Infectivity of blood products from donors with occult hepatitis B virus infection

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BACKGROUND: Occult hepatitis B virus (HBV) infection (OBI) is identified in 1:1000 to 1:50,000 European blood donations. This study intended to determine the infectivity of blood products from OBI donors.

STUDY DESIGN AND METHODS: Recipients of previous donations from OBI donors were investigated through lookback (systematic retrieval of recipients) or traceback (triggered by clinical cases). Serologic and genomic studies were undertaken on consenting donors and recipients. Multiple variables potentially affecting infectivity were examined.

RESULTS: A total of 45 of 105 (42.9%) donor-recipients pairs carried antibodies to HBV core (anti-HBc) as evidence of previous HBV infection. Subtracting 15% of anti-HBc population background, the adjusted transmission rate was 28%. Anti-HBc prevalence increased to 28 of 44 (63.8%) in unvaccinated recipients receiving anti-HBs–negative OBI blood products. In contrast, four of 26 (15.4%) recipients of anti-HBs–positive products were anti-HBc positive. Transmission with anti-HBs–negative products depended on volume of plasma transfused (85%-100% with 200 mL of fresh frozen plasma [FFP], 51% with 50 mL in platelet concentrates [PCs], and 24% with 20 mL in red blood cells [RBCs], $p < 0.0001$ FFP vs. RBCs). The 50% minimum infectious dose of OBI HBV DNA was estimated at 1049 (117-3441) copies. Donor and recipient strains sequence homology of at least 99% confirmed transfusion-transmitted infection in 10 cases and excluded it in one case.

CONCLUSION: Blood products from donors with OBI carry a high risk of HBV transmission by transfusion. This risk is dependent on presence of anti-HBs and viral dose. This may justify safety measures such as anti-HBc and HBV nucleic acid test screening depending on epidemiology.

In the past 5 years, nucleic acid testing (NAT) of hepatitis B virus (HBV) genome has been implemented in most countries of Europe applied either in plasma pools of various sizes (6-96) or in individual donations.[1] Preliminary data collected in Europe, South Africa, and South East Asia indicated that, to be effective, HBV NAT needed maximum sensitivity since for the majority of both window period infection and late stage occult HBV infection (OBI), levels of circulating viral

ABBREVIATIONS: ID_{50} = 50% minimum infectious dose; OBI = occult hepatitis B virus infection; PC(s) = platelet concentrate(s); VL = viral load.

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genome were very low (<100 IU/mL).[2-4] OBI is defined as the presence of circulating HBV DNA in the absence of detectable HBV surface antigen (HBsAg) excluding the window period. Little data are available on the clinical relevance of these HBV DNA–positive donations in the presence of antibodies to HBV. In a context of anti-HBc screening associated with NAT in minipools of 50, the Japanese Red Cross estimated OBI infectivity by transfusion at 3%.[5] In Western Europe, a few cases of clinical transmission of HBV led to traceback studies suggesting infectivity of anti-HBc only–positive donors[6] and in one case with anti-HBs.[7] In high endemic areas where both HBV natural contact and systematic HBV vaccination are frequent,[8-10] several studies reported the difficulties of lookback exercises (identification and testing of recipients of donations preceding an identified seronegative HBV NAT–positive donation).

In this study, lookback and traceback cases from five European countries were assembled to evaluate the still uncertain level of infectivity of blood products prepared from OBI blood donations. Factors potentially influencing infectivity and pathogenicity such as viral load (VL) transfused, the presence of anti-HBs in donor or recipient blood, and the potential role of chemotherapeutic or immunosuppressive drugs taken by recipient were evaluated.

MATERIALS AND METHODS

Testing sites

The collaboration was established between seven blood centers from Croatia, Denmark, Germany, Poland, and Spain and three virology laboratories in Giessen and Langen, Germany, and in Cambridge, UK. HBV DNA testing was implemented in 2005 in Poland, 2008 in Spain, and 2009 in Denmark. Croatia and Germany traceback cases were identified before implementation of HBV DNA testing between 2003 and 2010. Prevalence of OBI in blood donations was previously reported.[2]

Diagnosis of OBI

Blood donor samples were screened for HBsAg using the PRISM system or Architect (Abbott Laboratories, Delkenheim, Germany) or Vitros (Ortho Clinical Diagnostics, Johnson & Johnson, Sollentuna, Sweden) and for HBV DNA with Ultrio on Tigris (Novartis, Emeryville, CA) applied to individual-donor samples, the Cobas s201 (Roche, Basel, Switzerland) as previously described,[11] or validated in-house NAT applied to pools of 6 to 96 samples. Reactive samples were retested with virus-specific probes identifying the virus responsible for the initial signal. HBV-reactive samples were confirmed with real-time polymerase chain reaction (PCR), single-round or nested PCR assays. Donor samples carrying HBV DNA but HBsAg negative were further tested for antibodies to HBV core (anti-HBc) and anti-surface antigen (anti-HBs). Antibody-negative donors carrying HBV DNA were not included in this study because the infectivity of window period cases has been previously demonstrated in humans.[5]

Selection of blood product recipients for infectivity studies

All studies conducted had received local ethical approval. In lookback, donations from repeat donors preceding the OBI index donation were identified as well as the recipients of the corresponding products. The index blood identified as carrying OBI was discarded and the donor deferred from donation. Blood centers contacted the hospitals and collected information regarding the identity, records, and personal details of the patients. Living patients were contacted and informed of a potential HBV transmission by transfusion. They were informed and asked to sign an informed consent to collect a blood sample to test for HBV markers.

