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Journal of Bioscience and Bioengineering
VOL. 115 No. 1, 104–110, 2013

www.elsevier.com/locate/jbiosc

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

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Received 27 June 2012; accepted 31 July 2012
Available online 24 August 2012

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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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2012.07.021

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 nonideality 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).

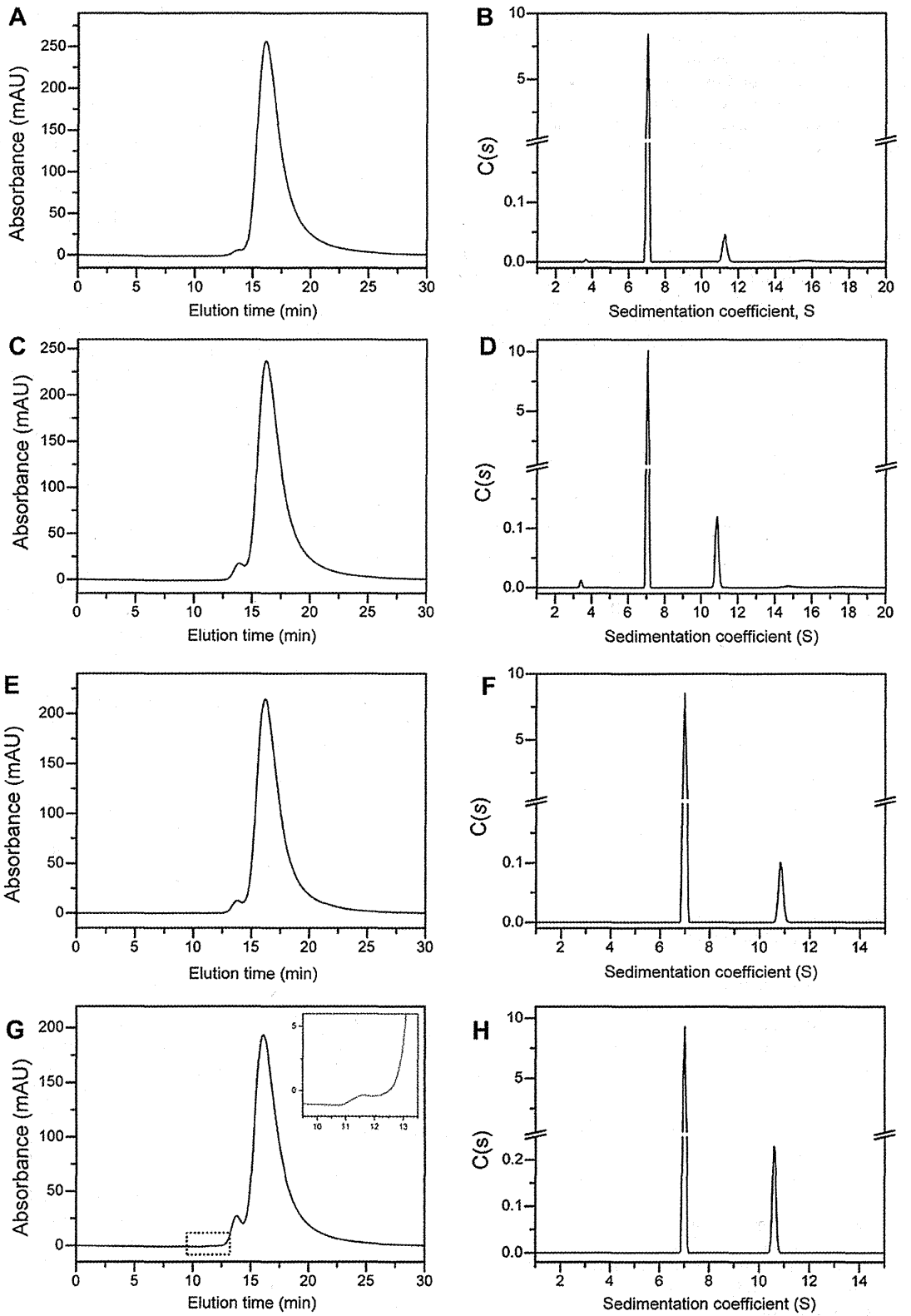

FIG. 1. Size-exclusion chromatograms and sedimentation coefficients distributions C(*s*) obtained for the following human immunoglobulin preparations: (A, B) Polyglobin, (C, D) Venoglobulin, (E, F) Hebsbulin, (G, H) Tetanobulin, (I, J) Glovenin, (K, L) Gammagard, and (M, N) Sanglopor. The insets in panels 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.

