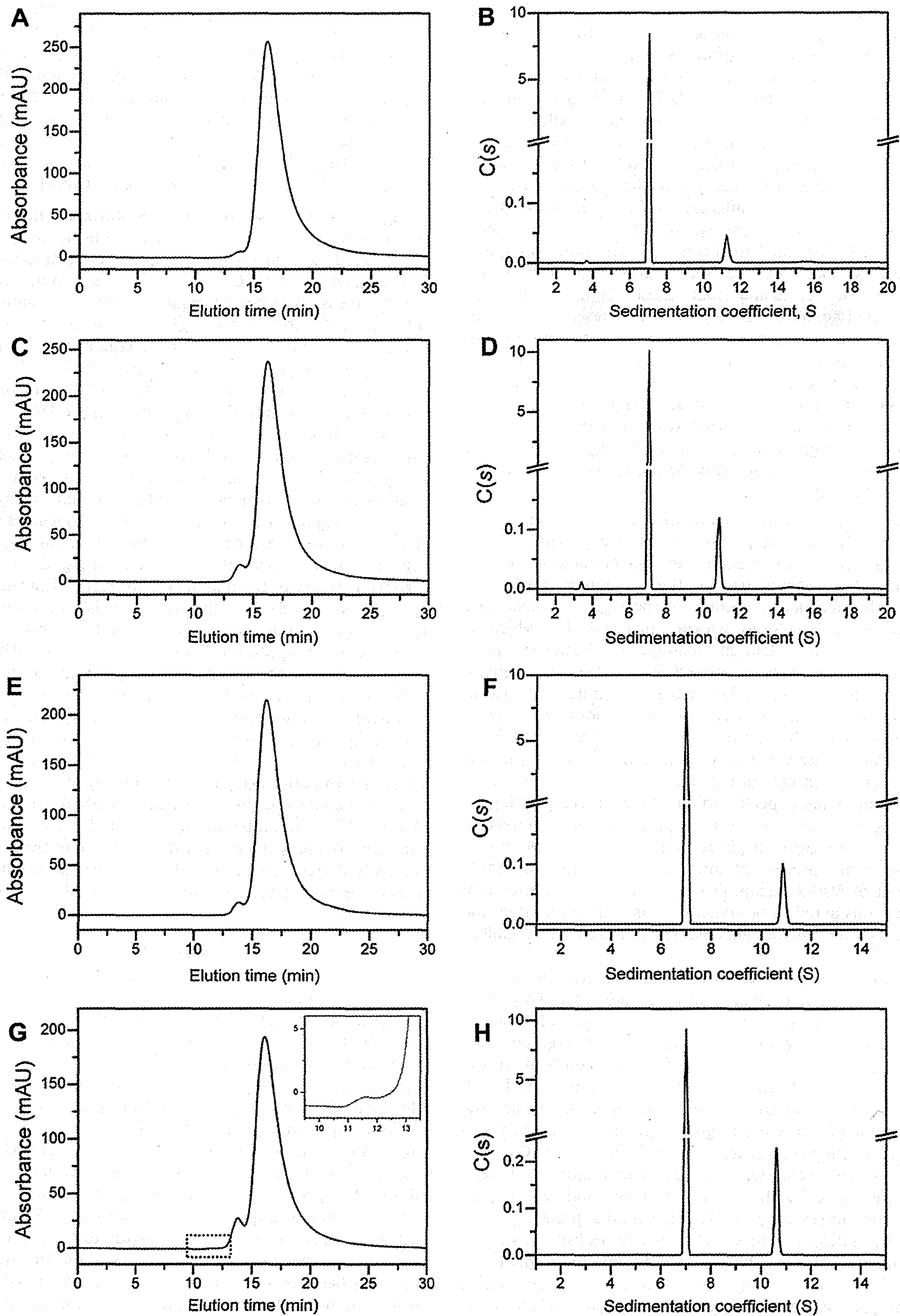

FIG. 1. Size-exclusion chromatograms and sedimentation coefficients distributions C(*s*) obtained for the following human immunoglobulin preparations: (A, B) Polyglobin, (C, D) Venoglobulin, (E, F) Hebsbulin, (G, H) Tetanobulin, (I, J) Glovenin, (K, L) Gammagard, and (M, N) Sanglopor. The insets in panels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

