Hepatitis B virus transmission by blood transfusion during 4 years of individual-donation nucleic acid testing in South Africa: estimated and observed window period risk

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BACKGROUND: Since October 2005, a total of 2,921,561 blood donations have been screened by the South African National Blood Service for hepatitis B virus (HBV) by individual-donation nucleic acid testing (ID-NAT). Over 4 years, 149 hepatitis B surface antigen–negative acute-phase HBV NAT–positive donations were identified (1:19,608). The lookback program identified one probable HBV transmission.

STUDY DESIGN AND METHODS: The complete genomes of HBV isolated from the donor and recipient were sequenced, cloned, and analyzed phylogenetically. The HBV window period (WP) transmission risk was estimated assuming a minimum infectious dose of 3.7 HBV virions and an incidence rate correction factor of 1.34 for transient detectability of HBV DNA.

RESULTS: Of 149 acute-phase HBV NAT yields, 114 (1:25,627) were classified as pre–antibody to hepatitis B core antigen (anti-HBc) WP and 35 (1:83,473) as post–anti-HBc WP. The acute-phase transmission risk in the HBV DNA–negative pre- and post–anti-HBc WPs (of 15.3 and 1.3 days, respectively) was estimated at 1:40,000 and 1:480,000, respectively. One HBV transmission (1:2,900,000) was identified in a patient who received a transfusion from an ID-NAT–nonreactive donor in the pre–anti-HBc WP. Sequence analysis confirmed transmission of HBV Subgenotype A1 with 99.7% nucleotide homology between donor and recipient strains. The viral burden in the infectious red blood cell unit was estimated at 32 (22-43) HBV DNA copies/20 mL of plasma.

CONCLUSION: We report the first known case of transfusion-transmitted HBV infection by blood screened using ID-NAT giving an observed HBV transmission rate of 0.34 per million. The estimated pre–acute-phase transmission risk in the ID-NAT screened donor population was 73-fold higher than the observed WP transmission rate.

In October 2005, the South African National Blood Service (SANBS) implemented individual-donation nucleic acid testing (ID-NAT) to screen all blood donations for human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV).[1] Although the development of systems to screen blood donations using NAT has significantly reduced the residual risk of transfusion-transmissible infections (TTIs),[1-5] it has not eliminated it completely.[4,6-12] Factors that contribute to the transmissibility of infections by blood transfusion during the window period (WP) include the level of viremia, the sensitivity of the screening assay, and the minimal infectious dose.[13,14] The length of the WP is determined by the doubling time of viremia and the analytical sensitivity of the testing system used for screening.[13,15,16] ID-NAT is generally more sensitive and detects

ABBREVIATIONS: ID = individual donation; ID_{50} = 50% minimum infectious dose; LOD = limit of detection; MP = mini-pool; OBI = occult hepatitis B infection; SANBS = South African National Blood Service; TTI(s) = transfusion-transmissible infection(s); WP(s) = window period(s).

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lower levels of nucleic acid compared to minipool (MP) NAT, although for HBV, three head-to-head comparison studies showed no significant difference in analytical and clinical sensitivity between the Ultrio assay (Novartis Diagnostics, Emeryville, CA) in ID-NAT format and the TaqScreen assay (Roche Molecular Systems, Pleasanton, CA) utilized in MP6 configuration.[17-19]

A review of the literature revealed that to date there has been no documented case of transfusion-transmitted HBV where these latter systems have been used to screen donated blood. However, there have been a number of cases of HBV transmission that have been reported in blood screened by NAT using larger pool sizes.[7,20-22] Interestingly, when some of these donations were tested by ID-NAT they were also not reactive, but the diagnostic polymerase chain reaction (PCR) methods used in these cases might have been less sensitive than the current triplex NAT blood screening assays.

The benefit of implementing a more sensitive ID-NAT system (as opposed to an MP system) is expected to be greater in regions of high HBV incidence and prevalence, where the rate of donations in the WP and in cases of occult hepatitis B infection (OBI) is high.[23]

Before the implementation of hepatitis B vaccination in the immunization program of infants in 1995, it was reported that more than 70% of the South African population had been exposed to HBV, with an estimated 10% being hepatitis B surface antigen (HBsAg) carriers.[24] Interestingly, when the background prevalence of HBV was examined in an adult population 5 years after introduction of infant immunization, the HBsAg- and anti-HBc–positive rate was reported to be lower (3.3 and 36.7%, respectively), which indicated that beneficial factors other than HBV vaccination play a role in reducing HBV transmission in a community.[24] The HBsAg and anti-HBc prevalences in a South African blood donor population were reported to be 0.54 and 6.0%,[1,25] six times lower than in the general population.[24] Even though the potential transmission of HBV infection by blood transfusion in South Africa has been significantly reduced by ID-NAT, the residual risk of HBV transmission by donations in the window phase remains relatively high.[1] SANBS operates a comprehensive lookback program to identify possible cases of transfusion-transmitted HIV, HCV, and HBV infections. Donor-triggered lookback investigations are undertaken on all HBV DNA– and/or HBsAg-positive donations, including OBI, for donations procured within 1 year of the positive (index) donation. SANBS does not routinely screen for anti-HBc; however, should recent seroconversion to anti-HBc be identified, a lookback investigation will be conducted. Recipient triggered lookback investigations are carried out whenever there is a reported case of a possible transfusion-transmitted infection.

In this study, we report the first case of transmission of HBV by a blood donation in the HBV DNA–negative WP, which occurred during the fourth year of ID-NAT screening and which was confirmed by comprehensive nucleic acid sequencing and phylogenetic analyses. Moreover, we compare the observed HBV WP transmission rate with the estimated transmission risk caused by donors with acute HBV infection undetectable by ID-NAT.

MATERIALS AND METHODS

Screening and confirmation of HBV infection

All blood donations in South Africa are donated by voluntary, nonremunerated donors and are routinely screened for HIV, HBV, and HCV by both serologic testing and ID-NAT. Screening for HBsAg was performed on a chemiluminescent immunoassay system (Abbott PRISM ChLIA system, Abbott, Delkenheim, Germany) and, for HBV DNA, on a multiplex NAT assay system (Procleix Ultrio multiplex system on TIGRIS, Novartis Diagnostics). Serology and ID-NATs were performed concurrently using two donor samples, a citrated plasma sample for HBsAg and an ethylenediaminetetraacetic acid gel-separated plasma sample for NAT. Initial reactive donations in the Ultrio assay are tested in duplicate on the primary test tube as well as in the discriminatory probe assays. Donors that are concordantly repeat reactive in Ultrio or dHBV assays and the HBsAg assay are considered HBV infected. HBsAg-negative donations that are Ultrio repeat reactive or reactive in the dHBV assay are tested for viral load by quantitative PCR (on the Cobas TaqMan, Roche Molecular Diagnostics, Pleasanton, CA) assay as well as by triplicate or fivefold Ultrio and dHBV assays on samples taken from the frozen plasma unit. These potential HBV NAT–yield samples are also tested for immunoglobulin (Ig)M anti-HBc, total anti-HBc, and anti-HBs titer (Elecsys, Roche Molecular Systems, Pleasanton, CA).

