Detection of occult hepatitis B and window period infection among blood donors by individual donation nucleic acid testing in a tertiary care center in South India

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With the introduction of highly sensitive hepatitis B surface antigen immunoassay, transfusion associated HBV infection have reduced drastically but they still tend to occur due to blood donors with occult hepatitis B infection (OBI) and window period (WP) infection. Sera from, 24338 healthy voluntary blood donors were screened for HBsAg, HIV and HCV antibody using Vitros Enhanced Chemiluminescent Immunoassay. The median age of the donor population was 30 (range 18–54) with male preponderance (98%). All serologically negative samples were screened by nucleic acid testing (NAT) for viral DNA and RNA. NAT-positive samples were subjected to discriminatory NAT for HBV, HCV, and HIV and all samples positive for HBV DNA were tested for anti-HBc, anti-HBs, HBeAg. Viral load was determined using artus HBV RG PCR Kit. Of the 24,338 donors screened, 99.81% (24292/24338) were HBsAg negative of which NAT was positive for HBV DNA in 0.0205% (5/24292) donors. Four NAT positive donors had viral load of <200 IU/ml making them true cases of OBI. One NAT positive donor was negative for all antibodies making it a case of WP infection. Among OBI donors, 75% (3/4) were immune and all were negative for HBeAg. Precise HBV viral load could not be determined in all (5/5) NAT positive donors due to viral loads below the detection limit of the artus HBV RG PCR Kit. The overall incidence of OBI and WP infections was found to be low at 1 in 6503 and 1 in 24214 donations, respectively. More studies are needed to determine the actual burden of WP infections in Indian blood donors.

Keywords: HBV, Nucleic acid testing, Occult hepatitis B virus infection, Anti-HBc

Introduction

Blood donor screening using serological assays alone cannot rule out the risk of Hepatitis B virus (HBV) transmission. With 37 million healthy HBV carriers in India it becomes imperative to screen blood donors using a highly sensitive assay to curtail its transmission to recipients.[1] Although, introduction of highly sensitive hepatitis B surface antigen (HBsAg) immunoassay, has resulted in drastic reduction of transfusion-associated HBV infection, they still tend to occur due to blood donors with occult hepatitis B infection (OBI). OBI has been defined by the Taormina expert panel as the absence of HBsAg and the presence of HBV DNA in liver tissues with (<200 IU/ml) or without HBV DNA in the serum.[2] They further defined true OBI when viral loads were <200 IU/ml and false OBI when viral loads were comparable to those with overt infection. Serological pattern 'anti-HBc only' (absence of

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HBsAg and anti-HBs) has been used as a surrogate marker for chronic HBV infection, after reports of HBV infection following transfusion of such blood and blood components (units).[3] This resulted in the implementation of anti-HBc screening of blood donors with recommendations to discard positive units. The major drawback of this policy was the high discard rates in geographic areas endemic to HBV. Window period (WP) HBV infections are classified as pre-anti-HBc or pre-HBsAg WP if all serum markers are non reactive or, as post-anti-HBc or post-HBsAg WP when IgM anti-HBc is positive coinciding with early recovery phase. When HBV DNA is detected in the presence of anti-HBs as the sole serological marker, it is classified as vaccine breakthrough infection or abortive HBV infection. The factors that determine the length of the WP are doubling time of viremia and the analytical sensitivity of the screening test. This residual risk has been reduced by implementation of nucleic acid testing (NAT) assay which has shortened the core window period. Previous Indian studies in anti-HBc positive donors have reported a wide range of prevalence of OBI with the lowest of 0.92% reported from Vellore to as high as 30% reported from Odisha.[4,5] The most plausible reason for such variability would be the use of assays with varying sensitivity in different studies for screening the donors as well as the nature of study population. However, a lone study from Delhi done on all HBsAg negative donors reported a prevalence of OBI and WP infections at 0.046 and 0.018%, respectively.[6] There is absence of any data on OBI and WP infections from the state of Kerala to determine its incidence among all HBsAg negative blood donors.