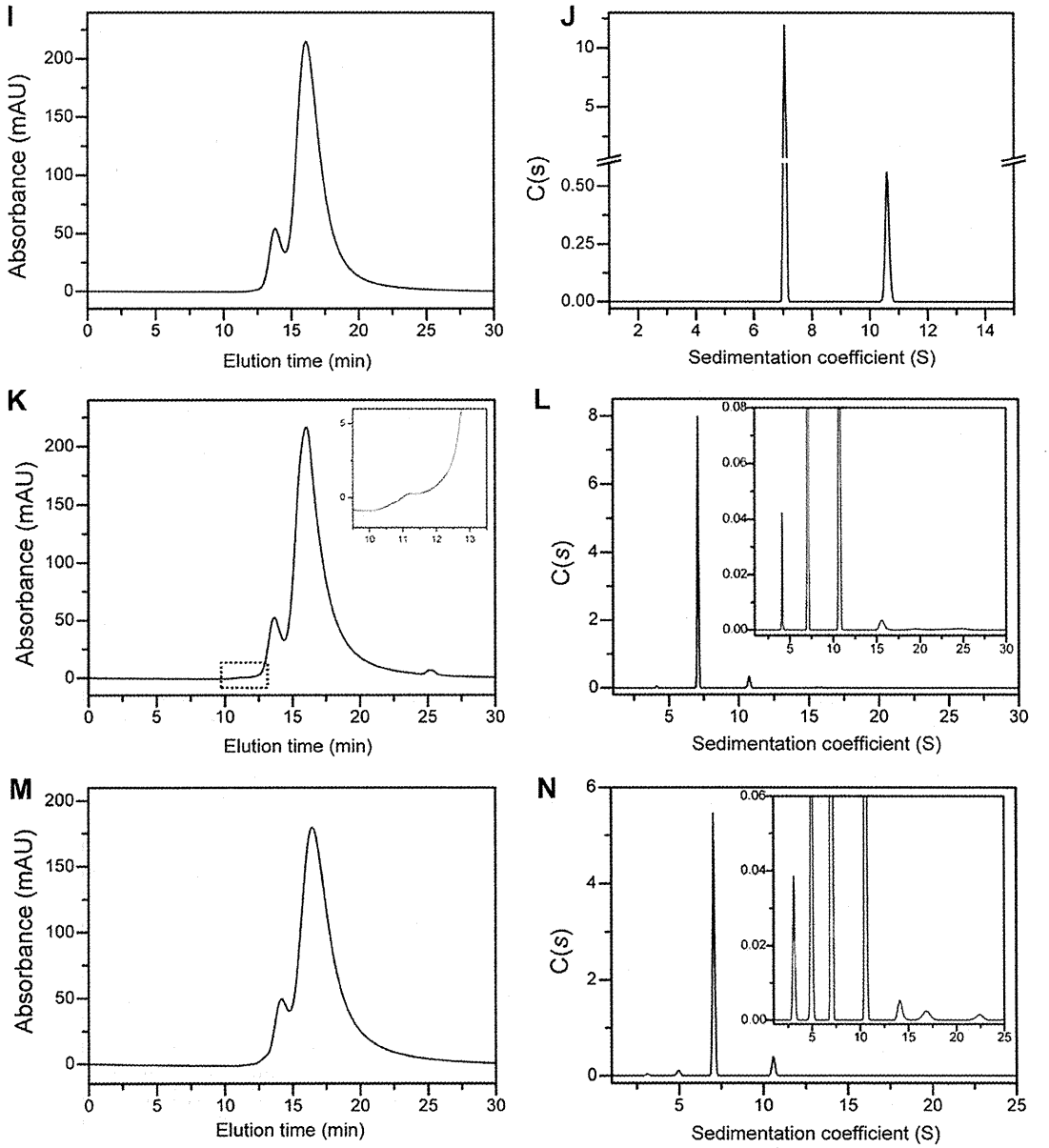

FIG. 1. (*continued*).

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.

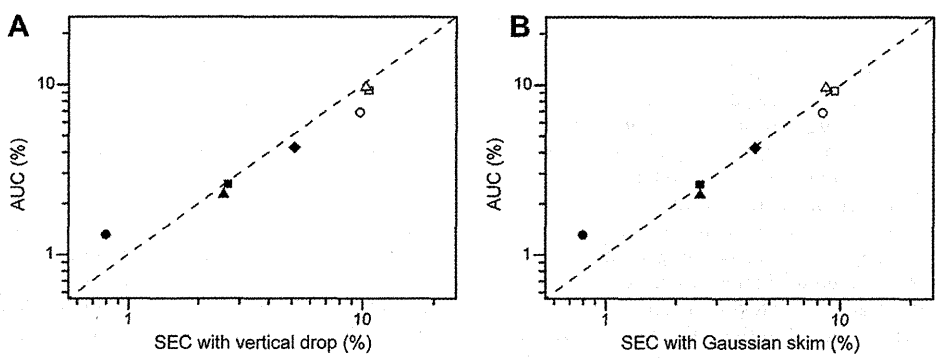

FIG. 2. Comparison of the dimeric aggregate amounts detected using AUC-SV and SEC with either (A) the vertical drop method or (B) the Gaussian skim approach. In each panel, the results for Polyglobin (filled circle), Venoglobulin (filled square), Hebsbulin (filled triangle), Tetanobulin (filled diamond), 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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Transfusion and Apheresis Science 48 (2013) 95–102

Contents lists available at SciVerse ScienceDirect

Transfusion and Apheresis Science

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

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

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ARTICLE INFO

Article history:
Received 17 May 2012
Accepted 30 July 2012

Keywords:
Adverse effects
Blood transfusions
Haemovigilance
Online system
Pilot study

ABSTRACT

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Participants in the pilot study

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

2.2. Online system

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

2.3. Statistics

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

3. Results

3.1. Reporting system and classifications

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

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

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

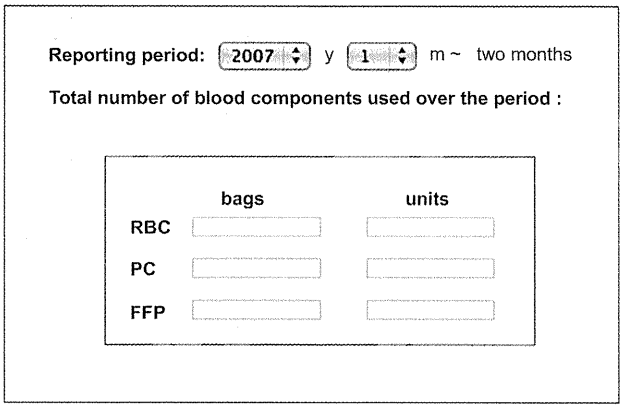


Fig. 1. Online surveillance system (1): Screenshot of the total number of the three labile blood components (bags and units) used over each reporting period. RBC: red blood cells; FFP: fresh frozen plasma; PC: platelet concentrates.

