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Original Article

Nationwide Nucleic Acid Amplification Testing of Hepatitis B Virus, Hepatitis C Virus and Human Immunodeficiency Virus Type 1 for Blood Transfusion and Follow-Up Study of Nucleic Acid Amplification Positive Donors

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SUMMARY: This study described a program for and the results of a nationwide nucleic acid amplification testing (NAT) screening for hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus type 1 (HIV-1) by multiplex reagent with a pooled system. After routine serological screening, this test was used in order to be in time for blood transfusions. The Japanese Red Cross currently supplies donated blood all over Japan for blood transfusion. As of January 2000, 2,140,207 units (5,093 pools) were tested by a pool size of 500 and 19 HBV DNA-positive cases and 8 HCV RNA-positive cases were found. Since February 2000, the pool size was switched to 50 and among 420,770 units (8,564 pools), 7 HBV DNA-positive cases and 1 HCV RNA-positive case were found. HIV RNA was not detected in any of the tested pools. Among the 26 HBV DNA positives, 22 were wild type; of these, 6 (23%) had hepatitis B surface antigen (HBsAg) that was undetectable by overnight enzyme immunoassay (EIA). Except for one case, in which coexisting antibody inhibited the immune reaction, all 17 cases that were followed later showed seroconversion. In 10 of these cases, HBV DNA disappeared below the level of detection and seroconversion of IgM anti-HBc and anti-HBc antibody occurred during the observation period. The remaining 4 cases were precore mutants and all had an undetectable level of HBsAg by EIA. Three cases did not show IgM anti-HBc seroconversion, which should be observed during the early stage of HBV infection. As for the HCV RNA, the following types were identified: 2 genotype II (1b), 3 genotype III (2a), and 4 genotype IV (2b). A weak anti-HCV positive reaction was observed in two cases and strong seroconversion in one case among 4 of the cases that were followed. Although it is not 100%, NAT narrows the window period in early-stage infection, resulting in an exponential reduction of the virus load that escapes serological screening tests for blood destined for blood transfusions. In the case of HBV, NAT screening detects HBV DNA in persistently infected individuals with extremely low levels of HBV antigen and antibody often observed in the case of HBV mutants.

INTRODUCTION

During the period from November 1997 to November 1999, 5.6 million serologically negative donations were tested manually by nucleic acid amplification testing (NAT) in order to reduce the virus load from source plasma for plasma derived products. We found 78 donations positive for hepatitis B virus (HBV) (1:72,000), 10 for hepatitis C virus (HCV) (1:560,000) and 2 for human immunodeficiency virus type 1 (HIV-1) (1:2,800,000); the risk of HBV window-period donations is highest among these three viruses (1). These data indicate that blood transfusions have become safer in contemporary Japan than they ever were before. The Japanese system is a 100% voluntary blood donation system and serological screening is performed at nationwide Japanese Red Cross (JRC) blood centers by automated agglutination tests using PK7200 (2). Residual risk does persist due to blood donations made during the window period in early stages of infection. In addition, another risk is due to late-stage infection with virus mutants or viral loads that are undetectable by current serological screening methods. In the United States, the risk of giving blood during an infectious window period has been estimated to be 1:63,000 in the case of HBV, 1:103,000 for HCV, and 1:641,000 for HIV-1; there, too, the risk of HBV is the highest (3). Therefore, newer laboratory assays to detect directly the presence of virus genome with high sensitivity and specificity such as NAT are necessary.

Currently, tests are being performed based on cost considerations, and should be done as rapidly as possible in order to provide blood for transfusion of cellular components. Sacher et al. (4) have noted that HCV is particularly suited for minipool NAT because of the high levels of virus load observed during the window period. However, the effects of HBV DNA screening are more complex because HBV seems to be present in low titers during the window period. However, we quantified HBV genomes in 6 hepatitis B seroconversion panels of Boston Biomedica, Inc. (Bridgewater, Mass., USA) during the early stage of HBV infection; the virus growth curve showed an exponential straight line that proceeded against the date of donation. Doubling time was 2.0 days and log time was 6.5 days (5). Therefore, a highly sensitive NAT assay with an appropriate pool size could narrow the window period of early-stage HBV infection, thus resulting in an exponential reduction of the virus load that escapes from present screenings.

In December 1998, a highly sensitive multiplex HBV/HCV/HIV-1 reagent (MPX) was developed for NAT for HBV, HCV

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and HIV-1 infections, which can thus be screened at one time. Therefore, the cost of screening is reduced and the time required for testing is also less than that needed for screening individual viruses. With the development of a rapid transportation system, computer networks, an automatic sample pooling system, and an automated viral nucleic acid detection system, we started nationwide NAT screening of serologically negative, voluntarily donated blood at two NAT centers in Japan since July 1999. After the resolution of MPX-positive samples into individual virus, each JRC blood center passed on the results to the donors and a follow-up study of NAT-positive cases was initiated in order to distinguish between transient or chronic infection. An additional goal was the improvement in the health care of voluntary blood donors.

