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

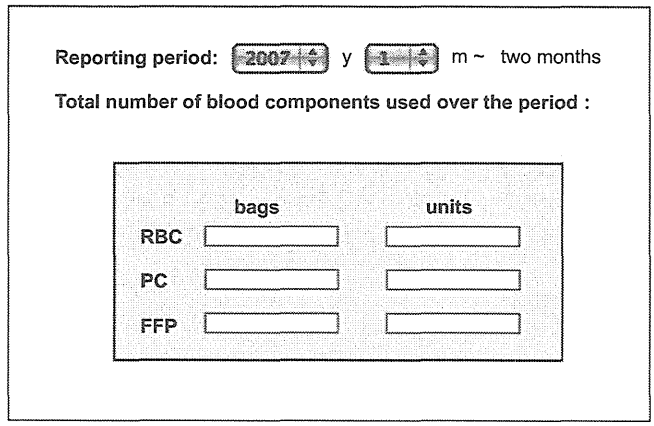


Fig. 1. Online surveillance system (1): Screenshot of the total number of the three labile blood components (bags and units) used over each reporting period. RBC: red blood cells; FFP: fresh frozen plasma; PC: platelet concentrates.

Clinical signs	RBC	PC (Number of cases)	FFP
1) Fever			
2) Chill · Rigor			
3) Feverishness			
4) Pruritus			
5) Rash			
6) Urticaria			
7) Respiratory distress			
8) Nausea · Vomiting			
9) Headache			
10) Chest, flank or back pain			
11) Hypotension			
12) Hypertension			
13) Tachycardia			
14) Vein pain			
15) Disturbance of consciousness			
16) Hemoglobinuria			
17) Others			
17) Others			

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%,

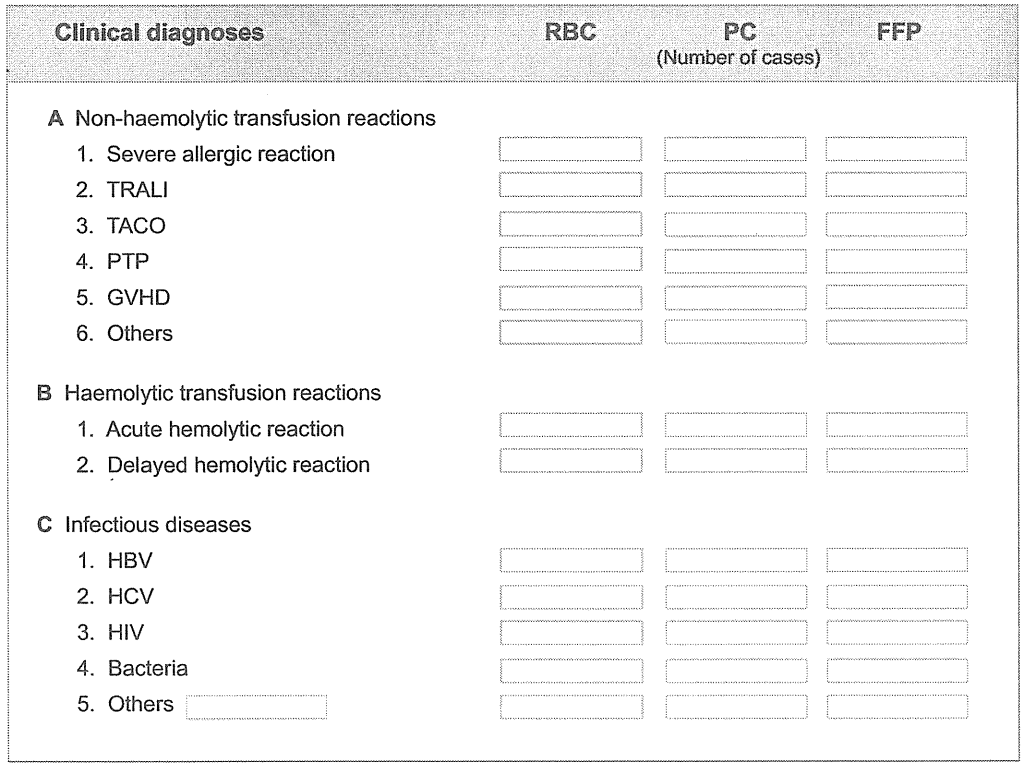


Fig. 3. Online surveillance system (3): The total number of transfusion reactions by clinical diagnoses for the three blood components over the period is presented. Clinical diagnoses are classified into the three categories indicated. Among non-haemolytic transfusion reactions, the events not included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

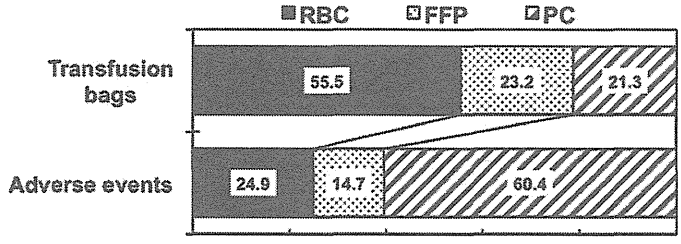
We compared the incidence of adverse events in seven large-scale university hospitals with that in five small-scale hospitals with fewer than 300 beds. Seven large-scale hospitals participated in this pilot study since 2007 and the data reported by these hospitals from 2007 to 2010 were analyzed (Fig.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

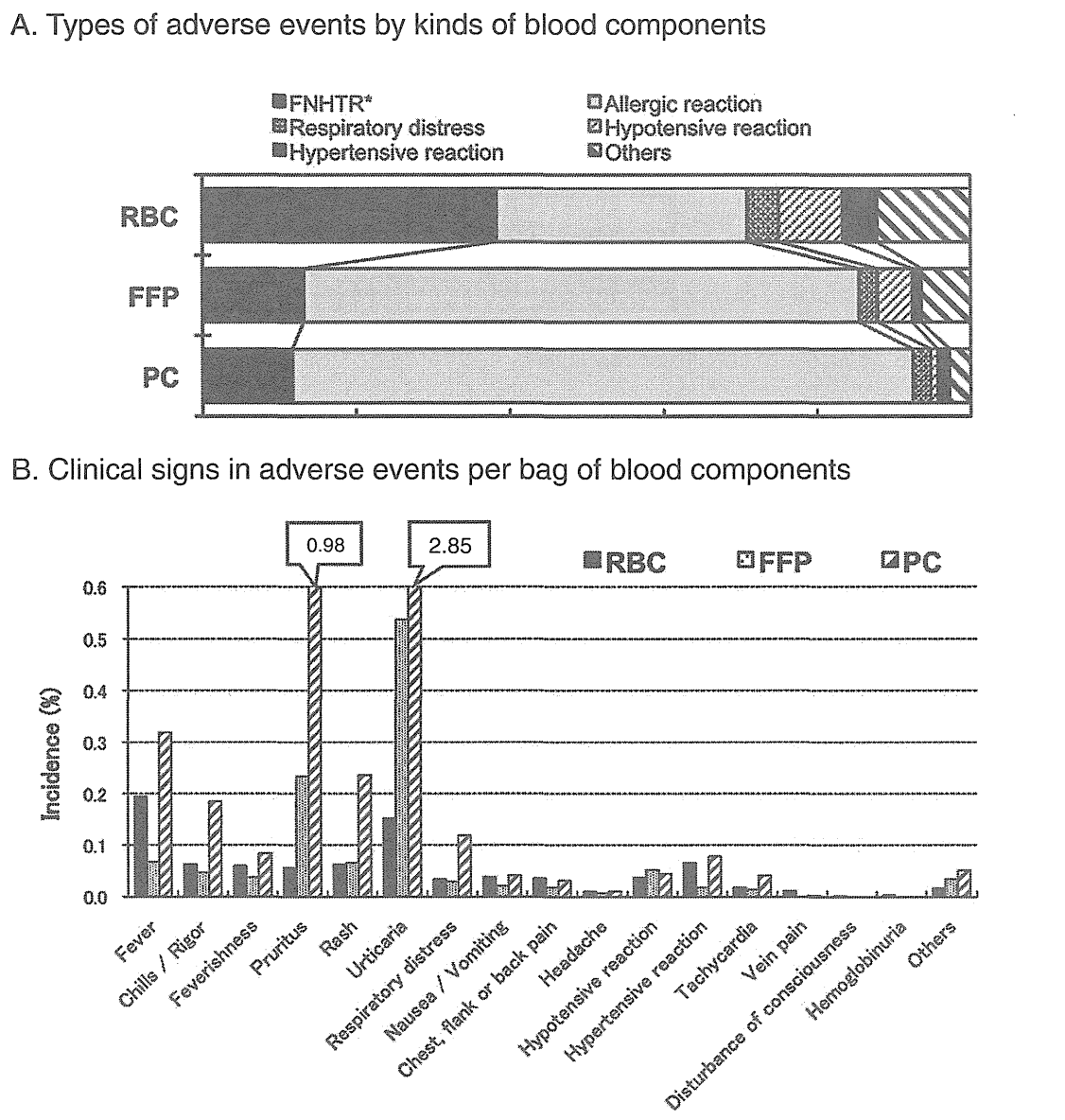


Fig. 5. Types of adverse events and clinical signs of adverse events by blood component. (A) Proportions of adverse events by type of blood component. (B) Incidence of clinical signs of adverse events by type of blood component. FNHTR: febrile non-haemolytic transfusion reaction.

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.

