For the candidate WHO standard, 4256 vials were lyophilized; the coefficient of variation of the fill volume was 1.1%. In the case of the candidate Japanese National Standard, 2154 vials were lyophilized; the coefficient of variation of the fill volume was 1.0%. In both cases, measurements were made for a total of 26 vials. For analysis of residual moisture, vials filled with 0.5 ml volumes of plasma diluent were distributed throughout the freeze-drier. Residual moisture was 0.73%, as determined by testing of 12 vials (Karl Fischer analysis). The freeze-drying process did not affect the HEV RNA titre of the lyophilized samples when compared to aliquots of the respective bulk preparations which were stored at -80°C (data not shown).
Vials of the candidate WHO standard are held at the Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany. The vials are kept at -20°C with continuous temperature monitoring.
All manufacturing records are held by PEI and are available on request by the ECBS.

Collaborative study

The collaborative study comprised 24 laboratories from 10 countries. The participants in the collaborative study who returned data are listed in Appendix 1.
The samples analysed in the study were labelled as Sample 1, Sample 2, Sample 3 and Sample 4. Sample 1 and Sample 2 were replicates of the candidate WHO standard; and Sample 3 and Sample 4 were replicates of the candidate Japanese National Standard. The collaborative study materials were shipped to participants at ambient temperature.
Participants were asked to test the panel using their routine assay for HEV RNA, testing the samples in four separate assay runs, using fresh vials of each sample for each run. Where laboratories performed quantitative tests, they were requested to report results in copies/ml, testing samples in the linear range of the assay. In the case of qualitative assays, participants were requested to assay each sample by a series of one $\log_{10}$ dilution steps, to obtain an initial estimate of an end-point. For the three subsequent assays, they were requested to assay half-$\log_{10}$ dilutions around the end-point estimated in their first assay. Participants reported diluting the materials using plasma, water or phosphate buffered saline. Data sheets and a method form were provided so that all relevant information could be recorded.

Statistical Methods

Quantitative Assays

Evaluation of quantitative assays was restricted to dilutions in the range between 0.0 $\log_{10}$ and -2.5 $\log_{10}$ where the assays of most participants seem to produce comparable data. For comparison of laboratories, the replicate results of each laboratory, corrected for the dilution factor, were combined as 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 with *laboratory* and *(log) dilution* as random factors.

Qualitative Assays

The data from all assays were pooled to give series of number positive out of number tested at each dilution. For each participant, these pooled results were evaluated by means of probit analysis to estimate the EC50 i.e. the concentration at which 50% of the samples tested were positive (for assays where the change from complete negative to complete positive results occurred in two or fewer dilution steps , the Spearman-Kaerber method was applied for EC50 estimation). The calculated end-point 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

Potencies of Samples 2, 3 and 4, for the quantitative assays, were estimated relative to Sample 1 using parallel line analysis of log transformed data. In the case of the qualitative assays, the relative potencies were determined using parallel line analysis of probit transformed data.

The statistical analysis was performed with SAS®/STAT software, version 9.2, SAS System for Windows. Estimation of end-point dilution and relative potencies were done with CombiStats Software, version 4.0, from EDQM/Council of Europe.

Stability studies

Stability of the candidate WHO standard is under continuous assessment, through both real-time and accelerated thermal degradation stability studies. Vials of the candidate WHO standard have been stored at -20°C (the normal storage temperature) and -80°C (to provide a baseline if there is any suggestion of instability at higher temperatures). For the accelerated thermal degradation, vials have been incubated at +4°C, +20°C, +37°C and +45°C for up to 4 months. After incubation at the respective temperatures, the contents of the vials were reconstituted in 0.5 ml of nuclease free water and analysed by real-time PCR (Jothikumar *et al.*, 2006).

Data Received

Data were received from a total of 23 participating laboratories; one laboratory failed to complete the study within the specified time frame. Data from 20 qualitative and 14 quantitative assays were reported. The types of assays used by participants are listed in Table 2; all assays were developed in-house. The assays used by participants were mainly based upon real-time PCR, although some conventional PCR methods were also used.
For the purposes of data analysis, each laboratory has been referred to by a code number allocated at random and not representing the order of listing in Appendix 1. Where a laboratory performed more than one assay method, the results from the different methods were analyzed independently, as if from separate laboratories, and coded, for example, laboratory 16a and laboratory 16b. In the case of 9 assays, quantitative values were reported covering the linear range of the respective assays; in addition, further dilutions have been performed allowing end-point determination. These data have been analysed separately and the number of estimates therefore exceeds the number of assay sets returned by the participants.