Materials and methods

A cross-sectional study was done on blood donors, who donated to the blood bank from March 2014 to October 2015. The study was approved by the institutional ethics committee. Samples were collected after getting informed consent from the subjects. Serum samples from the donors were tested for HBsAg, HIV and HCV antibody using Vitros Enhanced Chemiluminescent Immunoassay (Ortho-Clinical Diagnostics, Raritan, NJ, USA). All negative samples were screened, by individual donor nucleic acid testing (ID-NAT) for viral DNA and RNA using Procleix Ultrio Plus kit (Chiron, USA). NAT testing was done in batches and with each batch nine calibrators comprising of two positive calibrators for HIV-1, HCV and HBV, respectively and three negative calibrators, were also included. To enhance the disruption of HBV viral particles, target enhancer reagent is used. The internal control included in the target capture reagent ensures the accurate processing and amplification of each specimen. Measures to prevent false positive results like the use of magnetic separation and wash steps to remove the extraneous components, Selection reagent to inactivate the chemiluminescent label on the unhybridized probes were part of the testing protocol. ID-NAT-positive samples were tested again to rule out contamination or false positivity. All repeat reactive samples by ID-NAT were subjected to discriminatory NAT (dNAT) using the Procleix Discrimination test kit (Chiron, USA) to differentiate between HBV, HCV, and HIV nucleic acids. All dNAT HBV DNA positive samples were tested for Anti-HBc, Anti-HBs and HBeAg using Vidas ELFA assays (BioMérieux, Marcy-l'Etoile, France). DNA was extracted from 200 µl of plasma using QIAamp MinElute Virus Spin kit (Qiagen GmbH, Hilden, Germany) and HBV viral load was determined using CE marked artus HBV RG PCR Kit (Qiagen GmbH, Hilden, Germany) in Rotor gene Q. The assay targets 134 bp region of HBV core gene and amplifies all genotypes of Hepatitis B (A-H). The analytical detection limit of the artus HBV RG PCR Kit was 0.02 IU/µl. An internal control DNA provided in the *artus* HBV RG PCR Kit was included in the isolation procedure. It served as a control for DNA isolation procedure and to check for possible PCR inhibition. All PCR reactions included one no template control (NTC), a positive control (sample known to contain HBV DNA) and internal controls. NTC contained all reagents used in the PCR reaction mixture except the template DNA and ensured that the amplification observed in the samples was not due to contamination. Internal control PCR reaction ensured that the DNA isolation has worked and there are no PCR inhibitors in the sample. Positive control in the PCR ensured that the HBV specific amplification was not hampered. The data were tabulated using MS excel and analyzed using SPSS v20.

Results

A total of 24338 donors were tested using serological methods during the study period and 99.49% (24214/24338) were found negative for all serological markers (HBsAg, HIV, HCV, malaria and syphilis). The median age of the donor population was 30 (range 18–54) with male preponderance (98%). Of the donors 27 (0.11%) were anti-HIV1/2 positive, 51 (0.21%) were anti-HCV positive and 46 (0.19%) were HBsAg positive. ID-NAT and dNAT testing of serologically negative samples identified five HBV DNA positive donors of which four were positive for anti-HBc and were classified as true OBI. One donor was seronegative (negative for anti-HBc and anti-HBs) and classified as WP donor. Overall, the prevalence of OBI and WP was found to be 0.017% (4/24214) i.e. 1 in 6053 donations and 0.004% i.e. 1 in 24214 donations, respectively. Among the OBI donors 75% (3/4) were immune (anti-HBs ≥10 IU/L). All (4/4) OBI cases were negative for HBeAg. Of the five HBV DNA positive samples, viral load for 60% (3/5), including the case of WP donation, were below the detection limit of the artus HBV RG PCR Kit while in 40% (2/5) cases DNA was detected but could not be quantified by the artus HBV RG PCR Kit due to very low viral load. Therefore, in the absence of quantifiable viral load genotyping of HBV could not be carried out.

