Efficacy of hepatitis B virus (HBV) DNA screening and characterization of acute and occult HBV infections among blood donors from Madrid, Spain

Rocio González, Pilar Torres, Emma Castro, Luz Barbolla, Daniel Candotti, Marco Koppelman, Hans L. Zaaijer, Nico Lelie, Jean-Pierre Allain, and Jose-Manuel Echevarría

BACKGROUND: Screening of blood units for hepatitis B virus (HBV) DNA identifies donations collected during the window period (WP) of the acute infection and may improve viral safety of the blood supply. It also leads to the detection of occult hepatitis B infection (OBI).

STUDY DESIGN AND METHODS: From January 2005 to December 2006, a total of 383,267 blood units were screened for hepatitis B surface antigen (HBsAg) and HBV DNA in two transfusion centers in Madrid, using either individual-donation nucleic acid testing (ID-NAT) or minipool (MP-NAT) of eight donations (MP8). Samples positive for HBV DNA and negative for HBsAg were confirmed by a second molecular test, the viral DNA was quantified, and a genome fragment including the region encoding the major hydrophilic region (MHR) of HBsAg was sequenced.

RESULTS: The overall yield of HBV DNA–positive, HBsAg-negative units was 1 in 21,282 (18 cases), higher when using ID-NAT than MP8-NAT (1:9862 vs. 1:51,011; $p < 0.01$). Four donations (1/95,817) were collected during the infectious pre-HBsAg WP, one during an early recovery stage, and the remaining 13 (1/29,482) were OBIs, six of whom had no detectable antibody to HBsAg. Low-level Genotype D HBV DNA was detected in all OBI cases; the frequencies of this genotype and MHR amino acid substitutions were significantly higher than reported from unselected Spanish HBsAg carriers. Donors with OBI had normal aminotransferase levels and were significantly older than donors carrying HBsAg.

CONCLUSIONS: Blood donors in the WP and with OBI are not uncommon in Madrid and are detected at a higher frequency with ID-NAT than MP-NAT.

Prevention of hepatitis B virus (HBV) transmission in the Spanish transfusion setting is based on screening of blood units by sensitive assays for hepatitis B surface antigen (HBsAg) detection. HBsAg screening cannot, however, identify blood donations given during the window period (WP) of the acute primary infection, which extends for approximately 2 months after contact with HBV. Since such blood units contain infectious virus and may transmit HBV to recipients, a residual risk for transfusion-associated HBV transmission estimated at 1 in 74,000 persists in Spain after HBsAg screening.[1] Introduction of HBV nucleic acid testing (NAT) in the screening of blood units was intended

ABBREVIATIONS: anti-HBc = antibody to HBV core antigen; anti-HBs = antibody to HBsAg; dHBV = discriminatory TMA for HBV; ID-NAT = individual-donation nucleic acid testing; MHR = major hydrophilic region; MP-NAT = minipool nucleic acid testing; MP8 = minipool of eight donations; OBI(s) = occult hepatitis B virus infection(s); Q-PCR = quantitative polymerase chain reaction; SRCTC = Spanish Red Cross Transfusion Center; TCCM = Transfusion Center of the Community of Madrid; TMA = transcription-mediated amplification; WHV = woodchuck hepatitis virus; WP = window period.

From the Spanish Red Cross Blood Transfusion Center and the Transfusion Center of the Community of Madrid, Madrid, Spain; the National Health Service Blood and Transplant, Cambridge, UK; Sanquin Research, Amsterdam, The Netherlands; Chiron, Novartis Vaccines and Diagnostics, Suresnes, France; the Department of Haematology, University of Cambridge, Cambridge, UK; and the National Center for Microbiology, Instituto de Salud Carlos III, Majadahonda, Spain.

Address reprint requests to: Dr J.M. Echevarría, National Center for Microbiology, Instituto de Salud Carlos III, Majadahonda, Spain; e-mail: jmecheva@isciii.es.

Received for publication February 9, 2009; revision received June 8, 2009, and accepted June 8, 2009.

doi: 10.1111/j.1537-2995.2009.02343.x

TRANSFUSION 2010;50:221-230.

to reduce such a risk,[2,3] and implementation of HBV NAT as a mandatory test for the Spanish blood supply is currently being evaluated. Many Spanish transfusion centers are performing routine HBV NAT voluntarily.

In addition to blood units collected during the WP, routine NAT may identify blood donors experiencing other modalities of HBV infection with potential impact on the recipient's health, collectively designated as "occult HBV infection" (OBI).[4] OBI is characterized by the presence of HBV DNA in blood and/or liver tissue in the absence of reactivity for HBsAg, both in index and in follow-up samples. Most cases carry antibody to HBV core antigen (anti-HBc), and many also carry antibody to HBsAg (anti-HBs), but cases lacking both anti-HBc and anti-HBs have been reported.[5,6] The first report of OBI involved a blood donor who transmitted HBV to one recipient,[7] but OBI was mainly described in patients with liver disease, and since introduction of HBV NAT blood screening the first reports involving blood donors have been published.[8-16] A study performed in Poland on 1 million blood units identified two cases of low-level, HBsAg-positive chronic HBV infection, five cases of WP infection, and 21 cases of OBI.[9] The frequency of OBI in that study was therefore 1 in 47,619 units, being approximately four times higher than the frequency of WP (1/200,000 units). In a recent report from Italy involving more than 3 million units, the frequency of OBI was 1 in 18,019 units, 24 times higher than the frequency of WP blood donations (1/435,000).[16]

The biologic meaning of OBI and its significance for the donor's health, as well as its infectivity to recipients, remain to be established. OBI has been found in relation to chronic hepatitis, liver cirrhosis, and primary liver cancer, but long-term follow-up studies on healthy individuals carrying OBI have not yet been reported. Multiple amino acid changes in the major hydrophilic region (MHR) that includes the major antigenic determinant of HBsAg have been reported from sequences of Genotype A2 and D strains involved in cases of OBI found among blood donors in Europe, and these findings suggested that selection of escape mutants under the host immune pressure may play a role in generating this type of viral persistence.[14,15] Such findings were not, however, observed in a similar study involving Genotype E strains in West African blood donors with OBI.[13]

The aim of this study was 1) to investigate the efficacy of the Procleix Ultrio assay in either individual-donation (ID-) or minipool (MP)-NAT configuration in interdicting potentially infectious donations, 2) to determine the frequency of OBI and WP infections among blood donors from a central region of Spain, and 3) to characterize the viral strains involved in OBI, including genotype identification, HBsAg subtype prediction, and identification of viral mutants present in the strains as dominant viral populations.