Results

Quantitative Assay Results

Initially evaluation of quantitative assays was performed without removing any outlying data; subsequently the data was restricted to a range between 0.0 $\log_{10}$ and -2.5 $\log_{10}$ where reproducible results were obtained across dilutions. The laboratory mean estimates in copies/ml ($\log_{10}$) are shown in histogram form in Figure 1. Each box represents the mean estimate from an individual laboratory, and is labelled with the laboratory code number. The individual laboratory means are given in Table 3. The relative variation of the individual laboratory estimates is illustrated by the box-and-whisker plots in Figure 2.

Qualitative Assay Results

The NAT-detectable units/ml ($\log_{10}$) for the qualitative assays are shown in histogram form in Figure 3. Each box represents the mean estimate from an individual laboratory and is labelled with the laboratory code number. The individual laboratory means are given in Table 4. From Figure 3, it can be seen that the estimates of NAT detectable units/ml ($\log_{10}$) from the qualitative

assays are more variable than the quantitative assays, reflecting the different sensitivities of the various assays. This observation is not unexpected and is in line with other studies.

Determination of Overall Laboratory Means

The overall means for the laboratories performing quantitative assays are shown in Table 5a. The means for both Sample 1 and Sample 2, replicates for the candidate WHO standard, are 5.58 $\log_{10}$ and 5.60 $\log_{10}$ copies/ml HEV RNA respectively, which demonstrates excellent 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 combined mean values for the replicate samples are shown in Table 5b.
The overall means for the qualitative assays are shown in Table 6a; there is good agreement between the duplicate samples as seen previously for the quantitative assays. The combined mean values for the replicate samples are shown in Table 6b. The qualitative assays show 0.3 $\log_{10}$ lower mean estimates than the quantitative assays.

Relative Potencies

Based upon the data from both qualitative and quantitative assays, the candidate WHO standard was estimated to have a potency of 5.39 $\log_{10}$ units/ml (95% confidence limits 5.15 – 5.63). This value was estimated with a combined end-point evaluation of qualitative and quantitative (restricted to dilutions in the range of 0.0 $\log_{10}$ to - 2.5 $\log_{10}$) data 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 are shown in Tables 7 and 8 for the quantitative and qualitative assays, respectively. For the quantitative data from laboratory 9, no potency was estimable since there was only one dilution tested for each sample. The data is plotted in histogram form (Figures 4-6). The data demonstrate that expressing the results as potencies relative to Sample 1, as a standard with an assumed unitage of 5.39 $\log_{10}$ units/ml gives a marked improvement in the agreement between the majority of methods and laboratories. These data provide some evidence for commutability of the candidate standard for evaluation of HEV from infected individuals, since Samples 1 and 2 represent a different strain of HEV compared to Samples 3 and 4.

Results of Stability Studies

Vials of the candidate WHO standard were incubated at +4°C, +20°C, +37°C and +45°C for up to four months and tested by real-time PCR for HEV RNA. The heat-treated vials were assayed concurrently with vials that had been stored at -20°C and at -80°C. All samples were tested in duplicate and were compared to a standard curve prepared using vials of the candidate WHO standard stored at -80°C.
There was no evidence of instability of the samples stored at -20°C when compared to samples stored at -80°C. After 4 months incubation at +20°C a small loss of titre was observed.The observed drop in titre at higher temperatures (+37°C and +45°C) may be related to problems with reconstitution of the samples rather that actual degradation and has previously been observed for some other preparations, particularly for RNA viruses formulated in pooled plasma. The potency of the reconstituted material, after freezing and thawing, has not been investigated. Further stability studies (both real-time and accelerated) are on-going and will be communicated to the WHO.

All raw data for the collaborative study and stability analysis are held by PEI and are available on request by the ECBS.

