cells that were freshly seeded onto another 96-well plate. Culture plates inoculated with virus-spiked DMEM2 were fixed with acetone for DIFA. Subsequently, cells that were inoculated with original culture fluid were incubated for 72 h at 35 °C (first-subculture), and 50-μl aliquots of culture fluid were transferred onto newly seeded Neuro-2a cells in 96-well plates. The plates that were inoculated with the original culture fluid were fixed as above. Plates inoculated with first subculture fluid were incubated for 72 h at 35 °C and were fixed (second subculture). Table 1 shows data from three independent experiments. The number of virus antigen-positive wells increased in subculture with culture fluid. The number of positive wells in the first-subculture was same as that in the second subculture. Hence, the first subculture was used for further analyses.

3.4. *In vitro cell-based immunofluorescence assay*

From the results above, we developed a novel *in vitro* cell-based immunofluorescent assay, in which 2 ml of reconstituted rabies vaccine is mixed with 10 ml of DMEM2 and is then dispensed onto Neuro-2a cells cultured in 96-well plates. Culture medium is then removed and 120-μl aliquots of the mixture are dispensed per well. Cells are then incubated at 35 °C for 3 days, and 50-μl aliquots of culture supernatant are transferred onto another 96-well plate containing fresh cells. Plates are used for DIFA after incubation for 72 h at 35 °C.

3.5. *Comparison of sensitivity between in vitro and in vivo assays*

Sensitivities of *in vivo* and *in vitro* assays were compared. As shown in Fig. 4, reconstituted vaccine products were spiked with the infectious HEP-Flury strain at doses of 150, 30, 6, 1.2, 0.24, and 0.048 ffu/20 μl using serial 5-times dilutions of the reconstituted vaccine product. For *in vitro* assays (left), 2-ml doses of sample were mixed with 10 ml of DMEM2 and were distributed onto Neuro-2a cells in 96-well plates (120 μl/well containing 20-μl virus spiked vaccine product). For *in vivo* assays (right), each sample dose was inoculated into 8 suckling mice by intracerebral injection (20 μl/mouse). Experiments were performed 3 times on separate occasions and data are presented as the mean in Fig. 5. Although detection frequencies were identical in *in vitro* and *in vivo* assays with 0.048 ffu/well or mouse, positive detection rates after doses of 0.24, 1.2, and 6 ffu/well or mouse were significantly higher in *in vitro* than *in vivo* assays. Median lethal dose (LD_{50}) values in suckling mice and median cell culture infective dose ($CCID_{50}$) values from *in vitro* assays were calculated using probit analyses [21,22] with the Bioassay Assist software (NIID, Japan), and indicated by 50% positive doses (Table 2). $CCID_{50}$ values in the *in vitro* assay were significantly lower than equivalent LD_{50} values in the *in vivo* assay ($p < 0.001$). Moreover, narrower 95% confidence intervals were produced in *in vitro* assays than *in vivo* assays, probably due to small numbers of mice. Average $CCID_{50}$ and LD_{50} values in each assay were 0.465 ± 0.097 and 2.650 ± 0.99 ffu/well or mouse, respectively, suggesting that the *in vitro* assay is 5.7 times more sensitive than the *in vivo* assay.

Table 1
Effects of subcultures on detectability of residual virus.

Exp. No.	Number of positive wells/plate		
	Original culture	First subculture	Second subculture
1	8	11	11
2	7	11	11
3	5	10	10

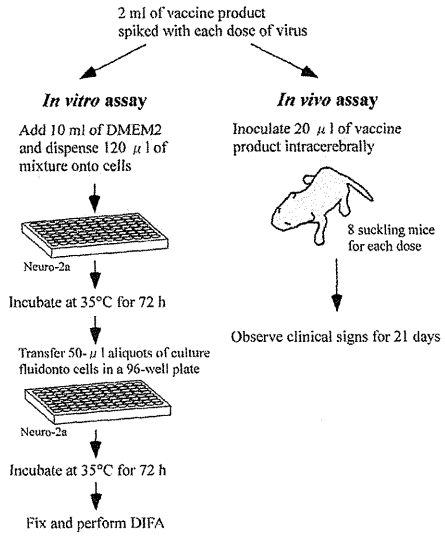


Fig. 4. Schematic of assay design for comparisons of *in vitro* and *in vivo* assays. The *in vitro* assay was performed using Neuro-2a cells cultured in 96-well plates. Reconstituted vaccine spiked with each dose of virus was mixed with the culture medium and added to Neuro-2a cells in 96-well plates. After 72 h incubation, 50-μl aliquots of culture fluids were transferred onto fresh Neuro-2a cells on 96-well plates and further incubated for 72 h. The plates were tested for virus antigen using DIFA (left panel). The *in vivo* assay was performed in 8 suckling mice intracerebrally inoculated with vaccine suspensions. Clinical signs were observed for 21 days (right panel).

3.6. *Detection limits of the in vitro assay*

To determine the detection limits of the *in vitro* assay, we examined its sensitivity after spiking with very small quantities of virus in vaccine product. The HEP-Flury strain was spiked into the vaccine product at 0–0.125 ffu/well using serial 2-times dilutions of reconstituted vaccine product, and was inoculated into Neuro-2a cells in 96-well plates (Fig. 6). For each dose, 96 wells were inoculated. Only one positive well was detected at a dose of 0.008 ffu/well (Table 3). When 0.016 ffu of active virus was added per well (20 μl), one or more positive wells were detected in 8 of 10 tests. The detection limit for the *in vitro* assay was 0.02 ffu/well, which was 3.3 times the SD of the regression line *y*-intercept and the slope of the low concentration curve according to the VICH Guideline 2

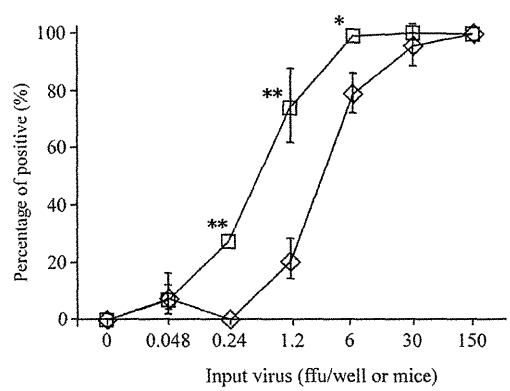


Fig. 5. Percentages of positive replicates in *in vitro* and *in vivo* assays. Percentage of positive wells in the *in vitro* assay was calculated as (number of positive wells/96) × 100 (open square) and that in the *in vivo* assay was calculated as (number of dead mice/8 suckling mice) × 100 (open rhombus). Data were expressed as the mean ± SD of three independent experiments. Significant differences were identified using Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 2
50% positive dose in *in vitro* and *in vivo* assays.

Exp. No.	Number of positive wells/plate	
	In vitro assay[a]	*In vivo* assay[b]
1	0.363 (0.295–0.447)	2.536 (0.949–6.852)
2	0.556 (0.434–0.707)	2.707 (1.241–5.881)
3	0.477 (0.388–0.589)	2.707 (1.241–5.877)

Each dose was tested *in vitro* and *in vivo* in 96 wells and 8 suckling mice, respectively.
[a] $CCID_{50}$ of *in vitro* assay: ffu/well (95% CI).
[b] LD_{50} of *in vivo* assay: ffu/mouse (95% CI).

[23], and was equivalent to 1 ffu/ml of vaccine product. Virus input strongly correlated ($r^2 = 0.96$) with numbers of positive wells.

4. Discussion

Confirmation of inactivation and potency of rabies vaccines is necessary before release for human use. Because inactivation failure can cause death [2], vaccine inactivation test are crucial for vaccine quality assurance. In Japan and across the world, manufacturers are required to perform such testing after harvesting inactivated and final products according to the WHO TRS, the Japanese MRBP, and other regulatory authorities [4–8,12].

The WHO and EP allow NRAs and manufacturers to use *in vitro* cell-based assays for inactivation testing [4,12]. Mitchell et al. [24] developed a sensitive inactivation test for rabies vaccines. Although this assay still uses animals, the vaccine is passed through primary hamster kidney cell cultures before mouse inoculation. Using this combination method, the virus is detected with at least 100 times more sensitivity than direct mouse inoculation methods. Because they used immunocompetent adult mice for mouse inoculation test, this significant enhancement may reflect immunological effects. Equivalent sensitivity of cell-based and *in vivo* assays for residual infectious West Nile virus [25] has been reported, suggesting that cell-based methods are equally or more sensitive than suckling mouse inoculation methods. Furthermore, implementation of intracerebral inoculation requires extensive training and skills; in addition, accidental death of suckling mice occurs in approximately 9% cases in our laboratory. However, animal-based rabies inactivation tests before batch release are still used by manufacturers and NRAs in several countries, including Japan, partly because no practical *in vitro* inactivation test is available for rabies vaccines. Although an excellent report was published by Blum et al. [14], the article is available only in German. Nonetheless,

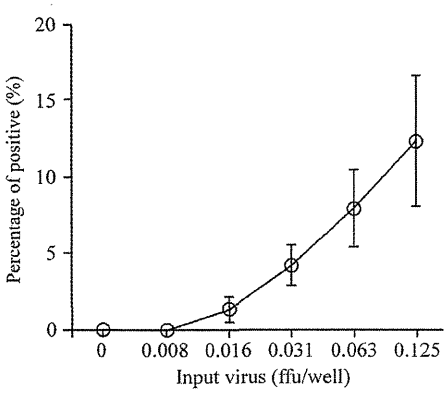


Fig. 6. Percentages of positive wells in the *in vitro* assay at very low doses. Percentages of positive wells in very low-dose the *in vitro* assays were calculated as shown in Fig. 5. Data are presented as the mean ± SD from 10 independent experiments.

Table 3
Number of positive wells in *in vitro* assay at very low doses.

Exp.No.	Input virus (ffu/well)					*y*-Intercept[a]	Slope[b]	DL[c]
	0.008	0.016	0.031	0.063	0.125			
1	0	2	2	4	9	−0.04	71	0.029
2	0	2	6	11	21	−0.50	175	0.012
3	0	2	5	9	9	1.46	73	0.028
4	0	1	4	6	8	0.63	66	0.031
5	1	2	6	11	16	0.96	129	0.016
6	0	0	3	5	10	−0.58	86	0.024
7	0	2	4	9	14	0.13	117	0.017
8	0	2	5	5	12	0.33	92	0.022
9	0	1	3	9	12	0.29	89	0.023
10	0	0	3	7	7	0.29	64	0.032
Average	0.1	1.4	4.1	7.6	11.8	0.30	96	0.023

[a] *y*-Intercept of the linear regression.
[b] Slope of the linear regression.
[c] Detection limit was calculated as 3.3 × SD of y-intercepts/slope.

summaries of their protocols appear in WHO TRS [12] and EP monographs [4]. In particular, the WHO TRS method involves cell adsorption of the vaccine, trypsinization on day 7, and observation of cultures for at least 21 days [12]. The protocol in EP monographs is similar to that in the WHO TRS, but the observation period is 14 days [4]. These methods require large volumes of cultured cells and take over 14 days for completion. In the present study, we developed a new *in vitro* assay for the confirmation of rabies virus inactivation.

The WHO recommends tests to be conducted on those cell lines that are used for production or on others that have demonstrated greater sensitivity in inactivation tests [12]. Primary chick embryo cells, which are used for production of the rabies vaccine in Japan, were not examined in this study because primary cell cultures are more complicated to use than immortalized cell lines and are considered unfavorable for constant use in tests of inactivation. Nonetheless, purified Vero cell vaccine (PVRV) and human diploid cell vaccine (HDRV) must be examined using Vero cells and human diploid cells, respectively. In the present study, subculture of supernatants from inoculated cells enhanced residual virus detection efficiency and was easier to perform than the reported cell trypsinization methods [4,12,26].

Sensitivity was significantly higher in the present *in vitro* assay than in the *in vivo* assay, and provides a novel inactivation test for rabies vaccines with sufficient reliability to be licensed as a standard assay. Small mouse numbers (8 suckling mice/dose) potentially lead to comparative insensitivity of the *in vivo* assay. Indeed, the probability of infection in the *in vitro* assay is heightened by an increased number of replicates, and positive detection was observed at a dose of 0.048 ffu/well or mouse. Increasing mice numbers are not practical; thus, giving rise to a significant advantage of the *in vitro* approach. A large number of samples also contributed to the high precision of the *in vitro* assay.

