Journal of Clinical Virology 119 (2019) 60–67

Contents lists available at ScienceDirect

Journal of Clinical Virology

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

Development of a World Health Organization International Reference Panel for different genotypes of hepatitis E virus for nucleic acid amplification testing

Sally A. Baylis[a,*], Kay-Martin O. Hanschmann[a], Keiji Matsubayashi[b], Hidekatsu Sakata[c], Anne-Marie Roque-Afonso[d], Marco Kaiser[e], Victor M. Corman[f,g], Saleem Kamili[h], Rakesh Aggarwal[b,i,1], Nirupma Trehanpati[j], Thomas Gärtner[k], Emma C. Thomson[l], Christopher A. Davis[l], Ana da Silva Filipe[l], Tamer T. Abdelrahman[l,m], Johannes Blümel[a], Eriko Terao[n], the HEV collaborative study group[2]

[a] Paul-Ehrlich-Institut, Langen, Germany
[b] Central Blood Institute, Japanese Red Cross Society, Tokyo, Japan
[c] Japanese Red Cross Hokkaido Block Blood Center, Sapporo, Japan
[d] Virologie AP-HP Hôpital Paul Brousse, Villejuif, France
[e] GenExpress Gesellschaft für Proteindesign mbH, Berlin, Germany
[f] Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institute of Virology, Berlin, Germany
[g] German Centre for Infection Research (DZIF), Berlin, Germany
[h] Division of Viral Hepatitis, Centers for Disease Control and Prevention, Atlanta, USA
[i] Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India
[j] Institute of Liver and Biliary Sciences, New Delhi, India
[k] Octapharma, Frankfurt am Main, Germany
[l] University of Glasgow Centre for Virus Research, UK
[m] Microbiology Department, King Fahad Medical City, Riyadh, Saudi Arabia
[n] European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France

ARTICLE INFO

Keywords:
Hepatitis E virus
HEV
NAT
NAAT
Standardization
Genotype
World Health Organization

ABSTRACT

Background: Globally, hepatitis E virus (HEV) is a major cause of acute viral hepatitis. Epidemiology and clinical presentation of hepatitis E vary greatly by location and are affected by the HEV genotype. Nucleic acid amplification technique (NAT)-based assays are important for the detection of acute HEV infection as well for monitoring chronic cases of hepatitis E.

Objectives: The aim of the study was to evaluate a panel of samples containing different genotypes of HEV for use in nucleic NAT-based assays.

Study design: The panel of samples comprises eleven different members including HEV genotype 1a (2 strains),

* Corresponding author at: Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, 63225 Langen, Germany.
E-mail address: Sally.Baylis@pei.de (S.A. Baylis).

[1] Present address - Jawaharlal Institute of Postgraduate Medical Education & Research, Puducherry 605006, India.

[2] HEV Collaborative Study Group (listed alphabetically by country): Dieter Pullirsch, Brigitte Hottowy, AGES PharmMed, Vienna, Austria; Lia Laura Lewis-Ximenez, Marcelo Alves Pinto, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; Youchun Wang, Weijin Huang, Chenyan Zhao, National Institutes for Food and Drug Control, Beijing, China; Zizheng Zheng, James Wai Kuo Shih, Zi-Min Tang, Wen-Fang Ji, Xiamen University, Fujian, China; Jacques Izopet, Sébastien Lhomme, Martine Dubois, Institut Fédératif de Biologie Purpan, Toulouse, France; Anne-Marie Roque-Afonso, Virologie AP-HP Hôpital Paul Brousse, Villejuif, France; Thomas Gärtner, Maike Schönborn, Christiane Beckort, Octapharma, Frankfurt am Main, Germany; Markus Hess, Manuela Tillack, Daniel Brischke, altona Diagnostics, Hamburg, Germany; Tanja Vollmer, Jens Dreier, Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum Nordrhein-Westfalen, Bad Oeynhausen, Germany; Jürgen Wenzel, Jasmin Klein, University of Regensburg Regensburg, Germany; Joan O'Riordan, Jessica Murphy, Fiona Boland, Irish Blood Transfusion Service, Dublin, Ireland; Barbara Pacini, Kedrion Biopharmaceuticals, Bolognana, Lucca, Italy; Giulio Pisani, Matteo Simeoni, Sara Fabi, Istituto Superiore di Sanità, Rome, Italy; Keiji Matsubayashi, Japanese Red Cross Hokkaido Block Blood Center, Sapporo, Japan; Saeko Mizusawa, National Institute of Infectious Diseases, Tokyo, Japan; Shigeharu Uchida, Central Blood Institute, Japanese Red Cross Society, Tokyo, Japan; Elisabet Ekvärn, Octapharma, Stockholm, Sweden; Boris Hogema, Sanquin Blood Supply Foundation, Amsterdam, the Netherlands; Tim Schuurman, Hubert Niesters, University Medical Center Groningen, Groningen, the Netherlands; Oliver Schär, Roche Diagnostics International Ltd. Rotkreuz, Switzerland; Samreen Ijaz, Steven Dicks, Becky Haywood, Public Health England, London, UK; Tonya Mixson-Hayden, Saleem Kamili, Centers for Disease Control and Prevention, Atlanta, USA; Jeffrey Linnen, Edgar Ong, Robin Cory, Hologic Inc., San Diego, USA.

