Significance of anti-HBc screening of blood donors & its association with occult hepatitis B virus infection: Implications for blood transfusion


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Background & objective: Expansions of blood donor screening and improved laboratory detection of viral markers have remarkably reduced the risk for infection with transfusion-transmitted viruses. This study was aimed to evaluate the presence of anti-HBc and to determine the presence or absence of HBV DNA in the serum samples from HBsAg negative, anti-HBc positive blood donors in a tertiary care hospital blood bank from Delhi.

Methods: A total of 2175 HBsAg negative, first time volunteer blood donors were included in the study from blood bank, Lok Nayak Hospital, New Delhi. The blood specimens from all these subjects were evaluated for anti-HBV-core antigen (anti-HBc) serology, anti-HBV-surface antigen (anti-HBs) titres and HBcAg. The presence of HBV DNA was evaluated by testing, through polymerase chain reaction (PCR) techniques.

Results: Of the 2175 HBsAg negative voluntary blood donors, 413 (19.8%) were tested to be positive for anti-HBc alone. Of these, 153 (group-I) were anti-HBs negative whereas group-II comprises a total of 260 anti-HBs positive cases i.e. 89 out of 413 had anti-HBs titres of 10-99 IU/l and the remaining 171 had anti-HBs titres of 100-500 IU/l. HBV DNA was detected in 7.5 per cent anti-HBc positive samples irrespective of anti-HBs status.

Interpretation & conclusions: Our results showed that 18.9 per cent of our donor population was anti-HBc reactive, and hence inclusion of anti-HBc testing will lead to a high discard rate. The presence of HBV DNA in fairly high percentage of anti-HBc positive samples highlighted the need for a stringent and better screening system to prevent occult HBV infection.

Key words Anti-HBc screening - anti-HBs titres - occult HBV infection - polymerase chain reaction (PCR) - volunteer blood donors

The risk of hepatitis B virus infection through transfusion has been reduced subsequently with the introduction of hepatitis B surface antigen (HBsAg) screening in blood donors. Generally, HBV infection
is diagnosed by the detection of HBsAg in the serum or plasma of an individual\(^1\). Detection of HBsAg in blood is a diagnostic marker for infection with HBV and in the blood banks screening for HBsAg is carried out routinely to detect HBV infection. Occult HBV infection is defined as the presence of HBV DNA in blood or liver tissues in patients negative for HBsAg but who may or may not be positive for HBV antibodies\(^2\). It is possible that, donors with occult HBV infection, who lack detectable HBsAg might have exposure to HBV infection indicated by positive anti-HBc positive for antibodies against HBV core antigen and HBV DNA, are a potential source of HBV infection\(^3\). Low levels of viraemia have been shown to continue long after clinical recovery from acute, self-limiting HBV infection\(^4\). HBV is also transmitted very frequently when liver is transplanted from HBsAg negative, anti-HBc positive blood donors which shows that liver harbours infectious HBV in some persons negative for HBsAg but positive for anti-HBc\(^5\). However, some HBsAg negative individuals with positive anti-HBc and/or positive for antibodies against HBsAg (anti-HBs) continue to be positive for HBV DNA. Due to limitations in current blood screening practices in developing countries, donation by such individuals is a potential source of HBV transmission to the recipients\(^6\).

There is higher risk of HBV DNA detection in persons who lacked anti-HBs compared to those with detectable anti-HBs levels\(^7\). The infectivity of anti-HBs-positive, HBV DNA-positive blood components is low, with only 10 per cent transmission of HBV infection\(^8\). All blood donors with occult HBV infection may not transmit the disease in blood recipients. Factors, such as viral load in the blood component and immune status of patient, may play a role in viral transmission.

Routine anti-HBc screening of individual blood donations and nucleic acid amplification testing (NAT) by pooling of sera is done in some countries to exclude these donations\(^9\). In India, detection of HBV infection among blood donors is carried out by HBsAg screening while detection of anti-HBc is rarely done\(^10\). Screening blood donors for anti-HBc is not mandatory in India; and blood reactive for anti-HBc would normally be transfused to patients. Recommendations for India include not transfusing blood with high titre anti-HBc, although the titre is not defined\(^11\). Therefore, there is an urgent need to clarify the prevalence of viraemia among HBsAg negative, anti-HBc positive cases and to evaluate the infectivity of blood components from such donors because anti-HBc screening is not mandatory in many countries including India\(^12\). Hence, the aim of this study was to evaluate the presence of anti-HBe amongst the first time blood donors from Delhi and to determine the presence or absence of HBV DNA in the serum samples from HBsAg negative, anti-HBc positive blood donors by PCR method to assess the magnitude of occult HBV infection in these subjects.

