Enhanced detection of hepatitis B virus in Hong Kong blood donors after introduction of a more sensitive transcription-mediated amplification assay

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BACKGROUND: A total of 517,072 and 399,326 consecutive donations were screened for hepatitis B virus (HBV) by individual-donation nucleic acid testing (ID-NAT) using Ultrio and Ultrio Plus assays (Novartis Diagnostics), respectively. The impact of more sensitive HBV detection by the latter assay version was established by comparing NAT yield and transmission risk.

STUDY DESIGN AND METHODS: Donations were screened simultaneously for HBV serologic markers and ID-NAT, followed by discriminatory assay and confirmatory test algorithms. Window period (WP) reduction and residual HBV transmission risk were computed using mathematical modeling.

RESULTS: HBV NAT-yield rates for both WP and occult HBV infection (OBI) increased significantly from 1:34,471 to 1:17,362 (p = 0.036) and from 1:5120 to 1:2450 (p < 0.0001), despite a 1.2- and 1.6-fold decrease in hepatitis B surface antigen (HBsAg) incidence and prevalence rates respectively. After adjusting for this bias, the WP and OBI NAT-yield improvement factors were 2.3 and 3.4, respectively, higher than a less than 1.5-fold increase estimated from analytical sensitivity studies on HBV Genotype A and C standards. The current WP transmission risk with Ultrio Plus screening was estimated at 1:55,000 compared to 1:22,000 with HBsAg testing.

CONCLUSION: The observed greater than twofold enhanced WP NAT yield with the Ultrio Plus assay can be explained by greater than 10-fold increased analytical sensitivity in detecting the HBV Genotype B and C strains in Hong Kong. Direct comparison studies of the two assay versions on dilutions of HBV NAT-yield samples are required to confirm this hypothesis.

The population in Hong Kong has a high prevalence of hepatitis B virus (HBV) infection, mostly acquired vertically during early childhood. Surveillance of viral hepatitis in the community during the past 10 years reported a hepatitis B surface antigen (HBsAg) carrier rate of approximately 8% and a prevalence of antibody against hepatitis B core antigen (anti-HBc) of approximately 44% in population groups without apparent risk, such as antenatal women. Genotyping studies of HBV revealed that Genotype C was the commonest (62.6%) and was followed by Genotype B (32.5%) in chronic hepatitis B patients. A universal neonatal HBV vaccination program was launched in 1988 and long-term protective efficacy of immunization had been demonstrated. This has explained at least in part the temporal decline of the prevalence of HBsAg detected in first-time donors over the past two decades from 8.0% in 1990 down to 1.2% in 2010.

HBV infection is a continuing threat to transfusion safety and it has been suggested that blood recipients may be at higher risk than the general population on account of higher ages and comorbid conditions. Blood donations collected during the seronegative window period (WP) of HBV infection are associated with high risk.

ABBREVIATIONS: cps = copies; dHBV = hepatitis B virus–specific discriminatory testing; HKRCBTS = Hong Kong Red Cross Blood Transfusion Service; ID = individual donation; LOD(s) = limit(s) of detection; MP = minipool; OBI = occult hepatitis B virus infection; TMA = transcription-mediated amplification; WP = window period.
of transfusion-transmitted HBV infection. With subsequent development of antibodies against hepatitis B core and surface antigens (anti-HBs) in HBV carriers, the transmission risk lessens depending on the levels of viremia and immune neutralization. Like other areas with high HBV endemicity, it is not cost-effective to screen donations for anti-HBc for past HBV infection and discard all anti-HBc-positive donations. To ensure maximal blood safety, the Hong Kong Red Cross Blood Transfusion Service (HKRCBTS) implemented individual-donation nucleic acid amplification testing (ID-NAT) to screen all blood donations for human immunodeficiency virus (HIV), hepatitis C virus (HCV), and HBV in April 2007. This decision was made in consideration that ID-NAT would detect lower levels of virus than minipool (MP)-NAT and therefore would further reduce the transmission risk by blood from donors in the WP or with occult HBV infection (OBI). All donations were then screened by ID-NAT using triplex PROCLEIX ULTRIO assay (Ultrio) on the automated TIGRIS platform (Novartis Diagnostics, Emeryville, CA). This assay was later replaced by the new version of the triplex transcription-mediated amplification (TMA) assay, known as PROCLEIX ULTRIO Plus (Ultrio Plus) assay, with enhanced analytical sensitivity for HBV DNA detection. It was implemented on the same platform with hardware and software upgrades after a validation study in September 2009. Here we report the results of screening 517,072 donations by Ultrio assay from April 2007 to September 2009 and 399,326 donations by Ultrio Plus assay from October 2009 to June 2011. In this study, we compared the analytical and clinical sensitivity of the Ultrio and the Ultrio Plus assays and explored the impact of a more sensitive assay on the HBV NAT yield rate and the residual HBV transmission risk.