MATERIALS AND METHODS

Blood samples

From January 2005 to December 2006, a total of 383,267 blood units were collected by the Transfusion Center of the Community of Madrid (TCCM) and the Spanish Red Cross Transfusion Center (SRCTC) of Madrid. Among them, 85,769 units (22.4%) were from first-time donors, and the remaining 297,498 units (77.6%) were from repeat donors.

Testing

HBsAg screening was performed on plasma samples from individual units by an automated, chemiluminescence immunoassay (PRISM HBsAg, Abbott Laboratories, North Chicago, IL). Reactive samples were retested in duplicate by the same method, and those displaying results above the cutoff value in at least one of the duplicates were considered repeatedly reactive and were submitted to further testing. It included detection of HBV "e" antigen (HBeAg), anti-HBe, total and immunoglobulin M (IgM) anti-HBc, and anti-HBs quantified in mIU/mL, using automated chemiluminescence immunoassay methods (ARCHITECT, Abbott Laboratories).

Screening tests for HBV DNA were performed either on individual plasma samples (SRCTC, 128,212 units) or on plasma pools of eight samples (TCCM, 255,055 units). A multiplex, transcription-mediated amplification (TMA) assay for detection of HBV DNA, hepatitis C virus (HCV) RNA, and human immunodeficiency virus type 1 (HIV-1) RNA (Procleix Ultrio HIV-1, HBV, and HCV TMA, Chiron, Novartis Vaccines and Diagnostics, Emeryville, CA) was used. The presence of HBV DNA was investigated in reactive samples and in individual samples from reactive pools by discriminatory TMA for HBV (dHBV; Procleix HBV, Chiron). Low-viral-load samples with discrepant Ultrio and dHBV reactivity were further tested in repeat Ultrio and dHBV assays.

Quantification of HBV DNA

Samples reactive for HBV DNA were confirmed by viral DNA quantification. Quantification of HBV DNA of minipool of eight donations (MP8)-NAT yield samples from TCCM was performed by a quantitative polymerase chain reaction (Q-PCR; TaqMan, Roche, Indianapolis, IN) assay or by an in-house developed investigational real-time PCR method (Chiron). The HBV DNA load in the ID-NAT yield samples from SRCTC was determined by real time Q-PCR assay as described[13] and by limiting dilution analysis using a modified TMA assay of enhanced sensitivity for HBV DNA. Briefly, serial threefold dilutions of each sample were prepared in HBV-negative plasma, and each dilution was tested 10 times by HBV TMA assay with

modified target capture chemistry together with a HBV DNA standard dilution panel (PeliCheck, code S2184, Sanquin-VQC, Alkmaar, The Netherlands). A dose-response curve was constructed for each sample, and each curve was compared by probit analysis (SPSS statistical package, SPSS, Inc., Chicago, IL) to the curve drawn by the PeliCheck panel members, which are calibrated in genome equivalents (geq/mL). Results were expressed in international units per milliliter (IU/mL) or were transformed from copies/mL into IU/mL using the conversion formula 1 IU = 5 copies.[17]

Confirmation and classification of HBV infections

Samples that were concordantly HBV DNA and HBsAg repeat reactive were further characterized for HBV serum markers (see above) and considered confirmed HBV infections. HBsAg-negative, Ultrio- and dHBV-reactive NAT yield donor samples were also tested for HBV serum markers and donors were recalled for follow-up testing. Seronegative donors who seroconverted to HBsAg and/or anti-HBc and anti-HBs were classified as "pre-HBsAg WP." HBV NAT yield donations that were HBsAg negative, IgM anti-HBc (and anti-HBs) reactive were called "post-HBsAg WP." IgM anti-HBc–negative, but total anti-HBc–positive, HBV NAT yields were classified as occult HBV infection (OBI) if HBV DNA was confirmed by Q-PCR or by the nested PCR used for sequence analysis. HBsAg repeat reactive donors that were HBV DNA negative in Ultrio or dHBV assay were classified as HBV DNA–negative HBsAg carriers if the presence of anti-HBc (or in some cases also anti-HBe and/or anti-HBs) confirmed HBV infection.

Sequence analysis

Amplification and sequencing of a fragment from the HBV pre-S/S or S gene encoding the MHR was performed after ultracentrifugation of 1 mL (or in some cases 10 mL) of plasma by a previously described method, and the viral genotype was identified by phylogenetic analysis of the sequence obtained.[13,18] DNA sequences were translated into amino acid sequences in the HBV S open reading frame, and the HBsAg subtype was predicted from the amino acids found at specific positions.[19] Amino acid changes in Positions 116 to 150 were identified by comparison with the predicted amino acid sequence with wild-type reference sequences of the corresponding HBV genotype-subtype association.