Conclusions

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, the majority of assays were able to detect the two HEV strains consistently. Based upon the data from the qualitative and the quantitative assays, the candidate WHO standard was estimated to have a potency of 5.39 $\log_{10}$ units/ml. Since the unitage assigned to the 1st WHO standard of a preparation is essentially arbitrary, for practical purposes, the candidate International Standard has been assigned a unitage of 250,000 International Units/ml. Since there was only a negligible difference in the overall means for the candidate Japanese National Standard compared to the WHO preparation, the two materials have therefore been assigned the same value i.e. 250,000 International Units/ml. In the case of the quantitative assays, laboratories reported values in HEV RNA copies/ml. The participants 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 which had been calibrated against *in vitro* transcribed HEV RNA. Another laboratory prepared standard using stool-derived virus, the titre of which was determined by end-point dilution and analysis by Poisson distribution. No standard method or common quantitation standard material was used, and this is reflected in the variation observed for the quantitative results, with a variation in the order of 2 $\log_{10}$, which were improved by expressing the results against Sample 1 as a common standard. In the case of the qualitative assays, the variation in NAT-detectable units was at least 3 $\log_{10}$, and again expressing potencies relative to Sample 1 improved the agreement between the different laboratories and methods.
The collaborative study materials have been dispatched at ambient temperature, replicating the intended shipping conditions. Initial accelerated thermal degradation analysis indicates a reduction in the levels of HEV RNA at higher incubation temperatures. On-going studies on the real-time stability under normal storage conditions as well as studies concerning thermal degradation are in progress.
The standard will be of value for comparison of results between laboratories, determination of assay sensitivities and for validation. It is anticipated that the standard will find application in clinical laboratories, particularly hepatitis reference laboratories performing diagnosis and monitoring HEV viral loads in chronically infected transplant patients, research laboratories, blood and plasma centres which implement HEV NAT screening, regulatory agencies and organizations developing HEV vaccines as well as manufacturers of diagnostic kits.

Each vial of the HEV RNA standard contains the lyophilized residue of 0.5 ml of HEV RNA positive plasma. Predictions of stability indicate that the standard is stable and suitable for long-term use when stored as directed in the accompanying proposed "Instructions For Use" data sheets for the panel (Appendix 2).

Recommendations

Based upon the results of the collaborative study, it is proposed that the genotype 3a HEV strain (Samples 1 and 2, in this study) should be established as the 1st International Standard for hepatitis E virus RNA and be assigned a unitage of 250,000 International Units/ml. The standard has been given the code number 6329/10; 3800 vials are available to the WHO and custodian laboratory is the Paul-Ehrlich-Institut.

Comments from participants

After circulation of the draft report for comment, replies were received from all participants. The majority of the comments were editorial in nature and the report has been amended accordingly. All participants were in agreement with the conclusions of the report.

One participant commented on the possible incorrect estimation of the viral load by the participants who used DNA standards (synthetic oligonucleotides or plasmid DNA) due to lack of control for reverse transcription of virus RNA into cDNA. This might be better controlled using *in vitro* transcribed RNA or a virion-based preparation.

Another participant remarked that many laboratories have used the same method, showing quite different sensitivities, possibly due to differences in extraction and amplification/detection reagents and instrumentation and its set up.

Acknowledgements

The viraemic HEV donations used to prepare the candidate standards were generously provided by Keiji Matsubayashi of the Japanese Red Cross Hokkaido Blood Center. We thank all the laboratories who took part in the study and Roswitha Kleiber and Christine Hanker-Dusel for assistance.

