EU Official Control Authority Batch Release

Human Vaccine and Blood Derived Medicinal Products

EU Administrative Procedure for Official Control Authority Batch Release (OCABR) of Centrally Authorised Immunological Medicinal Products for Human Use and Medicinal Products Derived From Human Blood and Plasma

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European Commission

European Directorate for the Quality of Medicines & HealthCare

Document title	Procedure For Official Control Authority Batch Release (OCABR) of Centrally Authorised Immunological Medicinal Products For Human Use And Medicinal Products Derived From Human Blood And Plasma
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Previous titles and other references	This procedure has been approved by the European Commission, the EMEA and was finalised and approved by the Official Control Authority Batch Release Network and the EDQM on the occasion of their annual meeting 2001 under PA/PH/OMCL (2000) 83, DEF; editorial revisions to update reference to EU legislation under PA/PH/OMCL (2004) 143 DEF.
Custodian organisation	The present document was elaborated by the EDQM in the OMCL network and finalised under PA/PH/OMCL (10) 24 DEF

PROCEDURE FOR OFFICIAL CONTROL AUTHORITY BATCH RELEASE (OCABR) OF CENTRALLY AUTHORISED IMMUNOLOGICAL MEDICINAL PRODUCTS FOR HUMAN USE AND MEDICINAL PRODUCTS DERIVED FROM HUMAN BLOOD AND PLASMA

This procedure is aimed at defining the special steps that are applied specifically to Centrally Authorised Products when submitted to Official Control Authority Batch Release. For all other steps, the procedure for OCABR, as defined in the general EU Administrative Procedure for Official Control Authority Batch Release apply.

- For medicinal products that will be subjected to OCABR (i.e. covered under Article 114 of Directive 2001/83/EC as amended by 2004/27/EC) the Marketing Authorisation Holder (MAH) proposes an OMCL(s) which would carry out OCABR for the product in question. This should be highlighted at Pre-submission or Scientific Advice meetings with the European Medicines Agency (EMA) relevant group. The collaboration with the OMCL(s) should begin at least one year before Submission in order to allow for input to be considered in the development of suitable testing methodology for potency assays and batch release. It is proposed that the information on the chosen OMCL by the Applicant will be recorded in pre-submission meeting minutes and be passed on to the appointed Rapporteur Teams. The EMA should also inform EDQM of any upcoming start of a MAA procedure with official batch release.

- Proposed OMCL(s) determine the feasibility with input from EDQM and the EU/EEA OCABR network as needed and make a decision on their participation, informing the MAH and EMA as soon as possible if they are unable to participate so that alternative arrangements can be made. The main issue is whether or not technical capacity is available to carry out the proposed tests or can be developed in the necessary time frame.

- At the time of submission the OMCL(s) eligible for OCABR, in close collaboration with the Rapporteur and Co-rapporteur, signal the need to prepare an appropriate guideline to the relevant OCABR drafting group and/or the OCABR Advisory group

- This includes proposal of a list of tests to be performed during OCABR and highlighting of any special issues related to the model protocol

- The drafting group/Advisory group determines if a new guideline is needed, if an existing guideline can be revised to cover the needs or if existing guidelines are already sufficient to cover the new product and reacts appropriately. If necessary the appropriate guideline is developed according to the general OCABR procedure for implementation of technical documents.

- The draft OCABR guideline should be ready for adoption at the time the Community Marketing Authorisation is granted. The guideline will be formally adopted by the OMCL batch release network once the Community Marketing Authorisation has been finalised.

- When the first batches are marketed OCABR will be carried out as described in the general EU administrative procedure for OCABR.

Glossary:

EDQM: European Directorate for the Quality of Medicines & HealthCare
EEA: European Economic Area
EMA: European Medicines Agency
EU: European Union
MAA: Marketing Authorisation Application
MAH: Marketing Authorisation Holder
OCABR: Official Control Authority Batch Release
OMCL: Official Medicines Control Laboratory

III. 研究成果の刊行に関する一覧表

Ⅲ．研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Shuetsu Fukushi, Mina Nakauchi, Tetsuya Mizutani, Masayuki Saijo, Ichiro Kurane, Shigeru Morikawa	Antigen-capture ELISA for the detection of Rift Valley fever virus nucleoprotein using new monoclonal antibodies	Journal of Virological Methods	180	68-74	2012

Kazunari Kondo, Asami Uenoyama, Ryo Kitagawa, Hajime Tsunoda, Rika Kusumoto-Matsuo, Seiichiro Mori, Yoshiyuki Ishii, Takamasa Takeuchi, Tadahito Kanda Iwao Kukimoto	Genotype Distribution of Human Papillomaviruses in Japanese Women with Abnormal Cervical Cytology	The Open Virology Journal	6, (Suppl 2: M14)	277-283	2012
内藤　誠之郎	ワクチン・レギュレーションの新展開 - 国家検定へのSLP審査制度の導入	PHARM TECH JAPAN	Vol.28 No.10	25-31	2012

IV. 研究成果の刊行物・別刷

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

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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.
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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

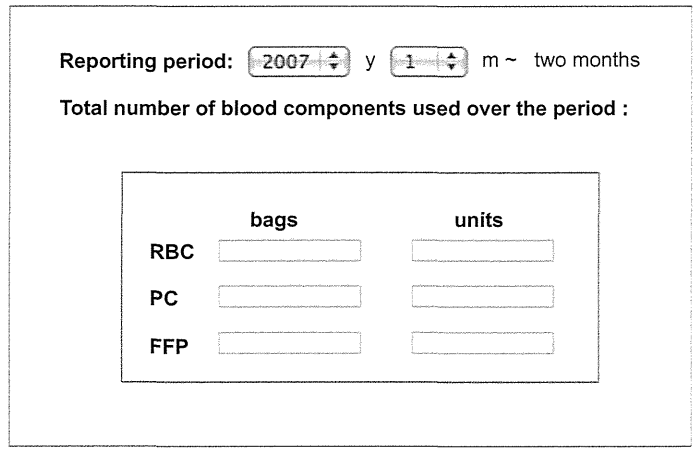


Fig. 1. Online surveillance system (1): Screenshot of the total number of the three labile blood components (bags and units) used over each reporting period. RBC: red blood cells; FFP: fresh frozen plasma; PC: platelet concentrates.