In this study, we showed detection limits as 10 times lower than those previously reported [14]. This increased sensitivity may reflect cell types (Neuro-2a or BHK-21), virus strains (HEP-Flury or CVS), incubation conditions, or other differences. In the previous study, BHK-21 cells were cultured in 96-well micro titer plates and were inoculated with 30-μl samples of the CVS rabies virus strain in 100 μl of MEM without FBS. After 2 days incubation at 34 °C, cells were fixed and immunofluorescence assays were performed using anti-Rabies G protein-FITC-conjugated antibodies. In agreement with the present data, the previous study showed no inhibition of active virus, even in the presence of high concentrations of inactivated virus. Although adjuvants and preservatives reportedly affect *in vitro* test detection limits, we did not verify this in direct comparisons of the present vaccine and preservative-free test samples.

The present *in vitro* assay takes only 6 days to perform, whereas current suckling mouse inoculation methods require 14–21 days [4–8,12]. In addition to improvements in animal welfare, the *in vitro* assay is easier, more cost-effective, and less time-consuming than the established *in vivo* assay that is performed according to Japanese MRBP [8], EP monographs [4], and WHO technical reports [12].

In summary, we developed a novel *in vitro* assay and confirmed its sensitivity in comparison to the *in vivo* suckling mouse assay. Although further validation of each vaccine product is required to investigate the influences of adjuvants and other additives, the present *in vitro* assay may be a useful method for testing of both human and veterinary vaccines. The present data warrant further consideration of this *in vitro* assay for routine quality assurance of inactivated rabies vaccines.

Acknowledgments

All animal studies were performed in accordance with the Research Animal Care Policies of the NIID (approval code H23-110068). This work was supported by JSPS KAKENHI, Grant Number 23790516, and a Grant in Aid from the Ministry of Health Labour and Welfare, Japan; Grant Numbers H21-IYAKU-IPPAN-010 and H24-IYAKU-IPPAN-002. The authors would like to thank Enago (www.enago.jp) for the English language review.

References

[1] Knobel DL, Cleaveland S, Coleman PG, Fèvre EM, Meltzer MI, Miranda MEG, et al. Re-evaluating the burden of rabies in Africa and Asia. Bull World Health Organ 2005;83:360–8.
[2] Pará M. An outbreak of post-vaccinal rabies (rage de laboratoire) in Fortaleza, Brazil, in 1960. Residual fixed virus as the etiological agent. Bull World Health Organ 1965;33:177–82.
[3] CDC. Notice to Readers: manufacturer's recall of human rabies vaccine. Morb Mortal Wkly Rep 2004;53:287–9.
[4] European Pharmacopoeia. Rabies vaccine for human use prepared in cell cultures. In: Monograph 0216 2008. pp. 699–701.
[5] U.S. Pharmacopoeia. Rabies vaccine. USP29-NF24; 2012. p. 1887.
[6] British Pharmacopoeia. Rabies veterinary vaccine, inactivated. In: Monograph 0451 2012.
[7] The Japanese Pharmacopoeia. Freeze-dried inactivated tissue culture rabies vaccine: in official monographs. 16th ed.; 2011. p. 1333.
[8] National Institute of Infectious Diseases. Freeze-dried inactivated tissue culture rabies vaccine. In: Minimum requirements for biological products 2006. pp. 40–3.
[9] Gifford G, Agrawal P, Hutchings D, Yarosh O. Veterinary vaccine post-licensing safety testing: overview of current regulatory requirements and accepted alternatives. Procedia Vaccinol 2011;5:236–47.
[10] Stokes W, Kulpa-Eddy J, McFarland R. The international workshop on alternative methods to reduce, refine, and replace the use of animals in vaccine potency and safety testing: introduction and summary. Procedia Vaccinol 2011;5:1–15.
[11] Kulpa-Eddy J, Srinivas G, Halder M, Brown K, Draayer H, Galvin J, et al. Alternative methods and strategies to reduce, refine, and replace animal use for veterinary vaccine post-licensing safety testing: state of the science and future directions. Procedia Vaccinol 2011;5:106–19.
[12] World Health Organization. Annex 2 Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs. In: WHO Expert Committee on biological Standardization, Fifty-sixth report, technical report Series, vol. 941; 2007. pp. 83–132.
[13] Bruckner L, Cussler K, Halder M, Barrat J, Castle P, Duchow K, et al. Three Rs approaches in the quality control of inactivated rabies vaccines. Altern Lab Anim 2003;31:429–54.
[14] Blum SA, Braunschweiger M, Krämer B. How to prove complete virus inactivation in rabies vaccines. A comparison of an in vivo to an in vitro method. Altex 1998;15:46–9.
[15] Yamada A, Kuniaki S, Sako M, Nonaka S. Production and quality control of rabies vaccine. In: Fukai K, editor. Virus vaccines in Asian countries. Tokyo: University of Tokyo Press; 1986. pp. 83–90.
[16] Kondo A. Growth characteristics of rabies virus in primary chick embryo cells. Virology 1965;27:199–204.
[17] Arai YT, Ogata T, Oya A. Studies on Japanese-produced chick embryo cell culture rabies vaccines. Am J Trop Med Hyg 1991;44:131–4.
[18] Koprowski H, Black J. Studies on chick-embryo adapted rabies virus. II. Pathogenicity for dogs and use of egg-adapted strains for vaccination purposes. J Immunol 1950;64:185–96.
[19] Inoue KI, Shoji Y, Kurane I, Iijima T, Sakai T, Morimoto K. An improved method for recovering rabies virus from cloned cDNA. J Virol Methods 2003;107:229–36.
[20] Madhusudana SN, Sundaramoorthy S, Ullas PT. Utility of human embryonic kidney cell line HEK-293 for rapid isolation of fixed and street rabies viruses: comparison with Neuro-2a and BHK-21 cell lines. Int J Infect Dis 2010;14:1067–71.
[21] Bliss CI. The calculation of the dosage-mortality curve. Ann Appl Biol 1935;22:134–67.
[22] Bliss CI. The determination of the dosage-mortality curve from small numbers, Quart. J Pharm Pharmacol 1938;11:192–216.
[23] Validation of analytical procedures: methodology. Implemented in October 1999. Available at: http://www.vichsec.org/pdf/gl02_st7.pdf.
[24] Mitchell JR, Everest RE, Anderson GR, Control D. Sensitive procedure for detecting residual viable virus in inactivated rabies vaccine. Appl Microbiol 1971;22:600–3.
[25] Koldijk MH, Bogaards JA, Kostense S, de Vocht M, Gijsbers L, Ter Haak M, et al. A sensitive cell-based assay for the detection of residual infectious West Nile virus. Vaccine 2007;25:6872–81.
[26] Mondal SK, Neelima M, Reddy KSR, Rao KA, Srinivasan VA. Validation of the inactivant binary ethylenimine for inactivating rabies virus for veterinary rabies vaccine production. Biologicals 2005;33:185–9.

Degenerate polymerase chain reaction strategy with DNA microarray for detection of multiple and various subtypes of virus during blood screening

Kazuya Takizawa, Tatsuo Nakashima,* Takuo Mizukami,* Madoka Kuramitsu, Daiji Endoh, Shigeto Kawauchi, Kohsuke Sasaki, Haruka Momose, Yoshiharu Kiba, Tetsuya Mizutani, Rika A. Furuta, Kazunari Yamaguchi, and Isao Hamaguchi*

BACKGROUND: The risk of transferring blood-borne infections during transfusion is continually increasing because of newly emerging and reemerging viruses. Development of a rapid screening method for emerging viruses that might be transmitted by transfusion is required to eliminate such pathogens during blood donor screening. Owing to increased use of human materials in organ transplants and cell therapy, the risk of donor-transmitted viral infections is also increasing. Although nucleic acid amplification technology (NAT) is dedicated to blood screening, a small, convenient detection system is needed at the laboratory and hospital level.

STUDY DESIGN AND METHODS: We developed a new pathogen detection system that can detect multiple viruses simultaneously, using originally designed degenerate polymerase chain reaction primers to amplify a wide range of viral genotypes. Amplified samples were identified using a DNA microarray of pathogen-specific probes.

RESULTS: We detected very low copy numbers of multiple subtypes of viruses, such as human hepatitis C virus (HCV), human hepatitis B virus (HBV), human parvovirus B19 (PVB19), and West Nile virus (WNV), using a single plate. We also detected all genotypes of human immunodeficiency virus (HIV) but sensitivity was less than for the other viruses.

CONCLUSION: We developed a microarray assay using novel primers for detection of a wide range of multiple pathogens and subtypes. Our NAT system was accurate and reliable for detection of HIV, HBV, HCV, PVB19, and WNV, with respect to specificity, sensitivity, and genotype inclusivity. Our system could be customized and extended for emerging pathogens and is suitable as a future NAT system.

Quality and safety in blood products are major public health concerns. In addition to general quality control (QC) testing, introduction of good manufacturing practice and routine screening of blood material and products have assured consistency and quality in production and increased blood transfusion safety in recent decades. Newly developed serologic tests and nucleic acid technology (NAT) have markedly reduced the risk of transmitting human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) from infected blood.[1] Currently, several Food and Drug Administration (FDA)-licensed NAT assays are available to screen blood donors for HIV, HCV, HBV, and West Nile virus (WNV). However,

ABBREVIATIONS: DLC-chip = diamond-like carbon-coating microarray chip; dPCR = degenerate polymerase chain reaction; IC = internal control; NIBSC = National Institute for Biological Standards and Control; OE-PCR = overlap-extension polymerase chain reaction; PVB19 = parvovirus B19; TMA = transcription-mediated amplification; WNV = West Nile virus.

From the Department of Safety Research on Blood and Biologics and the Department of Virology, Medicine, National Institute of Infectious Diseases, Tokyo, Japan; the Department of Pathology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan; the Department of Veterinary Medicine, Rakuno Gakuen University, Hokkaido, Japan; Osaka Red Cross Blood Center, Osaka, Japan; and the Nihon Parkerizing Hiroshima Works Co. Ltd, Hiroshima, Japan.

Address reprint requests to: Isao Hamaguchi, Department of Safety Research on Blood and Biologics, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan; e-mail: 130hama@nih.go.jp.

*These authors contributed equally.

This study was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan.

Received for publication September 25, 2012; revision received January 26, 2013, and accepted January 27, 2013.

doi: 10.1111/trf.12193

TRANSFUSION 2013;53:2545-2555.

TABLE 1. Pathogen-specific dPCR primers for microarray detection*

Pathogens	Primer sequence (5′-3′)	GenBank Accession Number	Position (NT)	Gene name	Amplicons (bp)
HIV	RARAGGGGGGATTGGGGGGTA	NC_001802	4336-4356	Integrase	129
	YTGTCYCTGWAATAAACCCGA		4444-4464		
HCV	GAAAGCGYCTAGCCATGGCGT	D90208	59-327	5′-UTR	269
	TGCACGGTCTACGAGACCTCC		307-327		
HBV	AYTAYCAAGGTATGTTGCCCG	X70185	450-470	S	266
	GGAAAGCCCKRCGMACCACTG		695-715		
PVB19	AGTGGTGGTGAAAGCTCTGAA	NC_000883	2148-2168	NS1	122
	TCTCCTGAACTGGTCCCG		2252-2269		
WNV	GGHTGTTGGTATGGNATGGA	NC_009942	3451-3590	NS1	141
	CTCCTGGGTGRCCAAGAAC		3573-3591	NS2A	
IC	TCGAAGACGATCAGATACCGT	M10098	1147-1157	18S rRNA	129
	ATACTCCCCCCGGAACC		1259-1275		

* Code base description: M, A/C; R, A/G; W, A/T; S, C/G; Y, C/T; K, G/T; V, A/C/G; H, A/C/T; D, A/G/T; B, C/G/T; N, A/T/C/G.

the continuous development of a highly sensitive screening system is a challenging task for NAT. The focus is mainly on assay sensitivity rather than the range and diversity of viral species detected; therefore, the current NAT systems only ensure detection of a restricted range of viruses and their subtypes and not newly diverged, emerging, or reemerging viruses.[2] It has been reported that the NAT sensitivity for HCV detection differs slightly in relation to virus subtype.[3] Recent advances in organ transplantation and cell therapy have also increased the risk of donor-transmitted viral infections, such as cytomegalovirus, Epstein–Barr virus, WNV, and lymphocytic choriomeningitis virus.[4] Further development of multiple virus detection systems is required to increase coverage of a range of virus strains and subtypes. We have experienced pandemics, such as WNV in the United States in 2003 and the chikungunya virus on Reunion Island in 2006; thus, there is a need to develop a rapid virus detection system that uses a more flexible blood-testing platform and meets the safety requirements for transfusion.