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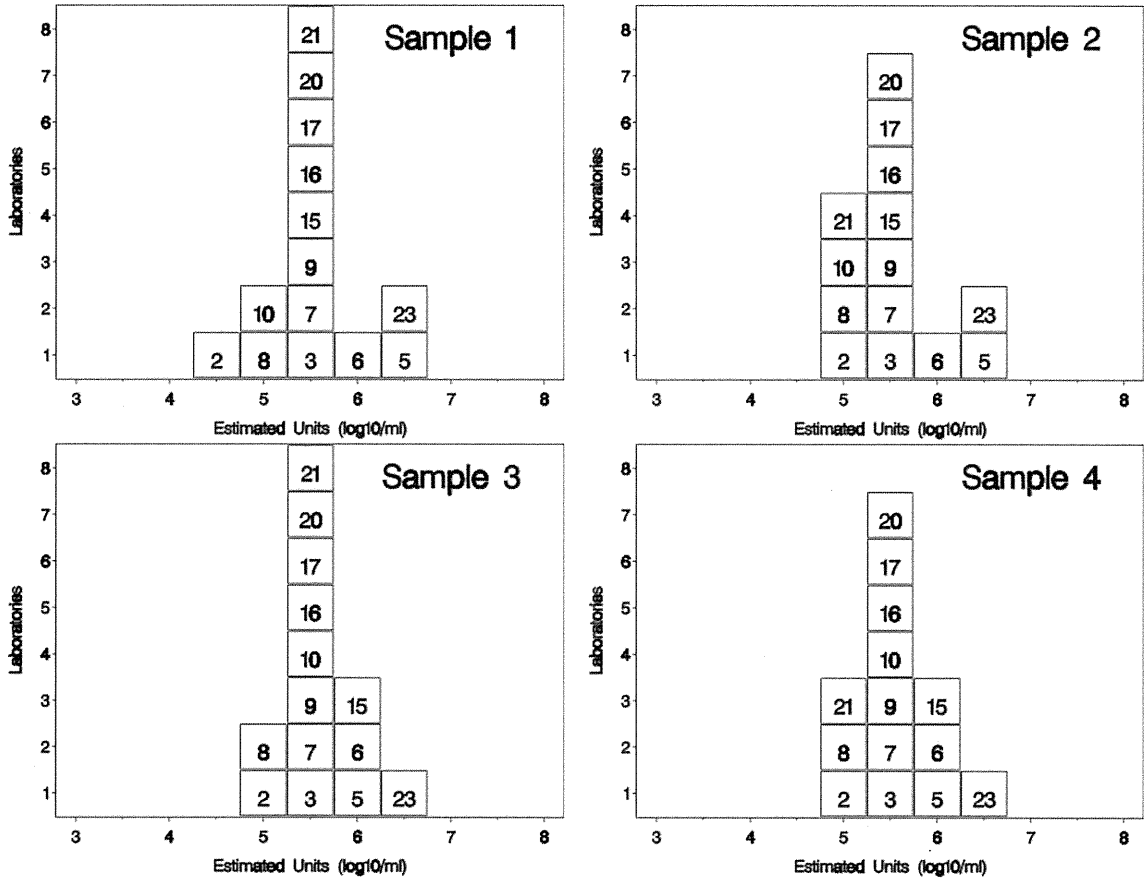
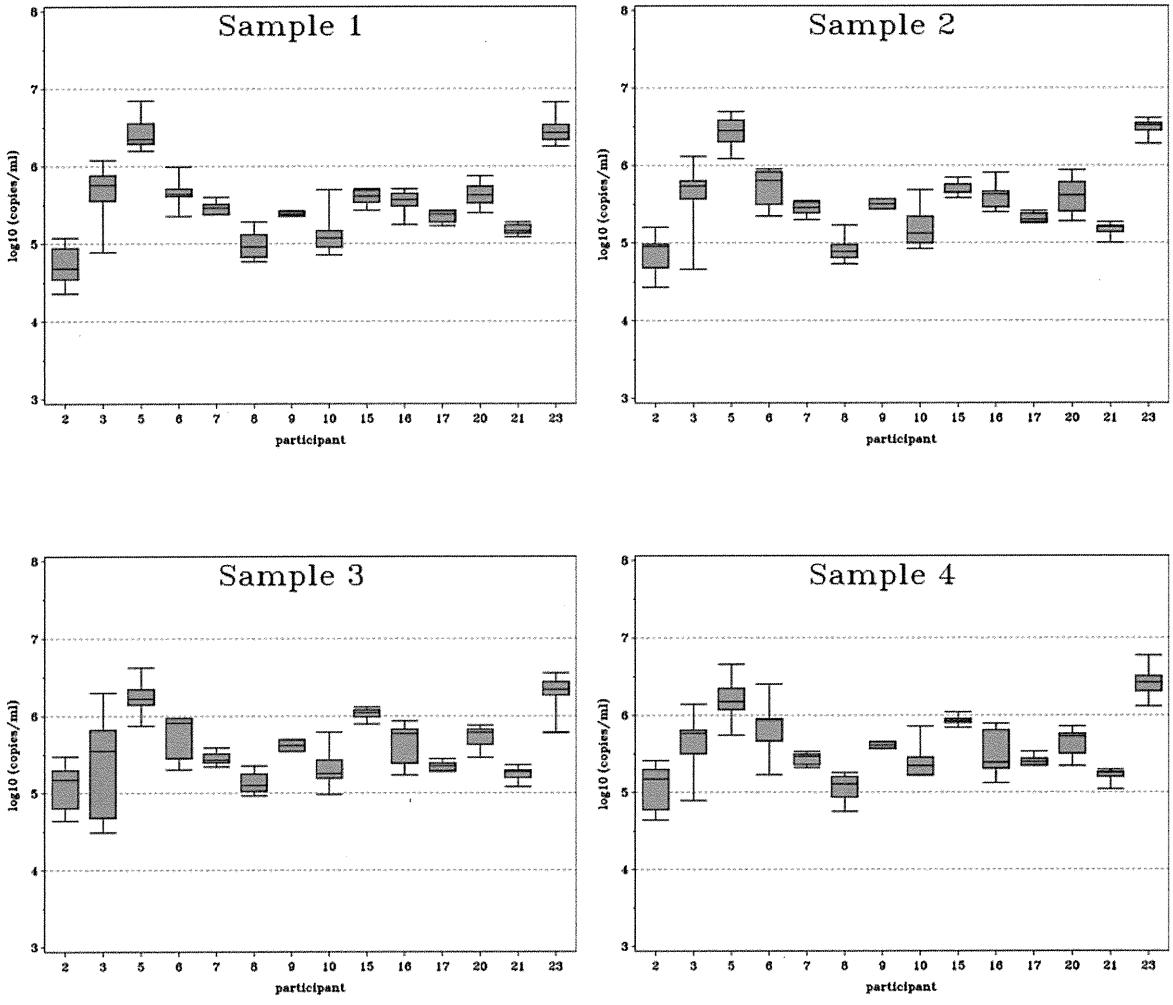
Figure 1 Estimates for quantitative assays



Histograms of the quantitative results for participating laboratories for Sample 1, Sample 2, Sample 3 and Sample 4. Estimates of $\log_{10}$ copies/ml are indicated on the x-axis. Data are shown for laboratory 16a.