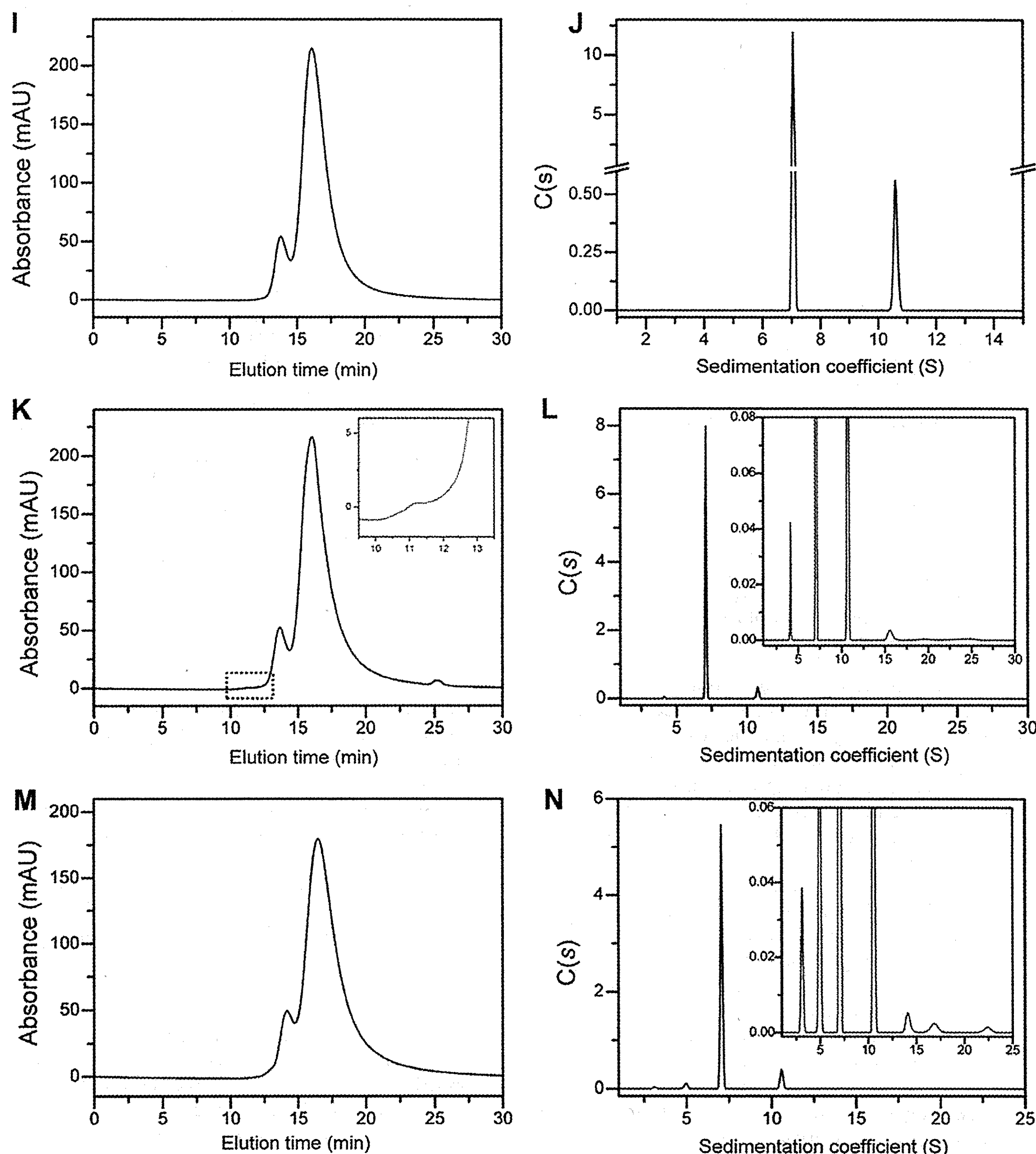

FIG. 1. (*continued*).

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.