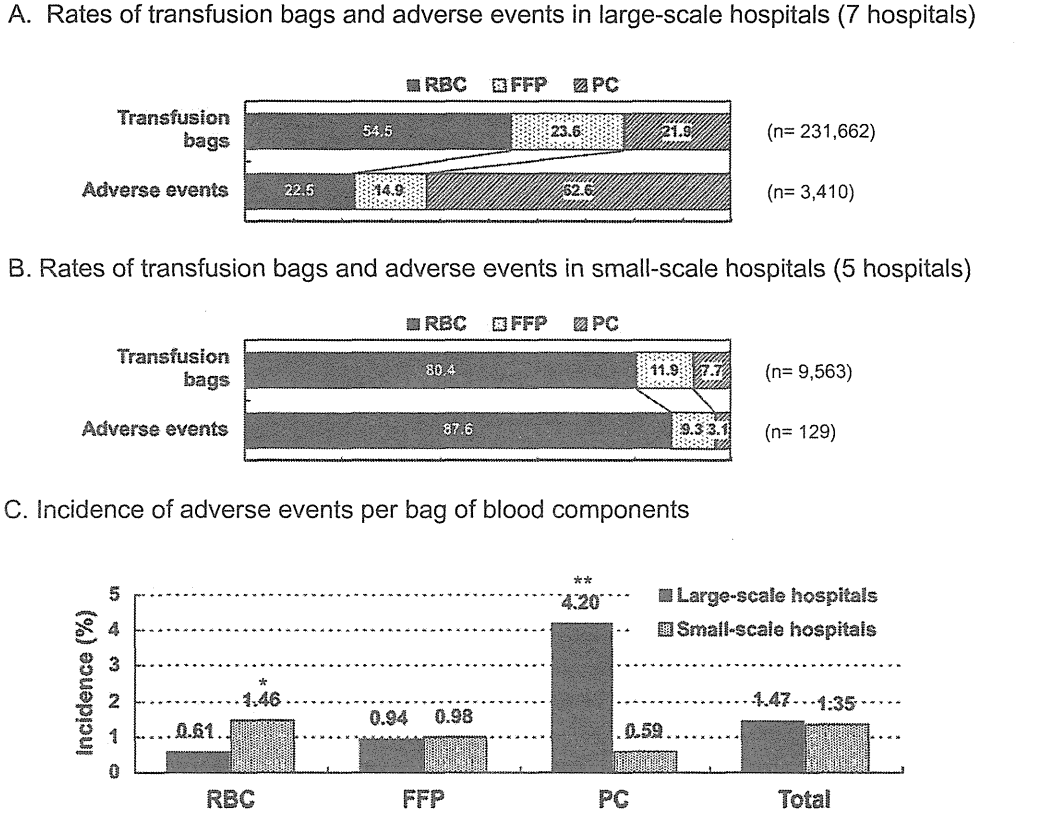


Fig. 6. Comparison of use of transfusion bag type, adverse events and incidence between large-scale and small-scale hospitals. Proportions of type of blood component and adverse events by type of blood component in seven large-scale university hospitals (A) and in five small-scale hospitals (fewer than 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.

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Aggregation analysis of pharmaceutical human immunoglobulin preparations using size-exclusion chromatography and analytical ultracentrifugation sedimentation velocity

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In the pharmaceutical industry, analysis of soluble aggregates in pharmaceutical formulations is most commonly performed using size-exclusion chromatography (SEC). However, owing to concerns that aggregates can be overlooked by SEC analysis, it has been suggested that its results should be confirmed with orthogonal methods. One of the main alternative methods for SEC is analytical ultracentrifugation sedimentation velocity (AUC-SV), which has been indicated as an important tool for the measurement of protein aggregation. The present study aimed to show that AUC-SV can be effectively applied for the characterization of marketed immunoglobulin pharmaceutical preparations to support the results obtained by SEC. In addition, the present research aimed to assess the appropriateness of two integration approaches for the quantitative analysis of the SEC results. Thus, the aggregates were measured in seven different preparations of human immunoglobulins by AUC-SV and SEC, and the acquired chromatographic data were processed by using either the vertical drop method or the Gaussian skim approach, implemented in the Empower II chromatography data software (Waters, Tokyo, Japan). The results of aggregation measurements performed using AUC-SV were in good agreement with those obtained using SEC. As expected, the Gaussian skim integration approach inherently provided lower estimates of aggregation content than the results of the vertical drop method. The finding of this study confirmed the complementary nature of AUC-SV to SEC for aggregate composition analysis and underscored the important role that the different integration methods can play in the quantitative interpretation of chromatographic results.

[**Key words:** Immunoglobulin; Size-exclusion chromatography; Vertical drop method; Gaussian skim; Analytical ultracentrifugation sedimentation velocity]

Antibody aggregation is a common problem in the pharmaceutical industry, encountered during the manufacturing process and long-term storage of antibody products. It was suggested that the presence of antibody aggregates in a therapeutic product can affect drug efficacy or may even cause immunogenic reactions when administered to patients (1). To ensure that the biotherapeutic product is consistent with quality standards and meets all regulatory requirements, an accurate measurement of aggregation content is necessary.

Size-exclusion chromatography (SEC) is currently employed for the quantification of soluble antibody aggregates as a quality control method. SEC separates molecules based on their hydrodynamic volume, provides highly reproducible results, is easy to perform, and is a relatively fast technique for the characterization of pharmaceutical formulations. The elution profiles generated by SEC are analyzed, and the fractional amount of each solute detected in the sample is estimated from the area under the peak, which is calculated using chromatographic software. In general, the area under unresolved peaks is determined by the vertical drop method. This method involves the addition of a vertical line from the valley between the peaks to the horizontal baseline. However, the perpendicular separation of overlapping peaks has previously been shown to introduce significant errors in peak area estimation (2–4). Alternative approaches implemented in chromatographic data analysis packages allow more sophisticated approaches for the identification of unresolved peaks, such as the Gaussian skim method implemented in the Empower II software. This algorithm fits the shapes of the peaks observed in the chromatogram using a Gaussian profile and is assumed to better represent the shape of the parent peak in the overlapping peaks group. Nevertheless, the vertical drop method remains the most commonly applied approach for the integration of chromatographic peaks (5). Another problem with SEC is related to a nonspecific binding of protein aggregates to the column matrix, as recently discussed (6).

The above-mentioned issues potentially affect the accuracy of SEC measurements. Thus, it has been suggested that the results of

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the SEC method should be verified using different analytical techniques (7). Among alternatives to SEC method, analytical ultracentrifugation sedimentation velocity (AUC-SV) was found to be very suitable for this purpose (8).

The improvements in AUC instrumentation and data analysis packages have promoted an increase in the number of potential applications of AUC-SV (9–12). Particularly, it has been indicated as a valuable tool for monitoring antibody aggregation (8,13–16). Nevertheless, owing to the lower degree of reproducibility of AUC-SV results compared with SEC results, the implementation of AUC-SV to a routine characterization of pharmaceutical antibodies has not been successful until now. In the recent study presenting the summary opinion of the members of the protein characterization subcommittee of the European Immunogenicity Platform, it has been suggested that throughout the pharmaceutical development process, AUC should not be used for validation of SEC results but rather should be used as a complementary method for SEC (17).

The purpose of this study was to demonstrate that AUC-SV can very effectively be used for the characterization of marketed immunoglobulin preparations and to confirm the performance of SEC. Over the years, a number of studies have been performed using AUC-SV and SEC, as applied to custom monoclonal antibody formulations (13,14). In contrast, the present research was conducted using a wide range of available marketed products, consisting of four liquid formulations and three lyophilized formulations of pharmaceutical human immunoglobulin preparations. Based on the previous studies (18–20) and our own results (21), experimental and data analysis procedures for precise aggregation content measurement in immunoglobulin formulations using AUC-SV were developed. Following the established protocol, we confirmed that AUC-SV can very effectively be used to characterize marketed pharmaceutical products. To address the uncertainty that can result from application of different methods for chromatographic peak identification, we applied the vertical drop method and the Gaussian skim approach, implemented in the Empower II software to analyze SEC data. Although integration of chromatographic peaks using the vertical drop method consistently indicated a slightly greater amount of aggregates compared with the value estimated by using the Gaussian skim algorithm, we achieved good overall agreement between AUC-SV and SEC results.

MATERIALS AND METHODS

Human immunoglobulin preparations In the present study, four liquid and three lyophilized preparations of human immunoglobulins were used. Polyglobin-N 5% for intravenous injection (0.5 g/10 ml), a pH 4-treated acidic human normal immunoglobulin, was purchased from the Japanese Red Cross Society (Tokyo, Japan). Venoglobulin IH 5% for intravenous injection (0.5 g/10 ml), a polyethylene glycol-treated human normal immunoglobulin; Hebsbulin IH for intravenous injection (1000 units), a polyethylene glycol-treated human anti-HBs immunoglobulin; and Tetanobulin IH for intravenous injection (250 units), a polyethylene glycol-treated human tetanus immunoglobulin, were purchased from Benesis Corporation (Osaka, Japan). Glovenin-I for intravenous injection (500 mg), a freeze-dried polyethylene glycol-treated human normal immunoglobulin G, was purchased from Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan). Gammagard for intravenous injection (2.5 g), a freeze-dried ion-exchange resin-treated human normal immunoglobulin, was purchased from Baxter Limited (Tokyo, Japan). Sanglopor for intravenous infusion (2.5 g), a freeze-dried pH 4-treated human immunoglobulin, was purchased from CSL Behring (Tokyo, Japan). For all lyophilized products, the immunoglobulin concentration in the reconstituted formulation was 50 mg/ml.