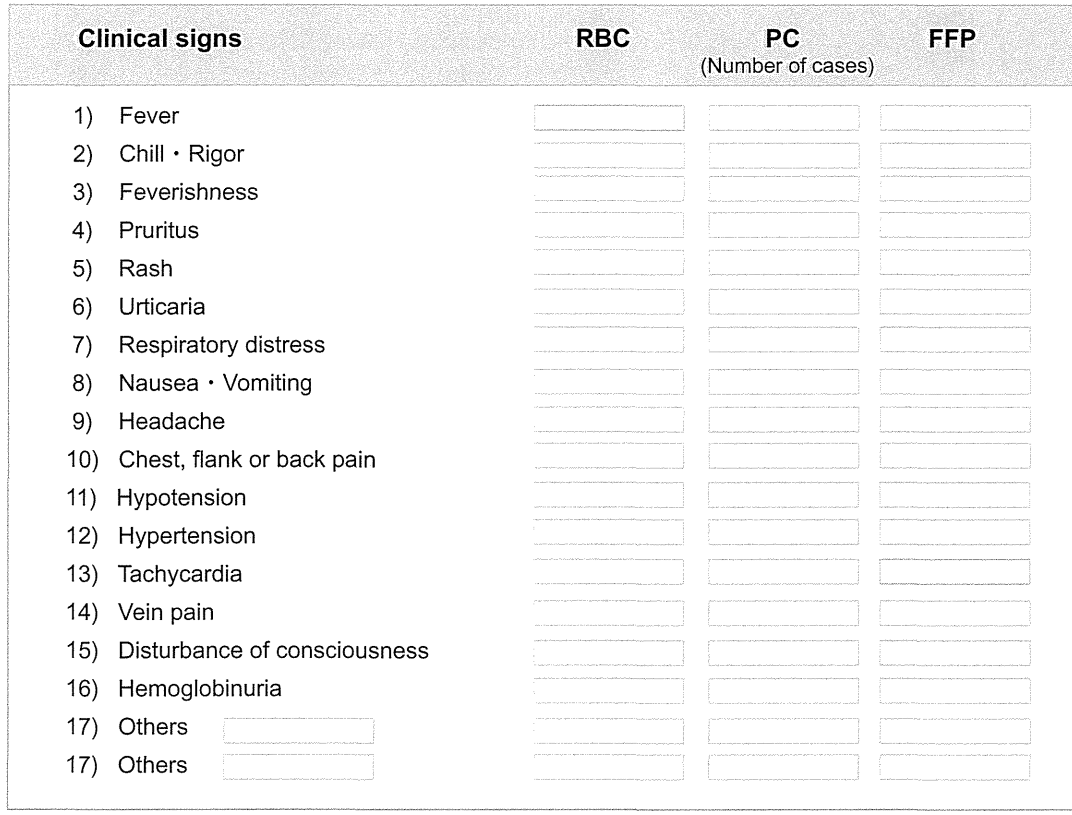


Fig. 2. Online surveillance system (2): The total number of transfusion reactions by clinical signs for the three blood components used over the reporting period is presented. Clinical signs are classified into the 16 categories indicated. Fever: more than 38 °C or a 1 °C or more increase from the baseline; 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%,