RESULTS

HBsAg and HBV DNA NAT yield rates

Table 1 summarizes the distribution of different categories of HBV infections identified by HBsAg and NAT screening, confirmation, and follow-up testing in the two participating centers, and Table 2 compares the data obtained for first-time and repeat donors. A total of 193 units (1:1986) were confirmed HBsAg positive. A total of 172 of these samples (89.1%) were positive for HBV DNA by NAT. The rate of HBsAg-positive blood units during the 2-year period of the study was significantly higher at the TCCM (1:1614) than at SRCTC (1:3663). In 2005, the

TABLE 1. HBV infections stratified according to infection stages observed in 2005 and 2006 in two blood centers of Madrid using MP8- and ID-NAT, respectively*

	TCCM	SRCTC	All Madrid
All donations	255,055	128,212	383,267
HBV DNA screening system	MP8-NAT	ID-NAT	
HBV NAT yield	5 (1:51,011)†	13 (1:9,862)†	18 (1:21,293)
HBV infection category			
Pre-HBsAg WP	1 (1:255,055)	3 (1:42,737)	4 (1:95,817)
Post-HBsAg WP	0	1 (1:128,212)	1 (1:383,267)
HBsAg+/DNA+ (ID-NAT)	139 (1:1,835)‡	33 (1:3,885)	172 (1:2,228)
HBsAg+/DNA– carrier	19 (1:13,424)	2 (1:64,106)	21 (1:18,251)
HBsAg–/DNA+ OBI	4 (1:63,764)§‖	9 (1:14,246)§**	13 (1:29,482)
All HBV infected	163 (1:1,565)	48 (1:2,671)	211 (1:1,816)

* Data are reported as number (rate). Pre-HBsAg WP defined as HBV DNA positive before HBsAg and antibody to HBV. Post-HBsAg WP defined as HBV DNA as only maker of HBV infection except IgM anti-HBc after undetectability of HBsAg. HBsAg+/DNA+ corresponds to the status of most chronic HBV infections. A small proportion of HBsAg-positive samples contain HBV DNA below limit of detection (DNA–), others contain HBV DNA but no detectable HBsAg (HBsAg–).
† $p < 0.005$.
‡ Thirty-five of 158 HBsAg+ donations (22.2%) were MP8-NAT negative, of which 16 (10.1%) were dHBV reactive in ID-NAT.
§ $p < 0.05$.
‖ Three in 101,708 donations (1:33,903) in 2005.
** Eight in 61,865 donations (1:7733) in 2005.

TABLE 2. HBV infections stratified according to stages observed in 2005 and 2006 in first-time and repeat donors in the two blood centers of Madrid combined*

	First-time donors	Repeat donors	All donors
Number of donors	82,729	184,548	267,277
HBV-DNA+/HBsAg– (NAT yield)	8 (1:10,341)	10 (1:18,455)	18 (1:14,849)
HBV infection category			
Pre-HBsAg WP	2 (1:41,365)	2 (1:92,247)	4 (1:66,819)
Post-HBsAg WP	0	1 (1:184,548)	1 (1:267,277)
HBsAg+/DNA+	164 (1:504)	8 (1:23,068)	172 (1:1,554)
HBsAg+/DNA– carrier	21 (1:3,939)	0	21 (1:12,727)
HBsAg-/DNA+ OBI	6 (1:13,788)	7 (1:26,364)	13 (1:20,560)
All HBV infections	193 (1:429)	18 (1:10,252)	211 (1:1,267)

* Data are reported as number (rate).

frequency of HBsAg carriers among first-time donors was higher in TCCM than in SRCTC (1:362 and 1:1506, respectively), and, in 2006, the frequency increased to 1:274 in TCCM and 1:1025 in SRCTC. The median age of donors positive for HBsAg was 39.8 years.

Eighteen blood units (1:21,293) nonreactive for HBsAg were positive by NAT. Five of them were found at the TCCM where donations were tested in pools of eight plasma samples and 13 at the SRCTC where individual testing was performed. The NAT yield rate was significantly higher at the latter site (1:51,011 vs. 1:9862, $\chi^2 = 10.47$, $p < 0.005$; Table 1). NAT-positive, HBsAg-negative units were significantly more frequent in year 2005. Data from the first year of NAT screening showed that the MP8 and ID-HBV NAT yield rates in TCCM and SRCTC were 4 in 101,708 (1:25,427) and 11 in 61,865 (1:5624) donations, respectively. The HBV NAT yield rate was significantly higher among blood donations from first-time donors than among blood units from repeat donors (1/10,721 vs. 1/29,750, $p < 0.05$). However when the HBV NAT yield rate was compared between the first-time and repeat donor population (on a per-donor basis) the difference was no longer significant (1:10,341 vs. 1:18,455).

A total of 211 donations contained HBV viral markers. In 172 samples (81.5%), both HBsAg and DNA were detected, in 21 samples (10%) only HBsAg was detected, and in 18 samples (8.5%) only HBV DNA was found.

Testing for HBV markers of follow-up samples identified five acute HBV infections (1:76,653 units, 1:53,455 donors, Tables 1-3), with four donations collected in the early pre-HBsAg WP and one in the late acute clearance phase. Two of these five cases have been previously reported in detail.[3] Three cases seroconverted to HBsAg and two of them to anti-HBc and anti-HBs (Table 3). One WP case identified by MP8-NAT at TCCM had a high viral load of 15,500 IU/mL, but was nevertheless HBsAg negative with PRISM (S/CO = 0.63). This case rapidly seroconverted to anti-HBc and anti-HBs 32 days after the index donation and was classified as a primary occult HBV infection.[5] The characteristics of this acute occult HBV infection with high viral load have recently been reported.[20] HBV genotyping could be performed in three cases, and two Genotype D (donors TCCM5, SRCTC 10) and one Genotype F (Donor SRCTC 11) strains were identified. The frequency of donations collected during WP was similar in years 2005 and 2006 and was not significantly different in first-time and in repeat donors (1:41,365 vs. 1:61,516, respectively, Table 2). The WP-NAT yield rate in donations was higher, but not significantly ($p = 0.08$), at SRCTC using ID-NAT (1:32,053) than at TCCM using MP8-NAT (1:255,055).