https://doi.org/10.1016/j.jcv.2019.05.006
Received 6 March 2019; Received in revised form 10 May 2019; Accepted 13 May 2019

1e, 2a, 3b, 3c, 3e, 3f, 4c, 4g as well as a human isolate related to rabbit HEV. Each laboratory assayed the panel members directly against the 1st World Health Organization (WHO) International Standard (IS) for HEV RNA (6329/10) which is based upon a genotype 3 a strain.

Results: The samples for evaluation were distributed to 24 laboratories from 14 different countries and assayed on three separate days. Of these, 23 participating laboratories returned a total of 32 sets of data; 17 from quantitative assays and 15 from qualitative assays. The assays used consisted of a mixture of in-house developed and commercially available assays. The results showed that all samples were detected consistently by the majority of participants, although in some cases, some samples were detected less efficiently.

Conclusions: Based on the results of the collaborative study the panel (code number 8578/13) was established as the "1st International Reference Panel (IRP) for all HEV genotypes for NAT-based assays" by the WHO Expert Committee on Biological Standardization. This IRP will be important for assay validation and ensuring adequate detection of different genotypes and clinically important sub-genotypes of HEV.

1. Background

Worldwide, hepatitis E virus (HEV, species *Orthohepevirus A, Hepeviridae*) is a major cause of acute hepatitis. Diagnosis of acute hepatitis E relies upon detection of HEV RNA by nucleic acid amplification techniques (NAT or NAAT) [1]. The lack of assay standardization [2] led to the development and the establishment of the 1st World Health Organization (WHO) International Standard (IS) for HEV RNA (code number 6329/10) [3]. The WHO IS is a measurement standard with an internationally agreed concentration. The introduction of this reference material has facilitated the comparison of quantitative HEV RNA assays and determination of analytical sensitivities by reporting of results in international units (IU) in line with other WHO ISs [4]. This WHO IS represents an HEV sub-genotype 3a HEV strain from a Japanese blood donor diluted in HEV RNA negative human plasma.

A member of the *Hepeviridae* family of viruses, HEV is represented by a single serotype with four main genotypes infecting humans [5]. HEV genotypes 1 and 2 can be found in humans, whilst genotypes 3 and 4 are found in both humans as well as a range of animal species, particularly pigs [1]. The geographical distribution of HEV genotypes is complex. HEV genotype 1 consists of strains circulating in Africa and Asia. Genotype 2 has been found in Mexico and in some African countries. Genotype 3 is widely distributed, mainly being reported in the USA, Europe and Japan and more recently South America. Genotype 4 tends to be restricted to China, Japan and East Asia. However, genotype 1 viruses, and more recently genotype 4 viruses, have also occasionally been found in patients in Europe, North America and elsewhere after travelling to endemic areas and are mainly imported cases. Epidemics and sporadic cases of hepatitis E occur in areas of endemicity (genotypes 1, 2 and 4); more isolated clinical cases are diagnosed mostly among asymptomatic seropositive residents in developed countries (mainly genotype 3). Chronic infection, almost exclusively with genotype 3 HEV, occurs in immunocompromised patients and, more rarely, those with HIV infection [1]. At the nucleotide level, HEV strains, more rarely, can vary by ~26% between genotypes and up to ~15% between sub-genotypes. Given the levels of diversity and the importance of the epidemic strains in terms of the global disease burden of hepatitis E, the preparation of an International Reference Panel (IRP) panel for HEV genotypes was endorsed by the WHO Expert Committee of Biological Standardization. Such panels are important to help ensure consistent detection of viral variants and are complementary resources to WHO ISs.

Here, we present the results of a study evaluating the candidate panel which was evaluated simultaneously with a biological reference preparation (BRP) prepared on behalf of the Biological Standardisation Programme of the European Directorate for the Quality of Medicines & HealthCare (EDQM), Council of Europe and the EU Commission. The calibration of the genotype 3f [6] BRP in IU/ml, used as a control for the testing of HEV RNA in solvent/detergent-treated plasma is described elsewhere [7].

2. Study design

2.1. Preparation of materials

The HEV strains selected for the preparation of the panel are shown in Table 1. The strains were derived from a mixture of HEV RNA-positive plasma and stool samples. The strains were selected to cover the four main genotypes of HEV infecting humans as well as clinically

Table 1
Details of HEV strains used in the development of the panel.