Figure 2 Box and whisker plots of the quantitative data (log_{10} copies/ml)



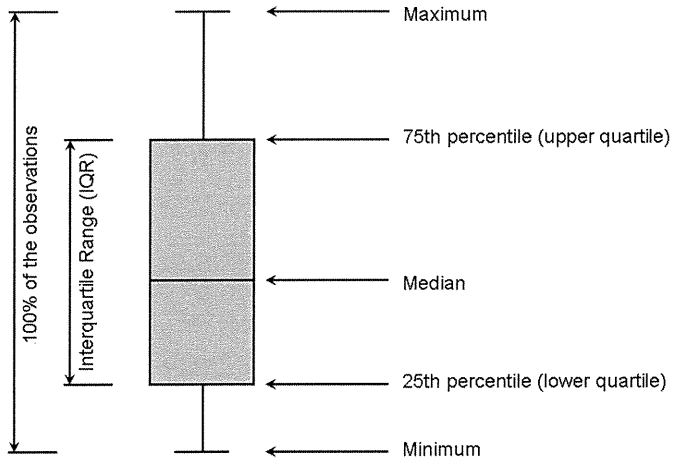
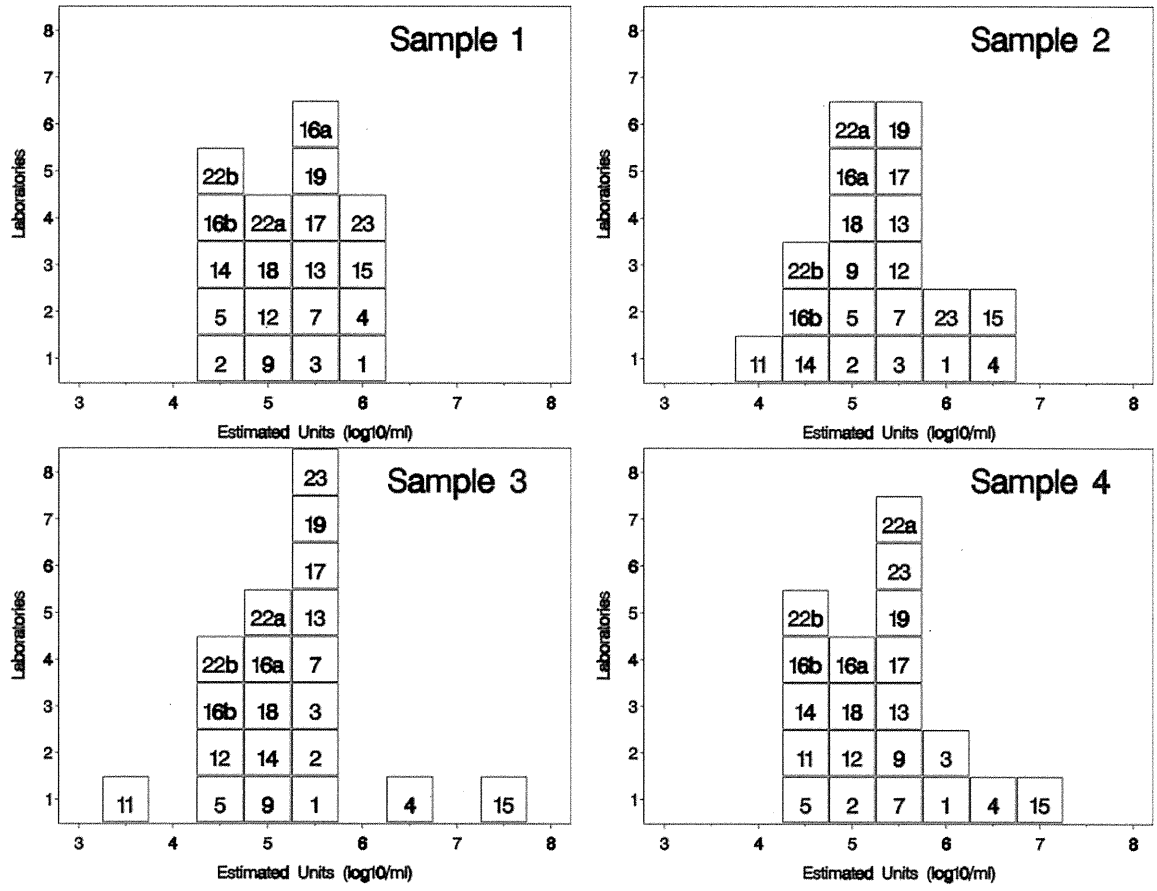
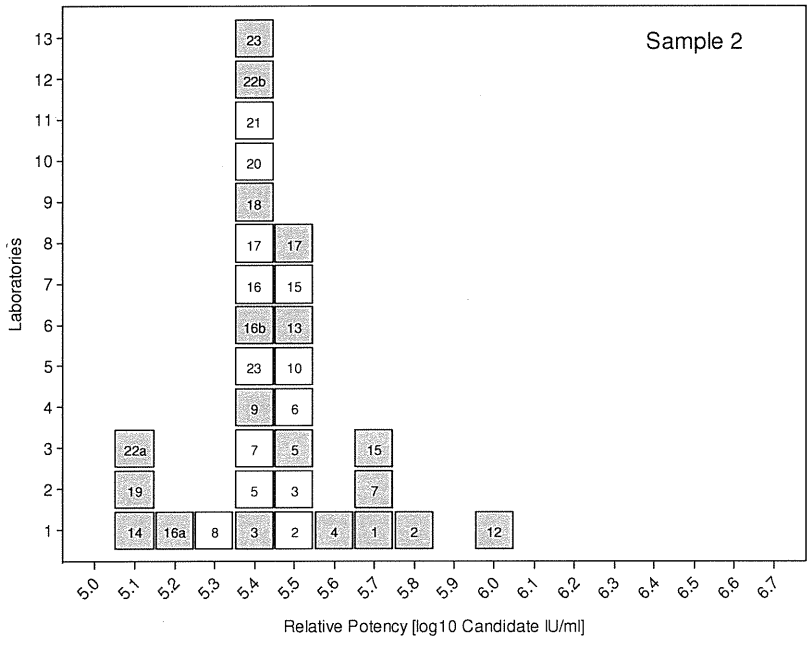