**MATERIALS AND METHODS**

Screening and supplementary testing

The HKRCBTS collects 100% of the blood supply from voluntary nonremunerated donors in the territory. All the eligible blood donors must meet the established donation criteria of the Hong Kong Hospital Authority. All the donations to the HKRCBTS are tested negative for the regular blood-screening markers, including HBsAg, using Abbott PRISM chemiluminescent immunoassay (Abbott, Abbott Park, IL) before they are released for clinical transfusion. Repeatedly reactive samples for HBsAg are confirmed with neutralization assay, and if HBV DNA is nonreactive, more recently also for additional hepatitis B serum markers (see below). All donations are also screened by ID-NAT using HIV-1/HCV/HBV triplex Ultrio Assay on automated TIGRIS platform (Novartis Diagnostics) from April 2007 to September 2009 and thereafter by the Ultrio Plus assay. Initial reactive samples are retested with discriminatory assays to determine the viral specific reactivity. Blood components derived from donations with initial reactive ID-NAT results are not used for transfusion. Seronegative samples with nonreactive discriminatory test for HBV DNA (dHBV) are retested for ID-NAT (on alternative samples of the same donation if available). Donors with nonreactive retested ID-NAT are eligible for further donations and those with reactive retested ID-NAT will be followed up for HBV markers. Concordant HBsAg and ID-NAT/dHBV-reactive donations are considered as confirmed positive. Potential HBsAg-negative NAT-yield donations with initial triplex TMA- and dHBV-reactive results are subjected to further testing for additional HBV serology markers, that is, HBsAg (Architect, Abbott), immunoglobulin (Ig)M, and total anti-HBc (Vitros, Ortho-Clinical Diagnostics, Amersham, UK), HBeAg (Murex HBeAg/anti-HBe) and anti-HBe (Monolisa, Bio-Rad Laboratories), anti-HBs titer (Architect, Abbott) and HBV DNA confirmation test on alternative samples of the index donation. If available, archived samples of repeat donors or lapsed donors and follow-up samples are also tested for HBV DNA, based on a modified Cambridge protocol using COBAS TaqMan HBV test (Roche Molecular Systems, Branchburg, NJ) and in-house polymerase chain reaction. Potential NAT-yield cases that cannot be confirmed are considered as indeterminate and followed up medically.

Analytical sensitivity

Limit of detection (LOD) of Ultrio Plus and Ultrio running on TIGRIS was determined by testing an independently prepared dilution series of the WHO HBV International Standard 97/750 in parallel in 24 replicates by each assay. The 50 and 95% LODs were determined by probit analysis in parallel line mode with statistical software (SPSS package, SPSS, Inc., Chicago, IL). The results were also expressed in copies (cps)/mL using a conversion factor of 5.3 cps/ IU and compared with those reported by P. Grabarz and colleagues (personal communication, Institute of Hematology and Transfusion Medicine, Warsaw, Poland) on the Eurohep standard (from which the WHO standard is derived) and with Japanese chimpanzee Genotype A and C plasma samples with a known infectivity titer provided by Prof. H. Yoshizawa and Prof. J. Tanaka (Hiroshima University, Hiroshima, Japan).

Implementation of new version of triplex TMA for ID-NAT

ID-NAT donation screening for HIV-1, HCV, and HBV using Ultrio Plus on TIGRIS was launched in October 2009. The screening algorithm was not different from the previous Ultrio protocol. Donations with discrepant HBsAg and HBV NAT results were further investigated for
additional HBV serum markers and/or with alternative molecular procedures as described above.

Categorization of NAT-yield cases
Based on the pattern of results of the index donations, archived and follow-up samples, confirmed HBV DNA yield cases from first-time donors, repeat donors (coming back for donation within 1 year since the last donation), and lapsed donors (>1 year since the last donation) were classified into either acute or chronic NAT-yield cases. The acute yield group included: 1) early WP, when all the serology markers in the index donation were negative and follow-up samples showed seroconversion to HBsAg and/or anti-HBc; 2) vaccine breakthrough WP infection or abortive HBV infection, when anti-HBs was the sole serum marker detected in the index sample with subsequent rise in anti-HBs titer and later seroconversion to anti-HBc on follow-up samples if available; and 3) late WP or early recovery phase, when IgM anti-HBc was positive. In some cases early WP infections could be further classified as possible primary OBIs according to the criteria of a consensus conference organized by Raimondo and colleagues. Primary or acute occult infection was considered when HBV DNA remained reactive in the absence of detectable HBsAg and anti-HBc in a follow-up sample but showing the usual postinfection anti-HBc and anti-HBs profile in later follow-up samples. Like the anti-HBs breakthrough infections, these possible primary occult cases were included in the early WP category. If no follow-up samples were available and diagnosis could not be defined because anti-HBc testing had not been performed, these cases were grouped as unclassifiable. For the chronic yield group, they were largely cases of chronic OBI carriers with positive total anti-HBc with or without anti-HBs. Some anti-HBc-nonreactive cases were classified as “anti-HBs only OBI carriers,” if presence of HBV DNA was confirmed in the index donation and sometimes also in follow-up samples without increasing anti-HBs titer.

Estimation of WP transmission risk
The refined viral transmission model of Weusten and coauthors was applied to estimate the residual risk of HBV infection from transfusion of red blood cells (RBCs) donated by repeat and/or lapsed donors. The following variables were entered into a preformatted computer spreadsheet (Excel, Microsoft Corp., Redmond, WA): 1) the respective total numbers of repeat and/or lapsed donations during the study periods of Ultrio and Ultrio Plus assays; 2) the respective numbers of acute HBV infections (all HBV NAT acute yield and HBsAg conversions taken together) encountered in repeat and/or lapsed donors; 3) harmonic mean of preconversion interdonation intervals in lapsed and repeat donors; 4) mean HBV replication doubling time (2.6 days); 5) estimated number of virions present in a RBC unit leading to infection in 50% of cases or ID₅₀ of 3.16 (1-10) copies; and 6) properties of the probit curve (concentrations corresponding to 50 and 95% hit rates of Ultrio and Ultrio Plus reagents, respectively). We used a half-life of HBV DNA of 1.6 days and an ID₅₀ of 316 (100-1000) copies. To correct for underestimation of the incidence of HBV infections as a result of the transient nature of HBsAg antigenemia and viremia (in vivo HBV DNA), an incidence rate adjustment factor was estimated and applied in the residual risk model according to the formulae previously described. When all these variables have been entered, the formulae set in the spreadsheet provide automatic calculations about the number of WP risk day equivalents and the residual risk expressed as number of infections per million transfusions in the early and late WPs, respectively.