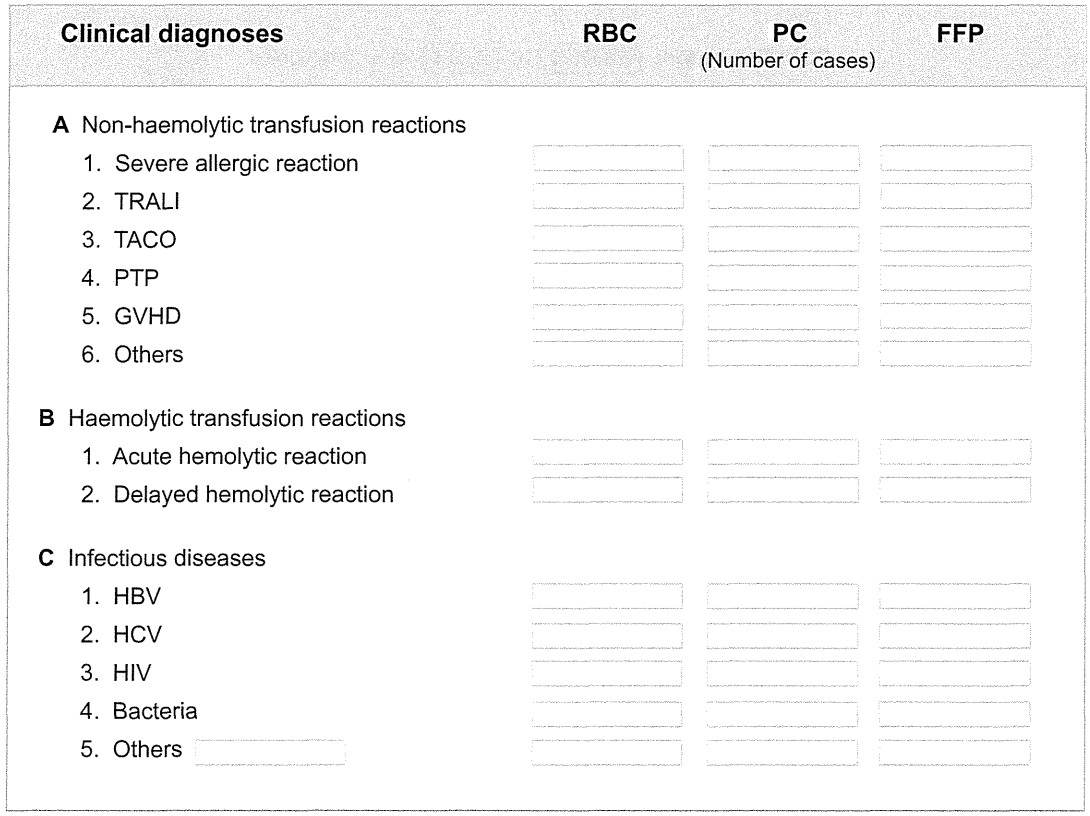


Fig. 3. Online surveillance system (3): The total number of transfusion reactions by clinical diagnoses for the three blood components over the period is presented. Clinical diagnoses are classified into the three categories indicated. Among non-haemolytic transfusion reactions, the events not included 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

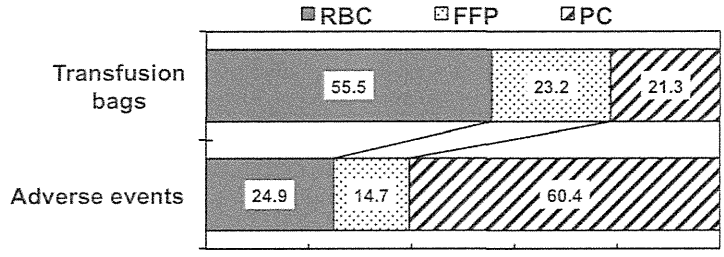
We compared the incidence of adverse events in seven large-scale university hospitals with that in five small-scale hospitals with fewer than 300 beds. Seven large-scale hospitals participated in this pilot study since 2007 and the data reported by these hospitals from 2007 to 2010 were analyzed (Fig. 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

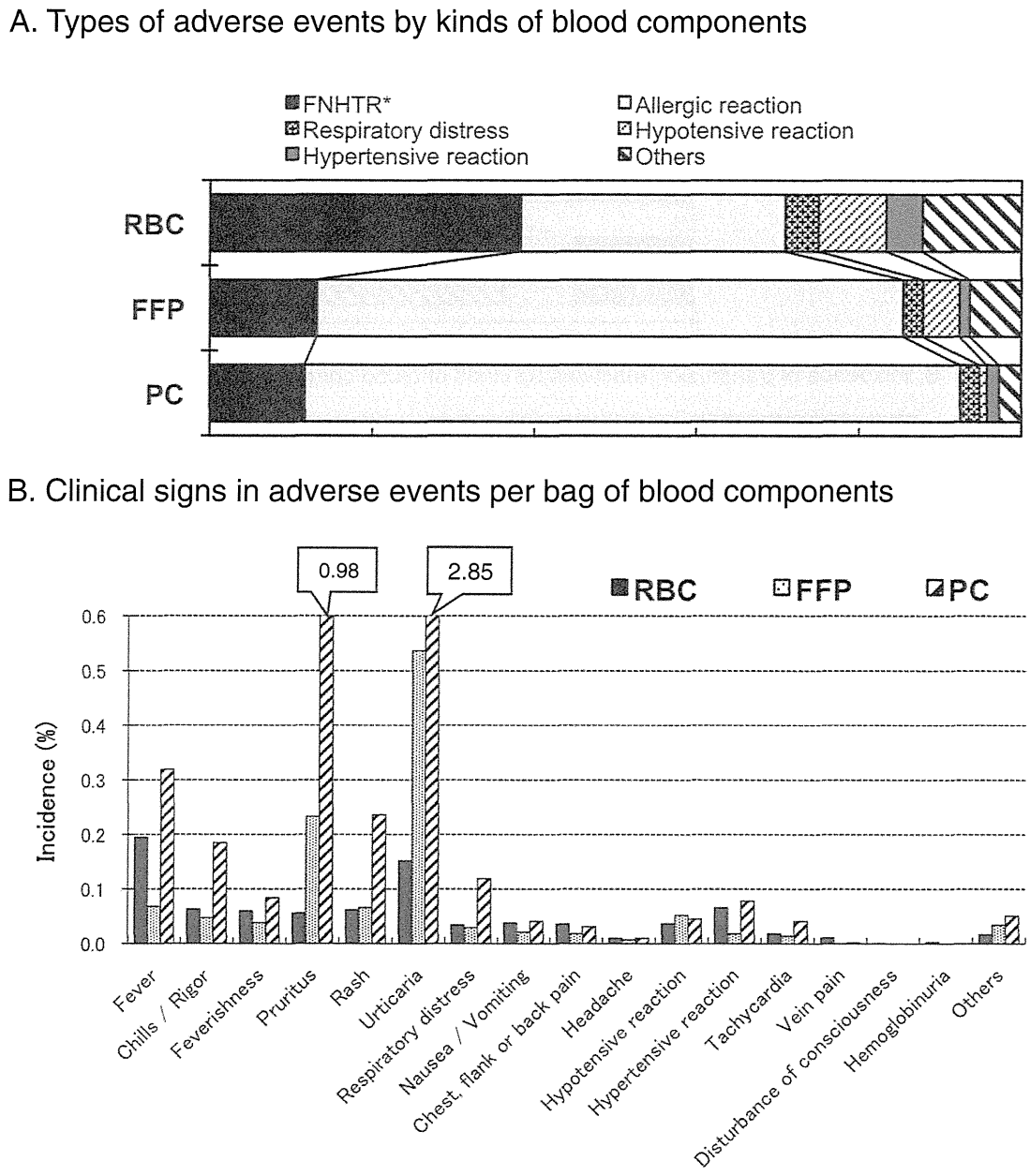


Fig. 5. Types of adverse events and clinical signs of adverse events by blood component. (A) Proportions of adverse events by type of blood component. (B) Incidence of clinical signs of adverse events by type of blood component. FNHTR: febrile non-haemolytic transfusion reaction.