Figure 3 Estimates for qualitative assays



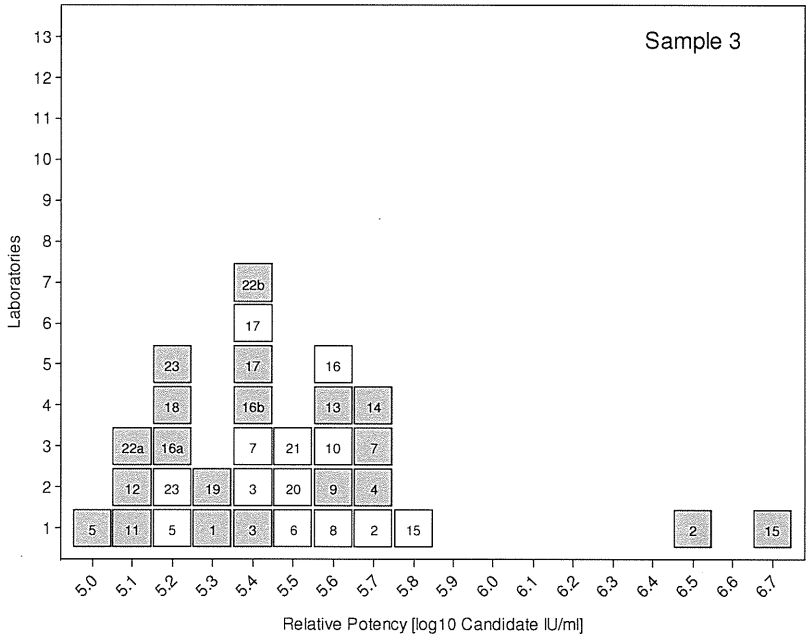
Histograms of the qualitative results for participating laboratories for Sample 1, Sample 2, Sample 3 and Sample 4. Estimates of $\log_{10}$ NAT-detectable units/ml are indicated on the x-axis. In the case of laboratory 11, data for Sample 1 have been omitted due to a 2 $\log_{10}$ higher cut-off.

