TABLE 2. Determination of sensitivity of two PCR systems by replicate testing and probit analysis

PCR	Ref* (geq/mL)	D 1	D 2	D 3	D 4	Total	95% LOD (geq/mL)	50% LOD (geq/mL)
Artus CMV	1	1/5	1/5	1/5	1/5	4/20	41.6	5.3
	5	5/5	3/5	2/5	5/5	15/20		
	10	5/5	5/5	4/5	4/5	18/20		
	50	5/5	5/5	5/5	5/5	20/20		
In house (UL83)	1	0/5	1/5	0/5	1/5	2/20	29.6	5.4
	5	5/5	3/5	5/5	4/5	17/20		
	10	5/5	5/5	4/5	5/5	19/20		
	50	5/5	5/5	5/5	5/5	20/20		

* NATtrol as CMV reference was diluted in 5% BSA.
artus = artus CMV TM PCR kits (Qiagen); D = day; in house (UL83) = in-house PCR targeting CMV UL83 sequence; LOD = limit of detection.

TABLE 3. CMV DNA–positive samples and PCR procedures

PCR results	Number of samples
UL83 positive and artus positive	29
UL83 repeatedly positive	2
artus repeatedly positive	2
UL83 positive and UL139 positive	3
artus positive and UL139 positive	1
Indeterminate*	4
Negative	2359
Total	2400

* Positive in only one PCR analysis.
artus = artus CMV TM PCR kits (Qiagen); UL139 = PCR targeting CMV UL139 sequence; UL83 = in-house PCR targeting CMV UL83 sequence.

the risk of TT-CMV caused by transfusion with window period–derived blood components because we did not focus on blood samples obtained at the time of acute primary infection when CMV replication is most likely to be active. In fact, Ziemann and colleagues[8] found that two (2.9%) samples were DNA positive among 68 plasma samples obtained from final seronegative donations during the course of seroconversion. Collectively, a risk of TT-CMV related to window period donation exists but the frequency seems very low.

The frequency of detecting CMV DNA was 4.3% among donors in their 60s, compared with 1.0% (0.8%-1.3%) in the population aged from 16 to 59 years. Considering that the specific IgG prevalence has already peaked by age 60 years in Japan, the notion that the DNA-positive individuals in their 60s were nonimmune to CMV and emitted CMV virions during the course of primary CMV infection is inconceivable. Latent CMV more likely became reactivated in those elderly individuals. The reactivation of CMV in elderly persons is thought to represent "immunosenescence" caused by chronic CMV infection.[21,22] The current concept of immunosenescence in relation to CMV infection is that terminally differentiated memory T cells accumulate with ageing in the limited peripheral "immunologic space," which causes a progressive decline in the generation of naive T cells that protect against new pathogens. In addition, a considerable portion of the accumulated memory T cells were specific for CMV.[23-25] Thus, CMV infection is considered a driving force or risk biomarker for the constitution of a skewed peripheral T-cell repertoire. Despite conflicting results and ideas about epidemiology and immunologic mechanisms, the clinical impact of the CMV infection on individuals who are not immunocompromised has remained a central question.[22] Whether or not all persons with CMV infection acquire skewed T-cell phenotypes with aging, the kinds of socioeconomic or physical factors that facilitate this process, and when this process starts to compromise the immune system should be addressed. We established statistical evidence of CMV reactivation occurring in the peripheral blood of voluntary blood donors in their 60s. Viral load did not significantly differ between donors in their 60s and those aged less than 60 years. Blood donors in Japan are supposedly healthy individuals who have all been qualified by questionnaires and consultation with physicians. Our results therefore suggest that CMV reactivation is a constitutional event in CMV carriers and starts to occur during the sixth decade of life, although the possibility remains that donors positive for CMV DNA recently might have had specific illnesses or behaviors that are related to CMV reactivation. The findings of animal experiments suggest that lytic viral reactivation is necessary to establish the peripheral T-cell repertoire skewed for CMV.[26] Stowe and colleagues[27] detected CMV in 57% of urine samples from elderly individuals (66 to 83 years) but in none of those from younger individuals (25 to 55 years). This would also suggest that CMV reactivation occurs more frequently among elderly, than younger, individuals, although they did not detect CMV in any blood samples from both groups. However, this might have resulted from the small sample size studied (11 elderly individuals compared with 400 aged ≥60 years in this study). The rather clear cutoff of the reactivation frequency between the 50s and 60s is reminiscent of a Swedish study[28] showing increased 10-year mortality

TABLE 4. Association between CMV serostatus and CMV DNA positivity

Serology status	Number of samples	DNA+ (n)	Ratio (%)	Viral load Mean/median Range (geq/PBL unit)
IgM−/IgG−	562	0	0	
IgM+/IgG−	7	0	0*	
IgM+/IgG+	91	6	6.6*	670/62† <40-3400
IgM−/IgG+	1740	31	1.8*	170/80† <40-920
Total	2400	37	1.5	

* DNA positivity ratio significantly higher in IgM+ than in IgM−/IgG+ samples (6 [6.1%] of 98 vs. 31 [1.8%] of 1740); chi-square test ($p < 0.03$).
† Viral load in IgM+/IgG+ samples significantly higher than that in IgM−/IgG+ samples; t test ($p < 0.03$).
DNA+ = DNA positive.

TABLE 5. Age distribution of CMV DNA positivity in cellular and plasma fractions*

Age (years)	Cellular fraction DNA+	Cellular fraction Ratio (%)	Plasma fraction, DNA+
16-19	4 (2)	1.0	1 (1)
20-29	5	1.3	1
30-39	5 (1)	1.3	0
40-49	3 (2)	0.8	0
50-59	3	0.8	1
16-59 total	20 (5)	1.0	3 (1)
60-69	17 (1)	4.3	2†
Total	37 (6)	1.5	5

* Numbers of donors specifically positive for both IgM and IgG are shown in parentheses.
† In addition to these two samples, two others from donors in their 60s were positive for one PCR analysis.
DNA+ = DNA positive.

rates among individuals with immune risk profiles at the age of 65 years but not at the age of 55 years.

We identified five (13.5%) samples that were positive for CMV DNA in the plasma fraction of 37 blood samples that contained CMV DNA in the cellular fraction. This result is comparable to the report by Ziemann and coworkers[8] in which 44% of blood samples from 82 recently seroconverted donors were CMV DNA positive in the plasma fraction. Drew and colleagues[6] also reported that three of 384 samples obtained from 192 seroconverted blood donors contained low plasma levels of CMV DNA. We quantified CMV DNA levels in three plasma products derived from donations that had CMV DNA in plasma samples. The DNA levels (1.9×10^2 to 9.7×10^3 geq/mL) were comparable to those measured in the plasma samples. Because all blood components including apheresis-derived plasma components are leukoreduced in Japan, this finding indicated that leukofiltration cannot reduce levels of free CMV DNA in the plasma fraction.

The identification of blood donations with plasma fractions containing CMV DNA raises concerns about the safety of blood components. The residual risk of TT-CMV under the current blood program that applies both seroscreening and universal leukoreduction could be focused on blood with plasma viremia that is provided during the window period because plasma viremia might not be appropriately managed by leukofiltration. This leads to the notion of Ziemann and colleagues[8] that leukoreduced components would be safer when obtained from seropositive donors at least 1 year after seroconversion. This is also based on the finding that plasma viremia has barely been detectable among donors who remain seropositive for more than 1 year. However, our study showed that a proportion of latently infected individuals presents with free CMV DNA in plasma fractions. Free CMV DNA in plasma could not be effectively diminished by prestorage leukoreduction, which was verified by assays of leukoreduced plasma products. Therefore, the strategy suggested by Ziemann and colleagues, while eliminating window period–related risk, might generate another risk associated with blood containing free plasma CMV DNA that is provided mainly by elderly donors. Although we identified only two samples from donors in their 60s that were plasma DNA positive, one of them related to a plasma product containing 1.6×10^3 geq/mL CMV DNA, a viral load that was comparable to that for window period donation provided by the teenager (9.7×10^3 geq/mL). Moreover, we found two other samples with possible plasma DNA among donors in their 60s, although they were DNA positive only for a single PCR analysis, suggesting low DNA concentrations. Whereas we found that whole blood CMV DNA positivity among donors in their 60s was 4.3%, that identified in an elderly US population with a mean age of 84.5 years was 42.3%.[29] Thus, since the frequency of whole blood CMV increases dramatically after 60 years, we can speculate that the frequency of plasma CMV also increases with age. In this context, serious problems could arise in countries that accept donors over 70 years of age if seropositive donations are accepted for transfusion into patients at risk. Although leukoreduced blood components have been advocated as an alternative when seronegative blood is not available, they might carry a higher risk of TT-CMV

than seronegative blood, which might partly explain higher TT-CMV frequency among patients transfused with leukoreduced blood compared with seronegative blood.[2,4]

Further study is required to compare possible TT-CMV risks between persistently seropositive blood that might contain free CMV virions due to reactivation and seronegative blood that might incidentally contain such virions due to window period donation. Ziemann and coworkers concluded from a recent extensive study of more than 22,000 samples that TT-CMV risk is essentially comparable between window period donations among seronegative donors and donations with reactivation among long-term seropositive donors.[30] Before assessing the TT-CMV risk in Japan, the degree to which window period donation constitutes the blood donor population will need to be determined. Other basic issues also need to be resolved. Because we used techniques involving DNA amplification but not viral culture for plasma study, whether CMV DNA identified in plasma with this technique constitutes replication-competent virions remains unknown.[31,32] Whether plasma products containing free CMV virions is infectious through blood transfusion also needs to be resolved.[13,33] Whether blood components containing CMV virions possibly derived from reactivation in latently infected blood donors are as infectious as those derived from donations provided during acute primary infection also requires investigation. The clinical relevance of CMV neutralizing antibody that can be found in latently infected individuals also must be considered. Finally, the minimal infectious dose of CMV virions acquired through blood transfusion should be determined for each type of blood component.