Code	Genotype	Strain	Origin	Sample Type	Anti-HEV - IgM/IgG	Accession number
8567/13	1a		India	Plasma	n.d.	n.a.#
8568/13 s	1a	Kol-15	India	Stool	n.a.	n.a.#
8569/13	1e		Sudan	Plasma	+/-	n.a.#
8570/13	3b	JRC-HE3	Japan	Plasma	+/-	AB630971
8571/13	3c	Oct 8	Sweden	Plasma	-/-	JN995569#
8572/13	3e	Oct 3	Germany	Plasma	-/-	JN995564#
8573/13	3f*	Oct 12	Sweden	Plasma	-/-	JN995573#
8574/13 s	3 (rabbit)		France	Stool	n.a.	MG211750
8575/13	4c	HRC-HE15	Japan	Plasma	-/-	LC387631
8576/13	4 g	HRC-HE58	Japan	Plasma	-/-	LC387632
8577/13	2	Mex 14	Mexico	Stool	n.a.	KX578717
8577/13 s	2	Mex 14	Mexico	Stool	n.a.	KX578717

n.d. not determined
n.a. not applicable
N.B. XXXX/13 s, denotes panel members derived from stool samples
*3f – tentatively assigned as 3l using the HEVnet criteria: https://www.rivm.nl/en/Topics/H/HEVNet
Sample 8577/13 was not formulated using stabilizers and contains the same strain of HEV as sample 8577/13 s. The concentration of HEV in the two samples is different.
#Partial sequence data included in original study report for 1a and 1e http://apps.who.int/iris/bitstream/handle/10665/197775/WHO_BS_2015.2264_eng.pdf;jsessionid = 31DC19F99562D2722A9B88594D83AF51?sequence = 1, whole genome sequencing of panel has been performed

important sub-genotypes prevalent in different regions.

For panel preparation, the HEV RNA-positive samples were diluted using pooled citrated human plasma which tested negative for HBV, HCV and HIV-1/2 using the cobas® TaqScreen MPX Test v 2.0 (Roche Molecular Systems Inc., Branchburg, NJ, USA) and was negative for HEV RNA (testing described below) and anti-HEV IgM and anti-HEV IgG by enzyme immunoassays (Wantai, Beijing, China). All HEV RNA-positive plasma samples used in the preparation of the candidate panel were tested and found negative for the presence of HBV, HCV and HIV-1/2 using the cobas® TaqScreen MPX Test v 2.0. In the case of 8567/13 and 8569/13, the samples were diluted 1:500 prior to testing.

Stool samples were diluted in Dulbecco's Modified Eagle's Medium, filtered through 1 μM and then 0.2 μM filters and mixed with an equal volume of negative plasma (described above) and frozen at −80 °C prior to processing. The stool samples were further diluted in plasma prior to lyophilization, and in order to stabilize the HEV strains, trehalose and magnesium chloride were added at final concentrations of 5% and 150 mM, respectively. The formulation of the final matrix was found not to inhibit a range of PCR and transcription-based NAT assays.

To confirm the (sub-)genotype, sequencing and phylogenetic analysis was performed in the HEV open reading frame 1 region (ORF1) by a highly sensitive and broadly reactive nested reverse transcription-PCR (RT-PCR) assay amplifying a 283-nucleotide fragment of the RNA-dependent RNA-polymerase (RdRP) gene as described previously [8]. Furthermore, for 11 of the 13 complete HEV genomes information was obtained by next generation sequencing (manuscript in preparation) or was available from previous studies (Table 1). No discrepancies between the RdRP sequences and the complete genome sequences were observed. Therefore, complete genome data was used for phylogenetic reconstructions, using a GTR nucleotide substitution model and a Maximum Likelihood algorithm with 1000 bootstrap in Geneious 11 (http://www.geneious.com).

Filling and lyophilization of 0.5 ml volumes of the bulk samples was as previously described [3]. Vials were stored at −20 °C.

2.2. Collaborative study

In the collaborative study, the candidate IRP members were evaluated for potency in parallel with the WHO IS.

Study materials, sufficient for three assay runs, were shipped to participants on dry ice and then stored at ≤ −20 °C. Participants received 12 samples representing different HEV (sub-) genotypes (including one strain prepared in two different types of matrices together with the WHO IS (Table 1). Of the samples, eleven were candidate IRP members and one further sample (8577/13) was included as a matrix control for candidate IRP member 8577/13 s. Both 8577/13 and 8577/13 s were formulated with the same strain, however, they were formulated in plasma and plasma containing stabilizers, respectively. Participants were requested to perform testing using their routine HEV RNA assays using fresh vials of each sample for each assay run. Samples reconstitution was performed using 0.5 ml molecular biology grade water per vial with agitation for ~20 minutes prior to use.

For quantitative tests, participants were requested to use the WHO IS 6329/10 to create a standard curve (testing the IS neat and by three ten-fold serial dilutions i.e. 250,000 IU/ml (neat) to 250 IU/ml) and data reported directly in IU/ml. The panel of samples were tested without prior dilution.