Figure 4 Potency of Sample 2 relative to Sample 1



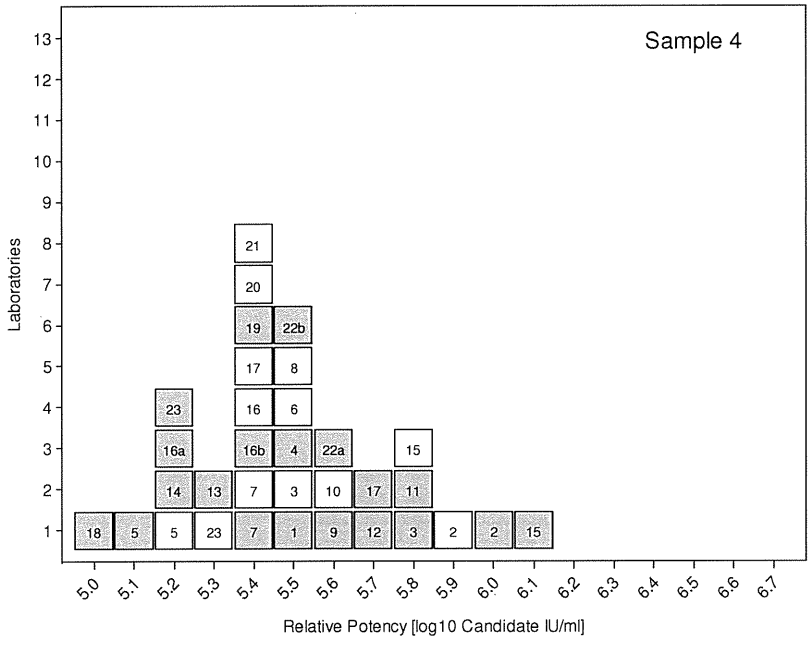
Histogram of the potency of Sample 2 relative to Sample 1 (=5.39 $\log_{10}$ units/ml); qualitative data (grey boxes) and quantitative data (white boxes). No relative potency is shown for laboratory 11 for sample 2, since no value had been determined for Sample 1 (i.e. the data were outlying and did not perform as the replicate i.e. Sample 2).

Figure 5 Potency of Sample 3 relative to Sample 1



Histogram of the potency of Sample 3 relative to Sample 1 (=5.39 $\log_{10}$ units/ml); qualitative data (grey boxes) and quantitative data (white boxes). In the case of Laboratory 11, the data have been calculated relative to Sample 2.

Figure 6 Potency of Sample 4 relative to Sample 1



Histogram of the potency of Sample 4 relative to Sample 1 (=5.39 $\log_{10}$ units/ml); qualitative data (grey boxes) and quantitative data (white boxes). In the case of Laboratory 11, the data have been calculated relative to Sample 2.

Table 1 Details of HEV strains lyophilized as candidate standards

Virus strain	HEV RNA (copies/ml)*	Genotype	Accession No.**	Anti-HEV IgM/IgG	ALT (IU/L)
HRC-HE104	1.6×10^7	3a	AB630970	-/-	36
JRC-HE3	2.5×10^7	3b	AB630971	+/-	398

*Concentrations determined by the Japanese Red Cross Hokkaido Blood Center
**Full length sequence

Table 2 Assay protocols used by participants

Laboratory code	Assay type (qualitative or quantitative)	Extraction method	NAT method	Assay target	Reference
1	Qual.	QIAamp MinElute Virus Spin kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
2	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2	Adlhoch *et al.* 2009
3	Qual./Quant.	High Pure Viral Nucleic Acid kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
4	Qual.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
5	Qual./Quant.	QIAamp DNA Mini Blood kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
6	Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
7	Qual./Quant.	QIAamp MinElute Virus Spin kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Matsubayashi *et al.* 2008
8	Quant.	SMI-TEST EX-R&D (Medical Biological Laboratories Co., Ltd.)	Real-time RT-PCR (TaqMan)	ORF2/3	Tanaka *et al.* 2007
9	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
10	Quant.	COBAS AmpliPrep Total Nucleic Acid Isolation kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
11	Qual.	COBAS AmpliScreen Multiprep Specimen Preparation and Control kit (Roche)	Conventional one step RT-PCR; analysis by agarose gel electrophoresis	ORF1	
12	Qual.	QIAamp MinElute Virus Spin Kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
13	Qual.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
14	Qual.	Viral DNA/RNA Isolation kit (GenMag Biotechnology)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	
15	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006 (modified)
16a	Qual./Quant.	MagNA Pure LC (Roche)	Real-time PCR (SYBR Green)	ORF2/3	Jothikumar *et al.* 2006

