Anti-HBc & HBV-DNA detection in blood donors negative for hepatitis B virus surface antigen in reducing risk of transfusion associated HBV infection

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Received July 7, 2004

Background & objectives: Though sensitive screening assays for detection of hepatitis B virus surface antigen (HBsAg) are available, occasional cases of post-transfusion hepatitis B virus infection (PTH) still occur. The present study was undertaken to assess the prevalence of anti-hepatitis B core (anti-HBc) positivity and presence of HBV-DNA in serum sample of healthy blood donors negative for both HBsAg and anti-HCV antibody in Shiraz, Iran. Since anti-HBc detection is not mandatory in Iran, we evaluated whether anti-HBc detection could be adopted as a screening assay for safety of donated blood.

Methods: Two thousands serum samples negative for both HBsAg and anti-HCV collected from healthy blood donors were tested for the presence of anti HBc antibody. All samples positive for anti-HBc antibody were then investigated for determination of anti-HBc titre, anti-HBs titre, 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: Of the 2000 samples tested, 131 (6.55%) blood samples were found to be positive for anti-HBc. HBV DNA was detected among 16 of 131 (12.2%) anti-HBc positive specimens. Further, there was an association between the titration of anti-HBc antibody and the intensity of expected PCR product band. The liver function test results were all in normal range except in 4 of 16 HBV-DNA positive subjects. The mean levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in HBV-PCR positive subjects were 14 IU/l and 23.7 IU/l respectively.

Interpretation & conclusion: Anti-HBc antibody should be tested routinely on blood donors volunteers and if the sample found positive regardless of anti-HBs titre, the blood should be discarded. Further testing for HBV-DNA would be appropriate to follow up the donor for HBV infection.

Key words: Anti-HBc - HBV-DNA - healthy blood donors - hepatitis B virus infection
The safety of blood products is one of the major issues in the area of transfusion medicine. Transmission of hepatitis B virus (HBV) infection through donated blood is more common than hepatitis C virus (HCV) infection (1:60000 vs. 1:103000)\(^1\). In spite of availability of sensitive screening assay for detection of hepatitis B virus surface antigen (HBsAg), occasional cases of post-transfusion hepatitis B virus infection (PTH) are common\(^2\). There are three possible explanations for 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 superinfection with hepatitis C virus (HCV) may induce clearance of hepatitis B. This could be due to the dominant role of HCV in eliciting an immune response\(^6\). Antibodies to hepatitis B core (HBc) antigen are marker of acute, chronic, or resolved HBV infection and remain detectable for life. These can be 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. Anti-HBc is therefore detected in anyone who has been infected with HBV\(^7\).

It has been demonstrated that some HBsAg negative individuals and those positives for anti-HBc continue to replicate HBV\(^8,9\). These findings suggest that recovery from acute hepatitis B virus infection may not result in complete virus elimination, but rather the immune system keeps the virus at a very low level. A positive correlation has been shown between anti-HBc titre and detection of HBV-DNA in serum samples of HBsAg negative individual\(^10\).

Currently, a number of countries, including United States screen all donations for anti-HBc, which is not mandatory in some other countries\(^11\). However, detection of HBV-DNA by polymerase chain reaction (PCR) has the same significance as detection of HBsAg, and indicates current HBV infection. All blood donations in Iran are collected from healthy donors, and tested for HBsAg as a marker of transmissible HBV. Though these measures have resulted in low rates of transmission by transfusion but have not eliminated it fully.

Despite the availability of an effective vaccine, HBV infection continues to be an important problem in Iran and nearly 8000 to 10000 deaths occur each year due to this sequelae. In Iran, the rate of asymptomatic hepatitis B carriers (HBsAg positive) varies between 0 and 3.9 per cent with an average of 1.7 per cent\(^12,13\). The prevalence of HBV carriers in asymptomatic healthy blood donor in Fars province; the southwest of Iran was about 1 per cent\(^13\). In a study on 4930 healthy blood donors in Hamadan province in Iran, 5.1 per cent were only positive for anti-HBc, without any detectable HBsAg\(^14\), but the presence of HBV-DNA has not been determined in this study.

We undertook this study to assess the anti-HBc positivity and presence of HBV-DNA in serum sample of healthy blood donors negative for both HBsAg and anti-HCV antibody. Further, we investigated the relationship between detection of HBV-DNA and anti-HBc titre. Since anti-HBc detection is not mandatory in Iran, we evaluated whether anti-HBc could be adopted as a screening assay for the donated blood.