In conclusion, the seroprevalence of CMV among a Japanese population of blood donors was 76.6%. The prevalence among donors in their 20s and 30s has not changed over the past 15 years. We detected CMV DNA in 1.7% of 2400 samples. None of the 562 seronegative samples was DNA positive. We detected CMV DNA more frequently in blood from donors aged in their 60s than from younger donors. Among DNA-positive samples, 14% contained DNA in the plasma fraction, and this frequency might be higher among donors in their 60s than younger donors. CMV DNA persists in a portion of seropositive blood even after prestorage leukoreduction and leukoreduced blood without seroscreening might not be as safe as seronegative blood in terms of TT-CMV risk. The risk of TT-CMV in blood with detectable CMV DNA in the plasma fraction should be determined.

CONFLICT OF INTEREST

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Interstitial fluid shifts to plasma compartment during blood donation

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BACKGROUND: A vasovagal reaction (VVR) occurs in 0.8% to 0.9% of voluntary blood donors in Japan. However, they generally tolerate the acute loss of 400 mL of whole blood rather well, perhaps because several circulatory defense mechanisms compensate for the loss. This study aimed to determine the extent to which an interstitial fluid shift contributes to the development of a VVR.

STUDY DESIGN AND METHODS: Blood hemoglobin (Hb) was measured upon admission, at venipuncture, and immediately after collecting 400 mL of whole blood from 736 donors. Shifted fluid volume was calculated using a formula that included Hb levels and estimated total blood volume.

RESULTS: By the end of blood collection, 188 ± 80 and 211 ± 82 mL of fluid, which is equivalent to almost half of the total amount of withdrawn blood, had entered the intravascular space in male and female donors, respectively. The difference between the sexes was significant despite the lower body weight and circulating blood volume of the female donors. Body weight increased, whereas age decreased the volume of shifted fluid in female donors.

CONCLUSION: Blood loss after donation is quickly compensated by an interstitial fluid shift into the intravascular space and may not be the only direct cause of VVR in the setting of a whole blood donation of 400 mL.

Adverse reactions that occur among blood donors during or shortly after blood donation seriously impair the motivation of repeat donors. A vasovagal reaction (VVR) indicated by light-headedness, dizziness, and weakness is the most frequent reaction, occurring in 0.8% to 0.9% of blood donors in Japan (Japanese Red Cross Blood Centers data). Although a VVR in most individuals has a relatively moderate clinical course and spontaneously resolves, a small proportion (0.015%) of donors might faint, fall, and possibly sustain serious injuries after a donation. From this perspective, investigation into the mechanisms of VVR and the development of preventive or relief measures has been crucial for blood collection institutions.[1-4]

During the presymptomatic phase of VVR, donors have a hyperdynamic circulatory status with moderately elevated blood pressure and increased cardiac output. The symptoms of VVR coincide with a sudden decrease in arterial blood pressure and heart rate.[4-6] Although a reflex activation and/or depression of the autonomous nervous system has been implicated in the pathophysiology of the abrupt transition from hyper- to hypodynamic status,[7,8] the time course of symptoms can be explained from the viewpoint of venous return. Excessive blood pooling in capacitance vessels results in decreased venous return, followed by insufficient left ventricle filling and hence decreased cardiac output. Among the primary factors that cause blood pooling is fear of needles or pain accompanying venipuncture, which often results in the withdrawal of the sympathetic tone of blood vessels. Standing shortly

ABBREVIATION: VVR = vasovagal reaction.

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after blood donation might also contribute to blood pooling.[9-12] In the setting of blood donation in Japan, 400 mL of whole blood takes less than 10 minutes to withdraw, and this corresponds to a moderate grade of hemorrhage. Thus, routine blood donation presents a possibly problematic series of events that could facilitate insufficient venous return to the ventricles of a proportion of blood donors. A recent study has elucidated the extent to which primary vasodilation, blood volume loss, and standing posture contributes to the development of syncope.[4]

Several physiologic mechanisms could compensate for acute blood loss.[13-15] The mobilization of interstitial body fluid from organs that have a large tissue mass and fluid reservoir, such as skeletal muscle and skin, contributes to the restoration of plasma volume.[16,17] By measuring hemoglobin (Hb) concentrations before and after collecting blood from 736 blood donors, we calculated plasma volume recovery after a short period of blood collection and investigated the role of the refilling mechanism in the restoration of blood volume.

MATERIALS AND METHODS

Blood donor criteria

Individuals ranging in age from 16 to 69 years are eligible to donate blood in Japan, where 200 and 400 mL of whole blood may be collected. Males aged 17 years and all individuals aged at least 18 years are permitted to donate 400 mL of whole blood. The standard Hb concentrations in donations of 400 and 200 mL from males and females are at least 13.0 and at least 12.5 and at least 12.5 and at least 12.0 g/dL, respectively. Body weight should be at least 50 kg for 400-mL donations and at least 45 and at least 40 kg for 200-mL donations from males and females, respectively. The total volumes of whole blood withdrawn at one donation, including that for initial flow diversion, are 425 and 225 mL for 400- and 200-mL donations, respectively. Males and females may donate an annual maximum volume of 1200 and 800 mL, respectively. Only donors with systolic blood pressure of at least 90 mmHg are eligible for blood donation.

Blood donors and blood donation in this study

We analyzed data obtained only from those who donated 400 mL of whole blood between July 2008 and May 2009. All such donors who presented at the Tachikawa (a suburb of Tokyo) blood donation facility in the morning between Monday and Friday were included in this study. Changes in peripheral blood volume during the collection of 400 mL of whole blood were estimated on the basis of the Hb concentration measured at three time points during the donation process.

After an interview with a physician was completed, venous blood samples were collected from donors to determine Hb and count blood cells. Donors judged eligible for donation according to the Hb standard described above were encouraged to consume as much liquid as possible and instructed to urinate before donation. A 17-gauge intravenous catheter was placed in an antecubital vein of recumbent donors to collect whole blood. A small portion of blood for Hb measurements and blood testing and a repository sample were obtained from a diversion bag. The Hb values obtained in this manner were regarded as those at the start of blood collection. Blood collected over a period of more than 10 minutes was regarded as invalid. After the catheter was removed at the completion of blood collection, Hb was measured in 2 to 3 mL of blood that remained in the tubing of the collection bag system. This volume is usually around 7 mL and does not contain any of the anticoagulant included in the collection bag. This sample was regarded as blood in the peripheral vein at the endpoint of blood collection. Those who completed donations remained recumbent for at least 5 minutes thereafter. After it was confirmed that their blood pressure had returned to a safe range, the donors rested in another room for at least 10 minutes and consumed a beverage.

Estimation of blood volume and statistics

The baseline blood volume at admission (V_1) of each donor was calculated using Ogawa's equation[18] as $0.168H^3 + 0.050W + 0.444$ for adult males and $0.250H^3 + 0.0625W - 0.66$ for adult females, where H and W indicate donor height (m) and weight (kg), respectively. Blood volume is routinely calculated in Japan using this equation. V_1, V_2, and V_3 and Hb_1, Hb_2, and Hb_3 in the following equations represent blood volume (V) and Hb concentrations upon admission and at the start and end of blood collection, respectively. Assuming that the absolute amount of Hb remains constant and is estimated as $V \times Hb$, the relationships among them are expressed as:

$$V_1 \times Hb_1 = V_2 \times Hb_2,$$

$$V_3 \times Hb_3 = V_2 \times Hb_2 - 0.425 \times (Hb_2 + Hb_3)/2,$$

and

$$\text{shifted volume} = V_3 - (V_2 - 0.425),$$

where $0.425 \times (Hb_2 + Hb_3)/2$ represents the amount of Hb present in the donated blood. The Hb concentration was measured using an automated cell counter (Sysmex 3000, Sysmex, Tokyo, Japan) immediately after sampling at the donation site. Hb was measured daily in three samples per donor using a single-cell counter that was validated every day.

Data were statistically analyzed using software (SSRI, Excel Statistics, Version 8, Social Survey Research Informa-

TABLE 1. Profiles of blood donors enrolled in this study and volume of fluid that shifted from interstitium to plasma compartment during blood collection

Characteristic	Mean	SD	Min	Max
Males (n = 447)				
Age (years)	42.0	13.2	18	69
Height (cm)	171	6	154	189
Body weight (kg)	68.7	9.8	47.0	130.0
Blood volume (mL)	4721	534	3590	7923
Body mass index	23.5	2.9	15.9	40.1
Hb_1*	15.0	1.0	13.0	18.3
Hb_2	14.9	1.0	12.7	18.5
Hb_3	14.3	1.0	11.9	17.7
Shifted volume (mL)	188	80	25	601
V_2 (blood volume at start of blood collection): 4751 ± 547 mL				
V_3 (blood volume at the end of blood collection): 4514 ± 566 mL				
$p < 0.03$ between Hb_1 and Hb_2 and between Hb_2 and Hb_3				
Females (n = 289)				
Age (years)	39.6	14.0	18	69
Height (cm)	159	5	143	173
Body weight (kg)	57.9	6.6	47.0	82.0
Blood volume (mL)	4003	449	3062	5566
Body mass index	22.9	2.7	17.7	32.8
Hb_1	13.5	0.7	12.5	16.1
Hb_2	13.4	0.7	12.0	16.1
Hb_3	12.7	0.7	11.3	15.0
Shifted volume (mL)	211	82	31	540
V_2: 4031 ± 456; V_3: 3817 ± 478; $p < 0.03$ between Hb_1 and Hb_2 and between Hb_2 and Hb_3.				