In traceback, hospitals contacted the blood centers when a clinical case of acute HBV infection was identified in a transfused patient. Archived samples of implicated blood donations were tested for HBV markers. If a donor was suspected, complete HBV work-up was conducted and recipients of other donations from the suspected donor were also tested for the presence of HBV markers. The recipients of all identified blood components from these donations were traced and tested if alive and consenting.

Donor and recipient sample supplemental testing

Recipient samples were tested for HBV markers: HBsAg, anti-HBc, anti-HBs, and HBV DNA and, when reactive, were confirmed in Giessen, Langen, or Cambridge laboratories as described.[2,12] Whenever possible, VL was obtained by real-time PCR assay or in-house PCR and estimated in copies or international unit (IU)/mL (ratio 5 to 1). Standard or nested PCR was used to amplify full genome, basal core promoter/precore, pre-S/S region, or S region. Amplicons were sequenced to identify strain genotype and specific mutations, particularly in the S protein major hydrophilic region and determine the genetic relationship between donor and recipient strains as previously described.[13,14]

Additional information collected from recipients

Hospitals were asked to provide additional information: date of transfusion and the type of product transfused, the date of blood sample collection within the lookback or traceback exercises, possible occurrence of clinical hepatitis, and the underlying disease. Drugs received were also recorded to evaluate the immune status of the patient and

TABLE 1. Overall scope of the study

Items	Number
OBI donors	24
Lookback donors	19
Traceback donors	5
Median (range) age (years)	46.5 (29-69)
Male donors	19
Female donors	5
Number of donations	251
Number of recipients identified	224*
Number of recipients tested	104 (46.9%)
Number of blood products transfused	105†
FFP	19
PC	12
RBCs	74

* In 73 cases, either the recipient or the product transfused was not identified. In most cases, when the recipient was identified as having died (46 patients), further search was not undertaken.

† In one case, one RBC unit and one PC from the same donor went to the same recipient.

classify them into immunocompetent or immunodeficient. Presence of anti-HBs without anti-HBc before transfusion was considered a marker of vaccination. Recipients older than 30 years without anti-HBs were assumed to be unvaccinated since HBV vaccine became common in exposed groups, newborn, or adolescents in the late 1980s unless explicit data on vaccination was available.

Statistical analysis

Categorical variables were compared using Fisher's exact test and, for continuous variables, the nonparametric Mann-Whitney test. A logistic regression was performed (using the glm function from the R statistical software, http://www.r-project.org/). The 50% minimum infectious dose (ID_{50}) was estimated by Probit analysis[15] using a statistical package in Probit analysis (SPSS, SPSS, Inc., Chicago, IL), a statistical treatment of the sigmoid response curve. The log value of the estimated amount of virus infused is plotted on the X-axis and the infection rate on the Y-axis. The infection rate was assumed 1.0 or 100% in each of the anti-HBc–positive recipients and 0% in each of the anti-HBc–negative recipients. From the probability curve the ID_{50} (and 95% confidence interval [CI]) can be estimated.

RESULTS

Table 1 shows the distribution of OBI blood donors identified in the collaborating centers through either lookback or traceback process. Each donor gave a mean of 10.5 donations (range, 2-22 donations) that were transfused to 224 recipients. In a large proportion of cases, the plasma from these donations was directed toward fractionation rather than therapeutic fresh-frozen plasma (FFP). Red blood cells (RBCs) were transfused in 79% of cases. Blood products from these donations were transfused to recipients of whom 46.4% were identified as alive and consenting to being tested for HBV markers. Forty-six recipients had died by the time the lookback or traceback exercise was initiated and no information on the cause of death was available, five declined participating, and 69 could not be found.

All 24 index donor samples were reactive with the NAT screening assay and identified as HBV DNA carriers by discriminatory assay. All index samples contained anti-HBc confirmed by two different assays in two independent laboratories and nine samples contained anti-HBs with levels ranging 20 to 160 IU/L. In 10 of the 24 donor strains, sequences were obtained in the pre-S/S region classifying four strains as genotype A2 and six strains as Genotype D1. In 11 donor samples, VL ranged between less than 10 and 198 IU/mL. In nine donors of whom multiple samples were available, HBV DNA was consistently detected in six donors and fluctuated in three donors ranging between negative and 75 IU/mL, negative and 12 IU/mL, and 1.8 and 56 IU/mL, respectively.

