Absence of occult hepatitis B among blood donors in southern Brazil

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ABSTRACT

Background: Occult hepatitis B virus (HBV) infection is characterized by the detection of HBV DNA in serum and/or in liver in the absence of detectable hepatitis B surface antigen (HBsAg). The reported prevalence of occult hepatitis B varies markedly among populations and according to the sensitivity of the HBV DNA assay. The aim of the present study was to describe the prevalence of occult hepatitis B among HCV-infected and non-infected blood donors in Porto Alegre, Southern Brazil, using a highly sensitive real time polymerase chain reaction (PCR) method. **Methodology:** Between 1995 and 1997 a sample of 178 blood donors with two positive anti-HCV ELISA tests were consecutively selected as cases, and 356 anti-HCV negative donors were selected as controls. Blood donors were randomly selected from eight blood centers in Porto Alegre, Southern Brazil, representative of the whole blood donor population. Blood samples were kept at 70°C and defrosted for the first time for the analysis of this report. Tests previously performed in the laboratory using the same real time PCR for HBV DNA had sensitivity for detecting as low as 9 copies/mL. Among 158 blood samples from HBsAg-negative blood donors, five were anti-HBc positive, 53 tested positive for anti-HCV and 105 had anti-HCV negative. The samples analysis was performed in duplicate and all blood samples tested negative for HBV DNA. **Conclusion:** The result reflects a very low prevalence of occult hepatitis B in our setting.

Keywords: hepatitis B; hepatitis B virus; blood donors; seroepidemiologic studies.

INTRODUCTION

Occult hepatitis B virus (HBV) infection is a well-recognized clinical entity characterized by the detection of HBV-deoxyribonucleic acid (DNA) in serum and/or in liver in the absence of detectable hepatitis B surface antigen (HBsAg). Occult HBV infection has been described not only in patients who have resolved an acute or chronic HBV infection, but also in patients without any serological markers of past HBV infection.[1] The prevalence of occult hepatitis B found in 15 different studies ranged from 1 to 95%,[1] which might be accounted to differences between endemic and not endemic areas, presence of risk factors and to the sensitivity of the HBV-DNA assay.

Hepatitis B and C coinfection is commonly reported all over the world and high coinfection rates are attributed to the viruses sharing the same modes of transmission and being endemic in some regions.[2] Since this coinfection is highly prevalent, occult hepatitis B has been extensively investigated in hepatitis C virus (HCV) infected patients. It has been estimated a rate of 20-30% of occult hepatitis B in international studies,[3] but in some areas occult HBV infection has not been detected.[4,5]

In the report by Shetty *et al*, the authors described 28% prevalence of occult hepatitis B based on serum samples, and 50% on liver specimens of a liver transplant population.[6] Association between hepatocellular carcinoma and high prevalence of occult hepatitis B raises concerns regarding the detection of the occult hepatitis B infection.

The aim of the present report was to describe the prevalence of occult hepatitis B among HCV-infected blood donors (cases) and non-infected controls, in Southern Brazil, using a highly sensitive real-time polymerase chain reaction (PCR) method.

METHODS

Ethics statement

The study was approved by the Ethics Committees of the following institutions invited to participate in the study: *Hospital de Clínicas de Porto Alegre, Santa Casa de Misericórdia de Porto Alegre, Hospital Moinhos de Vento, Hospital Ernesto Dorneles, Laboratório Marques-Pereira, Hospital Nossa Senhora da Conceição, Hospital São Lucas, Hemocentro do Rio Grande do Sul.* All participants signed a consent form in accordance to the ethical guidelines of the 1975 Declaration of Helsinki.

Participants

A random sample of blood donors was selected from eight blood centers in Porto Alegre, Southern Brazil, between 1995 and 1997, in order to represent the blood donor population. Blood samples were taken from all participants for the pre-donation testing and a 2 mL sub-sample was kept apart for further analysis. From the initial sample, 178 blood donors with two positive anti-HCV ELISA tests were consecutively selected as cases, and 356 anti-HCV negative donors were selected as controls.

Description of procedures

The samples were centrifuged less than 4 hours after the collection and frozen at -70°C. The samples were defrosted at a single time for the analysis of this study. All tests were carried out at Simbios Biotechnology in Brazil, a reference laboratory for molecular biology in our region. Sampling details and other results of the study were described elsewhere.[7]

DNA extraction method and real-time PCR detection assay

The DNA extraction was made as described by Kramvis *et al.*[8] For phenol-chloroform DNA extraction, a 200 μL aliquot of serum was incubated at 70°C for 2 hours in the presence of 400 mg of proteinase K, 1% sodium dodecyl sulfate, and 2.5 mM disodium EDTA. The suspension was sequentially extracted with phenol and then chloroform. DNA was precipitated with 0.3 M sodium acetate and then with absolute ethanol, washed with 70% ethanol, vacuum dried, and then dissolved in 50 μL of best-quality water (BQW). The material was than exposed to GeneReleaser, which is a proprietary reagent that can release DNA from serum. Lysis is accomplished directly in the amplification tube in a thermocycler. A 20 μL volume of GeneReleaser was added to 5 μL of serum, and the extraction was performed in a programmable thermal cycler according to the manufacturer's directions as follows: 65°C held for 30 seconds, 8°C held for 30 seconds, 65°C held for 90 seconds, 97°C held for 180 seconds, 8°C held for 60 seconds, 65°C held for 180 seconds, 97°C held for 60 seconds, 65°C held

for 60 seconds, and 80°C held until the PCR mixture was added. The end product (25 μL) was used as the template for the 100 μL first-round PCR mixture.