* Hb_1, Hb_2, and Hb_3: Hb concentrations upon admission and at start and end of blood collection, respectively.

tion Co. Ltd, Tokyo, Japan) for Windows (Microsoft Excel 2007, Tokyo, Japan). Statistical analysis was performed using one-sided t tests with paired or unpaired samples and chi-square test. Factors associated with shifted volumes were determined by multiple regression analysis.

RESULTS

We analyzed data derived from samples of whole blood (400 mL) donated by 736 individuals (male, n = 447; female, n = 289) during the study period. Among the male and female donors 4.7% (21/447) and 12.5% (36/289), respectively, were donating for the first time. Four male and five female donors had developed a VVR during previous blood donations. The mean interval between admission and venipuncture for blood collection averaged 19 minutes, while 93 and 90% of male and female donors, respectively, consumed 200 to 400 mL of water. We first analyzed the difference in blood volume estimated upon admission (V_1) and at the start of blood withdrawal (V_2) to determine the effect of the consumed water on the circulating blood volume. Although the Hb concentration significantly decreased from the time of admission (Hb_1) to the start of collection (Hb_2) (Table 1), the mean (±SD) increase in blood volume ($V_2 - V_1$) was only 30 ± 87 mL for males and 28 ± 88 mL for females.

The Hb concentration significantly decreased between the start and the end of collection in both sexes ($p < 0.03$, Table 1). The volumes of fluid that shifted from the interstitial space to the plasma compartment during blood collection were 188 ± 80 (mean ± SD) and 211 ± 82 mL for males and females, respectively (Table 1). The difference in the shifted volume between the two groups was significant ($p < 0.01$) and remarkable considering that the women had a lower mean body weight and lower mean circulating blood volume than men (57.9 kg vs. 68.7 kg and 4003 mL vs. 4721 mL, respectively; Table 1). Figure 1 shows a greater shifted volume in females than in males within the same range of body weight ($p < 0.01$ for all body weight groups) and that the volume increased more sharply in females than in males accompanied by increasing body weight. The difference in the shifted volume between male and female donors tended to be greater in younger donors, significant between the ages of 30 and 49 years ($p < 0.03$), and minimal at ages between 50 and 69 years (Fig. 2). The shifted volume tended to decrease with increasing age for both sexes, and the difference was significant between females in the age ranges of 30-49 (217 mL) and 50-69 (186 mL, $p < 0.01$) years. No other donor age categories were associated with a significant decrease in the shifted volume. Body mass index was not associated with the volume of shifted fluid (data not shown). Multiple regression analysis using donor age, height, and body weight as predictor variables revealed only body weight in males as an independent predictor ($p < 0.01$) of an increase in shifted volume, whereas donor age and body weight in females were independent factors for a decrease and an increase ($p < 0.01$), respectively, in shifted volume. The mean amounts of time required to collect 400 mL of blood were 8.0 and 8.8 minutes and the mean rates of fluid shift during blood collection were 0.38 ± 0.19 and 0.45 ± 0.22 mL/min/kg (mean ± SD, $p < 0.01$) for males and females, respectively. Figure 3 shows the distribution of the shifted volume by time and weight. The distribution curve for females shifted to the right of that for males (that is, greater skewness for volume).

We also investigated whether any specific features caused a lower shifted volume by assigning the donors to categories based on a shifted volume of either more or less than 150 mL (Table 2). Greater height, body weight, and blood volume, but not donor age in males, and a greater body weight, blood volume, and younger age in females were associated with a greater shift in volume (univariate

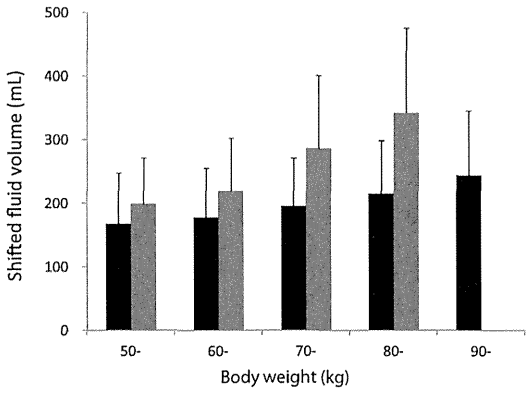

Fig. 1. Volume of shifted fluid relative to body weight of donors. (■, ▩) Mean volumes of shifted fluid in male and female donors, respectively.

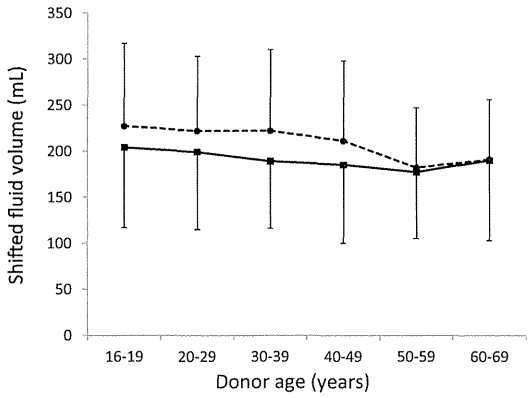

Fig. 2. Volume of shifted fluid relative to donor age. (—▪—, ····) Mean volumes of shifted fluid in male and female donors, respectively.

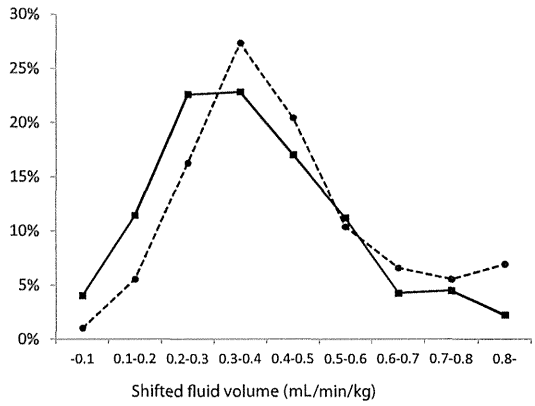

Fig. 3. Distribution of shifted fluid volume by time and weight. (—▪—, ····) Ratios of male and female donors, respectively, with indicated shifted volumes.

TABLE 2. Profiles of donors with shifted fluid volumes of less than or at least 150 mL*

Characteristic	Shifted volume (mL) <150	Shifted volume (mL) ≥150	Difference
Males (n)	155	292	
Age (years)	42.8 (±13.6)	41.6 (±13.0)	>0.05
Height (cm)	169.7 (±5.7)	171.4 (±5.8)	<0.01
Body weight (kg)	66.4 (±9.0)	70.0 (±10.1)	<0.01
Blood volume (mL)	4598 (±504)	4787 (±539)	<0.01
Females (n)	68	221	
Age (years)	42.5 (±14.0)	38.8 (±14.0)	<0.05
Height (cm)	159.0 (±4.9)	159.3 (±4.9)	>0.05
Body weight (kg)	56.6 (±5.5)	58.4 (±6.8)	<0.05
Blood volume (mL)	3903 (±374)	4034 (±465)	<0.05

* Data are reported as mean (±SD).

analysis, Table 2). The proportions of male and female donors with a shifted volume of less than 100 mL was 11.0% (49/447) and 5.5% (16/289), respectively (significant difference, $p < 0.01$). The shifted fluid volume in four donors who developed relatively mild VVR symptoms shortly after donation during this study (Table 3) was relatively low at 99, 158, 163, and 197 mL.

DISCUSSION

The acute loss of 400 mL of whole blood in the blood donation setting is generally tolerated well by voluntary donors. In fact, a blood donation resulting in organ failure or systemic inflammation in Japan has never been reported. This may be because either the depletion of such moderate volume has no noticeable effect on systemic blood circulation and/or because several circulatory defense mechanisms collaborate to restore physiologic blood distribution. Nevertheless, VVR indicated by temporary hypotension accompanied by decreased cardiac output does arise in a proportion of healthy blood donors.

Although the precise physiologic mechanism of VVR observed during the blood donation setting has not been fully elucidated, it might be initiated by the primary withdrawal of sympathetic vasoconstrictor tone in blood vessels. Although arterial blood pressure can be momentarily maintained due to the autonomous reflex mechanism, impending hypotension finally becomes uncompensated and symptomatic. The decrease in blood pressure could be a result of the combined effects of a vasodepressive reflex with or without a cardioinhibitory reflex and a failure to increase venous return. Impaired venous return would be caused by the dilation of capacitance vessels, an orthostatic effect after donation,[4,9] and decreased circulating blood volume as a result of blood withdrawal. Whole blood is collected in quantities of 200

TABLE 3. Demographics of four blood donors with VVR

Characteristic	Donor 1	Donor 2	Donor 3	Donor 4
Sex	Male	Male	Female	Female
Age (years)	19	51	18	23
FTD/RD	FTD	RD	RD	RD
VVR history	No	Yes	No	Yes
Height (cm)	160	168	166	166
Body weight (kg)	62	73	57	58
Blood volume (mL)	4232	4890	4072	4135
Water intake (mL)	400	400	200	100
Shifted volume (mL)	197	158	99	163
Symptoms	Nausea, paleness	Nausea, paleness	Nausea, paleness, yawning	Nausea
VVR onset				
Blood pressure	91/58	98/62	94/62	97/58
Pulse rate	61	55	68	77
Treatment	Recumbent rest	Recumbent rest	LRI (500 mL)	Recumbent rest
Recovery time (min)	15	>30	30	20

FTD = first-time donor; LRI = Lactate Ringer infusion; RD = repeat donor.

and 400 mL in Japan, and the frequency of VVR among female donors is higher after collecting 400 mL (1.2% vs. 0.74%; 2010 data from Japanese Red Cross Blood Centers). Thus, the decrease in the circulating blood volume generated by removing a larger volume of blood might contribute to the occurrence of VVR during donation. We therefore investigated the effect of moderate hypovolemia (400 mL of collected blood) on circulating blood volume during blood collection to help identify factors involved in VVR to develop strategies to decrease the occurrence and severity of VVR.