**Material & Methods**

**Patients and samples:** Serum samples negative for Treponemal infection, HBsAg, anti-HCV and anti-HIV I, II antibodies were collected randomly from 2000 healthy blood donor volunteers, who were referred to blood transfusion center, Shiraz, Iran during December 2001 to December 2002. Of them, 1820 were male (age range 17-66 yr, mean: 42 ± 14 yr) and 180 were females (age range 23-66 yr, mean: 46 ± 11 yr). Donors were interviewed and medically examined before transfusion. Those with high risk behaviour including intravenous drugs abusers or with any medical problem and those who received HBV vaccination were excluded from the study population. The samples were stored at -20°C until
use. Shiraz University of Medical Sciences ethical committee granted ethical approval for the study. Informed written consents were also obtained from all participants.

Serological assay: All serological tests were performed following manufacturers’ instructions. HBsAg, anti-HBs, anti-HBc-IgG, HBeAg and anti-HBe antibody were measured using commercially available one step enzyme immunoassay technique (MonoLISA, Bio-RAD, France). Anti-HCV (third generation assay) was measured by enzyme immunoassay (EIA) according to manufacture instruction (Innogenetics, Belgium).

Using MonoLISA anti-HBc test, the presence or absence of anti-HBc antibodies was determined by comparing for each sample the recorded absorbance with that of the calculated cut-off value. Samples with an optical density less than the cut-off values were considered to be negative. However, those just below the 10 per cent cut-off value were retested in duplicate according to manufacture instruction. Samples with optical density higher than, or equal to, the cut-off value were considered to be positive and retested in duplicate before the final interpretation.

MonoLISA anti-HBs 3.0 was used for the detection and/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. Samples showed an OD below or equal the cut-off value were considered to be negative. For samples showed an OD within the range of the cut-off value, the HBs antibody concentration was determined from the standard curve.

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 assay.

PCR assay: Preparation of DNA samples from the sera - Strict measures were adopted to prevent any contamination. Samples found positive for anti-HBc, were subjected for detection of HBV-DNA by PCR. Fifty µl serum was diluted 1:5 with 250 µl NaOH (50 mM) for DNA denaturation and RNA inactivation. Samples were then heated at 95°C for 20 min to denature proteins. Forty µl of Tris 1 mM (pH = 7.3) was then added and centrifuged at 14000 g for 5 min. Supernatants were then removed and transferred into a new tube. Forty µl of phenol - chloroform (1:1 v/v) was added and centrifuged at 14000 g for 7 min. Ten µl sodium acetate (3M) and 300 µl absolute ethanol were added to each tube. After incubation at -70°C for 40-60 min tubes were centrifuged at 14000 g for 10 min. Supernatant was removed and 50-100 µl of TE buffer (pH = 7.8) was added to the pellets.

PCR amplification: PCR amplification was performed using a published oligonucleotide primer set selected from the highly conserved HBV surface gene, whereby primer 1 and primer 2 flank 597 base-pair fragment.

Five microlitre 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 MgCl₂]. Samples were denatured for 5 min at 94°C and then subjected to 40 cycles of 1 min at 58°C, 1 min at 72°C and 1 min 94°C in an Eppendorf thermal cycler (Master cycler 5330 Germany).

Detection of PCR product: Ten µl of reaction product was electrophoresed in a 1.5 per cent agarose gel made in Tris-acetated-EDTA (TAE) buffer, pH=8-8.5 and visualized by UV illumination after ethidium bromide (10 µg/ml) staining. Positive and negative controls were also treated as samples. The intensity of expected PCR product band, were then compared with serial dilution of standard control after electrophoresis and ethidium bromide staining.

Biochemical tests: For all the PCR positive samples biochemical factors including aspartate aminotransferase (AST), alanin aminotransferase (ALT), total protein, albumin and total bilirubin were also performed using standard methods.
**Determination of the sensitivity of PCR:** To assess the sensitivity of the PCR assay, template DNA was extracted from 10-fold serial dilutions of plasmid DNA that had been seeded into serum samples negative for HBV-DNA. Conventional gel electrophoresis and ethidium bromide staining were then used to analyze the PCR amplification products from these templates.

**Statistical analysis:** Using SPSS software version 10.0, chi-square and two tail fisher’s exact test were performed to analyze the data.

