Characterisation and follow-up study of occult hepatitis B virus infection in anti-HBc-positive qualified blood donors in southern China

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Background. Most major Chinese blood centres look for hepatitis B surface antigen (HBsAg) and perform nucleic acid testing to screen blood for hepatitis B virus infection. The search for antibodies to the core of hepatitis B virus (anti-HBc) has not been implemented because it would lead to a high rate of discarded blood units. The aim of this study was to evaluate the prevalence of occult HBV infection among anti-HBc-positive qualified blood donors in southern China.

Materials and methods. We tested anti-HBc-positive blood donations negative for HBsAg and HBV DNA by standard NAT from Shenzhen for the presence of HBV DNA by sensitive nested and quantitative polymerase chain reactions. Anti-HBs titres were quantified. HBV DNA-positive donors were traced and followed-up.

Results. Of the 1,033 qualified donors, 47.4% (95% CI: 44.4 to 50.5%) carried anti-HBc as evidence of exposure to HBV. The rate of anti-HBc positivity increased steadily with age, ranging from 32.6% in the age group <30 years to 69.8% in the age group ≤50 years ($p<0.001$). Of the 1,033 donors, 777 (75.2%; 95% CI: 72.4 to 77.8%) carried anti-HBs (>10 IU/L). HBV DNA was detected in 14 donors who were anti-HBc-positive, HBsAg-negative and negative by routine NAT. Seven of those 14 specimens had an anti-HBs titre above 100 mIU/mL. The prevalence of OBI in anti-HBc-positive qualified blood donors was 2.86% (95% CI: 1.57 to 4.75%). Eight of the 14 OBI cases were genotype B and one was genotype C; 7/14 cases were followed-up, one case converted to anti-HBe. HBV DNA became undetectable in all follow-up samples.

Discussion. A small proportion of anti-HBc-positive qualified donors carry HBV DNA after HBsAg and NAT screening. This finding suggests the possibility of HBV transmission from asymptomatic donors, especially in areas of high HBV prevalence. More sensitive NAT rather than anti-HBc testing should be considered to improve blood safety.

Keywords: blood donors, antibodies to the core of hepatitis B, HBV DNA, occult hepatitis B infection.

Introduction

Hepatitis B virus (HBV) is one of the most frequent and detrimental causes of liver infection in humans[1]. One third of the world's population is estimated to have been in contact with HBV. Each year approximately 620,000 individuals die from HBV-related illnesses, including acute fulminant infection, cirrhosis and hepatocellular carcinoma[2,3]. In addition, approximately 4.5 million new HBV infections occur worldwide each year, of which a quarter progresses to liver disease[4]. In China, chronic hepatitis B ranked first among the 27 infectious diseases reported by the Chinese government for more than 10 years, and about 50% of the Chinese population has a history of HBV infection, and 7.18% are chronic carriers of hepatitis B surface antigen (HBsAg)[5]. HBV remains a major threat to the blood safety.

Screening for HBsAg, implemented 40 years ago, massively decreased the risk of transfusion-related transmission of HBV. The sensitivity of HBsAg screening assays has improved dramatically but these assays remain unable to detect infection in the pre-seroconversion window period and in samples with very low viral load after decades of chronicity or clinical recovery[6]. The availability of HBV nucleic acid testing (NAT) in blood for transfusion enabled the screening of donated blood and the identification of a variable prevalence of HBV DNA carriers in asymptomatic donors negative for HBsAg. However, a proportion of chronic low-level carriers would not be identified even by individual sample polymerase chain reaction (PCR) due to insufficient sensitivity[7-10]. In contrast, anti-HBc screening can individuate nearly all HBV present in