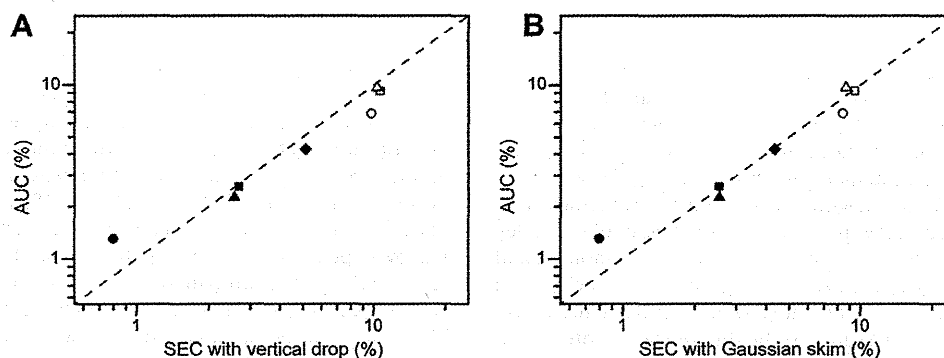

FIG. 2. Comparison of the dimeric aggregate amounts detected using AUC-SV and SEC with either (A) the vertical drop method or (B) the Gaussian skim approach. In each panel, the results for Polyglobin (filled circle), Venoglobulin (filled square), Hebsbulin (filled triangle), Tetanobulin (filled diamond), 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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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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[1]Members of the HEV Collaborative Study Group are listed at the end of this article.

[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).

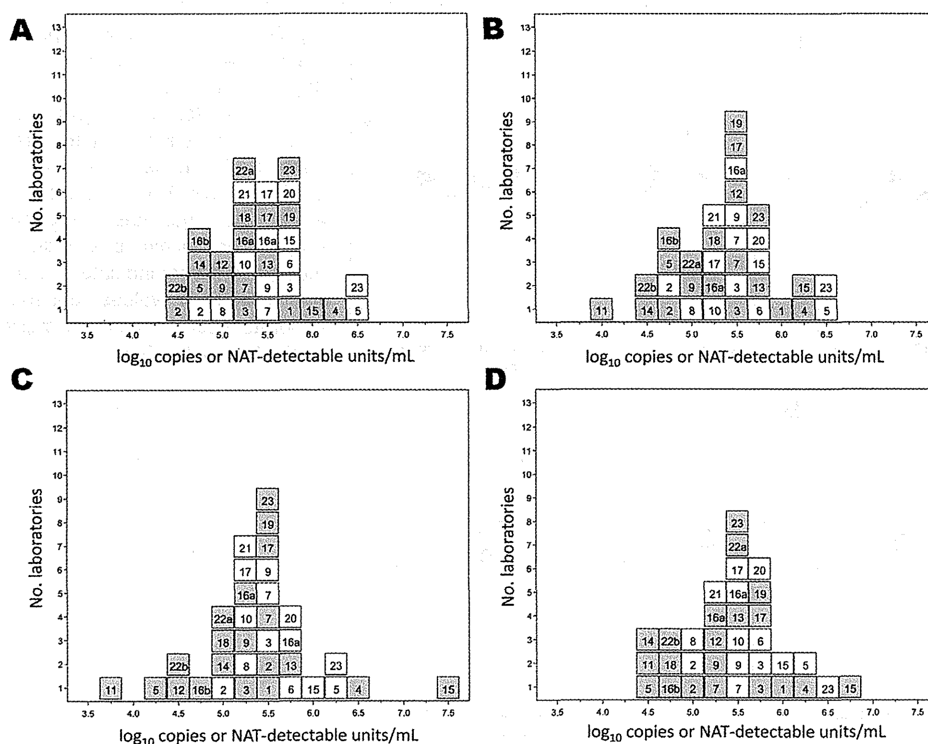

Figure 1. Histograms showing results for quantitative and qualitative assays conducted by 23 laboratories for the determination of the hepatitis E virus (HEV) RNA content of sample 1 (A), sample 2 (B), sample 3 (C), and sample 4 (D). White indicates quantitative assays ($\log_{10}$ copies/mL); gray indicates 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