Potential HBV NAT–yield donors, as well as HBsAg neutralization–positive donors with Ultrio-nonreactive or nonrepeatable reactive results, are recalled to confirm HBV infection markers in a follow-up sample. On the basis of the pattern of results in index and follow-up samples, HBV NAT–yield infections in first-time, lapsed, and repeat donors are categorized into acute and chronic NAT-yield cases. The first group is further classified as pre-HBsAg or pre–anti-HBc WP if all serum markers in the index donation are nonreactive or as post HBsAg or post–anti-HBc WP (or early recovery phase) when IgM anti-HBc is positive (Elecsys, Roche Molecular Systems). If two sequential samples were HBV DNA reactive followed by seroconversion to anti-HBc (and anti-HBs) in a later follow-up sample, we classified the infection as primary OBI (Fig. 1). In some of these cases, it is unlikely that HBsAg would have been detectable at some point in time because of the length of the sampling intervals. A second subcategory of WP infections could be classified as either abortive HBV infection or, more likely, vaccine breakthrough infections,

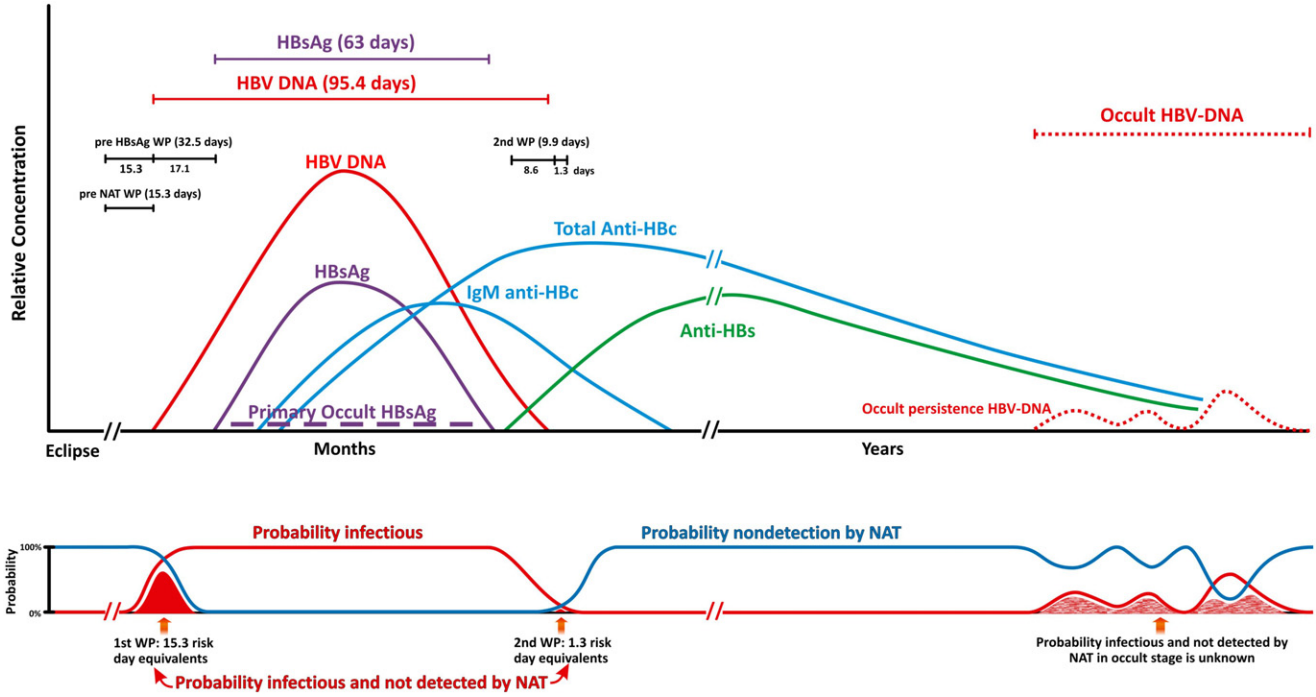

Fig. 1. Course of serum markers in acute resolving hepatitis B infection. The curves in the upper part of the diagram show the relative concentration of the markers in a typical infection. The lines above the curves show the mean lengths of the detection periods of HBV DNA and HBsAg as estimated from the numbers of HBV NAT yields with and without detectable HBsAg. The lengths of the pre- and post-HBsAg WPs and pre- and post-NAT (Ultrio) WPs were calculated for RBCs (20 mL of plasma) from the 50% LODs of 9.5 and 1000 copies/mL for HBV DNA and HBsAg by the transmission risk model of Weusten and coworkers,[16] assuming a 50% minimum infectious dose of 3.7 copies in the ramp-up viremia phase and 370 copies in the declining viremia phase. The resulting probability curves that RBCs are infectious and the probability of nondetection by Ultrio based on 95 and 50% LODs of 98.5 and 9.5 copies/mL determined by comparison against the Eurohep standard are shown at the bottom part of the diagram. The shaded area shows the probability that RBCs are infectious but not detected by NAT and give the number of risk-day equivalents in the first and second WPs, which were estimated to be 15.3 and 1.3 days, respectively, for Ultrio and 12.6 and 0.74 days, respectively, when the more sensitive Ultrio Plus assay with 95 and 50% LODs of 46.9 and 4.5 copies/mL would be used. In a later stage of OBI when anti-HBs titers have declined to below 10 to 100 mIU/mL occult persisting HBV DNA in the liver can reappear in plasma and the blood can become potentially infectious again. The probability that blood from occult HBV carriers is infectious but not detected by NAT is unknown and not addressed in this article.

when HBV DNA was detectable in the presence of anti-HBs as the sole detectable serum marker, followed by an increase in anti-HBs titer and delayed anti-HBc seroconversion. If, however, no follow-up sample was available or no increase in anti-HBs titer was observed, it cannot be excluded that the donor was a chronic occult HBV carrier with anti-HBs as the sole detectable marker. Since there were only a few of these, we classified them as acute NAT yields.

Analyses of the HBV transmission case

A 47-year-old regular male blood donor tested positive for HBsAg and HBV DNA on his 53rd whole blood donation in January 2009. The donor had given a whole blood donation 60 days before the positive index donation (X) and this donation had tested negative for all markers of TTI. The previous whole blood donation (X – 1) had been processed into a red blood cell unit (RBC), a random-donor platelet (PLT) unit, and a fresh-frozen plasma (FFP) unit.