The same spreadsheet formulae were also applied in deriving the lengths of the infectious WPs as well as the residual transmission risk in the absence of NAT screening, in different scenarios for Genotype A and Genotype C. For Genotype A, we used the regression analysis in seroconversion panels reported by Assal and colleagues showing a 50% HBsAg seroconversion point in PRISM at 960 cps/mL in the HBV DNA ramp-up phase according to quantification in a viral load assay (Versant bDNA 3.0, Bayer, Tarrytown, NY). For Genotype C, we used the regression analysis on Japanese blood donors, which showed a HBsAg seroconversion point at 2100 cps/mL in the Roche TaqMan assay. Using a conversion factor of 1.62 from bDNA to TaqMan copies (see below), the Genotype C seroconversion point in Abbott PRISM was estimated at 1273 cps/mL in the HBV DNA ramp-up phase for Genotype C. For comparison, we also performed a regression analysis on data provided by Prof. J. Tanaka (Hiroshima University, Hiroshima, Japan) who tested ramp-up phase samples of three chimpanzees infected with one Genotype A strain (Chimps C-246, C-279, C-280) and three chimpanzees infected with one Genotype C strain (C-272, C-285, C-269). We found HBsAg seroconversion points at 3222, 3863 and 2960 cps/mL for Genotype A and much higher values of 70,407, 14,995, and 21,765 cps/mL for Genotype C in the Roche TaqMan assay. Based on conversion factors of 2.06 and 1.62 from bDNA to TaqMan copies as found by recalibration experiments of Van Drimmelen and coworkers (personal communication, BioQControl-DDL, Rijswijk, the Netherlands) in the respective Genotype A and C challenge plasma samples used to infect
these chimpanzees, we converted the geometric mean seroconversion point from 3327 TaqMan cps/mL into 1615 bDNA cps/mL for Genotype A and from 28,430 TaqMan cps/mL to 17,549 bDNA cps/mL for Genotype C. For chimpanzee Genotype A and C, Komiya and coworkers\(^2\) found viral doubling times of 3.4 and 1.9 days, respectively, and after recalibration in bDNA copies the 50% minimum chimpanzee infectious doses were estimated at 4.0 (1.3-12.6) and 5.9 (1.8-18.5) copies of HBV virions for Genotypes A and C, respectively. These latter variables were used to estimate the WP risk day equivalents in the inoculated chimpanzees. As described above, we assumed a viral doubling time of 2.6 days and an ID\(_{50}\) of 3.16 copies for the risk calculations in Genotype A and C seroconversion in humans.

**Estimation of WP reduction times**

The WP reduction after introduction of the Ultrio Plus assay can be estimated by subtracting the number of risk day equivalents calculated by the “Weusten spreadsheet” (see above) with the latter assay from those estimated for the Ultrio assay. The length of the WPs is dependent on the 95 and 50% LODs (expressed in cps/mL) with the Ultrio and the Ultrio Plus assays. These are rather consistent for the Ultrio Plus assay but are highly variable for the Ultrio assay depending on the HBV genotype and strains used for the analytical sensitivity studies (P Grabarczyk et al., personal communication, Institute of Haematology and Transfusion Medicine, Warsaw, Poland). Therefore, we estimated the WP risk day equivalents and WP reduction times from analytical sensitivity studies on different HBV Genotype A and C standards (see above).

**Comparison of WP NAT-yield rates and WP reduction times**

The increase of the early WP NAT-yield rate after conversion to Ultrio Plus should be a reflection of the average ID-NAT WP reduction times compared to HBsAg with the Ultrio and the Ultrio Plus assays, respectively. To adjust for a lower HBV incidence in the Ultrio Plus screening period, the ratio of the HBsAg seroconversion rates in repeat donations in the Ultrio Plus and the Ultrio screening periods was determined and multiplied with the pre-HBsAg WP NAT-yield ratio. The so-obtained “incidence rate adjusted WP NAT-yield improvement factor” was compared with the ratio of the estimated lengths of the WP reduction times with the two TMA assay versions as deduced from the LODs on different Genotype A and C standards.

**Statistical analysis**

The HBV NAT-yield rates in acute and chronic phases of infection and the occurrence rates of cases with discordant serological and ID-NAT results during the Ultrio and Ultrio Plus screening periods were compared by chi-square test for first time, lapsed, or repeat and all donations. Calculated values less than 0.05 were considered as significant. The 50 and 95% LODs on the WHO HBV DNA standard dilution panels were compared by parallel line probit analysis and from the shift in the probability curves the sensitivity of Ultrio Plus relative to Ultrio and the 95% confidence interval (CI) was calculated using a statistical software package (SPSS, SPSS, Inc., Chicago, IL).

**RESULTS**

**Analytical sensitivity on WHO HBV standards**

Table 1 shows the 50 and 95% LODs obtained by testing the same WHO HBV standard dilution panels with both the Ultrio and the Ultrio Plus assay. The data are compared with those reported in the package inserts. In a parallel line probit analysis, the Ultrio Plus assay was 3.0 (1.3-11.4)-fold more sensitive than the Ultrio assay.

