



RESEARCH PAPER

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Molecular and serological analysis of surface antigen negative hepatitis B-virus infection among Jordanian blood donors

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Abstract

Occult hepatitis B virus (HBV) infection is characterized by the presence of viral DNA circulating blood and/or in liver tissue without detectable HBV surface antigen (HBs Ag). This study aims to investigate the molecular basis of occult hepatitis B virus (HBV) infection (OBI) among Jordanian blood donors. OBI blood donors were tested for HBV serological marker and they had normal alanine aminotransferase (ALT). In this study, we examined a total 578 HBsAg donated healthy individual from the blood bank unit/Islamic Hospital Amman-Jordan, enrolled during 2013. The people age between 20 – 48 years. The test was performed on serum for anti HBC by ELISA and plasma for PCR/NAT. Out of 578 HBsAg negative anti HBc antibody was found positive in 30 donors (5.2%). The positive samples for anti HBC antibody were then investigated to determine the presence of hepatitis B virus DNA using PCR- technique. In conclusion, to be potentially safe of blood donations HBsAg and nucleic acid amplification testing should be implemented even if anti HBsAg negative before blood and organ donation, transplantation, and chemotherapy and in hemodialysis to reduce the risk of HBV transmission among people who donate blood.

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Introduction

Hepatitis B is a viral infection of the liver. HBV infection may be acute or chronic. Chronic hepatitis B virus (HBV) infection is a complex entity frequently associated with cirrhosis, hepatocellular carcinoma (HCC) and liver failure. Death from chronic liver disease occurs in 15-25% of chronically infected persons.

Hepatitis B virus (HBV) infection is a major global public health problem. Of the approximately 2 billion people who have been infected worldwide, more than 350 million are chronic carriers of HBV. There are eight genotypes of hepatitis B virus (A-H) which show a distinctive geographical distribution and subgroups are recognized. Genotyping can be accomplished based on a partial sequence of HBV genome and was classified into subtypes according to the antigenic determinants of HBsAg (Abdel-Rahman *et al.* 2007). The genotypes show a distinctive distribution between and even within regions and are proving to be an invaluable tool in tracing the molecular evolution and patterns and modes of spread of HBV. Structural and functional differences between genotypes can influence the severity of infection [Kramis *et al.* 2005; Mello *et al.* 2007; Abdel-Rahman *et al.* 2007]. Genotyping of chronic HBV infections can help to identify those at risk of disease progression and determine optimal antiviral therapy [Tanwar and Dusheiko 2012]. Pattern of infection are variable and are influenced by age, sex, and state of immune system.

The persistence of HBV genomes in the absence of detectable surface antigenemia is termed occult HBV infection. Occult hepatitis B virus infection is defined as the presence of viral DNA in circulating blood without detectable HBV surface antigen (HBsAg). Occult hepatitis B virus infection might allow the release of viremic units into the blood supply network if blood is tested only for hepatitis B surface antigen (HBsAg) [Manzini *et al.* 2007]. The prevalence and clinical significance of occult hepatitis B virus infection have been reviews elsewhere [Torbenon and Thomas 2002; Romeno *et al.* 2011; Raimonodo *et*

al. 2013; Zobeiri 2013; Hiroki *et al.* 2013]. The screening for hepatitis B virus infection varies markedly in different geographic areas of the world as well as different population subgroups [Lavenchy *et al.* 2004; Kumar *et al.* 2006; El-Sherif *et al.* 2007; Sofiana *et al.* 2010; Yuan *et al.* 2010; Zheng *et al.* 2011; Romano *et al.* 2013; Said *et al.* 2013; Al-Naamani *et al.* 2013].

Occult HBV may be transmitted by blood transfusion or organ transplantation to human inducing acute hepatitis B in the recipients and the occult HBV carriers may show an acute reactivation of the infection with reappearance of the typical hepatitis B serological profile. The mechanisms responsible for the inhibition of HBV activities are suggested that the host's immune response, co-infection with other infections agents and epigenetic factors may play important roles in inducing the occult status [Raimondo *et al.* 2007; Salami *et al.* 2012].