Once it was confirmed that the X donation was positive for HBsAg and HBV-DNA, a donor- triggered lookback investigation was initiated. This investigation established that the RBC unit and PLT unit of the X-1 donation had been transfused and that the fresh frozen plasma (FFP) unit had been forwarded to the South African National BioProducts Institute (Pinetown SA), a plasma fractionation facility.

To confirm transmission of HBV by the X-1 donation, follow-up blood samples were taken from the recipient and from the donor at 110 days and 120 days post-transfusion, respectively. The follow-up samples were

sequenced and genotyped (see below) and tested for HBsAg, anti-HBc (IgM), anti-HBc (total), and anti-HBs titer. Liver function tests—alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH) and total bilirubin—were also performed on donor and recipient samples.

Written informed consent to perform additional laboratory tests, including tests to characterize the virus, was obtained from both the donor and the recipient. Approval to molecularly characterize the HBV isolates was obtained from the Human Ethics Committee of the University of the Witwatersrand.

Viral load estimation

The FFP unit from the X – 1 donation was recovered from the National Blood Fractionation Centre and aliquoted into 3.5 mL samples and frozen at –80°C. Probit analysis can be used to estimate the HBV concentration and the number of viral particles in the units transfused, which are below the detection limit of the Ultrio assay. This was undertaken by testing the donor's FFP unit in 30 replicate tests on the Ultrio and the new generation Ultrio Plus assay and comparing the proportions of positive results with those found on the Eurohep HBV DNA Subgenotype A2 standard dilutions calibrated in copies/mL,[26] comparable to the copies/mL quantified by the Versant bDNA 3.0 viral load assay (Siemens, Tarrytown, NY).[16] The Eurohep standard has subsequently been used for preparation of batches of the WHO standard and it has been estimated that one IU of the lyophilized material in the international standard is equivalent to approximately 5 HBV DNA copies or virions.[26,27] An aliquot of plasma from the FFP unit was also provided to Gen-Probe for testing 30 replicates on the Procleix Ultrio Plus assay. Another aliquot was sent to the Paul Ehrlich Institute for testing 12 replicates in the Ultrio and TaqScreen assays (Roche Molecular Systems) in ID format.

HBV amplification

For both the donor and the recipient, total DNA was extracted from the plasma samples using a DNA mini kit (QIAamp, QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. The surface and basic core promoter regions of both the donor and the recipient HBV isolates were amplified directly from DNA extracted from serum. A nested PCR procedure was carried out to amplify the complete S open reading frame: primers S1F 5′-TCAATCGCCGCGTCGCAGAAGAT CTCAATC-3′ (2410-2439 from *Eco*RI site) and S1R 5′-TCCAGACCXGCTGCGAGCAAAACA-3′ (1314-1291 from *Eco*RI site) were used for the first round (denaturation 94°C for 60 sec, annealing 66°C for 3 min, extension 72°C for 3 min, 40 cycles) and S2F 5′-AATGTTAGTATTCCTT GGACTCATAAGGTGGG-3′ (2451-2482 from *Eco*RI site) and S2R 5′-AGTTCCGCAGTATGGATCGGCAGAGGA-3′ (1280-1254 from *Eco*RI site) for the second round (denaturation 94°C for 60 sec, annealing 68°C for 3 min, extension 72°C for 3 min, 40 cycles).

The basic core promoter/precore region was amplified using a slight modification of a previously published nested PCR:[28] primers BCP1F 5′-GCATGGAGAC CACCGTGAAC-3′ (1606-1625 from *Eco*RI site) and BCP1R 5′-GGAAAGAAGTC**C**GA**G**GGCAA-3′ (1974-1955 from *Eco*RI site) were used for the first round (denaturation 94°C for 60 sec, annealing 55°C for 1 min, extension 72°C for 2 min, 40 cycles) and BCP2F 5′-CATAAGAGGA CTCTTGGACT-3′ (1653-1672 from *Eco*RI site) and BCP2R 5′-GGCAAAAAA**C**AGAGTAACTC-3′ (1959-1940 from *Eco*RI site) for the second round, with the identical cycling conditions as the first-round PCR.

The complete HBV genome was amplified using a single amplification,[29] with modified primers P1 5′-CTTTT TCACCTCTGCCTAATCA-3′ (1820-1841 from *Eco*RI site) and P2RM 5′-AAAAAGTTGCATGRTGMTGG-3′ (1825-1806 from *Eco*RI site).

Cloning

The complete genome amplicons were gel purified using a gel DNA recovery kit (Zymoclean, Zymo Research, Irvine, CA) and cloned individually into a cDNA vector (pSMART, Lucigen, Middleton, WI) according to the protocol provided by the manufacturer and the clones sequenced directly.[30]

Sequencing

The amplicons and clones were prepared for direct sequencing using a cycle sequencing ready reaction kit (BigDye Terminator v3.1, Applied Biosystems) and sequenced on a genetic analyzer with 16 capillaries (ABI3130xl, Applied Biosystems). In addition to the primers used for amplification, vector-specific primers as well as HBV-specific primers (1959F 5′-CTTCTGACTT CTTTCCTTC-3′ [1959-1977 from *Eco*RI site]; 2837R 5′-CCAAGAATATGGTGACC-3′ [2837-2821 from *Eco*RI site]; 2454F 5′-GTTAGTATTCCTTGGACT-3′ [2454-2471 from *Eco*RI site]; 185R 5′-GTCCTAGGAATCCTGATG-3′ [185-168 from *Eco*RI site]; 60F 5′-CTGGTGGCTCC AGTTC-3′ [60-75 from *Eco*RI site]; 734R 5′-CTGAAAG CCAAACAGT-3′ [734-719 from *Eco*RI site]; 595F 5′-CACCTGTATTCCCATC-3′ [595-610 from *Eco*RI site]; 1431R 5′-GACGTAAACAAAGGACG-3′ [1431-1415 from *Eco*RI site]; 1258F 5′-CTGCCGATCCATACTG-3′ [1258-1273 from *Eco*RI site]; and 1769R 5′-CAATTTATGCCTACAGCCTC-3′ [1769-1777 from *Eco*RI site]) were used for sequencing. All sequences were analyzed in both the forward and the reverse directions. The accession numbers of HBV isolates sequenced in this study have been deposited in GenBank/EMBL/DDBJ: JN182318-JN182334.