chronically infected or recovered subjects who carry detectable HBV DNA. Anti-HBc testing is implemented in several countries in which the prevalence of hepatitis B is low: it is used as a marker for HBV carriers in cases in which HBsAg is undetected and results in a decrease in the risk of post-transfusion HBV infection[11]. However, in areas in which the anti-HBc prevalence is >2-5%, the deferral of anti-HBc-positive donors is impractical. The deficit in blood donations that would be created by deferring anti-HBc-positive donors is considered too great to be able to maintain an adequate blood supply[12,13]. Many countries with medium and high HBV endemicity such as Italy, Greece, Spain and various Asian nations chose not to test donors for anti-HBc[14]. The major risk of HBV transmission by transfusion in the absence of screening for anti-HBc stems from carriers of occult hepatitis B infection (OBI), which is characterised by the presence of HBV DNA in blood or tissues with undetectable HBsAg, with or without antibodies to hepatitis B core (anti-HBc) or hepatitis B surface (anti-HBs), outside the pre-seroconversion window period[6]. Hence, HBV DNA screening becomes the main option in addition to testing for HBsAg in these regions, including China.

Shenzhen is a modern city of immigrants in Southern China. Blood donors are relatively young and proportionally well educated. With government budget support, Shenzhen Blood Centre began to implement mini-pool NAT as an option in routine blood screening from 2003, and in 2009 decided to use individual donation NAT as a mandatory test to identify more low-level viral carriers, the window period and occult infections of HBV which were identified by several NAT assays from blood donations in a previous study[15,16]. We undertook this study to investigate the improved detection of HBV infection in plasma samples negative for both HBsAg plus routine NAT and to determine the frequency and load of HBV DNA in anti-HBc-positive samples using sensitive nested PCR and real-time PCR (qPCR) assays.

Materials and methods

A total of 1,033 blood samples were collected randomly at the Shenzhen Blood Centre between April and June 2014. All donors in this study were voluntary and non-remunerated, and passed the pre-donation questionnaire, then underwent rapid pre-donation HBsAg testing at the collection sites (colloidal gold strip method, Abon Diagnostics, Hangzhou, China) and assay of alanine aminotransferase (ALT) (Roche Refletron, Roche Diagnostics GmbH, Mannhein, Germany). Qualified donors (negative for the pre-donation tests) proceeded to donate blood. At the blood centre, donations were re-tested for HBsAg (Diasorin S.P.A., Amersham, UK) and screened for anti-hepatitis C virus (HCV) (Ortho Clinical Diagnostics, Buckingham, UK), anti-human immunodeficiency virus (HIV1/2) (Bio-Rad, Marnes-la-Coquette, France), Syphilis (Diasorin S.P.A.) and ALT with a kinetic method (AusBio Biotech, Yantai, China). Only donors non-reactive for serological tests and with a normal ALT level (<50 IU/L) were tested with NAT for HBV, HCV and HIV-1 by the multiplex Procleix Ultrio assay (ID NAT, Novartis Diagnostics, Emeryville, CA, USA). Negative donors for all tests were enrolled randomly in this study.

Blood donors were stratified into four groups according to age. Hepatitis B e antigen (HBeAg), anti-HBeAg, and hepatitis B core antibodies (anti-HBc) were tested by commercially available enzyme immunoassay (Kehua Biotech, Shanghai, China). The anti-HBc-positive samples were re-tested with another commercially enzyme immunoassay (Wantai Biotech, Beijing, China). Results positive with both assays were accepted as "anti-HBc-positive". Anti-HBs was quantified by a commercial quantitative enzyme immunoassay (Wantai Biotech) in accordance with the manufacturer's instructions.

HBV DNA was extracted from 2,500 µL of plasma with anti-HBc positive by HighPure Viral Nucleic Acid Large Volume Kits (Roche Diagnostics GmbH), and were confirmed by a combination of qPCR and nested PCR amplifying the basic core promoter/pre-core (BCP/PC) and S regions as previously described[13,14]. The experiments were performed in a standard PCR laboratory to avoid any contamination. To prevent carryover or cross-contamination during the extraction of DNA from plasma and PCR, each step of the procedure was performed in separate areas with dedicated equipment. Negative controls, including plasma DNA from normal subjects without HBV infection and distilled water, and a positive control were always included in every nested PCR test.