HBV is one of the most geographically widespread viruses and is subdivided into eight main genotypes (A–H),[5,6] causing liver cirrhosis and hepatocellular carcinoma. Although most infectious blood units are removed by screening for hepatitis B surface antigen (HBsAg), there is clear evidence that transmission by HBsAg-negative components occurs during the serologically negative window period and late stages of infection.[7] In addition to the window period of infection, HBV blood screening is required to detect all virus genotypes. Similar to HBV, several false-negative results in minipool NAT screening were reported after the introduction of WNV NAT because of the low viral load. Moreover, WNV continues to diverge rapidly from the originally isolated strain.[8,9] Multiplex NAT assays have become the modern method for detecting several viruses, and in conjunction with automated systems, they have the potential to improve processes that ensure blood safety. Candotti and colleagues[10] have reported the feasibility of a multiplex real-time quantitative reverse-transcriptase polymerase chain reaction (PCR) for HBV, HCV, and HIV-1, suggesting that simultaneous amplification of multiple pathogens is an effective approach for improving pathogen detection methods. The flexibility provided by multiplex PCR is limited, however, because the PCR primers are designed in commonly preserved regions of the viral genomes. To increase the detectable range for multiple pathogens, PCR using degenerate primers has been developed. Recently, bioinformatics has strongly improved the design of degenerate primers, allowing the coverage of a wide range of virus subtypes. We have developed a new method for designing degenerate primers.[11] Here, we used the diamond-like carbon-coating microarray chip (DLC-chip) to reduce background noise and increase the detection sensitivity of the system.[12,13] We combined two newly developed technology platforms for a multiple pathogen detection system using a degenerate PCR–based NAT system (dPCR-NAT).

MATERIALS AND METHODS

Design of dPCR primers and microarray oligoprobes

We designed dPCR primers that hybridized with HIV, HCV, HBV, human parvovirus B19 (PVB19), and WNV genomic sequences (Table 1). We used the CoCoMo (Coordination of Common Motifs) algorithm (www.geneknot.info/cocomo)[14] for dPCR primer design. CoCoMo determines primer regions in commonly conserved nucleotide regions in the assembled nucleotide sequences of virus strains. In each case, all viral sequences were identified from GenBank and EMBL, and a low degeneracy primer set was selected as a candidate using the CoCoMo algorithm. We collected data on 2072 HIV-1 nucleotide sequences and selected 14 complete genome sequences that corresponded to each genotype of HIV-1. We designed dPCR primer sets for detecting 14 complete genome sequences, resulting in approximately 3897

TABLE 2. Oligonucleotide probe sequences of DNA microarray assay

Virus	Probe name	Sequence (5′-3′)	Tm (°C)*
HIV	IR1-1	ACTATTCTTTCCCCTGCACTGTACCCCCCAATCC	78
	IR1-2	TCTGTTGCTATTATGTCTACTATTCTTTCCCC	66
	IR1-3	CTTTAGTTTGTATGTCTGTTGCTATTATGTCTAC	63
	IR1-4	GTAATTTGTTTTTGTAATTCTTTAGTTTGTATGTCTG	66
	IR3-1	GGGATTGTAGGGAATTCCAAATTCCTGCTTGATT	76
	IR3-3	CTTTAATTCTTTATTCATAGATTCTACTACTCCTTGACTTTG	69
HCV	CF1-1	AACCGGTGAGTACACCGGAATTGCCAGGAC	77
	CF1-2	TTTCTTGGATCAACCCGCTCAATGCCTGGAGATTTGGGCG	88
	CF1-3	TGCCCCCGCAAGACTGCTAGCCGAGTAGTGTTGGG	85
	CF2-1	AGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGA	86
	CF2-2	CTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTG	81
	CF2-3	GCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCT	82
	CR2-1	TCCGGTGTACTCACCGGTTCCGCAGACCACTATGGCTCT	86
HBV	BF4-1	CTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGG	75
	BF4-2	CTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCCTC	78
	BF4-3	TGTCCTCTAATTCCAGGATCAACAACAACCAGTAC	73
	BF4-4	ATTCCCATCCCATCGTCCTGGGCTTTCGCAAAATACC	84
	BF4-5	CCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTC	84
	BF4-6	GTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAG	80
	BR4-1	CCAGAAGAACCAACAAGAAGATGAGGCATAGCAG	75
PVB19	PVB19F-1	GGCGCCTGGAACACTGAGACCCCGCGCTCTAGTAC	85
	PVB19F-2	GGCGCCTGGAACACTGAAACCCCGCGCTCTAGTAC	84
	PVB19F-3	GAACTCAGTGAAAGCAGCTTTTTCAACCTCATCACTCC	78
	PVB19R-1	GTACTAGAGCGCGGGGTCTCAGTGTTCCAGGCGCC	85
	PVB19R-2	GTACTAGAGCGCGGGGTTTCAGTGTTCCAGGCGCC	84
	PVB19R-3	GGAGTGATGAGGTTGAAAAAGCTGCTTTCACTGAGTTC	78
WNV	WNVF-1	ATGATTGATCCTTTTCAGCTGGGCCTTCTGGT	77
	WNVF-2	ATGATTGACCCTTTTCAGTTGGGCCTTCTGGTCG	80
	WNVF-3	ATGATTGATCCTTTTCAGCTGGGCCTTCTGGT	77
	WNVF-4	ACGCCGACATGATTGATCCTTTTCAGTTGGGCCT	81
	WNVR-1	ACCAGAAGGCCCAGCTGAAAAGGATCAATCAT	77
	WNVR-2	CGACCAGAAGGCCCAACTGAAAAGGGTCAATCAT	80
	WNVR-3	ACCAGAAGGCCCAGCTGAAAAGGATCAATCAT	77
	WNVR-4	AGGCCCAACTGAAAAGGATCAATCATGTCGGCGT	81
IC	IC-1	GTCGTAGTTCCGACCATAAACGATGCCGACCGG	81
	IC-2	GGCGATGCGGCGGCGTTATTCCCATGACCC	86
	IC-3	CCGCCGGGCAGCTTCCGGGAAACCAAAGTCTTTG	87
	IC-4	TCGAAGACGATCAGATACCGTCGTAGTTCCGACC	78
QC	QC	TTGGCAGAAGCTATGAAACGATATGGG	69

* The melting temperature (Tm) was calculated using NetPrimer (PREMIER Biosoft International, http://www.premierbiosoft.com/).

primer sets. For HCV, we collected 978 sequences and selected 167 complete genome sequences to design dPCR primer sets, generating 31 primer sets. For HBV, 1461 sequences were collected and 1344 complete genome sequences were selected to generate the dPCR primer sets, generating approximately 29 primer sets. For WNV, we collected 17,172 sequences and used 111 complete genome sequences to design the dPCR primer sets, generating 1649 primer sets. For PVB19, we collected 1145 sequences and selected seven complete genome sequences to design the dPCR primer sets, generating 2517 primer sets. Selected primers are listed in Table 1. The sequences of the oligonucleotide detection probes on the DLC-chip are indicated in Table 2. The probes were manually designed from regions amplified by the degenerate primers. Thirty to 42 oligomers that had a GC content between 50 and 60% were selected. The resultant melting temperature values were 62 to 88°C. The hybridization stability of the PCR fragments was biased according to strand; therefore, we designed probes on each strand of the PCR products (sense strand—same sense as forward primer).

Synthesis of genotype panel oligomers for screening primers

Genotype panel oligomers of HIV-1, HBV, PVB19, and WNV were prepared by overlap-extension PCR (OE-PCR;[15] Fig. 1A). Regions for OE-PCR were selected according to the nucleotide sequences amplified with our primers for each virus genotype. The nucleotide sequences of HBV subtypes B and C panel oligomers had the same sequence. The joining oligonucleotides listed in Supplemental Table S1 (available as supporting information in the online version of this paper) were designed using DNA works (http://helixweb.nih.gov/dnaworks/).[16] The nucleotide sequences of each genotype panel oligomers are listed in Supplemental Table S2 (available as supporting information in the online version of this paper). OE-PCR

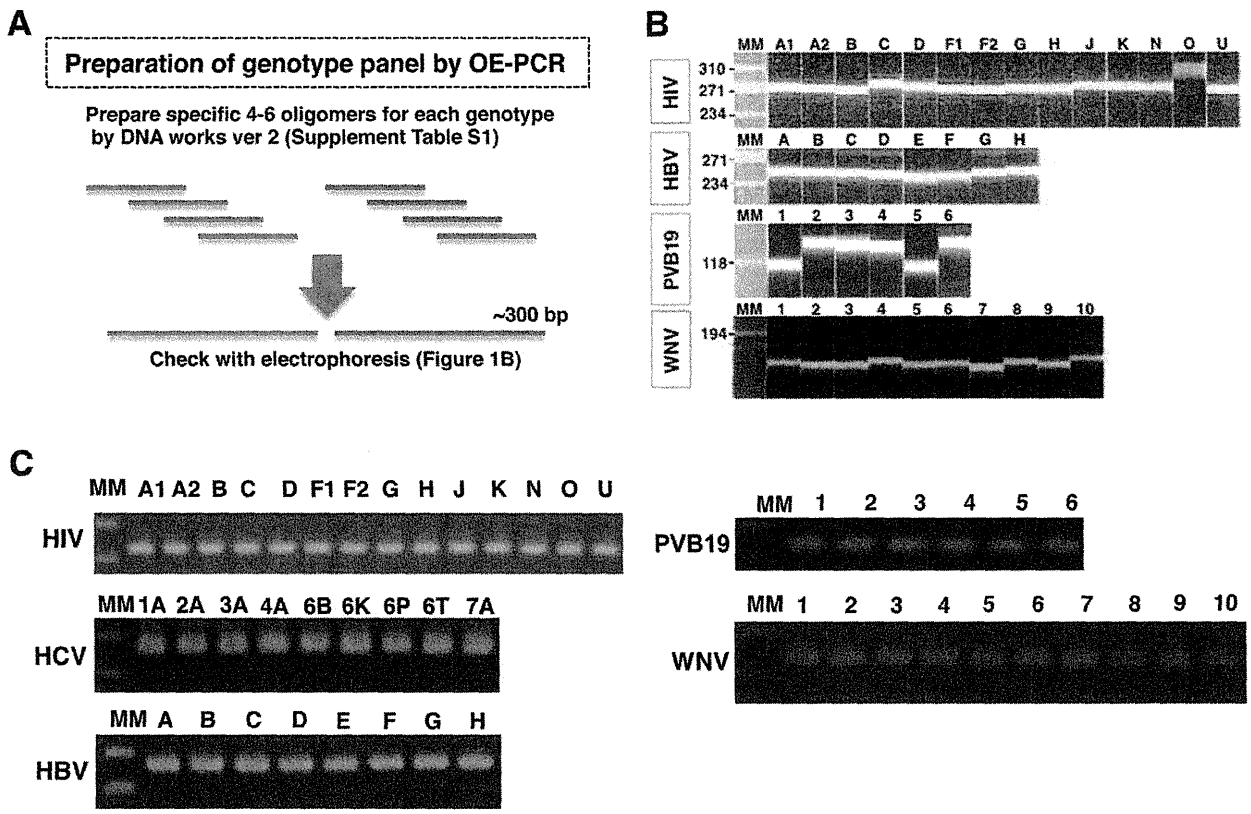


Fig. 1. Synthesis of genotype panel oligomers for HIV, HCV, HBV, PVB19, and WNV. (A) Preparation of genotype panel oligomers by PCR-based gene synthesis, OE-PCR. dPCR primers were validated with genotype panel oligomers. (B) Genotype panel oligomers were synthesized against 14 subtypes of HIV-1, eight genotypes of HBV, nine genotypes of HCV, six genotypes of PVB19, and 10 genotypes of WNV. (C) Each of the HIV-1 subtype oligomers was amplified by dPCR primers for HIV-1. The top panel shows the agarose gel electrophoresis analysis of PCR products to identify the 14 HIV-1 subtypes. Similar results were obtained from HCV, HBV, PVB19, and WNV genotype panel oligomers with specific dPCR.

was carried out according to a two-step reaction method by using a PCR kit (Prime Star, Takara-bio, Otsu, Japan; Fig. 1). The first reaction was carried out using a mixture of OE-PCR oligomers in 30 cycles of 98°C for 15 seconds, 55°C for 10 seconds, and 72°C for 15 seconds. One microliter of the first PCR products was transferred to the second PCR solution, which included 5 pmol/μL each of the 5′- and 3′-end primers. The second reaction consisted of 30 cycles at the temperature conditions used in the first reaction. The molecular weights of the OE-PCR products were checked on a chip electrophoresis system (Multina 202, Shimazu, Kyoto, Japan; Fig. 1B). HCV genotype panel oligomers were synthesized and obtained from Invitrogen (Carlsbad, CA; custom DNA oligonucleotide synthesis service).