Phylogenetic analyses

Complete and subgenomic HBV sequences were compared with corresponding sequences belonging to the eight genotypes of HBV (A-H) from GenBank. Multiple sequence alignments and nucleotide divergence calculations were carried out using Dambe.[31] The alignments were edited manually in GeneDoc[32] and fed into PHYLIP (Phylogeny inference package) version 3.69.[33] DNAML (maximum likelihood) alone and DNADIST consecutively with NEIGHBOR (neighbor-joining) were used to generate dendograms. SEQBOOT, DNADIST, and NEIGBOR were used for bootstrapping of 1000 data sets. CONSENSE was used to compute a consensus tree. Trees were visualized using TreeView Win 32 Version 1.6.6.[34]

WP transmission risk analysis

We used the recently refined transmission risk model of Weusten and colleagues[16] to estimate the HBV residual transmission risk posed by RBC transfusions from repeat donors. Using a preformatted calculation spreadsheet the relevant variables were entered, that is, the MP size, the amount of plasma in a RBC unit (estimated at 20 mL), the total number of repeat donations over 4 years of testing, the number of acute HBV infections or HBV DNA and/or HBsAg seroconversions in the repeat donors, the mean preseroconversion interdonation interval, the mean viral doubling time of 2.56 days for the ramp-up phase, and the mean half-life of HBV (1.6 days) in the recovery phase.[35] For the Ultrio assay the recently established 95% and 50% detection limits on the Eurohep genotype A2 standard in a SANBS validation study were used for the calculations (98.5 and 9.5 copies/mL, respectively). The 50% minimum infectious dose (ID_{50}) estimated in the pre–ID-NAT WP was estimated at 3.7[15] but in the second HBV DNA–negative WP the ID_{50} was estimated to be approximately 100-fold higher (and set at 370 copies) as could be deduced from infectivity studies in human liver chimera mice.[36]

The formula below describes the WP transmission risk for HBV in repeat donors:

$$\text{risk} = \frac{r_{\text{days}}}{t_{\text{between}}} \frac{D_{\text{conv}}}{D_{\text{total}}} \times \text{incidence rate adjustment factor},$$

where the total number of risk-day equivalents in the acute phase is the sum of that in the first and second WP, $t_{between}$ is the mean preseroconversion interdonation interval in repeat donors with interdonation intervals less than 12 months, D_{conv} is the number of seroconverting repeat donors, and D_{total} is the total amount of repeat donations. Because of the transient detectability of HBsAg and HBV DNA in the acute phase, the observed incidence rate is an underestimation since seroconversion to anti-HBc and anti-HBs, without detectable HBsAg and HBV DNA, remains unrecognized. Korelitz and colleagues[37] estimated a mean HBsAg detection period of 63 days on the basis of previous follow-up studies. The length of the HBV DNA detection period in acute infection can be estimated from the number of concordant HBsAg and HBV DNA–reactive donations in repeat donors and the additional number of acute NAT-yield infections in this study. The NAT detection period can then be estimated by dividing the total number of viremic acute infections by the number of acute HBsAg-positive infections and multiplying the estimated HBsAg detection period with this factor.[35]

The incidence rate adjustment factor can then be deduced by dividing the mean preseroconversion interdonation interval by the estimated HBV DNA detection period. To estimate the risk of HBV infection in first-time, lapsed, and all donations, the acute-phase NAT-yield rate in repeat donors was compared with those in first-time, lapsed, and all donors. The HBV transmission risk in first-time, lapsed, and all donors was then determined by multiplying the residual risk in repeat donors with the NAT-yield rate ratios in the respective donation categories.

RESULTS

Classification of acute HBV infections

To estimate the risk of HBV transmission by WP donations, it is important to classify the HBV NAT–yield donations as acute or chronic infections. Table 1 shows the number of acute and chronic HBV infections in first-time, lapsed, and repeat donations as detected by HBV DNA and HBsAg screening during 4 years of ID-NAT screening. Over 4 years, 170 repeat donors seroconverted to both HBsAg and HBV DNA, while another 84 seroconverted to HBV DNA without detectable HBsAg. The mean preseroconversion interdonation interval (and range) in the total of 254 acutely infected repeat donors was 127 (35-364) days.

Of the 149 acute-phase NAT-yield cases found in first-time, lapsed, and repeat donors, 114 were classified as early WP donations in the pre-HBsAg or pre–anti-HBc seroconversion phase. Seventy-nine (69%) of these WP infections were confirmed by the presence of HBsAg, HBV DNA, and/or anti-HBc in a follow-up sample, whereas in 35 (31%) no follow-up sample was available. These latter seronegative donations were classified as being in the WP because the TaqMan viral load assay was positive and/or HBV DNA was reactive in multiple replicate Ultrio and dHBV assays on aliquots taken from the frozen plasma unit. Six infections were classified as probable primary OBIs because HBV DNA was reactive in at least two follow-up samples several weeks apart without seroconversion to HBsAg, followed by seroconversion to anti-HBc and anti-HBs in a later sample. Thirteen infections were classified as possible abortive or vaccine breakthrough infections because of presence of HBV DNA in anti-HBs–

TABLE 1. Acute and chronic HBV infection rates as detected by HBV-DNA and HBsAg screening

	Number HBV infections in donations			
HBV infection classification	First time	Lapsed	Repeat	All
Number donations	315,488	302,970	2,303,103	2,921,561
HBsAg+, DNA+	2,302	113	170	2585
(rate)	(1:137)	(1:2,681)	(1:13,548)	(1:1,130)
Acute NAT yield	46	19	84	149*
(rate)	(1:6,858)	(1:15,946)	(1:27,418)	(1:19,608)
Chronic OBI NAT yield	89	27	61	177†
(rate)	(1:3,545)	(1:11,221)	(1:34,375)	(1:16,506)
HBsAg+/DNA–	86	0	0	86
(rate)	(1:3,668)			(1:33,972)
Unclassified	3	1	6	10
All HBV infections	2,523	159	315	3,007
(rate)	(1:125)	(1:1,905)	(1:7311)	(1:972)

* Of 149 acute-phase NAT yields, 95 donors were in the pre–anti-HBc WP, six were likely to be primary occult infections, 13 were possible vaccine breakthrough infections, and 35 were in the IgM anti-HBc+ early recovery phase.

† Of 177 donors with chronic OBI, 75 were potentially infectious (anti-HBc+, anti-HBs–) and 102 were not infectious (anti-HBc+, anti-HBs+). Another five donors could have been classified as OBI with anti-HBs as the sole marker, but we chose to classify these as acute phase NAT yields.

positive (or negative) donations, followed by a significant increase in anti-HBs titer in the second sample (and in four cases followed by delayed anti-HBc seroconversion detected in a third or fourth sample obtained from the donor). In four of the possible vaccine breakthrough infections no follow-up sample was available. Theoretically, these donations could also be classified as occult carriers with anti-HBs as the sole detectable serum marker, but for the purposes of this study they were assumed to be acute infections. One of these four infections was a repeat donor. Misclassification of this case as an acute phase NAT yield would negligibly affect the incidence rate of 254 in 2.3 million repeat donations.

