Hepatitis B virus DNA viral load determination in hepatitis B surface antigen–negative Swiss blood donors

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BACKGROUND: Nucleic acid test (NAT) hepatitis B virus (HBV) screening for all blood donations with a sensitivity limit of 25 IU/mL in the individual donation is mandatory in Switzerland since 2009. The aims of the two studies were to define the percentage of antibody to hepatitis B core antigen (anti-HBc) or anti-HBc and antibody to hepatitis B surface antigen (anti-HBs)-positive donors bearing HBV DNA and to gather HBV viral load data on HBV NAT yields during the routine screening since the introduction of the HBV NAT.

STUDY DESIGN AND METHODS: Archive samples from anti-HBc–positive donors (Group I) were analyzed with a quantitative HBV DNA test and further with anti-HBc and anti-HBs assays. In addition, all the HBV NAT-only–yield samples (Group II) from the routine donor screening performed between July 2007 and May 2013 were included in the study.

RESULTS: From the 667 samples investigated (131 donors), three donors (2.3%) had donated eight samples (1.2%) with detectable HBV DNA; however, all had very low viral loads (≤10 IU/mL). From the 1,160,426 donations screened with the routine HBV NAT assay, 16 HBV NAT yields were detected: two window period (WP) and 14 occult hepatitis B infection (OBI) cases. In eight of these positive donations (two WP and six OBI), the HBV viral loads were not more than 10 IU/mL, in three cases between 10 and 25 IU/mL, and in the remaining five donations between 37 and 166 IU/mL.

CONCLUSION: The highly sensitive HBV NAT assay with a threshold significantly below 10 IU/mL is a valuable alternative to anti-HBc and a less sensitive HBV NAT screening in blood donor screening.

ABBREVIATIONS: BTS = blood transfusion service; ID(s) = individual donation(s); MP(s) = minipool(s); OBI = occult hepatitis B infection; WP = window period.

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TRANSFUSION **, ** **.
This study was set up to investigate the presence of HBV DNA in archive samples from anti-HBc– or anti-HBc– and antibody to hepatitis B surface antigen (anti-HBs)-positive donors (Group I). The aims of the study were first to define the percentage of anti-HBc–positive or anti-HBc– and anti-HBs–positive donors bearing HBV DNA in the Swiss donor population; second, to gather HBV viral load data on HBV NAT yields during the routine screening (Group II); and third, to determine range of HBV NAT yields measured since the introduction of the HBV NAT screening. This information will provide additional mosaic stones to define an optimal sensitivity limit for HBV NAT screening within the Swiss donor population.

MATERIALS AND METHODS

Group I: archive anti-HBc samples

Samples were selected from a study performed in 2006, in which more than 20,000 donors were screened for anti-HBc. All these donations were HBsAg negative. At that time all the anti-HBc–positive donors were confirmed with two additional anti-HBc assays targeting different regions of the HBc antigen and were subsequently indefinitely deferred. From these donors, 262 donors were considered for this study. This number was further reduced to 167 donors as at least four archive samples were requested from 7 years before their deferral. Furthermore several archive samples did not qualify as they had been stored inadequately or insufficient serum was available. Finally 667 archive samples from 131 donors were available to be included in the study and were tested for HBV DNA as well as tested for anti-HBc and anti-HBs. Donors were additionally stratified according to their anti-HBs status (Table 1).

Immediately after thawing the archive samples were tested for the presence of HBV DNA followed by testing for anti-HBc (anti-HBc Axsym, Abbott, Delkenheim, Germany) and anti-HBs (anti-HBs Axsym, Abbott). HBV viral load was determined with a quantitative real-time HBV assay (Abbott), which has a 4.3 IU/mL (range, 3.6-5.6 IU/mL) 95% limit of detection determined by Probit analysis and a linear range from 4 to $1 \times 10^{10}$ IU/mL. Due to limited sample volume the HBV NAT positives could only be identified by single NAT from 500-μL sample extracts.