**Material & Methods**

**Volunteer blood donors:** A total of 2175 HBsAg negative blood specimens were collected from randomly selected first time volunteer blood donors between January 1, 2005 and November 30, 2006 at the blood bank of Lok Nayak Hospital, New Delhi. The majority of contributions to this blood bank are voluntary donations which are primarily obtained from blood donation camps. Informed consent was obtained from all the donors at the time of blood donation. The major end points were to determine and assess the clinical significance of isolated anti-HBc positivity in the donor population and the seroprevalence of occult HBV infection amongst the healthy blood donor population. Studies of biochemical parameters, blood counts and coagulation, as well as a clinical-epidemiological interview were performed in all the patients. The present study was approved by the ethical committee of Maulana Azad Medical College, New Delhi.

**Laboratory methods:**

Specimen collection and donor screening - Ten milliliter of blood specimen was collected from each subject in plain vial for liver function profile, serological testing and PCR amplification of HBV DNA. The specimens were kept at room temperature for half an hour, and then centrifuged at 489 x g for 15 min. Serum was separated and then stored at -40°C until tested.

All the donor samples were examined using commercial EIA kits for anti-HIV (General Biologicals, Taiwan), anti HCV (Innotest HCV antibody III kit, Innogenetics, Belgium), HBsAg (Qualpro Diagnostics, India), and serologic test for syphilis. Blood donations from individuals who were found to be positive for any of the above infections previously were deferred. Liver function tests [LFT, aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, albumin, globulin and alkaline phosphatase] were performed on all anti-HBc positive samples.
In a subset of 2175 donors negative for the above mentioned tests, supplementary testing of HBV markers and HBV DNA was done for the detection of occult HBV infection. They were assayed for HBeAg, anti-HBc and anti-HBV surface antigen (anti-HBs) (RADIM, Italy) followed by HBV DNA detection. All anti-HBc positive samples were retested in duplicate for HBsAg as well as for anti-HBc. Only repeat HBsAg negative/anti-HBc positive samples were considered to be positive for anti-HBc. The sensitivity of anti-HBc was evaluated by testing a serial dilution of anti-HBc standards supplied by Paul Ehrlich Institute (Germany). The minimal anti-HBc concentration detected by the ELISA kit used was 0.5 U/ml and the specificity was calculated on 1500 blood donors’ samples, with a result of 99.7 per cent. Anti-HBs levels were also quantitated using a standard curve according to the instructions provided by the manufacturer. For better understanding, the levels of anti-HBs were stratified into two groups i.e., 10-100 IU per liter (low level) and > 100 IU per liter (high level). Further, these HBsAg negative/anti-HBc positive samples were also tested for HBeAg (RADIM, Italy).

HBV DNA extraction and PCR amplification - DNA was extracted using phenol chloroform method15. Briefly, 100 μl of serum sample was diluted to 500 μl with reagent-grade distilled water. An equal volume of Tris EDTA (TE)-saturated phenol (pH 8) was added, mixed thoroughly and incubated at 65°C for 2 h. The tubes were centrifuged at 11,269 x g at 4°C for 20 min. The supernatant was collected carefully and extracted once with an equal volume of TE-saturated phenol followed by an equal volume of chloroform and isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase with two volume of ethanol and one-third volume 7.5 M ammonium acetate at -20°C for overnight, washed with 70 per cent ethanol, air dried, and dissolved in 25 μl of Tris-EDTA (pH 8.0).

HBV DNA was detected by an in-house nested PCR technique, amplifying two different regions of the HBV genome as described earlier by Dutta et al14. For the first stage PCR, 50 μl of reaction mixture containing 4 μl of the DNA sample, 1X PCR buffer (10 mM tris-HCl, pH 9.0, 50 mM KCl, 2.5 mM MgCl2, 0.01% gelatin and 0.1% triton X-100), 2.5 mM of each dNTP, 50 pM of each outer primer and 2.5 U of Taq DNA polymerase was amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 35 cycles. After the first amplification, 1 μl of the PCR products was re-amplified for another 35 cycles with 50 pM of each inner primer. The amplified DNA product was run on 2 per cent agarose gel, stained with ethidium bromide at 150V and analyzed with a U.V transilluminator (Vilber Lourmat, Germany). Control samples included normal sera, HBV positive sera, and negative controls. The sensitivity of this PCR assay was also evaluated using the cloned HBV DNA for amplification which served as positive control. Positive and negative controls were run at HBV DNA extraction step as well as for PCR amplifications.