HBV DNA sequencing and genotype determination

To confirm the HBV-DNA specificity, the amplified products obtained from the PCR of the BCP/PC and S-regions were purified using an AxyPrep DNA gel extraction kit (Axygen Scientific, Hangzhou, China) to remove the excess primers. The purified BCP/PC S products were sent for sequencing to Shanghai Invitrogen Co., Ltd. (Guangzhou, China). Nucleotide sequences and reference genotype sequences from Genebank were aligned and manually adjusted using the BioEdit programme (Ibis Biosciences, Carlsbad, CA, USA). Genotype was determined by phylogenetic analysis using the MEGA5.1 programme (Informer Technologies, Inc., San Francisco, CA, USA). The neighbour-joining method with 1,000 bootstrap replicates was applied.

Follow-up study of donors positive for both anti-HBc and HBV DNA

Donors who were positive for both anti-HBc and HBV DNA were monitored by taking follow-up samples for further testing within 1 year. HBsAg, anti-HBc, anti-HBe, HBeAg and anti-HBs were tested. HBV DNA was extracted from 2,500 µL of plasma, qPCR and nested PCR were performed.

Statistical analyses

Ninety-five percent confidence intervals (95% CI) for the observed yield rate were derived using the binomial exact proportion method. Categorical variables were compared using Fisher's exact test whereas continuous variables were compared using the non-parametric Mann-Whitney test. A p-value of <0.05 was statistically significant.

Results

Anti-HBc detection in HBsAg and NAT-negative blood units

Of 1,033 blood samples negative for HBsAg and by NAT screening, 703 (67.9%) came from males and 332 (32.1%) from females (Table I). The gender distribution in different age groups was significantly different (χ^2=24.1, p=0.000). Four hundred and ninety samples were anti-HBc-positive (47.4%; 95% CI: 44.4 to 50.5%), 456 samples (44.1%) carried both anti-HBc and anti-HBs and 34 (3.3%) had only anti-HBc (Table II). There was a clear increase of anti-HBc prevalence with age, ranging from 32.6% in the age group <30 years to 69.8% in the age group <50 years (χ^2=68.8, p=0.000).

Anti-HBs titre distribution in age groups

Of the 1,033 collected samples, 256 (24.8%) were negative for anti-HBs, and 777 (75.2%, 95% CI: 72.4 to 77.8%) were anti-HBs-positive (>10 IU/L). Anti-HBs was the only antibody detected in 321 samples (31.1%). The situation of these donors regarding HBV vaccination was unknown. Amongst anti-HBs-positive donors, 221 (21.4%) had a titre between 10-100 IU/L and 556 (53.8%) had a titre >100 IU/L (Table III). The anti-HBs titre distribution was significantly different among the age groups (χ^2=18.1, p=0.006).

Table I - Age groups and gender distribution in 1,033 blood donors.

Age groups	Female	Male	Total (%)
18-30 years (%)	181 (39.9)	273 (60.1)	454 (43.9)
31-40 years (%)	74 (24.2)	232 (75.8)	306 (29.6)
41-50 years (%)	65 (29.5)	155 (70.5)	220 (21.3)
>50 years (%)	12 (22.6)	41 (77.4)	53 (5.1)
Total (%)	332 (32.1)	703 (67.9)	1,033 (100)

χ^2=24.1, p=0.000.

Serology, biochemistry, HBV DNA level, and virological characteristics in HBV DNA-positive cases

Serum samples from 490 of the 1,033 anti-HBc-positive donors were available for HBV DNA and HBV genotype analysis. HBV DNA was detected in 14 of 490 anti-HBc-positive samples (Table IV). Overall, the prevalence of HBV-DNA positivity in anti-HBc-positive healthy blood donors was 2.86% (95% CI, 1.57 to 4.75%). The percentage of HBV DNA among the different age groups was significantly different between age groups (χ^2=9.8, p=0.02). Of 14 identified HBV DNA-positive donors, nine were repeat donors. In all cases, the HBV DNA load was less than 50 IU/mL and in six cases it was <5 IU/mL. Regarding the other HBV viral markers, of these 14 samples, two had anti-HBc alone (LC114 and LC426) and 12 were positive for both anti-HBc and anti-HBs (Table V). Six out of the 12 anti-HBs-positive samples had titres over 100 IU/L and the other six had titres between 10-100 IU/L.