Size-exclusion chromatography SEC analysis was performed in triplicate using a high-performance liquid chromatography (HPLC) workstation (Alliance 1100 HPLC system) with a TSK gel G3000SW$_{XL}$ column (Tosoh Bioscience, Tokyo, Japan) under standard conditions. The separation was conducted at a flow rate of 0.5 ml/min at room temperature and was monitored by UV detection at 280 nm. The elution buffer consisted of 1 mM potassium phosphate, 3 mM sodium phosphate, and 155 mM sodium chloride at pH 7.4. A minimum reproducible volume of 5 µl of antibodies at formulation concentrations was injected into the HPLC system for analysis. This prevented excessive antibody dilution, as SEC itself is known to produce a high dilution of the sample that will tend to dissociate the reversible aggregates (22). An antibody mass recovery of 94% and higher was confirmed for all studied samples and was in agreement with the values from previous studies (14,23). Chromatographic data were processed by the Empower II chromatography data software (Waters, Tokyo, Japan) using the ApexTrack integration algorithm combined with either the vertical drop method or the Gaussian skim method. The integration parameters were set at default, and Detect Shoulders event was enabled. To estimate the fractional amount of each peak, the calculated peak area was divided by the total area that was obtained by summation of the areas of the peaks, including the solvent peak, where it was present.

Analytical ultracentrifugation sedimentation velocity AUC-SV analysis was performed according to the experimental routine especially designed for the present study. It addresses specific requirements to conduct the measurement of aggregation content in immunoglobulin preparations in a very precise manner. Thus, the sedimentation experiments were conducted in a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter) equipped with a 4-hole An60 Ti rotor. Beckman Coulter 12-mm double-sector charcoal-filled epon centerpieces manufactured after July 2008, when an improved manufacturing process was implemented by Beckman Coulter (19), and quartz windows were used for the experiments. The cells filled with 430 µl of the prepared samples were placed in the rotor and thoroughly equilibrated at 20°C and 0 rpm for approximately 1 h before beginning data acquisition. Data were recorded at 40,000 rpm and 20°C using absorbance optics at 280 nm. The scanning was performed as quickly as possible between radial positions 5.9 and 7.2 cm, with a step size of 30 µm until the sample was completely sedimented.

AUC-SV analysis of the selected preparations was complicated by the non-ideality of the formulations, containing high concentrations of excipients such as sugars and sugar alcohols. Therefore, the antibody formulations were diluted to the concentration of approximately 0.5 mg/ml using buffer consisting of 1 mM potassium phosphate, 3 mM sodium phosphate, and 155 mM sodium chloride at pH 7.4. The antibody samples were prepared immediately before the AUC-SV measurement. In this way, any potential decrease in the amount of aggregates due to reversible dissociation was minimized. AUC-SV runs were performed in triplicate, with three data sets collected in each run. The same combination of rotor hole position, cell housing, windows, and centerpiece was used for all consecutive runs, as recommended previously (19). This practice helped us to identify the micro-deformation of the centerpiece systematically affecting the quality of the data acquired for the cell placed in rotor hole 3. Therefore, these data were excluded from further analysis.

The SEC analysis of the reconstituted lyophilized preparations of immunoglobulins indicated relatively slow time-dependent change in the distribution of monomeric/dimeric forms of antibody, which was negligible compared with the time of the first sedimentation experiment, performed immediately after reconstitution. However, as the time interval between the reconstitution and the beginning of the second and third experiments was longer, the distribution was significantly affected. Thus, the results of the AUC-SV analysis are presented as the mean values of six measurements performed in three independent runs for the liquid formulations and as the mean values of two measurements performed in one run for the freeze-dried formulations.

The data were analyzed using the C(s) method of SEDFIT ((24); http://www.analyticalultracentrifugation.com). For the analysis, the meniscus was set to the midpoint position of the absorbance spike corresponding to the air-sample boundary and was floated during the fit. It was confirmed that the fitted meniscus position was physically relevant and was still located in the vicinity of the maximum of absorbance spike. The sedimentation coefficient (s) range was chosen so that no partial peaks were presented at the edges of the s-range and was 1–15 S, 1–20 S, 1–25 S, or 1–30 S, respectively. A grid resolution was selected in a way that resolution of s values corresponded to 0.05 S. The frictional ratio was initialized at 1.4 and floated during the fitting procedure. A regularization level of 0.68 was used. The buffer density and viscosity were calculated using the SEDNTERP software and were 1.00516 g/ml and 1.0175 cP, respectively. The partial specific volume was kept at the SEDFIT default value of 0.73 cm^3/g, which in general provides a good estimate of the partial specific volume of proteins. The actual values could not be estimated owing to the polyclonal nature of the studied antibody formulations. The goodness of the obtained fits was evaluated by comparing the rmsd values of the resulting fits with the values obtained for the empty cells before the experiments. In this way, it was verified that all the fits had only a randomly distributed noise and that no systematic errors were introduced during the fitting routine. In addition, it was confirmed that no visible diagonal lines were present on the residuals bitmap. To estimate the relative abundance of the different species present in the samples, integration of the C(s) distributions was performed. The percentages of antibody monomers, oligomers, fragments, and albumin were calculated by dividing the corresponding peak area by the sum of the areas under all peaks.

RESULTS

Experimental routine for AUC-SV analysis of immunoglobulin preparations The development of an experimental routine for AUC-SV analysis of immunoglobulin preparations

followed two main phases: a systematic review of the available literature on the subject and testing of a theoretically designed protocol. First, based on the previous studies (18–20), a set of experimental parameters regarding rotor and cell components, sample concentration used for the analysis, optics applied for the detection, and software package for the data analysis were chosen. At the next step, the optimum rotational speed from the recommended range of 40,000–60,000 rpm was selected for AUC-SV analysis. Our previous study has shown that the hydrodynamic parameters of antibodies are affected by high rotational speed during sedimentation experiments, whereas the amount of dimeric antibody aggregate remained unaffected by the rotational speed (21). Thus, we excluded rotational speeds faster than 50,000 rpm from consideration, assuming that these high speeds can contribute to the imprecision of aggregation analysis. High-quality centerpieces, which have previously been shown to improve the precision of aggregates measurements, were Beckman Coulter charcoal-filled epon centerpieces. The Beckman Coulter Buyer's Guide recommends using the epon charcoal-filled centerpieces at speeds slower than 42,000 rpm. Finally, the possible presence of fast-sedimenting high-molecular weight aggregates was considered; thus a rotational speed of 40,000 rpm was selected for the AUC-SV experimental setup.

Aggregation analysis of liquid human immunoglobulin preparations The SEC chromatogram of Polyglobin (Fig. 1A) showed a major peak corresponding to the monomeric form of the antibody. The asymmetry of the monomer peak was attributed to the nonspecific binding of the highly concentrated antibody to the SEC column packing material (6), which is a common problem in protein chromatography. A shoulder peak eluted before the major peak suggested the presence of a dimeric component in the solution. When either integration algorithm was applied for the data analysis, the area under the dimeric peak was estimated to be approximately 0.8% of the total signal (Table 1). This estimate was lower compared with that obtained by AUC-SV analysis. In C(s) distribution (Fig. 1B), in addition to monomeric and dimeric peaks, minor peaks corresponding to antibody fragments and trimeric aggregates were observed. Nevertheless, the results of triplicate measurements showed that these species were present at amounts below the commonly accepted limit of AUC-SV quantification of 1% (25) and therefore could not be considered to be reliably measured. In addition, the standard deviation of the obtained values indicated low reliability of these estimates.

The fraction of dimeric aggregates present in the Venoglobulin formulation was higher than that estimated for Polyglobin (Table 1). Similar to Polyglobin, the AUC-SV analysis of Venoglobulin indicated the presence of trace amounts of fragments and trimeric aggregates below the accepted limit of quantification. The estimates of the total quantity of aggregates derived from AUC-SV measurements and integration of chromatogram using the Gaussian skim approach were in good agreement. The results of peak separation using the vertical drop method and the Gaussian skim approach were consistent, although the amount of dimeric aggregates calculated by the vertical drop method was slightly higher than that obtained by the Gaussian skim approach.

The results of AUC-SV and SEC obtained for Hebsbulin were in good agreement, indicating the presence of only monomeric and dimeric forms of the antibody (Fig. 1E and F). The amount of dimeric aggregates was estimated to be the highest by SEC with the vertical drop method (2.62%), followed by SEC with the Gaussian skim approach (2.54%), and AUC-SV (2.25%; Table 1).

The C(s) distribution of Tetanobulin showed two peaks corresponding to the monomeric and dimeric forms of the antibody (Fig. 1H). The amount of dimeric aggregates derived from the AUC-SV analysis was lower than the value obtained using the vertical drop method for chromatographic data analysis (Table 1). The SEC analysis detected a minor peak in the chromatogram corresponding to trimer/higher aggregates, which was not detected by AUC-SV (Fig. 1K). Integration of the chromatographic peaks showed that these species were present at amounts below the estimated limit of AUC-SV detection of 0.2% (25). Moreover, the obtained value was close to the limit of detection previously determined for SEC (TSK gel SEC Brochure – Tosoh Bioscience GmbH).