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.

Table 1
Clinical diagnosis of transfusion-related adverse events from 2007 to 2010.

	RBC cases (%)	FFP cases (%)	PC cases (%)
Non-haemolytic transfusion reaction			
Severe allergic reaction	4 (0.5%)	7 (1.3%)	8 (0.4%)
TRALI	4 (0.5%)	3 (0.6%)	3 (0.1%)
TACO	4 (0.5%)	1 (0.2%)	0
PTP	0	0	0
GVHD	0	0	0
Others	861 (97.7%)	509 (97.9%)	2127 (99.5%)
Haemolytic transfusion reaction			
Acute hemolytic reaction	3 (0.3%)	0	0
Delayed hemolytic reaction	1 (0.1%)	0	0
Infectious diseases			
HBV	1 (0.1%)	0	0
HCV	0	0	0
HIV	0	0	0
Bacteria	0	0	0
Others	0	0	0
Total all cases	881	520	2138

The number of events and their frequency for each blood component are shown. TRALI, transfusion-related acute lung injury; TACO, transfusion associated circulatory overload; PTP, transfusion purpura; GVHD, graft-versus-host disease; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

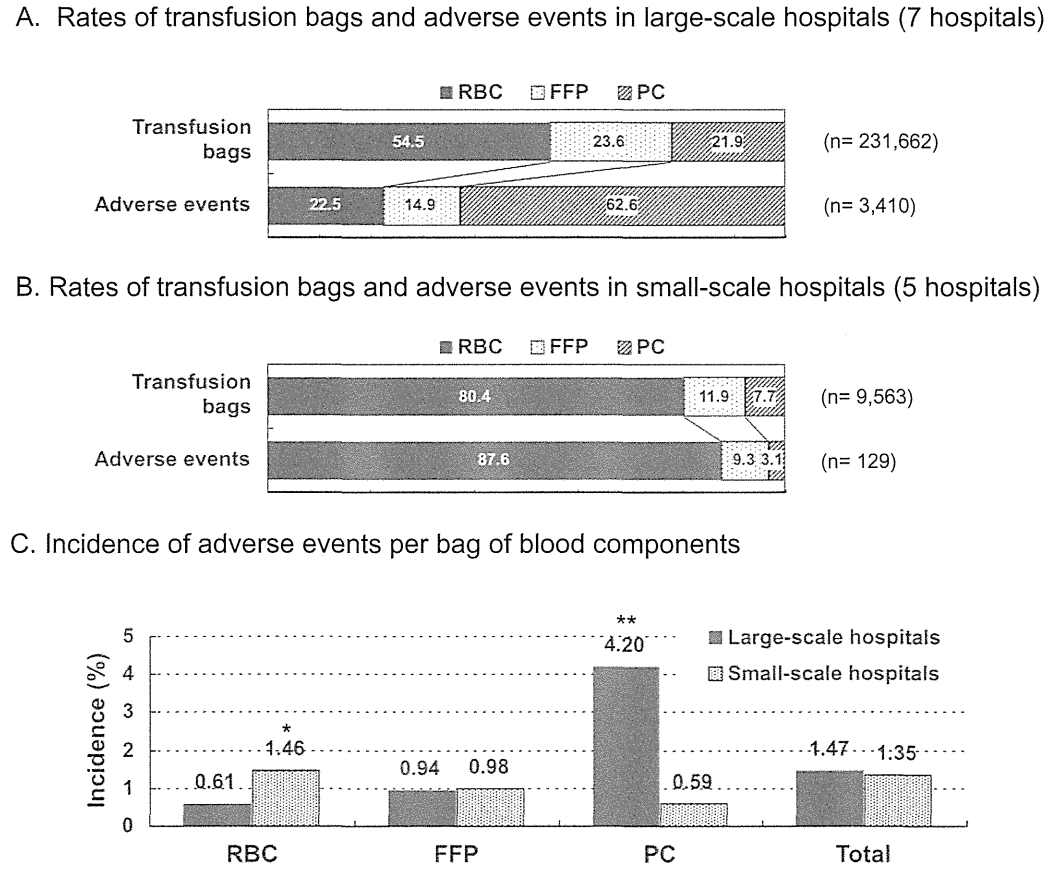


Fig. 6. Comparison of use of transfusion bag type, adverse events and incidence between large-scale and small-scale hospitals. Proportions of type of blood component and adverse events by type of blood component in seven large-scale university hospitals (A) and in five small-scale hospitals (fewer than 300 beds) (B). (C) The incidence of adverse events per bag of each blood component in large-scale and small-scale hospitals. $^{*}p < 0.05$ compared with large-scale hospitals; $^{**}p < 0.01$ compared with small-scale hospitals.

5. Conclusions

We have developed a comprehensive online system for the collection of all adverse reactions in recipients related to blood transfusion. Despite the limitation of our current system described above, this system is effective for collection and analysis of actual adverse events in recipients and can be used to enhance the existing surveillance system in Japan.

Conflict of interest statement

The authors declare no competing financial interests.