Another group of acute NAT yields, most of them with very low viral loads (below the detection limit of the viral load assay), were the IgM anti-HBc–positive donations in the early recovery phase. In 33 of the 35 early recovery NAT-yield donations, the anti-HBs titer was determined. In 23 of 33 (70%), anti-HBs was reactive and in 19 (58%) anti-HBs titers were above 100 mIU/mL. Ten of the IgM anti-HBc–reactive early recovery phase donations (30%) were anti-HBs negative (<10 mIU/mL) and potentially infectious. A total of 177 (54%) of the HBV NAT yields were OBIs, of which 75 (42%) had no detectable anti-HBs and could potentially be infectious.

HBV transmission case

The blood donor was confirmed positive for HBsAg and HBV DNA on his 53rd whole blood donation. It was determined that the RBC component had been transfused to a 28-year-old male undergoing surgery after a motor vehicle accident. This recipient developed clinical signs and symptoms of acute hepatitis B, 84 days after transfusion, with markedly abnormal liver function test results: ALT 866 IU/L (normal range <50 IU/L), AST 470 IU/L (normal range <38 IU/L), ALP 254 µmol/L (normal range 40-130 µmol/L), GGT 222 IU/L (normal range <60 IU/L), LDH 322 IU/L (normal range 100-250 IU/L), and total bilirubin of 60 µmol/L (normal range 5-21 µmol/L). A further sample taken 110 days after transfusion showed that the recipient was positive for HBV DNA, HBsAg, and IgM anti-HBc. Six months after transfusion the recipient was clinically asymptomatic but did not avail himself for repeat serologic and liver function testing. The PLT unit was issued to a patient with an unreported diagnosis, who, despite concerted efforts, remains untraceable.

The donor remained clinically asymptomatic throughout the follow-up period and upon questioning did not reveal any risk behavior for HBV. He was retested at 2 and 5 months after the X (positive) donation (Table 2). Two months after the X donation, although he was HBsAg negative, HBV DNA was still detectable by ID-NAT. This sample was anti-HBc IgM positive and anti-HBs negative. Five months after the X donation, the donor was anti-HBc and anti-HBs positive and HBV DNA negative.

Viral load estimation

Table 3 shows the proportion of reactive results in replicate Ultrio, Ultrio Plus, and TaqScreen assays in the HBV transmission sample as found by three laboratories. Three of 30 (10%) replicate Ultrio and 10 of 30 (33%) replicate Ultrio Plus assays performed on the recovered FFP unit tested positive for HBV DNA by SANBS. The sample was also tested in Ultrio Plus assays by Gen-Probe and was found reactive in 7 of 30 (23%) replicates. In the Paul Ehrlich Institute the infectious HBV WP sample tested reactive in 2 of 12 (17%) replicates in both the TaqScreen and the Ultrio assay. Using probit analysis, the 95 and 50% detection limit on the Eurohep standards were 98.5 (59-189) and 9.5 (6.5-13.9) copies/mL for the Ultrio and 46.9 (28.4-89) and 4.5 (3.0-6.7) copies/mL for the Ultrio Plus assays, respectively, showing a 2.1 (1.2-4.0)-fold enhancement in analytical sensitivity of the Ultrio Plus assay. From the probit curves the HBV DNA concentration in the plasma unit was estimated to be 1.5 (1.2-1.8) and 1.6 (1.1-2.1) copies or virions/mL in Ultrio and Ultrio Plus, respectively. From this estimation of the low viral load the RBC unit would have contained 32 (22-43) HBV virions in 20 mL of plasma. According to the infectivity risk formula

TABLE 2. HBV markers in samples from both donor and recipient post transfusion

	Donor (47-year-old regular blood donor)			
Marker	Index donation	(X + 2 months)	(X + 5 months)	Recipient (110 days after transfusion)
HBsAg (S/CO ratio)	Positive (352)	Negative (0.27)	Negative	Positive (743.45)
HBV DNA (S/CO ratio)	Positive*	Positive†	Negative	Positive (14.3)
dHBV (S/CO ratio)	Positive (23.1)		Negative	Positive (25.2)
Anti-HBc (IgM)		Positive	Negative	Positive
Anti-HBc (total)		Positive	Positive	Positive
Anti-HBs		Negative	Positive (310 U/L)	Negative

* Positive on three replicates (S/CO ratios of 15.3, 15.6, and 14.9).
† Positive on two replicates (S/CO ratios of 13.3 and 12.8) and negative on one replicate (S/CO ratio 0.12).
S/CO = signal to cutoff.

TABLE 3. Proportion of reactive test results on WP sample in different laboratories*

Laboratory	cobas TaqScreen MPX test	Ultrio assay	Ultrio Plus assay
Paul Ehrlich Institute	2/12 (17)	2/12 (17)	
SANBS		3/30 (10)†	10/30 (33)
Gen-Probe			7/30 (23)†

* Data are reported as number (%) reactive.
† Estimated concentration in probit analysis against the Eurohep Genotype A2 standard was 1.5 (1.2-1.8) and 1.6 (1.1-2.1) copies/mL in Ultrio and Ultrio Plus, respectively.

of Weusten and colleagues,[16] the transmission risk of the amount of HBV virions in the RBC unit would have been 99.7% (98.4%-100%).

Sequence analysis of HBV in donor and recipient

The complete genome of HBV isolated from both the recipient and the donor was amplified and cloned. The full 3221 nucleotides of the HBV genome were successfully sequenced from five clones from the donor and three clones from the recipient. In addition, less than genome length sequences were obtained for four clones from the donor and five clones from the recipient because nucleotides were lost from the 5′ and 3′ ends during the cloning process. These sequences were compared phylogenetically to reference sequences from public databases. Neighbor joining trees for both the complete HBV genome (Fig. 2) and a 3141-nucleotide subgenomic fragment (Nucleotides 1855-1775; Fig. 2 inset) show the isolates from both the donor and the recipient cluster together and belong to Subgenotype A1.

The intragroup divergence of the recipient and donor clones did not differ significantly from each other or from the donor-recipient clone intergroup divergence. On the other hand, the mean intragroup nucleotide divergence for the donor-recipient HBV clones (0.31 ± 0.0006%, mean ± standard deviation [SD]) was significantly lower than intragroup divergences for the South African (2.1% ± 0.006, mean ± SD), African (2.33% ± 0.006, mean ± SD), and global (2.81% ± 0.85, mean ± SD) Subgenotype A1 sequences, respectively ($p < 0.05$).

The consensus sequence of the donor HBV clones was identical to that of the recipient HBV clones but differed at 28 nucleotide positions from the consensus generated from 33 Subgenotype A1 sequences. Fifteen of these mutations were synonymous and 13 were nonsynonymous (Fig. 3).