For qualitative assays, participants were requested to assay each sample alongside the IS. In the first assay, a series of one $\log_{10}$ dilution steps, were used to obtain estimates of end-points. Half-$\log_{10}$ dilutions around the end-point were performed for the subsequent two assay runs. Results were reported as either positive or negative. Dilutions were prepared using diluent in regular use by the respective laboratories e.g. HEV-negative plasma. All participants used plasma as a diluent with the exception of Laboratory 3 where water was used.

Relevant information (e.g. C_T values for the respective dilutions where real time PCR methods were used or signal to cut-off (S/Co) values - e.g. for transcription-mediated assays) were reported by participants using electronic reporting sheets.

2.3. Statistical methods

2.3.1. Quantitative assays

Potency estimates determined for each sample relative to the WHO IS (measured in $\log_{10}$ IU/ml) based on quantitative data were derived using a mixed linear model with random factors *laboratory* and *assay run*.

2.3.2. Qualitative assays

For qualitative data analysis, results from all the three assays for each laboratory were pooled to give the number of positives out of the total number tested at each dilution. Assuming that a single 'detectable unit' will give a positive result, with the probability of a positive result following a Poisson distribution, the EC63 (the dilution at which 63% of the samples are expected to be positive) was chosen as the end-point. For each dilution series, this end-point was estimated by means of a probit analysis. Within the same evaluation, relative potencies were also estimated.

For assays reporting C_T values, these were evaluated for both qualitative and quantitative methods (relative to WHO IS) using a parallel line model for each laboratory and assay run, as well as combined for all evaluable (i.e. valid) assay runs.

Qualitative sample cut-off values from Laboratory 5 and 7 were evaluated by means of a sigmoid dose-response model. Samples with less than 3 dose values and/or non-linear or non-parallel behaviour were excluded from the analysis.

Parallel line and sigmoid evaluation model as well as a combination of assays were performed according to methods as described in chapter 5.3, "Statistical analysis of results of biological assays and tests", of the Ph. Eur. The statistical analysis was performed with SAS®/STAT software, version 9.4, SAS System for Windows, and CombiStats, version 5.0, EDQM, Council of Europe.

2.4. Stability studies

Vials of the candidate panel samples were stored at −20 °C (the normal storage temperature) and −80 °C (to provide a baseline). For the accelerated thermal degradation, vials were incubated at +4 °C, +20 °C and +37 °C for up to 6 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 as previously described [3].

3. Results

3.1. Data received

Data were received from 23 participating laboratories. A total of 32 data sets were returned; 17 from quantitative assays and 15 from qualitative assays. Some laboratories reported results for more than one type of assay. The types of methods used by the participants are listed in Table 2 [9–15]. The majority of assays designs are based on primers targeting HEV ORF2/3 region.

Phylogenetic analysis of the panel strains, the WHO IS, and all reference Orthohepevirus A strains and subtypes defined by Smith et al. [16], are shown in Fig. 1.

The different HEV genotypes and sub-genotypes were detected by all participants, with a single exception – Laboratory 12 was unable to detect sample 8567/13 (HEV genotype 1a) in any of the three assay runs. In some cases, differences in the efficiency of detection were observed for some of the candidate panel members. Some of the other samples were inconsistently detected by Laboratory 12. Laboratory 6

Table 2
Assay protocols used by participants.

Laboratory code	Assay type[a]	Extraction method	NAT method	Reference
1	Quant.	High Pure Viral Nucleic Acid Kit Large Volume (Roche)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
2	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
3A/3B	Quant./Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (HepatitisE@ceeramTools™)	
4	Quant.	EZ1 Virus Mini Kit v2.0 (Qiagen)	Real-time RT-PCR (TaqMan)	9
5	Qual.	Automated proprietary magnetic target capture method – Procleix Panther System	Transcription mediated amplification and hybridization protection assay (Procleix HEV Assay, Hologic Inc.)	
6A/B	Quant./Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
6C/D	Quant./Qual.	chemagic Viral DNA/RNA Kit (PerkinElmer)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
6E	Qual.	NucliSENS® easyMag® (bioMérieux)	*ampli*Cube HEV 2.0 (Mikrogen GmbH)	
6F	Qual.	chemagic Viral DNA/RNA Kit (PerkinElmer)	*ampli*Cube HEV 2.0 (Mikrogen GmbH)	
7	Qual.	Automated proprietary magnetic target capture method – Procleix Panther System	Transcription mediated amplification and hybridization protection assay (Procleix HEV Assay; Hologic Inc.)	
8	Qual.	QIAamp MinElute Virus Spin Kit/QIAcube (Qiagen)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
9	Quant.	QIAsymphony DSP Virus/Pathogen Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	
10	Quant.	QIAamp MinElute Virus Spin Kit (Qiagen)	Real-time RT-PCR (TaqMan)	10
11	Qual.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
12	Quant.	SMI-TEST EX-R&D (Medical Biological Laboratories Co., Ltd.)	Real-time RT-PCR (TaqMan)	11
13	Qual.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	
14	Qual.	Roche cobas® 6800/8800	Real-time RT-PCR (cobas® HEV, Roche Molecular Systems)	
15	Quant.	QIAamp MinElute Virus Spin Kit/QIAcube (Qiagen)	Real-time RT-PCR (TaqMan)	
16	Quant.	MagNA Pure 96 Viral NA Large Volume Kit (Roche)	Real-time RT-PCR (TaqMan)	12
17	Quant.	MagNA Pure LC (Roche)	Real-time RT-PCR (SYBR Green)	13
18	Quant.	MagNA Pure 96 Viral NA Large Volume Kit (Roche)	Real-time RT-PCR (TaqMan)	13, 14
19A/B	Quant./Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (TaqMan)	15
20	Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (TaqMan)	
21A/B	Quant./Qual.	GenMag Viral DNA/RNA Kit (GenMagBio)	Real-time RT-PCR (TaqMan)	13
22	Qual.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (Diagnostic kit hepatitis E virus RNA, Beijing Kinghawk Pharmaceutical Co., Ltd.)	15
23A	Quant.	High Pure Viral Nucleic Acid Kit (Roche)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
23C	Quant.	High Pure Viral Nucleic Acid Kit (Roche)	Real-time RT-PCR (TaqMan)	13

