IJMS
Vol 30, No 1, March 2005

Original Article

Indication of Anti-HBc Antibody Screening and HBV-DNA Detection in Diagnosing Latent Hepatitis B Virus Infection

A. Behzad-Behbahani,[1] A. Mafi-Nejad, [1]
S.Z. Tabei,[1] K.B. Lankarani,[2] M. Rashidi,[3]
M. Rasouli,[3] B. Pourabbas,[3] A. Torab,[4]
A.R. Salah [4]

Abstract

Background: In spite of available, and sensitive screening assay for detection of hepatitis B virus surface antigen (HBsAg), occasional cases of post-transfusion hepatitis B virus infection are still observed. The aim of the present study was to assess the prevalence of positive anti hepatitis B core (anti-HBc) and presence of HBV-DNA in serum sample of healthy blood donors negative for both HBsAg and anti-HCV antibody. We evaluated whether anti-HBc could be adopted as a screening assay for blood donation.

Material and Methods: Two thousands sera negative for both HBsAg and anti-HCV collected from healthy blood donors tested for presence of anti-HBc antibody. All sera positive for anti-HBc antibody were then investigated for determination of anti-HBc and anti-HBs titers, HbeAg and anti-HBe antibody by enzyme immunoassay (EIA). Every sample that tested negative for HBsAg but positive for anti-HBc alone or in combination with other serological markers was also examined for the presence of HBV-DNA by polymerase chain reaction (PCR).

Results: Out of 2000 HBsAg negative blood samples, 131 samples (6.55%) were positive for anti-HBc. HBV-DNA was detected in 16 of 131(12.2%) anti-HBc positive specimens. The liver function test results were all in normal range except in 4 (25%) of 16 HBV-DNA positive subjects.

Conclusion: Anti-HBc antibody should be tested routinely on blood donor volunteers, and if the sera become positive regardless of anti-HBs titer, the blood should be discarded. Further testing for HBV-DNA is appropriate to follow up the blood donor patient for HBV infection.
Iran J Med Sci 2005; 30(1): 28-33.

Keywords ● hepatitis B virus infection ● blood donors ● Iran ● anti-HB**c** ● HBV-DNA

[1]Clinical virology section,
Organ Transplant Research Center,
[2]Gastroenterohepatology Research Center,
[3]Professor Alborzi Clinical Microbiology Research Center,
Nemazee Hospital,
Shiraz University of Medical Sciences,
[4]Iran Blood Transfusion Center,
Shiraz, Iran.

Correspondence:
Abbas Behzad-Behbahani, PhD,
Organ Transplant Research Center,
Nemazee Hospital,
Shiraz University of Medical Sciences,
Shiraz, Iran.
Tel: +98 711-6276211
Fax: +98711-6276211
E-mail: Behbahani_2000@yahoo.com

Introduction

The safety of blood products is one of the major problems facing transfusion medicine. Transmission of hepatitis B virus (HBV) infection through donated blood is more common than hepatitis C virus (HCV) infection (1: 60,000 vs. 1: 103,000).[1] In spite of available sensitive screening assay for

detection of HBV surface antigen (HBsAg), occasional cases of post-transfusion hepatitis B virus infection (PTHBV) are still observed. [2]

There are three possible explanations of false-negative results in commercial assays. Blood donors infected with HBsAg mutants and those circulating low-level of viral protein may escape detection by screening assay and therefore, may affect the safety of blood supply.[3] Another explanation is that virus variants yield sequences that are not recognized by the antibodies employed in the assays.[4] There are variants in other parts of the genome that down regulate the production of HBsAg.[5]

Occasionally, a super-infection with hepatitis C virus may even induce clearance of hepatitis B. This could be due to the dominant role of HCV in eliciting an immune response.[6]

Antibody to hepatitis B core (HBc) antigen is a marker of acute, chronic, or resolved HBV infection and remains detectable for life. They may present in the absence of both HBsAg and anti-HBs antibodies, during the convalescent period following acute hepatitis B, before the appearance of anti-HBs antibodies, or in patients who resolved infection but lost detectable anti-HBs antibodies.[7] Hence, anti-HBc may be detected in anyone who is infected with HBV.[7]

Polymerase chain reaction (PCR) assay has demonstrated that some HBsAg negative individuals and those positives for anti-HBc continue HBV replication. [8,9]These findings suggest that recovery from acute hepatitis B virus may not result in complete virus elimination, but rather the immune system keeps the virus at very low level. It has been reported that there is a direct correlation between anti-HBc titer and detection of HBV-DNA in serum samples of HBsAg negative individual. [10] Thus, it is suggesting that HBV-PCR would be positive in patients with higher titer of anti-HBc than others would.