In 2009 HBV NAT in ID or minipools (MPs) NAT was declared mandatory for all Swiss blood donations. The Swiss guidelines were also modified to allow anti-HBc–positive individuals who had an anti-HBs titer of more than 100 mIU/mL and were HBV DNA negative (<10 IU/mL) to donate blood. After these changes, all previously deferred donors were invited to determine their anti-HBs status and if they fulfilled the new guidelines were requested to donate blood again. Fifty-five of the 131 eligible donors could be motivated to donate blood again.

Group II: yield cases

All consecutive blood donations between July 2007 and May 2013 were screened with a commercially available NAT assay (Procleix Ulitrio, Novartis Diagnostics, Basel, Switzerland) with a 95% limit of detection of 7.6 IU/mL (range, 6.0-11.1 IU/mL). In addition, they were tested with various HBsAg testing systems (Enzygnost HBsAg Siemens, Marburg, Germany; Axsym HBsAg, Abbott; Architect HBsAg, Abbott; Monolisa HBsAg, Bio-Rad, Marnes la Coquette, France). The presence of viral DNA was confirmed with a HBV discriminatory assay (Procleix Ulitrio, Novartis Diagnostics) and a quantitative real-time HBV assay (Abbott). Serologic confirmation of the HBV status was determined by testing for anti-HBc (anti-HBc Axsym, Abbott), anti-HBs (Axsym, Abbott), anti-HBe (anti-HBe Axsym, Abbott), and HBeAg (HBeAg, Axsym, or HBeAg Architect, Abbott). HBV DNA–positive samples were identified in replicate NAT assays (Ultrio and Abbott).

RESULTS

Group I samples

Eight (1.2%) of the 667 archive samples taken from the anti-HBc study performed in 2006 (in total 131 donors) were HBV DNA positive. These eight samples originated from three donors (2.3%). Four, three, and one archive samples from these three donors, respectively, were HBV DNA positive. The donors and their respective number of archive samples according to their anti-HBs status are listed in Table 1. The three HBV DNA–positive cases derived from different categories according to their anti-HBs titer (i.e., one negative, one between 100 and 999, and one ≥1000 mIU/mL), respectively, are shown in Table 2. More detailed data from these three donors and their respective archive samples are described.

| Table 1. Number of donors and archive samples according to their anti-HBs status |
|---------------------------------|------------------|----------------|
| Anti-HBs (mIU/mL) | Number of donors | Number of samples |
| ≥1000 | 48 | 57 |
| 100-999 | 45 | 324 |
| 10-99 | 17 | 113 |
| Negative | 23 | 173 |
| Total | 131 | 667 |
the whole period as anti-HBc and anti-HBs were both positive. The anti-HBs titers were the following: 850 mIU/mL (1999), 520 mIU/mL (2000), 572 mIU/mL (2001), 680 mIU/mL (2002), 580 mIU/mL (2004), and 556 mIU/mL in 2005. The donor was female, 56 years old, and was not vaccinated against HBV. She first donated in 1993; however, unfortunately until 1999 no archive samples were available.

Donor 2
Three of the 18 archive samples available from this donor were HBV DNA positive with viral loads 5 IU/mL (2005), 4 IU/mL (2010), and 2 IU/mL (2010), respectively. His serologic status remained unchanged all over the study period. All samples were anti-HBc positive but none were anti-HBs positive. The donor was male, 65 years old, and had not been HBV vaccinated. He first donated in 1987 but at another regional BTS and thus no archive samples were available from these early donations.

Donor 3
In total 21 consecutive archive samples from this donor were analyzed. The initial 10 samples (1992 until 1998) showed no sign of an HBV infection, but the following 11 samples (1998 until 2005) were all anti-HBc reactive and had anti-HBs titers of more than 1000 mIU/mL. In total, four samples (two in 2002, one in 2003, and one in 2005) were HBV DNA positive with very low HBV levels of approximately 1 IU/mL. The donor was male, 36 years old, and had never been HBV vaccinated. He first donated in 1987 but at another regional BTS and thus no archive samples were available from these early donations.