Viral samples for dPCR-based NAT

For more accurate analysis, we purchased PVB19 NAT-based assays genotype panel (First International Standard; Category Number, 09/110 National Institute for Biological Standards and Control [NIBSC], UK); HIV-1 RNA genotype panel (Category Number 08/358 NIBSC); HCV RNA genotype panel (Category Number 08/264 NIBSC); HCV for NAT (Fourth WHO International Standard; Category Number 06/102 NIBSC); HBV for NAT (Third WHO International Standard; Category Number 10/264 NIBSC); and PVB19 DNA NAT assays (Second International Standard; Category Number 99/802 NIBSC). To evaluate the specificity of our dPCR-NAT system, we diluted each genotype panel with defibrinated plasma (Basematrix 53, SeraCare BBI Diagnostics, Milford, MA) to give a final concentration of 151 to 9722 copies/mL (HIV), 500 to 1500 IU/mL (HCV), 5754 to 123,027 IU/mL (HBV), and 870,964 to 954,933 IU/mL (PVB19), respectively. To evaluate the sensitivity of our dPCR-NAT system, we diluted each international standard with defibrinated plasma to give a final concentration of 1 to 10,000 IU/mL. Samples containing the New York strain of WNV RNA (NY 2001-6263; NATtrol, Category Number NATWNV-0005,

1 mL, 50,000 copies/mL) were purchased from Zepto-Metrix (Buffalo, NY) and were diluted with defibrinated plasma to give a final concentration of 1 to 1000 copies/mL. Blood specimens from healthy volunteer donors who were confirmed as negative for HCV, HBV, and HIV were provided from Japan Red Cross and used as a negative control.

DNA and RNA extraction, reverse transcription, and PCR amplification

Each viral DNA and RNA was extracted from 200 μL of diluted sample with a viral nucleic acid kit and a viral RNA kit (High Pure, Roche, Basel, Switzerland). Total RNA of HCV, HIV-1, and WNV were all reverse transcribed with a cDNA synthesis kit (Superscript III RT, Invitrogen) according to the manufacturer's protocol. Twenty-microliter cDNA samples were prepared for PCR amplification of each virus. The PCR was carried out with PCR mixture (GoTaq, Promega, Madison, WI). In the PCR mixture, diluted nucleic acid and 50 μmol/L of each degenerate primer were included. The reaction consisted of 50 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. We used 5 ng of human cDNA fragment as an internal control (IC).

Microchip fabrication

We purchased 3-mm^2 silicon DLC-chip from Toyo Kohan (Tokyo, Japan). Each probe was spotted by Spotarray 72 (Perkin-Elmer, Waltham, MA) with a 250-μm spot distance and 100-μm-diameter spots. Spotted probes were baked for 60 minutes at 80°C. We made DNA chips to evaluate probe sensitivity and for detection of viral samples, including WHO International Standards and WHO genotype panels. Each sequence for detection probes on the chip is listed in Table 2.

Synthesis and hybridization of fluorescently labeled DNA samples

PCR amplification of extracted DNA or cDNAs was performed for fluorescent labeling using polymerase (GoTaq, Promega) with Cy-5 dCTP. The PCR mixture included template DNA or cDNA, 50 μmol/L primers, 0.5 μL of Cy-5 dCTP (Perkin-Elmer), 1 μL of dNTP mixture (2.5 mmol/L each, 0.25 mmol/L dCTP), 5 μL of 5× PCR buffer (GoTaq, Promega), and 0.25 μL of polymerase (GoTaq, Promega). The 50-cycle PCR profile was 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Amplification was carried out in a PCR system (GeneAmp 9700, Applied Biosystems, Foster City, CA). Two microliters of the PCR-amplified reaction mixture was hybridized with the chip for 30 minutes at 50°C. The hybridized chip was washed with saline-sodium citrate buffer and scanned with a fluorescent scanner (FLA-8000, Fujifilm, Tokyo, Japan). Geographic origin was estimated from the obtained fluorescence patterns, thereby indicating specific genotypes.

Transcription-mediated amplification assay

To validate our assay sample preparation including viral DNA or RNA, we performed transcription-mediated amplification (TMA) assays for HBV, HCV, and HIV-1 by using an assay kit (Ultrio, Novartis Pharma, Tokyo, Japan) according to the manufacturer's protocol.

RESULTS

Synthesis of genotype panel oligomers by OE-PCR

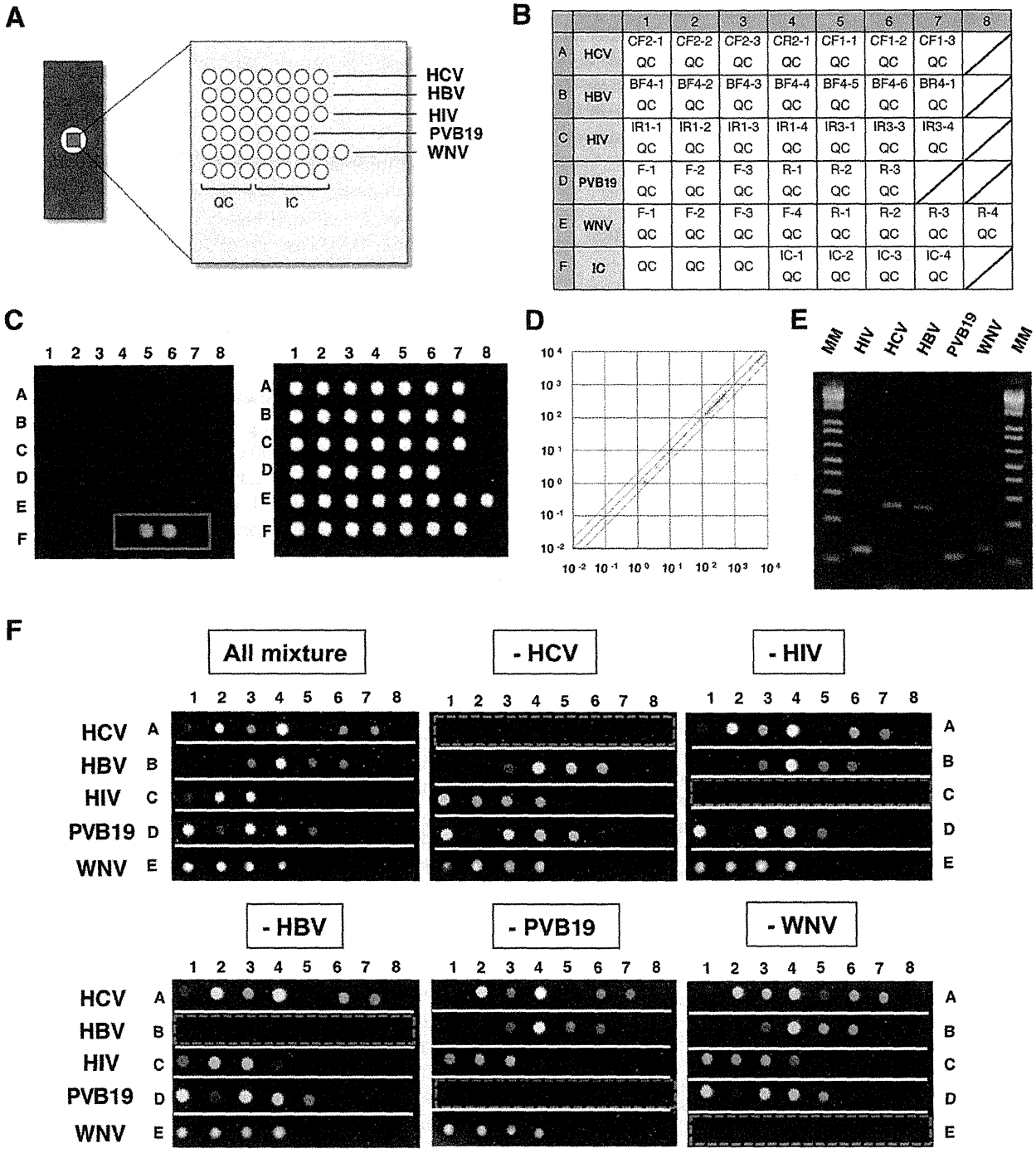
To verify our designed dPCR primers, we prepared genotype panel oligomers (100-300 bp) as viral genotype reference materials for HIV (A1, A2, B, C, D, F1, F2, G, H, J, K, N, O, and U), HBV (A–H), WNV (1-10), and PVB19 (1-6) made by OE-PCR (Fig. 1A). The target regions for OE-PCR were selected according to the nucleotide sequences of the standard strain for dPCR and the DLC-chip detection system (Supplemental Table S2). The joining oligonucleotides were designed using an online computer program (DNAWorks, Version 2, http://helixweb.nih.gov/dnaworks/). The nucleotide sequences of each genotype panel oligomer are listed in Supplemental Table S1. OE-PCR was carried out according to a two-step reaction method. The OE-PCR products were checked on a chip electrophoresis system (Multina 202, Shimazu). As expected, we could detect our OE-PCR products at appropriate molecular size (Fig. 1B). For HCV (1A, 2A, 3A, 4A, 6B, 6K, 6P, 6T, and 7A), genotype panel oligomers were designed and made by custom service (Invitrogen).

Validation of dPCR primer for detection of virus genotype panel oligomers

We confirmed whether our designed primers could specifically amplify each virus subtype by PCR. The PCR products for all HIV, HCV, HBV, PVB19, and WNV subtypes were detected at the expected size by gel electrophoresis (Fig. 1C).

QC of our pathogen detection DNA microarray system

To evaluate specificity of each probe in our DLC-chip, the detection ability of our probes was analyzed. We selected 36 specific probe sets that had high detection abilities (>50% of genotypes) from the 53 originally designed evaluated probes (data not shown) and then spotted them on DLP-Chip (Fig. 2A, B). DNA microarray images were



		1	2	3	4	5	6	7	8
A	HCV	CF2-1 QC	CF2-2 QC	CF2-3 QC	CR2-1 QC	CF1-1 QC	CF1-2 QC	CF1-3 QC	
B	HBV	BF4-1 QC	BF4-2 QC	BF4-3 QC	BF4-4 QC	BF4-5 QC	BF4-6 QC	BR4-1 QC	
C	HIV	IR1-1 QC	IR1-2 QC	IR1-3 QC	IR1-4 QC	IR3-1 QC	IR3-3 QC	IR3-4 QC	
D	PVB19	F-1 QC	F-2 QC	F-3 QC	R-1 QC	R-2 QC	R-3 QC		
E	WNV	F-1 QC	F-2 QC	F-3 QC	F-4 QC	R-1 QC	R-2 QC	R-3 QC	R-4 QC
F	IC	QC	QC	QC	IC-1 QC	IC-2 QC	IC-3 QC	IC-4 QC	

captured by an image analyzer (FLA-8000, Fuji Photo Film, Tokyo, Japan). Hybridized slides were inserted in the FLA-800, and the scan conditions were set as 10 μm resolution, standard scan mode, and photomultiplier tube high-value 100% laser. Saved DNA microarray images were analyzed, and fluorescent intensity of each spot was measured using a computer program (ArrayGauge Software, Fuji Photo Film). After removing the background signal, we defined the positive signal standard as radio intensity more than five times higher than background. The signal intensity was calculated as the total pixel value minus the global background. The signal intensities were then normalized to the mean for all the spots in the array.