HBV WP transmission risk

ID-NAT screening interdicted an additional 84 of 254 (33%) acute viremic infections in repeat donors. From the relative proportions of detection of HBsAg and HBV DNA it was estimated that the mean HBsAg detection period of 63 days[37] was extended to 95.4 days (Fig. 1) by multiplying the 63-day period by a factor of 254 in 170 or 1.49. Assuming a 50% HBsAg seroconversion point at 1000 copies/mL[18] in HBsAg PRISM and a ID_{50} of 3.7 copies[15] the infectious pre-HBsAg WP is estimated at 32.5 days, which can be reduced to 15.3 days by Ultrio and 12.6 days with the Ultrio Plus assay in ID-NAT format, when modeled on the detection limits found on the Eurohep standard. The WP reduction times by introduction of ID-NAT are then calculated to be 17.1 days for Ultrio and 19.9 days for Ultrio Plus. Using the same seroconversion risk model of Weusten and colleagues[16] in a reversed manner in the declining HBsAg and HBV DNA clearing phase it can be estimated that with a half-life of 1.6 days[35] and a 100-fold reduced infectivity of HBV because of immune neutralization,[36] still 1.29 and 0.74 risk-day equivalents remain in the second WP for Ultrio or Ultrio Plus, respectively (Fig. 1). If the relative sensitivity of HBsAg and HBV DNA assays were to be the same in the pre-HBsAg ramp-up phase as in the HBsAg declining phase, the reversed Weusten model estimates a post-HBsAg infectious WP of 9.9 days. This means that Ultrio has shortened the second post-HBsAg WP by 8.6 days. However, when the risk-day equivalents are estimated on the basis of the proportion of IgM anti-HBc–positive NAT yields we can calculate a shorter reduction period as follows: In 14 repeat donors HBV DNA was detectable

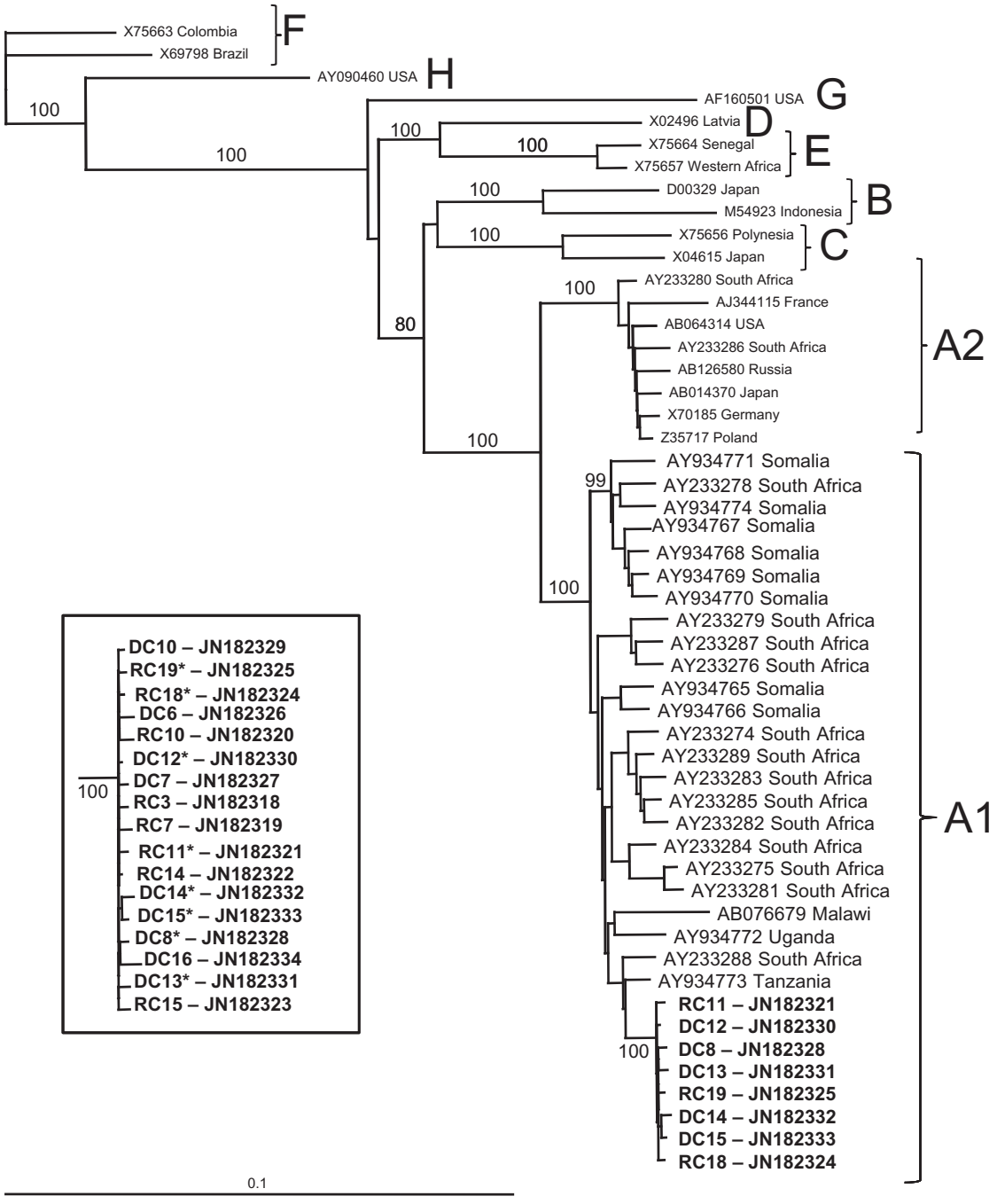

Fig. 2. Phylogenetic relationship of eight HBV clones, shown in bold (five from the donor and three from the recipient) to full-length sequences of other HBV isolates obtained from GenBank, established using neighbor-joining methods. Bootstrap statistical analysis was performed using 1000 data sets with the numbers on the nodes indicating the percentage of occurrences. Clones are designated DC (donor clone) or RC (recipient clone) followed by their GenBank accession number. Other sequences are designated by their GenBank accession number followed by the country of origin. (Inset) Phylogenetic relationship of a 3141-nucleotide sub-genomic fragment (minus nucleotides 1775-1855) from an additional nine shorter than full-length HBV clones (four from the donor and five from the recipient) relative to the full-length clones (*), established using neighbor-joining methods.