Acknowledgments

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Antigen-capture ELISA for the detection of Rift Valley fever virus nucleoprotein using new monoclonal antibodies

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Monoclonal antibodies (MAbs) raised against the nucleoprotein (NP) of Rift Valley fever virus (RVFV) were developed, and an antigen-capture enzyme-linked immunosorbent assay (Ag-capture ELISA) system was developed for the detection of RVFV NP. The assay detected RVFV antigen from culture supernatants containing as little as 7.8–31.3 pfu per 100 μl. Reactivity with various truncated NPs indicated that MAb C10-54 bound only to the full-length NP, probably due to recognition of a conformational epitope, whereas MAbs G2-36 and D5-59 bound to a linear epitope ranging from amino acid residues 195–201 in the C-terminal region. Based on the alignments of the amino acid sequence of RVFV NP, the epitope regions of MAbs G2-36 and D5-59 were completely conserved among all RVFV strains. These results suggest that the MAbs are applicable to the Ag-capture ELISA for the diagnosis of RVFV infections.

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1. Introduction

Rift Valley fever (RVF), a mosquito-borne zoonotic disease that affects domestic animals and humans, is caused by infection by the RVF virus (RVFV). The disease is found in sub-Saharan areas in Africa, as well as in Egypt, the Comoros Islands, Madagascar, and the Arabian Peninsula (Shimshony and Barzilai, 1983; Shoemaker et al., 2002; Sissoko et al., 2009). Infection of RVFV causes abortions or resorption of the fetus in pregnant domestic ruminants, with newborn mortality approaching 100%, and thus can cause catastrophic economic losses. Transmission of RVFV to humans, either through contact with bodily fluids of infected animals or mosquito bites, may result in mild to moderate influenza-like symptoms and severe retinitis, encephalitis and hemorrhagic fever (Alrajhi et al., 2004; Gerdes, 2004; Shimshony, 1999). During an RVF outbreak, confirmed cases are defined as suspected or probable cases by laboratory confirmation of the presence of anti-RVFV IgM by enzyme-linked immunosorbent assay (ELISA), RVFV antigen by antigen-captured (Ag-captured) ELISA, or viral RNA by RT-PCR in serum or blood samples (Al-Hazmi et al., 2003; Bird et al., 2008; Madani et al., 2003; MMWR, 2007).

RVFV belongs to the *Phlebovirus* genus of the *Buniyaviridae* family. Like other members of the family, RVFV possesses a single-stranded tripartite RNA genome composed of three segments, namely, S, M, and L. The S segment codes for nucleocapsid protein (NP) in negative sense, and non-structural NSs protein in positive sense, using an ambisense strategy. The M segment codes for a precursor of glycoproteins Gn and Gc and two non-structural proteins of 78 kDa and 14 kDa. The L segment codes for an L protein. The nucleotide sequence of the NP gene is highly conserved among various RVFV isolates (Bird et al., 2007b). Serum antibodies against NP are detected readily early after infection and in the convalescent phase, providing a basis for the diagnosis of RVF (Fafetine et al., 2007; Jansen van Vuren et al., 2007). An Ag-capture ELISA for detecting viral NP has been applied commonly to detect RVFV, as well as various viruses, since it is the most abundant viral antigen (Al-Hazmi et al., 2003; Bird et al., 2008; Jansen van Vuren and Paweska, 2009; Ji et al., 2011; Saijo et al., 2005, 2006, 2007; Madani et al., 2003; Nakauchi et al., 2009; Velumani et al., 2008). Monoclonal antibodies (MAbs) are used often as a capture antibody for Ag-capture ELISA since they have a high specificity for antigens, and identification of the epitopes of MAbs is of crucial importance for the assessment of cross-reactivity of the assay (Saijo et al., 2005, 2006, 2007; Nakauchi et al., 2009). In this study, MAbs were raised against recombinant RVFV NP (RVFV rNP). Epitope mapping showed that these MAbs recognized highly conserved epitopes on RVFV NP, suggesting their potential application for the detection of all RVFV isolates. By using these MAbs as capture antibodies, an Ag-capture ELISA for detecting an RVFV antigen was developed.

2. Materials and methods

2.1. Recombinant baculoviruses

Ac-His-RVFV-NP baculoviruses, expressing RVFV rNP, were generated using the same strategy as previously described (Saijo et al.,

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2002). Briefly, an entire cDNA clone of NP from RVFV-MP12 was used to construct a transfer vector. RVFV NP cDNA was amplified by PCR. The amplified DNA was digested with BamHI and subcloned into the BamHI site of pQE30 vector DNA (QIAGEN GmbH, Hilden, Germany) to construct pQE30-RVFV NP. An RVFV NP DNA fragment with a six-histidine (His) tag was isolated from the pQE30-RVFV NP plasmid by digestion with EcoRI and SalI. Each extremity was then blunted with T4 DNA polymerase and subcloned into the blunt-ended BamHI site of pAcYM1 (Saijo et al., 2002) to generate pAcYM1-His RVFV rNP.

Tn5 insect cells were transfected with mixtures of linearized BacPAK6 DNA (Clontech, Mountain View, CA) and the recombinant transfer vector pAcYM1-His RVFV rNP according to the manufacturer's instructions and the procedures described by Kitts and Possee (1993). The resulting recombinant baculovirus, which expresses a His-tagged recombinant NP of RVFV (His-RVFV rNP), was designated as Ac-His-RVFV NP.

2.2. Expression and purification of rNPs

Ac-His-RVFV NP-infected Tn5 cells were incubated at 26 °C for 72 h. Then, the cells were washed twice with cold phosphate-buffered saline (PBS) solution and lysed in cold PBS solution containing 1% Nonidet P-40 (NP-40). The cell lysate was centrifuged at 13,000 × *g* at 4 °C for 10 min. The supernatant fraction was collected as a source of His-RVFV rNP for purification. The His-RVFV rNP was purified by Ni^{2+} column chromatography (QIAGEN GmbH), as previously described (Saijo et al., 2002). Sabia virus (SABV) rNP as a control was expressed and purified, as described previously (Nakauchi et al., 2009).