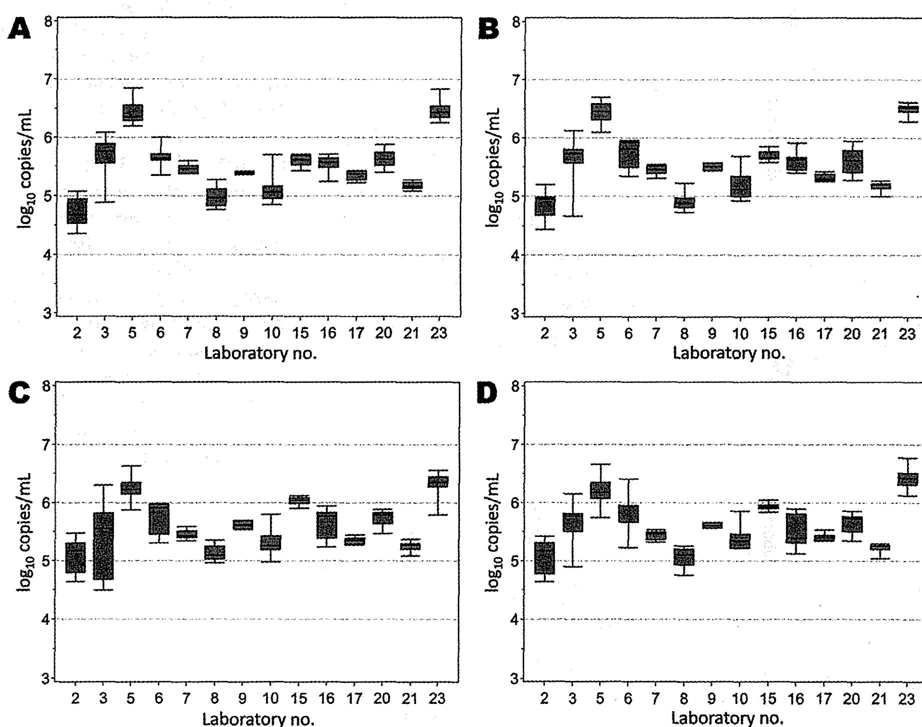

Figure 2. Box and whisker plots of the results for quantitative assays ($\log_{10}$ copies/mL) conducted by laboratories for the determination of the hepatitis E virus (HEV) RNA content of sample 1 (A), sample 2 (B), sample 3 (C), and sample 4 (D). Box indicates interquartile range; line within box indicates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

Table 2. Overall mean estimates from quantitative and qualitative assays of HEV samples in study to establish a WHO International Standard for HEV RNA NAT-based assays*

Assay type and sample	No.	Mean (95% CI)†	SD	% CV
Quantitative				
1	123	5.58 (5.32–5.85)	0.54	98
2	125	5.60 (5.33–5.87)	0.53	94
1 + 2	248	5.59 (5.33–5.86)	0.55	99
3	124	5.66 (5.40–5.93)	0.45	77
4	125	5.66 (5.40–5.93)	0.44	76
3 + 4	249	5.66 (5.40–5.93)	0.44	76
Qualitative				
1	19	5.25 (5.01–5.50)	0.51	150
2	20	5.26 (4.97–5.56)	0.62	179
1 + 2	39	5.26 (5.08–5.44)	0.56	163
3	20	5.27 (4.90–5.64)	0.79	226
4	20	5.31 (5.02–5.61)	0.64	183
3 + 4	40	5.29 (5.07–5.52)	0.71	202

*Samples 1 and 2, replicate samples of the candidate WHO International Standard; samples 3 and 4, replicate samples of the candidate Japanese national standard. HEV, hepatitis E virus; WHO, World Health Organization; NAT, nucleic acid amplification technique; no., no. dilutions analyzed (in linear range for quantitative assays); % CV, geometric coefficient of variation.
†Values are $\log_{10}$ copies/mL for quantitative and $\log_{10}$ NAT–detectable units/mL for qualitative assays.

dilution was tested for each sample. The data are plotted in histogram form in Figure 3.

The data demonstrate that expressing the results as potencies relative to sample 1 (set as a standard with an assumed unitage of 5.39 $\log_{10}$ units/mL) results in a marked improvement in the agreement between the majority of methods and laboratories, as evidenced by the reduction in SDs. Furthermore, these data provide some evidence for commutability of the candidate standard for evaluation of HEV from infected persons, because samples 1 and 2 represent a different strain of HEV compared with samples 3 and 4.