MATERIALS AND METHODS

Serological tests for HBV, HCV and HIV-1 infections and measurement of serum alanine aminotransferase (ALT): All voluntarily donated blood in JRC was qualified by a questionnaire administered by the JRC blood centers throughout Japan. These samples were screened for hepatitis B surface antigen (HBsAg) by reverse passive hemagglutination (RPHA), for anti-HBV core antibody (anti-HBc) by hemagglutination inhibition (HI), and for anti-HBsAg antibody (anti-HBs) by passive hemagglutination (PHA) using reagents prepared by the JRC, as described elsewhere (6, 7). Anti-HCV antibody (anti-HCV) was tested by PHA (Dainabot Co. Ltd., Tokyo) or by particle agglutination (PA) (Fujirebio Inc., Tokyo) (8). Anti-HIV-1/2 was tested by agglutination of gelatin particles coated with recombinant HIV-1/gp41, HIV-1/p24 and HIV-2/gp36 (Fujirebio) (9). All of these agglutination screenings were carried out using an automatic PK7200 (Olympus Co. Ltd., Tokyo) and were confirmed by specific inhibition tests. The endpoint of the 2-fold dilution of test samples was expressed as an exponent of 2^n versus the end titer of the specific inhibition test; these results of the tests are shown in Table 3 and 4. Serum ALT levels were measured by the method of Wroblewski and LaDue (10). All these tests were carried out in each blood center. Serologically positive and ALT-elevated (>61 IU) samples were excluded from NAT screening.

Shipment: Five milliliters of each donated blood sample were taken into a polyethylene terephthalate (PET) tube containing 7.5 mg of EDTA • 2K and were sent to two NAT centers, in Tokyo or in Chitose, Hokkaido. The most direct route by air-flight or surface transportation was chosen. Blood arrived within 1 day of donation. Samples were kept at 2-8°C during shipping and storage so that the viral titers could remain unchanged as indicated (11).

Pooling of samples and NAT of pool samples by simultaneous MPX: For sample preparation, 0.1 ml of every 50 serologically negative samples was harvested, and 5 ml collected is given the term of 50 pool. A pool size of 500 consisted of ten of each 50 pool. When the MPX-positive 500 pool was found, 10 samples from the 50 pool were tested separately. Individual units from the MPX-positive 50 pool were identified. Since February 2000, all tests are switched to a pool size of 50 in order to increase the test's sensitivity and to shorten time necessary for pooling and resolving. Pooling was performed using ALOKA automatic pooling system, APS-NAT (Aloka Co. Ltd., Tokyo), with a capacity to pool 10,000 units successively in 6 h. For the preparation of specimens for NAT from pooled plasma, target-specific biotinylated probes co-captured HBV, HCV, HIV-1 and Internal Control (IC) on streptavidin-coated paramagnetic microparticles (12) using GT-12/GT-X (Roche Diagnostics K.K., Tokyo). Coamplification and real-time detection of HBV, HCV, HIV-1 and IC were performed by TaqMan PCR (12). A detailed account of the procedure and its principles was reported by Chaka Impraim et al. (12). Carry-over was prevented by excluding samples by serological prescreening in the blood centers and by use of AmpErase (13) in the NAT centers. Multiplex HBV/HCV/HIV-1 reagent (Roche Diagnostics and Roche Molecular Systems, Inc., Pleasanton, Calif., USA) possessed a 95% detection limit for HBV:25 copies/ml, HCV:100 IU/ml and HIV-1:60 copies/ml and was used as the first-step NAT screening reagent without target discrimination. Results were provided within 5-6 h. Non-template controls (NTC) were employed to determine the cutoff value with specifically-designed data reduction software. The results were reported as positive, negative, or inhibitory (12). In this manner, 20,000 units were tested every day by the use of limited human resources. All samples were handled and controlled automatically by a bar-code computer system. We also identified individual samples with a double-check system.

Reporting NAT results to blood centers: NAT results with MPX were reported immediately by the NEC computer network (NEC Corp., Tokyo) to each blood center and MPX-negative units were released for blood transfusions or as source plasma for plasma-derived products. When an MPX-positive pool was found, the release was withheld until the positive unit could be identified. After the MPX-positive unit was identified, other MPX-negatives were released.

Later MPX-positive units were resolved into HBV DNA by the method of Iizuka et al. (6), HCV RNA by the method of Okamoto et al. (14), and HIV-1 RNA by the method of Matsumoto et al. (15) at two JRC NAT Centers.