Analysis of HBV markers

The presence of anti-HBc with or without anti-HBs in recipients was taken as indicator of HBV transmission by transfusion, irrespective of potential recipient infection before transfusion or of otherwise acquired infection during the interval between transfusion and recipient sample collection. This interval ranged between 2 months and 8.5 years (median, 3 years). In the lookback group, 25 of 68 recipients (36.8%) carried anti-HBc with or without anti-HBs and 54.1% (20/37) in the traceback group (Table 2). Forty-five of 105 recipients (42.9%) of OBI products carried anti-HBc, 17 of 62 (27.4%) tested for HBV DNA carried this marker (five HBsAg positive), and 15 of 105 (14.3%) carried anti-HBs as only HBV marker. Information regarding HBV vaccination was available in seven of these 15 cases and they had all been vaccinated.

Factors potentially influencing HBV transmission

The number of HBV genome copies in the plasma transfused (viral dose) was a prime factor. The volume of plasma each recipient received with the transfused product was estimated according to European standards at 200 mL in FFP, 50 mL in platelet concentrate (PC) from whole blood, and 20 mL per RBC unit. The rate of possible HBV infection (anti-HBc with or without anti-HBs) increased from 23/74 (31.1%) to 6/12 (50%) to 16 of 19 (84.2%) for RBCs, PCs, and FFP, respectively (Table 3). The difference in transmission rate between RBCs and FFP was highly significant ($p \leq 0.0001$), even more when only anti-HBs–negative blood products were considered

TABLE 2. HBV serologic markers in recipient according to mode of presentation and product transfused

Mode	Product	Donor-recipient pairs (%)	Anti-HBc+, anti-HBs+	Anti-HBc+, anti-HBs–	Anti-HBc–, anti-HBs+	Anti-HBc–, anti-HBs–	Partial data	Possible infection (%)
Lookback	RBCs	46 (48.4)	8	2	7	24	5	10 (21.7)
	PC	6 (70.0)	1	1	1	3	0	2 (33.3)
	FFP	16 (73.9)	9	4	1	2	0	13 (81.3)
Total		*68 (54.0)*	*18*	*7*	*9*	*29*	*5*	*25 (36.8)*
Traceback	RBCs	28 (70.0)	11*	2	5	9	1†	13 (42.9)
	PC	6 (100.0)	4	0	1	1	0	4 (66.7)
	FFP	3 (60.0)	1	1	0	0	1†	3 (100.0)
Total		*37 (72.5)*	*16*	*3*	*6*	*10*	*2*	*20 (54.1)‡*
Grand total		105	34 (32.4)	10 (9.5)	15 (14.3)	39 (37.1)	7 (6.7)	45§ (42.9)

* One patient received both RBCs and PC from the same donor; possible infection was attributed to PC.
† One patient was anti-HBc positive but anti-HBs not tested.
‡ Considering 21 anti-HBc–positive recipients.
§ Includes 33 anti-HBc+/anti-HBs+, 10 anti-HBc only, and two anti-HBc+ with untested anti-HBs.

(Table 4). Fifteen of 15 FFP (100%), four of six PCs (67%), and nine of 23 (39%) RBC unvaccinated recipients of anti-HBs–negative products carried HBV markers.

In 17 transfused products VL was estimated. In 13 products, although HBV DNA was detected, the quantitative assay was either negative or below the assay limit of quantification (5 IU/mL). In the corresponding products, VL was considered below the assay limit of quantification and assigned this value divided by two (between 2 and 200 IU/mL). When multiple index and archived samples from a donor were quantified the geometric mean value was utilized to estimate the VL of products lacking direct quantification. Considering only unvaccinated recipients (n = 63) of anti-HBs–negative products, the projected viral dose transfused was 1032 copies (n = 28) in anti-HBc–negative and 2694 copies (n = 35) in anti-HBc–positive recipients, respectively (Fig. 1; $p = 0.003$) and the estimated ID_{50} as calculated by probit analysis was 1049 (117-3441) copies. However, in 17 recipient samples with directly quantified infused amount of HBV the ID_{50} was 535 copies (95% CI cannot be calculated) and the difference between the viral dose of 1500 copies (range, 180-56,000) observed in nine infected and 900 copies (range, 200-19,800) in eight noninfected patients was not significant ($p = 0.29$).

The levels of anti-HBs in donors ranged between 20 and 160 IU/L (median, 40 IU/L). As shown in Table 4, four of 26 (15%) and 41 of 80 (51%) recipients of blood products from OBI donors with or without anti-HBs, respectively, carried anti-HBc ($p = 0.013$), and an even larger difference in potential transmission rate was found when only unvaccinated recipients were taken into account (15% vs. 64%, $p < 0.0001$). Unlike in recipients of anti-HBs–negative OBI blood the amount of transfused plasma had no impact on the anti-HBc prevalence in recipients of anti-HBs–positive OBI blood. Only three of 19 RBC recipients (17.6%) carried anti-HBc and, significantly, none of three FFP recipients were infected.