The phylogenetic analysis identified genotype B in eight samples and genotype C in one. The amino acid sequences of the S region of these nine cases showed that three cases (LC114, LC281, LC346) had amino acid substitutions, G145K/R and S132F. Sequences analysis of nine cases of BCP/PC showed that five (LC22, LC134, LC340, LC450 and LC285) had nucleotide mutations compared to genotype B and C reference consensus sequences.

Follow-up study of donors with occult HBV infection

A total of seven cases that were HBsAg-negative but HBV DNA-positive and anti-HBs-positive were successfully followed-up. All samples remain anti-HBc-positive and HBsAg-negative. One anti-HBe case converted to positive. In three cases anti-HBs became undetectable after more than 100 days. The viral loads of the index and follow-up samples show that the majority of samples were near or below the limit of quantification, while only one case (LC311) had a viral load higher than 10 IU/mL occasionally. In the end, viraemia became undetectable in all follow-up samples. Three cases presented identical sequences for BCP/PC and one case for the S region.

Discussion

OBI is one of the most challenging issues in the field of viral hepatitis. The frequency of detection of OBI is directly dependent on the sensitivity of assays for either or both HBV markers[17], In China, HBV NAT has been preliminarily introduced in many major blood centres since 2003 and the detection yield ranged between

Table II - Distribution of HBV seromarkers according to age groups.

Seromarkers	Age				Total (%)
	<30 years	*31-40 years*	*41-50 years*	*>50 years*	
C+S+	140	158	126	32	456 (44.2)
C+S–	8	11	10	5	34 (3.5)
C–S+	180	77	51	13	321 (31.1)
C–S–	126	60	33	3	222 (21.5)
Total (%)	454 (43.9)	306 (29.6)	220 (21.3)	53 (5.1)	1,033 (100)

C+: anti-HBc-positive; S+: anti-HBs-positive; C–: anti-HBc-negative; S–: anti-HBs-negative. χ^2=82.8, p=0.000. Anti-HBc: antibody to hepatitis B virus core antigen; anti-HBs: antibody to hepatitis B virus surface antigen.

Table III - Distribution of anti-HBs titres in different age groups.

Age	<10 IU/L (%)	10-100 IU/L (%)	>100 IU/L (%)	Total (%)
<30 years	134 (29.5)	79 (17.4)	241 (53.1)	454 (43.9)
31-40 years	71 (23.2)	70 (22.9)	165 (53.9)	306 (29.6)
41-50 years	43 (19.6)	59 (26.8)	118 (53.6)	220 (21.3)
>50 years	8 (15.1)	13 (24.5)	32 (60.4)	53 (5.1)
Total	256 (24.8)	221 (21.4)	556 (53.8)	1,033 (100)

Serum anti-HBs titres <10 IU/L were considered negative. χ^2=18.1, p=0.006. Anti-HBs: antibody to hepatitis B virus surface.

Table IV - Frequency of HBV serological markers and HBV DNA in age groups.

Age	No	Anti-HBc+ (%)	Anti-HBc– (%)	HBV DNA (%)
<30 years	454	148 (32.6)	306 (67.4)	1 (0.67)
31-40 years	306	169 (55.2)	137 (44.8)	5 (2.96)
41-50 years	220	136 (61.8)	84 (38.2)	7 (5.04)
>50 years	53	37 (69.8)	16 (30.2)	1 (2.63)
Total	1,033	490 (47.4)	543 (52.6)	14 (2.86)

HBV: hepatitis B virus; anti-HBc: antibody to hepatitis B virus core antigen.

