ORIGINAL ARTICLE

Low-level viraemia of hepatitis B virus in an anti-HBc- and anti-HBs-positive blood donor

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SUMMARY. In many countries, screening of hepatitis B virus (HBV) in blood donors is limited to HBsAg testing. However, if anti-HBc testing and sensitive HBV nucleic acid amplification testing (NAT) for routine screening are not prescribed, HBV viraemia might remain unrecognized. A clinically inconspicuous HBsAg-negative 35-year-old female blood donor was detected with anti-HBc antibodies following the introduction of anti-HBc screening of donors. Based on her history, she had seroconverted to anti-HBs positive (titre >7000 IU/L) after vaccination. Blood donations were routinely tested HBV-DNA negative by minipool NAT. The individual donor samples were reinvesti-

gated by an ultrasensitive NAT with a lower detection limit of 3·8 IU/mL. Intermittent HBV viraemia was detected over a 7-year period from this donor, with a concentration ranging from 8 to 260 IU/mL. In the subsequent donor-directed lookback study, no post-transfusion hepatitis was detected. Low-level HBV viraemia in simultaneous anti-HBc- and anti-HBs-positive blood donors could only be identified with enhanced sensitivity individual polymerase chain reaction assays and is not detectable by pool HBV NAT.

Key words: anti-HBc, anti-HBs, HBV-DNA, real-time PCR.

Hepatitis B virus (HBV) testing of blood donors in several European countries is focused on HBsAg. Anti-HBc for routine screening is not prescribed. Moreover, previous HBV-infected donors are authorized to donate blood 5 years after hepatitis has been resolved (Richtlinien zur Blutgruppenbestimmung und Bluttransfusion, 2000).

Blood donor screening for anti-HBc has been implemented in some countries to decrease the non-A, non-B viral hepatitis and HBV transfusion risk (e.g. in the US and Japan). However, as anti-HBc testing would not identify donors in the pre-seroconversion window phase, some German blood banks introduced HBV nucleic acid amplification testing (NAT) for donor screening utilizing minipool NAT with up to 96 blood donations (Jilg *et al.*, 1995; Cardoso *et al.*, 1998; Roth *et al.*, 1999, 2002). This test strategy is not suitable to

reach the sensitivity necessary to identify low-copy HBV viraemia as occurred in occult HBV infection (Kleinman *et al.*, 2001, 2003; Roth *et al.*, 2002).

Reactivity to anti-HBc is observed infrequently in the absence of either HBsAg or anti-HBs (called isolated anti-HBc). The prevalence of this serological constellation is estimated to be about 0·08% in the German population, and at least 3% of the cases are low-level viraemic and need further diagnostic procedures (Jilg *et al.*, 1995; Hennig *et al.*, 2002; Weber, 2002). These cases can be explained by false-negative test results of other HBV markers, a phase of late immunity with loss of anti-HBs, the passive transfer of anti-HBc or a nonspecific cross reaction (Weber, 2002).

In fact, blood donors who were negative for HBsAg but positive for anti-HBc have been reported to transmit HBV, leading to acute hepatitis (Hoofnagle *et al.*, 1978; Thiers *et al.*, 1988; Elghouzzi *et al.*, 1995; Allain *et al.*, 1999).

Here, we describe HBV viraemia in a primarily isolated anti-HBc-positive blood donor, who seroconverted to anti-HBs after vaccination. The low viral load could only be detected by HBV PCR with enhanced sensitivity but not by minipool testing.

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MATERIALS AND METHODS

Case report

Antibodies to hepatitis B core antigen (anti-HBc) were detected in a clinically inconspicuous 35-year-old German, Caucasian female blood donor on March 6, 2002, following implementation of annual screening of donors for this marker. The positive anti-HBc results were verified by alternative anti-HBc assays. Additional investigations on March 6 and 12, 2002 confirmed the positive anti-HBc results (Table 1). Before this incident, the last donation had taken place at June 26, 2001. The donor had a consistently normal alanine aminotransferase (ALT) below 20 U/L (exclusion threshold $\leq$45 U/L) and had been tested HBsAg negative on every previous donation. Although the first donation by this donor took place in 1988, archived samples for reinvestigation were only available for the donations since 1995. The time and origin of the occult HBV infection remains unclear, because of the asymptomatic course. The donor did not report specific infection risks in the questionnaire and denied such risks in the entry interview. Informed consent was provided by the donor.