Patterns characteristic of OBI were found in the remaining 13 cases (Table 3), 11 of which were identified in 2005 and another two among first-time donors in 2006. In the first year of NAT screening, the rate of OBIs identified by MP8 and ID-NAT in the two blood centers was 3 in 101,708 donations (1:33,903) and 8 in 61,865 donations (1:7733), respectively. Three of these 13 cases had been included in a prior report.[14] The overall frequency of OBI during the 2 years of observation was 1 in 29,482 units and was higher at the SRCTC ($\chi^2 = 5.95$, $p < 0.05$, Table 1) where HBV DNA was screened in individual donor samples. OBI yield was significantly higher in year 2005 (11/13). The lower yield of OBI in 2006 (two cases in first-time donors) was attributable to the deferral of HBV-infected repeat donors the previous year. The OBI rate was significantly higher in units from first-time donors than in units from repeat donors (1:13,788 vs. 1:26,364, respectively). However, when the prevalence of OBI was compared on a per-donor basis the difference was not significant (Table 2). No cases of OBI negative for total anti-HBc were found.

There was a striking difference in the proportions of HBV DNA– and HBsAg-reactive HBV infections between first-time and repeat donors. The number of OBIs among HBV-infected first-time donors was 6 in 193 (3.1%), whereas the number in repeat donors was 7 in 18 (38.9%). The proportion of HBV DNA–negative HBsAg carriers among first-time donors was 21 in 193 (10.9%). All 11 incident HBV infections in repeat donors contained detectable HBV DNA, of whom 8 in 11 (72.7%) were HBsAg reactive, two (18.2%) were in the pre-HBsAg WP, and one (9.1%) in the IgM anti-HBc–positive early recovery phase.

Characterization of NAT yield samples

Results obtained on blood units from donations collected during the WP and with OBI are shown in Table 3. Alanine aminotransferase (ALT) serum levels in OBI cases were consistently below 30 IU/L, and anti-HBs was found in seven cases, with titers ranging between 17 and 149 IU/L (mean, 43.8 UI/L). HBV DNA concentration was always below 200 IU/mL and was lower than 40 IU/mL in most cases. The median age of donors with OBI was 53.6 years and was significantly higher than the median age of HBsAg-positive donors (39.8 years; t test, $p < 0.001$).

HBV DNA sequences were obtained in 12 samples from donors with OBI, and all were Genotype D strains (Table 4). Three of them belonged to the T125M cluster.[21] HBsAg Subtype ayw2 or Subtype ayw3 was predicted from the amino acid sequences, but an amino acid change at Residue 122 (K/R122P) prevented Serotype d/y prediction in one case.

Two strains were Genotype D/ayw2 wild type (Table 4). Single-amino-acid substitutions were predicted in four strains, and six strains presented with two or more (up to 10) predicted substitutions. Substitutions P120S, T126I, G145A, or G145R, which have been described in association with vaccine escape mutants,[19] were found in

TABLE 3. HBV markers in index and follow-up samples from 18 donors with HBsAg-negative/HBV DNA–positive index blood donations

Donor	Follow-up (days)	HBsAg	Total anti-HBc	Anti-HBc IgM	Anti-HBs (IU/L)	Classification	HBV DNA (IU/mL)	Age (years)	ALT level (IU/L)
TCCM1*	0	Neg	Pos	Neg	22.3	OBI	6	50	26
	40	Neg	Pos	ND	Pos				15
TCCM2*	0	Neg	Pos	Neg	17	OBI	10	43	12
	18	Neg	Pos	ND	Pos				14
TCCM3*	0	Neg	Pos	Neg	Neg	OBI	20	65	13
	4	Neg	Pos	ND	Neg				12
TCCM4	0	Neg	Pos	Neg	57.5	OBI	8	64	10
TCCM5	0	Neg	Neg	ND	Neg	Acute OBI WP	15500	52	23
	32	Neg	Pos	ND	Pos				122
	57	Neg	Pos	ND	Pos				10
SRCTC1	0	Neg	Pos	Neg	Neg	OBI	ND	64	15
	75	Neg	Pos	Neg	Neg				11
SRCTC2	0	Neg	Pos	Neg	17	OBI	32	42	13
	80	Neg	Pos	Neg	Pos				12
SRCTC3	0	Neg	Pos	Neg	149	OBI	21	62	16
	30	Neg	Pos	Neg	Pos				13
SRCTC4	0	Neg	Pos	Neg	22	OBI	46	58	29
	15	Neg	Pos	Neg	Pos				20
SRCTC5	0	Neg	Pos	Neg	Neg	OBI	8	53	23
	16	Neg	Pos	Neg	Neg				17
SRCTC6	0	Neg	Pos	Neg	Pos	OBI	178	51	14
	5	Neg	Pos	Neg	Pos				13
SRCTC7	0	Neg	Pos	Neg	Pos	OBI	ND	58	23
	22	Neg	Pos	Neg	Pos				ND
SRCTC8	0	Neg	Pos	Neg	Neg	OBI	27	68	19
	5	Neg	Pos	Neg	Neg				15
SRCTC9	0	Neg	Pos	Neg	22	OBI	25	19	ND
	16	Neg	Pos	Neg	Pos				
SRCTC10*	0	Neg	Neg	Neg	Neg	Pre-HBsAg WP	6	25	9
	33	Pos	Neg	Neg	Neg				8
	77	Neg	Pos	Pos	Pos				12
SRCTC11*	0	Neg	Neg	Neg	Neg	Pre-HBsAg WP	168	30	22
	51	Pos	Pos	Pos	Neg				264
	91	Neg	Pos	Pos	Pos				74
SRCTC12	0	Neg	Pos	Pos	Pos	Post-HBsAg WP	<6	29	20
	44	Neg	Pos	Pos	Pos				25
SRCTC13	0	Neg	Neg	Neg	Neg	Pre-HBsAg WP	ND	63	ND
	16	Pos	Neg	Neg	Neg				
	44	Pos	Neg	Neg	Neg				

* Cases reported elsewhere.[3,12]
HBeAg was negative in all cases but SRCTC 11 and SRCTC 13.
ND = not done.