While 15 of 15 (100% of FFP recipients of anti-HBs–negative OBI blood) were infected, none of three FFP recipients of anti-HBs–positive OBI blood had markers of HBV infection. We therefore assumed that four of 26 (15.4%) recipients of anti-HBs–positive OBI blood products carried anti-HBc because of HBV infection unrelated to the OBI transfusion. When this percentage of 15.4% was taken as the background anti-HBc prevalence, the transmission rate of anti-HBs–negative OBI blood products given to the unvaccinated recipients (>30 years) could be estimated by subtraction of 15.4% from the anti-HBc prevalence. The 15% background prevalence in the older recipient population is in line with epidemiologic data (see discussion). With this assumption being compatible with our data assuming negligible contribution of transmission risk of anti-HBs–positive OBI blood, the transmission rate by anti-HBs–negative OBI blood products

TABLE 3. Serologic reaction patterns in recipients of blood products from donors with OBI

Product	Tested recipients	Possible transmission anti-HBc+, anti-HBs+/–	Probable immunity anti-HBc–, anti-HBs+	No infection anti-HBc–, anti-HBs–
RBCs	74	23 (31.5)*	12	39
PC	12	6 (50.0)	2	4
FFP	19	16 (84.2)†‡	1	2§
Total	105§	45 (42.9)	15 (14.3)	45 (42.9)‖

* This number includes one recipient with incomplete data but one recipient having received RBCs and PC from the same donor was subtracted.
† One recipient with incomplete serology but anti-HBc positive was considered possible transmission.
‡ Highly significant difference between infectivity of RBCs and FFP $\chi^2 = 21.7$; $p = 0.0003$.
§ One patient carried 13 IU/L anti-HBs and was probably vaccinated to HBV; the other received anti-HBs–positive FFP.
‖ Eight recipients with incomplete serologic data are not included but being either anti-HBc or anti-HBs negative are considered not infected.

TABLE 4. Impact of anti-HBs in transfused products or in vaccinated recipients on OBI infectivity

Category	Anti-HBc positive in recipients/total (%)			
	All products	RBCs	PC	FFP
All*	45/105 (42.9)	23/74 (31.5)†	6/12 (50.0)	16/19 (84.2)
All receiving anti-HBs–positive blood	4/26 (15.4)	3/19 (17.6)	1/4 (25.0)	0/3 (0)
All receiving anti-HBs–negative blood	41/80 (51.3)	20/55 (35.7)	5/8 (62.5)	16/17 (94.1)
Unvaccinated patients receiving anti-HBs–negative blood‡	28/44 (63.6)	9/23 (39.1)	4/6 (66.7)	15/15 (100)
Adjusted rate (%) of transmission after deduction of 15% background anti-HBc	48	24	51	85

* All donors identified as OBI carried anti-HBc and all recipients considered infected were anti-HBc positive.
† One RBC unit and PC unit were transfused to the same recipient; the RBCs were not counted.
‡ Recipients for whom no information regarding vaccination was available were excluded.

increases from 24, 51, and 85% in recipients of RBCs, PCs, and FFP, respectively (Table 4).

Immunodeficiency related to young age (premature infants or newborn) or to hematologic disorders and/or the drugs received for these conditions may cause susceptibility to lower infectious dose of HBV. Immune status was known for 61 of 105 recipients (58.1%) as estimated by the collaborators but very few details regarding the drug regimen were available. Of 36 recipients classified as "immunocompetent," 24 (66.7%) were anti-HBc positive after transfusion (six with anti-HBc only). In the "immunodeficient" group eight of 24 (33.3%) were anti-HBc positive after transfusion. This difference was significant ($p = 0.01$).

Prediction of OBI transfusion risk

A logistic regression was performed with presence of anti-HBc in the recipient as outcome. Potential predictors were presence or absence of anti-HBs in donors, vaccination, immunodeficiency (each with two levels), transfused product type (encoded as 1 for RBCs, 2 for PCs, and 3 for FFP), and donor HBV DNA (in IU/mL). Since copies of viral dose received is highly correlated with product, which is a much better predictor, it was dropped from further analysis and replaced by donor HBV DNA in the logistic regression model (Table 5). Only two variables are significant predictors of infection: donor anti-HBs ($p = 0.012$) and transfused product ($p = 0.00078$). As shown in Table 5 the presence of donor anti-HBs reduces the risk of infection by approximately fivefold, while PC over RBCs or FFP over PC increases risk by approximately threefold (and consequently FFP over RBCs by approximately ninefold). Donor HBV DNA load, although not significant, contributes to improved prediction. The logistic regression can be used to predict whether a recipient will be positive for anti-HBc based on the above predictors. The performance of such predictor can be measured by the area under the receiver operator characteristic curve, which is 0.738. This means that the predictor score of an anti-HBc–positive individual will be above that of an anti-HBc–negative individual in 73.8% of cases making this a reasonably useful predictor in practice.

Genetic evidence of HBV infection by transfusion

In only 11 donor samples, sufficient length of the genome in the pre-S/S region was sequenced to determine HBV genotype. In 11 recipients from seven of these 11 donors, sufficient HBV DNA was present to amplify, sequence, and determine genetic homology between donor and recipient sequences (Table 6). In 10 of these paired sequences, nucleotide sequences were at least 99% homology. In one case, two donor and five recipient sequences were obtained (Fig. 2) forming a unique cluster with more than 99% homology. The 11th pair (D4569) donor and recipient strains were of different genotypes (A2 and C), excluding transfusion as the origin of the recipient infection.