Viral serology

Initial screening for HBsAg was performed using the AxSYM kit (Abbott, Wiesbaden, Germany). The sensitivity of this assay is 0·17–0·60 ng HBsAg/mL. Until July 1998, HBsAg screening was carried out with AUSZYME monoclonal on the Abbott Commander system (Abbott). Anti-HBs quantification on the plasma samples was estimated from a standard curve according to the manufacturer's recommendations (AUSAB, Abbott).

HBeAg, anti-HBe, total anti-HBc (CORE) and anti-HBc IgM (Core-M) were assayed using commercial assays (AxSYM, Abbott). A second total anti-HBc assay (Vidas Anti-HBc Total II, BioMérieux, Nürtingen, Germany) was used for repeat testing of reactive samples in an alternative test environment. Until 1998, testing for anti-HBc was performed using the Corzyme assay (Abbott). The patient was tested negative for antibodies against hepatitis C virus and human immunodeficiency virus 1/2 (HIV-1/2).

Hepatitis B virus NAT

Prior to December 1999, we used an in-house TaqMan PCR for the HBV-DNA screening of blood donations. Total nucleic acid was prepared from 300 µL of ethylenediaminetetraacetic acid plasma from minipools of up to 10 single specimens by using the High Pure Viral Nucleic Acid Kit according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

The TaqMan PCR core reagents (Applied Biosystems, Darmstadt, Germany) were used with primers TM4-A (5′-AAG GTA TGT TGC CCG TTT GT-3′) and TM4-B (5′-GTT CCT TGA GCA GGA GTC GT-3′) and probe TM4-S (5′-FAM-CAA CCA CCA CCA GCA CGG GAC CA-TAMRA) for HBV PCR on the sequence detection system (ABI Prism 7200 SDS, Applied Biosystems). This screening TaqMan PCR assay has a detection limit about 60 IU/mL (approximately 500 geq/mL).

For ultrasensitive HBV NAT, the QIAamp Ultrasens Virus Kit was used to isolate nucleic acid from 1 mL plasma samples. DNA was eluted finally in 60 µL AVE buffer, and 15 µL was used for real-time PCR in a reaction volume of 50 µL. This individual donor HBV NAT was performed as follows: the reaction mixture consisted of ×1 HotMaster Taq buffer (Eppendorf GmbH, Hamburg, Germany), 5 mM MgCl$_2$, 300 nM of each primers RG-R (5′-ATA TGA TAA AAC GCC GCA GAC AC-3′) and RG-F (5′-CAA CCT CCA ATC ACT CAC CAA C-3′), 200 nM probe RG-S (5′-FAM-TCC TCC AAT TTG TCC TGG TTA TCG CT-BHQ1), 300 µM dATP, dCTP, dGTP, 600 µM dUTP, 1·25 U Hot Master Taq DNA polymerase and 0·01 U µL^{-1} uracil-N-glycosylase (AmpErase-UNG; Applied Biosystems). Cycle conditions were 37 °C for 10 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 65 °C for 40 s. Amplification, detection and data analysis were performed with the Rotorgene 3000 cycler (Corbett Research, Sydney, NSW, Australia). To determine the detection limit of this HBV PCR, we investigated semilogarithmic dilutions in 24 replicates of the first WHO international standard for HBV-DNA NAT assays, 97/746 (Saldanha et al., 2001) described above. The 95% detection limit was determined by probit analysis as 3·8 IU/mL (approximately 30 geq/mL) for HBV at a 95% probability with 2·7 IU/mL (21 geq/mL) lower and 7·3 IU/mL (58 geq/mL) upper 95% confidence limit. HBV-DNA was quantified by comparison with the standard curve obtained with the WHO standard.

Positive NAT results were confirmed by an alternative hemi-nested primer system with outer primers P1 and P2 and inner primers P1 and P4W (Repp et al., 1993). In addition, the DNA sequence of the amplicons was compared to that of the positive control. The complete sequence of the pre-S1/S2 and S region of the donor's HBV isolate was deposited in the GenBank database (NCBI, Rockville Pike, Bethesda, MD, USA) with the accession number AY221115.

Table 1. Hepatitis B virus (HBV) status of the blood donations and results of the lookback procedure