Aggregation analysis of lyophilized immunoglobulin preparations The two major peaks corresponding to the monomeric and dimeric forms of the antibody were detected by SEC and AUC-SV analyses of the Glovenin formulation. In the chromatogram (Fig. 1I), these peaks co-eluted and were baseline-unresolved. From integration of the chromatogram by using the vertical drop method, the amount of dimeric aggregates was estimated to be 1.18% higher compared with the estimate produced by the Gaussian skim integration algorithm and 1.39% higher compared with the estimate determined by the C(s) analysis of the AUC-SV data.

The chromatographic profile obtained for Gammagard indicated the presence of four major peaks corresponding to solvent, monomeric, dimeric, and trimeric forms of the antibody. The AUC-SV analysis also detected albumin and trace amounts of high-molecular weight aggregates. The amount of dimeric aggregates estimated by the vertical drop method of SEC chromatogram was significantly higher compared with that calculated using the Gaussian skim algorithm. However, the Gaussian skim algorithm failed to accurately resolve a minor peak corresponding to solvent preventing accurate quantification of the monomeric form of the antibody (Table 1).

There was a significant difference between the AUC-SV and SEC results obtained for Sanglopor independent of the integration approach applied to the SEC data analysis. Similar to other immunoglobulin preparations, the dimeric aggregates amount determined by the Gaussian skim algorithm was lower than that obtained by the vertical drop method. It is interesting that the amount of dimeric aggregates estimated by AUC-SV was lower than that calculated using the vertical drop method but was higher than that resulting from integration using the Gaussian skim approach. The AUC-SV analysis revealed the presence of two antibody fragments and trace amounts of high-molecular weight aggregates, which were not detected in the elution profile.

DISCUSSION

In the present study, the aggregate compositions of different preparations of human immunoglobulins were analyzed using AUC-SV and SEC with the vertical drop method and the Gaussian skim approach. Although AUC-SV and SEC degrees of precision differed, these two analytical techniques provided similar results in the quantification of aggregates confirming the complementary relationship between AUC-SV and SEC. As has been discussed (6,14,16,17), both SEC and AUC-SV methods can be used to quantify the aggregates in the pharmaceutical formulations. Due to its simplicity, speed, and reproducibility of obtained results, SEC is routinely used as a quality control method to evaluate the aggregation of pharmaceuticals. In contrast to SEC, AUC-SV does not conform to the requirements specified for the quality control methods because of the relatively low precision and repeatability. However, AUC-SV offers a significant advantage over SEC as it provides matrix-free separation of the solutes, and therefore, it can be performed to ensure that the sample's composition has not changed owing to interaction with the column packing material. In addition, larger soluble aggregates eluted in the void volume of the SEC column can be detected and characterized by AUC-SV (14).

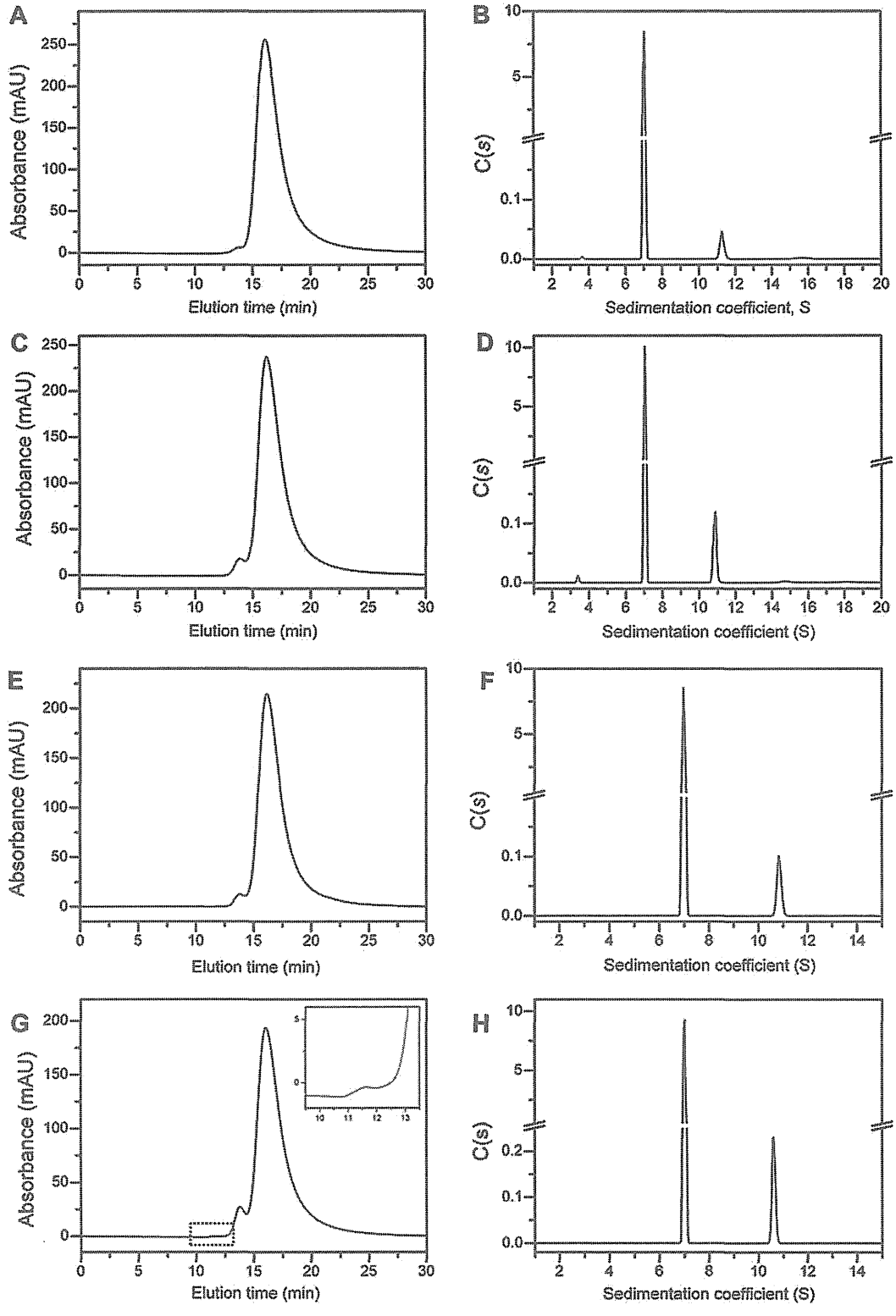

FIG. 1. Size-exclusion chromatograms and sedimentation coefficients distributions C(*s*) obtained for the following human immunoglobulin preparations: (A, B) Polyglobin, (C, D) Venoglobulin, (E, F) Hebsbulin, (G, H) Tetanobulin, (I, J) Glovenin, (K, L) Gammagard, and (M, N) Sanglopor. The insets in panels G and K show enlarged view of the boxed areas. The insets in panels L and N show the C(*s*) distributions with expanded vertical scale. The size-exclusion chromatograms and the continuous sedimentation coefficient distributions C(*s*) consistently showed the same number of peaks, with the exception of Sanglopor, for which the C(*s*) distribution indicated the presence of two peaks corresponding to antibody fragments, whereas these peaks were not seen in the SEC chromatogram.

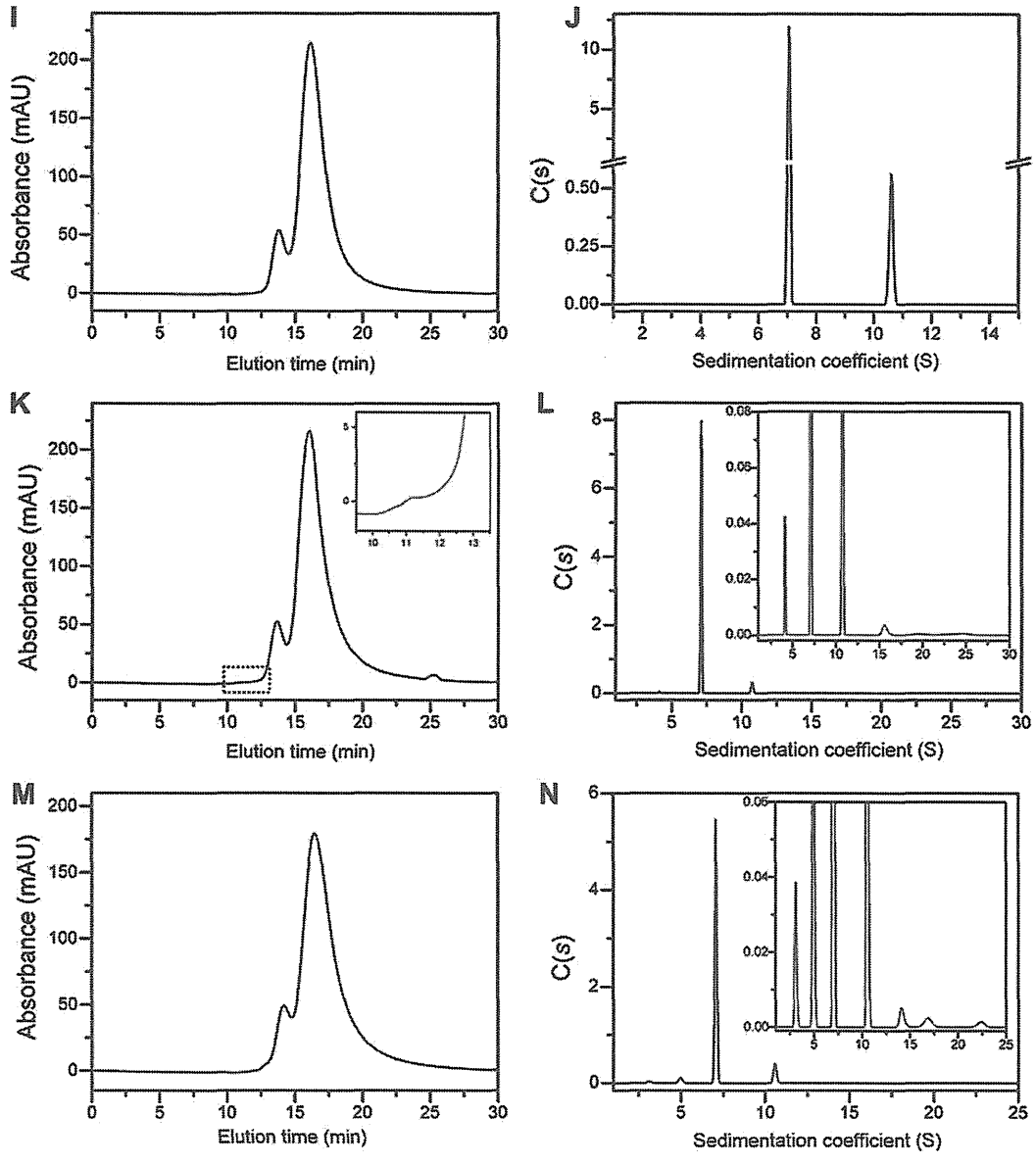

FIG. 1. (*continued*).