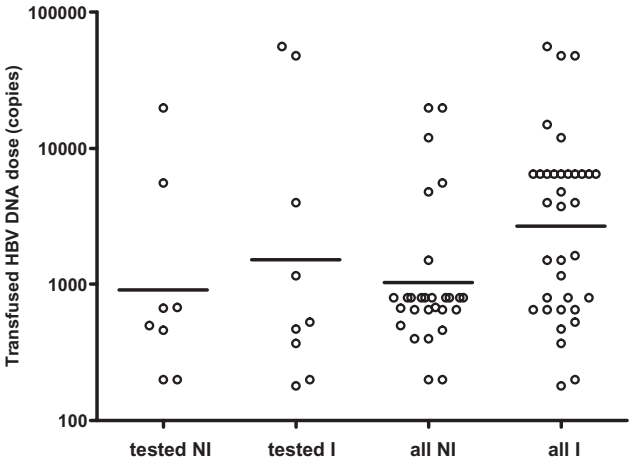

Fig. 1. Distribution of HBV DNA load transfused in noninfected (NI) and potentially infected (I) blood product recipients. Only recipients receiving anti-HBs–negative blood products and not vaccinated were included. The first group indicated as "tested" corresponded to donation samples involved in the case actually quantified by quantitative PCR. The second group indicated as "all" includes those of the first group and those where the VL of the involved donation was not actually quantified but extrapolated from other samples from the same donor that were quantified. Estimation of viral dose transfused is indicated under Materials and Methods. The horizontal bar indicates the geometric mean of the viral dose transfused for each subgroup. It is 1032 HBV DNA copies for noninfected recipients and 2694 copies for recipients carrying HBV markers (p t test = 0.0033).

HBV mutants in OBI donors

Three of seven donors (G4-G6, Table 6) had mutations in the S gene, which were very relevant for the antigenicity of HBsAg. In G4, the mutation K160N changed one of the two HBsAg subtype determinants from wild type to an unknown specificity. In Case G5, four subsequent substitutions from Amino Acids 142 to 145 altered the group-specific determinant "*a*" profoundly. This caused false-negative results with some HBsAg assays, but most assays detected the transmitted mutant in the infected recipient who had a much higher viremia than the donor. The donor G6 had donated 42 times over 10 years. The five available FFPs from the past 10 months were analyzed for HBV quasi-species composition by cloning of the amplified DNA and sequencing eight to 20 clones. All clones coded for seven amino acid substitutions in the HBsAg loop, four of them altering determinant *a* and transiently up to four additional variable amino acid substitutions.

Clinical consequences of HBV transmission

The lookback procedure did not identify any of the 62 anti-HBc–positive recipients who had suffered from clinically apparent hepatitis or chronic HBV infection with persistence of HBsAg over the average 2 years until the posttransfusion sample was collected. The only recipient who was acutely infected had a HBV genotype different from the Donor D4569 (Table 6). Thus, transmission of HBV from OBI leads in most cases to asymptomatic resolving infection with subsequent development of anti-HBc and anti-HBs. However, four index donor cases shown in Table 6 (G3-G5) were identified because recipients developed acute liver failure with all signs of acute HBV infection that was fatal in three of them. All three patients had some degree of immunologic disturbance. One patient had a sepsis soon after the transfusion of the infectious donation. The second patient had an unidentified type of hematologic disorder, and the third patient had autoimmune hepatitis. In two cases the interval between transfusion and fulminant hepatitis was 4 (G4) and 3 (G5) months after transfusion, strongly suggesting the connection between transfusion and hepatitis. Further, in all four cases transfusion transmission was confirmed by more than 99% identity between donor and recipient strain sequences.

DISCUSSION

Although HBV DNA was identified in 1:1000 to 1:50,000 donations depending on the epidemiology of HBV in different areas,[1-4,9,11,16] the infectivity of blood units from OBI donors was not determined except in Japan where it seemed to be very low.[5] In Europe clinical traceback cases indicated a relatively high rate of transmission.[6,12] Except for a preliminary report,[6] no lookback data related to OBI infectivity have been reported in Europe. In high-prevalence Taiwan or Hong Kong, lookback studies emphasized the reliance on donor and recipient strain sequence homology to establish transfusion transmission when no pretransfusion sample was available.[8-10]

In this study, both lookback and traceback cases have been examined to compare the data collected in each situation. In relation with the large proportion of elderly patients and of patients with oncologic underlying conditions, nearly 40% of recipients identified died shortly after transfusion or did not accept to participate as previously reported (Table 1).[17,18]

A pretransfusion sample being unavailable and molecular evidence of HBV donor-recipient strains identity being available in only 10% of cases, identification of possible HBV transfusion transmission relied on post-transfusion detection of anti-HBc. The concomitant presence of anti-HBs supported the likelihood of recent transmission, as this marker level tends to decline with time, particularly in older people.[19] Such likelihood was lower when anti-HBc was the only marker of HBV infection (27% of cases). The sole reliance on the presence of anti-HBc in recipients without preransfusion sample and tested

TABLE 5. Logistic regression analysis of factors influencing recipient infection status