Real-time PCR detection assay was made as described by Pas *et al.*[9] PCR primers and probe were designed using Primer Express software (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Amplification was performed in a 50 μL reaction mixture containing 2x TaqMan Universal MasterMix (PE Biosystems), 45 pmol of forward primer (59-GGA.CCC.CTG.CTC.GTG. TTA. CA-39, nucleotides 184 to 203), 45 pmol of reverse primer (59-GAG.AGA.AGT. CCA.CCM.CGA.GTC.TAG.A-39, nucleotides 273 to 249), 15 pmol of TaqMan probe (59-FAM-TGT.TGA.CAA.RAA. TCC.TCA.CCA.TAC.CRC. AGA-TAMRA-39, nucleotides 218 to 247), and 10 μL of isolated DNA. Primers and probe were selected in the pre-S gene of the HBV genome and generated a product of 89 bp. All isolations and amplification reactions were performed in duplicate. After preparation of the reaction mixtures in 96-well plates, the plates were centrifuged at 1,200 rpm for 1 min in a Rotina 48R swing rotor to remove small air bubbles in the vessels. Amplification and detection were performed with an ABI Prism 7700 Sequence Detection System (PE Biosystems). After incubation for 2 min at 50°C, which enables uracil N9-glycosylase (present in the 23 Universal MasterMix) to inactivate possible contaminating amplicons, incubation for 10 min at 95°C allowed AmpliTaq Gold polymerase to activate and inactivate the uracil N9-glycosylase. The PCR cycling program consisted of 45 two-step cycles of 15 seconds at 95°C and 60 seconds at 60°C. For standardization of the real-time PCR detection assay, a standard curve of the plasmid ranging from 10 million to 670 copies/mL and a run control of 30,000 copies/mL (CLB) were included in each run. Validation of the plasmid controls was done with the VQC panel (CLB).

Diagnostic properties of the test

For clinical purposes, this technique is able to detect 200 viral copies/mL and it has high reproducibility. For research purposes, we considered any viral load detected as a positive PCR. Previous testing performed by Simbios laboratory, using the same technique, detected viral loads as low as 9 copies/mL.

RESULTS

In this analysis, 158 samples of blood donors from one of the main blood centers were tested. All samples were from HBsAg negative participants and five blood donors tested positive for hepatitis B core antibodies (anti-HBc). Fifty three individuals tested positive for anti-HCV and 105 were anti-HCV negative. The analysis of samples was performed in duplicate and all samples tested negative for HBV-DNA. Since all 158 samples tested were negative, we decided to stop testing the remaining samples.

DISCUSSION

The zero prevalence of occult hepatitis B among blood donors, detected in our study, is far from the 28% serum HBV DNA prevalence among the liver transplant population reported by Shetty et al.[6] Our result is in accordance with other studies carried out in Brazil to detect HBV in serum among hepatocellular carcinoma patients[5] and among liver transplant patients.[10] Even considering that occult HBV might be more prevalent in end-stage liver disease population, such as that studied by Shetty et al.,[6] than in healthy blood donors, it was anticipated at least a few occult hepatitis B cases.

The screening procedures in the blood centers included an interview of candidates to donate, precluding from donation the unhealthy or high risk individuals. This screening might have lowered the occult hepatitis B prevalence in the sample in comparison to the real prevalence in the general population. However, HBV and HCV share routes of transmission and risk factors, and blood donor candidates were detected as HCV cases. Even so, 53 HCV cases and 105 anti-HCV negative controls might have not enough statistical power to detect a case of occult HBV.

False-negative results obtained from HBV-DNA detection could be another reason for not finding a single positive case. In our study, the lacking of occult hepatitis B cases might not be attributed to false-negative tests. The DNA extraction method and the real-time PCR technique, used for HBV-DNA detection, is the one currently adopted in referent laboratories and very low viral loads have been detected in Simbios laboratory on another ongoing study. However, there are theoretical problems related to the storage of the samples. Our samples had been maintained frozen and stored at -70°C on average for seven years. There were no voluntary or involuntary thaws in the period, what gives our sample an additional guaranty of quality. Because HBV is a DNA virus, the possibility that the freezing time could have spoiled the samples is remote. Studies that had used samples with multiple freezing and thawing cycles have not had their results affected.[11]

Whether individuals with occult HBV infection are at risk for unfavorable endpoints still is an unresolved issue. Considerable data suggest that occult infection may contribute to chronic liver damage,[12-14] the development of hepatocellular carcinoma,[15-18] transmission[19,20] and reactivation of chronic hepatitis B.[21-23] Therefore, despite some controversy concerning its clinical significance, improving the knowledge on different aspects of occult HBV infection is a matter of concern worldwide, as we can see by the high number of new published papers on this topic. Our paper helps researchers and clinicians to better understand how relevant (or not) is occult hepatitis B in our setting, and, probably in other low-prevalence HBV settings.

The main reasons for the differences in occult hepatitis B rates among studies seem to be the genuine variability on the hepatitis B prevalence – either occult or not – in several regions of the world, in regions of the same country, and according to characteristics of the studied population. Therefore, we believe that the result of our study reflects a true negative result in a sample with extremely low prevalence of occult hepatitis B. As our sample size (158 individuals) is only able to detect prevalence rates over 0.6%, we may estimate from 0 to 0.6% the prevalence of occult hepatitis B in blood donors in southern Brazil.

Limitations

The results refer to blood samples collected 12 to 14 years ago, an issue that may rise concerns about how updated are the information we present. Considering that the prevalence of hepatitis B and C and their risk factors have not changed much in this period, we have no reason to believe the prevalence of occult hepatitis B would be different if more recent samples were tested.

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