Although screening all donations for anti-HBc is not mandatory world wide, in some western countries, such as United States, screening is obligatory.[11] However, detection of HBV-DNA by PCR has the same significance as detection of HBsAg and indicates current HBV infection. All blood donations in Iran are collected from healthy donors. All donations issued for transfusion is being tested for HBsAg as a marker of transmissible HBV. These measures have resulted in low rates of transmission by transfusion but have not eliminated all infectious donations from the blood supply.

In spite of the availability of an effective vaccine, HBV infection continues to be an important problem in Iran and about 8-10 thousand deaths occur each year due to this sequelea. In Iran, the rate of hepatitis B carriers (HBsAg positive) varies from 0 to 3.9% with an average of 1.7%, and all are asymptomatic.[12,13] Amini and his colleague performed a study on 4930 healthy blood donors in Iran, and found that 5.1% were only positive for anti-HBc without having any detectable HBsAg, however, they did not determine the presence of HBV-DNA.[14]

The present study, therefore, assessed the prevalence of anti-HBc positivity and the presence of HBV-DNA in serum samples of healthy blood donors negative for both HBsAg and anti-HCV antibody. Furthermore, we investigated the relationship between detection of HBV-DNA and anti- HBc titer. Finally, since anti-HBc detection is not mandatory in Iran we evaluated whether anti-HBc could be adopted as a screening assay for blood donation.

Materials and Methods

After initial tests, 2000 serum samples negative for Treponemal infection, HBsAg, anti-HCV and anti HIV I, II antibodies were collected from the same number of healthy blood donor volunteers, who referred to blood transfusion center, Shiraz, Iran during December 2001 to December 2002. Of them 1820 were male with mean age 42±14 yrs (range 17-66 yrs) and 180 female with mean age 46±11yrs (range 23-66 yrs). Before transfusion, donors were interviewed and medically examined and those with high-risk behaviors, such as intravenous abusers, had medical problems, and those received HBV vaccination were excluded from the study population. The specimens were stored at –20°C until use. The local ethics committee granted ethical approval for the study and a written consent was obtained from all participants.

Serological assays

All serological tests were performed as instructed by the manufacturers. HBsAg, anti-HBs, anti-HBc-IgG antibody, HBeAg and anti-HBe antibody were measured using standard commercially available one step enzyme immunoassay technique (MonoLISA, Bio-RAD, France). Anti-HCV (third generation assay) was measured by enzyme immunoassay (EIA) according to the manufactures instruction (Innogenetics, Belgium). The presence or absence of anti-HBc antibodies were determined by using MonoLISA anti-HBc test, and comparing for each sample the recorded absorbance with that of calculated cut-off values. Samples with an optical density (OD) less than the cut-off values were considered as negative. However, the results below the cut-off value of less than 10% OD were retested in duplicate according to the manufacture instruction. Samples with

OD higher than, or equal to, the cut-off value were also considered as positive and retested in duplicate before the final interpretation.

The third generation of MonoLISA anti-HBs was used for the detection or quantitative determination of antibody to hepatitis B surface antigen. A mixture of HBsAg of the “ad” subtype and “ay” subtype of human origin has been used for anti-HBs antibody detection. Assays of undiluted samples showed an OD below or equal the cut-off value was considered to be negative. The HBs antibody concentration was determined from the standard curve for samples showing an OD within the range of the cut-off value and 150 mIU/ml standard (undiluted or at 1: 10 dilution). In the case of diluted samples, their concentrations were calculated by multiplying the obtained results by the dilution factor. Each sample that tested negative for HBsAg but positive for anti-HBc alone or in combination with other serological markers was also examined for the presence of HBV- PCR assay.

Preparation of DNA samples from the sera

We strictly followed the guidelines elaborated by Kowk and Higushi to prevent any contamination.[15] PCR was used for positive anti-HBc samples to detect HBV-DNA. 50 µl of serum was diluted 1: 5 with 250 µl NaOH (50 mM) for DNA denaturation and RNA inactivation. Samples were heated for 20 min at 95^{o}C for protein denaturation and then 40 µl of tris buffer (l mM, pH=7.3) was added and finally centrifuge for 5 min at 14000 g. Supernatants were then removed, transferred into a new tube, mixed with 40 µl of phenol–chloroform (1:1), and then centrifuged for 7 min at 14000 g. Ten µl sodium acetate (3M) and 300 µl absolute ethanol was added to each tube and incubated at–70^{o}C for 40–60 min. Then the tubes were centrifuged at 14000 g for another 10 min. Supernatant was removed and 50–100 µl tris-EDTA buffer (pH = 7.8) added to the pellets.