| | HBV assay | | | | | | HBV status (HBsAg, anti-HBs, anti-HBc) of blood recipient | |
Date of donation	ALT (U/L)	HBs antigen (Abbott, AxSYM)*	Anti-HBs (Abbott, AxSYM) (IU/L)	Anti-HBc (Abbott, AxSYM)†	HBV-DNA (Minipool)‡	Individual HBV-DNA (IU/mL)§	RBC recipient	FFP recipient
October 22, 2002	9	Not tested	Not tested	Not tested	Not tested	-	Not transfused	Not transfused
July 24, 2002	10	Not tested	Not tested	Not tested	Not tested	-	Not transfused	Not transfused
July 8, 2002	12	0·37	2861	0·843	Not tested	-	Not transfused	Not transfused
March 12, 2002	12	0·36	Not tested	0·778	-¶	+ (134)	Not transfused	Not transfused
March 6, 2002	13	0·29	2534	0·752	-¶	+ (260)	Not transfused	Not transfused
June 26, 2001	15	0·31	2726	0·874	-¶	+ (56)	Not transfused	Not transfused
February 1, 2001	12	0·33	Not tested	0·805	Not tested	-	Not transfused	Not transfused
October 25, 2000	11	0·39	Not tested	Not tested	Not tested	-	No sample available (patient deceased)	Not tested
July 18, 2000	10	0·50	3373	Not tested	Not tested	-	No sample available (patient deceased)	No sample available (patient deceased)
April 11, 2000	9	0·45	Not tested	Not tested	-¶	+ (10)	Not tested	Not tested
January 5, 2000	9	0·61	Not tested	0·693	Not tested	-	-	-
September 2, 1999	11	0·41	4717	Not tested	-	-	Not transfused	Not transfused
May 14, 1999	19	0·45	Not tested	0·708	-	-	No sample available (patient deceased)	No sample available (patient deceased)
February 5, 1999	13	0·43	Not tested	0·653	-	-	-	-
November 6, 1998	11	0·37	Not tested	0·633	-	-	-	-

Table 1. Continued

		HBV assay					HBV status (HBsAg, anti-HBc) of blood recipient	
Date of donation	ALT (U/L)	HBs antigen (Abbott, AxSYM)*	Anti-HBs (Abbott, AxSYM) (IU/L)	Anti-HBc (Abbott, AxSYM)†	HBV-DNA (Minipool)‡	Individual HBV-DNA (IU/mL)§	RBC recipient	FFP recipient
July 23, 1998	10	0·53	3928	Not tested	-	-	No sample available (patient deceased)	No sample available (patient deceased)
April 24, 1998	9	0·42	Not tested	Not tested	-	+ (8)	No sample available (patient deceased)	-
January 22, 1998	11	0·53	5421	Not tested	-	-	No sample available (patient deceased)	No sample available (patient deceased)
October 9, 1997	10	0·43	Not tested	0·556	-	-	-	No sample available (patient deceased)
July 8, 1997	7	0·47	7371	Not tested	-	-	No sample available (patient deceased)	No sample available (patient deceased)
March 23, 1997	8	0·34	Not tested	0·587	-	-	-	+ **
January 20, 1997	8	0·38	7096	Not tested	-	-	No sample available (patient deceased)	-
March 2, 1995	13	0·41	Not tested	0·961	-	-	Not tested	Not tested

*Reactive: >1 sample-to-cutoff (S/CO), Gray zone: 0·8–1 S/CO.

†Reactive: <1, negative: >1.

‡95% detection limit of the minipool NAT: 60 IU/mL pooled plasma (10 samples).

§95% detection limit of the individual NAT: 3·8 IU/mL.

¶Re-evaluation of minipool NAT of 1/10 dilution of plasma.

**Known cured hepatitis B before transfusion.

FFP, fresh frozen plasma.

Lookback process

Recipients of previous donations from the corresponding donor during the previous 5 years were traced and tested for markers of HBV infection. According to the German guidelines, donor-directed lookback procedures were initiated, and blood donations between January 1997 and June 2001 were included in the study. A total of 30 blood components had been collected from the donor including 14 units of fresh frozen plasma (FFP) and 16 units of red blood cells (RBCs), each transfused to different recipients. Of these recipients, 50% (15) were deceased due to the primary disease. Of the 15 recipients who were alive and notified, we were able to ascertain HBV test results for 11.

RESULTS

Anti-HBc- and anti-HBs-positive blood donation

In addition to the prescribed HBsAg screening of blood donations in Germany, we introduced yearly anti-HBc testing of all donors in 2002. On this examination, antibodies to hepatitis B core antigen (anti-HBc) were detected in a clinically inconspicuous 35-year-old German female blood donor on March 6, 2002. HBV minipool PCR with a maximum of 10 samples per pool had not detected HBV-DNA in any sample because HBV NAT testing had been implemented at the end of 1999. Recently, when the archived donor samples were re-evaluated in a more sensitive individual donation NAT, HBV viraemia was detected, excluding a false-positive anti-HBc result (Table 1).

The HBV isolate was characterized by DNA sequencing as genotype A and HBsAg subtype *ayw* without mutations in the functional domains. Further sequence analysis revealed no core promoter or pre-core region mutations.