Table 1 Demographics of published Indian studies on occult hepatitis B virus infection in blood donors

Place/year	Study population (n)	Male (%)	Anti-HBc seropositive			Overall prevalence of OBI (%)	HBV DNA system	HBsAg assay
			Anti-HBc (%)	Anti-HBs (%)	HBV-DNA (%)			
Delhi/2003[17]	Blood donors, (*n* = 24694 and 6159)	–	10.8 and 3.7	5.3 and 1	27.2 and 20.9	0.16 and 0.78	Nested PCR (in-house)	Organon Teknika, Boxtel, The Netherlands
Kolkata/2007[19]	Blood donors, (*n* = 1027)	–	18.3	–	21.3	3.9	Nested PCR (in-house)	Span Diagnostics, India
Chandigarh/2008[25]	Blood donors, (1700)	–	8.4	50.7	1	0.05	Nested PCR (in-house)	General Biologicals, Taiwan. Monolisa ULTRA Bio-rad
Delhi/2010[4]	Blood donors, (*n* = 2175)	70.43	18.9	63	7.5	0.015	Nested PCR (in-house)	Qualisa, Qualpro Diagnostics, India
Orissa/2010[18]	Blood donors, (*n* = 729)	94.78	30.1	18.2	30	9	Nested PCR (in-house)	Biomerieux, Boxtel, The Netherlands
Delhi/2012[24]	Blood donors, (*n* = 94247)	98.39	9.19	–	0.15	0.013	PROCLEIX® ULTRIO® Assay (Gen-Probe Inc.)	Monolisa HBsAg ULTRA Bio-rad
Vellore/2012[5]	Blood donors, (*n* = 1300)	89	16.7	60.3	1.1	0.15	Artus HBV RG RT-PCR (Qiagen GmbH, Hilden, Germany)	Vitros ECi (Ortho-Clinical Diagnostics, USA)
Delhi/2014[6*]	Blood donors, Delhi (*n* = 28465)	100	–	–	–	0.09	PROCLEIX® ULTRIO® Assay (Gen-Probe Inc.)	Vitros ECi (Ortho-Clinical Diagnostics, USA)
Kochi/2016 (Present study)*	Blood donors, (*n* = 24338)	98	–	–	–	0.017	PROCLEIX® ULTRIO® Assay (Gen-Probe Inc.) Artus HBV RG RT-PCR	Vitros ECi (Ortho-Clinical Diagnostics, USA)

*Blood donors were screened using nucleic acid testing (NAT) and anti-HBc testing was performed only in NAT-positive samples.

Discussion

OBI is generally broadly defined as the presence of HBV DNA in the absence of HBsAg.[2] In 2008, Allain provided a more specific definition as the presence of HBV DNA, without HBsAg, with or without HBV antibodies, outside the acute phase window period.[7] Acute phase of HBV infection before the appearance of HBsAg is not considered OBI as the patient eventually develops all the markers of HBV infection.[8] Subsequently in 2008,international workshop on OBI defined it as the presence of HBV DNA in the liver of an individual testing negative for HBsAg with a cut off value for serum HBV DNA (<200 IU/ml).[9] This new definition will exclude patients with HBV DNA level comparable to overt HBV infections due to HBV escape mutants and are classified as false OBI. Some authors have classified OBI as seropositive (anti-HBc alone or together with anti-HBs) or seronegative (no antibodies).[10] The probability of detecting HBV DNA is highest in subjects who are anti-HBc positive and anti-HBs negative and such individuals are more likely to transmit infections.[11] The several reasons for OBI include recovery from a past infection indicated by the presence of anti-HBs, infections due to surface escape mutants and chronic carriers with anti-HBc alone. There is a close association between prevalence of OBI and the endemicity of HBV infection leading to varying rates in different geographical regions. Subjects living in highly endemic countries are more likely to develop OBI.[12] The nature of biological material tested also determines the prevalence as high positivity is seen for liver when compared to serum.[12]