Although, serological test of hepatitis B virus infection markers are well documented, but it may not be good indicator for viral infection. It is generally accepted that diagnosis of infection by hepatitis B virus is based on the presence of HBsAg in blood stream. In addition, it has been admitted that the blood bank donors screening does not fully eliminate the risk of HBV infection through blood transfusion, since the absence of this marker in serum does not exclude the presence of HBV related DNA [Roberio *et al.* 2006]. Occult HBV infection is usually associated with very low levels of HBV DNA that can be identified by detection HBV DNA by different genotyping methods including PCR or other amplification assays [Norder *et al.* 1992; Conjeevaram *et al.* 2001; Chandotti *et al.* 2006; Dettori *et al.* 2009; de Oliveira *et al.* 2014].

A few hepatitis B-virus (HBV) infections are characterized by the presence of HBV-DNA in serum or liver tissue or both, in the absence of detectable hepatitis B-surface antigen (HBsAg) in serum, in the present study 30 patient who had no detectable HBsAg in their serum were studied, in these 30 cases

HBV-DNA was detected using polymerase chain reaction (PCR). However, such infections have rarely been described in Jordanian patients.

Our study was conducted to determine the prevalence of hepatitis B surface antigen (HBsAg), hepatitis B core antibodies (anti-HBc) and HBV-DNA among group of Jordanian blood donors.

Materials methods

Blood Sample Collection

Five hundred and seventy eight samples were collected from healthy, volunteer's donors (having HBsAg negative) from the Blood Bank Unit/ Islamic Hospital. The patients age range between 20-48 years. The test was performed on serum for anti-HBc by ELISA, and whole blood, plasma or serum for PCR. The whole blood was kept in refrigerator at 4 °C up to 5 days and serum and plasma were kept in deep freeze until analysis.

Serological Test (Enzyme Linked Immuno-Sorbent Assay)

Special wells coated with monoclonal anti- HBc were used. The wells were identified carefully by labeling each one with patient number and also identified positive and negative control. After all samples were diluted to 1:11, 220 µl of each sample and control were distributed in the wells, and then the microplate was incubated in thermostat-controlled for 30 min at 37 °C. After that, the contents of all wells were aspirated and 370 µl of washing solution were added to each well, then the solution was aspirated. This step was repeated three times. Then, 200 µl of washing solution was added to each well and the microplate was incubated for 60 min at 37°C. The wells were aspirated and were washed three times as previous and 100 µl of the substrate was added to each well. The color changed to dark blue in positive control and positive samples and pink in others. The microplate was incubated in dark for 30 min at room temperature. Finally, 100 µl of stop solution was added, the color of positive control and positive samples wells were converted from blue to yellow but others from pink to colorless. The optical density for

all samples was measured at 450/620 nm by using plate reader.

DNA Extraction

DNA from plasma was extracted using ZR Genomic DNA II Kit™. The DNA was extracted according to the manufacture's instruction.

Polymerase Chain Reaction (PCR) Analysis

The genomic DNA that was extracted by the previous method was amplified exponentially by the polymerase chain reaction (PCR) to facilitate analysis. In standard PCR setup, a master mix was prepared with a volume to account for the number of samples to be analyzed, and a blank to check for contamination.

The following volumes were used for single PCR reaction: (15.5 µl) of nuclease-free water, (2.5 µl) of (10X) buffer, (0.5 µl) from (5U/ µl) Taq polymerase. Then, (5 µl) of each genomic DNA sample was added to different PCR tubes; final volume in each reaction was (25 µl). Then the samples were run in a thermal cycler (iCycler; BIO-RAD, USA) according to the following program: 45 cycles of 95°C for 45 sec., 55°C for 45 sec. and 72°C for 1 min.