In total 21 consecutive archive samples from this donor were analyzed. The initial 10 samples (1992 until 1998) showed no sign of an HBV infection, but the following 11 samples (1998 until 2005) were all anti-HBc reactive and had anti-HBs titers of more than 1000 mIU/mL. In total, four samples (two in 2002, one in 2003, and one in 2005) were HBV DNA positive with very low HBV levels of approximately 1 IU/mL. The donor was male, 36 years old, and had never been HBV vaccinated.

In addition to the presented data, all in the archive study implicated donors were requested to donate blood again during 2010 to 2012. Of the 131 implicated donors, 55 could be further investigated. From all these donations no HBV DNA was detected.

Group II samples
Since the introduction of the HBV ID-NAT in July 2007, a total of 1,160,426 donations were screened in the BTS Berne over the following 6 years. Eighty-four concordant HBsAg and HBV DNA–positive donors were detected. In addition, 16 HBV NAT yields and three HBsAg confirmed-positive but HBV DNA–negative donors were detected. Two of the 16 donations were window period (WP) cases, whereas the remaining 14 were occult hepatitis B infection (OBI) cases.

The first WP case was from a 66-year-old male, clinically inconspicuous donor (47 donations since 1978). The donor declared that he did not have any risk behavior. The twice repeated-reactive NAT screening result was confirmed with a quantitative HBV DNA assay (approx. 1 IU/mL). A sample taken 1 month later (X + 1 blood drawing) had a viral load of 514 IU/mL and was HBsAg confirmed positive. The X − 1 archive sample (135 days earlier) was negative in the HBV DNA confirmation assay, HBsAg, anti-HBc, and anti-HBs. The recipient of the corresponding RBC unit was a 70-year-old man who underwent orthopedic surgery and needed a RBC unit because of postoperative anemia. Before transfusion he was seronegative for HBsAg, anti-HBc, and HBV DNA. Two months later he was HBV DNA positive with a viral load of 146 IU/mL.

The second WP case was a 62-year-old male donor (75 donations), not declaring any risk behavior. The index donation was retested five times with the screening assay; three of them were positive, and two were negative. The HBV discriminatory assay (Ultrio), the real-time PCR, and all serologic assays were negative. In a sample taken 14 days afterward, the Procleix HBV discriminatory assay was positive. In the X + 2 sample (40 days) again the screening assay (Ultrio) and the discriminatory assay were positive, but the serologic markers were negative. The X + 3 (7 days) sample showed a borderline reaction for anti-HBc and had an anti-HBs concentration of 8 mIU/mL. All other HBV markers including HBV DNA were negative. The X + 4 (155 days) follow-up sample was anti-HBc positive with a concentration of 12 mIU/mL. There were no data available from the corresponding recipients.

In nine donations (two WP and seven OBI) the HBV viral loads were not more than 10 IU/mL. In the other seven OBI cases the viral load was also very low, ranging from 11 to 166 IU/mL (Table 3). Within these 14 OBI cases three were anti-HBc alone, eight were anti-HBc and anti-HBe positive, and one was solely positive for anti-HBs (not vaccinated). The three HBsAg confirmed-positive but DNA-negative donations were all anti-HBc and anti-HBe positive but negative for anti-HBs. While two were also HBsAg positive, the third case was not determined due to insufficient plasma (Table 3).

### TABLE 2. Summary of all HBV DNA–positive archive samples

<table>
<thead>
<tr>
<th>Donor</th>
<th>Amount of archive samples tested</th>
<th>HBV DNA–positive samples</th>
<th>Viral load (IU/mL)*</th>
<th>Anti-HBs titer (mIU/mL)</th>
<th>Stage of HBV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>520–&gt;1000</td>
<td>OBI</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>3</td>
<td>4, 2 and 5</td>
<td>Negative</td>
<td>OBI</td>
</tr>
<tr>
<td>3</td>
<td>21 (11)†</td>
<td>4</td>
<td>&gt;1000</td>
<td></td>
<td>OBI</td>
</tr>
</tbody>
</table>

* Viral loads below 10 IU/mL lie outside the linear range of the quantitative HBV DNA assay and therefore only approximate values are given.

† The first 10 samples were also anti-HBc negative.