2.3. Establishment of MAbs

BALB/c mice were immunized subcutaneously three times with the purified His-RVFV rNP. Spleen cells were obtained 3 days after the last immunization and fused with P3/Ag568 cells using polyethylene glycol (Invitrogen). The culture supernatants of the hybridoma cells were screened by ELISA with purified His-RVFV rNP as an Ag in the presence of 2 M urea in order to exclude MAbs with a low-avidity. MAbs, designated as D5-59, C10-54, and G2-36, were purified from the culture supernatants of the respective hybridoma clones using protein-G column chromatography, as described previously (Nakauchi et al., 2009).

2.4. Polyclonal antibodies

Polyclonal antibodies were raised in rabbits by immunization with the purified His-RVFV rNP expressed in the baculovirus system, as described previously (Saijo et al., 2002). Protocols for animal experiments were approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases, Tokyo, Japan.

2.5. Indirect immunofluorescence assay

Vero E6 cells were infected with RVFV-MP12 (MOI = 0.1). After 18 h, the cells were fixed with a mixture of methanol and acetone [1:1 (v/v)]. Binding of the RVFV infected cells was performed by immunofluorescence assay (IFA), as described previously (Saijo et al., 2005).

2.6. Ag-capture ELISA

The Ag-capture ELISA was performed essentially as described elsewhere (Saijo et al., 2005, 2006, 2007; Nakauchi et al., 2009). Purified MAb D5-59, C10-54, or G2-36 was coated on 96-microwell immunoplates (Falcon; Becton Dickinson Labware) at 100 ng/well in 100 μl of PBS at 4 °C overnight, followed by blocking with PBS containing 0.05% Tween-20 and 5% skim milk (PBST-M) for 1 h at room temperature. After the plates were washed three times with PBS containing 0.05% Tween-20 (PBST), 100 μl samples containing serially diluted His-RVFV rNP or culture supernatants of Vero cells, either infected with RVFV MP12 or uninfected, were added, and the plates were incubated for 1 h at 37 °C (viruses were inactivated by treatment with 1% NP40 followed by UV irradiation for 15 min). The plates were then washed with PBST, and 100 μl of rabbit anti-serum raised against His-RVFV rNP diluted 1:500 with PBST-M was added to each well. After 1 h of incubation at 37 °C, the plates were washed with PBST, and horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) was added. The plates were further incubated for 1 h at room temperature. After another extensive washing with PBST, 100 μl of ABTS substrate solution (Roche Diagnostics) was added and the OD405 was measured with a reference wavelength of 490 nm after 30 min of incubation at room temperature. As a negative control, the ODs of wells inoculated with control Ag (SABV rNP or culture supernatants of mock-infected Vero cells) were measured. Means and standard deviations were calculated from the OD405 values of 12 negative control wells, and the cutoff value for the assay was defined as the mean plus three standard deviations.

2.7. Expression of truncated rNPs of RVFV

In order to determine the epitope region reacted with the MAbs, a series of truncated RVFV rNPs were expressed as fusion proteins with glutathione S-transferase (GST), as previously described (Nakauchi et al., 2009). Briefly, the cDNA corresponding to each of the truncated NP fragments was amplified by PCR with specifically designed primer sets. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). The GST-tagged full-length RVFV NP or truncated forms of RVFV NP were expressed in *Escherichia coli* (*E. coli*) BL21.

2.8. Western blotting

The MAbs were tested for reactivity to the GST-tagged RVFV NP fragments expressed in *E. coli* by Western blotting, as described previously (Saijo et al., 2005). Goat anti-GST antibody (GE Healthcare, Piscataway, NJ) was used for detection of GST-tagged proteins in the assay.

2.9. Plaque assay

VeroE6 cells prepared in 12-well plates were inoculated with 50 μl of 10-fold serially diluted virus samples and incubated at 37 °C for 1 h. Then the cells were cultured with 1.0 ml per well of DMEM containing 1% FCS and 1% methyl cellulose (Sigma) for 5 days. Cells were fixed with 10% formalin, irradiated under a UV lamp, and stained with crystal violet. Plaques produced by RVFV were counted under light microscopy. Titration was done in duplicate and infectivity was displayed by plaque-forming units (pfu).

3. Results

3.1. Generation of MAbs

In order to obtain MAbs against RVFV NP, BALB/c mice were immunized with purified RVFV rNP. The hybridomas were cloned and their culture supernatants were tested for reactivity to RVFV rNP by IgG ELISA. Three MAbs, designated D5-59, C10-54, and G2-36, reacted with the His-RVFV rNP by IgG ELISA, even in the presence of 2.0 M urea (data not shown). The reactivity of MAbs

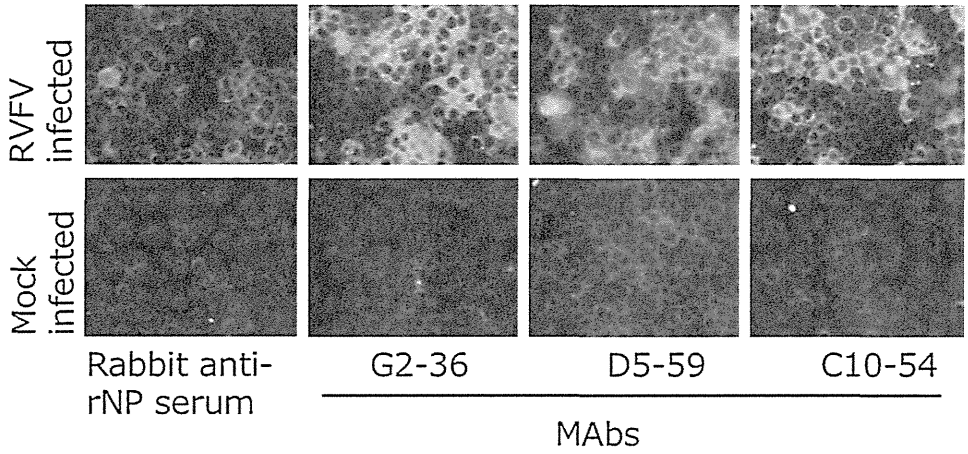

Fig. 1. Reactivity of MAbs in IFA. Vero E6 cells infected with RVFV MP12 were stained by indirect immunofluorescence with MAbs D5-59, C10-54, and G2-36. Rabbit anti-rNP serum was used as a positive control.