To determine the specificity of the new system, we applied two different QCs: an IC probe (oligonucleotide complementary to the Cy5-labeled amplicon of the 18S

Fig. 2. (A) Schematic design of DLC-chip, including HIV, HCV, HBV, WNV, and PVB19 probes (B) and their relative position in the slide layout. Spotted probes of seven subtypes of HIV-1, seven genotypes of HCV, seven genotypes of HBV, six genotypes of PVB19, and eight genotypes of WNV were selected. All of the probes were spotted together on the chip, along with the QC probes. (C) Evaluation of the detection system using the IC (left panel) and QC (right panel). Anti-IC probes specifically hybridized to and amplified 18S rRNA PCR product from human DNA. (D) QC by using same amplified sample. Correlation coefficients using scatter plot indicated that each DLC-chip was highly reproducible, with a correlation of 0.99905. (E) Multiple detection system for five virus genomes. Agarose gel electrophoresis analysis of PCR products to detect HIV-1, HCV, HBV, PVB19, and WNV. These PCR products were positive on the microarrays. (F) Multiple detection system for five viruses by DLC-chip. The PCR products of five viruses were mixed at the following concentrations: HCV, 10 IU/mL; HBV, 10 IU/mL; HIV-1, 10,000 IU/mL; PVB19, 10 IU/mL; and WNV, 10 copies/mL. Each panel used a mixture of the five viruses that lacked HIV-1, HCV, HBV, PVB19, or WNV. The red line indicates the row where excluded virus was not detected. Cross-hybridization between the five viruses was not confirmed.

rRNA gene; Fig. 2C, left) and a QC probe (oligonucleotide complementary to the Cy3-labeled QC probe; Fig. 2C right). This allowed us not only to monitor the spot uniformity, but also to detect potential irregularities during the hybridization process. To evaluate the reproducibility of our DLC-chip, we hybridized the same PCR-amplified samples to different DLC-chips and measured each signal intensity. Correlation coefficients using scatter plots indicated that each DLC-chip was highly reproducible, showing a correlation of 0.99905 (Fig. 2D).

Multiple detection of five viruses in one test

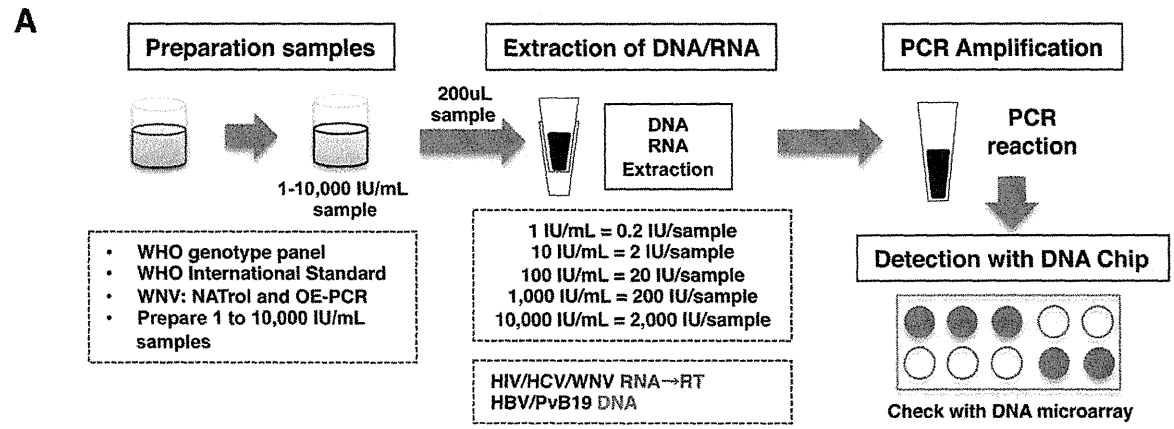
We determined whether our designed probes could detect the PCR products of all the HIV genotypes. On the DLC-chip, seven different HIV-1–specific probes were aligned. After being labeled with Cy5, PCR products were detected by hybridization to HIV-specific probes on the DLC-chip. We considered that a sample was positive if at least two different probes showed a positive signal. These HIV-1–specific degenerative primer and probe sets detected all of the following genotypes: A1, A2, B, C, D, F1, F2, G, H, J, K, N, O, and U (data not shown). Similar to the HIV detection system, different probe sets detected all the HCV, HBV, PVB19, and WNV genotypes (data not shown). To determine the ability to detect multiple viruses on one DNA chip, we separately performed virus-specific genome amplification (Fig. 2E) and mixed each PCR product in one tube and hybridized the PCR products onto the DLC-chip. The mixed viral PCR product (HIV, HCV, HBV, PVB19, and WNV) was readily detected as a hybridized spot on the DCL-chip (Fig. 2F). We prepared a mixed sample minus one virus amplicon as a negative control for cross-hybridization and nonspecific binding. We confirmed that no cross-hybridization occurred with HIV-1, HCV, HBV, PVB19, and WNV.

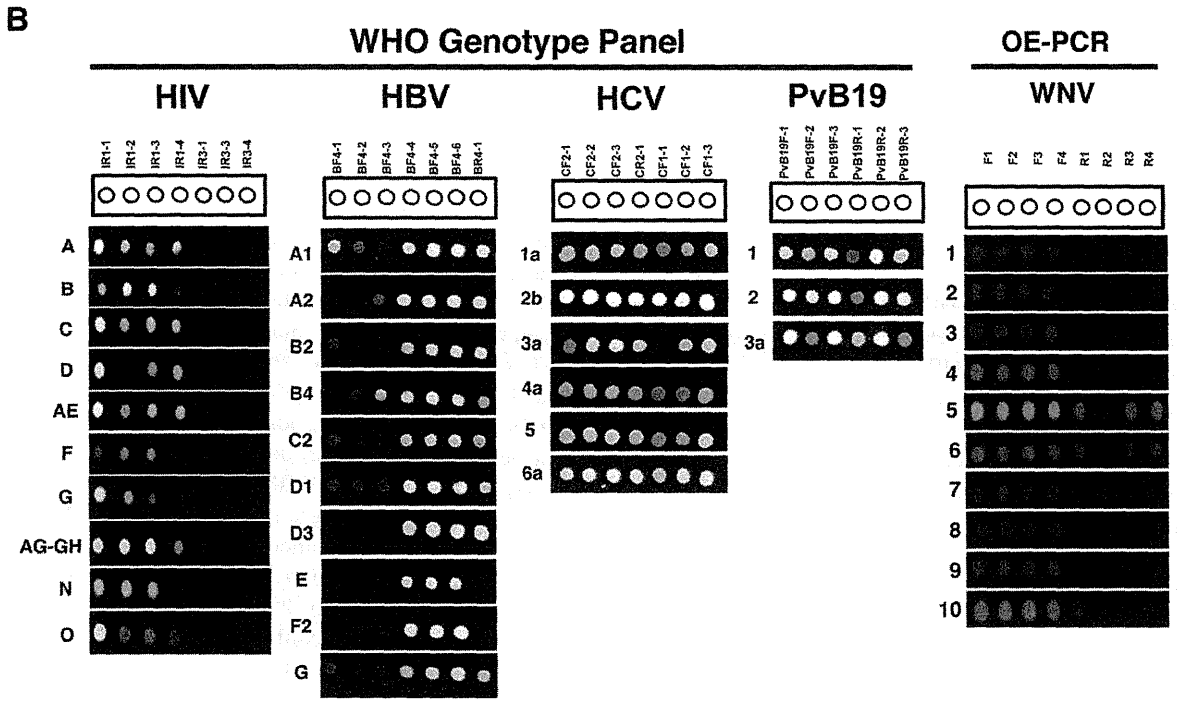
Specificity and sensitivity of our dPCR-NAT system by using WHO genotype panels and international standards

To determine the specificity of our DNA microarray system to detect each virus genotype, we prepared the WHO genotype panel samples for each virus. For WNV, we used the genotype panel oligomer described in Figure 1 as an NAT genotype panel because there was no commercially available panel. We extracted DNA or RNA from each genotype panel for HIV, HCV, HBV, PVB19, and WNV. Each template DNA or cDNA was amplified with each dPCR primer listed in Table 1. The amplified PCR products for all genotypes of each virus were confirmed on 3% agarose gels and DNA chips. We detected all the HIV, HCV, HBV, PVB19, and WNV genotypes (Fig. 3B). Our DNA microarray data are summarized in Supplemental Table S4 (available as supporting information in the online version of this paper). To validate the sensitivity of our NAT system, we used WHO International Standards as reference materials. To check our reference samples, we performed FDA-licensed NAT assays using a TMA assay (Supplemental Table S3, available as supporting information in the online version of this paper) before analysis. For sensitivity assay, we prepared HCV RNA, HBV, and PVB19 from NIBSC. These materials were used as international standards for NAT quality assurance. We prepared each sample to give a final concentration of 1 to 10,000 IU/mL and isolated DNA or RNA from 200-μL samples. Thus, each sample was assumed to contain 0.2 to 2000 IU virus if extraction efficiency was 100%. DNA and RNA were extracted. RNA samples were all reverse transcribed with a cDNA synthesis kit (Superscript III RT, Invitrogen), and all cDNA samples were used for PCR. We detected 1 IU/mL HCV, 1 IU/mL HBV, 1 IU/mL PVB19, and 1 copy/mL WNV (Fig. 3C). Similar results were obtained from at least three independent experiments. For HIV, we estimated the detection limit in at least five independent tests. We detected 10,000 IU/mL for 100%, 1000 IU/mL for 77%, 100 IU/mL for 7%, 10 IU/mL for 0%, and 1 IU/mL sample for 0%. Thus we conclude that our system could detect 1000 IU/mL equivalent to 200 IU/PCR sample for HIV. Detection limits for each virus are listed in Table 3.

DISCUSSION

We investigated the performance of the new NAT system using dPCR primers and a DLC-chip. We showed that our NAT system was specific for HIV-1, HCV, HBV, PVB19, and


A
Preparation samples
Extraction of DNA/RNA
PCR Amplification
200uL sample
DNA RNA Extraction
PCR reaction
1-10,000 IU/mL sample
• WHO genotype panel
• WHO International Standard
• WNV: NATrol and OE-PCR
• Prepare 1 to 10,000 IU/mL samples
1 IU/mL = 0.2 IU/sample
10 IU/mL = 2 IU/sample
100 IU/mL = 20 IU/sample
1,000 IU/mL = 200 IU/sample
10,000 IU/mL = 2,000 IU/sample
HIV/HCV/WNV RNA→RT
HBV/PvB19 DNA
Detection with DNA Chip
Check with DNA microarray


B
WHO Genotype Panel
OE-PCR
HIV
HBV
HCV
PvB19
WNV
A
B
C
D
AE
F
G
AG-GH
N
O
A1
A2
B2
B4
C2
D1
D3
E
F2
G
1a
2b
3a
4a
5
6a
1
2
3a
1
2
3
4
5
6
7
8
9
10

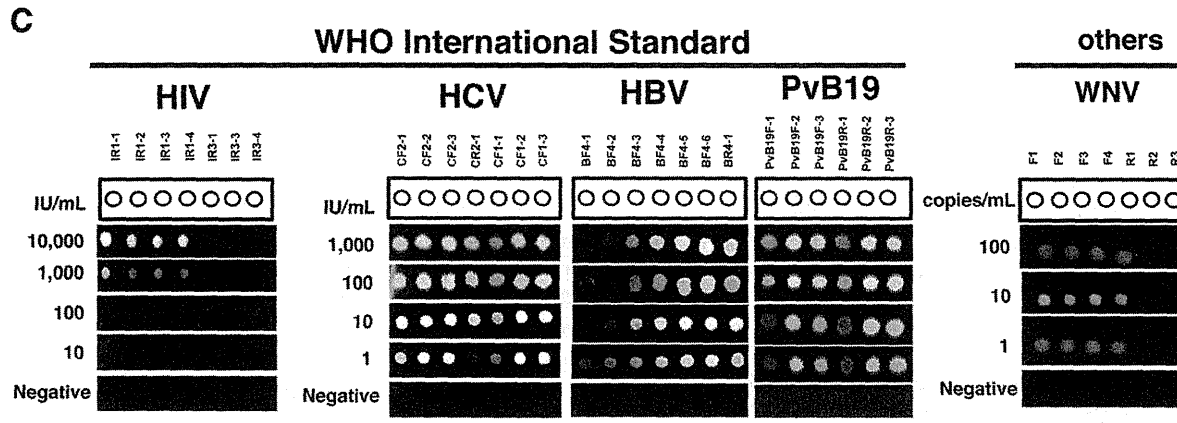

C
WHO International Standard
others
HIV
HCV
HBV
PvB19
WNV
IU/mL
10,000
1,000
100
10
Negative
IU/mL
1,000
100
10
1
Negative
copies/mL
100
10
1
Negative

Fig. 3. (A) Schematic illustration of sensitivity analysis of our detection system, including extraction process. Detection of the isolated virus genomes from human plasma sample. Samples containing HIV-1, HCV, and HBV were diluted with defibrinated plasma (Basematrix 53; SeraCare) at 1 to 10,000 IU/mL. (B) For more accurate analysis, we prepared a WHO genotype panel for HIV-1, HBV, HCV, and PVB19. We detected all genotypes by using our designed degenerated primer. (C) For sensitivity analysis of our NAT system, we prepared WHO International Standards for HIV-1, HCV, HBV, and PVB19. We diluted these materials with negative sera at 1 to 10,000 IU/mL and extracted DNA and RNA from 200-μL samples. All RNA samples were transcribed with Superscript III. We detected HIV at 1000 IU/mL, HCV at 1 IU/mL, HBV at 1 IU/mL, and PVB19 at 1 IU/mL.