Factor	Estimate	Relative risk (OR)	SE	p Value
Intercept*	–1.25	0.28 (0.10, 0.80)	0.53	0.018†
Anti-HBs (donor)	–1.51	0.22 (0.07, 0.72)	0.60	0.012†
Vaccination (recipient)	–0.65	0.52 (0.12, 2.23)	0.74	0.38
Product transfused	1.09	2.97 (1.57, 5.59)	0.32	0.0008‡
Viral dose (IU/mL)	–0.01	0.99 (0.97, 1.01)	0.01	0.20
Immunosuppressive drugs	0.04	1.04 (0.31, 3.52)	0.62	0.94

* The intercept indicates the baseline relative risk in this study, the OR of positive people in the study after taking account of predictors.
† $p < 0.05$.
‡ $p < 0.01$.

often several years after transfusion seems a major weakness in this study. However, it was the only feasible solution and several elements indicate that it has been a valid approach. 1) The study was conducted in populations where HBV infection is relatively low (<8% of anti-HBc and <1% of HBsAg prevalence); the risk of nosocomial infection is therefore limited. The recipient population being relatively old (median, 46 years) makes the risk of transmission through intravenous drug abuse or promiscuous sexual activity after hospitalization and transfusion relatively low. 2) The background anti-HBc prevalence in the population has been eliminated by the deduction of 15% anti-HBc originating from infection contracted before the implicated transfusion after correction for recipient age (see results and discussion below). 3) The data collected showing the close correlation between volume of plasma transfused and the presence of anti-HBc in recipients strongly supports the responsibility of infection by transfusion of anti-HBs–negative OBI blood. Further, this correlation did not exist in recipients of anti-HBs–positive OBI blood. 4) Finally in the limited number of cases where HBV DNA could be sequenced in donor and recipient more than 99% of homology clearly incriminated the OBI-carrying donor who, in all cases, was anti-HBs negative. The identification of one of 11 cases where sequence homology excluded transfusion as origin of the infection is in line with the 15% deduction applied to estimate the transmission risk of OBI blood in our study population.

For traceback cases infectivity of the transfused unit was not in doubt and this could have created a bias overestimating OBI infectivity. However, the rate of potential infection observed in a larger number of lookback cases was not considerably lower (36.8% vs. 54.1%) and the difference was not significant ($p = 0.16$, Table 2). Overall, the 42.9% rate of potential infection was unexpectedly high and infections from other sources were considered. No specific risk groups were identified. The background prevalence of anti-HBc in Croatia, Denmark, Germany, and Spain ranges between 5 and 8% in the general population.[20,21] In Germany and Spain the prevalence of anti-HBc reaches 15% in the age group older than 65 years.[22] Since the majority of the patients in this study were of older age, a 15% lower adjustment seemed appropriate. Such adjustment was consistent with the prevalence of HBV markers in recipients of anti-HBs–containing blood components (15.4%). Therefore, the conservatively estimated rate of HBV OBI transmission is 28% overall and 22% for lookback cases. This is compatible with the 18.2% rate (two of 11 cases) identified in a Taiwanese study.[8] The much lower transmission rate of 3% (one of 33) observed in Japan for transfusion of blood components collected from OBI donors[5] may be explained by the fact that in Japan donors with HBV DNA levels detectable by minipool NAT or with anti-HBc hemagglutination inhibition titer of greater than 16 were already excluded and many recipients were immune.

Three main specific elements potentially interfering with HBV OBI transmission are: 1) the viral dose (number of HBV genome copies/mL multiplied by the volume of plasma transfused); 2) the presence of anti-HBs either in the donor blood or passively acquired by simultaneously transfused blood components from anti-HBs–carrying donors or actively acquired in a vaccinated patient; or 3) the recipient immune status, which is variably impaired in neonates or patients receiving chemotherapy or immunosuppressive treatments.[23] For instance, cyclosporine or fludarabine in combination with anti-CD20 are known to cause severe immunodeficiency and HBV reactivation.[24,25] These factors were examined overall.

The viral dose transfused to the recipient resulted in two independently significant factors: plasma volume in components ($p = 0.0003$) and VL. The projected geometric mean value of viral DNA received by transfusion in possibly infected and noninfected recipients was significantly higher in anti-HBc–positive recipients ($p = 0.003$). However, when only the actually measured viral dose infused in 17 recipients was compared, the difference in geometric mean viral dose between infected and noninfected recipients was not significant while the dose ranges overlapped (Fig. 1). The ID_{50} calculated by probit analysis (1049; 117-3441) was, however, not significantly different (Tables 3 and 4; Fig. 1). Several possible factors, both viral- and host-related, might play a role in the infectivity of OBI HBV and for the not so clear distinction between infectious and noninfectious VL.