PCR amplification

PCR amplification was performed using a published oligonucleotide primer set,[16] selected from a highly conserved HBV surface gene. Five µl of sample was added to 45 µl of reaction mixture (2.5 unit Taq polymerase, 22.5 picomol/µl of each primer, 200 µm of each deoxynucleotid triphosphate, 5 µl of reaction buffer [50 mM KCl, 10 mM tris–HCl, pH = 8.3] and 1.5 mM $MgCl_2$). Samples were denatured for 5 min at 94^{o}C and then subjected to 40 cycles of 1 min at 58^{o}C, 1 min at 72^{o}C and 1 min at 94^{o}C in an Eppendorf thermal cycler (Master cycler 5330).

Detection of PCR product

Ten µl of reaction product was electrophoresed in a 1.5% agarose gel made in Tris–acetated–EDTA (TAE) buffer (pH=8.0-8.5) and visualized by UV illumination after ethidium bromide (10µg/ml) staining. Positive and negative controls were also treated as samples.

Biochemical tests

Aspartate amino transferase (AST), alanin amino transferase (ALT), total protein, albumin and total bilirubin were performed for all the PCR positive samples biochemical factors including.

Results

Serological results

The "cut-off" points for detection of anti-HBc antibody in serum samples between positive and negative was $OD_{450/620}$ (nm) 0.62. 131 out of 2000 (6.55%) HBsAg negative blood samples were positive ($OD_{450/620}$ (nm)>0.62) for anti-HBc antibody. 110 male (84%) and 21 female (16.6%) had positive sera.

Molecular findings

Ten fold serial dilution of HBV-DNA was used in PCR reaction to determine the limits of sensitivity of this assay. The sensitivity of PCR assay was equal to about 80 copies (+, ++ as shown in Table 1) of HBV genome in PCR mixture. HBV-DNA was detected in 16 out of 131anti-HBc positive specimens (12.2%). All PCR positive samples were obtained from male blood donors. Furthermore, after electrophoresis and ethidium bromide staining, there was an association between the titration of anti-HBc antibody and the intensity of expected PCR product band, HBV-DNA was not detected in about 87.8% of anti-HBc positive individuals. Anti-HBs antibody (>10 mIU/ml) was detected in serum samples obtained from 6 out 16 individuals (37.5%) who had positive HBV-DNA and anti-HBc antibody.

Other HBV markers

The laboratory data of 16 individuals with positive PCR results are displayed in Table 1. HBeAg, anti-HBe and liver function tests (LFT) including AST, ALT, bilirubin, albumin, globulin and alkaline phosphatase, were performed for HBV-PCR positive samples. Only two out of 16 (12.5%) of the sera were positive for anti-HBe antibody. However, the LFT results were all in normal range except in four subject (25%) in whom the level of AST was more than normal (>33 IU/l). The mean amounts of ALT and AST in HBV-PCR positive subjects were 14 IU/l and 23.7 IU/l respectively. Because anti-HBc positive blood donors had normal ALT levels, a

Table 1: Laboratory results of 16 male blood donors with positive anti-HBc and HBV-DNA.

#	Age (Yr)	Anti-HBc*	Anti-HBs**	HBV[!]
21	18	1.48	0.64/45	++
60	44	1.53	0.32/20	++
24	29	0.86	0.25/14	+
25	38	0.94	0.63/42	+
30	61	0.95	0.81/63	+
26	53	0.84	0.92/70	+
31	40	1.49	0.05/<10	++
87	50	1.57	0.12/<10	++
77	57	1.63	0.09/<10	++
127	48	0.76	0.08/<10	+
100	35	0.65	0.16/<10	+
103	27	0.73	0.05/<10	+
99	65	0.66	0.18/<10	+
129	48	0.64	0.20/<10	+
89	18	0.84	0.08/<10	+
131	58	0.71	0.16/<10	+

*= anti-HBc optical density (OD) cut-off value: 0.62 [$OD_{450/620}$ (nm)];
**= Anti-HBs OD cut-off: value 0.20 [$OD_{450/620}$ (nm)/mIU/ml]
!= the density of ethidium bromide staining of HBV-PCR-products is compared with products obtained from diluted plasmid. For more detail see text.

liver biopsy for the investigation of HBV-DNA was considered unethical.