TABLE 3. Detection limit of our dPCR-NAT system*

IU/mL	HIV (%)	HCV (%)	HBV (%)	PVB19 (%)
10,000	100	100	100	100
1,000	77	100	100	100
100	7	100	100	100
10	0	100	100	100
1	0	50	25	75

* % positive: Reactive/Tested (Percent Reactive). The measurement obtained in each specimen was tested with two sample lots in two independent test assays. For HIV, we tested five sample lots in five independent test assays.

WNV at low viral loads. In addition, we showed that our system detected various virus genotypes. Degenerate primers are useful not only for detecting unknown genes, but also for the simultaneous amplification of mutated genes.[17] In the case of viruses, many mutated strains appear in a particular geographical area and at a specific time.[18] Recently, we used CoCoMo primers, a fully automatic design pipeline for PCR primers, according to the CodeHop primer design strategy, by which others can analyze the oligonucleotide motif incidence.[14] The CoCoMo program is available online (www.geneknot.info/cocomo). We utilized CoCoMo primers to design the primer sequences in this study. The algorithm-designed primers were confirmed to fit most subtypes or genotypes of the target viruses (Table 1) and enabled efficient detection of a wide range of viruses. In general, PCR procedures with the degenerate primers had lower sensitivity than that of the specific primers. To overcome this disadvantage, we used fluorescence detection on the DLC-chip, which provided higher sensitivity.[12] Additionally, the degenerate primer set was designed to detect the polymorphic region of the viral genome; therefore, subtypes or genotypes could be discriminated on the DLC-chip. The combination of the primers and DLC-chips was therefore validated. These results suggest that the combination of dPCR and DLC-chips is beneficial for blood-borne virus detection. To increase the safe use of the system, automation of our detection system will be required in the future.

A low level of HBV may proliferate in transfused recipients who are immunocompromised or immunosuppressed. In addition, the window period of HBV is relatively long, but the presence of HBV DNA without detectable HBsAg outside the window period, known as occult HBV infection, has been reported.[7] This suggests that the development of a highly sensitive detection system for HBV is particularly important. Although the current TMA sensitivity corresponds to 1 to 5 IU/mL at the reproductive level, using the same viral samples in this study we detected 1 to 10 IU/mL HBV. These data suggest that the sensitivity of our analysis system is equivalent to that of the TMA assay (Fig. 3C). Thus far, HBV Genotype C is the most prevalent genotype in Japan (85%), while the prevalence of Genotypes A and D is 1.7 and 0.4%, respectively.[19-21] Currently, the level of Genotype A is increasing in the younger generation because of horizontal infection.[11,22,23]

For HIV-1 detection and quantification, various methods have been developed, but most real-time techniques involve their sensitivity to point mutations within primer and probe target sequences. Our dPCR-NAT system could detect a wide range of HIV genotypes by using dPCR primers. Despite the wide range of genotype detection, sensitivity was not high. We could detect 200 IU HIV/PCR procedure. Improvement is needed for HIV dPCR sensitivity. Similarly for HBV and HCV, the next-generation virus detection system must be able to cope with this situation, namely, by possessing a wide detectable viral genotype range and a low detection level. With our detection system, all of the virus genotypes were detected at 1 to 1000 IU/mL sample. Previous studies by Hsia and coworkers[24] combined multiplex PCR and DNA chips and detected three different viruses in a single sample. Our mixed PCR product data (Fig. 2F) indicated that our system could simultaneously detect five different viruses in one DNA chip. These data suggest that our system is suitable for multiple pathogen testing.

In conclusion, the dPCR-NAT system is an accurate and reliable test for HIV, HBV, HCV, PVB19, and WNV detection with respect to specificity, sensitivity, and genotype inclusivity and a reproducible assay for the detection of multiple blood-contaminating pathogens.

ACKNOWLEDGMENT

We are grateful for the advice and support of Dr Makoto Handa (Keio University).

CONFLICT OF INTEREST

All authors concur with submission of this manuscript, and we affirm that the material submitted has not previously been reported, and is not under consideration for publication elsewhere. We do not have any conflicting financial interests.

REFERENCES

1. Tabor E, Epstein JS. NAT screening of blood and plasma donations: evolution of technology and regulatory policy. Transfusion 2002;42:1230-7.
2. Stramer SL, Hollinger FB, Katz LM, Kleinman S, Metzel PS, Gregory KR, Dodd RY. Emerging infectious disease agents and their potential threat to transfusion safety. Transfusion 2009;49(Suppl 2):1S-29S.
3. Lelie PN, Cuypers HTM, van Drimmelen AAJ, Quint WGV. Quality assessment of hepatitis C virus nucleic acid amplification methods. Infus Ther Transfus Med 1998;25:102-10.
4. Fischer SA. Emerging viruses in transplantation: there is more to infection after transplant than CMV and EBV. Transplantation 2008;86:1327-39.
5. Kramvis A, Kew M, François G. Hepatitis B virus genotypes. Vaccine 2005;23:2409-23.
6. Norder H, Couroucé AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 2004;47:289-309.
7. Allain JP. Occult hepatitis B virus infection: implications in transfusion. Vox Sang 2004;86:83-91.
8. Pealer LN, Marfin AA, Petersen LR, Lanciotti RS, Page PL, Stramer SL, Stobierski MG, Signs K, Newman B, Kapoor H, Goodman JL, Chamberland ME. West Nile Virus Transmission Investigation Team. Transmission of West Nile virus through blood transfusion in the United States in 2002. N Engl J Med 2003;349:1236-45.
9. Grinev A, Daniel S, Stramer S, Rossmann S, Caglioti S, Rios M. Genetic variability of West Nile virus in US blood donors, 2002-2005. Emerg Infect Dis 2008;14:436-44.
10. Candotti D, Temple J, Owusu-Ofori S, Allain JP. Multiplex real-time quantitative RT-PCR assay for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1. J Virol Methods 2004;118:39-47.
11. Kobayashi M, Ikeda K, Arase Y, Suzuki F, Akuta N, Hosaka T, Sezaki H, Yatsuji H, Kobayashi M, Suzuki Y, Watahiki S, Mineta R, Iwasaki S, Miyakawa Y, Kumada H. Change of hepatitis B virus genotypes in acute and chronic infections in Japan. J Med Virol 2008;80:1880-4.
12. Gao Y, Chen X, Gupta S, Gillis KD, Gangopadhyay S. Magnetron sputtered diamond-like carbon microelectrodes for on-chip measurement of quantal catecholamine release from cells. Biomed Microdevices 2008;10:623-9.
13. Iwafune Y, Tan JZ, Ino Y, Okayama A, Ishigaki Y, Saito K, Suzuki N, Arima M, Oba M, Kamei S, Tanga M, Okada T, Hirano H. On-chip identification and interaction analysis of gel-resolved proteins using a diamond-like carbon-coated plate. J Proteome Res 2007;6:2315-22.
14. Jimba M, Takeshima SN, Matoba K, Endoh D, Aida Y. BLV-CoCoMo-qPCR: quantitation of bovine leukemia virus proviral load using the CoCoMo algorithm. Retrovirology 2010;7:91.
15. Hoover DM, Lubkowski J. DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis. Nucleic Acids Res 2002;30:e43.
16. Dong B, Mao R, Li B, Liu Q, Xu P, Li G. An improved method of gene synthesis based on DNA works software and overlap extension PCR. Mol. Biotechnol 2007;37:195-200.
17. Moonka D, Loh EY. A consensus primer to amplify both alpha and beta chains of the human T cell receptor. J Immunol Methods 1994;169:41-51.
18. Ha C, Coombs S, Revill PA, Harding RM, Vu M, Dale JL. Design and application of two novel degenerate primer pairs for the detection and complete genomic characterization of potyviruses. Arch Virol 2008;153:25-36.
19. Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590-4.
20. Sugauchi F, Kumada H, Sakugawa H, Komatsu M, Niitsuma H, Watanabe H, Akahane Y, Tokita H, Kato T, Tanaka Y, Orito E, Ueda R, Miyakawa Y, Mizokami M. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. Clin Infect Dis 2004;38:1222-8.
21. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, Gish RG, Tanaka Y, Kato T, Orito E, Ueda R, Miyakawa Y, Mizokami M. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811-20.
22. Yoshikawa A, Gotanda Y, Suzuki Y, Tanaka M, Matsukura H, Shiraishi T, Matsubayashi K, Kon E, Suzuki K, Yugi H; Japanese Red Cross HBV Genotype Research Group. Age- and gender-specific distributions of hepatitis B virus (HBV) genotypes in Japanese HBV-positive blood donors. Transfusion 2009;49:1314-20.
23. Matsuura K, Tanaka Y, Hige S, Yamada G, Murawaki Y, Komatsu M, Kuramitsu T, Kawata S, Tanaka E, Izumi N, Okuse C, Kakumu S, Okanoue T, Hino K, Hiasa Y, Sata M, Maeshiro T, Sugauchi F, Nojiri S, Joh T, Miyakawa Y, Mizokami M. Distribution of hepatitis B virus genotypes among patients with chronic infection in Japan shifting toward an increase of genotype A. J Clin Microbiol 2009;47:1476-83.
24. Hsia CC, Chizhikov VE, Yang AX, Selvapandiyan A, Hewlett I, Duncan R, Puri RK, Nakhasi HL, Kaplan GG. Microarray multiplex assay for the simultaneous detection and discrimination of hepatitis B, hepatitis C, and human immunodeficiency type-1 viruses in human blood samples. Biochem Biophys Res Commun 2007;356:1017-23.

SUPPORTING INFORMATION

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

Table S1. Sequence of each oligomer set for preparing genotype panel oligomers.

Table S2. Sequence of each genotype panel oligomers.

Table S3. Validation of our sample using current NAT system.

Table S4. Summary of DLC-chip analysis of genotype panels and international standards.

Transfusion and Apheresis Science 48 (2013) 95–102

Contents lists available at SciVerse ScienceDirect

Transfusion and Apheresis Science

journal homepage: www.elsevier.com/locate/transci

Online reporting system for transfusion-related adverse events to enhance recipient haemovigilance in Japan: A pilot study

Chikako Odaka [a], Hidefumi Kato [b], Hiroko Otsubo [a], Shigeru Takamoto [b], Yoshiaki Okada [a], Maiko Taneichi [a], Kazu Okuma [a], Kimitaka Sagawa [c], Yasutaka Hoshi [d], Tetsunori Tasaki [d], Yasuhiko Fujii [e], Yuji Yonemura [f], Noriaki Iwao [g], Asashi Tanaka [h], Hitoshi Okazaki [i], Shun-ya Momose [j], Junichi Kitazawa [k], Hiroshi Mori [l], Akio Matsushita [m], Hisako Nomura [n], Hitoshi Yasoshima [o], Yasushi Ohkusa [p], Kazunari Yamaguchi [a], Isao Hamaguchi [a,*]

[a] *Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, Tokyo, Japan*
[b] *Department of Transfusion Medicine, Aichi Medical University, Aichi, Japan*
[c] *Department of Laboratory Medicine, Kurume University, Fukuoka, Japan*
[d] *Department of Transfusion Service, Tokyo Jikei University, Tokyo, Japan*
[e] *Department of Blood Transfusion, Yamaguchi University School of Medicine, Yamaguchi, Japan*
[f] *Department of Transfusion Medicine and Cell Therapy, Kumamoto University, Kumamoto, Japan*
[g] *Department of Transfusion Medicine and Cell Therapy, University of Yamanashi, Kumamoto, Japan*
[h] *Department of Blood Transfusion, Tokyo Medical University Hachioji Medical Center, Tokyo, Japan*
[i] *Central Blood Institute, Blood Service Headquarters, Japanese Red Cross Society, Tokyo, Japan*
[j] *Blood Service Headquarters, Japanese Red Cross Society, Tokyo, Japan*
[k] *Kuroishi General Hospital, Aomori, Japan*
[l] *Minami Tama Hospital, Tokyo, Japan*
[m] *Shibetsu City Hospital, Hokkaido, Japan*
[n] *Sanraku Hospital, Tokyo, Japan*
[o] *Yao General Hospital, Osaka, Japan*
[p] *Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan*

ARTICLE INFO

Article history:
Received 17 May 2012
Accepted 30 July 2012

Keywords:
Adverse effects
Blood transfusions
Haemovigilance
Online system
Pilot study

ABSTRACT

Background: A surveillance system for transfusion-related adverse reactions and infectious diseases in Japan was started at a national level in 1993, but current reporting of events in recipients is performed on a voluntary basis. A reporting system which can collect information on all transfusion-related events in recipients is required in Japan.
Methods: We have developed an online reporting system for transfusion-related events and performed a pilot study in 12 hospitals from 2007 to 2010.
Results: The overall incidence of adverse events per transfusion bag was 1.47%. Platelet concentrates gave rise to statistically more adverse events (4.16%) than red blood cells (0.66%) and fresh-frozen plasma (0.93%). In addition, we found that the incidence of adverse events varied between hospitals according to their size and patient characteristics.
Conclusion: This online reporting system is useful for collection and analysis of actual adverse events in recipients of blood transfusions and may contribute to enhancement of the existing surveillance system for recipients in Japan.