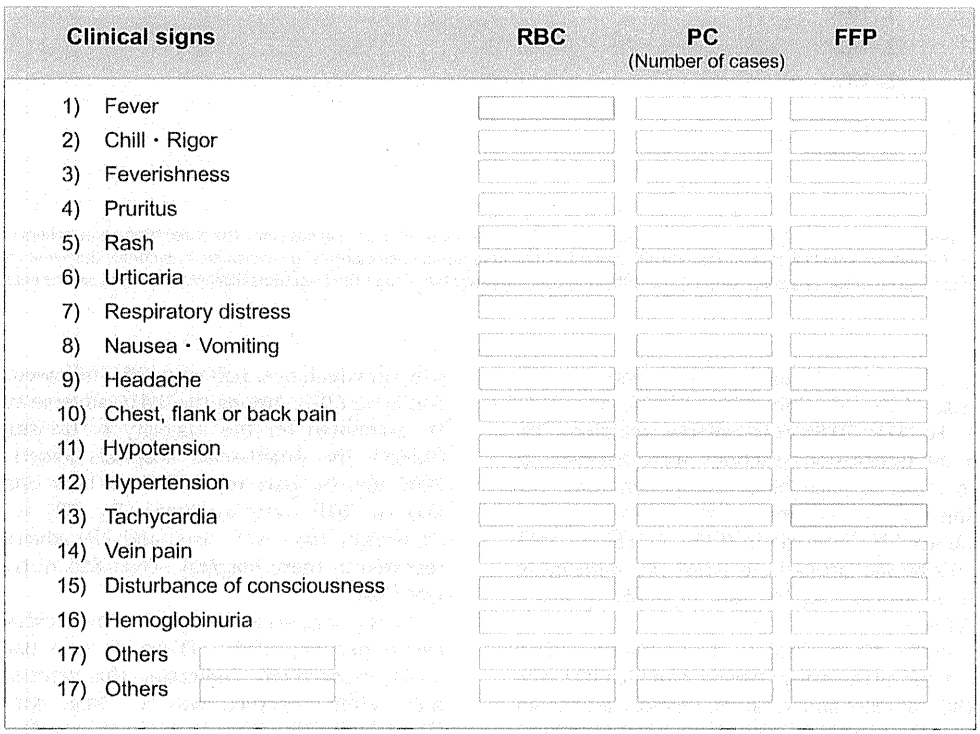


Fig. 2. Online surveillance system (2): The total number of transfusion reactions by clinical signs for the three blood components used over the reporting period is presented. Clinical signs are classified into the 16 categories indicated. Fever: more than 38 °C or a 1 °C or more increase from the baseline;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

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

3.3. Types, clinical signs and diagnoses of adverse events

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

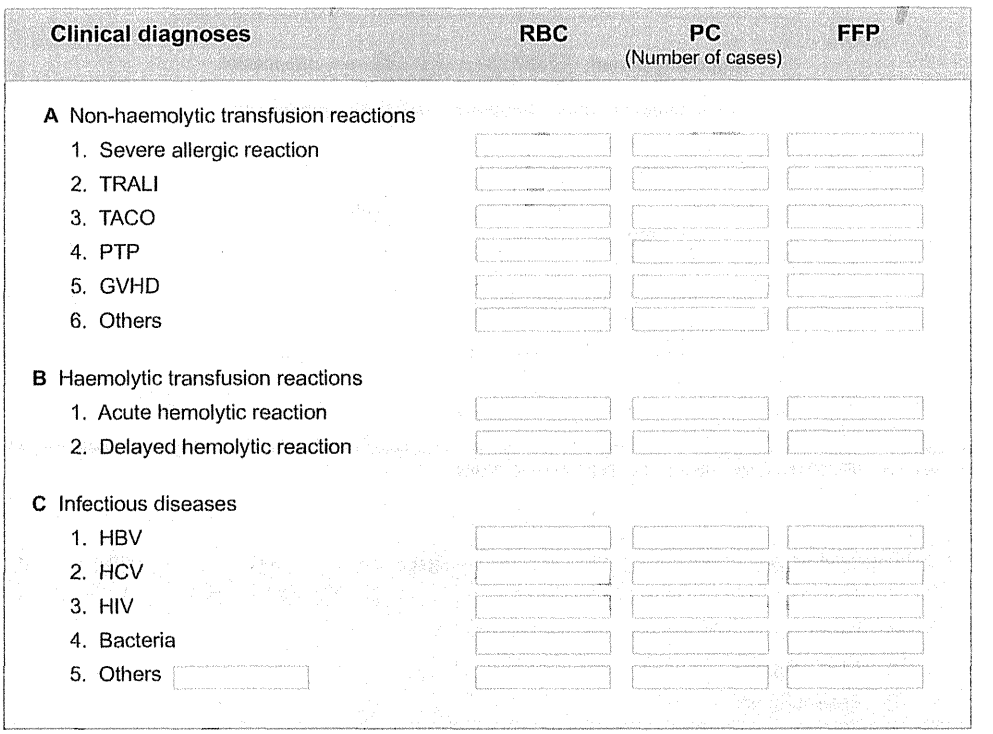


Fig. 3. Online surveillance system (3): The total number of transfusion reactions by clinical diagnoses for the three blood components over the period is presented. Clinical diagnoses are classified into the three categories indicated. Among non-haemolytic transfusion reactions, the events not included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