Discussion

At present, HBsAg detection is the only diagnostic screening test for HBV infection in blood transfusion centers in Iran. The prevalence of anti-HBc positivity and presence of HBV-DNA in sera of healthy blood donors negative for both HBsAg and anti-HCV antibody was investigated. Since anti-HBcAg detection is not mandatory in Iran, we molecularly and serologically evaluated if anti-HBc could be adopted as a screening assay for blood donation. To address these issues, we examined 2000 HBsAg negative sera obtained from healthy blood donors and found that 6.55% of them were positive for anti-HBcAg, which was lower than 16.4% that reported by Bernvil et al.[17] Different explanations have been suggested for the reason of the scarcity of HBsAg in anti-HBc positive individual.[11] They had not mentioned what percentage of the donated blood also was positive for anti-HCV antibody.[17]

Most studies on occult HBV infection have reported higher rates of HBV-DNA detection in liver or peripheral mononuclear cells as compared with those of serum or plasma.[18] In our study population the overall prevalence of occult HBV infection (DNA in serum) in healthy blood donors was 12.2%, among anti-HBc positive individuals. It is interestingly to note that there was an association between anti-HBc titration and the intensity of expected PCR product. Nevertheless, no association was found between the presence of anti-HBc and positivity of HBV-DNA.

The frequency of PTHBV is apparently due to the fact that HBsAg is circulating at very low and undetectable level for screening assays, nonetheless, anti-HBc antibody screening tests are able to eliminate some of these donor units.[2] HBV is not highly endemic in Iran and it may be practical to introduce anti-HBc screening in blood banks. Since we did not test anti-HBc negative samples for the presence of HBV-DNA, we do not know whether the removal of anti-HBc positive units would lead to elimination of PTHBV.

According to the hypothesis offered by Brechot et al,[19] we divided our blood donor population to two specific groups of HBsAg positive and HBsAg negative. Seronegative individuals were also divided into two sub-groups (i) anti-HBc positive and (ii) anti-HBc negative individuals. The sub-group (i) which consisted of 6.55% of blood donor population was further divided into two groups: with ani-HBs antibody (37.5%) and without anti- HBs (62.5%) individuals respectively. it has been reported that HBV-DNA is found in HBsAg negative, anti-HBc positive and anti-HBs positive donors.[20,21]

In this study the highest rate of DNA detection was detected in individuals who were positive for anti-HBc but negative for anti-HBs antibody (62.5%). Whereas, the rest of them (37.5%) were positive for both anti-HBc and anti-HBs. These individuals may have recovered from previous infection but may have persistent low level of HBV. Symptomatic hepatitis B has almost never been observed in immunized persons who develop anti-HBs more than 10 IU/ml. Some vaccine recipients may develop anti-HBc, which is indicative of HBV infection; but they usually do so in the absence of disease.[11] The protective anti-HBs are directed against the "a" determinant of HBsAg. In some cases, the antibodies are directed against one of the determinants other than "a" determinant and are unable to neutralize the circulating virion. These cases should therefore be regarded as chronic infection.[22] It should be noted however that in Iran 58% of HBV infections are pre core mutants.[23]

Detection of HBV-DNA in sera of individuals with both anti-HBc and anti-HBs positive may be due to chronic and persistent HBV infection. The exclusion of anti-HBc positive donors is impractical in countries where HBV infection is prevalent and greater than 20% of the populations are anti–HBc positive.[24] However, in our study only 6.5% of blood donor populations was found to be positive for anti-HBc. Although HBV-DNA testing is too difficult and unreliable to perform it would be preferred to perform on anti-HBc positive donors serum samples regardless of aminotransferase levels. No HBV-

DNA was detected in 115 out of 131 (87.7%) patients with anti HBc positive. In organ transplant, anti-HBc test can be used as an indicator for HBV infection. In case of positive results, sera should be subjected to PCR in order to detection of HBV DNA.

In conclusion, anti-HBc antibody should be tested routinely on blood donor volunteers and if the sera become positive regardless of anti-HBs titer, the blood should be discarding. Further testing for HBV-DNA is appropriate to follow up the blood donor patient for HBV infection.

In conclusion, latent HBV infection present in most instances is associated with very low levels of HBV rather than HBV mutants. The prevalence of latent HBV infection is apparently more common among seropositive individuals (anti-HBc positive with or without anti-HBsAg). Thus anti-HBc antibody must be tested routinely on blood donor volunteers, and in case of positive sera, regardless of anti-HBs titer, the blood should be tested for the presence of HBV-DNA. In case of positive HBV-DNA the donated blood must be discarded and the blood donor volunteer examined for HBV infection.

Acknowledgment

This project was financially supported by grant provided by Vice Chancellor for Research of Shiraz University of Medical Sciences.