1:1,000 to 1:10,000 of which approximately 20% were pre-seroconversion window period and 80% OBI[15,16]. This study was conducted to investigate OBI in the qualified blood donor population of Shenzhen, southern China after screening for HBsAg and NAT. It was found that 47.5% of the blood donors were anti-HBc-positive and had, therefore, been exposed to HBV. Of these 2.86% were found to be HBV DNA-positive, HBsAg-negative and anti-HBc–positive with or without other viral markers. The frequency of HBV DNA in anti-HBc-positive qualified donor is consistent with that in the Brazilian Amazon[18], but lower than that found in Pakistan (5/167), Nigeria (8%), Egypt (6.25%) and Indonesia (8.1%)[19-22], and higher than in Germany (1.56%) or Italy (0.56%)[23,24] due to the different epidemiological situations. A prevalence of 2.86% (14/490) shows that the policy adopted in the state of China does not guarantee the safety of blood transfusion. The different rates of OBI reported across the country may be due to the varying sensitivity of HBsAg assays used for donor screening and different protocols for preparing and detecting HBV DNA. Furthermore, contamination in the nested PCR approach for nucleic acid detection or non-specific amplification of other targets is possible and replicate testing to discriminate true and false positive HBV DNA results is required. The data confirm that high volume extraction and very sensitive molecular methods for virus detection are able to reveal the presence of HBV-DNA in healthy individuals such as blood donors, who are expected to have completely cleared a previous HBV infection[25].

The presence of anti-HBs and anti-HBc is usually indicative of immunity after infection. In some countries such as Germany, Austria and Japan, blood units with anti-HBs levels greater than 100 IU/L are considered to be safe[26]. However, a study on blood component infectivity by Allain *et al.*[27] reported that the presence of anti-HBs (titre: 20-160 IU/L) in donors just reduces the risk of HBV infection by approximately five-fold. Cases of hepatitis occurred as the result of transfusion of anti-HBc-positive, anti-HBs positive (12 IU/L), HBV DNA-positive (180 IU/mL) blood products from one donor[28]. There is evidence that transmission of HBV from subjects with OBI occurred in the concomitant presence

Table V - The HBV DNA results and serological markers in the index and follow-up samples in 14 donors with occult HBV infection.

Samples	Age	F/R	Sex	Seromarkers				BCP/PC	S	Virus load (IU/mL)	Genotype
				anti-HBs (IU/L)	*anti-HBc*	*anti-HBe*	*HBeAg*				
Lc22*	35	R	M	40.03	+	–	–	+	–	<5	/
103d#				>300	+	–	–	–	–	–	
Lc114	42	R	M	<10	+	–	–	–	+	–	B
Lc134	49	F	M	>300	+	–	–	+	–	–	-
Lc161	45	R	M	145.7	+	–	–	–	+	<5	B
35d				161.5	+	–	–	–	–	–	
LC178	23	R	M	189.6	+	–	–	+	–	–	/
166d				93.7	+	–	–	+	–	–	
271d				59.9	+	–	–	–	–	–	
Lc200*	48	R	F	73.71	+	–	–	–	+	<5	B
72d				92.3	+	–	–	–	–	<5	
104d				<10	+	–	–	–	–	–	
Lc249	55	F	M	223.4	+	–	–	–	+	–	B
94d				176.2	+	+	–	–	–	–	
183d				52.3	+	+	–	–	–	–	
Lc281*	32	F	F	101.9	+	–	–	–	+	–	B
Lc285	38	R	M	34.3	+	–	–	+	–	<5	/
76d				47.4	+	–	–	–	–	–	
91d				15.6	+	–	–	–	–	–	
121d				<10	+	–	–	–	–	–	
135d				17.9	+	–	–	–	–	–	
155d				24.2	+	–	–	–	–	–	
169d				23.7	+	–	–	–	–	–	
214d				<10	+	–	–	–	–	–	
Lc311	37	R	F	56.8	+	–	–	+	+	<5	C
62d				116.8	+	–	–	–	+	–	C
152d				56.5	+	–	–	+	+	10.5	C
232d				17	+	–	–	–	–	–	
Lc346	47	R	M	81.2	+	–	–	+	+	–	B
Lc366	32	R	F	219.5	+	–	–	+	+	12.9	B
Lc426	41	F	M	<10	+	–	–	+	–	–	/
Lc450	41	F	M	149.1	+	–	–	+	+	<5	B