Characterization of NAT-positive samples: HBV DNA and HCV RNA, identified by two JRC NAT centers, were sent to the Research Laboratory of JRC Saitama Blood Center. Virus genomes of both viruses were quantified. Furthermore, subtype, genotype and mutation in the precore region of HBV and the genotype of HCV were identified.

Samples from HBV NAT-positive donors were further tested for HBsAg by overnight enzyme immunoassay (EIA) by Auszyme II (Abbott Laboratories, North Chicago, Ill., USA) for IgM-type anti-HBcAb (IgMHBcAb) by Corzyme-M (Abbott Laboratories). Hepatitis B e antigen antibody was tested by a commercial kit (HBeAg/Ab; Institute of Immunology, Co. Ltd., Tokyo). HBV DNA was quantified by the method of Iizuka et al. (6) and sequenced to determine subtype, genotype, and mutation in the precore region (16). As for samples from HCV NAT-positive donors, HCV RNA was quantified by ABI PRISM 7700 (PE Applied Biosystems, Faster City, Calif., USA) and genotype was determined by genotype-specific primers (17).

RESULTS

Positive rate of HBV DNA, HCV RNA and HIV-1 RNA in NAT-positive donors: From July 1999 to February 2000, 2,560,977 serologically negative donors were screened by MPX in two NAT centers. All 54 JRC blood centers in Japan participated in this program. MPX-positive units were resolved into either HBV DNA and HCV RNA. The results of the 500-pool test and the 50-pool test are shown separately

in Table 1. HBV DNA- and HCV RNA-positive cases numbered 26 (1/98,499) and 9 (1/284,553), respectively. No HIV-1 RNA was found. We were able to withhold the release of infected units from proceeding to blood transfusion. The HBV DNA-positive rate of the 50-pool group (1/60,110) was higher than that of the 500-pool group (1/112,642), but a statistical difference between these two groups was not be observed on the χ^2 test (Table 1); this result is due to the very low number of NAT-positive cases.

Quantification and characterization of HBV DNA in NAT-positive donors: As shown in Figure, among the 26 HBV DNA positives, 22 were wild type and 4 were precore

Table 1. Positive rate of HBV DNA, HCV RNA and HIV-1 RNA of MPX positive donors

NAT Center	Participating Blood Centers	Pool Size	No. of Blood Units Tested	(No. of Pools Tested)	NAT Positive Cases				
					HBV DNA		HCV RNA		HIV-1 RNA
Tokyo	27	500 pool	1,274,807	(3,008)	7	1/182,115	4	1/318,701	0
		50 pool	222,262	(4,514)	3	1/ 74,087	0	0	0
Chitose	27	500 pool	865,400	(2,085)	12	1/ 72,117	4	1/216,350	0
		50 pool	198,508	(4,050)	4	1/ 49,627	1	1/198,508	0
Total	54	500 pool	2,140,207	(5,093)	19	1/112,642	8	1/267,526	0
		50 pool	420,770	(8,564)	7	1/ 60,110	1	1/420,770	0
		Total	2,560,977	(13,657)	26		9		0