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

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

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

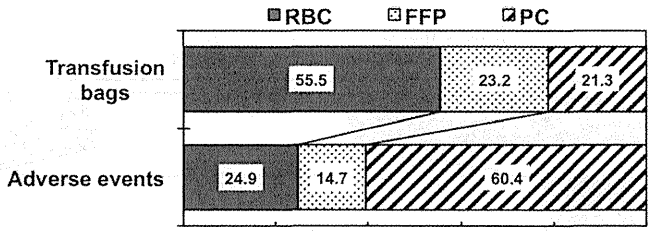
We compared the incidence of adverse events in seven large-scale university hospitals with that in five small-scale hospitals with fewer than 300 beds. Seven large-scale hospitals participated in this pilot study since 2007 and the data reported by these hospitals from 2007 to 2010 were analyzed (Fig.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

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

4. Discussion

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

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



B. Incidence of transfusion reactions by kinds of blood components

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

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

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

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

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

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

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

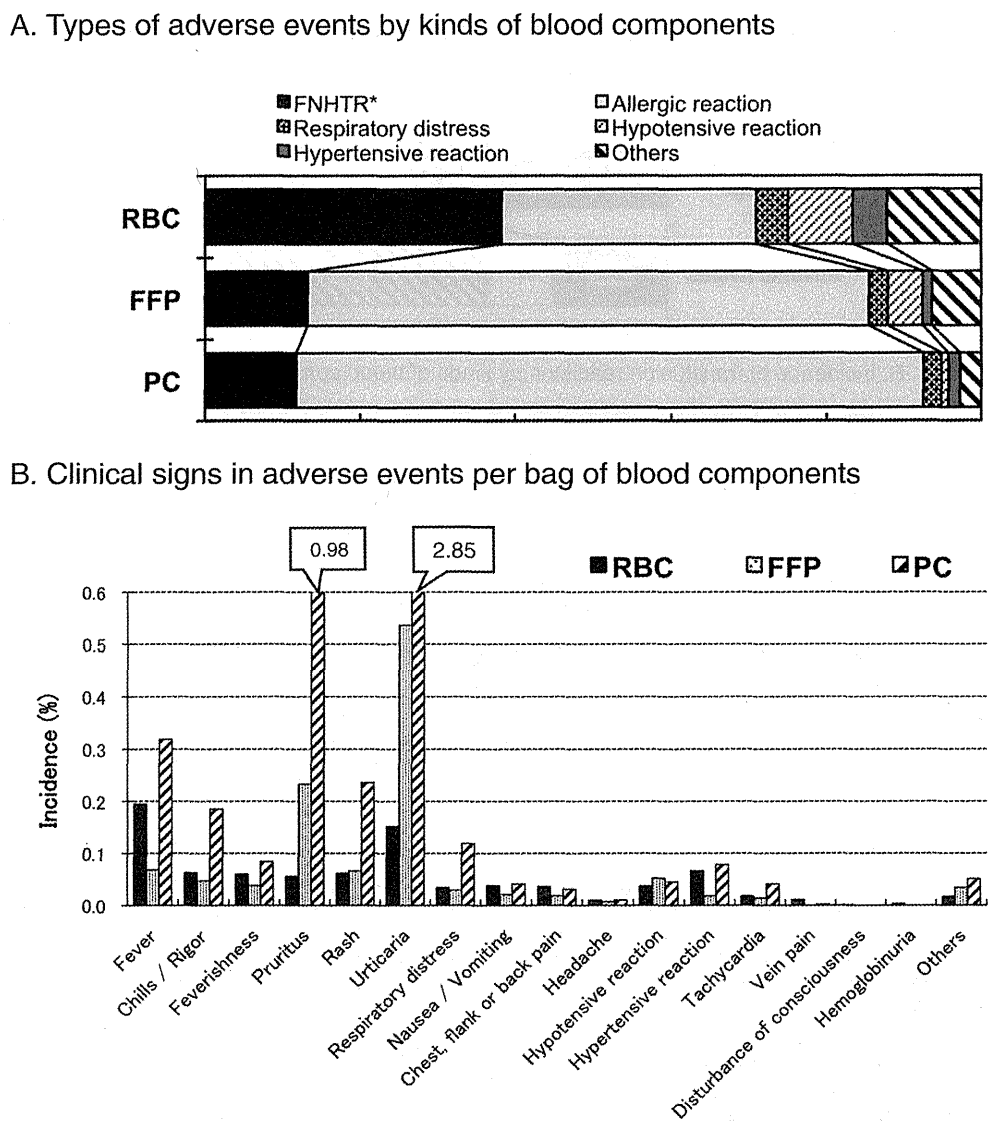


Fig. 5. Types of adverse events and clinical signs of adverse events by blood component. (A) Proportions of adverse events by type of blood component. (B) Incidence of clinical signs of adverse events by type of blood component. FNHTR: febrile non-haemolytic transfusion reaction.