**Confirmation and classification of HBV infections**

**HBV infections in the Ultrio screening period**

During the period from April 2007 to September 2009, a total of 517,072 consecutive donations were screened for HBsAg and HBV DNA with the use of Ultrio. Of these, 92,551 (17.9%) donations were from first-time donors, 278,543 (53.9%) from repeat donors, and 145,978 (28.2%) from lapsed donors. A total of 1814 HBsAg-positive cases

<table>
<thead>
<tr>
<th>Reagent</th>
<th>WHO HBV standard</th>
<th>Evaluation source</th>
<th>Number</th>
<th>50% IU/mL (CI)</th>
<th>95% IU/mL (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrio</td>
<td>97/746</td>
<td>HKRCBTS(^*)</td>
<td>24</td>
<td>1.57 (0.87-2.82)</td>
<td>11.8 (5.9-37.9)</td>
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<td>HKRCBTS(^*)</td>
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<td>0.51 (0.28-0.91)</td>
<td>3.9 (2.0-11.4)</td>
</tr>
<tr>
<td>Ultrio(^†)</td>
<td>97/746</td>
<td>Package insert‡</td>
<td>115</td>
<td>2.3 (1.5-3.4)</td>
<td>14.7 (8.4-40.7)</td>
</tr>
<tr>
<td>Ultrio Plus(^†)</td>
<td>97/750</td>
<td>Package insert‡</td>
<td>120</td>
<td>0.6 (0.5-0.7)</td>
<td>2.1 (1.7-3.0)</td>
</tr>
</tbody>
</table>

\(^*\) Dilution panel prepared by HKRCBTS.
\(^†\) LODs of Ultrio and Ultrio Plus calculated on the response data in package inserts are presented for the purpose of easy comparison.
\(‡\) Gen-Probe, San Diego, CA.
were confirmed by neutralization assay. With dHBV on the triplex ID-NAT (Ultrio) initial reactive HBsAg-negative samples, 132 potential NAT-yield cases were identified, of which 128 (97.0%) were confirmed with alternative molecular procedures. Of 1814 HBsAg-positive donations, 165 (9.1%) were HBV DNA negative in Ultrio. Unfortunately only a subset of 47 of these samples were tested for additional HBV serum markers (and all were anti-HBc reactive, see Supporting Information Table S1, available as supporting information in the online version of this paper), but it cannot be excluded that some of the unconfirmed samples were false HBsAg reactive due to contamination of test samples, particularly among the HBsAg-positive and DNA-negative donations in lapsed (n = 17) and repeat donors (n = 5), some of which had very low HBsAg S/CO values.

HBV infections in the Ultrio Plus screening period
From October 2009 to June 2011, a total of 399,326 consecutive donations were screened for HBsAg and HBV DNA with Ultrio Plus. Of these 80,565 (20.2%) donations were from first-time donors, 217,435 (54.5%) from repeat donations, and 101,326 (25.4%) from lapsed donations. A total of 977 HBsAg-positive donations were confirmed in the neutralization assay. After dHBV testing on the HBsAg-negative triplex ID-NAT (Ultrio Plus) initial reactive samples, 208 potential NAT-yield cases were detected, 192 (92.3%) of which were confirmed in subsequent alternative molecular procedures. Unlike the Ultrio screening period (see above), all HBsAg-positive but HBV DNA-negative donations in the Ultrio Plus screening period were tested with supplementary assays (see Supporting Information Table S1) Interestingly only four of the 47 HBsAg-positive and DNA-negative donations were from lapsed donors and none of the acute infections in repeat donors was HBV DNA negative in the Ultrio Plus assay.

Acute HBV NAT yields in both screening periods
In a combined analysis over 4 years encompassing both Ultrio and Ultrio Plus periods, 38 acute HBV NAT-yield cases were encountered, 32 of which were classified as early WP NAT yields. Among these, seroconversion to HBsAg was found in 10 cases on follow-up and 22 did not show HBsAg seroconversion but became anti-HBc positive on a follow-up sample taken 8 to 10 weeks later. Of 38 acute yields, 12 donors (31.6%) had a known vaccination history. Among the 32 early WP NAT yields, anti-HBs was found in the index donation samples in nine cases, three of which had history of HBV vaccination while the other six were nonvaccinated donors. Since in the follow-up samples, anti-HBc seroconversion and an increase in anti-HBs titer were found in both categories, these nine cases were considered as anti-HBs breakthrough infections. Six acute HBV NAT yields were classified as IgM anti-HBc positive late WP NAT yields in the early recovery phase, four of which carried anti-HBs and may have been no longer infectious.

Chronic HBV NAT yields in both screening periods
Apart from the 38 acute NAT yields and 18 unclassified cases, another 264 of all 320 NAT-yield cases (82.5%) were categorized as chronic HBV NAT yields. The age of these donors with OBI was significantly higher than that of the general donor population (see Supporting Information Table S1). Of the 264 OBI donors, 125 (47.3%) had no detectable anti-HBs and were potentially infectious and 60 (22.7%) were vaccinated suggesting that some of them could represent past vaccine breakthrough infections. Of the 264 chronic HBV NAT yields, 252 were total anti-HBc positive and thus confirmed as OBI. In another 12 donors, HBV DNA was reactive in the presence of anti-HBs as the sole detectable serum marker and in a number of cases this pattern was confirmed in follow-up samples.