TABLE 4. Amino acid analysis of the MHR of 13 OBI samples from two Madrid blood centers

Donor	Sample ID	Genotype	Predicted HBsAg serotype	Amino acid substitutions*
TCCM1†	3596585	D	ayw3	T123P T125I G130E Y134F P135A S143L C147Y
TCCM2	8323344 9977414	D	a?w3‡	C121Y R122P A128V Y134S S136Y T140I G145A
TCCM3	943514	D	ayw2	G119V P120S T125M S143T
TCCM4	5511422	D	ayw3	T115N T116N T118R P120- T125M T126I Q129L G130E Y134N C137W D144E
SRCTC1§	3596585			
SRCTC2	8323344	D	ayw2	T118K
SRCTC3	5585335	D	ayw2	C149Y
SRCTC4	5570587	D	ayw2	wt
SRCTC5	5580467	D	ayw2	T118A
SRCTC6	5585979	D	ayw2	S136Y
SRCTC7	5581462	D	ayw2	T125M D144E G145R
SRCTC8	5607076	D	ayw2	P120T T126I
SRCTC9	736586	D	ayw2	wt

* Amino acid numbering is from Genotype D consensus S sequence; consensus amino acid is in front, strain at the end.
† The entire HBV genome of sample TCCM1 and short sequences from samples TCCM2, and TCCM3 were previously reported.[14]
‡ Determinant d/y could not be identified due to a mutation at the site.
§ Despite being HBV DNA confirmed positive, the pre-S/S or S region could not be amplified.

four strains. In four strains, a normally occurring cysteine was substituted by another amino acid (121Y, 137W, 147Y, and 149Y). All these substitutions are likely to substantially modify the secondary structure and antigenic reactivity of the extramembrane epitopes. Other significant changes such as T118R, P120S, P120T, P120del, and T126I[19] were recorded in three strains. In total, seven strains (58%) displayed amino acid substitutions that likely reflect mutant selection under the pressure of the anti-HBs response. Substitution S143L, reported previously in association with a transfusion-transmitted HBV infection after failure of HBsAg screening,[22] was found in one additional case.

DISCUSSION

HBV infection by transfusion remains in most countries of Western Europe the main residual viral transmission risk since HCV and, more recently, HIV-1 NAT has been implemented in most of them.[23] It is with the availability of commercial multiplex NAT able to simultaneously detect HCV, HIV-1, and HBV genomes that this risk has been addressed, particularly in European Mediterranean countries where the relatively high prevalence of anti-HBc impaired the use of this marker to improve HBV safety. In this study, both HBsAg testing and HBV DNA testing were applied to blood donations instead of testing only the HBsAg-negative units by NAT. This approach provided an opportunity to study the total spectrum of HBV infections and the respective efficacy of these two direct viral markers.

The prevalence of HBsAg in donor blood was 1 in 1986 overall. Among the 193 donations containing HBsAg, 21 (10.9%) were not reactive for HBV DNA with an assay with a 95% detection limit estimated at 11 IU/mL.[24,25] The proportion of HBV DNA–negative HBsAg carriers was lower (2%) in a study with Ultrio in France.[26] In another study in the United States, 6% of HBsAg-positive donors were HBV DNA negative by Ampliscreen.[27] This proportion was lower in another study conducted in Taiwanese donations tested with the more sensitive TaqScreen method.[28] This comparison might suggest that not only assay sensitivity, but also the viral load distribution in the prevalent HBV genotypes in a country (A2, D, and F in Spain, A2 and D in France, A2 in the United States, and B and C in Taiwan) may account for differences in the percentage of HBsAg carrying blood donations that would remain undetected by HBV DNA assays currently available.

The frequency of blood units containing HBV DNA without HBsAg detectable with PRISM (sensitivity, 0.1 IU/mL) was overall 1:21,293 units, lower than that reported in Italy,[16] but higher than that reported in Poland.[9] However, this frequency was considerably influenced, as reported in Italy and Poland, by the testing strategy in small pools of 8 to 24 or in individual donations. In 2005, the first year of NAT screening, the NAT HBV yield was significantly higher in donations tested individually at SRCTC (1:5624) compared to TCCM tested in pools of eight samples (1:25,427; $p < 0.01$). The dilution factor of 8 was nearly reflected in a five-time decrease in yield between the two strategies in place in the two blood centers. The frequency of HBV NAT yield observed at SRCTC was in the range previously described in Barcelona and Valencia, two other major Spanish transfusion centers testing in individual donations.[15]

Minipools of 8 plasma samples clearly reduces HBV DNA detection for both WP and OBI, although the former was not significant (Tables 1 and 2). Such difference could be theoretically limited by reducing the pool size and testing the pools with a screening assay of higher sensitivity, but results from real comparisons have not been reported yet. There is clear evidence that missed WP units

are infectious irrespective of viral load, and this is a sound argument in favor of individual donation testing. OBI infectivity is, however, lower, particularly when anti-HBs is present,[29,30] and OBI is much more frequent than WP among blood donors. Another element in decision making is the cost, and pooling is clearly less expensive than individual-donation testing. It is therefore difficult to draw a general recommendation with regard to the screening strategy (i.e., minipool testing vs. individual donation testing), and the decision should be taken after analyzing different factors, including the epidemiologic features of the HBV infection in the blood donor population of the region. Anti-HBc testing is, in addition, an alternative utilized in North America and in some countries in Europe to exclude donors at risk of presenting OBI. In Spain, the prevalence of anti-HBc ranges between 4 and 8%, and such blood donation discarding is estimated to negatively impact the blood supply at an unsustainable level. In the Spanish epidemiologic circumstances, anti-HBc screening would not otherwise detect approximately 1 in 40,000 WP donors (Table 2).