The results of the chromatographic analyses obtained using different integration approaches showed minimal variability for the solutions of Venoglobulin, Polyglobin, and Hebsbulin. For the other formulations used in this study, integration of the peaks using the vertical drop method systematically provided higher values of dimeric aggregate compared with those from the Gaussian skim approach. It was noted that with increase in the height of the valley between the unresolved peaks corresponding to monomer and dimer, the differences between estimates produced by the vertical drop method and the Gaussian integration approach increased. These differences were attributed to the differences in integration algorithms, and it was suggested that the Gaussian skim approach was inherently more accurate for overlapping peak separation than the traditional, vertical drop method. This conclusion is also supported by the AUC-SV results that showed better agreement with the results obtained using the Gaussian skim method, but not with the vertical drop method. However, in the case of Gammagard, the Gaussian skim method failed to accurately estimate the area under the small peak corresponding to solvent (Table 1) preventing correct quantification of the monomeric form of the antibody. A reasonably accurate result was obtained when tangential skim (algorithm that performs valley-to-valley extrapolation) (5) was applied, which is known to perform well when it is used to skim a much smaller and narrower peak from the large parent peak. However, similar to the result of the vertical drop method, the amount of dimeric aggregates was overestimated when the tangential skim approach was used. The analysis of the Gammagard chromatogram was complicated by the fact that the peaks could not conform to a single mathematical model, and the asymmetry and further overlapping of the peaks increased the complexity of the chromatogram.

The best way to eliminate measurement error is to increase the resolution of the chromatogram to obtain baseline separated peaks.

TABLE 1. Detailed quantitative summary of the results obtained by C(*s*) SEDFIT analysis of the AUC-SV data and by the vertical drop method and Gaussian skim algorithm analysis of the SEC data.

Product	Method	Albumin, %	Fragment, %	Monomer, %	Dimer, %	Trimer, %	HMW, %
Polyglobin	AUC[a]	–	0.33 ± 0.17	98.26 ± 0.27	1.27 ± 0.16	0.19 ± 0.07	–
	SEC-vertical drop[b]	–	–	99.20 ± 0.01	0.80 ± 0.01	–	–
	SEC-Gaussian skim[b]	–	–	99.20 ± 0.03	0.80 ± 0.03	–	–
Venoglobulin	AUC[a]	–	0.14 ± 0.02	97.24 ± 0.21	2.60 ± 0.21	0.18 ± 0.07	–
	SEC-vertical drop[b]	–	–	97.32 ± 0.08	2.68 ± 0.08	–	–
	SEC-Gaussian skim[b]	–	–	97.46 ± 0.07	2.54 ± 0.07	–	–
Hebsbulin	AUC[a]	–	–	97.75 ± 0.20	2.25 ± 0.20	–	–
	SEC-vertical drop[b]	–	–	97.45 ± 0.04	2.56 ± 0.04	–	–
	SEC-Gaussian skim[b]	–	–	97.47 ± 0.02	2.54 ± 0.02	–	–
Tetanobulin	AUC[a]	–	–	95.74 ± 0.21	4.26 ± 0.21	–	–
	SEC-vertical drop[b]	–	–	94.75 ± 0.11	5.15 ± 0.10	0.10 ± 0.01	–
	SEC-Gaussian skim[b]	–	–	95.51 ± 0.06	4.35 ± 0.06	0.14 ± 0.00	–
Glovenin	AUC[c]	–	–	90.76 ± 0.20	9.24 ± 0.20	–	–
	SEC-vertical drop[b]	–	–	89.37 ± 0.16	10.63 ± 0.16	–	–
	SEC-Gaussian skim[b]	–	–	90.55 ± 0.02	9.45 ± 0.02	–	–
Gammagard	AUC[c]	0.53 ± 0.13	–	92.05 ± 0.28	6.86 ± 0.26	0.34 ± 0.11	0.22 ± 0.04
	SEC-vertical drop[b]	1.57 ± 0.00[d]	–	88.61 ± 0.20	9.75 ± 0.14	0.11 ± 0.01	–
	SEC-Gaussian skim[b]	6.25 ± 0.25[d]	–	85.02 ± 0.06	8.40 ± 0.30	0.32 ± 0.04	–
Sanglopor	AUC[c]	–	1.07 ± 0.08 3.02 ± 0.26	85.40 ± 0.09	9.64 ± 0.09	0.47 ± 0.17	0.41 ± 0.02
	SEC-vertical drop[b]	–	–	89.69 ± 0.28	10.32 ± 0.28	–	–
	SEC-Gaussian skim[b]	–	–	91.34 ± 0.04	8.66 ± 0.04	–	–

[a] The data are the mean values of six measurements performed in three independent runs ± SD.
[b] The data are the mean values of triplicate measurements ± SD.
[c] The data are the mean values of two measurements performed in one run ± SD.
[d] The albumin peak was not detected. The reported value is the result of the solvent peak integration.

In general, this can be achieved by modifications of the mobile phase. However, the choice of optimum mobile phase is a tradeoff between resolution and accuracy. As has been discussed (6,22), adjustments of the mobile phase can increase the resolution and at the same time may affect the original aggregate distribution in the antibody formulation. In addition, by increasing the resolution between monomer and dimer, the resolution of higher oligomers can significantly be altered.

AUC-SV was extensively used for the characterization of antibody samples (16) and, in particular, was successfully applied to the aggregation analysis of pharmaceutical antibodies (20). In the present study, a very high degree of agreement was observed between AUC-SV and SEC results for liquid formulations of immunoglobulin. In contrast, the agreement was relatively poor in the case of reconstituted preparations. In these formulations, SEC measurements performed on consecutive days suggested the loss of monomer due to formation of dimeric aggregates. This process was shown to be relatively slow compared with the time course of the sedimentation experiment. Surprisingly, the amount of dimeric aggregates estimated using AUC-SV was lower than the value obtained by SEC. We concluded that in the reconstituted formulations used for the AUC-SV measurements, the equilibrium of the monomer–dimer reaction was shifted toward monomer formation owing to a hundred-fold dilution required to analyze these solutions.

In the case of Sanglopor, the AUC-SV analysis detected the presence of two fragments, which were not visible in the chromatogram (Fig. 1M). It is suggested that the highly asymmetrical large monomer peak eluted before the smaller fragments' peaks caused this effect. Another hypothesis was that the relatively long centrifugation times could cause the degradation of monomer into antibody fragments.

In conclusion, the results of AUC-SV and SEC were consistent and the degree of agreement was higher when the chromatographic data were analyzed by using the Gaussian skim approach (Fig. 2). Thus, the results of this study confirmed that AUC-SV is an appropriate complementary to SEC method for aggregate composition analysis and underscored the important role that the different integration methods can play in the quantitative interpretation of chromatographic results.

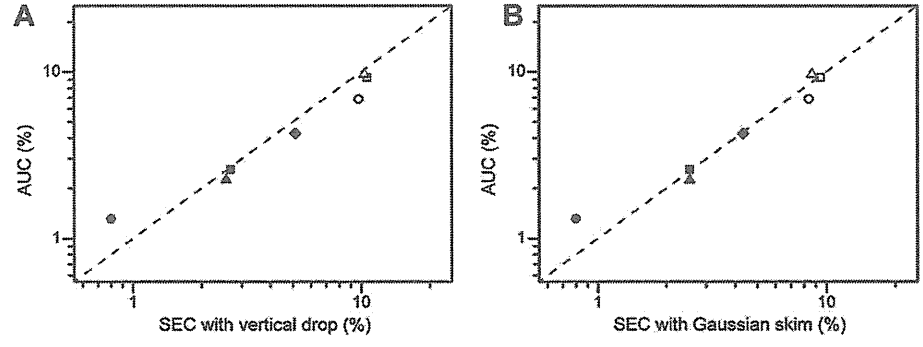

FIG. 2. Comparison of the dimeric aggregate amounts detected using AUC-SV and SEC with either (A) the vertical drop method or (B) the Gaussian skim approach. In each panel, the results for Polyglobin (filled circle), Venoglobulin (filled square), Hebsbulin (filled triangle), Tetanobulin (filled diamond), Gammagard (open circle), Sanglopor (open triangle), and Glovenin (open square) are shown. The degree of agreement between the AUC-SV and SEC results was higher when the Gaussian skim approach was applied for the chromatographic data analysis.