n.s.=not significant by χ^2 test

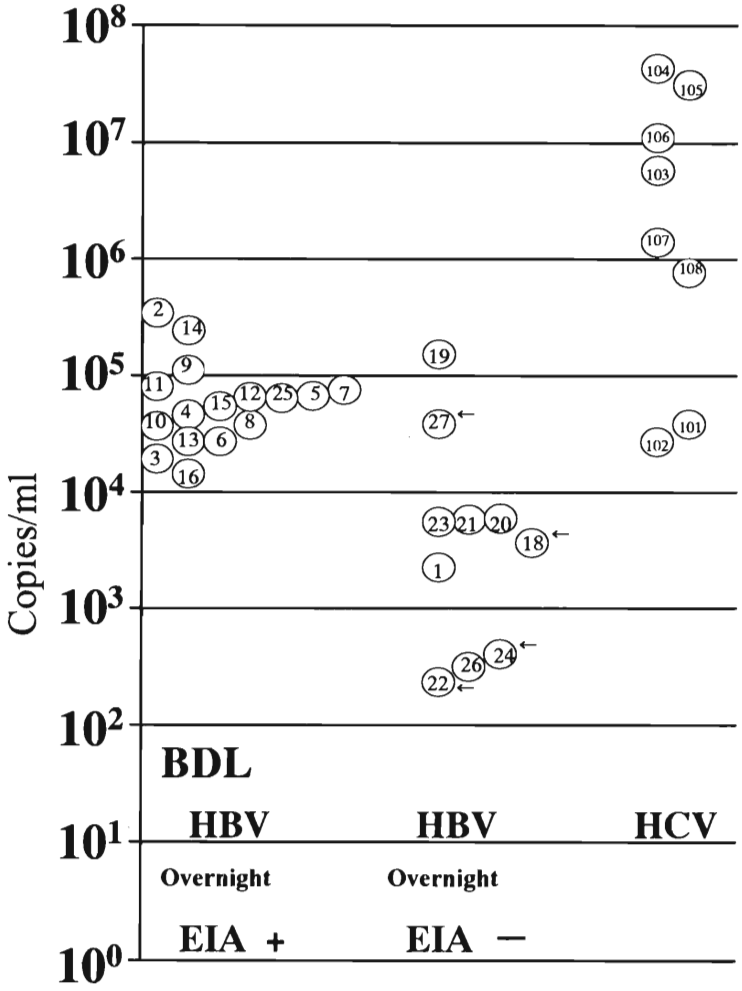

Figure. HBV DNA and HCV RNA copies in positive samples.
The numbers in the circles correspond to those of positive donors, shown in Tables 3-5.
Left column: HBV-positive cases by overnight EIA
Middle column: HBV-negative cases by overnight EIA
#19: HBsAg-positive and HBsAb-positive cases
#18, 22, 24, 27: Precore mutant cases, ←
Others: Wild type cases
Right column: HCV RNA window donation
BDL: below detection limit

mutant HBV. HBV DNA contents were distributed between 2.4×10^5 and 2.3×10^2/ml. Because our system aimed to shorten the serological screening time, we used RPHA for the HBsAg test by automatic PK 7200. We later reexamined the NAT-positive samples by overnight Auszyme II. Case #16, with a copy number of 1.4×10^4/ml, showed a faintly positive reaction (0.064/0.060). Seventeen cases of wild type HBV DNA with an even higher copy number, were overnight EIA-positive except for case #19. Case #19 showed rather high HBV DNA (1.5×10^5/ml), but had a coexisting high titer anti-HBc and anti-HBs, which gave negative results for HBsAg test by overnight EIA. These coexisting antibodies may interfere with the serological reactions of RPHA or EIA for the detection of HBsAg. The HBsAg-undetectable unit with both high-titer anti-HBc and anti-HBs and with normal ALT level was permissible to release for transfusion according to the criterion of serological screening before the introduction of NAT in Japan. The question remains whether or not such cases with high-titer anti-HBs are infectious, but transfusion of such units could be prevented by screening with NAT. Five cases with HBV DNA contents below 5.8×10^3/ml were all EIA-negative.

All of the four HBV precore mutants were negative for HBsAg by overnight EIA. Among these, three was below 3.6×10^3/ml; however, the copy number of #27 was 3.8×10^4/ml. As six wild type cases with the same level of HBV DNA contents (between $3.7\sim1.4\times10^4$/ml), as in case of #27, were all reactive by overnight EIA. It is to be noticed that all four precore mutants were negative by EIA.

Among the 22 wild type cases, 19 (86%) were subtype adr, genotype C, which is the predominant subtype in Japan (18). The subtype and genotype of four precore mutants were as follows: two adr C, one adw B, and one ayw C (#18). Antigen expression of #18, ayw was discordant to genotype C by mutation, which explains why it was hardly detectable on the antigen assay.

Therefore, by the JRC NAT system, we were able to exclude 19 RPHA, HI negative units out of 2,140,207 (1/112,642) total units by the 500-pool test. Seven out of 420,770 (1/60,110) units were excluded by the 50-pool test, as shown in Table 1. Among them, 10 units were undetectable by overnight EIA. Four of these had precore mutants, 5 were wild type (below 5.8×10^3/ml), and one was wild type with coexisting anti-HBc and anti-HBs.

Follow-up study of HBV DNA NAT-positive donors: It is important to know whether or not these NAT-positive donors are at the early pre-seroconversion stage (serological window period) of HBV infection or at a late stage of chronic infection with very low levels of viremia. Moreover, considering the health care of the voluntary blood donors, we started a follow-up study of these NAT-positive donors.

Through collaboration of the corresponding blood centers and the courtesy and consent of the blood donors, blood was taken into 5 ml PET tubes with EDTA from the NAT-positive donors and sent to NAT centers. HBsAg, anti-HBs, anti-HBc, IgM anti-HBc, HBeAg/anti-HBe, and the copy number of the HBV of each sample were measured in the Research Laboratory of the JRC Saitama Blood Center.

We were able to follow 18 cases of wild type HBV NAT-positive donors (Table 2, 3) and three cases of mutant HBV NAT-positive donors (Table 2, 4); IgM anti-HBc and anti-HBc in 17 wild type HBV donors (94%) were seroconverted after donation. Among them, HBV DNA disappeared below detection levels in 10 cases within the follow-up period. It appears to be the case that all 17 wild type HBV-infected cases were at an early stage of infection (i.e., in the serological window period).