1) Fifteen percent of anti-HBc–positive recipients were considered to have acquired the infection from another source. These may be among the recipients who received a low VL and would in this case blur the data. 2) Not all infected recipients may develop anti-HBc for a sufficiently long period of time or at high enough amounts to be detected. In fact, seronegative or primary OBI has been

TABLE 6. Characterization of donor and recipient HBV infection

Donor*				Recipient(s)				
ID	VL (IU/mL)	Genotype	HBsAg loop mutations	HBV DNA	VL	Genotype	Percent homology	Clinical liver disease
10 proven transmissions								
G3	8	D1	wt†	Pos	0.8E5	D1 S114P	99.6	Acute liver failure
G4	5.3	A2	M103I, K160N, Y161F	Pos	2E4	A2	100	Acute liver failure
G5	48	D1	P142L, S143L, D144E, G145K, I150T, F158L	Pos	3400	D1	100	Acute liver failure
O0118	<10	D2	wt	Pos	4.6E4	D2	99.8	Acute hepatitis
C1223	<10-75	D3	wt	Pos	19	D3	99.8	Asymptomatic
				Pos	5	D3	100	Asymptomatic
				Pos	<5	D3	100	Asymptomatic
				Pos	543	D3	100	Asymptomatic
				Pos	182	D3	99.8	Acute liver failure
D0283	<5	D1	wt	Pos	7	D1	100	Asymptomatic
Probable or possible transmissions‡								
V1320	<10	D	wt	Pos	<10			Asymptomatic
G6	9-56	A2	M103I, L109H, P120Q, G130S/N, N131K, S132F	Neg	ND			Nothing noted
No transmission								
D4569	69	A2	wt	Pos	4.8E6	C		Asymptomatic
O0121	10	D	No seq	Neg	Neg			Asymptomatic

* All donors were consistently anti-HBs and HBsAg negative.
† wt = wild type.
‡ Three more recipients of G6 donor without sequenced DNA were recorded.

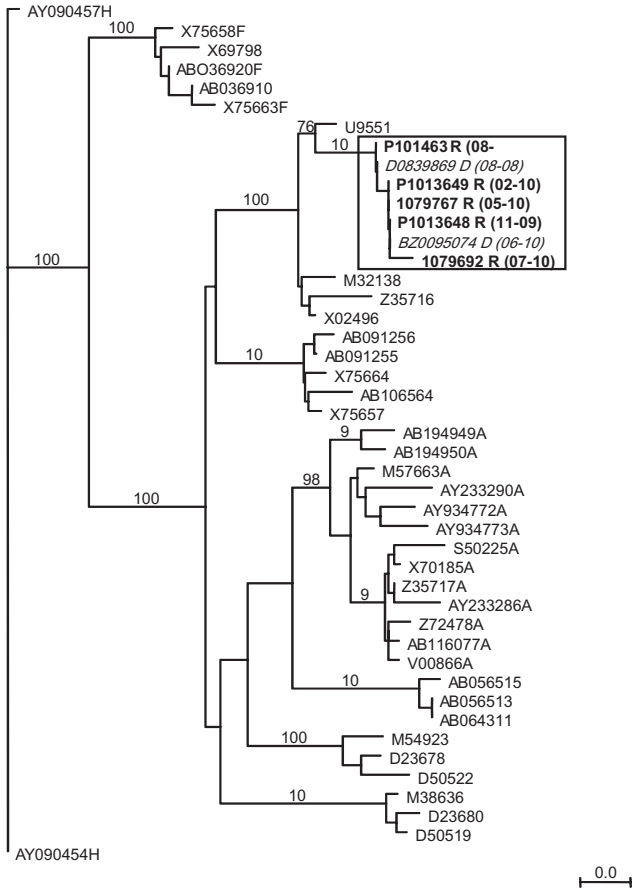

Fig. 2. Phylogenetic tree of OBI donor C1223-recipients pre-S/S sequences. Reference sequences were taken from GenBank and indicated by their accession code followed by the genotype. The two sequences from the donor (indicated by D in italics) collected at 2-year intervals (month and year in parentheses) and sequences from strains of five different recipients (indicated by R) form a specific cluster (boxed) separated by a bootstrap value of 100. Bootstrap values are indicated above tree main branches. The scale is indicated at the right bottom corner.

described[26] and occurs in approximately 15% of subjects with intrahepatic HBV DNA.[27] 3) The Poisson distribution of infectious particle estimated by probit analysis that the ID_{50} (95% CI) was approximately 1000 (100-3500) virions, which may explain that some recipients receiving relatively high doses of viral genomes remained anti-HBc negative. 4) Viral fitness might play a role in the apparently lower infectivity of HBV in OBI, although in four traceback cases, the transmitted strains replicated well and caused fatal liver disease in three of them. 5) Most important the detection of several mutations in the HBsAg loop of infectious donors without detectable anti-HBs (Table 6) suggests that unrecognized antibodies in OBI donors drive mutant selection.[2,28] Immune complexes frequent in chronic HBsAg carriers[29] may occur in OBI carriers.

Absence of such antibodies may be the major factor for greater than 100-fold higher infectivity of virus transfused during the window period.[5,30]

OBI transmission experiments in chimpanzees showed that detectable anti-HBs was protective.[31] In our study, anti-HBs in the OBI donor blood was exclusively found in lookback cases and in the presence of antibodies significantly less frequently transmission occurred ($p = 0.01$, Table 4) in agreement with previous data.[32] Infectivity of anti-HBs–positive OBI was previously reported but only when viral dose was beyond the neutralizing capacity of levels of anti-HBs below 30 IU/L.[7] The potential interference of components containing anti-HBs of natural of vaccine origin simultaneously transfused was not considered here since no systematic testing of archived samples was possible. Recent data have shown that the protective efficacy of vaccine-induced antibodies to Genotype A2 was relatively less protective against other genotypes such as Genotypes B, C, and D.[33] In this study, too few samples were genotyped to undertake verifying this aspect.