Among the 18 donations containing HBV DNA but no detectable HBsAg, 4 units did not carry HBV serologic markers and were classified as WP infections by the occurrence of HBsAg or HBV antibodies in follow-up samples (Table 3). One additional index sample was both IgG and IgM anti-HBc positive, and anti-HBs titer increased from 400 to 700 mIU/mL on follow-up, indicating recent infection. In this blood donor population, acute HBV infection undetected by HBsAg screening reached a frequency of 1 in 76,653, a yield considerably higher than observed with either HCV or HIV-1 NAT in Spain.[2] It is likely that TCCM screening in pools of eight donations might have missed additional, low-viral-load acute infections, as previously reported.[3] The single WP donation detected by MP8-NAT screening at TCCM had a relatively high viral load (15,500 IU/mL) and was classified as a primary occult HBV infection.[5,6] Molecular and physicochemical properties of HBV particles in this donation have been extensively characterized and were described elsewhere.[20] It is noteworthy that all 11 acute HBV infections in repeat donors were found HBV DNA reactive, whereas eight of them (73%) were also HBsAg positive. A similar proportion of acute HBV infections in repeat donors was found HBsAg positive in a study in South Africa.[31]

A majority of HBV DNA yield consisted of OBIs characterized by undetectable HBsAg and presence of anti-HBc. Compared to OBIs identified in blood donors from other regions or countries of Europe, the Madrid donors shared older age than both general donor population and HBsAg-positive donors, a dominance of males, normal ALT levels, and low viral load (<178 IU/mL; median, 21 IU/mL) in 11 quantified samples.[9,15,16] Similarly to OBIs described elsewhere in Europe, more than 50% of blood donations contained quantifiable anti-HBs (9/13, Table 3). Unlike observed in other studies, no significant difference in the prevalence of OBI was found between first-time and repeat donors.[16] This was probably related to the bias introduced by calculating rates per donation instead of per donor as done here.

Published studies of the HBV genotype distribution of HBsAg-positive patients or donors in Spain indicated the circulation of Genotypes D (66%), A2, and F,[32] but all OBI strains sequenced in this study were of Genotype D. This difference was significant ($p < 0.05$) and confirms previously published data from OBIs identified in Europe[15] indicating a significant excess of Genotype D OBIs compared to HBsAg-positive infections. In this small group of OBI strains, serotypes were a mixture of ayw2 and ayw3, as reported among Genotype D strains from HBsAg-positive cases.[32]

The overall frequency of mutations predicting amino acid changes in the MHR of strains found among blood donors with OBI was significantly higher than the frequency reported among chronic HBsAg carriers in Spain[33] (10/12, 83% vs. 106/272, 39%; $\chi^2 = 7.62$, $p < 0.01$), and mutations predicting dual or multiple changes in that region were also significantly more frequent among donors with OBI (6/12, 50% vs. 27/272, 9.9%; $\chi^2 = 17.97$, $p < 0.001$).

HBsAg variants found among these donors did not share, however, particular amino acid changes, but displayed changes likely selected under immune pressure, as previously reported,[15] with a significantly higher frequency than the variants found among HBsAg-positive carriers from Spain (7/12, 58% vs. 25/272, 9.2%; $\chi^2 = 27.76$, $p < 0.001$). In particular, cysteine residues 121, 137, 147, and 149 were found 100% conserved among such carriers, but found mutated in 4 of these 12 OBI strains (4/12, 33% vs. 0/272; $\chi^2 = 91.96$, $p < 0.001$). Cysteine residues are critical to achieve the secondary structure characteristic of HBsAg, and such substitutions are very likely to modify the expression of conformational epitopes and therefore the immunologic properties of the antigen. These data extend the findings from two prior studies performed among European blood donors[14,15] and suggest that establishment of OBI does not induce the emergence of specific HBsAg variants, but leads frequently to selection of HBsAg mutants under the pressure of the immune response. In agreement with such interpretation, donors with OBI were significantly older than donors displaying conventional HBV chronic carriage, and the single young donor with OBI found in the study carried one of the two wild-type HBV strains found in association with OBI. Although some of these amino acid changes have been associated with poor HBsAg detection by commercial assays, results from recent studies involving a relatively large number of clinical samples containing similar variants[34,35] suggest that the lack of serum HBsAg reactivity in

OBIs would not be the consequence of escape HBsAg mutants, but rather of a genuinely low level of circulating HBsAg.

Results from experimental infections of woodchucks with the woodchuck hepatitis virus (WHV) have shown that the establishment and maintenance of the occult WHV infection does not require the selection of escape WHV variants and that the presence of such variants is not required for transmission of occult WHV to susceptible animals.[36] Whether or not selection of escape HBV variants plays a role in the occurrence of OBI or may influence virus transmission is unknown and requires further investigation. Viral latency leading to episodes of periodic reactivation under immune control is one of the models that might explain both OBI and HBV reactivation among patients experiencing immunosuppressive therapy who carry markers of natural immunity against HBV.[29] Early neutralization of infectious virus resulting from partial and possibly temporary lack of tight immune control might participate in occasional episodes of such nature and lead to the selective accumulation of HBsAg mutants under the immune pressure. Such a model might also explain why donors with OBI are significantly older than donors with conventional HBV chronic infection and why they select HBsAg mutants in high frequency. This hypothesis would predict that donors with OBI should present with fluctuating low levels of viremia, which could be verified by performing long-term, follow-up studies in such donors.