PCR product detection by agarose gel electrophoresis

A 2.5% agarose solution was prepared in a glass bottle and heated in a microwave oven for 3 minutes until the agarose melted; after cooling, 1 µg/ml ethidium bromide was added. The solution was poured in a plastic mould, 9 well combs were inserted, and the gel was left to harden for 10 minutes at room temperature. When hard, the comb was removed and the gel was immersed in 1 X TBE. Ten µl of PCR products were loaded into the wells. Ladder (Promega, USA) was loaded in a separate well to allow the determination of molecular weight and the reaction quality. The gel was connected to the power supply in average 150 V for 20 minutes. The gel was then removed from the mould, and visualized under UV transilluminator using Gel Documentation System (BIO-RAD USA).

Results

Serological results

The cut-off between positive and negative for detection of anti-HBc antibody in serum samples was

calculated by dividing mean of optical density of three positive controls in on 5 that found to be $OD_{450/620}$ EQUAL 0.2676. Thirty samples were found positive for anti HBc antibodies (Table 1).

Table 1. The results are shown as an optical density reading for the color change in ELISA assay for anti-HBc antibodies. Wells A:1 & B:1 are negative control; wells C:1, D:1, & E:1 are positive control; the remaining are patients samples.

	1	2	3	4	5
A	0.035	2.451	1.996	1.514	1.452
B	0.035	1.324	1.692	1.409	2.358
C	1.394	1.324	2.2	1.63	2.2
D	1.364	2.749	1.872	1.691	0
E	1.394	1.265	2.357	2.342	0
F	2.53	2.229	1.123	0.617	0
G	2.527	0.52	2.034	1.39	0
H	0.951	2.393	2.425	2.281	0

Molecular Analysis by PCR

A few hepatitis B-virus infections are characterized by the presence of HBV-DNA in serum or liver tissue or both, in the absence of detectable hepatitis B surface antigen (HBsAg) in serum. However, such infections have rarely been described in Jordanian blood donors.

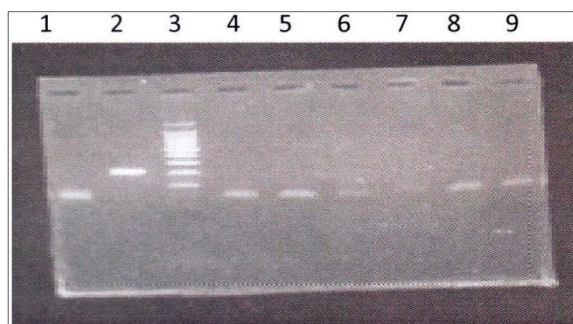


Fig. 1. HBV- DNA gel electrophoresis: 1. Negative control; 2. Positive control (DNA segment =250 bp); 3. DNA Marker; 4-9. Patient's Samples.

HBV-DNA from the 30 blood samples were subjected to PCR analysis using specific primers. These samples were tested serologically and show negative results with anti-HBc antibodies. As shown in figures 1, 2 and 3, the thirty samples show a positive result for the presence of HBV-DNA.

Discussion

Serological testing is widely used in controlling the risk of HBV transmission among people who donate blood but transmission of hepatitis B virus infections by transfusion continues to occur [Norder 1992]. Several studies recently reported that occult HBV infection is usually associated with very low levels of HBV DNA in the serum of patients with negative results of hepatitis Bs- antigen (HBsAg) test with or without serological markers of previous viral exposure [Torbenon and Thomas 2002; Kuhn and Bush 2006; Raimondo *et al.* 2007; Guirgis *et al.* 2010].