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

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

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.

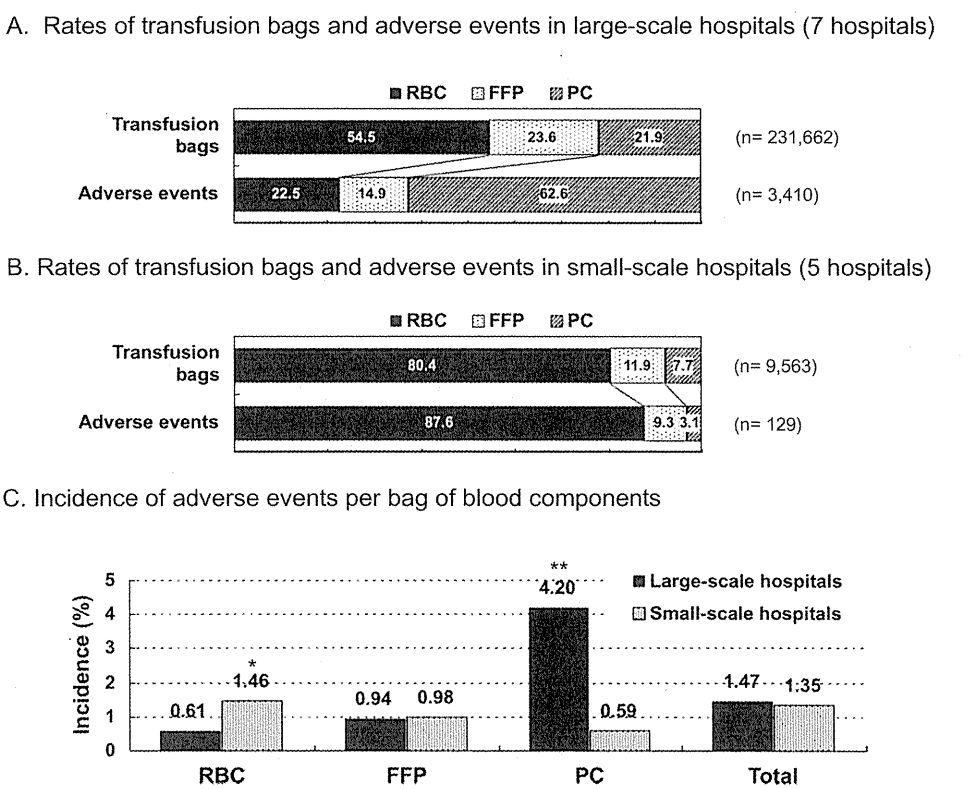


Fig. 6. Comparison of use of transfusion bag type, adverse events and incidence between large-scale and small-scale hospitals. Proportions of type of blood component and adverse events by type of blood component in seven large-scale university hospitals (A) and in five small-scale hospitals (fewer than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

This study was supported by a Grant from the Ministry of Health, Labour and Welfare of Japan and by The Japan Society of Transfusion Medicine and Cell Therapy.

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World Health Organization International Standard to Harmonize Assays for Detection of Hepatitis E Virus RNA

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Nucleic acid amplification technique–based assays are a primary method for the detection of acute hepatitis E virus (HEV) infection, but assay sensitivity can vary widely. To improve interlaboratory results for the detection and quantification of HEV RNA, a candidate World Health Organization (WHO) International Standard (IS) strain was evaluated in a collaborative study involving 23 laboratories from 10 countries. The IS, code number 6329/10, was formulated by using a genotype 3a HEV strain from a blood donation, diluted in pooled human plasma and lyophilized. A Japanese national standard, representing a genotype 3b HEV strain, was prepared and evaluated in parallel. The potencies of the standards were determined by qualitative and quantitative assays. Assay variability was substantially reduced when HEV RNA concentrations were expressed relative to the IS. Thus, WHO has established 6329/10 as the IS for HEV RNA, with a unitage of 250,000 International Units per milliliter.

Hepatitis E virus (HEV) is a nonenveloped, single-stranded RNA virus belonging to the family *Hepeviridae* (*1*,*2*). In developing countries, HEV is a major cause of acute hepatitis, transmitted by the fecal–oral route and associated with contamination of drinking water. In industrialized countries, reports of HEV infection have been uncommon but are being reported more frequently; some cases are imported after travel to HEV-endemic areas, but reports of autochthonous cases are also increasing, and infection with HEV appears to be more prevalent than originally believed (*3*). Prospects for control of HEV infection are encouraged by recent efforts in vaccine development (*4*,*5*).

Four main genotypes of HEV, representing a single serotype, infect humans. Genotype 1 viruses are found mainly in Africa and Asia and genotype 2 in Africa and Central America; it is in these areas that prevention of HEV infection by vaccination would be most beneficial. Genotypes 3 and 4 viruses are generally less pathogenic, although some exceptions have been reported, particularly for genotype 4; these genotypes infect not only humans but also animals such as swine, wild boar, and deer. Although genotype 4 strains have mainly been restricted to parts of Asia, genotype 3 viruses are found widely throughout the world. Zoonotic transmission of HEV genotypes 3 and 4 to humans can occur by consumption of contaminated meat or meat products or by contact with infected animals (*6*,*7*). Shellfish, such as bivalve mollusks, have also been shown to act as reservoirs for HEV (*8*).