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Laboratory and Epidemiology Communications

Virological Analysis of a Regional Mumps Outbreak in the Northern Island of Japan—Mumps Virus Genotyping and Clinical Description

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Mumps is an acute, contagious, vaccine-preventable disease caused by mumps virus (MuV) and is typically characterized by swelling of either or both parotid glands. A mumps epidemic occurs every 3–5 years in non-vaccinated populations, and humans are the only natural hosts for MuV, which is transmitted by the respiratory route and replicates primarily in the upper respiratory mucosal epithelium. Primary replication leads to viremia and triggers secondary viral replication (1). Parotitis is not, however, a necessary component of mumps symptoms. MuV can display neurovirulence. MuV can also enter the cerebrospinal fluid (CSF) through the choroid plexus, leading to a form of aseptic meningitis in up to approximately 10% of cases. More serious complications, such as deafness and encephalitis, occur less frequently and up to 30% of infections are asymptomatic or display only non-specific respiratory symptoms. Furthermore, reinfection with mumps has been confirmed in serological studies (2,3) and a significant portion of adult sudden sensorineural deafness is reportedly related to MuV infection (4).

In Japan, 5 mumps vaccines (genotype B) are currently licensed, and single dose is distributed on a voluntary basis (5), with a coverage rate of approximately 23% (6). However, herd immunity level is insufficient to prevent epidemics; therefore, mumps outbreaks in children repeat every several years.

MuV is a member of the genus *Rubulavirus* of the family *Paramyxoviridae*. Eight proteins, the nucleocapsid (N), V, phospho (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) proteins, are coded by 7 genes. The SH gene sequence is highly variable and is, therefore, used for MuV genotyping (7–9).

Fukagawa city (coordinates: 43°43′N, 142°2′E) is a relatively isolated rural region in Hokkaido, Japan, with a population of approximately 23,000. From April to August 2010, a total of 212 patients were diagnosed with mumps in the pediatric department of our hospital. In January 2011, 3 additional patients were diagnosed; the age range of these 215 patients was 1–19 years, and they had no significant medical history of immunodeficiency, malignancy, or developmental delay. Diagnosis was made on the basis of typical clinical manifestations and/or laboratory test results of serological study or amylase in the serum or urine. Seventeen patients were hospitalized; of these, 10 were diagnosed with meningitis, 3 with orchitis, and 1 with hepatitis. Two unrelated patients developed audiometry-con-

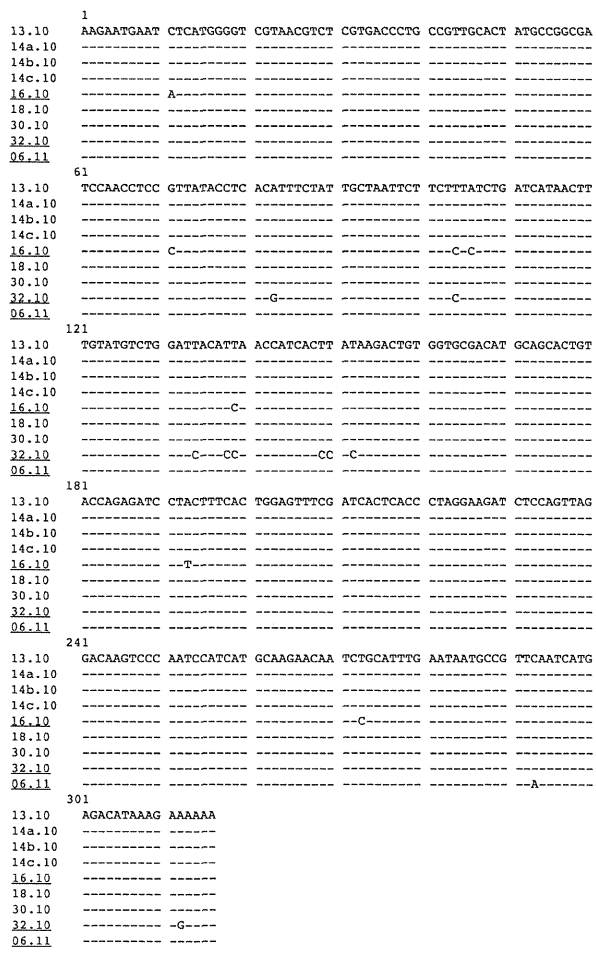

Fig. 1. Nucleotide sequences of SH gene detected in this study in chronological order. Left column shows week and year part of strain names. From top to bottom, AB725762.1, AB725761.1, AB725764.1, AB725766.1, AB725763.1, AB725760.1, AB725759.1, AB725765.1, and AB725767.1. Numbers above row denote nucleotide position. Underlined strains are not identical to index sequence.

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firmed unilateral hearing loss as a sequela. One of these patients who developed hearing loss was positive for meningitis despite a vaccine history 9 years prior to the episode. According to this patient's medical record, Torii strain vaccine was administrated. On admission, anti-mumps IgG and IgM titers were measured using an EIA mumps kit (Denka Seiken, Tokyo, Japan), in which the MuV Enders strain was used as an antigen. The IgG titer (reference range: positive at a titer of 4 or more) was 13.7, and the IgM titer (reference range: positive at a titer of 1.2 or more) was below the detectable limit. The other patient was not vaccinated but did not show clinical signs of meningitis. Because 2 patients with hearing loss were observed, virological analysis of several stored samples (13 CSF's and 3 throat swabs) was performed to discern any underlying factors. All analyses were anonymously performed and complaint with the national guidelines for clinical research.

Nucleic acid detection and genotyping of MuV were performed as described previously (7). In brief, using a standard technique, the SH gene was RT-PCR amplified from CSF or throat swab samples after RNA isolation (Roche Diagnostics, Tokyo, Japan), using the primer pair "tcaagtagtgtcgatgatctc" and "aggtggcatt gtctgacattg" corresponding to the nucleotides 6130–6150 and 6656–6636, respectively, of NC_002200.1. A specific 526-bp band was amplified and sequenced. Multiple sequence alignment and phylogenetic analysis by the neighbor-joining method were performed using GENETYX-MAC (GENETYX Co., Tokyo, Japan). Nine of 13 CSF samples were successfully analyzed (GenBank accession numbers, AB725759.1–67.1), but none of the throat swab samples were suitable for analysis.

The six samples collected in April, May, and August 2010 had identical sequences, while the other 3 samples collected in April, and August 2010 and January 2011 revealed slightly different sequences (Fig. 1). The latter sequences were detected at either the beginning or end of the outbreak. All of these sequences were determined as genotype G by phylogenetic analysis (Fig. 2). None of the samples from the 3 vaccinated patients were successfully analyzed due to amplification failure. Nonetheless, our success rate was comparable with that of previous reports (9,10).

Mumps outbreaks, even among vaccinated populations, have recently been reported worldwide, including in the UK, the US, the Netherlands, and Canada (10–14). In the present outbreak, we detected MuV genotype G, which has been the predominant genotype commonly detected in recent outbreaks. The majority of MuV strains we detected were identical at the SH

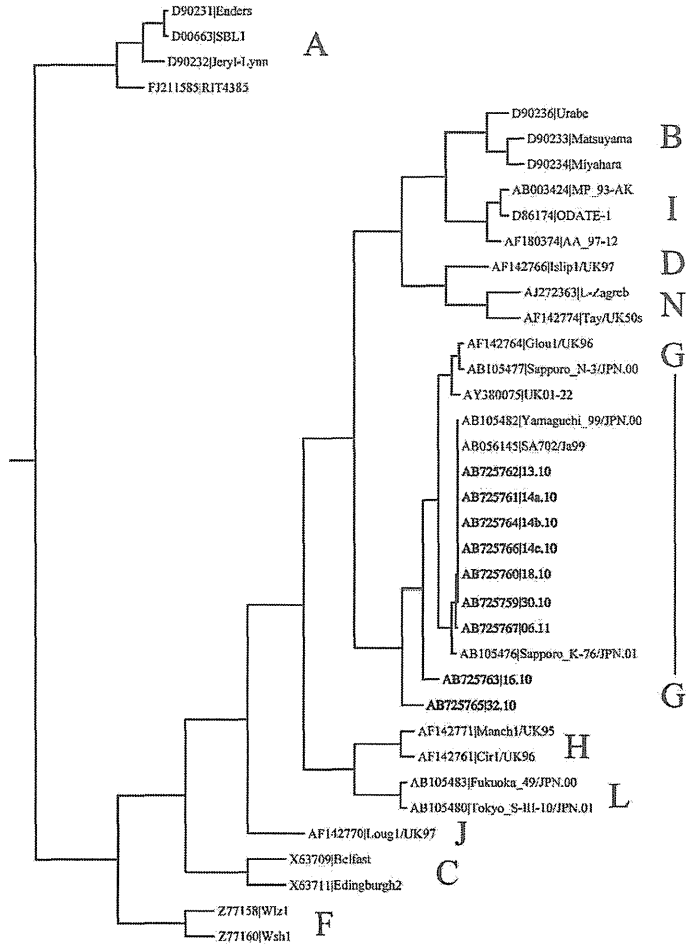

Fig. 2. Phylogenetic analysis of selected MuV strains. Strains detected in this study are in boldface. Capital letters denote MuV genotype.

gene level to those previously detected in geographically and chronologically distant areas of Yamaguchi (AB105482.1) and Saitama (AB056145.1), Japan (15,16). Although not identical, 2 other MuV genotype G strains (AB105476.1-77.1) were isolated in Sapporo, Japan, which is located approximately 100 km from Fukagawa on the same island. One finding of interest is that one strain isolated in the UK (AF142764.1) was phylogenetically similar to the strains we detected.