Table 2. Follow-up study of HBV NAT positive donors

A	Wild Type	18
	Seroconversion of IgM anti-HBc, anti-HBc	17(10)*
	HBV DNA with anti-HBc and anti-HBs	1
B	Precore Mutant	3
	IgM anti-HBc	0
	Low level of anti-HBc	2

*the number of cases that HBV DNA disappeared during observation period.

Wild type HBV DNA was detected in the anti-HBs and anti-HBc positive sample of case #19. As IgM anti-HBc were negative and the viremia level and high titer of anti-HBc ($>2^{13}$) continued, this was a case of HBV infection, however, HBsAg could not be detected because of coexisting antibody. As by JRC regulation, HBsAg negative units with high titers of anti-HBc are excluded from blood transfusion if anti-HBs is undetectable, but if the anti-HBs titer is higher than 2^4, the unit passes the guidelines for serological screening. Such units might be rare but can be screened out by the HBV DNA NAT.

As for the three HBV precore mutant cases (Table 4), case #24 and #18 showed borderline levels of anti-HBc and negative IgM anti-HBc after donation; case #22 completely negative for anti-HBc 21 days after donation and IgM anti-HBc was also negative. In all these cases, viremia levels did not change after donation, indicating that these three cases were not in the early stage of acute HBV infection; however, low levels of HBV DNA existed in later stages of subclinical HBV infection.

Here, our NAT screening system could detect HBV DNA in persistently infected individuals with extremely low levels of HBV antigen and antibodies which were often observed in the case of HBV precore mutant infection.

Quantification and characterization of HCV RNA in NAT-positive donors: HCV RNA concentration of serologically undetectable NAT-positive donors are shown in Figure and Table 5. The concentration of HCV RNA in NAT-positive donors in the serological window period are high ranging from $4.3\times10^7\sim2.7\times10^4$, indicating a prolonged, high titer viremia preceding seroconversion. Two cases of genotype II (1b), 3 genotype III (2a) and 4 genotype IV (2b) were identified.

Follow-up study of HCV RNA NAT-positive donors: Four HCV RNA NAT-positive cases were followed and described in Table 5. One case (#101) showed high-titer anti-HCV seroconversion, $2^0\rightarrow2^{12.5}$ after donation. The other two cases (#104 and #108) showed a weak anti-HCV response $2^3\sim2^8$ after donation and had significantly high ALT levels. Therefore these three cases were at the early stage of HCV infection prior to seroconversion of anti-HCV.

In case #102, the anti-HCV titer measured by PA was 2^4, which is within the threshold of negative results. When it was retested by PHA, the titer was 2^9, and thus we obtained discordant results. HCV RNA copies of 2.7×10^4 were found in this sample. After 87 days, the HCV RNA content slightly increased and it seemed that this was a case of persistent infection with normal ALT levels (Table 5).