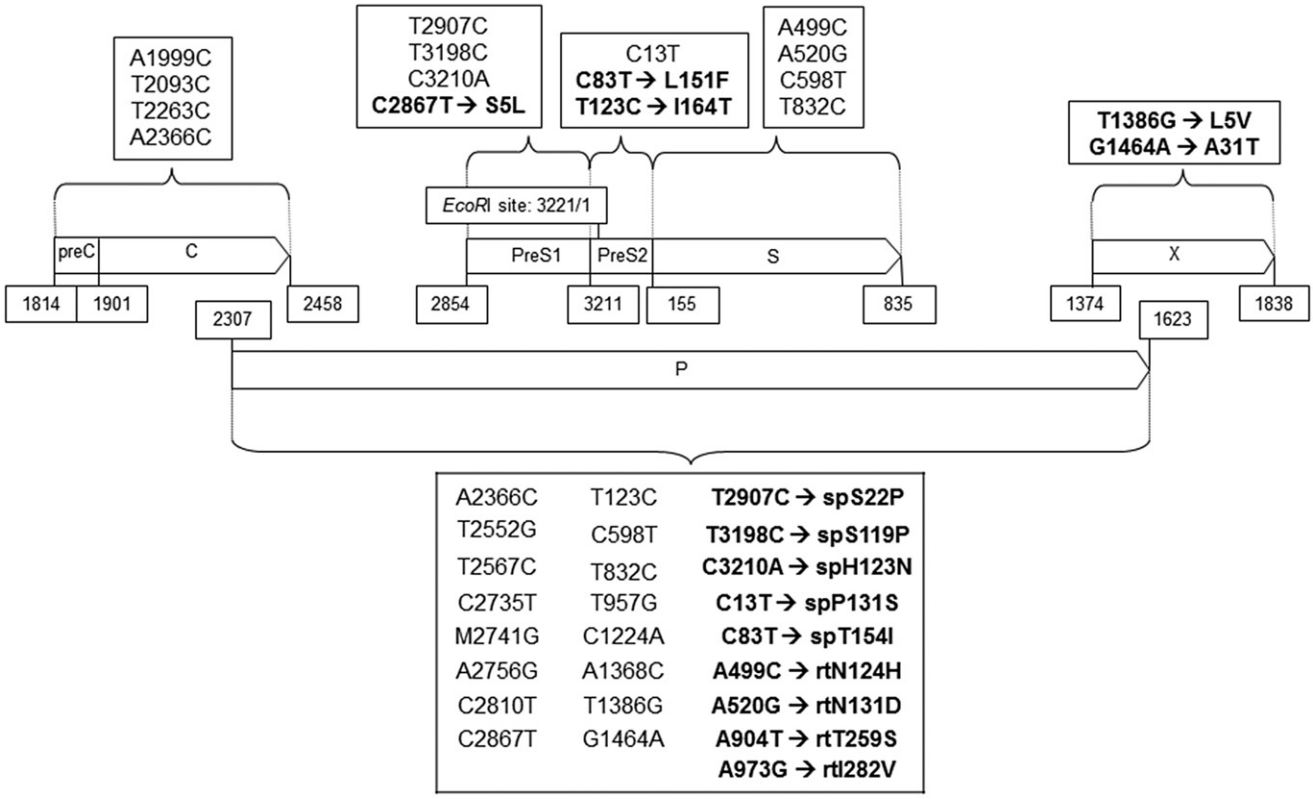

Fig. 3. Comparison of the genomic organization of the donor-recipient consensus sequence and Subgenotype A1 consensus sequence showing the differences found in each of the four open reading frames. Both the synonymous and the nonsynonymous (indicated by bold type) mutations are shown, with the corresponding amino acid changes for the nonsynonymous mutations included. The start and end sites of the four open reading frames are indicated: preC = precore; C = core; P = polymerase gene; S = surface gene; X = X gene. The first T of the *Eco*RI cleavage site (5′-GAATTC-3′) is Position 1.

longer than HBsAg in the IgM anti-HBc–positive early recovery phase. Since we found 70% of IgM anti-HBc–positive donors to be anti-HBs positive (>10 mIU/mL), 30% of the 14 repeat donors (equivalent to 4.2 cases) could be potentially infectious. Hence the proportion of HBsAg-negative donors in the anti-HBs–negative, but HBV DNA– and IgM anti-HBc–positive detection phase, can be estimated to be 4.2 in 254 (1.6%). Since the HBV DNA detection period by Ultrio in ID-NAT was estimated at 95.4 days the length of the HBV DNA–positive but HBsAg- and anti-HBs–negative second WP would be 0.016 × 95.4 days or 1.5 risk-day equivalents. This is less than the 8.6 risk-day equivalents determined by the reversed modeling according to Weusten and coworkers,[16] indicating that worst-case risk estimates for the second WP were calculated in this report.

Since the mean preseroconversion interval in repeat donors was 127 days and the length of the HBV DNA detection period was estimated at 94.5 days, one-third of the acute HBV infections would remain unrecognized. Therefore, the incidence rate in our study needs to be multiplied with an adjustment factor of 127 per 94.5 or 1.34. Using the refined risk analysis[15] for Ultrio in ID-NAT based on 15.3 and 1.3 risk-day equivalents for the first and second WP (total 16.6 risk-day equivalents) a residual HBV transmission risk of 17.9 per million donations in the first WP and 1.5 per million in the second WP was calculated (for a total residual risk of 19.4 HBV transmissions per million repeat donations). Since the HBV WP NAT-yield rate (Table 1) was found to be 4.00-, 1.72-, and 1.40-fold higher in first-time, lapsed, and all donations, respectively, we estimated the residual risk in these donations to be accordingly higher than in repeat donations, that is, 77.6, 33.4, and 27.1 per million, respectively. In other words, the residual HBV transmission risk from acute HBV infections was estimated at 1:12,880, 1:29,966, 1:46,164, and 1:36,800 donations in first-time, lapsed, repeat, or all donors, respectively. Note that these risk estimates do not take into account the unknown risk caused by ID-NAT–negative donors with OBI (see Fig. 1). During 4 years of observation the SANBS lookback program has revealed one clinical posttransfusion hepatitis B event in 2.9 million ID-NAT–screened donations, an observed HBV transmission rate of 0.34 per million.

DISCUSSION

This communication reports the first transfusion-transmitted HBV infection by blood that had been screened by ID-NAT. Definitive proof of a TTI can only be obtained by genomic analysis of both the donor and the recipient viral strains.[38] In this case, the clones of the HBV strains derived from the donor and the recipient were 99.7% homologous but had a number of nucleotide positions, which distinguished this strain from the consensus sequence of Subgenotype A1, the predominant subgenotype circulating in South Africa[39] (Fig. 3). Of the mutations depicted in Fig. 3, as far as we can ascertain, only the synonymous C1224A mutation in the polymerase open reading frame could possibly have any functional or regulatory significance. The C1224A mutation is within the NF-1 binding site of the HBV enhancer I[40] and may therefore affect transcription of the pregenomic and HBx mRNAs.[41] Further functional characterization would be required to confirm this. The high homology of the consensus sequences of the HBV strains from both the donor and the recipient excludes any possibility of a hospital-acquired infection or viral reactivation in the recipient. Furthermore, these strains form a unique branch within the Subgenotype A1 clade with 100% bootstrap support (Fig. 2).