Discussion

In this study, a wide range of quantitative and qualitative assays were used to determine the suitability and evaluate the HEV RNA content of the candidate standards. Although the methods used by the study participants were all developed in-house, most assays consistently detected the 2 HEV strains. On the basis of data from the qualitative and quantitative assays, the candidate WHO IS was estimated to have a potency of 5.39 $\log_{10}$ units/mL. For practical purposes, the candidate IS was assigned a unitage of 250,000 International Units (IU)/mL; because the difference in the overall mean for the candidate Japanese national standard was negligible compared with the WHO preparation, the 2 materials were assigned the same value. In the case of the quantitative assays, laboratories reported values in HEV RNA copies/mL. The participating laboratories used plasmid DNA containing HEV sequences, synthetic oligonucleotides, and in vitro–transcribed HEV RNA to control for copy number. In some cases, laboratories used HEV-containing plasma that had been calibrated against in vitro–transcribed HEV RNA. One laboratory prepared a standard by using stool-derived virus, the titer of which was determined by endpoint dilution and analysis by Poisson distribution. No standard method or common quantitation standard material was used; this fact is reflected in the variation observed for the quantitative results (in the order of 2 $\log_{10}$), which were improved by expressing the results against sample 1 as a common standard. For qualitative assays, the variation in NAT-detectable units was $\geq$3 $\log_{10}$, and as with quantitative assays, expressing potencies relative to sample 1 improved the agreement among the different laboratories and methods.

Many of the laboratories participating in the study used a real time RT-PCR developed in 2006 (*34*) that was designed to detect the 4 main genotypes of HEV. However, a recent study in the United Kingdom found a polymorphism in the probe-binding site in several HEV-infected patients who initially had negative test results using this assay (*40*). A modification of the probe, increasing the melting temperature, restored detection of the polymorphic virus strains. We identified a further polymorphism in an HEV strain (GenBank accession no. JN995566) from a plasma donor (*18*), located in the probe-binding site of the same assay; use of the modified probe improved the amplification curve for this virus strain (S. Baylis and T. Gärtner, unpub. data). Genetic variation and its potential effects on HEV RNA detection highlight the importance of confirmatory tests of different design, rather than reliance on single methods.

The WHO IS will be valuable for development of secondary standards traceable to the IU, which will facilitate comparison of results between laboratories and

Table 3. Overall mean potencies of samples 2, 3, and 4 relative to sample 1 from quantitative and qualitative analysis of HEV samples in study to establish a WHO International Standard for HEV RNA NAT-based assays*

Sample and assay type	No.	Mean (95% CI)†	SD	% CV
Sample 2				
Quantitative	19	5.46 (5.35–5.58)	0.23	3
Qualitative	13	5.42 (5.38–5.46)	0.07	1
Combined	32	5.45 (5.38–5.51)	0.18	2
Sample 3				
Quantitative	20	5.45 (5.27–5.65)	0.43	5
Qualitative	13	5.48 (5.37–5.59)	0.18	2
Combined	33	5.46 (5.35–5.58)	0.35	4
Sample 4				
Quantitative	20	5.51 (5.38–5.64)	0.29	3
Qualitative	13	5.47 (5.36–5.59)	0.19	2
Combined	33	5.49 (5.41–5.58)	0.25	3

*Mean potency values were determined by assigning a value of 5.39 $\log_{10}$ units/mL for sample 1. Samples 1 and 2, replicate samples of the candidate WHO International Standard; samples 3 and 4, replicate samples of the candidate Japanese national standard. HEV, hepatitis E virus; WHO, World Health Organization; NAT, nucleic acid amplification technique; no., no. dilutions analyzed (in linear range for quantitative assays); % CV, geometric coefficient of variation.
†Values are $\log_{10}$ copies/mL for quantitative and $\log_{10}$ NAT technique–detectable units/mL for qualitative assays.