The significant lower risk of OBI infection in potentially immunodeficient recipients and lack of significance in the logistic regression analysis (Table 5) was unexpected but the small number of cases and the relative inaccuracy of the stratification may have played a role. In addition, some cases might have been unable to mount an anti-HBc response. The therapeutic drug-induced potential immunodeficiency of a recipient has rarely been reported in previous studies of HBV transfusion transmission of OBIs. In Hong Kong, five recipients were considered susceptible to HBV infection since no HBV markers were detected after transfusion but were not infected.[9] These individuals had been exposed to fewer than 1000 to 19,000 copies of HBV DNA but may have been protected by anti-HBs present in other transfused components collected from recovered or vaccinated donors. A single presumably immunocompetent recipient of 49 considered confirmed transfusion infection by molecular homology (95%) received 1900 copies of HBV DNA.[9]

There were considerable fluctuations in donor VL over time (up to 100 times) in the follow-up samples as previously described.[7] This may play a role in HBV infection as illustrated by a report from Japan of a series of recipients of apheresis PCs from an OBI carrier donor over a period of 7 years. The viral dose received by the 10 recipients available for testing ranged between fewer than 10,000 and 40,000 copies but four were not infected, two had an asymptomatic HBV infection and four an acute, symptomatic infection.[34]

The multiple factors identified as potentially interfering with infectivity of transfused blood products from OBI donors were assembled to develop a matrix predictive of infectivity. Only two variables are significant predictors of infection: donor anti-HBs ($p = 0.016$) and transfused product ($p = 0.0008$). The donation VL, although not significant in the model, contributes to improve prediction and the estimated HBV dose received by infected recipients tended to be higher than in uninfected patients (Fig. 1).

As pointed out in several previous articles on OBI infectivity, the ultimate evidence of transfusion transmission is the identity of a long enough informative sequence between donor and recipient HBV DNA.[6,7,9,25] In one report, homology of at least 95% was considered sufficient evidence[9] but in this and other reports, more than 99% was observed. This percentage, however, is dependent on the region of the genome and the genetic diversity of the sequenced amplicons. Typically more than 500 nucleotides in the S region including the major hydrophilic region are reliably informative. Such genetic evidence was particularly important in endemic regions where four of five donor-recipient paired strains were less than 90% homologous and excluded transfusion responsibility.[9] In this study, the pre-S/S or S gene sequencing confirmed the identity of strains between donor and recipients in 10 cases but excluded this connection in one pair (D4569, Table 6). In several other cases (G4, G5, and G6) specific mutations leading to unusual amino acid substitutions in the HBsAg loop as frequently observed in OBIs from Europe[2] can be taken as unique flagging of the transmitted viral strains. This genetic variability in the HBsAg loop is particularly frequent in genotype D OBIs that are dominant in most of Europe.

In conclusion, anti-HBs–negative blood from OBIs in Europe is estimated at 25% up to 100% infectious by transfusion depending on the plasma volume transfused (Table 4). Since transfusion-transmitted infections are often a cause for legal procedures, the collection of a pre-transfusion recipient sample kept as archive as routinely done for donor samples would be critical in determining the transfusion origin of an HBV infection. The results may justify in European countries different blood safety measures, such as anti-HBc screening of blood donors where prevalence is less than 2% to 4% or, especially in higher anti-HBc prevalence countries, the introduction of sensitive HBV-NAT.

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JPA designed the study, analyzed the data, and wrote the manuscript; IM, MI, GF, KG, LHH, JMG, and EB collected data on donors and recipients, tested them for HBV markers, and reviewed the manuscript; CE collected data on donors and

recipients, tested them for HBV markers, and reviewed the manuscript; MS generated some of the virus quantification and molecular data on German samples and reviewed the manuscript; LW performed the statistical analysis, in particular the regression analysis of risks, and reviewed the manuscript; LB supervised the collection and testing of donor and patients from Valladolid and reviewed the manuscript; HU identified the donors and recipients, collected clinical data, and reviewed the manuscript; DC performed confirmation of OBI in samples, VL quantification, and molecular analysis of donors and recipients other than from Germany and revised the manuscript; NL contributed to the study design, analyzed some of the data, and contributed to the writing of the manuscript; WHG contributed to the study design, data analysis, and writing of the manuscript; and MC supervised the molecular testing and sequencing on the Paul Ehrlich Institute samples and reviewed the manuscript.

CONFLICT OF INTEREST

JPA is occasional speaker for Novartis and has received a research grant from Novartis; NL is consultant for Novartis; and WHG is consultant for Abbott Laboratories and occasional speaker for Abbott, Roche, and Diasorin. None of the other authors has any conflict of interest to declare regarding the subject of this study.

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