The viral load in the pre–ID-NAT WP donation was estimated by probit analysis to be 1.6 (1.1-2.1) copies/mL and was only detectable in 10% of replicate Ultrio assays. This translates to approximately 32 (22-43) HBV virions in the estimated volume of 20 mL of plasma in the transfused RBC unit. The viral load in the WP donation was also below the detection limit of the more sensitive Ultrio Plus and TaqScreen assays, which detected the sample 33 and 17% of the time in replicate assays, respectively.

The possibility that a unit of blood will transmit virus to a blood recipient is determined by the infectious WP. For HBsAg, using the Abbott PRISM ChLIA system, the infectious WP has been estimated to be 35.5 days if one viral particle or DNA copy in a RBC transfusion is infectious.[18] The WP reduction, using an HBV NAT assay system with a 50% limit of detection (LOD) of approximately 10 copies/mL, was independently calculated to be 17.9[13] and 14.9 days,[19] yielding a calculated WP for HBV by ID-NAT of 20.4 and 24.3 days, respectively. We previously estimated the 50% LOD to be 29 copies/mL and the infectious WP to be 24.3 days.[1] These reported WP estimates were all based on the assumption that one virus in a blood transfusion is enough to be infectious. The variation in the estimated lengths of the WPs in these reports is mainly caused by variation in the 95% and 50% detection limits observed in the analytical sensitivity studies. In this study the infectious WPs with Ultrio were considerably shorter because the refined transmission risk model of Weusten and colleagues[16] also brings the likelihood of infectivity of a low viral burden or the ID_{50} into the equation. Using a ID_{50} of approximately four HBV DNA copies (as found after recalibration of the viral load in a chimpanzee challenge plasma with a known infectivity titer[13] in multiple bDNA assays[15]), we estimated an infectious WP of 15.3 risk-day equivalents with the Ultrio assay and an additional 1.3 risk-day equivalents in the second anti-HBc–positive WP. Compared to the calculated infectious WP for HIV by ID-NAT (2.9 days) and the calculated infectious WP for HCV by ID-NAT (1.3 days),[15] it is evident that there is statistically a greater probability that a blood donation will be in an HBV infectious WP relative to those of HIV and HCV.

During the first 4 years of ID-NAT testing, SANBS detected 2523 HBV confirmed positive donations in 315,488 first-time donations, a prevalence of 0.80%. In the same period the incidence rate of acute HBV infections was 254 in 2,303,103 repeat donations (1:9067). In all 2,921,561 donations 3007 HBV infections were confirmed, yielding an infection rate of 0.10%. Of these confirmed positive HBV infections, 336 donations were detected by ID-NAT only and, of these, 149 were in the HBsAg-negative WP, 177 were OBIs, and 10 were unable to be classified. Therefore, for 149 WP donations interdicted by ID-NAT (rate, 1:19,600), only one donation was found to be in the pre–ID-NAT WP and caused infection in the recipient (1:2,900,000). We estimated the residual risk of a donation being in the first or second infectious WPs for HBV in South Africa to be 27.1 per million donations (1:36,800). This translates to 79.2 HBV transmission events in the 4-year ID-NAT screening period or an estimated 19.8 HBV transmissions per year.

Why is the estimated residual risk far higher using mathematical modeling than the observed transmission rate? Several reasons may account for the 80-fold lower observed transmission rate than the estimated WP transmission risk: 1) A proportion of approximately 40% of the transfusion recipients in South Africa have already been exposed to HBV and are immune;[24] 2) the younger population has, since 1995, been vaccinated in infancy against hepatitis B;[24] 3) low levels of HBV that escape ID-NAT screening can be easily neutralized by anti-HBs present in simultaneously administered blood products; 4) the infectious dose of HBV in ID-NAT–nonreactive WP donations may not lead to clinically recognized HBV infections in the majority of recipients since previous studies in prisoners showed that the incubation time and the occurrence of clinical hepatitis B was inversely correlated with the infectious dose;[42] 5) HBV infection in lookback programs may be underreported and underdiagnosed for several reasons among which is lack of pretransfusion sampling and fortuitous timing of HBV assays in posttransfusion samples; 6) the incubation time of HBV can be much longer when HBV is present in immune complexes;[11] and 7) possible reduction of the infectivity of HBV in stored RBC units.

Many studies have focused on the transmission risk posed by donors with chronic OBI.[23,43] So far, two lookback studies have been reported that indicate that the risk of HBV transmission by low viral load occult HBV carriers is 3% or less.[9,44] The HBV transmission case in this report is consistent with the premise that transfusion-transmitted HBV is greater than 10-fold more likely to occur when the donor is in a recent postexposure infectious WP compared to the presence of low-level viremia in OBI.[9] The risk of HBV transmission by ID-NAT–screened blood donations from donors with OBI is considered to be very low, but further systematic lookback studies are required to confirm this.

It has been suggested that the variation in analytical sensitivity of the current Ultrio test system may not only be HBV genotype dependent but also individual strain dependent.[17] In particular, a variation in relative sensitivities of HBV DNA detection on the Tigris system compared to the Roche s201 (Roche Molecular Systems) has been observed with Genotype A seroconversion panels.[17] This variation in sensitivity may have further contributed to the lack of detection of HBV DNA on the initial ID-NAT screening of the blood donation in this HBV transmission case. However, the comparison of the proportions of reactive results in the WP transmission sample did not demonstrate a large difference in sensitivity between the Ultrio, Ultrio Plus, and TaqScreen assays. The HBV transmission sample was not detectable 90% of the time in replicate Ultrio assays, but would also not be detectable 67% to 83% of the time by the Ultrio Plus and TaqScreen assays in ID-NAT configuration. Clearly, ID-NAT is not sensitive enough to prevent HBV transmission by donations in the early window phase. Further studies are required to determine whether ID-NAT is sensitive enough to prevent HBV transmission by donors with OBI. In the South African high-prevalence setting of hepatitis B a lookback program in recipients of donors, later identified as occult carriers, can only provide meaningful results if a control group of recipients that received blood from anti-HBc–negative donors is also subjected to the same lookback procedures and the proportion of anti-HBc–positive recipients in each group is compared to determine significant difference between the two groups. So far such a comparison study of prevalence of HBV markers, in particular anti-HBc, in recipients of blood from occult HBV carriers and a noninfected control group has not been undertaken.

This communication reports the first known case of transmission of HBV by blood that had been screened by ID-NAT and confirms that blood donations that test negative for HBV DNA may transmit the virus when the donation is in the infectious WP. Transmission was confirmed by the 99.7% sequence homology between the complete genome sequences of both the donor and the recipient viral strains. Our study has demonstrated that the introduction of ID-NAT has significantly enhanced the safety of the blood supply in South Africa, but does not completely eliminate the transmission risk.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to **TRANSFUSION**.

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