DISCUSSION

Blood safety as regards transfusion-transmitted viral infec-

Table 3. Infection with wild type HBV

No.	Bleeding Date	Age	Sex	ALT	HBsAg		anti-HBs	anti-HBc		HBe	HBV DNA			
					RPHA	EIA	PHA	HI	IgM-class	eAg/eAb	copies/ml	Subtype	Genotype	Precore
1	1999/ 9/20	18	M	13	0.5/0.5	0.009	0.5/0.5	0/0.5	0.021	−/−	2.2×10^3	adr	C	Wild
	106d			14	0/0	0.009	1/0.5	9/0*	0.665	−/+	$<10^2$			
	155d				0.5/0	0.011	1.5/0.5	8.5/0	0.328	−/+	$<10^2$			
7	1999/11/10	19	M	8	0/0	0.208	0/0	0/0	0.023	−/−	7.3×10^4	adr	C	Wild
	56d			28	1/1	0.007	2/1	10/0	0.977	−/+	$<10^2$			
8	1999/11/26	37	F	10	0/0	0.301	0.5/0.5	0/0	0.023	−/−	3.7×10^4	adr	C	Wild
	44d			8	1/1	0.006	2.5/1.5	5.5/1.5	0.467	−/−	$<10^2$			
	88d				0/0	0.023	2.5/0.5	4.5/0	0.189	−/−	$<10^2$			
4	1999/10/28	18	F	7	0/0	0.232	0/0	0/0	0.27	−/−	4.5×10^4	adr	C	Wild
	105d				0/0	0.001	8.5/2	6.5/0	0.526	−/−	$<10^2$			
12	1999/12/10	25	M	13	1/1	0.793	1/1	0/1	0.020	−/−	6.4×10^4	adr	C	Wild
	26d			23	0/0	0.004	2/0.5	6.5/0	0.414	−/+	$<10^2$			
13	1999/12/13	24	F	15	0/0	0.422	0/0	0/0	0.026	−/−	2.7×10^4	adr	C	Wild
	23d			41	1/1	0.007	3.5/2	7/0.5	0825	−/−	$<10^2$			
	56d			33	0.5/0	0.007	4.5/2	6.5/0	0.562	−/−	$<10^2$			
	85d			19	0/0	0.002	5.5/2	7/0	0.359	−/−	$<10^2$			
14	1999/12/13	19	F	13	1/1	0.214	1/1	0/0.5	0.030	−/−	2.4×10^5	adw	C	Wild
	25d			7	1/1	0.014	2.5/2	9/1	1.556	−/−	$<10^2$			
21	2000/ 1/30	31	M	12	0.5/0.5	0.008	1.5/1.5	0/1	0.024	−/−	5.7×10^3	adr	C	Wild
	13d			11	0/0	0.011	1/0	5.5/0	0.133	−/−	$<10^2$			
11	1999/12/10	22	M	22	0/0	0.090	0/0	0/0	0.027	−/−	7.9×10^4	adr	C	Wild
	26d			413	3.5/1	>2.000	0/0	5.5/0	0.372	−/−	3.3×10^6			
	80d			25	0/0	0.005	1.5/0.5	7.5/0	0.754	−/+	$<10^2$			
5	1999/11/ 4	26	M	18	0/0	0.139	0/0	0/0	0.025	−/−	6.6×10^4	adw	B	Wild
	123d			33	1.5/1.5	0.005	4/2	5/1.5	1.018	−/−	$<10^2$			
9	1999/11/30	27	M	16	0/0	0.167	1/1	0/0	0.039	−/−	1.1×10^5	adr	C	Wild
	40d			1029	13/7	>2.000	1/1	10/1.5	1.713	−/−	7.1×10^8			
23	2000/ 2/10	32	M	19	1/1	0.019	1.5/1.5	0/1	0.040	−/−	5.4×10^3	adr	C	Wild
	18d			32	0.5/0	0.463	0/0	0/0	0.021	−/−	9.7×10^4			
3	1999/10/12	24	F	7	0/0	0.257	0/0	0/0	0.032	−/−	1.9×10^4	adr	C	Wild
	91d			379	13.5/7.5	>2.000	1/1	9.5/2	1.665	−/−	2.9×10^4			
	100d			94	13.5/7.5	>2.000	0/0	9.5/0	1.248	−/+	9.8×10^3			
10	1999/12/ 4	30	M	13	0.5/0.5	0.164	1/1	0/0	0.022	−/−	3.6×10^4	adw	B	Wild
	36d			34	13/7	>2.000	1.5/1	0.5/0.5	0.025	+/−	6.0×10^8			
	44d			327	15.5/10	>2.000	0/0	5.5/0	0.223	+/−	6.9×10^8			
	58d			2877	11.5/3.5	>2.000	0.5/0.5	9.5/0	>2.000	+/−	4.6×10^6			
	72d			384	0/0	0.072	0/0	9/0	1.427	−/+	4.3×10^3			
	79d			214	0/0	0.034	0.5/0.5	9/0	1.273	−/+	3.7×10^3			
	86d				0/0	0.023	0/0	9/0	0.981	−/+	2.9×10^3			
15	1999/12/18	16	F	12	0/0	0.074	0/0	0/0	0.028	−/−	5.3×10^4	adr	C	Wild
	53d			15	1/1	0.008	2/1.5	5/2	0.151	−/−	1.3×10^4			
25	2000/ 2/12	25	M	46	0/0	0.198	0/0	0/0	0.029	−/−	6.3×10^4	adr	C	Wild
	14d			40	2/0	1.511	0/0	0/0	0.021	−/−	3.0×10^5			
	19d			77	0/0	0.248	0/0	3/0	0.071	−/+	1.3×10^4			
26	2000/ 2/15	16	M	17	0/0	0.009	0/0	0/0	0.027	−/−	3.1×10^2	adr	C	Wild
	20d			18	2/2	0.003	5/2	0/2	0.021	−/−	2.8×10^2			

NAT-positive case coexisting with anti-HBs and anti-HBc

No.	Bleeding Date	Age	Sex	ALT	HBsAg		anti-HBs	anti-HBc		HBe	HBV DNA			
					RPHA	EIA	PHA	HI	IgM-class	eAg/eAb	copies/ml	Subtype	Genotype	Precore
19	2000/ 1/26	22	M	27	0/0	0.013	4.5/1.5	12.5/0	0.002	+/−	8.8×10^5	adr	C	(Wild)
	24d			21	1.5/1.5	0.193	4.5/2.5	13/0	0.034	+/−	1.6×10^6			