Unclassified HBV NAT yields
Twelve HBV NAT yield cases could not be classified because anti-HBc test results were lacking. During the Ultrio period, seven of the 12 unclassifiable NAT yields could be WP cases on account of the relatively high viral loads, but inadequate follow-up data to conclude; during the Ultrio Plus period, two of the six unclassifiable NAT yields could be WP cases for the same reason.

Comparison of HBV detection rates in the Ultrio and the Ultrio Plus screening periods

HBV infections in all donations
Table 2 compares the HBV infection and NAT-yield rates in all donations in the Ultrio and the Ultrio Plus screening periods. The overall HBV NAT-yield rate doubled from 1:4040 to 1:2080 (p < 0.0001) after introduction of Ultrio Plus despite a decreasing HBsAg prevalence in the donations from 0.35% to 0.24%. The doubling of the NAT-yield rate was observed in both the acute and the chronic NAT yields, increasing from 1:34,000 to 1:17,000 (p = 0.036) and from 1:5100 to 1:2400 (p < 0.0001), respectively. Ultrio Plus was also significantly more sensitive than Ultrio in detecting HBV DNA among HBsAg carrier donors (90.9% vs. 95.2%, p < 0.0001).

HBV infections in first-time donors
Table 3 compares the HBV infection and NAT-yield rates found in first-time donors. The prevalence of HBsAg-positive infections in first-time donors decreased significantly (1.62-fold) from 1.83% in the Ultrio to 1.13% in the Ultrio Plus screening period (p < 0.0001). Notwithstanding the temporal decline trend of HBV prevalence from the Ultrio to the Ultrio Plus period, the acute-phase NAT-yield
rate slightly, though not significantly, increased from 1:23,000 to 1:16,000. However, the rate of OBI increased significantly from 1:7700 to 1:3000 (p < 0.005) on account of improved HBV DNA detection.

**HBV infections in lapsed and repeat donations**

The rate of HBsAg-positive infections in repeat and lapsed donations taken together decreased 1.33-fold from 1:3508 in the initial screening period with Ultrio to 1:4688 in the subsequent period with Ultrio Plus (Table 4). However, at least five HBsAg-positive and DNA-negative infections in repeat donors in the Ultrio screening period were considered to be chronic infections with fluctuating HBsAg around the cutoff or false positives since anti-HBc confirmation testing had not been systematically performed (see Supporting Information Table S1). Therefore, we took the rate of concordant HBsAg- and HBV DNA-positive infections to compare the incidence rate of acute HBsAg-positive infections and this decreased 1.17-fold in repeat donors from 1:10,316 (27/278,543) in the Ultrio screening period to 1:12,038 (18/217,435) in the Ultrio Plus screening period. Likewise, this incidence rate decreased 1.16-fold in lapsed and repeat donations together from 1:4288 to 1:4981 (Table 4). Nevertheless, both the acute WP and the chronic OBI NAT-yield rates had doubled from 1:38,593 to 1:17,709 (p = 0.036) and from 1:4770 to 1:2344 (p < 0.0001), respectively.

**Estimation of WP risk day equivalents by mathematical modeling**

When converting the 50 and 95% LODs in IU/mL in our analytical sensitivity studies (Table 1) to cps/mL using a factor of 5.3, we found 8.3 and 62.7 cps/mL for Ultrio and 2.7 and 20.6 cps/mL for Ultrio Plus, respectively. Similar 50 and 95% LODs were found by other investigators on dilutions of the Eurohep and chimpanzee Genotype A standards (Table 5). However, on a
chimpanzee HBV Genotype C infectivity standard, Ultrio was 6.7-fold less sensitive than Ultrio Plus when comparing the 50% LODs (32.8 and 4.9 cps/mL, respectively). The 50% LODs in Ultrio and Ultrio Plus on the various analytical standards can be translated to infectious WPs expressed in risk day equivalents (see Materials and Methods). The same holds for the HBsAg seroconversion points estimated by regression analysis in three seroconversion studies (see Materials and Methods). Table 5 gives an overview of the seroconversion studies in humans and chimpanzees as well as analytical sensitivity studies based on HBV Genotype A and C standards with the translation of the sensitivity data into the estimated lengths of the preseroconversion WPs. For estimating the mean WP risk day equivalents for Genotype A in Hong Kong blood donors, we took 50% HBV seroconversion points of 2.5, 7.5, and 960 cps/mL for Ultrio Plus, Ultrio, and HBsAg PRISM, respectively. For HBV Genotype C, we assumed 4.9, 32.8, and 1273 cps/mL, respectively, as the 50% seroconversion points (see references for these seroconversion points and corresponding lengths of WPs in Table 5). The geometric mean HBsAg seroconversion point in three chimpanzees inoculated with the same Genotype C strain was significantly higher than the one found by regression analysis in a large number of Japanese blood donors (17,546 cps/mL vs. 1273 cps/mL). We assumed that the human data on the circulating viruses in Japan were more representative than the chimpanzee data on this single Genotype C strain inoculated in three chimpanzees. Hence our modeling in Table 5 indicates that for a typical Genotype A seroconversion the Ultrio and the Ultrio Plus assays shorten the WPs compared to HBsAg PRISM with 18.2 and 22.2 days, respectively, whereas for a representative Genotype C strain, the WP reduction times were estimated at 13.7 and 20.8 days, respectively.