**Results**

**Serological results:** The cut-off points between positive and negative for detection of anti-HBc antibody in serum samples was found to be OD_{450/620} (nm) 0.62. One hundred and thirty one (110 males, 21 females) out of 2000 (6.55%) of the HBsAg negative blood samples were found to be positive for anti-HBc antibody. The cut-off points between positive and negative for detection of anti-HBs antibody in serum samples was found to be OD_{450/620} (nm) 0.20. Overall, 85 (64.9%) out of 131 anti-HBc antibody positive subjects were found positive for anti-HBs antibody (> 10 mIU/ml). HBV-DNA was detected in 6 (7%) of the 85 patients positive for anti-HBs antibody. Anti-HBs antibody was detected in 6 of the 16 (37.5%) subjects who were positive for both HBV-DNA and anti-HBc antibody.

**HBeAg and anti-HBe antibody** was not determined in any 16 HBV-DNA positive subjects.

**Biochemical markers:** Liver function tests [LFT, aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, albumin, globulin and alkaline phosphatase], were performed on HBV-PCR positive samples. LFT results were in normal range in all except in 4 in whom AST was more than normal (i.e., >33 IU/l). The mean levels of ALT and AST in HBV-PCR positive subjects were 14 ± 5.0 IU/l and 23.7 ± 9.9 IU/l respectively. Because our anti-HBc positive blood donors had normal ALT levels, a liver biopsy for the investigation of HBV-DNA in liver tissue was considered unethical.

**Molecular findings:** HBV DNA was detected among 16 (all male) out of 131 (12.2%) anti-HBc positive specimens. An association was seen between the titration of anti-HBc antibody and the intensity of expected PCR product band, after electrophoresis and ethidium bromide staining.

**Discussion**

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. At present, HBsAg detection is the major and only diagnostic screening test for HBV infection in blood transfusion centres in Iran. We found about 7 per cent of HBsAg negative donated blood positive for anti-HBcAg, which was lower than that reported by Bernvil et al in Saudi Arabia (16.4%). They had however, not mentioned what percentage of the donated blood also was positive for anti-HCV antibody.

Most studies on occult HBV infection have reported higher rates of HBV-DNA detection in liver or peripheral mononuclear cells compared with serum or plasma. In our study population the overall prevalence of occult HBV infection (DNA in serum sample) in healthy blood donors was 12.2 per cent, among anti-HBc positive individuals. Interestingly, there was an association between the titration of anti-HBc 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 post-transfusion hepatitis (PTH) is apparently due to the fact that HBsAg is circulating at undetectable levels for current screening assays. However, screening test for anti-HBc antibody can eliminate some of these donor units. HBV is not highly endemic in Iran and it may be practical to introduce anti-HBc screening in blood banks. Since we have not tested anti-HBc negative samples for the presence of HBV DNA, whether removal of anti-HBc positive units would lead to elimination of PTH-B cannot be ascertained.

According to hypothesis offered by Brechot et al we divided our blood donor population into two
specific groups: HBsAg positive and HBsAg negative individuals. Seronegative subjects were further divided into two sub-groups: anti-HBc positive and anti-HBc negative individuals. The anti-HBc positive subgroup which consisted of 6.55 per cent of blood donor population, can be further divided into two groups, with (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\textsuperscript{20,21}.

In our study the highest rate of HBV-DNA was detected in individuals positive for anti-HBc but negative for anti-HBs antibody (62.5%). These individuals may have recovered from previous infection but have persistent low level of HBV. Symptomatic hepatitis B has never been observed in immunized persons who develop anti-HBs titre 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\textsuperscript{11}. The protective anti-HBs antibody is normally directed against the “a” determinant of HBsAg. In some cases, the antibodies are directed against one of the determinants other than “a” and are unable to neutralize the circulating virion. These cases should therefore be regarded as chronic infection\textsuperscript{22}. Detection of HBV-DNA in anti-HBc and anti-HBs positive individuals may be due to chronic persistent HBV infection.

The exclusion of anti-HBc positive donors is impractical in countries where HBV infection is prevalent and more than 20 per cent of the population is anti-HBc positive\textsuperscript{23}. However, in our study only 6.5 per cent of blood donor population was found to be positive for anti-HBc. 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 to detect HBV DNA. In case of positive results, organ should not be used, or restricted to emergencies only.

In conclusion, anti-HBc antibody should be tested routinely on blood donor volunteers and if the found positive regardless of anti-HBs titre, the blood should be discarded. Further testing for HBV-DNA would be beneficial to follow up the blood donor for HBV infection.

Acknowledgment

Authors acknowledge financial support provided by Shiraz University of Medical Sciences (SUMS) Shiraz, Iran.

References


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