In conclusion, implementation of molecular methods for HBV DNA detection in the blood center on a routine basis may contribute significantly to improving the viral safety of the Spanish blood supply, and its efficacy would increase by testing individual units rather than pools. The finding of blood donors with "occult" HBV infection is a relatively frequent occurrence, both among first-time and among repeat donors, and its frequency would decrease with the deferral of repeat blood donors identified as occult carriers. Compared to HBsAg screening, HBV DNA added 27% of potentially infectious blood units from acutely infected repeat donors, and in first-time donors these two blood screening assays are clearly complementary. In terms of safety improvement, interdiction of WP units is highly efficient, while donations from donors with OBI are less frequently infectious.[10] Preliminary data suggest that anti-HBc– and anti-HBs–positive OBIs are rarely infectious.[30] Infectivity of anti-HBc only OBIs seems, in contrast, higher but remains to be precisely determined by lookback studies.[37,38]

ACKNOWLEDGMENTS

We are indebted to Jeff Linnen and Larry Mimms from Gen-Probe for performing limiting dilution tests for quantifying HBV DNA with a modified TMA assay. We also thank the technical staff of TCCM and SRCTC for testing of samples and administration of results.

CONFLICT OF INTEREST

Nico Lelie is employed by Chiron, Novartis Vaccines, and Diagnostics SAS in France, the company that distributes the Ultrio tests for blood screening.

REFERENCES

1. Álvarez M, Oyonarte S, Rodríguez PM, Hernandez JM. Estimated risk of transfusion-transmitted viral infections in Spain. Transfusion 2002;42:994-8.
2. Álvarez M, González R, Hernández JM. Residual risk of transfusion-transmitted viral infections in Spain, 1997-2002, and impact of nucleic acid testing. Eurosurveillance 2005;10:20-2.
3. González R, Echevarría JM, Avellón A, Barea L, Castro E. Acute hepatitis B window-period blood donations detected by individual-donation nucleic acid testing: a report of the first two cases found and interdicted in Spain. Transfusion 2006;46:1138-42.
4. Raimondo G, Pollicino T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. J Hepatol 2007;46:160-70.
5. Yoshikawa A, Gotanda Y, Mineshigi K, Aira R, Hino S, Tadokoro K, Ohnuma H, Miyakawa K, Tachibana K, Misoguchi H and the Japanese Red Cross NAT Screening Research Group. Lengths of hepatitis B viremia and antigenemia in blood donors: preliminary evidence of occult (hepatitis B surface antigen-negative) infection in the acute stage. Transfusion 2007;47:1162-70.
6. Manzini P, Abate ML, Valpreda C, Milanesi P, Curti F, Rizzetto M, Smedile A. Evidence of acute primary occult hepatitis B virus infection in an Italian repeat blood donor. Transfusion 2009;49:757-64.
7. Tabor E, Hoofnagle JH, Smallwood LA, Drucker JA, Pineda-Tamondong GC, Ni LY, Greenwalt TJ, Barker LF, Gerety RJ. Studies of donors who transmit post transfusion hepatitis. Transfusion 1979;19:725-31.
8. García-Montalvo BM, Farfán JA, Acosta KY, Puerto FI. Hepatitis B virus DNA in blood donors with anti-HBc as a possible indicator of active hepatitis B virus infection in Yucatán, México. Transfus Med 2005;15:371-8.
9. Brojer E, Grabarczyk P, Liszewski G, Mikulska M, Allain JP, Letowska M. Characterization of HBV DNA+/HBsAg- blood donors in Poland identified by triplex NAT. Hepatology 2006;44:1666-74.
10. Satake M, Taira R, Yugi H, Hino S, Kanemitsu K, Ikeda H, Tadokoro K. Infectivity of blood components with low hepatitis B virus DNA levels identified in a lookback program. Transfusion 2007;47:1197-205.
11. Chevrier MC, ST-Louis M, Perreault J, Caron B, Castilloux C, Laroche J, Delage G. Detection and characterization of hepatitis B virus of anti-hepatitis B core antigen-reactive