References

1 Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ. The risk of transfusion-transmitted viral infections. The Retrovirus Epidemiology Donor Study. *N Engl J Med* 1996; 334: 1685-90.
2 Saraswat S, Banerjee K, Chaudhury N, et al. Post-transfusion hepatitis type B following multiple transfusions of HBsAg-negative blood. *J Hepatol* 1996; 25: 639-43.
3 Jongerius JM, Wester M, Cuypers HT, et al. New hepatitis B virus mutant form in a blood donor that is undetectable in several hepatitis B surface antigen screening assays. *Transfusion* 1998; 38: 56-9.
4 Carman WF. The clinical significance of surface antigen variants of hepatitis B virus. *J Viral Hepat* 1997; 4: S11-S20.
5 Carman WF, Mimms LT. Pre-S/S gene variants of hepatitis B virus. In Rizetto M, Purcell RH, Gerin JL, Verne G, editors. Viral hepatitis and liver disease. Edizioni Minerva Medica; Torino, Italy; 1997. p. 108-15.
6 Tsai S, Liaw Y, Yeh C, Chu C, Kuo GC. Cellular immune responses in patients with dual infection of hepatitis B and C viruses: Dominant role of hepatitis C virus. *Hepatology* 1995; 21: 908-12.
7 Lee WM. Hepatitis B virus infection: *N Engl J Med* 1997; 337: 1733-45.
8 Ben Ayed M, Triki H, Cointe D, et al. The isolated presence of anti-HBc antibodies: prevalence and interpretation based on the results of viral DNA research and anti-HBs antibodies measurement after vaccination. *Ann Biol Clin (Paris)* 2001; 59: 53-60.
9 Gomes SA, Yoshida CF, Niel C. Detection of hepatitis B virus DNA in hepatitis B surface antigen-negative serum by polymerase chain reaction: evaluation of different primer pairs and conditions. *Acta Virol* 1996; 40: 133-8.
10 Iizuka H, Ohmura K, Ishijima A, et al. Correlation between anti-HBc titers and HBV DNA in blood units without detectable HBsAg. *Vox Sang* 1992; 63: 107-11.
11 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.
12 Malekzadeh R, Khatibian M, Rezvan H: Viral hepatitis in the world and Iran. J Iran Med Concil. 1997; 15: 183-200.
13 Merat Sh, Malekzadeh R, Rezvan h, Khatibian M. Hepatitis B in Iran. *Arch Iranian Med* 2000; 3: 192-201.
14 Amini S, Mahmoodi MF, Andalibi S, Solati AA. Seroepidemiology of hepatitis B, delta and human immunodeficiency virus infections in Hamadan province, Iran: a population based study. *J Trop Med Hyg* 1993; 96: 277-87.
15 Kwok S, Higushi R. Avoiding false positives with PCR. *Nature* 1989; 339: 237-8.
16 Gish RG, Lau JY, Brooks L, et al. Ganciclovir treatment of hepatitis B virus infection in liver transplant recipients. *Hepatology* 1996; 23: 1-7.
17 Bernvil SS, Andrews V, Kuhns MC, McNamara AL. Hepatitis B core antigen antibody as an indicator of a low-grade carrier state for hepatitis B virus in a Saudi Arabian blood donor population. *Transfus Sci* 1997; 18: 49-53.
18 Torii N, Hasegawa K, Joh R, Hayashi N. Configuration and replication competence of hepatitis B virus DNA in peripheral blood mononuclear cells from chronic hepatitis B patients and patients who have recovered from acute self-limited hepatitis. *Hepatol Res* 2003; 25: 234-43.
19 Brechot C, Thiers V, Kremsdorf D, et al. Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: clinically significant or purely "occult"? *Hepatology* 2001; 34: 194-203.

20 Hennig H, Puchta I, Luhm J, et al. Frequency and load of hepatitis B virus DNA in first time blood donors with antibodies to hepatitis B core antigen. *Blood* 2002; 100: 2637-41.
21 Roche B, Feray C, Gigou M, et al. DNA persistence 10 years after liver transplantation despite successful anti-HBS passive immunoprophylaxis. *Hepatology* 2003; 38: 86-95.
22 Seddigh-Tonekaboni S, Waters JA, Jeffers S, et al. Effect of variation in the common "a" determinant on the antigenicity of hepatitis B surface antigen. *J Med Virol* 2000; 60: 113-21.
23 Malekzadeh R, Mohamadnejad M, Rakhshani, et al. Reversibility of cirrhosis in chronic hepatitis B. *Clin Gastroenterol Hepatol* 2004; 2: 344-7.
24 Nandi J, Benerjee K. detection of hepatitis B virus DNA in donor blood by polymerase chain reaction. *Natl Med J India* 1992; 5: 5-7.