					(modified)
16b	Qual.	MagNA Pure LC (Roche)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	Meng *et al.* 2001
17	Qual./Quant.	QIAamp Virus BioRobot MDx kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Matsubayashi *et al.* 2008
18	Qual.	MagNA Pure LC Total Nucleic Acid Isolation kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
19	Qual.	easyMag (bioMérieux)	Real-time RT-PCR (TaqMan)	ORF2	
20	Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
21	Quant.	BioRobot Universal (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
22a	Qual.	QIAamp RNA Mini kit (Qiagen)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	Gyarmati *et al.* 2007
22b	Qual.	QIAamp RNA Mini kit	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
23	Qual./Quant.	QIAamp DNA Mini Blood kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Wenzel *et al.*, in press

Qualitative (Qual.) and quantitative (Quant.) assays

Table 3 Mean estimates from quantitative assays (log_{10} copies/ml)

Laboratory code	Sample			
	1	2	3	4
2	4.69	4.82	5.09	5.08
3	5.69	5.62	5.43	5.65
5	6.51	6.48	6.24	6.20
6	5.75	5.80	5.77	5.83
7	5.50	5.46	5.45	5.44
8	5.07	4.97	5.14	5.06
9	5.43	5.52	5.62	5.61
10	5.18	5.22	5.30	5.39
15	5.66	5.73	6.02	5.93
16a	5.59	5.62	5.64	5.51
17	5.40	5.34	5.35	5.41
20	5.70	5.65	5.74	5.65
21	5.25	5.23	5.25	5.23
23	6.54	6.53	6.31	6.41

Table 4 Mean estimates from qualitative assays (log_{10} NAT detectable units/ml)

Laboratory code	Sample			
	1	2	3	4
1	5.76	6.05	5.62	5.91
2	4.42	4.85	5.49	5.02
3	5.35	5.40	5.35	5.76
4	6.20	6.37	6.47	6.33
5	4.70	4.84	4.27	4.42
7	5.34	5.62	5.62	5.34
9	5.02	5.03	5.18	5.26
11		4.00	3.72	4.42
12	4.91	5.48	4.61	5.18
13	5.51	5.66	5.71	5.44
14	4.71	4.43	5.00	4.57
15	6.11	6.36	7.42	6.87
16a	5.32	5.17	5.17	5.17
16b	4.74	4.74	4.74	4.74
17	5.39	5.52	5.42	5.67
18	5.13	5.13	4.98	4.76
19	5.68	5.42	5.56	5.71
22a	5.21	4.92	4.91	5.44
22b	4.53	4.53	4.52	4.68
23	5.76	5.76	5.60	5.60

Laboratory 11, sample 1, omitted due to 2 log_{10} higher cut-off

Table 5a Overall mean estimates from quantitative assays (log_{10} copies/ml)

Sample	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
1	123	5.58	0.29	5.32	5.85	5.46	4.36	6.85	98%
2	125	5.60	0.28	5.33	5.87	5.46	4.43	6.69	94%
3	124	5.66	0.20	5.40	5.93	5.50	4.49	6.63	77%
4	125	5.66	0.20	5.40	5.93	5.48	4.64	6.77	76%

n – number of dilutions analysed (in linear range), sd – standard deviation, lowercl/uppercl – 95% confidence limits for the mean, cv_geo – geometric coefficient of variation [%]

Table 5b Combined mean estimates from quantitative assays (log_{10} copies/ml)

Candidate	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
WHO	248	5.59	0.30	5.33	5.86	5.46	4.36	6.85	99%
NIID	249	5.66	0.20	5.40	5.93	5.48	4.49	6.77	76%

Combined data for Samples 1 and 2, replicate samples of the candidate IS (WHO); combined data for Samples 3 and 4, replicate samples of the candidate Japanese National Standard (NIID)

Table 6a Overall means of estimates from qualitative assays (log_{10} NAT detectable units/ml)

Sample	n	mean	sd	Lower cl	Upper cl	median	min	max	cv_geo
1	19	5.25	0.51	5.01	5.50	5.32	4.42	6.20	150%
2	20	5.26	0.62	4.97	5.56	5.29	4.00	6.37	179%
3	20	5.27	0.79	4.90	5.64	5.27	3.72	7.42	226%
4	20	5.31	0.64	5.02	5.61	5.30	4.42	6.87	183%

n – number of tests, lowercl/uppercl – 95% confidence limits for the mean, cv_geo – geometric coefficient of variation [%]

Table 6b Combined means of estimates from qualitative assays (log_{10} NAT detectable units/ml)

Candidate	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
WHO	39	5.26	0.56	5.08	5.44	5.32	4.00	6.37	163%
NIID	40	5.29	0.71	5.07	5.52	5.30	3.72	7.42	202%

Combined data for Samples 1 and 2, replicate samples of the candidate IS (WHO); combined data for Samples 3 and 4, replicate samples of the candidate Japanese National Standard (NIID)