MuV genotypes H and D were detected in South Korea (17) and the Netherlands (18), respectively. In the present study, only genotype G was detected, with minor variations observed at the beginning and end of the outbreak. A short-time survey of vaccine coverage in the Fukagawa area revealed a rate of approximately 30%, which was slightly higher than the nationwide rate. Almost 30% of patients in this study were vaccinated (data not shown). Overall, the clinical courses of the vaccinated patients were mild, except for the patient who developed hearing loss. The anti-mumps serological study suggested secondary vaccine failure rather than primary vaccine failure.

The incidence of hearing loss in mumps patients is reportedly 0.001%-1.0% (19-21). The rate in the present study was 0.93% (2/215), which was comparable with the highest incidence thus far reported (11). Another study from Japan also reported a high rate of hearing problems. There are several other reported cases of hearing loss after mumps vaccination (20,22,23). We have been unable to conduct a survey to assess sensorineural deafness in this area.

Genotype mismatch between vaccine and epidemic virus strains has been often discussed in association with several outbreaks (24,25), demonstrating that antibodies against a vaccine strain failed to prevent infection by other strains. On the other hand, some publications have reported that antibodies raised against vaccine strains were able to neutralize other genotype strains (26,27). Antibodies raised against the Enders strain (genotype A) can be successfully employed in detection kits. We observed that a MuV genotype B vaccine failed to prevent the manifestations of mumps (genotype G) in a significant portion of a population, although the vaccine was able to ameliorate the clinical course of the disease. Our current mumps vaccine scheme may not be optimal. We were unable to discern any significant factors in either the host or viral side in this mumps outbreak with a high sequela rate. Further elucidation of the virological and epidemiological backgrounds of this occurrence was beyond the scope of this study.

Conflict of interest None to declare.

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TMPRSS2 Is an Activating Protease for Respiratory Parainfluenza Viruses

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Here, we show that human parainfluenza viruses and Sendai virus (SeV), like other respiratory viruses, use TMPRSS2 for their activation. The membrane fusion proteins of respiratory viruses often possess serine and glutamine residues at the P2 and P3 positions, respectively, but these residues were not critical for cleavage by TMPRSS2. However, mutations of these residues affected SeV growth in specific epithelial cell lines, suggesting the importance of these residues for SeV replication in epithelia.

In general, enveloped viruses initiate their infection by attaching to receptors on host cells, with subsequent membrane fusion between the virus envelope and the host cell membrane. The fusion process is mediated by a specialized surface glycoprotein, which is often synthesized as an inactive precursor and undergoes endoproteolysis by a host cell protease for activation. Recent studies have suggested that some type II transmembrane serine proteases (TTSPs), including TMPRSS2, human airway trypsin-like protease (HAT), TMPRSS4, and matriptase, are responsible for cleavage of the hemagglutinin (HA) protein of influenza A virus (IAV) in the human airway (1–6). Among these, TMPRSS2 has also been shown to proteolytically activate the F protein of human metapneumovirus (HMPV) (7). Severe acute respiratory syndrome (SARS) coronavirus (CoV), NL63 CoVs, and the novel human CoV (HCoV) EMC also use TMPRSS2 for their spike protein activation (8–13). Here, we studied the activation of respiratory parainfluenza viruses (human parainfluenza viruses [HPIVs] and Sendai virus [SeV]) by TMPRSS2.

HPIV1, HPIV4a, and HPIV4b generally require trypsin (trypsin dependent) to undergo multiple rounds of infection in most established cell lines, whereas some strains of HPIV2 and HPIV3 spread efficiently in cell lines in the absence of trypsin (trypsin independent) because they use the ubiquitous furin protease (14). HPIV1 (2272-Yamagata-2009 strain), HPIV4a (M-25 strain), HPIV4b (CH19503 strain), and trypsin-dependent HPIV2 (2331-Yamagata-2009 strain) and HPIV3 (1835-Yamagata-2009 strain) were used in the present study. In the absence of trypsin, none of these HPIV strains produced plaques in Vero cells, whereas all HPIVs showed plaque formation clearly in Vero/TMPRSS2 cells, which constitutively express TMPRSS2 (Fig. 1A). In a previous study (7), we generated Vero/TMPRSS2 cells by cotransfecting Vero cells with a TMPRSS2 expression vector, pCA7-TMPRSS2, in which EcoRI and NotI sites were used for cloning, and the *neo* gene-bearing vector pCXN2. For multistep growth experiments, Vero/TMPRSS2 and Vero cells were infected with HPIVs at a multiplicity of infection (MOI) of 0.01. All HPIVs replicated efficiently in Vero/TMPRSS2 cells, even in the absence of trypsin (Fig. 1B). Analyses by SDS-PAGE followed by immunoblotting demonstrated cleavage of the F proteins of HPIVs in Vero/TMPRSS2 cells, but not in Vero cells, in the absence of trypsin (Fig. 1C). In these assays, rabbit antisera raised against peptides corresponding to the cytoplasmic regions of the HPIV1, HPIV3, and HPIV4 F proteins (VRRLLVMINSTNNSPINAYTLESRMRN PYM, IIIIAVKYYRIQKRNRVDQNDKPYVLTNK, and EVKNVA RNQRLNRDADLFYKIPSQIPVPR, respectively) and a peroxidase-conjugated secondary antibody were used. Polyvinylidene difluoride (PVDF) membranes, on which polypeptides from cell lysates were blotted, were treated with the ECL Plus reagent (Amersham Biosciences), and chemiluminescence signals on the membrane were detected and visualized with a VersaDoc 3000 imager (Bio-Rad, Hercules, CA) (Fig. 1C). For analysis of SeV, a recombinant wild-type SeV expressing red fluorescent protein [SeV-RFP(wt)] was used. To generate SeV-RFP(wt), the RFP gene was inserted into the 3′-end-proximal first locus of the SeV genome, which is encoded by an expression plasmid, generating the full-length SeV genome plasmid pSeV-RFP (15, 16). SeV-RFP(wt) was then generated by using a reverse-genetics technique (15). In the absence of trypsin, SeV-RFP(wt) failed to undergo multiple rounds of infection in Vero cells, whereas it propagated efficiently in Vero/TMPRSS2 cells (Fig. 1D and E). Analyses by SDS-PAGE and immunoblotting also demonstrated cleavage of the F protein of SeV-RFP(wt) in Vero/TMPRSS2 cells, but not in Vero cells, in the absence of trypsin (Fig. 1F). In this assay, a rabbit antiserum raised against the cytoplasmic region of the SeV F protein (RIPR DTYTLEPKIRHMYTNGGFDAMAEKR) was used. Consistently with these data, SeV particles released from Vero/TMPRSS2 cells possessed cleaved F proteins, even in the absence of trypsin (Fig. 1G). On the other hand, SeV particles released from Vero cells cultured in the absence of trypsin possessed uncleaved F proteins (Fig. 1G). These virus particles with uncleaved F proteins were

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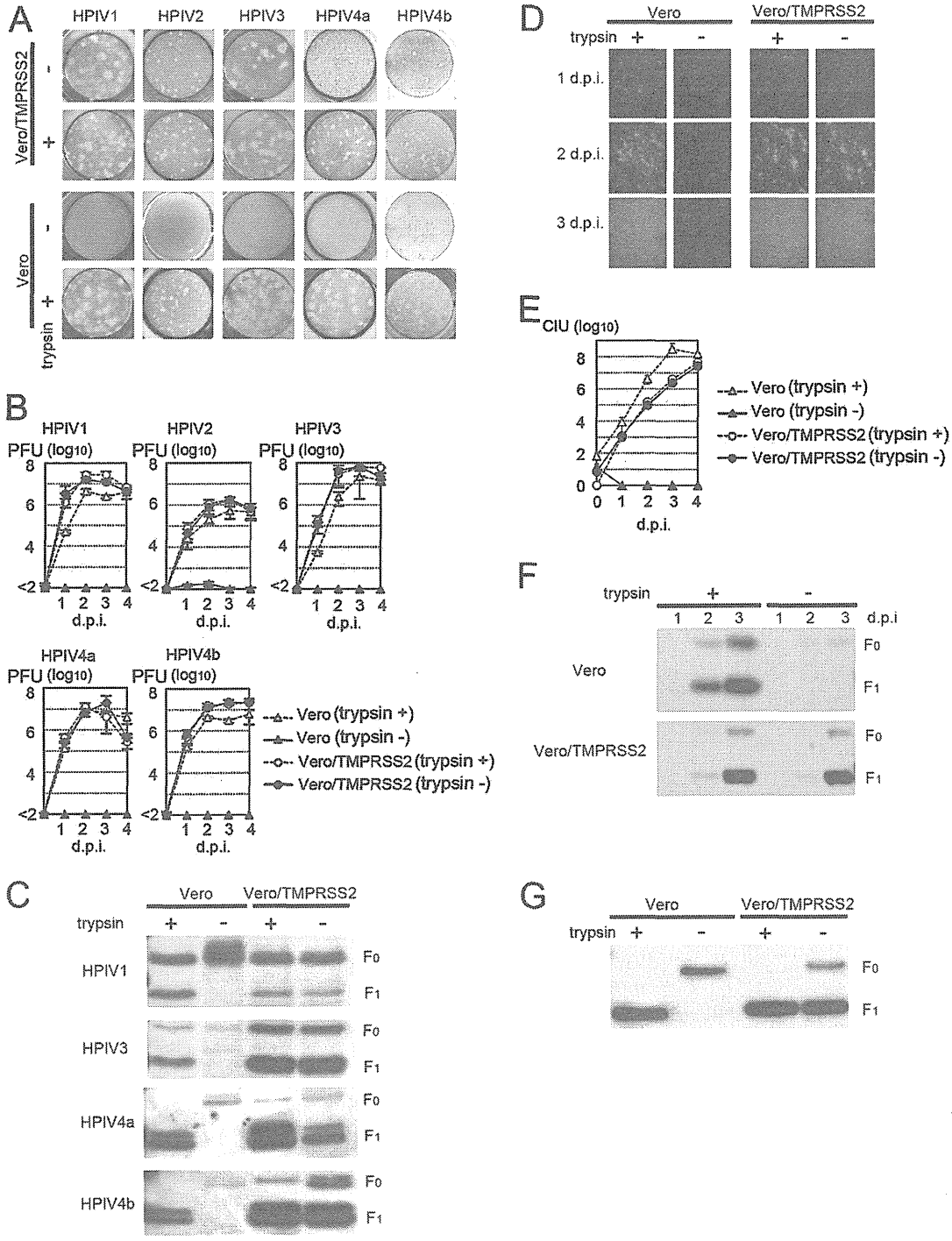