An alternate route of transmission of HEV by transfusion of blood components has been reported in Japan (9,10), the United Kingdom (11), and France (*12*,*13*). Studies in Japan (*14*) and the People's Republic of China (*15*) have identified acute HEV infections in blood donors, confirmed by the detection of HEV RNA. Analysis of blood and plasma donors in Europe has identified HEV-infected donors in Germany (*16*–*20*), Sweden (*18*), and England (*21*). Transmission of HEV by solid organ transplantation has also been reported (*22*). Rates of HEV infection may be underreported in some countries, and misdiagnosis of HEV infection also occurs. For example, in some cases of suspected drug-induced liver injury, HEV has been determined

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DOI: http://dx.doi.org/10.3201/eid1905.121845

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[2]In memory of Thomas Laue.

as the cause (*23*). In one such recent case, HEV was shown to have been transmitted by blood transfusion (*13*).

Infection with HEV may cause particularly severe illness in pregnant women and in persons who have preexisting liver disease. Chronic infection with HEV genotype 3 is an emerging problem among solid organ transplant recipients and may also occur in persons with HIV and certain hematologic disorders (*24*). In patients with chronic infection, viral loads are monitored to investigate the efficacy of antiviral treatment (*25*,*26*) and effects of reduction of immunosuppressive therapy (*27*).

HEV infection is diagnosed on the basis of detection of specific antibodies (IgM and IgG), but the sensitivity and specificity of these assays is not optimal (*28*–*30*). Analysis of HEV RNA by using nucleic acid amplification techniques (NATs) is also used for diagnosis; this method can identify active infection and help confirm serologic results (*31*). Several NAT assays have been reported for the detection of HEV RNA in serum and plasma or fecal samples: conventional reverse transcription PCR (RT-PCR) and nested protocols (*32*), real-time RT-PCR, and reverse transcription loop-mediated isothermal amplification (*33*). The NATs include generic assays designed for the detection of HEV genotypes 1–4 (*34*,*35*).

In 2009, the World Health Organization (WHO) Expert Committee on Biological Standardization endorsed a proposal by the Paul-Ehrlich-Institut (PEI) to prepare an International Standard (IS) for HEV RNA for use in NAT-based assays. PEI recently completed an initial study that investigated the performance of HEV NAT assays in detection of HEV infection (*36*). In that study, dilution panels of HEV genotype 3 and 4 strains underwent blinded testing in laboratories that had experience in detection of HEV RNA. Results demonstrated wide variations in assay sensitivity (in the order of 100- to 1,000-fold for most assays).

After the initial study, 2 virus strains included in the panel (*36*) were selected for further development of a candidate IS for the WHO, and a candidate Japanese national standard (done in collaboration with the National Institute of Infectious Diseases in Tokyo). These viruses belong to genotype 3, which is widely distributed, and were genotype 3a and 3b strains, which were equally well detected in the initial study. The strains were derived from plasma samples that had sufficient titers of HEV RNA to prepare standards of good potency. An international collaborative study was conducted to establish the respective standards, demonstrate suitability for use, evaluate potency, and assign an internationally agreed-upon unitage.

Methods

Preparation of Materials

The 2 HEV strains selected for the preparation of the candidate WHO IS and candidate Japanese national standard were genotype 3a strain HRC-HE104 and genotype 3b strain JRC-HE3, respectively. The HEV-positive plasma donations were kindly provided by the Japanese Red Cross Society Blood Service Headquarters (Tokyo, Japan). Characterization of the stock virus strains is shown in Table 1.

The samples were tested for IgG/IgM against HEV by using an HEV enzyme immunoassay (Institute of Immunology Co., Ltd., Tokyo, Japan). Full-length sequences of the HEV strains were determined as described (*37*). Phylogenetic analyses were conducted by using MEGA version 5.05 (*38*), and HEV genotype and subgenotype were determined as described (*39*). The nucleotide sequences of HRC-HE104 and JRC-HE3 were deposited into GenBank under accession nos. AB630970 and AB630971, respectively.

The target HEV RNA concentration for the 2 bulk standard preparations was ≈5.5 $\log_{10}$ HEV RNA copies/mL, on the basis of the concentrations determined in the initial study (*36*). The 2 virus strains were negative when tested for hepatitis B virus, hepatitis C virus, and HIV-1/2 by using the Cobas TaqScreen MPX test (Roche Molecular Systems Inc., Branchburg, NJ, USA). The samples were diluted by using pooled citrated plasma (*36*) that had tested negative by NAT for hepatitis B virus, hepatitis C virus, and HIV-1/2, and HEV and was also negative for antibodies against HEV by using the recomWell IgG and IgM enzyme immunoassays (Mikrogen GmbH, Neuried, Germany). The diluted plasma was placed into 4-mL screw-cap glass vials, freeze dried, filled with nitrogen, sealed with rubber stoppers, and stored at –20°C. Stability studies demonstrated no substantial change in HEV RNA concentration after freeze drying or after 10 months of storage at –20°C (the usual temperature), +4°C, and +20 to +26°C, compared with samples stored at ≤–80°C.