[a] Data returned in qualitative (Qual.) or quantitative (Quant.) format. The altona Diagnostics assay was used both qualitatively (end-point dilution) and quantitatively against the WHO IS.

reported inhibition of some of the undiluted samples, including some of the stool-derived materials; however this was not consistent across assay runs. Laboratory 6 used the NucliSENS easyMag (bioMérieux) extraction platform and other laboratories using this system did not report inhibition of any of the samples and it is possible that inhibition may have been due to the high proportion of extracted nucleic acid used in the amplification/detection reaction.

3.2. Quantitative assay results

The individual laboratory mean estimates ($\log_{10}$ IU/ml) for the candidate panel samples assayed directly against the WHO IS 6329/10 are shown in Tables S1a and S1b. The relative variation of the individual laboratory estimates is illustrated by the box-and-whisker plots in Figure S1. Greater variation was observed with the genotypes 1, 2 and 4, whereas better agreement was observed for the genotype 3 strains. The genotype 3b strain (sample 8570/13) had been evaluated in previous studies [2,3] and was established as the first HEV RNA National Standard in Japan.

The laboratory mean absolute estimates of IU/ml ($\log_{10}$) are shown in histogram form in Fig. 2. For the quantitative assay results, each white box represents the mean estimate from an individual laboratory, and is labelled with the laboratory code number. In general, good agreement was observed between the laboratories performing quantitative assays. The overall means from all laboratories for the quantitative assays are shown in Table S2.

3.3. Qualitative assay results

The individual laboratory relative potency estimates ($\log_{10}$ IU/ml), for the candidate panel samples assayed in parallel against the WHO IS 6329/10 are shown in Tables S3A and S3b (based upon end-point dilution analysis). The overall means from all laboratories for the qualitative end-point dilution assays are shown in Table S4. In addition, mean estimates were also evaluated by analysis of C_T and S/Co values for the qualitative assays (Tables S5a and S5b). The relative variation of the individual laboratory estimates for the qualitative assays is illustrated by the box-and-whisker plots in Figure S2. The overall means from all laboratories for the analysis of C_T and S/Co values for the qualitative assays are shown in Table S6.

The qualitative assays are much more variable than the quantitative assays, reflecting the different sensitivities of the assays performed in different participating laboratories. The potencies of the panel samples for the qualitative assay results are shown in Fig. 2, each dark grey box represents the mean estimate from an individual laboratory, and is labelled with the laboratory code number – these data represent the end-point dilution analysis; values determined by analysis of the C_T values are shown in the black boxes. In general, the results from the qualitative assay are in good agreement with those of the quantitative assays.

3.4. Determination of overall laboratory means – combined qualitative and quantitative results

The overall mean values, including range and standard deviations, for the candidate panel samples are shown in Table 3. The respective overall means are a combination of the quantitative data (IU/ml), the qualitative data (based on end-point dilution); potencies determined by analysis of reported C_T values or S/Co values from the qualitative assays for the panel samples relative to the WHO IS.

In general, the overall means are in line with the expected range of results that had been previously communicated to the participants (data not shown). No unitage was assigned to the panel samples, in keeping with other such panels and potencies are merely for guidance for users [4].