FIG 1 Proteolytic activation of HPIVs and SeV by TMPRSS2. (A) Plaque formation of HPIVs in Vero and Vero/TMPRSS2 cells in the presence or absence of trypsin. (B) Replication kinetics of HPIVs in Vero and Vero/TMPRSS2 cells. Cells were infected with HPIVs at an MOI of 0.01, cultured in the presence or absence of trypsin, and examined for their virus titers (PFU) daily. d.p.i., days postinfection. (C) Detection of HPIV F proteins by immunoblotting. Vero and Vero/TMPRSS2 cells infected with HPIVs in the presence or absence of trypsin were lysed in radioimmunoprecipitation assay (RIPA) buffer and subjected to SDS-PAGE and immunoblotting for detection of the F proteins (indicated as F_0 and F_1). (D) Detection of SeV-RFP(wt)-infected cells. Monolayers of Vero and Vero/TMPRSS2 cells were infected with SeV-RFP(wt) at an MOI of 0.01 and observed daily using a fluorescence microscope. (E) Replication kinetics of SeV-RFP(wt) in Vero and Vero/TMPRSS2 cells. Cells were infected with SeV-RFP(wt) at an MOI of 0.01, cultured in the presence or absence of trypsin, and examined for their virus titers (cell infectious units [CIU]) daily. (F) Detection of SeV F proteins in infected cells by immunoblotting. Vero and Vero/TMPRSS2 cells infected with SeV-RFP(wt) in the presence or absence of trypsin were lysed in RIPA buffer and subjected to SDS-PAGE and immunoblotting for detection of the F proteins. (G) Detection of SeV F proteins in virus particles by immunoblotting. Vero and Vero/TMPRSS2 cells were infected with SeV-RFP(wt) in the presence or absence of trypsin, and virus particles released from these cells were purified and subjected to SDS-PAGE and immunoblotting for detection of the F proteins.

TABLE 1 Residues of the cleavage site in virus membrane fusion proteins

Virus	Residue(s) at[a]: P7	P6	P5	P4	P3	P2	P1	P'1	P'2	P'3
HMPV	E	N	P	R	Q	S	R	F	V	L
SeV	A	D/G	A/V	P	Q	S	R	F	F	G
HPIV1	A/N	D	N/V	P	Q	S/T	R	F	F	G
HPIV2 (trypsin dependent)	T	A/K/T	P/T	R	Q	E	R	F	A	G
HPIV2 (trypsin independent)	T	K/T	T	R	Q	K	R	F	A	G
HPIV3 (trypsin dependent)	T	D	P	R	T	E	R	F	F	G
HPIV3 (trypsin independent)	T	D/N	P	R/T	T	K/R	R	F	F	G
HPIV4a	S	S	E	I	Q	S	R	F	F	G
HPIV4b	S	S	E	I	Q	S	R	F	F	G
IAV H1 subtype	I/V	P	S	I	Q	S	R	G	L	F
IAV H2 subtype	I/V	P	Q	I	E	S	R	G	L	F
IAV H3 subtype	I/V	P	E	K	Q	T	R	G	I/L	F
IAV H4 subtype	I	P	E	K	A	T	R	G	L	F
IAV H5 subtype	V	P	Q	R	E/K	T	R	G	L	F
IAV H6 subtype	I/V	P	Q	I	E	T	R	G	L	F
IAV H7 subtype	P	E	I	P	K	G	R	G	L	F
IAV H8 subtype	T	P	S	I/V	E	P	K/R	G	L	F
IAV H9 subtype	V	P	A	A/V	S	D	R	G	L	F
IAV H10 subtype	P	E	I	M	Q	G	R	G	L	F
IAV H11 subtype	V	P	A	I	A	S/T	R	G	L	F
IAV H12 subtype	V	P	Q	A/V	Q	D/N	R	G	L	F
IAV H13 subtype	V	P	A/T	I	A/S	N/S	R	G	L	F
IAV H14 subtype	I	P	G	K	Q	A	K	G	L	F
IAV H15 subtype	P	E	K	I	R	T	R	G	L	F
IAV H16 subtype	V	P	S	I/V	G/N/S/V	E	R	G	L	F
NDV (avirulent)	G	E/G	G	K/R	Q	G	R	L	I	G
Avian paramyxovirus type 2	F	D	K	P	A	S	R	F	V	G
Avian paramyxovirus type 3	Q	A/P	R	P	R/S	G	R	L	F	G
Avian paramyxovirus type 4	D/E	A/V	D	I	Q	P	R	F	I	G
Avian paramyxovirus type 6	H/N	P/S	A/I	P/R	E	P	R	L	I/V	G

[a] Serine and glutamine residues at P2 and P3 are underlined.

incapable of entering cells, even when the target cells expressed TMPRSS2 (data not shown). Thus, TMPRSS2 did not activate the F protein of the incoming SeV particles, as was observed for IAV (17). These data demonstrated that TMPRSS2 proteolytically activated the SeV F protein intracellularly during the process of SeV assembly. Therefore, the mechanism for SeV and IAV activation by TMPRSS2 is different from that for the SARS coronavirus, which is activated at the entry step after receptor binding (11).

The F proteins of HMPV, SeV, HPIV1, and HPIV4 (both HPIV4a and HPIV4b) and the HA protein of IAV (H1 subtype) possess serine and glutamine residues at the P2 and P3 positions, respectively (Table 1). These residues are relatively conserved among respiratory viruses (Table 1). An arginine or a lysine residue at P1 is mandatory for serine protease substrates, while the residues at P2 and P3 are much more flexible than the P1 residue, although they may also modulate the specificity of the protease substrate (18, 19). By site-directed mutagenesis of pSeV-RFP, five predicted amino acid substitutions (Q114A, Q114S, Q114V, S115R, and S115V) were individually introduced into the F protein of recombinant SeV. The mutated hexanucleotide sequences, which correspond to amino acid residues at positions 114 and 115 of the Q114A, Q114S, Q114V, S115R, and S115V mutants, were GCCTCG, AGCTCG, GTGTCG, ATCCGC, and ATCGTG, respectively (mutated nucleotides are underlined). Infectious SeV mutants were generated by using a reverse-genetics technique (15). The replication capacities of the mutant SeVs were analyzed using TMPRSS2-expressing Huh7 cells (Huh7/TMPRSS2-18 and Huh7/TMPRSS2m-4 cell lines) (M. Esumi et al., submitted for publication). Huh7/TMPRSS2-18 cells constitutively express an intact and active form of TMPRSS2, while Huh7/TMPRSS2m-4 cells constitutively express an enzymatically inactive mutant form of TMPRSS2 (TMPRSS2m) with an S441A mutation (Esumi et al., submitted). In Huh7/TMPRSS2m-4 cells, all of the SeVs, including SeV-RFP(wt), spread slowly, whereas in Huh7/TMPRSS2-18 cells, all of the viruses spread much more efficiently (Fig. 2A and B). However, in Huh7/TMPRSS2-18 cells, the growth of the mutant SeV with S115R (SeV-RFP/S115R) was severely deteriorated, and the infectious-virus production was more than 1,000 times lower than that of SeV-RFP(wt) (Fig. 2A and B). The other four mutants (SeV-RFP/Q114S, -RFP/Q114A, -RFP/Q114V, and -RFP/S115V) also showed impaired replication capacities that were reduced by ~10-fold (Fig. 2B). However, pulse-chase labeling followed by immunoprecipitation and SDS-PAGE showed little, if any, effects of the mutations on the cleavage of the F proteins of the recombinant SeVs in Huh7/TMPRSS2-18 cells (Fig. 2C).

Human bronchial epithelial Calu-3 cells and intestinal epithelial Caco-2 cells have an intrinsic ability to proteolytically activate IAV using endogenously expressed proteases (20–22). In Calu-3 cells, TMPRSS2 and matriptase were shown to contribute to IAV HA cleavage (6, 21). In Caco-2 cells, TMPRSS2 and TMPRSS4 are