Samples unable to follow-up

No.	Bleeding Date	Age	Sex	ALT	HBsAg		anti-HBs	anti-HBc		HBe	HBV DNA			
					RPHA	EIA	PHA	HI	IgM-class	eAg/eAb	copies/ml	Subtype	Genotype	Precore
2	1999/10/ 1	39	M	53	0.5/0.5	1.127	0.5/0.5	0/0	0.020	−/−	3.4×10^5	adr	C	Wild
6	1999/11/ 9	35	M	28	0/0	0.188	0/0	0/0	0.028	−/−	2.7×10^4	adr	C	Wild
16	1999/12/23	31	F	21	0/0	0.064	0/0	0/0	0.032	−/−	1.4×10^4	adr	C	Wild
20	2000/ 1/30	63	M	21	0/0	0.001	0/0	0/0	0.027	−/−	5.8×10^3	adr	C	Wild

*underline: indicates positive result.

Table 4. Infection with precore mutant HBV

No.	Bleeding Date	Age	Sex	ALT	HBsAg		anti-HBs	anti-HBc		HBe	HBV DNA			
					RPHA	EIA	PHA	HI	IgM-class	eAg/eAb	copies/ml	Subtype	Genotype	Precore
24	2000/ 2/11	64	M	18	0/0	0.003	0/0	3.5/0	0.035	−/−	4.0×10^2	adr	C	Mutant
	7d			18	2/2	0.002	2/2	3.5/2*	0.027	−/−	2.0×10^2			
	21d				0.5/0.5	0.010	1/1	3.5/0.5	0.025	−/−	4.9×10^2			
18	2000/ 1/11	56	M	26	1/1	0.000	1/1	3/1	0.023	−/−	3.6×10^3	ayw	C	Mutant
	25d			14	0.5/0.5	0.004	0.5/0.5	3/0	0.022	−/−	3.0×10^3			
22	2000/ 2/ 5	55	M	48	0/0	0.003	0/0	0/0	0.019	−/−	2.3×10^2	adr	C	Mutant
	21d			45	0/0	0.005	0/0	0/0	0.016	−/−	3.1×10^2			
27	2000/ 1/20	25	F	12	0/0	0.010	0/0	0/0	0.016	−/−	3.8×10^4	adw	B	Mutant

*underline: indicates positive result.

Table 5. Infection with HCV

No.	Bleeding Date	Age	Sex	ALT	anti-HCV(2^N)		copies/ml	Genotype
					PHA	PA		
101	1999/ 9/21	52	M	19	−	−	8.0×10^3	II (1b)
	119d			21	12.5	13	8.2×10^2	
	150d			32	14.5	15	3.5×10^3	
102	1999/10/12	16	F	20	9	−	2.7×10^4	II (1b)
	87d			29	9	−	4.7×10^5	
104	1999/12/15	37	M	19	−	−	4.3×10^7	IV (2b)
	23d			580	−	−	7.4×10^6	
108	2000/ 1/20	48	M	24	−	−	7.7×10^5	IV (2b)
	28d			355	5	−	5.4×10^4	

Samples unable to follow-up

No.	Bleeding Date	Age	Sex	ALT	anti-HCV(2^N)		copies/ml	Genotype
					PHA	PA		
103	1999/11/ 4	33	F	13	−	−	5.7×10^6	III (2a)
105	1999/12/22	21	M	26	−	−	3.1×10^7	IV (2b)
106	2000/ 1/ 8	16	M	9	−	−	1.1×10^7	III (2a)
107	2000/ 1/21	25	M	10	−	−	1.4×10^6	III (2a)
109	2000/ 2/15	34	M	52	−	−	6.5×10^5	IV (2b)

tion is of great concern. Currently much worldwide attention is focused on HBV, HCV and HIV-1 infections. As for plasma derivatives, clinical studies have verified a lack of transmission of these three viruses, since effective virus removal and inactivation procedures have been introduced for serologically screened source plasma (19, 20). However, residual risk remains due to blood transfusions from individuals with serologically undetectable levels of viremia; such cases are due to pre-seroconversion at an early stage of infection or to viral infection with immunological mutants. NAT screening of these virus complements current serological screening by direct measurement of viral genomes with high sensitivity. This results in narrowing the window period, as well as detecting immunological viral mutants. Therefore, NAT screening is urgently needed to minimize residual risk, especially in the case of blood transfusions. NAT should be done as rapidly as possible in order to be ready for transfusions of cellular components. Based on our experience of separate HBV, HCV and HIV-1 NAT of 5.6 million serologically negative units as a source plasma for plasma-derived products started in November 1997 by manual procedures, we set up the JRC NAT screening system for blood transfusion to be completed within 36-50 h after donation (21) since July 1999.