*Vaccinated in 2011, 2012, 2011. # number of days post index. –: negative.
BCP/PC: basic core promoter/pre-core; F/R: first-time donors/repeat donors; HBV: hepatitis B virus; anti-HBe: antibody to hepatitis B virus e antigen; HBeAg: hepatitis B e antigen.

of neutralising anti-HBs in the same specimen[29]. Data from organ transplantation also clearly proved that HBV DNA in the presence of anti-HBs could be infectious in immunosuppressed patients[30]. Detection of HBV DNA in some anti-HBs-positive samples in this study indicates that the absence of HBsAg and the presence of anti-HBs (<100 IU/L) do not totally guarantee the safety of blood donations. A similar report from Italy also supports the notion that some blood donors with an anti-HBs titre >100 IU/L can still contain detectable HBV DNA[31]. The subjects with OBI identified in this study also carry anti-HBs suggesting that these infections occur largely in individuals who have recovered from HBV infection but have been unable to develop totally effective immune control[32,14].

Initial HBV DNA quantification in plasma showed that six donors (42.9%) had HBV DNA below 5 IU/mL,

the detection limit of the qPCR assay used. Viral load was quantified in two donors as below 15 IU/mL. Consequently, HBsAg screening even with the sensitive NAT assay available may not detect these occult HBV carriers. In addition, viral load fluctuating over time was observed in only one case. Six follow-up samples showed the virus becoming undetectable, which was not consistent with the OBIs NAT yield[33], indicating that OBI with extremely low viral load could easily be missed or the virus have been cleared.

There are several potential biological explanations for the presence of HBV DNA in anti-HBc-positive individuals. These include the chronic carrier state in which HBsAg has declined to sub-detectable levels and the tail end of a resolving acute HBV infection in which, for a short period of time, HBsAg is no longer detectable but a low load of HBV DNA is still present. Mutations in the HBV genome may result in limitations of the replicative capacity or production of altered surface antigen so that it is not detectable by current HBsAg assays. Some experts favour the chronic carrier state as the most likely explanation for the presence of HBV DNA in anti-HBc-positive donors[34-37]. According to the follow-up data from our seven cases, all cases had anti-HBs and, in the end, the virus became undetectable, suggesting that the tail end of a resolving acute HBV infection was the most likely cause of the findings in these OBI with an extremely low viral load.

Conclusions

Our study underscores the high rate of exposure to HBV of the blood donors of Shenzhen in southern China. Indeed, 47.5% of qualified blood donors were HBsAg-negative/anti-HBc-positive and 2.86% of the anti-HBc-positive donors had an OBI, with the potential of transmitting HBV to transfusion recipients. Our results suggest that a higher sensitivity NAT should be adopted and that anti-HBc seems to be an alternative screening parameter. Although addition of anti-HBc testing would lead to the rejection of a large number of donor units, it would definitely exclude many HBV-infected donations. Both strategies would help to reduce transfusion-associated transmission due to OBI in this resource-poor setting with its potential consequences, especially among the immunocompromised population. The significance of a high rate of OBI should be monitored by further studies to evaluate the transmissibility and infectivity of these infections by blood transfusion. These data might lead to improvements in donor screening in the blood banks of China.

Acknowledgements

This work was supported by Shenzhen Scientific and Technological Innovation Commission Fund (Project Number: CYJ20150401092220224).

Authorship contributions

XY performed the research, analysed data and wrote the paper. TL, XX and PD performed the NAT research. JZ, WZ and BY contributed the serological studies. CL designed the research and analysed data. J-PA designed the research, analysed the data and corrected the paper.

The Authors declare no conflicts of interest.

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Arrived: 10 October 2015 - Revision accepted: 28 January 2016
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