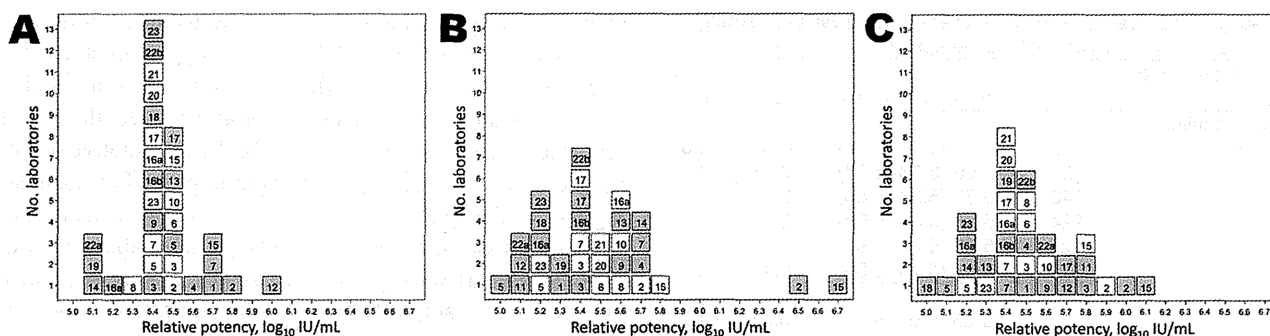

Figure 3. Histograms showing potencies of sample 2 (A), sample 3 (B), and sample 4 (C) compared with sample 1, the candidate World Health Organization International Standard for hepatitis E virus RNA for nucleic acid amplification technique (NAT)–based assays. White indicates quantitative assays (log_{10} copies/mL); gray indicates qualitative assays (log_{10} NAT–detectable units/mL). Number of laboratories is indicated on the vertical axis. Laboratory code numbers are indicated in the respective boxes.

determination of assay sensitivities and be helpful for validation purposes. We anticipate that the IS will find application in clinical laboratories, particularly in hepatitis reference laboratories that perform diagnosis and monitor HEV viral loads in chronically infected patients. The IS will also be helpful for research laboratories and blood and plasma centers that implement HEV NAT screening, regulatory agencies and organizations that are working to develop HEV vaccines, and manufacturers of HEV diagnostic kits.

The established WHO IS has been prepared by using a genotype 3a HEV strain. WHO has further endorsed a proposal by the PEI to prepare a genotype panel for HEV for NAT-based assays to continue standardization efforts for detection of this emerging infection. It is intended that the panel will contain representative strains of the 4 main genotypes of HEV that infect humans and notable subgenotypes. A new collaborative study will evaluate the IS against other genotypes and subgenotypes of HEV and investigate the commutability of the IS for standardization of assays for different genotypes of HEV. Laboratories that are able to provide high-titer HEV samples to aid in development of the proposed panel are requested to contact the authors.

In summary, WHO has established a genotype 3a HEV strain as the IS for HEV RNA (code number 6329/10), with an assigned a unitage of 250,000 IU/mL. The WHO IS for HEV RNA is available from PEI (www.pei.de).

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Dr Baylis is a scientist at the Paul-Ehrlich-Institut. Her work focuses on adventitious viruses in biological medicines, particularly with respect to blood and plasma-derived products.

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トピックス

輸血用血液における病原体不活化技術の現状と新規技術の開発

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TOPICS トピックス

輸血用血液における病原体不活化技術の現状と新規技術の開発

国立感染症研究所血液・安全性研究部
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はじめに

輸血用血液は，問診に加えて，病原体の血清学検査法とウイルス遺伝子を高感度に検出できる核酸増幅法の導入によって感染症の発生頻度は急激に低下したものの，スクリーニング法の限界や検査が実施されていない病原体などの感染リスクが存在する．そのため，輸血の安全性を確保するための対策として，病原体の不活化法が検討されるようになった．現在のところ，血漿製剤と血小板製剤の病原体不活化法が実用化され，欧州の一部の国や地域で導入されている．しかし，輸血用血液で最も使用量が多い赤血球製剤では，実用化された方法はない．