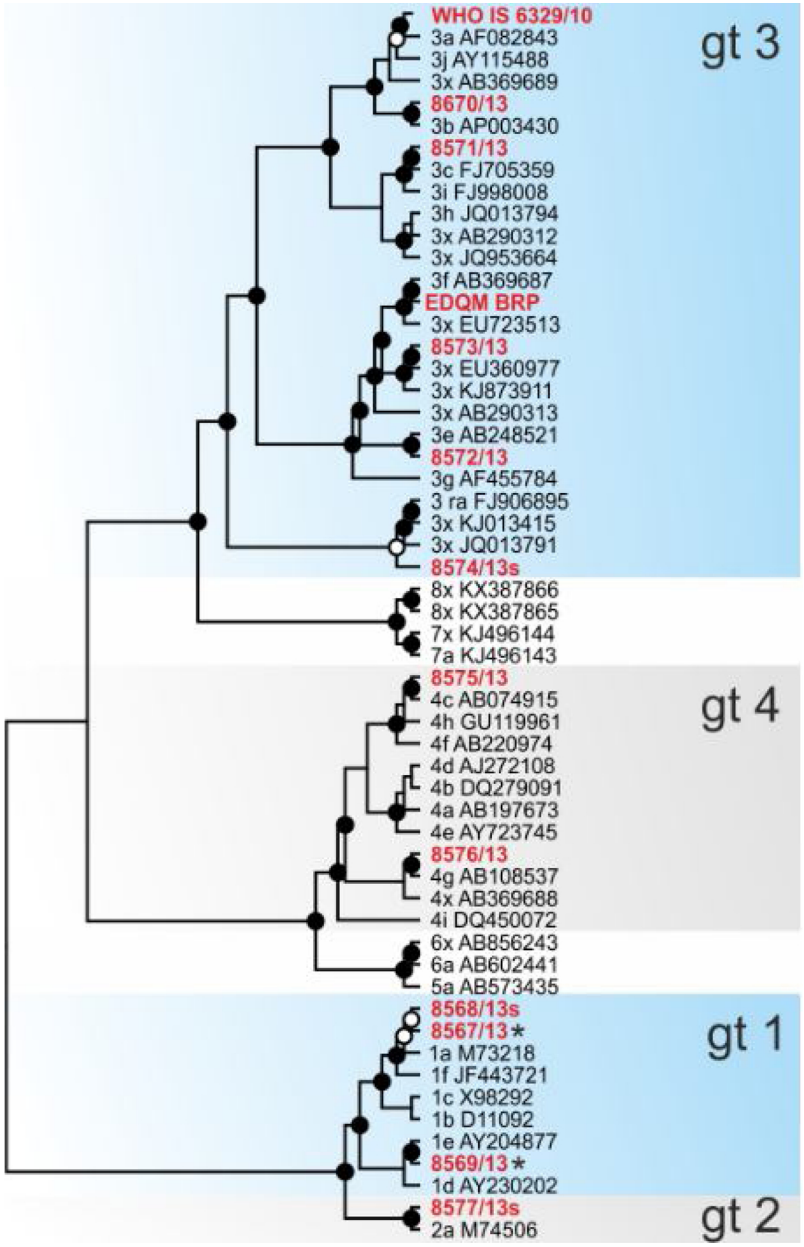

Fig. 1. Maximum-likelihood phylogeny of the complete coding sequences of reference Orthohepevirus A strains and genotype (gt) subtypes defined by Smith *et al.* [16], and HEV strains used in this study (given in red), the WHO IS strain is given in blue. Taxon names of all reference sequences include genotype, subtype ("x" if not available), and GenBank accession (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Black circles at nodes indicate bootstrap supports of > 90% and white circles > 75% (1,000 replicates). For the two viruses marked with an asterisk only a sequence fragment of ORF1 was available for analysis.

3.5. Stability studies

The plasma-derived viruses showed good stability comparable to that of the WHO IS when stored at elevated temperatures. However, in initial studies, it was found that all stool derived viruses (genotype 1a, 1e and 2a), diluted in plasma were unstable at 37 °C, such that in under 1 week, HEV RNA was barely detectable – Figure S3 (data shown for genotypes 1e and 2a). Lyophilization of plasma spiked with the same 3a HEV strain used to establish the WHO IS, treated with detergent (Triton X-100, 0.5% final concentration), demonstrated very similar inactivation kinetics to the HEV stool samples, this was not the case when no detergent was added (Figure S3). Because of the lack of stability at elevated temperatures, different concentrations/combinations of stabilizing agents were added to determine which formulation gave the best overall stability. It was found that trehalose and magnesium chloride added at final concentrations of 5% and 150 mM, respectively gave the best overall stability. Figure S4 demonstrates the difference on HEV genotype 2a RNA stability when formulated in the presence/absence of stabilizers at elevated temperature when compared to storage at −20 °C or baseline (-80°). The two stabilizers were most effective when used in combination than when used separately and lower concentrations were not as effective as those used in the final formulation.

For the real time stability studies, there was no loss of signal observed for any panel member under normal storage conditions i.e. −20 °C compared to baseline samples stored at −80 °C for ~3.5 years (data not shown). For the accelerated degradation studies, in the case of the individual panel members there was a drop of between 0.0-0.5 $\log_{10}$ IU/ml (Tables S7a and S7b) after storage of the samples at +20 °C for 3 to 6 months; greater loss of titre was observed at +37 °C.