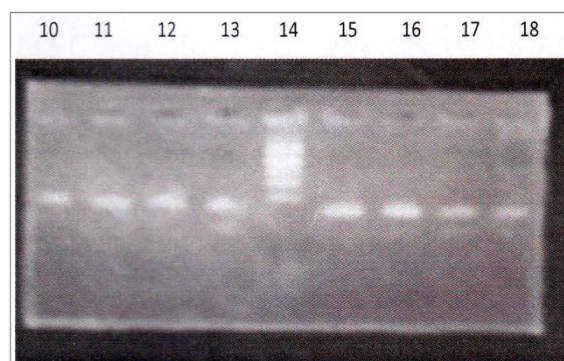


Fig. 2. HBV- DNA gel electrophoresis: 14. DNA Marker; 10-18 Patient's Samples.

Advances in molecular biology technique led to development of polymerase chain reaction (PCR) and other amplification assays in HBsAg negative individual for direct determination of hepatitis B virus DNA (HBV DNA) [Gutierrz *et al.* 2004; Dottori *et al.* 2009; Zheng 2011; Grabarczyk *et al.* 2010; said *et al.* 2013; de Oliveira *et al.* 2014].

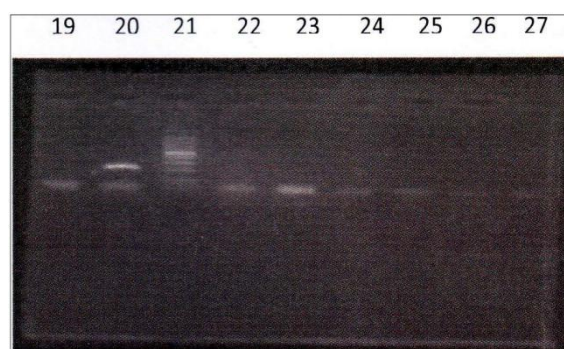


Fig. 3. HBV- DNA gel electrophoresis: 20. Positive Control; 21. DNA Marker; 19-27. Patient's Sample.

The criteria and protocol adopted in serological screening of blood bank donors have significantly reduced the possibility of HBV transmission. However, it's possible that, in a very recent phase of HBV infection, HBsAg sero-negative donor be able to transmit the virus. The persistence of HBV-DNA in the serum of HBsAg negative with presence of anti HBc antibodies is termed to occult HBV infection.

Our study showed that the overall prevalence of anti HBc antibodies only positive in 30 patients from 578 patients (5.2%) evaluated samples. Antibody to hepatitis B core antigen (anti- HBc) is the most sensitive marker of previous HBV infection. It appears in acute phase of HBV infection and usually

persists after the virus disappears. Lacking of detectable HBsAg in infectious blood may be due to a number of reasons such as co-infection with other viruses, for example hepatitis C virus (HCV), which may suppress HBsAg production, the presence of HBsAg at levels below the limit of detection of the assay or the presence of HBsAg mutants, particularly within the 'a determinant' region of the surface antigen.

Also, the presence of anti-HBcAg antibodies without any other HBV serological marker is frequently found in different population groups. Different situations may account for this result: (i) a false positive anti-HBcAg result; (ii) low levels of HBV replication inside the hepatocyte, without detectable production of HBsAg; (iii) the window phase of acute HBV infection; (iv) the loss of anti-HBsAg with time or failure to develop an antibody response against the antigen after infection; or (v) the presence of a vaccine escape mutant, not detected by most of the currently available HBsAg detection tests [Gutierrez *et al.* 1999].

In our study, PCR results were all negative, that may be due to the strong suppression of HBV activity is responsible not only for the HBsAg negativity but also for the very low, even undetectable levels of serum HBV DNA or indicate recovery from previous acute infection with HBV that left only HBc antibodies as an indicator.

The occult infection appears to be mostly due to a strong suppression of viral replication and gene expression affecting viruses whose genetic variability is comparable to that of HBV strains from individuals with "overt" chronic HBV infection [Behzad-Behbahani 2006].

Conclusion and recommendation

Anti-HBc antibody should be tested routinely on blood donor volunteers. Further testing for HBV-DNA would be beneficial to follow up blood donor for HBV infection this test should be implemented even if anti-HBs were negative especially before blood and

organ donation, transplantation, hemodialysis and chemotherapy.

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