本稿では，輸血用血液の不活化法の現状と問題点について述べる．

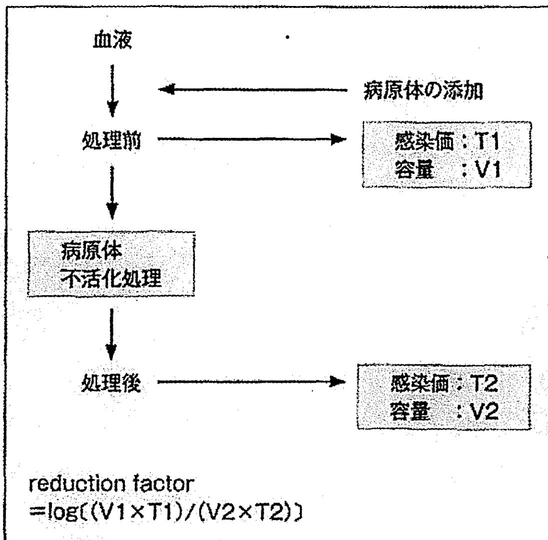

図1　病原体の不活化法の評価

病原体不活化法の評価法と効果について

不活化法を実施すれば，混入している病原体が全て不活化(感染力を失うこと)できるわけではない．不活化法にはそれぞれ不活化できる限界が存在する．不活化の能力を超えた量の病原体が混入していれば，感染価は減少するものの感染性を有した病原体が存在することになり，受血者の感染につながる．

日本では「不活化」という表現を使用するが，欧米では「低減化：リダクション」という言葉もよく使用されている．不活化効率は，図1に示すように，血液バッグに評価用の病原体を添加し，血液バッグに含まれる感染性を有する総病原体量(T1×V1)を計算する．次に不活化処理後の総病原体量(T2×V2)を計算し，リダクション値R=log(V1×T1/V2×T2)として求められる[1]．

ウイルスの場合，少量でも感染性ウイルスが

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表１　血漿および血小板製剤の不活化法

方法	S/D 処理法	メチレンブルー法	アモトサレン法	リボフラビン法	(参考)検疫
対象製剤	血漿	血漿	血漿 血小板	血漿 血小板	血漿
製剤を構成する供血者数	多数	単	単	単	単
添加薬剤	界面活性剤	メチレンブルー	アモトサレン	リボフラビン	不要
照射	不要	可視光	紫外線 A 320～400 nm	紫外線 B 265～370 nm	不要
添加薬剤の除去	＋	＋	＋	不要	不要
使用実績	◎	◎	○	△	◎

残存していれば感染する可能性があるので，血液中のウイルス量が少ないウイルスに対しては効果的であるが，ウイルス量が多い感染症に対しては，処理能力の範囲までスクリーニング法などを併用して混入するウイルス量を減らさないと，不活化法単独で完全に病原体の感染を防ぐことは困難である．実際に不活化法を導入している血液センターにおいては，HBV(hepatitis B virus)，HCV(hepatitis C virus)，HIV (human immunodeficiency virus)の３つの血清学的に加えて核酸増幅法も併用している．不活化法の導入でこれら３つのウイルスのスクリーニング検査を止めた施設はない．

以上の記載だけでは，不活化法単独では利用価値がない技術と誤解されるかもしれないが，血小板の細菌感染予防では大いに効果を発揮している．これについては，「血小板の病原体不活化法」の項で後述する．

血漿の病原体不活化法

現在，実用化されている血漿の病原体不活化法には，S/D(solvent/detergent)処理法，メチレンブルー法，アモトサレン法，リボフラビン法の４つがある[2]．

S/D 処理法は欧州で主に導入され，数十 L～数百 L の血漿を混ぜ合わせてから，界面活性剤と変性剤を加えて病原体を処理する方法である(一般的に S/D プラズマと呼ばれている)．S/D 処理法は，エンベロープを有するウイルス(HIV・HBV・HCV など)に対しては極めて有用な病原体不活化法である．その一方で，エンベロープをもたないウイルス(A 型肝炎ウイルス・E 型肝炎ウイルス・パルボウイルス B19 など)に対しては全く効果はない．

メチレンブルー法やアモトサレン法，リボフラビン法は，個々のバッグに化学物質を添加し，それぞれ可視光，紫外線 A，紫外線 B を照射することによって病原体を不活化する方法である．S/D 処理法と異なり，混ぜ合わせることなく個々のバッグで不活化処理できる大きなメリットがある．しかも，エンベロープを有するウイルスだけでなく，エンベロープをもたないウイルスに対しても不活化することができる(ウイルスによって，抵抗性を有していて全く不活化できないものや，不活化効率が低いものもある)[3]．問題点には，添加する化学物質の毒性に懸念があることや，凝固因子などの活性がある程度失活し，低下することであることが挙げられる[2]．各不活化法の特徴を表１に示す．