### TABLE 5. Estimation of WP risk day equivalents estimated from 50 and 95% LODs of Ultrio and Ultrio Plus assays on HBV Genotype A and C standard dilution panels and from HBsAg seroconversion cutoff crossing point in regression analyses on human and chimpanzee ramp-up samples (see Materials and Methods)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Marker</th>
<th>Panel/standard</th>
<th>Genotype</th>
<th>LOD in cps/mL in ramp-up phase</th>
<th>Risk day equivalents</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>50%</td>
<td>95%</td>
<td>Early WP</td>
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<tr>
<td>Prism</td>
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<td>1,615</td>
<td>45.2</td>
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<tr>
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<td>(10.6)*</td>
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<tr>
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<td>A</td>
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<td>15.3</td>
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<td>20.8</td>
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<td>0.54</td>
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<td>C</td>
<td>4.9</td>
<td>13.7</td>
<td>0.92</td>
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* Estimates assuming that HBsAg seroreversion point is at the same level as at seroconversion point.
† Chimpanzee infectivity plasma samples from Hiroshima University.‡ Personal communication.

### Comparison of observed and projected WP NAT yield rates and WP reduction times

After introduction of Ultrio Plus, the pre-HBsAg WP NAT-yield rate increased 1.9-fold (from 1:39,755 to 1:21,017), but the incidence of HBsAg-positive infections decreased 1.16-fold. After the lower incidence of HBsAg seroconversion was corrected for, the pre-HBsAg WP NAT-yield improvement factor would be 2.2-fold. The elongation of pre-HBsAg WP detection by Ultrio Plus over Ultrio according to modeling on analytical standards for HBV Genotype A and C was 1.2- and 1.5-fold, respectively (see above). This suggests that even the modeling on the Genotype C standard (Table 5) detected with 6.7-fold higher sensitivity by Ultrio Plus is not representative for our clinical findings. It therefore may be that the mean pre-HBsAg WP reduction time offered by Ultrio is shorter than the 13.7 days predicted from the analytical sensitivity data on the Genotype C infectivity standard (Table 5). A reverse analysis based on the clinical yield data would predict that the WP reduction time offered by Ultrio Plus (20.8 days) should be divided by a factor of 2.2 to obtain a mean WP reduction of 9.5 days provided by Ultrio. If this were true, the mean WP reduction time from Ultrio to Ultrio Plus would be 20.8 minus 9.5 or 11.3 days. With a doubling time of 2.6 days, this would mean that Ultrio Plus would on average be 20-fold more sensitive than Ultrio on the HBV strains circulating in Hong Kong and that the mean 50% LOD of Ultrio could be in reality as high as 20.5 × 4.9 or 101 cps/mL, much higher than the 33 cps/mL found in the chimpanzee genotype C infectivity standard. Figure 1 illustrates the estimated 50% seroconversion points (and range) in cps/mL in the Ultrio, Ultrio Plus, and PRISM assays during the log-linear ramp-up phase according to the reverse modeling on the clinical yield data in this study.
HBV WP transmission risk with Ultrio Plus assay

Because of the highly variable analytical sensitivity of the Ultrio assay and the uncertainty about the mean LODs in this assay version for the HBV strains in Hong Kong blood donors, we decided to calculate the residual HBV WP transmission risk for the more consistent Ultrio Plus assay version only and base the estimations on Genotype C detection limits (Table 5). During the Ultrio Plus screening period, 27 repeat donors converted to HBV DNA alone (n = 9) or to both HBV DNA and HBsAg (n = 18) with a harmonic mean preconversion interdonation interval of 128.2 days. Our modeling—assuming an ID50 of 3.16 virions/20 mL plasma in a RBC transfusion—estimated that the length of the infectious WP decreased from 34.5 days with PRISM HBsAg to 13.7 days with the Ultrio Plus assay yielding a WP reduction of 20.8 days (or 60% of the infectious pre-HBsAg WP). The acute HBV DNA–positive yield (n = 27) in repeat donors was 1.5-fold higher than the concordant HBsAg and HBV DNA–positive yield (n = 18). Thus, as a consequence of introduction of HBV DNA screening by Ultrio Plus, the mean length of the HBV detection period estimated at 63 days for the HBsAg assay by Korelitz and coworkers17 increased 1.5-fold to 94.5 days for HBV DNA detection with Ultrio Plus. The estimated length of the Ultrio Plus detection period was 1.36-fold shorter than the harmonic mean preconversion interdonation interval of 128.2 days. Therefore, the incidence rate adjustment factor for transient HBV DNA detection was estimated at 1.36 for the Ultrio Plus assay. According to 50% estimated seroconversion points in the WP ramp-up phase for Genotype C at 4.9 cps/mL for Ultrio Plus and 1273 cps/mL for PRISM HBsAg (Table 5), the WP transmission risk declined from 1:22,000 (45.4 per million donations) without NAT to 1:55,000 (18.0 per million donations) with ID-NAT (Ultrio Plus). The post ID-NAT WP for Ultrio Plus was estimated at 0.92 days because 100-fold lower infectivity of HBV was used in the modeling and the residual risk was calculated at 1:820,000 (1.2 per million). Taking both pre- and post–ID-NAT WP estimations together, the transmission risk was estimated at 1:52,000 (19.2 per million donations).