* Corresponding author. Address: Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Tel.: +81 42 848 7120; fax: +81 42 567 2790.
E-mail address: 130hama@nih.go.jp (I. Hamaguchi).

1473-0502/$ - see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.transci.2012.07.008

1. Introduction

Haemovigilance is defined as the surveillance of transfusion-related adverse reactions occurring in donors and in recipients. The ultimate purpose of haemovigilance is to prevent adverse events caused by blood products to ensure maximum safety. Various haemovigilance systems have been implemented around the world, with a different approach in different countries [1–6].

In Japan, the Japanese Red Cross Society (JRCS) is the sole provider of labile blood products, and controls blood collection, processing and supply nationwide. The JRCS, in cooperation with the national government, has been collecting data on transfusion-related adverse reactions and infections nationwide since January 1993 [7]. Epidemiological surveillance in donors is being performed to ensure their health as well as the safety and quality of blood components. For recipients, suspected adverse reactions, including infections related to the blood products, are reported from medical institutions to the JRCS on a voluntary basis, and nearly 2000 suspected cases were reported each year from 2004 to 2008 [7]. The JRCS investigates the relationship between transfusion and the reported adverse events. Based on the analysis, the JRCS evaluates blood safety with the government to take appropriate and immediate measures, as required, in JRC blood centers and medical institutions. The existing surveillance system for recipients has functioned well over a number of years, and most of the reported cases have been relatively moderate to severe. However, comprehensive data on adverse transfusion reactions in all recipients are unavailable. We therefore need to establish an improved system for monitoring recipients nationwide.

We have developed an alternative reporting system to collect data on all transfusion-related reactions in recipients. A pilot study of this online surveillance system has been performed since January 2007. Here, we describe our online system and present the data collected by 12 medical institutions from January 2007 to December 2010.

2. Materials and methods

2.1. Participants in the pilot study

Seven university hospitals (Aichi Medical University, 1014 beds; Tokyo Jikei University, 1075 beds; Yamanashi University, 600 beds, Tokyo Medical University Hachioji Medical Center, 621 beds; Yamaguchi University, 759 beds; Kurume University, 1186 beds; Kumamoto University, 843 beds) initially participated in the pilot study in 2007, and five small-scale hospitals with fewer than 300 beds (Kuroishi General Hospital, Minami Tama Hospital, Shibetsu City Hospital, Sanraku Hospital, Yao General Hospital) joined this study 2 years later.

2.2. Online system

In the participating hospitals, doctors or nurses monitored transfusion-related reactions at 0, 5, and 15 min after starting transfusion, at the end of transfusion, and within 6 h after finishing the transfusion. Severe adverse events and infections were determined after detailed diagnosis in JRC blood centers. These data were gathered in the hospital transfusion department. Doctors or transfusion specialists in the department reported the data every 2 months via the worldwide web (https://www.1597532.net/). Data were collected in the National Institute of Infectious Diseases, and analyzed statistically every 2 months. The online surveillance system was password-protected, and respondents were provided with an identification and password.

2.3. Statistics

All statistical analyses were performed by the Student *t* test. Probability values less than 0.05 were considered statistically significant.

3. Results

3.1. Reporting system and classifications

Our online surveillance system was designed to collect all transfusion-related reactions in recipients. The system monitored the total number of transfusions of three types of labile blood component: red blood cells (RBC), platelet concentrates (PC) and fresh-frozen plasma (FPP), in each reporting period (Fig. 1). The number of transfusion reactions, and clinical signs and symptoms were also collected. They were classified into 16 categories, as shown in Fig. 2. Additionally, information on diagnostic data was collected (Fig. 3). Transfusion-related adverse events were categorized into non-haemolytic reactions, haemolytic reactions and post-transfusion infectious diseases. The non-haemolytic reactions included: severe allergic reaction, transfusion-related acute lung injury (TRALI), transfusion associated circulatory overload (TACO), post-transfusion purpura (PTP) and transfusion-associated graft-versus-host disease (TA-GVHD). Definitions of these severe transfusion reactions were in accord with the International Society of Blood Transfusion [8]. For non-haemolytic reactions or infections, those events not covered by the diagnoses listed were assigned to the category "Others".

3.2. Number and frequency of adverse events from 2007 to 2010

We investigated transfusion reactions collected by 12 hospitals from January 2007 to end of December 2010 (Fig. 4). During the period, 241,225 bags of labile blood products were used in 12 hospitals: 133,993 bags of RBC, 55,861 bags of FFP and 51,371 bags of PC (Fig. 4B). The proportions of RBC, FFP and PC were 55.5%, 23.2% and 21.3%, respectively, of the total amount of blood bags (Fig. 4A). There were 3,539 transfusion-related adverse events reported during the period (Fig. 4B). Of the reported reactions, the blood product that accounted for highest proportion of adverse events was PC (60.4%), followed by RBC (24.9%) and FFP (14.7%) (Fig. 4A). When the frequency of transfusion reactions was calculated according to the total number of bags, the overall incidence of adverse events was 1.47% (Fig. 4B). PC was found to induce transfusion reactions at a

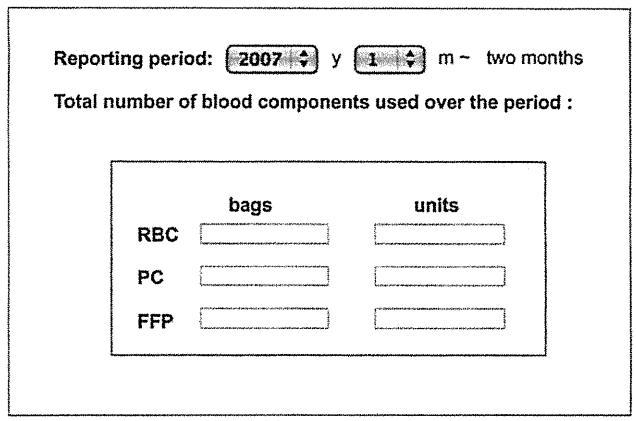


Fig. 1. Online surveillance system (1): Screenshot of the total number of the three labile blood components (bags and units) used over each reporting period. RBC: red blood cells; FFP: fresh frozen plasma; PC: platelet concentrates.

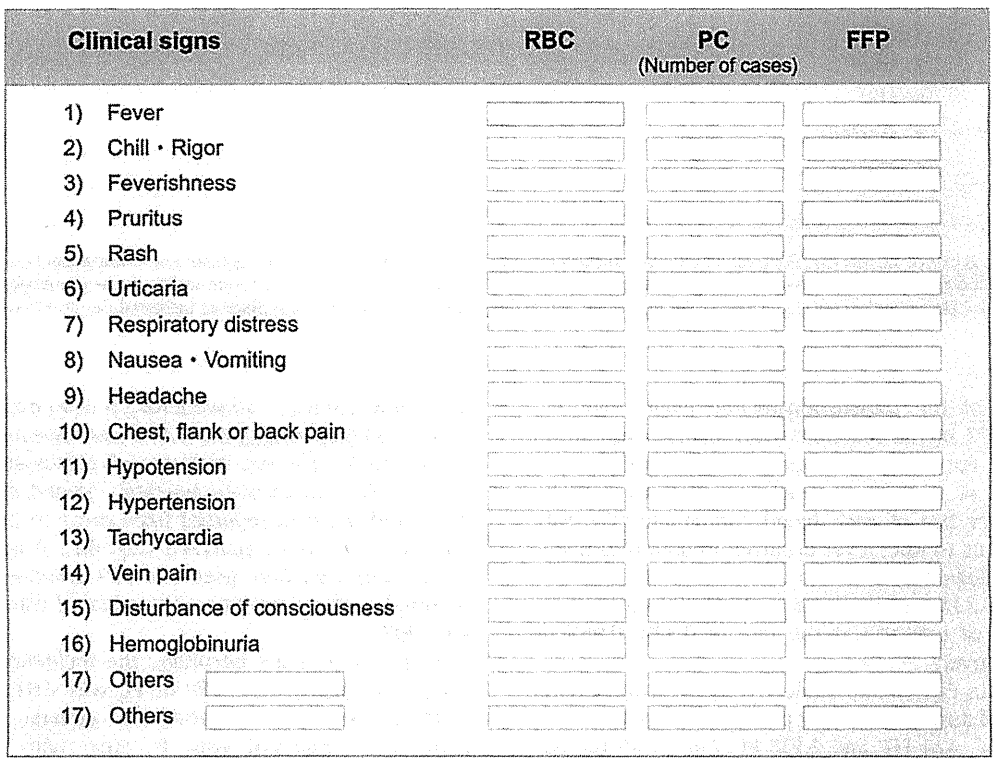


Fig. 2. Online surveillance system (2): The total number of transfusion reactions by clinical signs for the three blood components used over the reporting period is presented. Clinical signs are classified into the 16 categories indicated. Fever: more than 38 °C or a 1 °C or more increase from the baseline; hypotension: a decrease of more than 30 mmHg from the baseline; hypertension: an increase of more than 30 mmHg from the baseline; tachycardia: more than 100 times/min for adult, modified according to age for children. Any findings other than the 16 signs can be entered as free text in "Others".

rate of 4.16%. The incidence of transfusion reactions with RBC and FFP was 0.66% and 0.93%, respectively. The annual incidence of adverse events showed a similar tendency (RBC < FFP < PC) every year, as shown in Fig. 4C.

3.3. Types, clinical signs and diagnoses of adverse events

Next, we analyzed the types, clinical signs and diagnoses of adverse events collected from 12 hospitals over 4 years. The types of adverse events among the different blood components were diverse (Fig. 5A). Febrile non-haemolytic transfusion reactions (FNHTR) were more often found with RBC than with FFP or PC. Allergic reactions were observed significantly more often with FFP or PC than with RBC. In the reactions to RBC, 36.6% were FNHTR and 31.2% were caused by allergic reactions. Respiratory distress, a hypotensive reaction, and a hypertensive reaction accounted for 3.9%, 8.0% and 4.4%,