Statistical analysis: SPSS computer software (Version 10.0, SPSS Inc., Chicago, IL.) was used to perform data analysis. Descriptive statistics such as mean and standard deviation for the age of blood donors were calculated, and comparison between anti-HBe-positive and -negative groups was made by t-test. Statistical analyses were performed using a Chi square test and Fisher’s exact test for categorical variables. P<0.05 were considered as statistically significant.

Results

Over a period of two years (January 2005 - November 2006), a total of 3,62,094 blood units were collected at Blood Bank, L.N. Hospital, New Delhi, of which 67.2 per cent were collected from voluntary donors. Amongst them, a total of 2175 first time voluntary blood donors were potentially eligible for participation in the study (Fig. 1). The mean age of the study subjects was 27.3 ± 6.2 yr with age ranged from 16 to 45 yr and the male: female ratio of 2.1:1. Maximum donations were observed in the age group of 16-25 yr, with male donation 46.9 per cent and female donations 43.8 per cent, respectively (Table I). No statistically significant difference was observed amongst male and females with respect to age, anti-HBc and anti-HBs status (Table I). Of the 2175 HBsAg negative/anti-HBc positive samples were also tested for HBsAg and anti-HBc (RADIM, Italy).
negative blood samples screened, 413 (18.9%) were tested to be positive for anti-HBc (Table I). None of the samples was positive for HBeAg.

The anti-HBc positive cases (n=413) were further screened for anti-HBs levels, of whom 37.04 per cent (n=153) turned out to be negative for anti-HBs levels ≤ 10 IU/l (group-I) whereas 62.95 per cent (n=260) were anti-HBs positive (group-II), with 171 of 260 (65.7%) having anti-HBs titres ranging from 100-500 IU/l (high levels) and 89 (34.2%) having low titres (10-100 IU/l) anti-HBs (Fig. 2). In total, HBV DNA was positive in 7.5 per cent (31/413) anti-HBc positive cases. However, in group-I (anti-HBc only) cases, HBV DNA was positive in 12.4 per cent (19/153) cases whereas in group-II (anti-HBc and anti-HBs both positive), HBV DNA was positive in 4.6 per cent (12/260) cases (Table II). All twelve DNA positive cases in group-II, belongs to low titre anti-HBs positive anti-HBc positive cases.

LFT results were in normal range in all except in 12 in whom ALT was more than normal (i.e., >35 IU/l).

On comparison of the mean liver function profiles of anti-HBc alone (group-I) with anti-HBc plus anti-HBs positive cases (group-II), no statistically significant difference was observed (Table II).

Table II. Comparison of the mean liver function profile and detection of HBV DNA among group-I and group-II

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group-I n=153 (%)</th>
<th>Group-II n=260 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV DNA positive</td>
<td>19 (12.4)</td>
<td>12 (4.6)</td>
</tr>
<tr>
<td>AST (5-35 IU/l)</td>
<td>38.1 ± 5.87</td>
<td>36.5 ± 7.42</td>
</tr>
<tr>
<td>ALT (7-56 IU/l)</td>
<td>36.6 ± 7.32</td>
<td>38.9 ± 3.6</td>
</tr>
<tr>
<td>ALP (38-126 IU/l)</td>
<td>177 ± 42.7</td>
<td>178 ± 41.37</td>
</tr>
<tr>
<td>Total bilirubin (0.2-1.3 mg/dl)</td>
<td>0.56 ± 0.18</td>
<td>0.45 ± 0.23</td>
</tr>
<tr>
<td>Total protein (6-8 g%)</td>
<td>6.43 ± 0.3</td>
<td>6.38 ± 0.24</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. Group-I, anti-HBc alone; group-II, anti-HBc + anti-HBs positive. *P<0.05

DNA was positive in 4.6 per cent (12/260) cases (Table II). All twelve DNA positive cases in group-II, belongs to low titre anti-HBs positive anti-HBc positive cases. LFT results were in normal range in all except in 12 in whom ALT was more than normal (i.e., >35 IU/l). On comparison of the mean liver function profiles of anti-HBc alone (group-I) with anti-HBc plus anti-HBs positive cases (group-II), no statistically significant difference was observed (Table II).