HIV and HCV infection rates and residual transmission risk

During the entire Ultrio and Ultrio Plus screening period, 29 of 916,398 HIV infections were confirmed (1:31,600) and all were both HIV RNA and anti-HIV reactive. The infection rate was higher in first-time and lapsed donations (1:13,300 and 1:22,500, respectively) than in repeat donations (1:99,200). The prevalence of HIV infections in first-time donors decreased twofold from 1:10,300 in the Ultrio screening period to 1:20,100 in the Ultrio Plus screening period. Likewise the incidence in lapsed and repeat donations together decreased from 1:35,400 to 1:80,000. The residual WP transmission risk in lapsed and repeat donations together was estimated at 1:5,700,000 for the whole study period. Also for HCV, there were no WP infections interdicted by NAT and overall 191 infections were confirmed anti-HCV positive (1:4800) of which 174 were also HCV RNA positive. The vast majority of HCV infections (n = 177) was found in first-time donors (rate 1:978) and 13 (1:19,000) in lapsed donations. Only one concordant HCV RNA– and antibody-positive donation was found in repeat donations (1:496,000). The prevalence and incidence of HCV infections remained stable over the Ultrio and the Ultrio Plus screening periods. The residual HCV transmission risk in lapsed and repeat donations together was estimated at 1:26,000,000.

DISCUSSION

The HKRCBTS was the first in the Asia-Pacific area to replace the Ultrio assay by the more sensitive Ultrio Plus assay version for routine ID-NAT screening. The introduction of this new assay in October 2009 has had no impact on HCV and HIV-1 NAT yields (see Results) but has significantly enhanced detection of HBV. This was apparent in HBsAg-positive donors in whom the HBV DNA detection rate increased from 1649 of 1814 (90.9%) with the Ultrio assay to 930 of 977 (95.0%) with the Ultrio Plus assay.
yield rate was reported by Vermeulen and colleagues. The chronic OBI NAT-yield rate had increased 2.1-fold from 1:5100 to 1:2400 (p < 0.0001) even though the HBsAg prevalence in first-time donors had decreased 1.6-fold from 1.83% in the Ultro to 1.13% in the Ultro Plus screening period. If the OBI NAT-yield rate in the latter screening period is adjusted for the lower HBV prevalence, the improvement factor of 2.1 for the OBI detection rate might be as high as 3.4, comparable to that seen in Egypt (Faten Moftah, personal communication, National Blood Transfusion Service, Cairo, Egypt). Despite a 1.2-fold drop in the HBsAg seroconversion rate in repeat donors, the acute WP NAT-yield rate had also increased significantly from 1:34,500 in the Ultro to 1:17,400 (p = 0.036) in the Ultro Plus screening period. Hence, the HBV WP NAT yield became 2.0-fold higher after introduction of the Ultro Plus assay and when corrected for a 1.2-fold lower incidence of HBsAg conversion in repeat donors, the improvement would have been 2.3-fold. The incidence rate adjusted NAT-yield improvement factor was 2.2 when late (IgM anti-HBc positive) WP NAT yields were disregarded and only early (anti-HBc negative) WP NAT yields were taken into account. A similar 2.5-fold higher early WP NAT yield rate was reported by Vermeulen and colleagues who compared the two TMA assay versions in South African blood donors during two screening periods with comparable incidence and prevalence rates of HBsAg-positive infections.

How can a 2.2-fold higher early WP NAT yield be explained when the HBV DNA WP reduction compared to HBsAg is only 1.2-fold longer with Ultro Plus than with Ultro? This factor is predicted mathematically from analytical sensitivity studies on HBV Genotype A standards, such as the WHO International Standard, the Eurohep standard, or a Japanese chimpanzee infectivity standard (see Results, Table 5). Even when based on a chimpanzee HBV Genotype C infectivity standard detected with seven-fold higher analytical sensitivity by the Ultro Plus assay, the predicted pre-HBsAg WP NAT yield is estimated to be only 1.5-fold higher (see Results, Table 5). A 2.2-fold enhancement of the WP NAT yield can only be explained mathematically when Ultro would be on average 20-fold less sensitive than Ultro Plus in detecting HBV-DNA (see WP reduction model in Fig. 1). This would imply that the former TMA assay version is less efficient in detecting a large fraction of the HBV Genotype B and C strains circulating in Hong Kong (see Supporting Information Table S1 for further details of this hypothesis). Direct comparison studies of the two assay versions on dilutions of Ultro Plus HBsAg NAT-yield samples are required to prove that the Ultro assay is indeed underdetecting HBV DNA more than 10-fold. The most likely explanation for a diminished capacity of the Ultro assay to detect the local HBV strains is that the target region for the TMA capture probe is for the most part double stranded in the virions. The target enhancer reagent in the Ultro Plus assay giving an alkaline shock to the virus particles denatures the capsid proteins as well as the double-stranded portion of the HBV genome so that a larger amount of single-stranded DNA will be able to bind to the capture probe.

The highly variable analytical sensitivity of Ultro and the more consistent LODs found with Ultro Plus assay (P. Grabarczyk et al., personal communication, Institute of Haematology and Transfusion Medicine, Warsaw, Poland) made us decide to only estimate the WP reduction time and residual transmission risk for the latter assay. For this purpose we assumed that the 50% LOD of 4.9 cps/mL found on a Genotype C infectivity standard was representative for the mean 50% HBV seroconversion point in the ramp-up viremia phase in Hong Kong blood donors. For HBsAg we estimated a seroconversion point for Genotype C at 1270 cps/mL (see Results). When these estimates are representative for the HBV strains in Hong Kong the Ultro Plus assay reduces the early WP by 21 days from 35 to 14 days. The remaining infectious WP is based on a mean HBV viremia doubling time of 2.6 days and a 50% minimum infectious dose lying between 1 and 10 virions (3.16 HBV DNA copies). With these assumptions we estimated that the early WP transmission risk for RBC transfusions has been reduced from 1:22,000 with only HBsAg screening to 1:55,000 after implementation of ID-NAT screening with the Ultro Plus assay, reducing the early infectious pre-HBsAg WP by 60%.