was examined by an indirect immunofluorescence method (Fig. 1). The RVFV-infected (MOI = 0.1), but not mock-infected, cells were stained with each of these MAbs (Fig. 1). The staining pattern was consistently similar in all MAbs tested and was characterized by a diffuse granular cytoplasmic staining, similar to that observed previously (Billecocq et al., 1996), indicating specific recognition of MAbs against RVFV NP.

3.2. Development of Ag-capture ELISA

By using these MAbs and rabbit anti-rNP serum as capture and detection antibodies, respectively, an Ag-capture ELISA for detecting RVFV NP was developed. When the His-RVFV rNP antigen was used in Ag-capture ELISA, MAbs D5-59 and G2-36 detected as little as 0.16 ng per 100 μl of rNP, whereas MAb C10-54 was more sensitive in detecting the rNP, with a detection limit of 0.08 ng per 100 μl (Fig. 2). None of these MAbs reacted with rNP of the control virus, SABV, prepared from insect cells, even at high antigen concentration (20 ng per 100 μl). RVFV antigen in the culture supernatants from Vero E6 cells infected with RVFV-MP12 was also detected in the developed Ag-capture ELISA, whereas mock-infected cells showed a negative reaction, indicating that MAbs reacted not only with recombinant NP, but also with an authentic viral NP (Fig. 3). MAbs D5-59, C10-54, and G2-36 were able to detect as little as 15.6, 7.8, and 31.3 pfu per 100 μl of RVFV, respectively.

3.3. Epitope mapping of MAbs

To determine the binding regions including epitopes of MAbs, truncated NPs were expressed in *E. coli* and analyzed for MAbs reactivity by Western blot analysis. At first, five forms of truncated rNP, as well as full-length rNP, were examined for MAbs reactivity (Fig. 4A). MAbs G2-26 and D5-59 reacted with the full-length (NP1-245) and C-terminus region (NP177-245 and NP76-245) of rNP, whereas MAb C10-54 reacted only with the full-length rNP. These results indicated that the binding region for MAbs G2-36 and D5-59 mapped within the C-terminus one-third, corresponding to amino acid residues 177–245, and that full-length RVFV NP was required for MAb C10-54 to react. MAb C10-54 could recognize a conformational epitope since it reacts weakly by Western blot against full-length rNP, probably due to epitope renaturation during or after the transfer of the protein to a membrane as reported by Zhou et al. (2007).

To narrow the region recognized by MAbs G2-36 and D5-59, additional truncated rNPs from amino acid residues 177–245 were generated and tested for reactivity by Western blot analysis. Both MAbs reacted with NP177-201 and NP195-235, but not with NP177-200 and NP196-235, indicating that minimum region for these MAbs ranged from amino acid residues 195–201 (Fig. 3B). The results also suggested essential amino acid residues 195 and 201 for MAb binding. Although one truncated NP (NP177-200) was reacted with MAbs G2-36 and D5-59, the intensities of this protein bands were significantly lower than those of the NPs containing amino acid residues 195–201. The result might be attributed to the lack of critical amino acid residue 201 on NP177-200.

To ascertain whether MAbs G2-36 and D5-59 react broadly with various RVFV isolates, the amino acid sequence of the minimum epitope region ($_{195}$TFTQPMN$_{201}$) was aligned with corresponding amino acid sequences of all known RVFV isolates, as well as those of other Phleboviruses, deposited in the GenBank database (Fig. 4). The amino acid sequence ($_{195}$TFTQPMN$_{201}$) of the epitope was conserved completely among all RVFV isolates, but was not identical to those of other Phleboviruses. These results demonstrated that MAbs G2-36 and D5-59 recognized a highly conserved linear epitope in the RVFV NP.

4. Discussion

In diagnosing many virus infections, PCR assays have excellent analytical sensitivity, but the established techniques are limited by the need for expensive equipment and technical expertise. Since the sensitivity of Ag-capture ELISA is potentially comparable to that of RT-PCR (Ji et al., 2011; Saijo et al., 2006, 2007; Velumani et al., 2008), Ag-capture ELISA represents a sophisticated approach for the diagnosis of virus infections. As a prelude to the development of such a sensitive diagnostic test for RVFV infection, a recombinant RVFV NP from insect cells infected with recombinant baculovirus was purified and novel MAbs against them were developed. MAbs D5-59, G2-36, and C10-54 reacted with His-RVFV rNP and authentic viral antigen (NP) in RVFV MP12-infected cells. Furthermore, control assays with unrelated virus (SABV) rNP and mock-infected cells revealed that MAbs were specific to RVFV NP.

Although it is difficult to compare simply the detection limits among the various assay procedures, the Ag-capture ELISA developed in this study seems to have excellent sensitivity. The detection limit of the newly developed Ag-capture ELISA (0.08–0.16 ng of rNP per 100 μl, Fig. 2) was very similar to that of a previous report, in which an *E. coli* system for the expression of RVFV rNP was used, hyperimmune sheep anti-RVFV rNP serum was used as the capture antibody, and the detection limit of the assay was 0.11 ng per 100 μl of rNP (Jansen van Vuren and Paweska, 2009).