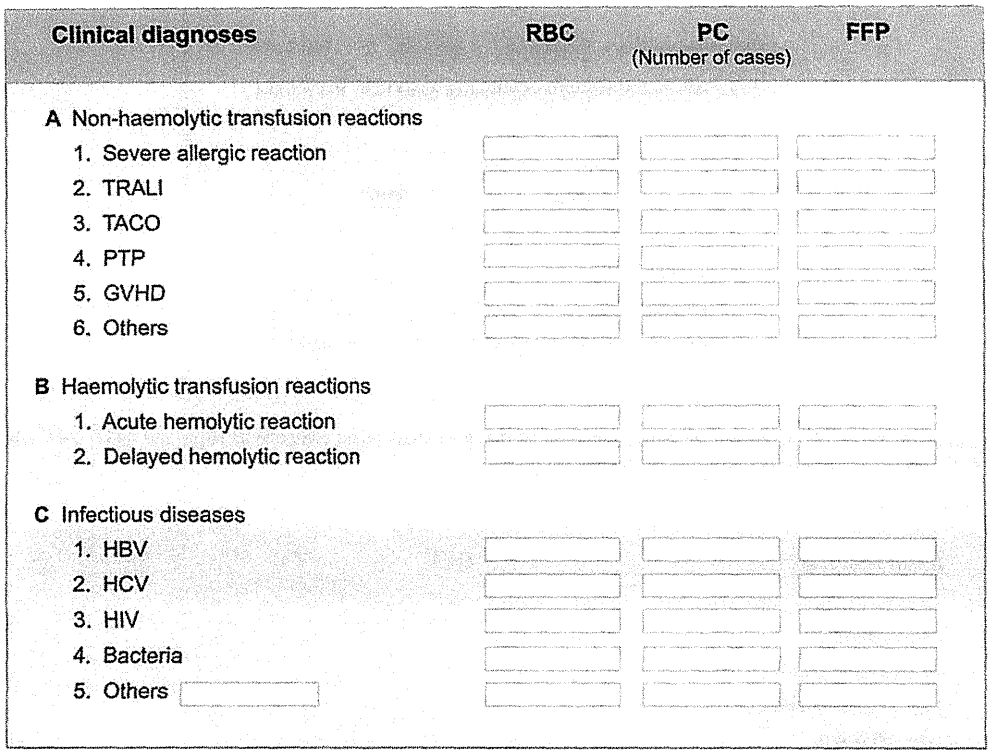


Fig. 3. *Online surveillance system (3): The total number of transfusion reactions by clinical diagnoses for the three blood components over the period is presented. Clinical diagnoses are classified into the three categories indicated. Among non-haemolytic transfusion reactions, the events not included in the diagnoses listed are placed in the category "Others". For infections, any findings other than the infectious diseases indicated can be entered as free text in "Others".*

respectively, of the transfusion-related events. For PC, more than 80% of the reactions were allergic and 11.6% were FNHTR. For FFP, 70.8% were allergic reactions. The clinical signs of transfusion reactions were assessed by the events per bag of each blood component (Fig. 5B). In the reactions to RBC, fever occurred in 0.2% of transfusion bags, followed by urticaria in 0.15%. In FFP, pruritus occurred in 0.23% and urticaria in 0.54%. PC induced fever, pruritus or urticaria at the rate of 0.32%, 0.98% or 2.85%, respectively.

As shown in Fig. 4B and Table 1, 3,539 reaction events were collected during the 4-year period, of which 881 were caused by RBC, 520 FFP and 2,138 PC. Almost all the adverse reactions reported were "Others" in non-haemolytic reactions. Severe allergic reaction, TRALI or TACO were reported at the rate of 0.1–1.3% for each blood component. In the adverse events for RBC, four cases of hemolytic reactions and one case of HBV infection were reported.

3.4. Variation in the incidence of adverse events by medical institutions

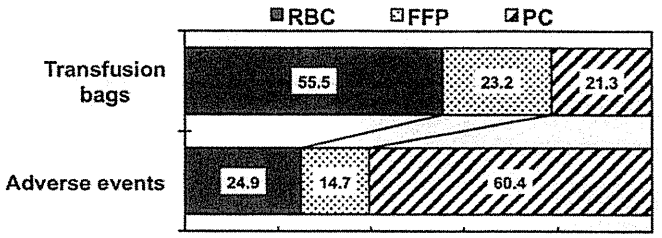
We compared the incidence of adverse events in seven large-scale university hospitals with that in five small-scale hospitals with fewer than 300 beds. Seven large-scale hospitals participated in this pilot study since 2007 and the data reported by these hospitals from 2007 to 2010 were analyzed (Fig. 6A). A total of 231,662 transfusion bags were used, of which over half were RBC, followed by FFP (23.6%) and PC (21.9%). Among the 3,410 adverse events reported, PC accounted for the majority of transfusion reactions (62.6%). Five small-scale hospitals joined this study in 2009, and the data reported from these institutions from 2009 to 2010 were analyzed (Fig. 6B). A total of 9,563 transfusion bags were used and 129 adverse events were reported in these hospitals. Over 80% of transfusion bags were RBC.

In the large-scale hospitals, the incidence of adverse events per bag of RBC, FFP or PC was 0.61%, 0.94% and 4.20%, respectively, indicating that adverse events were more often observed with PC than with FFP or RBC (Fig. 6C). On the other hand, in the small-scale hospitals, the incidence of adverse events per bag of RBC, FFP or PC was 1.46%, 0.98% and 0.59%, respectively, indicating that the adverse events were more often observed with RBC than with PC or FFP (Fig. 6C). There was a significant statistical difference in the incidence of transfusion-related adverse reactions per bag of RBC or PC in the large-scale vs. the small-scale hospitals.

4. Discussion

In our new reporting system, we analyzed the data collected from 12 medical institutions from 2007 to 2010. During the period, 241,225 labile blood products were used in these hospitals. Considering the number of blood

A. Rates of transfusion bags and adverse events by kinds of blood components



B. Incidence of transfusion reactions by kinds of blood components

	RBC	FFP	PC	Total
No. of transfusion bags	133,993	55,861	51,371	241,225
No. of adverse events	881	520	2,138	3,539
Incidence (%)	0.66	0.93	4.16	1.47

C. Annual incidence of adverse events by kinds of blood components

Year	RBC (%)	FFP (%)	PC (%)	Total (%)
2007	0.54	0.63	3.44	1.16
2008	0.61	0.69	4.22	1.45
2009	0.79	1.19	5.36	1.91
2010	0.70	1.30	3.77	1.49

Fig. 4. Proportions of transfusion bags and adverse events from 2007 to 2010. (A) The proportion of transfusion bags for each blood component and the proportion of adverse events ascribed to each component. (B) The incidence of transfusion reactions by type of blood component. (C) The annual incidence of adverse events by type of blood component.

products distributed nationwide during the 4 years, we monitored approximately 1% of the bags distributed in Japan for each blood component (data not shown). During this time, 3,539 transfusion-related adverse events were reported in this system, and the overall incidence of adverse events per bag was 1.47%. This incidence was higher than the reports from other countries which had 2.2–4.2 events per 1,000 blood products distributed [9–12]. We observed that the rate of reported cases varied considerably among seven university hospitals (data not shown). The true incidence of adverse events may be obscured by misdiagnosis. The lack of agreed definitions negatively affects data collection. The difficulty in the diagnosis of transfusion reactions also leads to misreporting. Therefore, sharing diagnostic criteria for transfusion-related reactions is required. Other studies in Japan have demonstrated similar incidences of adverse events by type of blood component (Kurata Y. et al., personal communication, 2007). Therefore, it is likely that our results reflect the real incidence of adverse events for blood products distributed in Japan.

PC (4.16%) gave rise to statistically more adverse events (6-fold) than RBC (0.66%) and FFP (0.93%). Our results were concordant with a previous report in Switzerland [12], although it should be noted that all products of PC in Japan are from single apheresis donor. PC was found to frequently induce fever, pruritus or urticaria. PC recipients, most of whom suffer from hematological diseases, tend to receive frequent blood transfusions. The repeated alloimmunization with PC may induce a high incidence of adverse events. We found that the incidence of adverse events varied between the university hospitals and the small-scale hospitals, based on the number of beds and patient characteristics. In Japan, most patients with hematological diseases have a check-up in large-scale hospitals including university hospitals. Actually, the five small-scale hospitals had no patients with hematological diseases, and their incidence of adverse events to PC was only 0.59%.

This online reporting system makes it possible to collect all transfusion-related adverse events in recipients rapidly. The database can perform calculations on the reported information automatically, and the results, such as the total number of adverse events or the incidence of adverse events, are fed back to participants continuously. This feedback should contribute to improving the safety of transfusion therapy in each medical institution. There are

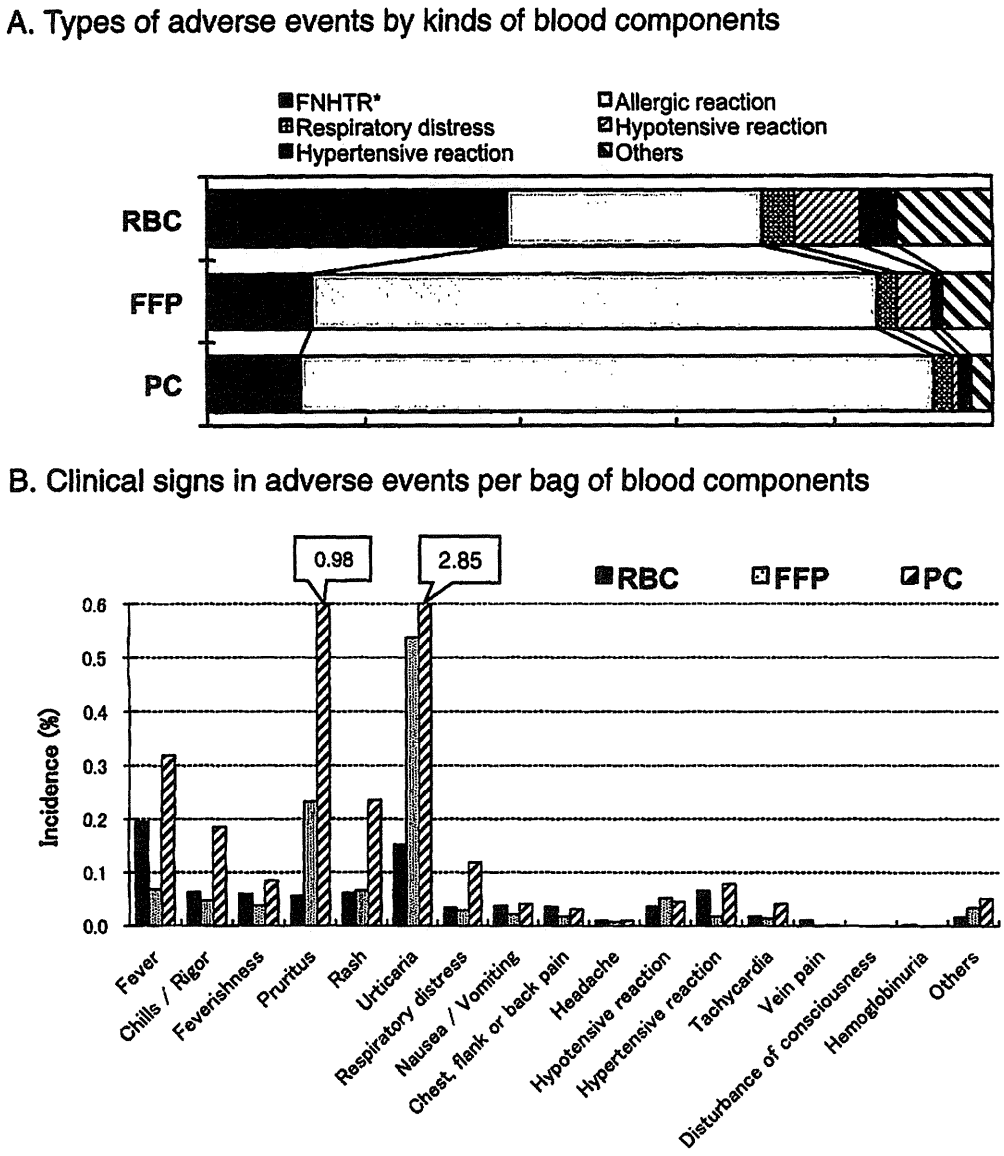


Fig. 5. Types of adverse events and clinical signs of adverse events by blood component. (A) Proportions of adverse events by type of blood component. (B) Incidence of clinical signs of adverse events by type of blood component. FNHTR: febrile non-haemolytic transfusion reaction.

a few limitations in this system. The focus of our study was only on three types of labile blood components. Information about the appearance of antibodies for each blood product was not collected. In addition, reporting of information on transfusion errors, including incorrect blood component transfusion and near-miss events, was out of the scope of the system. Almost all the adverse reactions collected for 4 years were "Others" in non-haemolytic reactions. As regards the severity of transfusion-related reactions, we speculated that the majority reactions were relatively mild. We did not confirm the individual cases of serious adverse events in this system during the period of the pilot study.

In the future, more detailed analyses of data collected by this system will be needed to determine how to improve the transfusion service and formulate new strategies to reduce adverse transfusion reactions. Almost all European Union countries have established a haemovigilance system and the number of haemovigilance systems outside Europe is steadily increasing. National haemovigilance systems linked to an international network will be indispensable to ensure the safety and quality of blood transfusions. Thus, an international standardized and centralized method for data collection and reporting is required. We have to continue to carefully monitor and compare the incidence of adverse events between Japan and other countries, in order to promote preventive measures in the manufacture of blood products in Japan, and other necessary steps to reduce transfusion-related events.