4. Discussion and conclusion

In this study, a wide range of quantitative and qualitative assay formats were used to evaluate the candidate HEV RNA genotype panel of samples in parallel with the WHO IS. Approximately half of the assays had been developed in-house with the rest being commercially available, this is in contrast to the initial study to evaluate laboratory performance where only one commercial assay was included [2] and

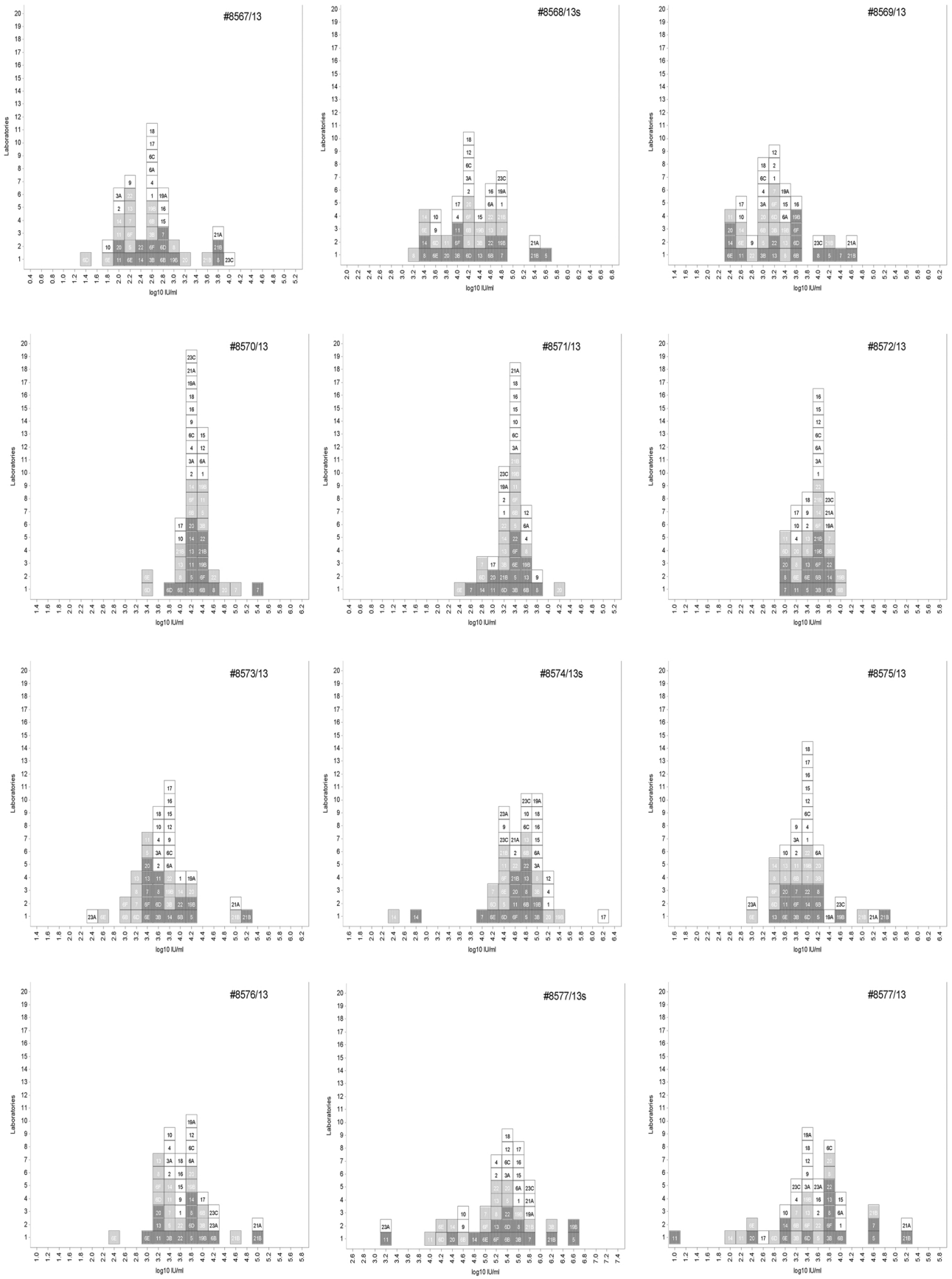

Fig. 2. Potency in $\log_{10}$ IU/ml based on quantitative methods (white boxes); potency based on analysis of C_T values from qualitative methods (black boxes); potency based on qualitative, end-point dilution analysis (dark grey boxes); potency based on analysis of C_T values from quantitative methods.
Values determined by Laboratory 23A were based on a single assay run, the samples were inconsistently detected.

Table 3
Overall laboratory means – combined qualitative and quantitative results (log_{10} IU/ml).