The physiologic mechanisms that control circulating blood volume during hypovolemia caused by acute blood loss are complex, but a rapid, compensatory shift of interstitial fluid from the skeletal muscle and skin into the circulation would increase plasma volume.[16,17] The exchange of fluid at the capillary–interstitium interface is largely determined by alterations in transcapillary Starling forces. The roles played by sympathetic activity and fluid shifts in the maintenance of arterial pressure are of equal importance during hemorrhage of 10% to 40% of total blood volume.[16]

Fluid shifts during blood donation have been described,[19,20] but only in relatively small cohorts. Considering the high variability of the shifted volume, we derived data from 736 donations. Moreover, the previous studies used the Hb concentration upon admission as the Hb value of the collected blood,[19,20] which contradicts the hypothesis that peripheral blood is diluted during donation, because the peripheral blood Hb concentration near the end of collection must be decreased according to the hypothesis. However, as we had no access to the actual Hb concentration in the collected blood, we used the median of concentrations at the beginning and end of blood collection, which should have been closer to reality. To avoid variability, we focused this study within a relatively short period of 11 months, when procedures among phlebotomists were uniform, and Hb was measured after blood sampling using the same instrument at the donation site. The reliability of the Hb measurement is suggested by the minimal difference in the Hb concentration (0.1 g/dL) between that at admission and that at the start of blood withdrawal compared with the large difference (0.6-0.7 g/dL) between that at the start and that at the end of blood withdrawal, although more time was required before starting to collect blood than to actually complete the process of collection.

The mean volume of fluid that shifted from the interstitium to the intravascular compartment during blood donation was 202 mL (adjusted for sex). Only about 200 mL of circulating blood volume was lost at the completion of blood collection, which is similar to described findings.[19,20] Given this finding, blood loss might not be a significant factor in the development of VVR in the setting of a whole blood donation of 400 mL. Moreover, the fluid shift would likely continue after completing the donation. If it continues at the same speed as that during blood collection, circulating blood volume would be restored in most donors within 10 minutes of catheter removal.

We propose that 400-mL donations may not be a direct causative factor, but rather only one of several factors that collectively contribute to low venous return. Donations of 400 mL might comprise a risk factor only when the release of capacitance vessel tone is higher, a change in posture causes a greater venous blood shift to lower extremities, the donor is extremely dehydrated, or the donor has a lower circulating blood volume. The difference in VVR frequency between 400 and 200 mL donations is higher indeed among donor populations with a circulating blood volume of less than 4000 mL. Neurally mediated hypotension might also be triggered more by a donation of 400 mL than of 200 mL. The 11.0% of males and 5.5% of females with a shifted volume of less than

100 mL might represent a subpopulation that is more vulnerable to VVR. This consideration would be applicable to donations of 400 mL, but not 200 mL, because the shifted and collected volumes are too small to elicit VVR.

We found that more interstitial fluid shifted to the intravascular compartment in females than in males. This was evident in differences between shifted volumes in male and female donors within the same body weight ranges (Fig. 1). The reason for this difference is not clear but it might relate to the fact that females have a larger interstitial space. More time was needed to collect blood from females than males (8.8 min vs. 8.0 min), which might have produced a larger shifted volume for females during blood collection. However, the rate of fluid shift is still significantly greater for females than for males when body weight is considered (0.45 mL/min/kg vs. 0.38 mL/min/kg), indicating a greater capacity of blood volume compensation in females than males. The shifted fluid volume in females tended to decrease with increasing age and reach the same level as that in males aged 50 to 69 years. Age-related changes such as increased blood vessel rigidity, decreased interstitial fluid volume, changes in endothelial permeability, or macromolecular composition of interstitial fluid compartments[21-23] might relate to the decreased fluid shift. Regardless of the mechanism, older female blood donors might be more prone to VVR than younger females.

This study underestimated the direct role of blood volume loss in the development of VVR,[20,24] suggesting that strategies for increasing circulating blood volume around the time of donation are ineffective. Several articles, however, have described that consuming water preferentially affects the occurrence of VVR.[25,26] The donors enrolled in this study consumed a mean of 215 mL of water during a mean 19-minute wait before venipuncture. The Hb concentration decreased from 14.4 to 14.3 g/dL during that period, implying that a mean of 29 mL of interstitial fluid shifted to the intravascular space. Consuming about 200 mL of water might be insufficient to elicit an effect, or it might not rapidly influence plasma volume. If an increase in plasma volume is predicted at the time of blood donation, then before donation, individuals should consume a large volume of a beverage with a high sodium concentration and a small amount of sugar, such as the oral rehydration solutions recommended for children with enteritis.[27,28] However, the increase in blood pressure that occurred soon after consuming water might not be a direct effect of an increase in plasma volume, but rather an outcome of stomach wall distension that stimulates the sympathetic tone of blood vessels.[29]

In summary, our findings revealed a rapid shift of interstitial fluid to the intravascular space during blood collection, thus restoring about half of the withdrawn volume by the end of blood collection. The shifted volume is lower in males than in females and tends to decrease with increasing age in females. The direct contribution of blood loss during a blood donation to the development of VVR might be lower in the setting of whole blood donations of 400 mL.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

This study received no support in the form of grants, equipment, or drugs. The authors have no conflict of interest regarding this article.

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Residual risk of transfusion-transmitted hepatitis B virus (HBV) infection caused by blood components derived from donors with occult HBV infection in Japan

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BACKGROUND: Nucleic acid amplification testing (NAT) for hepatitis B virus (HBV) during blood screening has helped to prevent transfusion-transmitted HBV infection (TT-HBV) in Japan. Nevertheless, 4 to 13 TT-HBV infections arise annually.

STUDY DESIGN AND METHODS: The Japanese Red Cross (JRC) analyzed repository samples of donated blood for TT-HBV that was suspected through hemovigilance. Blood donations implicated in TT-HBV infections were categorized as either window period (WP) or occult HBV infection (OBI) related. In addition, we analyzed blood from 4742 donors with low antibody to hepatitis B core antigen (anti-HBc) and antibody to hepatitis B surface antigen (anti-HBs) titers using individual-donation NAT (ID-NAT) to investigate the relationship between anti-HBc titer and proportion of viremic donors.

RESULTS: Introduction of a more sensitive NAT method for screening minipools of 20 donations increased the OBI detection rate from 3.9 to 15.2 per million, while also the confirmed OBI transmission rate increased from 0.67 to 1.49 per million. By contrast the WP transmission rate decreased from 0.92 to 0.46 per million. Testing repository samples of donations missed by minipools of 20 donations NAT showed that 75 and 85% of TT-HBV that arose from WP and OBI donations, respectively, would have been interdicted by ID-NAT. The ID-NAT trial revealed that 1.94% of donations with low anti-HBc and anti-HBs titers were viremic and that anti-HBc titers and the frequency of viremia did not correlate.

CONCLUSIONS: The JRC has elected to achieve maximal safety by discarding all units with low anti-HBc and anti-HBs titers that account for 1.3% of the total donations.

The prevalence of hepatitis B virus (HBV) surface antigen (HBsAg) in Japan is slightly higher than the average for developed countries. A recent screening of blood donors, local residents, and school pupils found an estimated national prevalence of HBsAg of 0.71%.[1] However, the prevalence was higher during the 1990s, being 1.5% among first-time blood donors aged in their 40s.[2] Taking into account horizontal transmission and a birth cohort effect, a relatively large cohort with historical HBV infection might persist among older individuals in Japan.

To prevent transfusion-transmitted HBV (TT-HBV) infection, Japanese Red Cross (JRC) blood centers introduced HBsAg screening for all blood donations in 1972. In 1989, antibody to hepatitis B core antigen (anti-HBc) testing was introduced to exclude donations by people with prior HBV infection. Because total elimination of anti-HBc–reactive donations might have seriously reduced the blood supply, donations with high antibody to hepatitis B surface antigen (anti-HBs) titers and those

ABBREVIATIONS: CLEIA(s) = chemiluminescence enzyme immunoassay(s); ID = individual donation; JRC = Japanese Red Cross; LOD = limit of detection; OBI = occult hepatitis B virus infection; PC(s) = platelet concentrate(s); S/CO = signal-to-cutoff ratio; TT-HBV = transfusion-transmitted hepatitis B virus infection; TTI = transfusion-transmitted infection; WP = window period.

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with low anti-HBc titers have been accepted, and only donations with low anti-HBs and high anti-HBc titers were excluded.