blood donors in Quebec with an in-house nucleic acid testing assay. Transfusion 2007;47:1794-802.
12. Manzini P, Girotto M, Borsotti R, Giachino O, Guaschino R, Lanteri M, Testa D, Ghiazza P, Vacchini M, Danielle F, Pizzi A, Valpreda C, Castagno F, Curti F, Magistroni P, Abate ML, Smedile A, Rizzetto M. Italian blood donors with anti-HBc and occult hepatitis B virus infection. Haematologica 2007; 92:1664-70.
13. Zahn A, Li C, Danso K, Candotti D, Owusu-Ofori S, Temple J, Allain JP. Molecular characterization of occult hepatitis B virus in genotype E-infected patients. J Gen Virol 2008;89: 409-18.
14. Zaaijer HL, Torres P, Ontañón A, Ponte LG, Koppelman MH, Lelie PN, Hemert FJ, Boot HJ. Multiple surface antigen mutations in five blood donors with occult hepatitis B virus infections. J Med Virol 2008;80:1344-9.
15. Candotti D, Grabarczyk P, Ghiazza P, Roig R, Casamitjana N, Iudicone P, Schmidt M, Bird A, Crookes R, Brojer E, Miceli M, Amiri A, Li C, Allain JP. Characterization of occult hepatitis B virus from blood donors carrying genotype A2 or genotype D strains. J Hepatol 2008;49:537-47.
16. Velati C, Romanò L, Fomiatti L, Baruffi L, Zanetti AR; SIMTI Research Group. Impact of nucleic acid testing for hepatitis B virus, hepatitis C virus and human immunodeficiency virus on the safety of blood supply in Italy: a 6-year survey. Transfusion 2008;48:2205-13.
17. Saldanha J, Lelie N, Heath A. Establishment of the first international standard for nucleic acid amplification technology (NAT) assays for HCV RNA. WHO Collaborative Study Group. Vox Sang 1999;76:149-58.
18. Koppelman MH, Zaaijer HL. Diversity and origin of hepatitis B virus in Dutch blood donors. J Med Virol 2004;73:29-32.
19. Echevarría JM, Avellón A. Hepatitis B virus genetic diversity. J Med Virol 2006;78:S36-S42.
20. Bremer CM, Saniewski M, Wend U, Torres P, Lelie N, Gerlich WH, Glebe D. Transient occult hepatitis B virus infection in a blood donor with high viremia. Transfusion 2009;49;in press.
21. Norder H, Couroucé AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes and HBsAg subtypes. Intervirology 2004;47:289-309.
22. Levicnik-Stezinar S. Hepatitis B surface antigen escape mutant in a first time blood donor potentially missed by a routine screening assay. Clin Lab 2004;50:49-51.
23. Coste J, Reesink HW, Engelfriet CP, Laperche S, Brown S, Busch MP, Cuijpers HT, Elgin R, Ekermo B, Epstein JS, Flesland O, Heier HE, Henn G, Hernandez JM, Hewlett IK, Hyland C, Keller AJ, Krusius T, Levicnik-Stezina S, Levy G, Lin CK, Margaritis AR, Muylle L, Niederhauser C, Pastila S, Pillonel J, Pineau J, van der Poel CL, Politis C, Roth WK, Sauleda S, Seed CR, Sondag-Thull D, Stramer SL, Strong M, Vamvakas EC, Velati C, Vesga MA, Zanetti A. Implementation of donor screening for infectious agents transmitted by blood by nucleic acid technology: update to 2003. Vox Sang 2005;88:289-303.
24. Koppelman M, Assal A, Chudy M, Torres P, de Villaescusa RG, Reesink HW, Lelie PN, Cuypers HT. Multi-center performance evaluation of a transcription-mediated amplification assay for screening of human immunodeficiency virus-1 RNA, hepatitis C virus RNA and hepatitis B virus DNA in blood donations. Transfusion 2005;45:1258-66.
25. Assal A, Barlet V, Deschaseaux M, Dupont I, Gallian P, Guitton C, Morel P, David B, De Micco P. Comparison of the analytical and operational performance of two nucleic acid test blood screening systems: Procleix Tigris and Cobas s 201. Transfusion 2009;49:289-300.
26. Bouchardeau F, Girault A, Razer A, Servant-Delmas A, Mercier M, LaPerche S. Sensitivity of hepatitis B virus transcription-mediated amplification testing in hepatitis B surface antigen-positive blood donations. Transfusion 2006;46:2047-52.
27. Kleinman SH, Strong DM, Tegtmeier GG, Holland PV, Gorlin JB, Cousins C, Chiacchierini RP, Pietrelli LA. Hepatitis B virus (HBV) DNA screening of blood donations in minipools with the COBAS Ampliscreen HBV test. Transfusion 2005;45:1247-57.
28. Li L, Chen PJ, Chen MH, Chak KF, Lin KS, Lin Tsai SJ. A pilot study for screening blood donors in Taiwan by nucleic acid amplification technology: detecting occult hepatitis B virus infections and closing the serologic window period for hepatitis C virus. Transfusion 2008;48: 1198-206.
29. Lubel JS, Testro AG, Angus PW. Hepatitis B virus reactivation following immunosuppressive therapy: guidelines for prevention and management. Intern Med J 2007;37:705-12.
30. Levicnik-Stezinar S, Rahne-Potokar U, Candotti D, Lelie N, Allain JP. Anti-HBs positive occult hepatitis B virus carrier blood infectious in two transfusion recipients. J Hepatol 2008;48:1022-5.
31. Vermeulen M, Lelie N, Sykes W, Crookes R, Swanevelder J, Gaggia L, Le Roux M, Kuun E, Gulube S, Reddy R. Impact of individual donation (ID) nucleic acid testing (NAT) on risk of HIV, HBV and HCV transmission by blood transfusion in South Africa. Transfusion 2009;49:1115-25.
32. Echevarría JM, Avellón A, Magnius LO. Molecular epidemiology of hepatitis B virus in Spain: identification of viral genotypes and prediction of antigenic subtypes by limited sequencing. J Med Virol 2005;76:176-84.
33. Avellón A, Echevarría JM. Frequency of hepatitis B virus "a" determinant variants in unselected Spanish carriers. J Med Virol 2006;78:24-36.
34. Mühlbacher A, Weber B, Burgisser P, Eiras A, Cabrera J, Louisirirotchanakul S, Tiller FW, Kim HS, v Helden J, Bossi V, Echevarria JM. Multicenter study of a new fully automated HBsAg screening assay with enhanced sensitivity for the detection of HBV mutants. Med Microbiol Immunol 2008;197:55-64.

35. Echevarría JM, Avellón A. Improved detection of natural hepatitis B virus surface antigen (HBsAg) variants by a new version of the VITROS® HBsAg assay. J Med Virol 2008;80: 598-602.
36. Michalak TI. Occult persistence and lymphotropism of hepadnaviral infection: insights from the woodchuck viral hepatitis model. Immunol Rev 2000;174:98-111.
37. Gerlich WH, Wagner FF, Chudy M, Harritshoj LH, Lattermann A, Wienzek S, Glebe D, Saniewski M, Schuttler CG, Wend UC, Willems WR, Bauerfeind U, Jork C, Bein G, Platz P, Ullum H, Dickmeiss E. HBsAg non-reactive HBV infection in blood donors: transmission and pathogenicity. J Med Virol 2007;79:S32-S36.
38. Mosley JW, Stevens CE, Aach RD, Hollinger FB, Mimms LT, Solomon LR, Barbosa LH, Nemo GJ. Donor screening for antibody to hepatitis B core antigen and hepatitis B virus infection in transfusion recipients. Transfusion 1995;35: 5-12.