Since HBV infectivity studies in human liver chimera mice demonstrated a 100-fold decrease of infectivity in the late acute anti-HBc–positive phase when HBsAg levels were declining in chimpanzees we estimated a late infectious WP of less than 1 day with an ID₅₀ of 316 virions. This ID₅₀ estimate between 100 and 1000 HBV DNA copies has been confirmed in a European lookback study in recipients of blood from anti-HBs–negative donors with OBI. In this latter study there was no indication of infectivity of anti-HBs–positive OBI blood, even when large fresh-frozen plasma volumes were transfused. It may therefore be that the estimated residual risk from Ultro Plus–negative donors in the late IgM anti-HBc–positive WP of 1:820,000 in our study is an overestimation because anti-HBs titers increase when HBV DNA levels are declining to undetectable levels. In this context, it is important to note that four of six IgM anti-HBc–positive NAT-yield cases were anti-HBs reactive with titers ranging from 24 to more than 1000 mIU/mL. These latter donations may not have been infectious because of antibody neutralization.

It should be stressed that the overall WP transmission risk estimate of 1:52,000 does not take into consideration the risk posed by donations collected from donors with OBI and with low viral load not being detected by Ultro
Plus ID-NAT. The anti-HBs–negative OBI detection rate was 1:11,500 for Ultrio and 1:5000 for Ultrio Plus. Vermeulen and coworkers22 used mathematical modeling on random distributed low viral loads in OBI to estimate that an additional 9% of anti-HBs negative OBI yield cases interdicted by Ultrio could still be missed by Ultrio Plus and also be infectious in RBC transfusions (assuming an ID$_{50}$ of 316 copies for OBI). If this analysis would be applied to our data the residual OBI transmission risk would be 1:128,000 for RBC transfusions, more than twofold lower than the risk from donations in the acute phase of infection.

OBI carriers in Hong Kong demonstrated the characteristics of male dominance, normal ALT levels (data not shown), low viral load (data not shown), and older ages than the general donor population (see Supporting Information Table S1), which did not differ from other OBIs reported worldwide.24-26 In Shenzhen, a city in China in close vicinity to Hong Kong, donors with OBI were surprisingly younger (median, 28 years old).27 When comparing the OBI rate (1:3471) in our study with those reported in other countries, the rate differences varied considerably depending on HBV epidemiology, proportion of repeat donors with OBI (Genotypes B and C) who were vaccinated but yet low viremic may indicate that HBV vaccine was efficacious in preventing clinical HBV disease but not infection38 (with the caveat that these donors may also have been infected before vaccination). In addition we found that 12 of 264 (4.5%) (unvaccinated) donors with OBI had anti-HBs as the sole detectable serum marker indicating that not all latently infected blood donors carry detectable anti-HBc. Although the presence of anti-HBs may neutralize infectivity of HBV in the vast majority of occult carriers22 this may not be the case at the time there is a burst of virus breaking through low levels of anti-HBs as was observed in a double-OBI transmission case in Slovenia.32 The implication for blood safety is then that, even in the presence of anti-HBs, donations collected from (vaccinated) donors with acute or chronic OBI cannot be considered safe for transfusion. It is then imperative to implement a sensitive HBV NAT system to exclude such infectious donations from entering into the blood inventory.

From the early WP risk estimates above, we projected a pre-HBsAg WP NAT–yield rate of 27.3 per million, whereas the observed yield rate was 32.2 per million. This small difference (16%) may be explained by either somewhat lower mean Ultrio Plus seroconversion points or more likely by higher mean HBsAg seroconversion points in the HBV DNA ramp-up phase than the values used in the modeling (Table 5). In this context, it may be relevant to note that five of 19 (27%) of early WP infections in our study were classified as anti-HBs breakthrough infections. Another explanation for the somewhat higher observed than projected Ultrio Plus WP NAT yield is the occurrence of acute HBsAg-negative infections with relatively high viral load. Some circulating Genotype C strains such as the one used for studying the minimum infectious dose in chimpanzees26 showed HBsAg seroconversion at a 10-fold higher HBV DNA level than that observed with typical Genotype A strains (Table 5). In some cases viral loads as high as 100,000 cps/mL were still HBsAg negative in acute infection and were classified as acute or primary OBIs.33 Bremer and colleagues34 investigated such a case in more detail and showed that the findings in this particular case could not be explained by amino acid changes in the HBsAg loops but by a low ratio of infectious HBV to subviral HBsAg particles (less than 1:10). This ratio is approximately 1:1000 during the acute phase of a typical HBV infection35 and greater than one in a million in HBeAg-negative HBsAg carrier donors with a low viral load, borderline detectable by the Ultrio Plus assay.36 It is reasonable to expect that the HBV transmission risk of such donations is comparable to that of ID-NAT–nonreactive OBI donations. In our study such ID-NAT–negative HBsAg carrier donors were mainly found in first-time donors and not in acutely infected repeat donors tested by Ultrio Plus. Therefore, if pathogen reduction was introduced for treatment of plasma and platelet products in Hong Kong, dropping of serologic screening of lapsed and repeat donations could be investigated in a new and more cost-effective blood safety scenario.

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CONFLICT OF INTEREST

WCT and CKL declare that they have no conflicts of interest relevant to the manuscript submitted to TRANSFUSION. NL is working as a private consultant for institutes in the blood transfusion and in vitro diagnostic industry, including Novartis Diagnostics, the company that distributes the Ultrio and Ultrio Plus assays, the performance of which were compared in the present study.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Table S1.** Donor demographic data of HBV NAT yield cases.