In addition to this serologic screening algorithm, the JRC implemented multiplex nucleic acid amplification testing (NAT) for HBV, hepatitis C virus (HCV), and human immunodeficiency virus Type 1 (HIV-1) in 1999.[3] Although NAT has greatly reinforced blood safety regarding TT-HBV infection, 4 to 13 TT-HBV infections continue to arise annually. While some occur as a result of transfusion with blood components obtained during the window period (WP), others arise due to components being derived from donors with occult HBV infection (OBI) defined as detectable HBV DNA in peripheral blood but no detectable HBsAg.[4,5] Although donations from donors with OBI have helped to maintain an adequate blood supply, such donations have also raised a concern about the risk of TT-HBV. Here, we describe the current status of TT-HBV under the NAT screening system as well as problems inherent in the current HBV screening algorithm, especially with regard to OBI-derived blood donations. We also discuss the feasibility of strategies that could increase HBV safety in countries such as Japan with a slightly elevated prevalence of HBV.

MATERIALS AND METHODS

Screening donated blood at JRC blood centers

The JRC blood centers are the only facilities authorized to handle blood collection, processing, testing, and delivery in Japan. Donated blood is screened at these centers for HBsAg, anti-HCV, anti-HIV-1 and -2, anti-human T-lymphotropic virus type 1, anti-*Treponema pallidum*, and human parvovirus B19 antigen. Whereas HBsAg-positive blood is rejected, HBsAg-negative samples are further tested for anti-HBc and anti-HBs (Table 1). Blood with a high anti-HBs titer (≥200 IU/L) is accepted irrespective of the anti-HBc titer and that with a low anti-HBc titer is also accepted irrespective of the anti-HBs titer. Blood with high anti-HBc and low anti-HBs titers (<200 IU/L) is disqualified. All blood had been serologically tested before 2008 using the agglutination method with the initial cutoff for a high anti-HBc titer being a dilution factor of 2^6, which was later revised to 2^5. All agglutination tests were replaced with chemiluminescence enzyme immunoassays (CLEIAs, CL4800 testing system, Fujirebio, Tokyo, Japan) in 2008 and the threshold for anti-HBc positivity is currently a signal-to-cutoff ratio (S/CO) of 12.0. This value was validated as being essentially equivalent to an agglutination titer of 2^5. Blood donations with elevated serum alanine aminotransferase (>60 IU/mL) are also rejected.

TABLE 1. HBV screening algorithm applied at JRC blood centers*

	Anti-HBc titer	
Anti-HBc reactive 4.9% 261,000, *49,000*	Low <2^6(2^5) or S/CO≥1.0 but <12.0	High ≥2^6(2^5) or S/CO ≥12.0
Anti-HBs ≥ 200 IU/L	Accepted 2.04% 108,000, *20,000*	Accepted 1.38% 73,000, *14,000*
Anti-HBs < 200 IU/L	Accepted 1.31% 69,000, *13,000*	Rejected 0.19% 10,000, *2,000*

* HBsAg-negative donations are tested for both anti-HBc and anti-HBs. Dilution factors for anti-HBc titers were applied for agglutination testing. Dilution factors in parentheses were applied between 1997 and 2007. The S/CO ranges are currently used for CLEIA. Ratios (%) of donations for each category are shown (2010 data). Observed number and number per million (italics) of donations are also included.

NAT

Samples that were qualified by the testing algorithm for anti-HBc and anti-HBs described above as well as by HBsAg testing are then screened using NAT. The JRC started NAT in 1999 using a real-time multiplex polymerase chain reaction system with a minipool format that originally comprised 500 samples (Ampli-NAT MPX system, Roche, Indianapolis, IN).[6] The pool size was decreased to 50 in 2000 and to 20 in 2004. The JRC implemented the Roche TaqScreen MPX system for NAT in 2008 with a pool size of 20, but with an approximately threefold increase in sensitivity because of the increased sample volume required for nucleic acid extraction and improvements in reagents. The screening sensitivity of HBV is 650, 260, and 76 copies/mL (50% limit of detection [LOD]; JRC data) for 50- (50p) and 20- (20p) sample pools using AmpliNAT and 20p using TaqScreen, respectively.

A trial screening using individual-donation NAT (ID-NAT) proceeded at the Tokyo Blood Center between December 2010 and May 2011 to verify the distribution of the rate of donations containing HBV DNA relative to anti-HBc titers and the residual TT-HBV risk that could arise from transfusion with blood donations that have low anti-HBc and anti-HBs titers. All available donations with both an anti-HBc titer between 1.0 and 12.0 S/CO and an anti-HBs titer of less than 200 IU/L were screened by ID-NAT using the Roche TaqScreen MPX system with a 50% LOD of 3.8 copies/mL (JRC data). The sensitivity of ID-NAT used in lookback studies (described below) was 13 copies/mL (50% LOD) using AmpliNAT until July 2008 and 3.8 copies/mL (50% LOD) using TaqScreen from August 2008.

Hemovigilance system

The JRC established a hemovigilance system in 1993 and has since collected reports on adverse effects caused by blood transfusion. Through blood screening the JRC obtains information about repeat donors who have

recently acquired infection.[7] Their previous donations are evaluated for transfusion-transmitted infection (TTI) risk by considering donation timing and performing ID-NAT on repository samples (lookback studies). If they are judged as harboring a TTI risk, the JRC notifies the relevant facilities that used the component at risk and requests that physicians investigate whether any patient who received a transfusion of the component has acquired the corresponding infection.

The JRC also obtains information about TTI in transfused patients through voluntary reports by physicians who are involved in blood transfusion at medical facilities.[7] Upon receiving such information, the JRC analyzes repository blood samples obtained from implicated donations using ID-NAT. The TTI risk of cocomponents derived from the implicated and previous donations provided by implicated donors is assessed. The JRC notifies the relevant medical facilities of the findings. Implicated blood components are interdicted if they have not yet been used for transfusion.

The JRC headquarters and central laboratory determine the causal relationship between the implicated donation and posttransfusion infection considering patient clinical course, results of virologic analysis including ID-NAT and sequence analysis, serologic viral markers, and donation timing. Even if all repository samples implicated for TTI are verified as being ID-NAT-negative, implicated donors are followed up for repeat donation thereafter for sero- or NAT conversion, because the possibility that the index donation was provided during the ID-NAT WP persists. All processes for lookback studies are defined in national guidelines[8] that describe in detail the test items and timing of testing for donated blood and at-risk patients in addition to the roles of the relevant physicians, blood centers, and blood authority.

Sequence analysis

The HBV genome sequence identity is assessed between implicated repository blood samples and patient samples by sequencing 1550 bp of the alpha region within the HBV pre-S and S regions using a genetic analyzer (ABI 3130XL, Life Technologies Japan, Tokyo, Japan). When the viral load is too low to sequence, viral nucleic acid is further extracted from larger plasma volumes if the accompanying plasma bag is available. When findings are ambiguous, HBV obtained from donor or patient samples is cloned, amplified, and sequenced.

Estimation of current risk of TT-HBV

Although universal pre- and posttransfusion testing of patient samples for TTI has been recommended, the likelihood that all transfused patients undergo this evaluation is low. Moreover, the JRC hemovigilance system described above is voluntary. Therefore, TTI might be underreported to JRC blood centers. The exact amount of TT-HBV infections that could occur under the current screening system must be defined to assess novel TT-HBV–mitigating strategies. This study therefore reevaluated the current risk of TT-HBV infection based on data obtained under current system.

The projected number of ID-NAT–positive donations derived from OBI donors was calculated using the ID-NAT positivity rate obtained in the ID-NAT trial screening described above and the number of donations with low anti-HBc and anti-HBs titers. The additional WP yield in donations determined by ID-NAT was calculated based on rates of detection of recently infected donors.[9,10] Assuming that the frequency of donation is constant at any time during the presymptomatic phase of acute infection, the yields by tests for an infection marker are in direct proportion to the length of time during which each test gives a yield. The potential ID-NAT yield (screening NAT negative) was calculated herein by multiplying the screening NAT yield by the ratio of the interval between ID-NAT detection and 20p-NAT detection (11.2 days) to that between 20p-NAT detection and HBsAg detection (9.7 days). The interval covered by each NAT strategy (11.2 and 9.7 days) was calculated using the value for the detection limit of each test (3.8, 76, and 1000 copies/mL for ID-NAT, 20p-NAT, and CLEIA detection, respectively) and the doubling time of HBV in human peripheral blood (2.6 days).[9,11] The number of donations that could appear in the ID-NAT–negative WP was similarly calculated separately for each component type taking into account both the interval between 1 copy/bag and ID-NAT detection deduced from the mean plasma volume of each component type and the number of each component issued to hospitals.

We estimated the number of TT-HBV infections with reference to our previous systematic lookback study.[7] The infectivity of ID-NAT–positive and screening NAT-negative components was calculated in that study as being 3% (95% confidence interval [CI], 0%-17.2%, n = 33) and 50% (95% CI, 28.2%-71.8%, n = 22) for OBI- and WP-derived components, respectively. The incidence rate for TT-HBV infections was thus obtained by multiplying the number of estimated at-risk donations deduced using the above method by the infection rates (0.03 or 0.5).

Statistical analysis

Data were statistically analyzed using computer software (SSRI for Windows, Excel Statistics Version 8, Social Survey Research Information Co. Ltd, Tokyo, Japan). Significance was determined using the chi-square test except for associations between total viral load in the components and alanine aminotransferase (ALT) levels in patients that were evaluated using the Mann-Whitney U test.