As described above, the system consists of 1) rapid serological prescreening by automated agglutination tests to avoid carry-over of high-titer virus loads to NAT; 2) immediate shipping of test samples from each blood center to NAT centers by airflight or surface transport all over Japan; 3) an automated pooling system excluding seropositives; 4) automated extraction, amplification, and simultaneous detection with a highly sensitive MPX; 5) a computerized reporting system connected to each blood center. Multiplex reagent-positive units were thereby excluded from availability for blood transfusions as well as from source plasma for plasma-derived products. Later, multiple NAT-positive samples were resolved into HBV, HCV and HIV by individual NAT. The results were then reported to the donors.

By the JRC NAT system, 26 HBV NAT-positives were detected from 2,560,977 serologically negative voluntary blood donors, and those units were excluded from blood transfusion or source plasma. As for the serological HBsAg tests (Figure), overnight EIA or the chemiluminescence method are probably more sensitive than RPHA. However, prescreening must be done as rapidly as possible for the blood to be available for transfusion of cellular components, and RPHA has been carried out in 54 blood centers nationwide. The main purpose of prescreening is to avoid carry-over of high titer virus loads to the NAT. Therefore, automated agglutination tests using PK7200 are currently the most practically suitable. Actually, since we started HBsAg screening

by RPHA and anti-HBc screening alone by HI (2, 6, 7), no fuluminant hepatitis has been reported. Serological screening of HBsAg and antibody is an indirect measurement of HBV itself. In cases of early-stage HBV infection, the ratio of HBsAg versus serum HBV DNA is lower (22). In cases with HBV-mutant infection, HBsAg is often undetectable by serological tests. It should be noted that five wild type HBV cases with a lower level of virus load (#1, 20, 21, 23, 26), four precore mutants (#18, 22, 24, 27), and one wild type HBV concomitant with both anti-HBs and anti-HBc (#19) were all negative when tested by overnight EIA.

A clinical follow-up study was carried out on 18 cases of wild type HBV infections. Case #19 was an exception, in which anti-HBV antibody might have interfered with the serological reaction. All other 17 cases showed seroconversion of IgM anti-HBc and anti-HBc by HI test, indicating that they were in early stages of primary HBV infection. In 10 of these latter cases, HBV DNA disappeared during the observation period. If negative conversion of HBV DNA is observed by further follow-up study, the donors may be able to reenter into the blood donation group.

On the contrary, in the three cases with precore mutant HBV infection, seroconversion of IgM anti-HBc was not observed and two of these cases showed continued extremely low levels of HBV DNA and anti-HBc. Thus, the JRC NAT system detected HBV DNA with undetectable levels of HBV antigen and antibody; this was observed in cases with HBV mutants, which might not be in an early stage of HBV infection. Some reports have suggested a possible linkage of precore mutant-HBV and fulminant hepatitis B in Japan (23-25) and Israel (26). Most of them may be screened out by sole high-titer anti-HBc screening (6). In Japan, the purpose of sole anti-HBc screening is to detect HBV viremia with low levels of HBs antigenemia in late stages of infection and not in window periods of early-stage infection. It is to exclude precore or core promoter mutants of HBV potentially linked to fulminant hepatitis B and is no longer a surrogate marker of HCV infection. Mutant HBV infection with undetectable levels of serological marker have been reported to cause infection in chimpanzee experiments (27, 28). Therefore, further characterization of these precore mutants is important.

Since our nationwide blood service is based on non-remunerated blood donation, the health care of voluntary donors is important. The follow-up study of NAT-positive donors in the very early stage of viral infection is necessary. We were able to differentiate between transient and chronic infection, thus disclosing viral dynamics at an early stage of infection. Results may be useful for reentry of previously NAT-positive donors into the group of blood donors. It was commented that the effect of NAT screening on the transmission of HBV is more complex (4, 29). Based on our experience since 1997, however, we are of the opinion that highly sensitive NAT screening of HBV together with that of HCV and HIV-1 NAT screening should be started with an appropriate pool size of blood units after serological routine screening (30). Triplet simultaneous NAT of HBV, HCV and HIV-1 is more favorable than individual NAT; the former may reduce the cost of reagents, shorten testing time with limited human resources.

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APPENDIX

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AUTHOR'S CORRECTION

Original Article

Nationwide Nucleic Acid Amplification Testing of Hepatitis B Virus, Hepatitis C Virus and Human Immunodeficiency Virus Type 1 for Blood Transfusion and Follow-Up Study of Nucleic Acid Amplification Positive Donors

Japanese Red Cross NAT Screening Research Group*

Volume 53, no. 3, p116-123, 2000: Page 117, column 1, line 27, "Fujirebio Inc., Tokyo" should read "Ortho Chemical Diagnostics KK, Tokyo".