To determine the state of HBV infection, additional serological markers were analysed. Anti-HBc-IgM, HBeAg and anti-HBe were negative in all samples tested (data not shown). According to historical data, a positive anti-HBs result has been observed subsequent to an HBV vaccination with three doses of hepatitis B recombinant vaccine (Engerix-B; Glaxo-SmithKline GmbH & Co. KG, Munich, Germany) in 1992. From that time, the HBV serology had tested negatively, with the exception of a positive result for anti-HBc on March 2, 1995, which was rated as a nonspecific, false-positive finding. At that time, there was a clear rise in the donor's anti-HBs titre (Table 1). The HBV infection of the donor was subsequently verified and classified as late immunity.

Lookback process

In the donor-directed lookback procedure, post-transfusion hepatitis B was not detected in any of the nine patients who received RBC or plasma from these HBsAg-negative, anti-HBc and anti-HBs-positive donations (Table 1). Only one tested recipient was anti-HBc positive, but because of a previously known resolved hepatitis B, no additional investigations were performed. One patient who received the FFP unit from the donation from April 24, 1998, which tested positive for HBV-DNA, was tested and found negative for HBsAg and anti-HBc. Therefore, even with levels of HBV detectable in individual PCR, no HBV transmission occurred.

DISCUSSION

Recently, it was controversially discussed whether HBV NAT achieves the same level of safety as anti-HBc testing of blood donations (Kleinman *et al.*, 2001, 2003). In our case, the individual HBV NAT could be successfully employed for the reliable detection of low viraemia in an anti-HBc-positive blood donor.

The serological constellation in our case can be interpreted as a resolved HBV infection, in which virions from hepatocytes were periodically secreted, as described previously (Rehermann *et al.*, 1996). As shown in our study, high levels of anti-HBs after vaccination do not exclude hepatitis B virus persistence. However, vaccination with recombinant HBs proteins are thought to immunize persons against all HBV subtypes (Shokrgozar & Shokri, 2002). Therefore, binding and neutralization of vaccine-induced anti-HBsAg to the donor's HBV particles is probable.

Our investigation supports previous findings that low-copy viraemia in anti-HBc-positive blood donations is not detectable by pool HBV NAT (Kleinman *et al.*, 2001, 2003; Roth *et al.*, 2002) and can only be identified by enhanced sensitivity individual PCR assays. However, several blood banks in Germany began to perform HBV NAT screening in 1997 using minipools of up to 96 samples, with a detection limit of 1000 geq/mL (95% probability) for each individual donation (Roth *et al.*, 1999). Minipool HBV PCR screening of 3·6 million blood donations from central Europe found only six HBV-positive samples. Low-level HBV carriers with HBV titres below the detection limit of minipool testing could only be detected in single-sample enrichment PCR (Roth *et al.*, 2002). Therefore, the routine NAT screening of blood donations cannot detect all viraemic carriers due to low titres at the beginning of the infection or low-level persistence of hepatitis B.

Post-transfusion hepatitis has been reported to occur despite negative PCR results of transfused blood components (Thiers *et al.*, 1988). In particular, as shown in this study, more emphasis has to be placed on the sensitivity of screening NAT to detect viraemia in isolated anti-HBc donations. Consequently, some blood centres have moved to smaller minipools for HBV, e.g. the Japanese Red Cross Blood centres reduced the pool size from 500 to 50 in February 2000 (Mitsunaga *et al.*, 2002), or have started to perform single-donor NAT with the highest sensitivity, as described for simultaneous NAT for HIV-1, HCV and HBV on fully automated systems (Kolk *et al.*, 2002).

HBV-DNA-positive blood units were thought to be infectious even in low titres (Tabor *et al.*, 1983). Our findings indicate a lack of infectivity in anti-HBc-positive and anti-HBs-positive blood components with low-copy HBV viraemia. Even if the blood components of anti-HB-positive donations tested HBV PCR negative, post-transfusion HBV transmission is possible because large volumes of blood components with a low viral load are transfused. In the chimpanzee model, it has been demonstrated that as few as one to ten HBV particles are infectious when inoculated intravenously (Tabor *et al.*, 1983). On the other hand, Prince *et al.* (2001) observed no HBV transmissions when chimpanzees were inoculated with serum and lymphocytes from HBsAg-negative, anti-HBc-positive patients with low-level HBV-DNA ranging from 204 to 1349 geq/mL. However, only small volumes of inocula were injected in this study.

Further studies with larger case numbers of transfused viraemic blood components should be conducted in immune-suppressed adults and children to further establish the infectivity of anti-HBc-only units with detectable HBV-DNA.

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