参考までに，不活化法のほかに検疫(quarantine)を紹介する．これは，製剤を一定期間保管(わが国では6カ月)し，供血者が次回の献血時の検査や問診で問題がないことが確認された場合や，同じ血液から製造された赤血球製剤を投与された受血者から異常の報告がなかった場合に，医療機関に凍結保存されていた血漿を供給する方法である．採血から供給まで長時間を要するが，不活化処理のための化学物質の毒性を考慮する必要はない．

血小板の病原体不活化法

現在，実用化されている方法にはアモトサレン法とリボフラビン法がある(**表1**)．方法は血漿と同様である．血小板は室温(20～24℃)で震とうしながら保存されるため，その間に細菌が増殖し，致死的な量まで増殖する可能性がある．細菌の混入する原因として，採血時に毛穴に存在する細菌が皮膚の断片と一緒に血液バックに入ることや無症候性の菌血症(健常人であっても口腔内などの菌が血液中に入ることがある)が挙げられる．対策として，最初の血液30 mLを検査用の検体として別のバックに取り，その後の血液を製剤用バックに取る(初流血除去)方法や細菌培養法が実施されている．しかし，初流血除去では，混入頻度は減らせても完全に予防はできない．さらに，無症候性の菌血症には無効である．

一方，細菌培養法では，混入する細菌数が少ないため〔1バッグ当たり10～100 CFU(colony-forming unit)〕，偽陰性となることがある．さらに，試験結果が出る前に使用せざるを得ないなど，無菌検査を導入しても細菌の混入を完全には検出することはできない．欧米では，採血日を入れないで有効期間が5～7日間と長い．さらに，5人前後の供血者の全血からの血小板を1つに集めて血小板製剤を製造しているため，細菌の混入する率が高くなる．一方，日本では採血日を入れて有効期間は4日なので，血小板への細菌感染は欧米に比べて少なく，初流血除去の導入後死亡例は報告されていない．

病原体の不活化が血小板の機能に与える影響は，臨床的に血小板輸血後の血小板増加数(corrected count increment, CCI：1時間後の補正血小板増加数$CCI_{1時間}$と24時間後の$CCI_{24時間}$)，血小板輸血回数，赤血球輸血回数・間隔，出血症状などから評価される．血小板の投与は，臨床的には予防的な投与もあることから不活化による機能低下の評価は難しいが，無処理と差はないという報告が多い．また，不活化された血小板輸血によって細菌感染が生じたという報告もない．

赤血球製剤の病原体不活化法

赤血球は輸血のなかで特に使用頻度・量とも多い製剤であるが，実用化された不活化法はない．血漿や血小板に用いられている方法は，化学物質に可視光，または紫外線を照射して病原体の核酸を破壊する方法であるため，赤血球製剤では，赤血球に照射された光が吸収されてしまい，病原体を不活化できる十分な光が病原体に到達しないためである．

近年，光を必要としない新しい不活化法が開発され，本年度フェイズⅢの治験が予定されるところまでになった[4]．この方法は，S-303と呼ばれるquinacrine mustard類似のアルキル化剤を使用し，アンカー，リンカー，エフェクターの3つの部分から構成されている．アンカーで核酸内に挿入されエフェクターが核酸に結合し核酸の複製を阻害する．添加された薬剤

はリンカー部分で加水分解される．ヘマトクリット60%でも幅広い病原体を不活化することが可能であるといわれている．この不活化法が実用化されると，3つの輸血用製剤にそれぞれ不活化法が実用化されたことになる．

おわりに

1. 輸血を介する感染症の頻度は急激に低下したが，リスクは0ではない．
2. 病原体（ウイルス）の不活化法には不活化可能な限界があるため，不活化法の導入によってウイルスのスクリーニング検査が全て廃止できるわけではない．
3. 病原体の不活化法によって血漿や血小板の機能は低下する．臨床的に容認できる範囲であることが必要である．
4. 血小板の細菌感染が輸血後感染症の大きなリスクとなっている国（地域）では，細菌感染予防のために不活化法は有用である．

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