RESULTS

Reports of possible TT-HBV infections

The JRC blood centers received 789 reports of possible TT-HBV infections between 2001 and 2010 (Fig. 1). The number of such reports obviously increased in 2004 and 2005 because a nationwide systematic retrospective study started in 2004 that also identified patients with TT-HBV infection that would have previously been unrecognized. Causality was investigated in all but two of these possible TT-HBV infections. The possibility of TT-HBV infection was precluded in 97 (12.3%) of the 789 reported patients without testing repository samples based on evaluation of the patient's clinical course and the transfusion setting for each. For all of the remaining patients, repository samples were tested serologically and by ID-NAT to detect the HBV genome. Of the 789 initial reports, 98 (12.4%) were determined to be TT-HBV infections after the introduction of 50p-NAT. The HBV sequence identity was established between donor and recipient in 88 of these cases, and TT-HBV was determined considering other HBV markers and the clinical setting in the remaining 10. An HBV genome was not detected in repository samples for 587 (74.4%) potential TT-HBV infections. Although HBV was detected in four repository samples, HBV sequence identity was not confirmed between donors and recipients. Forty-two (43%) of the established TT-HBV infections were discovered through lookback studies that were started based on risk information provided by JRC blood centers. The remaining 56 (57%) were initially recognized by physicians at medical facilities. The number of established TT-HBV infections ranged from 4 to 13 per year between 2006 and 2010.

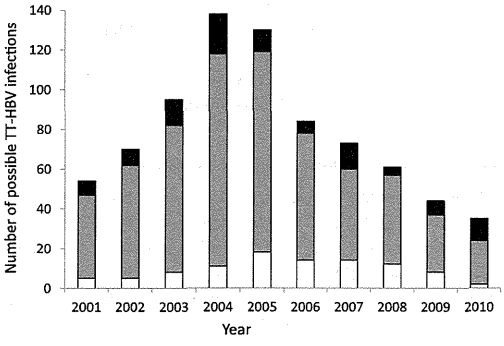

Fig. 1. Annual number of potential TT-HBV infections. (□) Patients in which possibility of TT-HBV was excluded (n = 97, 12.3%) without testing repository samples. (■, ▨) Patients in whom HBV DNA was identified or not, respectively, in the repository samples corresponding to a donation from the donor of the implicated blood components. Four patients are not included as HBV DNA sequence identity was not established.

Infection status of donors implicated in TT-HBV infection

The sensitivity of NAT screening improved through the three phases described above (50p-AmpliNAT, 20p-AmpliNAT, and 20p-TaqScreen). With the increased sensitivity of 20p-TaqScreen, the NAT yield of OBI donations increased from 3.9/million to 15.2/million, whereas the yield of WP donation decreased from 13.2/million to 5.7/million (Table 2). This was caused by the simultaneous introduction of CLEIA in 2008 for serologic screening including HBsAg detection, which effectively shortened the period that could be covered by 20p-NAT.

The established TT-HBV infections that occurred during each period were categorized based on the presence or absence of the HBV genome in the implicated component (that is, ID-NAT positive or negative) and the infection status of the donation (WP related [anti-HBc nonreactive] or OBI related [anti-HBc reactive]). Table 2 also shows the numbers of established TT-HBV infections associated with each group during each period. Figure 2 shows the incidence (per million donations) of estab-

TABLE 2. NAT yield and number of TT-HBV infections relative to three phases of screening NAT*

Screening system	50p-AmpliNAT	20p-AmpliNAT	20p-TaqScreen
Duration of screening period	Feb. 2000–Jul. 2004 (4.5 year)	Aug. 2004–Jul. 2008 (4.0 year)	Aug. 2008–Mar. 2010 (1.67 year)
Sensitivity of screening NAT (copies/mL)†	650	260	76
Sensitivity of ID-NAT used for lookback study (copies/mL)†	13	13	3.8
Number of donations tested	24,702,784	19,513,054	8,746,037
Confirmed WP donations (/million)	473 (19.1)	258 (13.2)	50 (5.7)
Confirmed OBI donations (/million)		76 (3.9)	133 (15.2)
Number of donations causing established HBV transmission			
ID-NAT–negative WP	5	6	1
ID-NAT–positive WP	28	12	3
ID-NAT–negative OBI	4 (1)‡	1 (0)‡	2 (1)‡
ID-NAT–positive OBI	13 (1)‡	12 (1)‡	11 (5)‡

* Yields by ID-NAT trial conducted from December 2010 are not included in the table.
† 50% LOD.
‡ Numbers of donations with anti-HBs of greater than 10 mIU/mL.

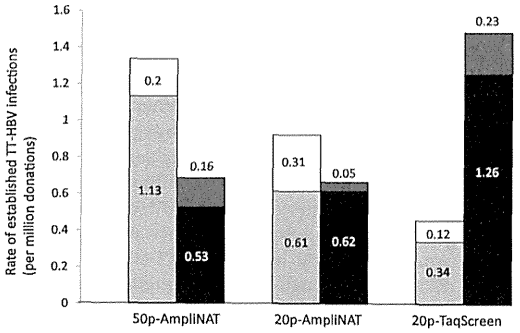

Fig. 2. Number of established TT-HBV infections grouped according to pool-based NAT screening systems. See Table 2 for intervals when indicated NAT systems were applied, sensitivities of NAT systems used, and actual yields for each category at each interval. (□, ▨) Infections caused by transfusion with ID-NAT–negative and -positive WP-derived components, respectively. (■, ■) Infections caused by transfusion with ID-NAT–negative and -positive OBI-derived components, respectively.

lished TT-HBV infections relative to the three periods. The rate of infections caused by transfusion with a WP-derived component notably decreased with increasing NAT sensitivity, but that caused by transfusion with OBI-derived components rather increased despite the increased NAT sensitivity. Current NAT screening protocols indicated that TT-HBV infections occur more frequently due to transfusion with OBI- than with WP-related components (1.49/million vs. 0.46/million donations; Fig. 2). Nine TT-HBV infections occurred as a result of transfusion with blood components containing more than 10 mIU/mL anti-HBs during the past decade (Table 2). Two of them were caused by donations with negative ID-NAT.

The number of TT-HBV infections caused by transfusion with ID-NAT–negative components accounts for 15% (2/13) and 25% (1/4) of OBI- and WP-related TT-HBV infections, respectively, according to the current NAT system (Table 2). These infections involving ID-NAT–negative donations were determined as TTI by analyzing repository blood samples obtained before the index donation and/or by following up with the implicated donors after the index donation. Details of the clinical course of a typical TT-HBV infection caused by ID-NAT–negative OBI-related blood components are shown in Tables 3 and 4.

Impact of blood product on transmission rate

Table 5 shows the numbers of implicated donations by either ID-NAT negative or positive for groups categorized by the type of component and WP/OBI status. During the past decade, ID-NAT–positive donations have caused 79 TT-HBV infections. Transfusion with red blood cells (RBC), fresh-frozen plasma (FFP), and platelet concentrate (PC) was associated with infections in 42, 22, and 15 of them, respectively. Of 19 TT-HBV infections associated with ID-NAT–negative donations, 2, 4, and 13 were caused by transfusion with RBCs, FFP, and PC, respectively. Transfusion with blood components containing a larger plasma volume (FFP and PC, but not RBCs) caused more frequent TT-HBV infections among patients who received ID-NAT–negative donations (17/19, 89%) than among those who received ID-NAT–positive donations (37/79, 47%; $p < 0.01$), which could be a reflection of the plasma volume effect on infectivity.

Table 5 also shows that if ID-NAT had been implemented during the screening, 81% of established TT-HBV infections would have been avoided. The introduction of ID-NAT would have been the most (95%) and least (54%) effective for preventing TTI caused by RBC- and PC-related transfusions, respectively. Under the current 20p-TaqScreen system, 75 and 85% of TT-HBV infections arising from WP and OBI donations, respectively, are ID-NAT positive and will be interdicted by ID-NAT. In particular, the effect of ID-NAT will be 100% for OBI-related infections caused by RBC transfusion.

Outcomes of patients with TT-HBV infection

ALT levels during TT-HBV infection were determined in 68 transfusion recipients who developed TT-HBV infection. Table 6 shows the maximal ALT values relative to WP or OBI donations and ID-NAT–positive or -negative donations. Almost half (47%, 32/68) of the patients had maximal ALT values of more than 1000 IU/L. The proportion of patients with maximal ALT of more than 1000 IU/L was greater in OBI-related (61%, 19/31) than in WP-related (35%, 13/37; $p < 0.05$) infections. Total viral load in the implicated components did not significantly differ between patients with ALT values above and below 1000, which was true for both WP- and OBI-related infections. Although barely insignificant, total infused viral load tended to be lower in OBI- than in WP-related patients among groups with maximal ALT values of more than 1000.

Three patients with TT-HBV infection died of fulminant hepatitis after the introduction of NAT. One was caused by transfusion with PC derived from an ID-NAT–negative, WP-related donation (Genotype A with wild type precore region). The other two developed hepatitis after transfusion with RBCs derived from ID-NAT–positive, OBI-related donations (Genotype B with a G1898A precore mutation and Genotype C with a G1896A precore mutation).