Table 1. HEV strains diluted and lyophilized as candidate standards in study to establish a WHO International Standard for HEV RNA NAT-based assays*

Virus strain	HEV RNA, copies/mL	Genotype	GenBank accession no.	IgM/IgG against HEV	Alanine aminotransferase, IU/L
HRC-HE104	1.6×10^7	3a	AB630970	–/–	36
JRC-HE3	2.5×10^7	3b	AB630971	+/–	398

*Strains were provided by the Japanese Red Cross Society Blood Service Headquarters, Tokyo, Japan. HEV, hepatitis E virus; WHO, World Health Organization; NAT, nucleic acid amplification technique.

Study Design

The collaborative study was conducted by 24 laboratories from 10 countries; each laboratory was randomly assigned a code number. The samples analyzed in the study were coded sample 1 and sample 2 (replicates of the candidate WHO IS) and sample 3 and sample 4 (replicates of the candidate Japanese national standard). Samples were shipped to participants at ambient temperature. Participants tested the samples by using the laboratory's routine assays for HEV RNA, in 4 separate assay runs, using fresh vials of each sample for each run. Quantitative assay results falling within the linear range of the assays were reported in copies/mL. For qualitative assays, participants assayed each sample by a series of 1.0-$\log_{10}$ dilution steps to obtain an initial estimate of an endpoint and then, in 3 subsequent runs, assayed 0.5-$\log_{10}$ dilutions around the endpoint determined in the first run.

Statistical Methods

Quantitative Assays

Evaluation of quantitative assays was restricted to dilutions of 0.0 $\log_{10}$ to -2.5 $\log_{10}$, a range over which the assays of most participants produced comparable data. For comparison of laboratories, the replicate results of each laboratory, corrected for the dilution factor, were combined as the arithmetic mean of $\log_{10}$ copies/mL. Furthermore, these estimates were combined to obtain an overall estimation for each sample by means of a mixed linear model, using laboratory and $\log_{10}$ dilution as random factors.

Qualitative Assays

The data from all assays were pooled to give a series of values for number positive/number tested at each dilution. For each participant, these pooled results were evaluated by means of probit analysis to estimate the concentration at which 50% of the samples tested were positive; for assays in which the change from complete negative to complete positive results occurred in $\leq$2 dilution steps, the Spearman-Kaerber method was applied for estimation. The calculated endpoint was used to give estimates expressed in $\log_{10}$ NAT-detectable units/mL, after correcting for the equivalent volume of the test sample.

Relative Potencies

For quantitative assays, potencies of samples 2, 3, and 4 were estimated relative to sample 1 by using parallel-line analysis of log-transformed data. For qualitative assays, relative potencies were determined by using parallel-line analysis of probit-transformed data. Statistical analyses were performed by using SAS/STAT version 9.3 (SAS Institute, Cary, NC, USA). Estimation of endpoint dilution and relative potencies was performed by using CombiStats version 4.0 (European Directorate for the Quality of Medicines and HealthCare/Council of Europe, Strasbourg, France).

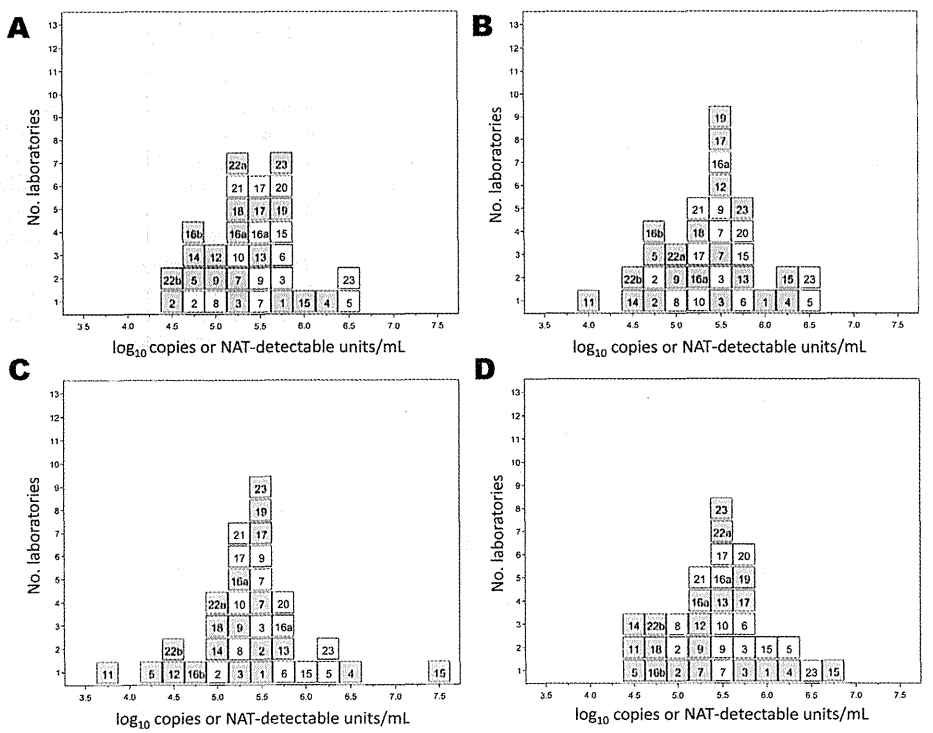

Figure 1. Histograms showing results for quantitative and qualitative assays conducted by 23 laboratories for the determination of the hepatitis E virus (HEV) RNA content of sample 1 (A), sample 2 (B), sample 3 (C), and sample 4 (D). White indicates quantitative assays ($\log_{10}$ copies/mL); gray indicates qualitative assays ($\log_{10}$ nucleic acid amplification technique (NAT)–detectable units/mL). Number of laboratories is indicated on the vertical axis. Laboratory code numbers are indicated in the respective boxes.