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The sensitivity limit for HBV DNA in blood donors is as follows: **Volume **, ** ** TRANSFUSION **
From the 16 NAT-yield donors, 12 were male and four were female. There was a considerable age variation in the donors, however, most were over 40 (eight >60, one >50, four >40, two >30, and one 19 years old; Table 3). Two of the three HBsAg-positive but NAT-negative donors were male and all three were first-time donors (Table 4).

**DISCUSSION**

Based on the data collected in three independent anti-HBc studies, a decision was taken not to introduce anti-HBc as a screening test for HBV in Switzerland (C. Steinemann, personal communication, 2006).\(^1\)\(^,\)\(^2\) The alternative approach with the addition of a highly sensitive HBV NAT was envisaged to increase the safety of the HBV screening without an unnecessary loss of potential donors. Thus, routine HBV NAT screening with the Procleix Ultrio in an ID-NAT format was introduced in BTS Berne in July 2007. Approximately 53% of the total Swiss blood donor population was analyzed with this routine HBV NAT screen. The HBV NAT yield calculated from the data collected from July 2007 until 2009 was 1 in 44,000.\(^3\) This information led to the decision taken by BTS of the Swiss Red Cross to declare HBV NAT mandatory for blood donor screening. A 95% sensitivity limit of 25 IU/mL for the single donation was set at that time point. This sensitivity limit was set due to the technical feasibility available in the six Swiss NAT centers and not based on solid scientific arguments. Three commercial HBV NAT systems were available at that time; the Procleix Ultrio on Tigris system, the Cobas MPX on s201 system, and the Cobas Ampliscreen on Amplicor system. With MPs of a maximum of six donations the 25 IU/mL sensitivity limit could be fulfilled by all three NAT systems. Since 2010 the Cobas Ampliscreen system has no longer been used for the donor screening in Switzerland. This study was thus devised to specifically address the question which HBV DNA viral load would be predominant in HBsAg-negative WP or OBI NAT–yield donations in the Swiss donor population. We chose two different approaches: first, the investigation of archive samples from known anti-HBc–positive donors, and second, the analysis of HBV NAT yields from the current routine HBV NAT screening at the BTS Berne.

Various studies have shown that HBV NAT can significantly reduce the WP of HBV infection. This is particularly the case when highly sensitive NAT assays are adopted and thus a number of potential WP donations were iden-
tified since the beginning of routine HBV NAT screening in blood. In this screening study, two WP NAT–yield cases in the total of 1,160,426 donations were detected (WP NAT yield 1:580,213). In both WP cases a follow-up sample confirmed the WP status. In one case a seroconversion was detected (anti-HBc and anti-HBs positive) and in the other case a viral load of 514 IU/mL and HBsAg positivity could be shown. In one case even a recipient was shown to be infected from the implicated RBCs. This sample confirms clearly that also blood products from WP cases with very low levels of HBV DNA are infectious.

Apart from shortening the WP, NAT screening has uncovered a relatively large number of HBsAg-negative occult HBV donors. The presence of HBV DNA detected in OBI cases appears to differ significantly depending on the geographical region (i.e., 0% to 15% between China, Japan, Saudi Arabia, Ghana, Egypt, and Germany). In Group I, the eight HBV DNA–positive samples from 667 total samples (1.2%) and the three from 131 anti-HBc–positive donors (2.3%) identified in our study suggest Switzerland lies within the lower region of this range. However, the analysis of the Group I samples could be suboptimal if very low HBV DNA loads are expected. Furthermore, the relatively limited number investigated samples may have contributed to a statistical bias. The investigated Group I samples were stored at −30°C for several years and had a limited volume of approximately 0.9 mL per sample; thus, only 500 μL could be used for the quantification of HBV DNA.