ID-NAT screening trial in donations with low anti-HBc and anti-HBs titers

During a 6-month ID-NAT trial, 4742 (0.74%) of 640,628 blood donations at the Tokyo Blood Center with low anti-

HBc and anti-HBs titers were analyzed by ID-NAT. The number of donations analyzed by ID-NAT decreased as the anti-HBc titer increased (Fig. 3). HBV DNA was detected in 92 (1.94%) of the 4742 donations. Figure 4 shows the frequency of ID-NAT–positive donations relative to the anti-HBc titer. The frequency of ID-NAT positivity for HBV did not correlate with the anti-HBc titer and did not tend to increase with an increasing anti-HBc titer. The proportions of anti-HBs–positive (>10 mIU/mL) donations among those that were ID-NAT positive and negative were 77 and 75%, respectively, and did not significantly differ. The proportion of anti-HBs–positive donations increased with increasing anti-HBc S/CO values among ID-NAT–negative donations (67.5, 82.0, and 87.5% for anti-HBc S/CO 1.0-3.9, 4.0-7.9, and 8.0-11.9, respectively; $p < 0.01$ between any two groups). The frequency of ID-NAT positivity between males (1.8%) and females (2.4%) did not significantly differ. Eighty-three (90.2%) of the 92 ID-NAT–positive donors were at least 50 years of age. Fifteen had a viral load of less than 100 copies/mL, whereas quantitative NAT could not detect HBV DNA loads in samples from the remaining 77. The distribution of HBV genotypes among the ID-NAT–positive donations did not differ from that among the general Japanese population: Genotypes A, B, C, and D, n = 1, 24, 45, and 1, respectively (21 were undetermined).

TABLE 3. Representative TT-HBV infection caused by OBI-derived, ID-NAT–negative blood component: clinical course of a patient who received an implicated blood component.

Date	Clinical events and test results
Nov. 10, 2008	Surgery to treat head injury*
	HBsAg negative, anti-HBc negative, HBV DNA negative, preoperatively
	Transfused until Nov. 20 with 21 RBC units, 5 PCs, and 11 FFP† including one derived from the donation of Mar. 27, 2008, shown in Table 4
Mar. 05, 2009	AST 15, ALT 32
Mar. 25, 2009	AST 517, ALT 1273
Mar. 30, 2009	AST 1312, ALT 3110, HBsAg positive, IgM-anti-HBc positive
	Reported to JRC blood center
Mar. 31, 2009	AST 695, ALT 2396
Apr. 01, 2009	HBsAg negative, anti-HBs positive, HBV DNA positive

* Recipient was a teenage boy who was injured in a traffic accident.
† HBV DNA was not detected based on ID-NAT for the repository samples from these 37 blood components transfused. These results were obtained in the first lookback study performed in April 2009.

Estimation of current TT-HBV risk in Japan

From the frequency of ID-NAT–positive (1.94%) donations among those with low anti-HBc and anti-HBs titers (69,000/year or 13,000/million; see Table 1 and below), we calculated that 1339/year or 252/million donations should be ID-NAT positive among screening NAT-negative donations with low anti-HBc and anti-HBs titers. Using an infectivity rate of 3%[7] among components derived from OBI donations that were screening NAT negative and ID-NAT positive, we calculated that 40/year or 7.6/million OBI-related TT-HBV infections should arise. If TT-HBV infections related to OBI-derived ID-NAT-negative donations are taken into account, then the total number of TT-HBV infections should be 47/year or 8.9/million. This estimate was based on the observation that TT-HBV infection caused by ID-NAT–negative components during the

TABLE 4. Representative TT-HBV infection caused by OBI-derived, ID-NAT–negative blood component: HBV marker profile of blood donor responsible for the outcome shown in Table 3

Date of donation	Date of testing	Test results
Oct. 17, 2007*	Oct. 17, 2007 (screening)	Pool NAT negative, anti-HBc 2^4 (negative), anti-HBs negative
	Feb. 24, 2010 (repository sample tested in second lookback study)	ID-NAT negative
Mar. 27, 2008† (index donation)	Mar. 27, 2008 (screening)	Pool NAT negative, anti-HBc 2^4 (negative), anti-HBs negative
	Apr. 7, 2009 (repository sample tested in first lookback study)	ID-NAT negative (negative result reported to corresponding facility)
Feb. 05, 2010‡	Feb. 05, 2010 (screening)	Pool NAT negative, anti-HBc 15.4 S/CO§ (positive), anti-HBs negative
	Feb. 10, 2010 (donated blood sample tested in second lookback study)	ID-NAT positive (high probability of TT-HBV infection in Patient A reported to corresponding facility)

* RBCs derived from this donation were transfused to an HBsAg-negative patient. Patient continued to be HBsAg-negative until May 2008 when he died.
† FFP derived from this donation was transfused to patient shown in Table 3. Cocomponent (RBCs) processed from this donation was transfused to a patient who died of the primary disease soon after transfusion. Whether TT-HBV occurred remains unknown.
‡ This donation was rejected due to anti-HBc seroconversion and a second lookback study was conducted on the donation of October 17, 2007.
§ Because of very low HBV load in donated blood sample of February 5, 2010, HBV sequence was assessed in donor blood only at 193 bp (Nucleotides 475-667) of S region. HBV sequence in that region was identical except for nt. 654 between the blood samples from donor and patient on April 01, 2009.

TABLE 5. Blood components implicated in established TT-HBV infection*

	ID-NAT+/ID-NAT–					
	WP transmissions established			OBI transmissions established		
Screening period	RBCs	FFP	PC	RBCs	FFP	PC
50p-AmpliNAT	15/0	6/1	7/4	5/0	5/1	3/3
20p-AmpliNAT	8/2	0	4/4	7/0	5/1	0
20p-TaqScreen	2/0	0	1/1	5/0	6/1	0/1
Total	25/2	6/1	12/9	17/0	16/3	3/4

	WP plus OBI				All components		
	RBCs	FFP	PC	FFP + PC	WP	OBI	Total
50p-AmpliNAT	20/0	11/2	10/7		28/5	13/4	41/9
20p-AmpliNAT	15/2	5/1	4/4		12/6	12/1	24/7
20p-TaqScreen	7/0	6/1	1/2		3/1	11/2	14/3
	100%	86%	33%		75%	85%	82%
Total	42/2	22/4	15/13	37/17	43/12	36/7	79/19
	95%	85%	54%		78%	84%	81%

* Ratios (%) in the two bottom rows represent rates of ID-NAT–positive events or effectiveness of ID-NAT implementation.

TABLE 6. Maximal values for ALT in patients with TT-HBV infection and total viral load contained in implicated components

	ALT	
	<1000	>1000
WP* (n‡)	24 (7)§	13
OBI† (n‡)	12 (2)§	19
Total viral load (copies/bag)‖		
WP		
n‡	21	10
Min	40	100
Max	260,000	560,000
Median	1,400	9,100
Mean	20,460	74,790
OBI		
n‡	8	16
Min	60	40
Max	6,240	19,200
Median	630	1,470
Mean	1,440	3,750
ID-NAT status		
Positive‡	30	28
Negative‡	6	4
Component types		
RBCs‡	19	16
FFP‡	7	13
PC‡	10	3

* Patients transfused with WP-related components include 11, 8, and 18 patients with malignant hematologic disorder, solid tumor, and others, respectively.
† Patients transfused with OBI-related components include 7, 11, and 13 patients with malignant hematologic disorder, solid tumor, and others, respectively.
‡ Numbers of patients.
§ Numbers in parentheses, patients with maximal ALT values of less than 100 IU/L.
‖ Total viral load was calculated using viral concentrations in implicated donations and average plasma volume of each component type. When viral load was less than 100 copies/mL, total viral load in the component was calculated assuming that viral concentration is logarithmically distributed between 1 and 100 copies/mL.

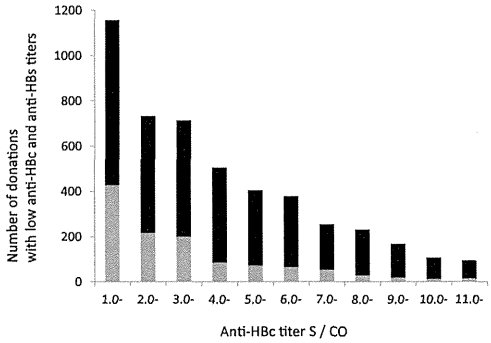

Fig. 3. Number of donations screened by ID-NAT trial categorized by anti-HBc titer. All donations tested had low anti-HBc (S/CO 1.0-11.9) and anti-HBs (<200 IU/L) titers and were qualified serologically based on algorithm applied at JRC blood centers. Donations verified to be ID-NAT-positive were disqualified. (■, ▨) donations with anti-HBs titers of more than and not more than 10 mIU/mL, respectively.

20p-TaqScreen period accounted for 15% (2/13) of all OBI-related infections (Table 2).