The high prevalence (Table 1) of 'anti-HBc alone' blood donors in India justifies the implementation of NAT for screening for transfusion-transmitted HBV infections.[5] However very few centers have actually migrated to NAT testing due to cost constraints. Although pre-conversion window period has been shortened by use of sensitive serological test, detection of newly infected cases still remains an Achilles heel. The risk of transmission has reduced considerably by shortening of the window period to 40–50 days and 15–34 days with minipool NAT and ID-NAT, respectively.[13] The potential of 'anti-HBc alone' blood units transmitting HBV was first reported in 1978.[14] It was in this background that anti-HBc screening was recommended to prevent HBV transmission from HBsAg negative blood donors.[15] Subsequent studies showed the proportion of 'anti-HBc alone' donors having HBV DNA was very low resulting in high discard rates of blood units. Therefore, the strategy of using anti-HBc screening to detect OBI is not practical in Indian set up and is recommended only in areas where seroprevalence is low (<2% anti-HBc positivity).[16]

The overall prevalence of HBV among blood donors in our study was very low (0.19%) classifying it as an area with low endemicity. As expected the frequency of OBI was also found to be very low at 0.017% which is similar to the studies from Delhi (Table 1).[4,6] Most of the Indian

studies on OBI were done on anti-HBc positive donors. Only one recent study from Delhi has actually looked for OBI in all HBsAg negative donors which detected additional cases of WP donors.[6] Indian studies have reported a wide range of OBI prevalence in anti-HBc positive donors (Table 1). In the year 2010, a study from Delhi reported a prevalence of 27.2% among anti-HBc positive donors.[17] Incidentally in the same year another study from Delhi reported a prevalence of only 7.5%.[4] Similar studies in OBI among anti-HBc positive donors from Kolkata and Odisha reported a prevalence of 21.3% and 30%, respectively (Table 1).[18,19] The most recent study from the state of Tamil Nadu found OBI in only 16.7% of the anti-HBc positive donors. These high HBV DNA positive rates in anti-HBc positive donors reported in various Indian studies is in total disagreement with the low anti-HBc positivity (6%) reported from studies from highly endemic countries.[20] The reason for such variation in the prevalence of OBI would be due to the use of HBsAg assay with varying sensitivity, HBV prevalence and the type of population in which the study was done. Among the five cases that were HBV DNA positive by NAT in our study one was a WP donor. The incidence of WP infection in our study was 1 in 24124 donations which is far less than the 1 in 4689 reported from Delhi and 1 in 5906 reported from China.[6,21] High incidence (1 in 1029) of window period donors have been reported from Taiwan.[22] Previous studies have reported that transmission by window period donors was higher (81%) than the occult cases with antibodies (19%).[13] The remaining four cases were true OBI, with three (75%) of them having immune titers (>10 IU/ml) for anti-HBs which is consistent with reports suggesting 90% of anti-HBc positive donors also carry anti-HBs thereby indicating recovered infection.[13] They also found that transmission was independent of HBV DNA load and the presence of anti-HBs (>100 IU/L) offered some protection.[13] It is noteworthy that transmission from donors positive for both anti-HBc and anti-HBs following acute hepatitis B has never been documented.[11] All our OBI donors were young males which are similar to the findings from Delhi, where the mean age of OBI cases was 27.5 years and all were males.[6] This is contrary to findings by Candotti et al. where OBI occurred mainly in older donors.[13] Of the four cases of OBI in our study, artus HBV RG PCR Kit was able to detect HBV DNA only in two, but the load was too low to be quantified. This is in agreement with previous studies that have shown that OBI cases are associated with low HBV loads (<200 IU/ml) [13] and nucleic acid detection requires an assay with highest sensitivity and specificity with lower limit of detection of <10 IU/ml.[23] High viral loads in the absence of HBsAg however have been reported in HIV co-infected individuals from South Africa.[24]

In Summary the incidence of OBI and WP infections was found to be low at 1 in 6503 and 1 in 24214 donations, respectively. The use of a very sensitive HBsAg assay detected most of the infected cases but the residual risk of transmission of OBI and WP infection still exists. Anti-HBc screening is not an option in Indian setting due to the high discard rates that it entails and its inability to detect WP infections. More studies are needed to determine the actual burden of WP infections in Indian donor population so as to implement the use of NAT-based screening for all HBsAg negative blood donations.

Conflicts of interest

The authors have no conflicts of interest to declare.

Ethics approval

The study received ethical approval Amrita Institute of Medical Sciences and Research Center Ethics Committee.

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