Discussion

After the introduction of reliable serologic screening of blood donations, post transfusion hepatitis has become rare. However, the identification of blood donors with occult HBV infection (donors who are negative for HBsAg but have detectable circulating HBV DNA) has created some concern with regards to the safety of blood supply. It is generally accepted that the diagnosis of infection by HBV is based on the presence of the HBsAg in the bloodstream. However, screening of blood bank donors for HBsAg does not totally eliminate the risk of HBV infection through blood transfusion, since the absence of this marker in the serum does not exclude the presence of HBV DNA. It is possible that, donors with occult HBV infection, who lacked detectable HBsAg but whose exposure to HBV infection was indicated by a positive anti-HBe and HBV DNA, are a potential source of HBV infection.

In many countries like India, anti-HBc screening is not mandatory. Hence, we have observed an 18.9 per cent (413 out of 2175) prevalence of anti-HBc positivity. A study from Eastern Indian reported 18.3 per cent anti-HBc prevalence in HBsAg negative blood donors, which corroborates with our finding. However, Banerjee et al reported higher prevalence of...
Persistent viremia after recovery from self-limited study did not establish complete lack of transmission HBc-reactive, HBsAg-negative donors. However, the O'Brien levels, have higher risk of HBV DNA detection. anti-HBs compared to those with detectable anti-HBs et al HBs titres in speculating the HBV infectivity. Brechot positive with low titre, showing significance of anti-HBc positive cases were anti-HBc positive and anti-HBs positive infection might be reactivated despite of high anti-HBs levels prior to allogeneic stem cell transplantation. The transfusion of anti-HBc positive blood with high levels of anti-HBs (titre >1 in 16 which has been reported to correspond to ≈ 200 IU/l) is permitted in Japan and no post-transfusion cases have been documented from such units. The same principle of lack of HBV transmission by units with high levels of anti-HBs has been referred to by many authors, although there does not appear to be agreement on the definition of low and high anti-HBs levels. We observed that 153 of 413 donors positive for anti-HBc (37.04%) lacked anti-HBs, 21.54 per cent had low anti-HBs levels and 41.4 per cent had high anti-HBs. Our findings do not match with the two earlier studies done in UK and US and the difference might be due to the sample size and the seroprevalence of HBV.

It is interesting to point out that HBV DNA was detected in 12.4 per cent of our ‘anti-HBc only’ positive group. Presence of occult HBV infection has also been reported in blood donors from earlier Indian studies showing 27.2 per cent HBV DNA positivity against anti-HBc positive and HBsAg negative cases from Delhi, northern India. Another study from eastern India reported 18.3 per cent HBV DNA positivity in ‘anti-HBc only’ group. Similar study from Chandigarh in north-western India showed 0 per cent prevalence of HBV DNA. In our study 4.6 per cent of the HBV DNA positive cases were anti-HBc positive and anti-HBs positive with low titre, showing significance of anti-HBs titres in speculating the HBV infectivity. Brechot et al also demonstrated that individuals who lacked anti-HBs compared to those with detectable anti-HBs levels, have higher risk of HBV DNA detection.

O’Brien et al did not provide proof of transfusion transmission of HBV from HBV DNA-positive, anti-HBc-reactive, HBsAg-negative donors. However, the study did not establish complete lack of transmission otherwise. Another study by Satake et al from Japan found that only 1 of 33 recipients of anti-HBc positive, HBV DNA positive blood became HBV infected, while 11 of 22 persons who were anti-HBc negative but HBV DNA positive became infected. These studies raise questions about the significance of finding HBV DNA in antibody-positive persons, although there appears to be some increased risk.

The present study highlighted serious concerns regarding the safety of the blood supply still after donor screening for HBsAg.

However, the usefulness of screening for anti-HBc as an additional screening test to improve the safety of the blood supply in India deserves further analysis. Since, exclusion of anti-HBc positive units from the donor pool is not practical in areas with intermediate HBsAg prevalence rates such as India where the anti-HBc positivity ranges from 4.2 per cent to 18.3 per cent in healthy donors. Discarding anti-HBc positive units would result in unacceptably high rates of donor rejection.

A national study, including a statistically significant number of blood donors from different blood donation centres across the country, should determine whether screening for anti-HBc in addition to HBsAg detection, and introduction of PCR based screenings like NAT be considered for the Indian blood donors.

In conclusion, our study supports the presence of occult HBV infections in blood donors positive for antibodies against hepatitis B core antigen and emphasizes on the need for establishing sensitive screening modalities for blood transfusion.

Acknowledgment

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References