We estimated how many more WP-related TT-HBV infections would be prevented by introducing ID-NAT. The current screening NAT yield (30 donations/year or 5.7/million, Table 2) was multiplied by the ratio of the interval between ID-NAT and 20p NAT detection (11.2 days) to that between 20p NAT and HBsAg detection (9.7 days). We then deduced that 34.6/years or 6.6/million more viremic donations would be captured by ID-NAT. The number of ID-NAT–negative WP donations was calculated separately for each component type. Based on the plasma volume of each component (20, 200, 240, 450, and 120 mL for RBCs, PC, FFP-3, FFP-5, and FFP1.5,

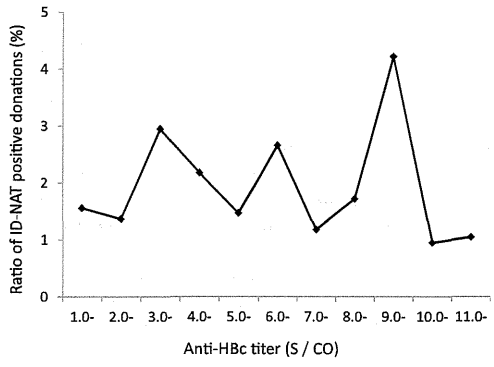

Fig. 4. Ratios (%) of ID-NAT–positive donations with low anti-HBc and anti-HBs titers relative to anti-HBc titer.

respectively), the deduced intervals between 1 copy/bag and ID-NAT detection were 16.3, 24.9, 25.5, 28, and 23 days, respectively. The ratio of the number of those components issued to hospitals is 6.3:2.2:2.1:0.7:0.1. The incidence of ID-NAT–negative WP donations calculated from these data was 59.5/year or 11.4/million. Adding ID-NAT–positive WP donations (34.6/year or 6.6/million), current risk related to WP donations amounts to 94.1/year or 18.0/million. The effect of ID-NAT on the reduction of all WP donation would be 37% (6.6/18.0). If the infectious risk (50%) of ID-NAT–positive, screening NAT-negative WP-related components is also applied to ID-NAT–negative WP-related components, the total number of WP-related TT-HBV infections would be 47.1/year or 9.0/million. Together, these estimates for WP- and OBI-related TT-HBV infections indicate that 94.1/year or 17.9/million TT-HBV infections are likely to occur in Japan.

DISCUSSION

Infection with HBV results in a wide spectrum of clinical manifestations ranging from asymptomatic liver dysfunction with only slightly elevated transaminase levels or acute self-limiting hepatitis to chronic hepatitis that in some patients progresses to cirrhosis, liver failure, or hepatic cell carcinoma. In rare cases, HBV infection can cause fulminant hepatitis that is associated with high mortality. Fulminant hepatitis in Japan is frequently associated with primary infection by HBV carrying precore or core-promoter mutations.[12,13] These HBV mutants are frequently found among chronic HBV carriers[14,15] who typically have an anti-HBc–positive serostatus. To prevent fulminant hepatitis arising as a result of blood transfusion,[16] the JRC incorporated anti-HBc testing into blood screening in 1989.

The agglutination method had been used for all serologic testing at JRC blood centers before 2008. Although this method was somewhat insensitive to HBsAg, it could semiquantify anti-HBc. Thus, the cutoff point for the anti-HBc titer had been set at 2^6, and donations with an anti-HBc titer of at least 2^6 and an anti-HBs titer of less than 200 mIU/mL were disqualified.[17] Although this anti-HBc testing had essentially prevented transfusion-transmitted fulminant hepatitis since 1989,[18] reports of fulminant or acute severe hepatitis continued for an additional 7 years. These conditions were attributed to transfusion with components with a 2^5 anti-HBc titer.[19] Consequently, the JRC lowered the anti-HBc cutoff from 2^6 to 2^5 in 1997. The agglutination method for serologic screening was replaced in 2008 with CLEIAs, which can also semiquantify anti-HBc. The policy described above is maintained in the algorithm for HBV screening with CLEIA; the range defined as a low anti-HBc titer includes S/CO values between 1.0 and 11.9, and donations with anti-HBc S/CO values within this range are currently accepted.

The highly sophisticated strategy of multiplex NAT was designed to decrease the incidence of TTI. Implementing HBV NAT into blood screening was important in Japan mainly because of the unsatisfactory sensitivity of the standard agglutination method to HBsAg. The JRC implemented multiplex NAT targeting HBV DNA, HIV RNA, and HCV RNA during 1999[6] and improved the sensitivity of the test at three points. The 98 infections described herein had been confirmed as TT-HBV since the introduction of 50p-AmpliNAT in 2000. The HBV genome was not detected in donor repository samples of 587 (74.4%) suspected TT-HBV infections. The JRC has informed the appropriate physicians of the ID-NAT results of viral detection that imply a low or high probability of TT-HBV infection.

In parallel with the increase in the screening NAT sensitivity, the incidence of WP-related TT-HBV infection has decreased as predicted, whereas that of OBI-related TT-HBV infection has not decreased (Fig. 2). To explain the increasing number of OBI-related TT-HBV infections, the increase in the sensitivity of NAT used in JRC laboratories for retrospective studies might have helped to identify TT-HBV infections, thus sustaining the number of OBI-related TT-HBV infections despite improvements in screening NAT sensitivity.[4]

This consideration could encourage the speculation that most of the 587 infection reports that had been excluded from established TTI (Fig. 1) based on negative results from repository samples might have been confirmed as TT-HBV had more sensitive NAT and a larger sample volume been analyzed. With regard to this notion, the outcomes of recent hemovigilance for TT-HBV are described below. During the 20p-Taqscreen period, the JRC received 61 clinical reports of possible HBV-TTI. Seventeen were determined as TTI, among which, three

repository samples were ID-NAT negative. Historical HBV infection was confirmed in 10 patients by retesting pretransfusion samples. Results from HBV tests of posttransfusion samples from five patients were false positive. The possibility of TT-HBV was ruled out in two patients related to ID-NAT–negative donations because repeated blood donations from two of two and three of three implicated donors were not sero- or NAT-converted. The remaining 27 patients related to ID-NAT–negative donations are inconclusive for TTI as follow-up studies have not yet been completed. Some of the 587 reported infections had been confirmed to be associated with passive anti-HBc transfer from infused components. Thus, it is unlikely that a considerable proportion of the infections excluded from the TTI category were real TT-HBV infections.

Among 19 patients with TT-HBV infections associated with ID-NAT–negative donations, 17 (89%) of them were caused by transfusion with FFP or PC that contained a larger plasma volume (120 to 450 mL) than RBCs (20 mL). In contrast, 37 (47%) were caused by FFP or PC among 79 infections associated with ID-NAT–positive donations (Table 5). This finding suggests that because HBV infectivity is extremely high, the relationship between infectivity and plasma volumes contaminated with HBV could only be established in the era of ID-NAT screening when the viral load in the donation is low enough to escape ID-NAT screening. This might explain why we could not previously establish such a relationship using viral loads around the sensitivity of the pool-based NAT system or serology.[7] If ID-NAT is introduced as routine screening, it will prevent 75 and 85% of WP- and OBI-related infections. In particular, all RBC-related TT-HBV could be prevented because of the small plasma volume involved. The finding also suggests that novel viral reduction technologies[20,21] could be an attractive strategy to decrease the incidence of TT-HBV because these technologies are presently more applicable to FFP or PC than to RBC.

The maximal ALT levels of patients with TT-HBV infection showed that transfusion with components harboring an extremely low HBV load that escaped NAT screening is not necessarily associated with mild clinical illness. This seems particularly true for OBI-related infection (Table 6). The frequency with which transfusion causes severe hepatitis (i.e., ALT > 1000) is significantly higher for OBI- than for WP-derived components. Moreover, OBI-derived components tend to cause severe hepatitis despite lower total viral loads compared with those in the WP-derived components. These findings should be further substantiated by analyzing samples from patients that are regularly obtained after transfusion because most of the maximal ALT values described in this article were found after occasional sampling.

Three patients died of TT-HBV fulminant hepatitis caused by transfusion with blood that had escaped NAT screening. Two of them were notably caused by transfusion of OBI-derived RBCs, and the other was caused by an ID-NAT–negative WP donation. Although a larger plasma volume might generally be required to establish TT-HBV infection under the NAT screening system, plasma volume or the total infused viral load might not be determining factors in fulminant hepatitis. Although viral genome mutations such as those in precore or core-promoter regions are frequently associated with the development of fulminant hepatitis in Japan,[12,13] other crucial factors have not clearly been demonstrated despite considerable investigation.

The JRC accepted 5.3 million donations in 2010, of which 4.9% (261,000) was anti-HBc reactive (Table 1), 0.19% (10,000) was rejected because of high anti-HBc and low anti-HBs titers. Another 3.4% (182,000) was accepted because of high anti-HBs titers (≥200 IU/L). The notion that blood components with an anti-HBs titer of more than 100 IU/L are not infectious is generally accepted.[22-25] The relationship between anti-HBs titer and TT-HBV infection will be discussed elsewhere (manuscript in preparation). Importantly, 1.3% of donations (69,000) with low anti-HBc and anti-HBs titers were accepted, and this category included all donations to which OBI-related TT-HBV infections were attributed. Our ID-NAT trial verified that 1.94% of the donations with low anti-HBc and anti-HBs titers were HBV DNA positive.[26] Accordingly, an estimated 1339/year or 252/million viremic OBI donors and 47/year or 8.9/million TT-HBV infections caused by OBI-derived components would be missed by the current screening algorithm. When estimates for WP-related TT-HBV infections are included, the calculated number of TT-HBV infections was 94.1/year or 17.9/million. Whole blood withdrawn from donors in Japan is split into RBCs and FFP, and the total number of components processed averages 23% more than the number of donations. However, because of outdating and rejection by testing or processing problems, the number of components finally issued by JRC becomes almost the same as the number of donations. Therefore, the calculated number of TT-HBV infections was not significantly influenced by the issue of splitting.

The considerable discrepancy between the estimated and established TT-HBV incidence per million (8.9 to 1.49 and 9.0 to 0.46 for OBI-related and WP-related infections, respectively) might be due to the following factors. A clinical manifestation of HBV infection is often unclear in patients transfused with blood components harboring a low viral load and low proliferative ability. Physicians might thus be likely to overlook infection under such circumstances. Medical practitioners are not compliant with national guidelines for lookback investigations. Indeed, only 30% to 40% of transfused patients were reportedly traced for TTI even after the guidelines were established.[27] A considerable proportion of patients who receive blood