape mutant of hepatitis B virus. Lancet 1990;336:325–9.
[25] Thomas HC. The emergence of envelope and precore/core variants of hepatitis B virus: the potential role of antibody selection. J Hepatol 1995;22:1–8.