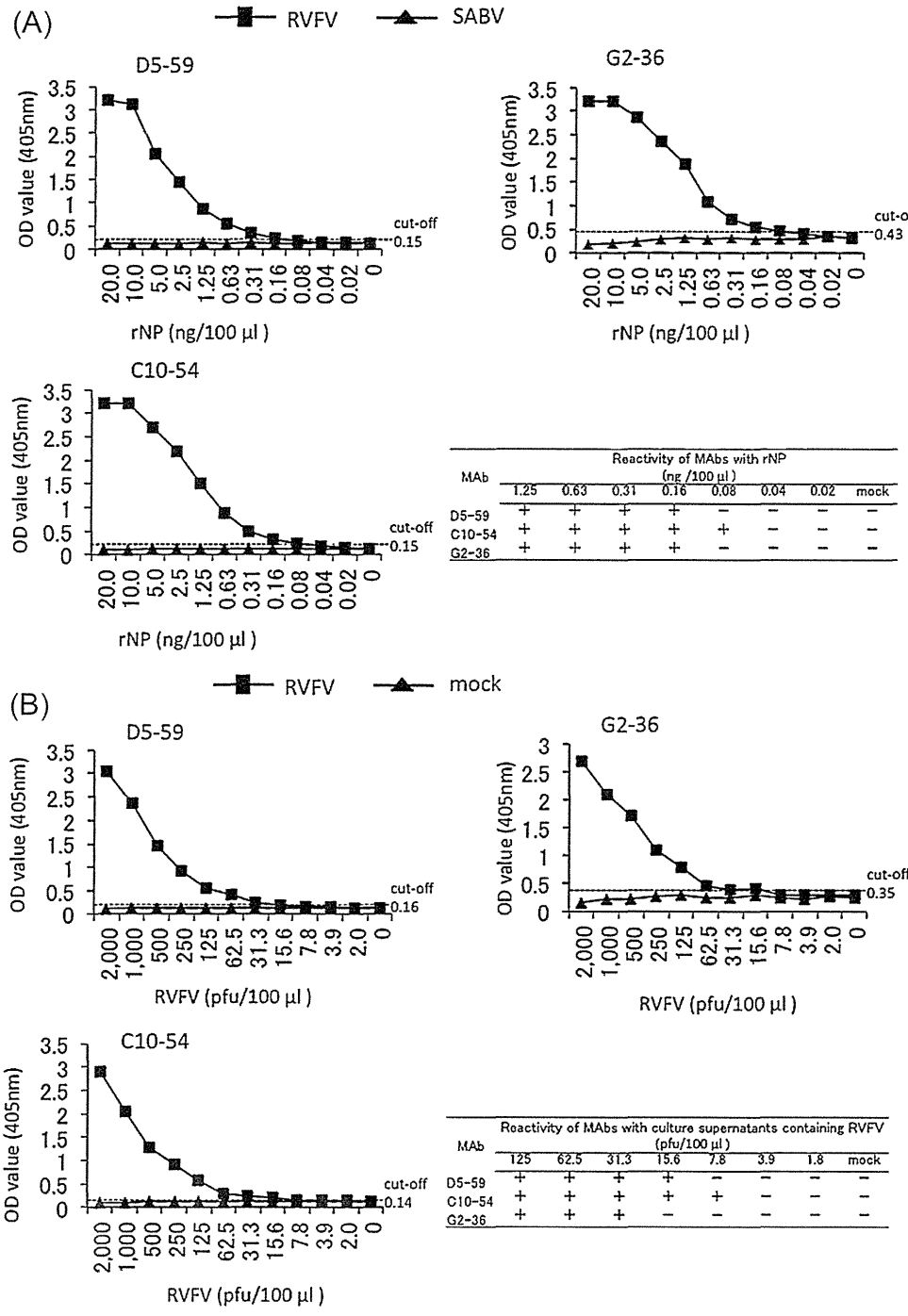

Fig. 2. Ag-capture ELISA for the detection of His-RVFV rNP (A) and authentic RVFV NP (B). MAbs D5-59, C10-54, and G2-36 were used as capture antibodies. Rabbit anti-rNP serum was used as the detecting antibody. Dashed lines indicate the cut-off for each antibody. Detection limits for each MAb are summarized in the table.

Using different amounts of authentic RVFV antigen obtained from the culture supernatants of cells infected with RVFV, as little as 7.8–31.3 pfu per 100 μl of RVFV was detected by ELISA. A real-time RT-PCR assay (Bird et al., 2007a) for detection of the RVFV genome from the same culture supernatant samples detected as little as 3.0 pfu per 100 μl (data not shown), indicating that the detection limit of the Ag-capture ELISA was slightly less than that of the real-time RT-PCR assay. However, the detection limit of this Ag-capture ELISA was approximately 10 times higher than that reported for detecting authentic RVFV antigen (Jansen van Vuren and Paweska, 2009). Thus, the newly developed Ag-capture ELISA might be useful in the diagnosis of RVFV infection.

MAbs directed against RVFV NP and their application for detecting RVFV have been reported, showing broad reactivity to RVFV strains (Martin-Folgar et al., 2010; Saluzzo et al., 1989). Since MAbs directed against RVFV NP might allow for the detecting RVFV antigen in the serological diagnosis, identification of the epitopes of MAbs is of crucial importance for the assessment of specificity of the assay system.

The Ag-capture ELISA using MAb C10-54 recognizing a conformational epitope on RVFV NP proved more sensitive than assays using MAbs D5-59 and G2-36 recognizing the linear epitope, indicating that, similar to the results shown by another diagnostic study (Velumani et al., 2008), conformation-specific MAb might