Results

Data were returned by 23 of the 24 participating laboratories; 20 sets of qualitative data and 14 sets of quantitative data were evaluated. The assays used by the participants are shown in online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/article/19/5/12-1845-Techapp1.pdf). All assays were developed in-house and were either conventional or nested RT-PCRs or based on real-time RT-PCR.

Quantitative and Qualitative Assay Results

Laboratory mean estimates for quantitative assays (in $\log_{10}$ copies/mL) and qualitative assays (in NAT-detectable $\log_{10}$ units/mL) for the HEV preparations are shown in histogram form in Figure 1, which shows that laboratory means are more variable for the qualitative assays than the quantitative assays, reflecting different assay sensitivities and lack of standardization. The individual laboratory means are given in online Technical Appendix Tables 2 and 3; relative variation of the individual laboratory estimates for the quantitative assays is illustrated by the box-and-whisker plots in Figure 2. Intralaboratory variation was lower than the interlaboratory variation for both types of assays (data not shown).

Determination of Overall Laboratory Means

The means for all the laboratories performing quantitative assays are shown in Table 2. The means for sample 1 and sample 2, replicates for the candidate WHO IS, were 5.58 $\log_{10}$ and 5.60 $\log_{10}$ copies/mL HEV RNA, respectively, with good agreement between the replicate samples. The candidate Japanese national standard showed identical mean results of 5.66 $\log_{10}$ copies/mL HEV RNA for replicate samples 3 and 4.

The means for all the laboratories performing qualitative assays are also shown in Table 2; again, there was good agreement between the duplicate samples. Results for the qualitative assays showed 0.3-$\log_{10}$ lower mean estimates and a higher SD than those for the quantitative assays. The combined mean values for the replicate samples for both types of assays are shown in Table 2.

Relative Potencies

On the basis of the combined data from both qualitative and quantitative assays, the candidate WHO standard was determined to have a potency of 5.39 $\log_{10}$ units/mL (95% CI 5.15–5.63). This value was calculated with a combined endpoint evaluation of qualitative and quantitative data (restricted to dilutions in the range of 0.0 $\log_{10}$ to -2.5 $\log_{10}$) by means of a mixed linear model.

The potencies of samples 2, 3, and 4 were calculated relative to sample 1, taking the value of sample 1 as 5.39 $\log_{10}$ units/mL. The relative potencies for the quantitative and qualitative assays are shown in online Technical Appendix Tables 4 and 5, respectively. Table 3 summarizes the overall mean potencies relative to sample 1, with the 95% CIs, SDs, and geometric coefficients of variation. For the quantitative data from laboratory 9, no potency could be estimated by endpoint evaluation because only 1

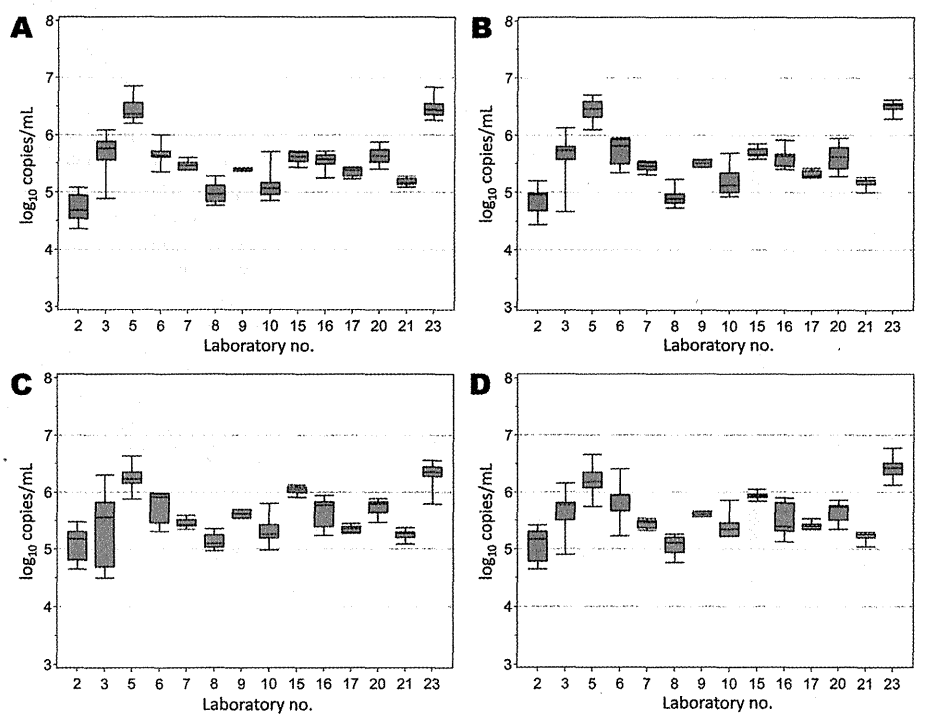

Figure 2. Box and whisker plots of the results for quantitative assays ($\log_{10}$ copies/mL) conducted by laboratories for the determination of the hepatitis E virus (HEV) RNA content of sample 1 (A), sample 2 (B), sample 3 (C), and sample 4 (D). Box indicates interquartile range; line within box indicates median; whiskers indicate minimum and maximum values observed. Laboratory code numbers are given on the horizontal axis.