The HBV NAT–yield cases from Group II measured from the analyzed donations was 1 in 72,526 (14 OBI and two WP in 1,160,426 tested donations). Approximately 2.5% of the Swiss donor population are anti-HBc positive. Extrapolated from the total 1,160,426 donations, 29,010 of these donations are theoretically anti-HBc positive. From these 29,010 theoretically anti-HBc–positive donations, 14 OBI were actually HBV DNA positive, which leads to a theoretical ratio of 1 in 2072 HBV DNA positive in the Swiss anti-HBc–positive donor population. This figure lies within the lower level of OBI estimations. In European blood donations OBI samples have been identified in 1 in 1000 to 1 in 50,000 donors. This figure is considerably higher than was found in studies conducted in United States where the probability for the presence of HBV DNA in anti-HBc–positive units led to between 1 in 37,000 and 1 in 54,000. The reason for this difference probably lies in the sensitivity of the HBV NAT assay used. In the studies presented by Glynn and coauthors, HBV DNA screening was performed in MPs of 16 donations or even greater and thus low-viral-load cases were certainly not detected.

A long-term persistent and intermittent viremia appears to occur frequently in isolated anti-HBc–positive individuals after they have recovered from their acute hepatitis B infection. This situation can often persist for up to 7 years and it has been proposed that these individuals are still potentially infectious and thus pose a risk for HBV transfusion-transmitted infections. Anti-HBc–positive but HBsAg-negative blood samples often have persistent very low HBV DNA levels ranging from fewer than 1 to 30 HBV genome copies/mL.

All the eight HBV-positive samples detected in this study (Group I samples) had viral loads clearly below 10 IU/mL. In addition, the routine NAT screening (Group II samples) over nearly 6 years revealed most of the 16 HBV NAT yields detected (nine) had viral loads below 10 IU/mL. The remaining seven NAT yields ranged from 11 to 166 IU/mL, with three of these below 25 IU/mL. It is thus apparent that most of the HBV-positive donations would not have been detected with the 25 IU/mL HBV NAT sensitivity limit currently endorsed by the Swiss BTS. The occurrence of viremia near the detection limit of the assays presents an additional risk as the level of HBV viremia is known to fluctuate in these individuals. Only by deploying highly sensitive HBV NAT assays would these low viral loads be adequately detected.

It has been demonstrated that HBV transfusion-transmitted infections often occur from WP cases with very low viral loads often below the linear range of the detection assay (<5 IU/mL). The risk of viral transmission, however, does not solely depend on the viral dose; other factors, such as the anti-HBs status of the donor, the immune status of the recipient, and the amount of plasma transfused, equally play a vital role in the viral transmission. It has been shown that the clinically observed HBV infection risk caused by blood components from OBI carriers with low anti-HBc titers is more than 10-fold lower than the transmission risk by donations in the pre-HBsAg and/or MP-NAT window phase. Nevertheless, the more sensitive HBV NAT assays are used, the more blood components with low HBV viral load will be detected and therefore fewer transfusion-transmitted HBV infections will occur, although it is unlikely that all transfusion-transmitted infections can be prevented, even when extremely sensitive HBV ID-NAT systems are introduced, particularly in WP cases, as has been shown in a recent publication.

In summary, the data collected in this study from the Swiss blood donor population, showed that 84 HBV-positive samples were concordant confirmed positive for HBsAg and DNA, three samples were HBsAg confirmed positive but HBV DNA negative, and 24 samples were HBV DNA positive but HBsAg negative. Seventeen of 24 HBV NAT DNA–positive but HBsAg-negative samples had viral loads clearly less than 10 IU/mL. Blood products with these very low HBV viral loads are potentially infectious, as was shown in several recent reports. Which sensitivity limit should be implemented in future donor screening programs still needs to be determined in a controlled study; however, based on our observations, a theoretical sensitivity limit below 5 IU/mL must be achieved.
commercially available NAT system is currently available that fulfills this requirement for HBV. It has been shown that this assay can detect more low-titer HBV-positive samples than the predecessor version and an additional NAT system with a sensitivity limit clearly less than 5 IU/mL will soon be available.\(^\text{50,41}\) Perhaps, in the not too distant future, it may be envisaged to abolish HBsAg and anti-HBc testing and rely solely on a HBV NAT approach in blood donor screening. Furthermore, in the foreseeable future pathogen reduction systems for all blood products will be available that will further reduce the requirement for retaining HBsAg and anti-HBc donor screening.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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