Sample	Mean[a]	95% CI[b]		Median	Std.	Min.	Max.	Range
#8567/13	2.57	2.37	2.78	2.57	0.58	1.47	3.91	2.45
#8568/13 s	4.28	4.09	4.48	4.24	0.55	3.23	5.56	2.34
#8569/13	3.25	3.05	3.45	3.16	0.56	2.40	4.66	2.26
#8570/13	4.24	4.13	4.36	4.21	0.34	3.36	5.49	2.13
#8571/13	3.32	3.22	3.43	3.36	0.33	2.36	4.17	1.81
#8572/13	3.47	3.39	3.56	3.54	0.26	2.91	3.96	1.06
#8573/13	3.69	3.50	3.88	3.67	0.54	2.40	5.14	2.74
#8574/13 s	4.73	4.52	4.94	4.73	0.57	2.40	6.10	3.70
#8575/13	3.96	3.80	4.13	3.93	0.47	3.00	5.34	2.34
#8576/13	3.68	3.51	3.85	3.65	0.48	2.37	5.06	2.69
#8577/13 s	5.22	4.98	5.47	5.33	0.71	3.25	6.69	3.45
#8577/13	3.48	3.22	3.74	3.48	0.75	1.02	5.23	4.21

Std. Standard deviation.
[a] Estimated mean accounting for laboratory and number of assays performed.
[b] 95% Confidence Interval.

the study to establish the WHO IS where all the assays had been developed in-house [2,3]. With a single exception, all participants were able to detect all the different HEV genotypes and sub-genotypes included in the panel. One in-house assay, used by a single laboratory, failed to detect 8567/13, a genotype 1a HEV strain which had the lowest titre of any of the panel members; this may be due to a lack of assay sensitivity for example, due to suboptimal extraction efficiency or possibly due to primer design.

There were noticeable differences in the potencies for some of the samples – particularly genotype 2 for which there are very few reported sequences. In contrast, the potencies reported for the genotype 3 strains were less variable and reflect the availability of a large number of sequences and evaluation of several of these strains in previous studies [2,3].

The sample 8577/13 (HEV genotype 2a) was included as a matrix control. Because of the instability of 8577/13 at ambient temperature, the formulation of the sample was revised to include stabilizers (8577/13 s). There was no evidence of inhibition of this revised matrix, either in testing prior to the collaborative study or during the collaborative study itself. All HEV samples prepared from stool were found to demonstrate instability. Interestingly, pre-treatment of the plasma-derived strain used to prepare the WHO IS with detergent, followed by lyophilization rendered the HEV unstable and showed similar degradation kinetics to the stool samples when RNA content was determined. This may reflect the quasi-enveloped form of HEV in blood and plasma [17] and the membranes most likely act to protect the HEV particles during the lyophilization process. Sample 8577/13 has not been included in the composition of the final panel. The other panel members showed 0-0.3 log_{10} loss of titre after storage at +20 °C, compared to the normal storage temperature of −20 °C.

Stability studies demonstrated 0-0-3 log_{10} change in HEV RNA concentration after 3 months of storage at +4 °C, and +20 to +26 °C, compared with samples stored at < −80 °C. No loss of titre was observed for samples stored at −20 °C (normal storage temperature). In accordance with WHO policy, no unitage has been assigned to the panel members, although the potencies and range, determined in the collaborative study are available to inform users of the panel performance.

The 1st International Reference Panel for Hepatitis E Virus RNA Genotypes (code number 8578/13) was established by the WHO ECBS in October 2015. The custodian laboratory is the Paul-Ehrlich-Institut. The panel is not intended to replace the WHO IS for HEV RNA but is intended to be used to ensure adequate detection of different HEV genotypes and sub-genotypes in nucleic acid testing.

Funding

The authors did not receive external funding for this study.

Ethical approval

Not required.

Competing interests

The authors declare no competing interests.

Acknowledgments

We thank all the laboratories who took part in the study and Roswitha Kleiber from the Paul-Ehrlich-Institut for excellent assistance and Heidi Meyer for helpful discussions.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.05.006.

References

[1] European Association for the Study of the Liver, EASL Clinical Practice Guidelines on hepatitis E virus infection, J. Hepatol. 68 (2018) 1256–1271.
[2] S.A. Baylis, K.M. Hanschmann, J. Blümel, CM Nübling; on behalf of the HEV Collaborative Study Group, Standardization of hepatitis E virus (HEV) nucleic acid amplification technique (NAT)-based assays: an initial study to evaluate a panel of HEV strains and investigate laboratory performance, J. Clin. Microbiol. 49 (2011) 1234–1239.
[3] S.A. Baylis, J. Blümel, S. Mizusawa, K. Matsubayashi, H. Sakata, Y. Okada, C.M. Nübling, K.M. Hanschmann, HEV Collaborative Study Group, World Health Organization International Standard to harmonize assays for detection of hepatitis E virus RNA, Emerg. Infect. Dis. 19 (2013) 729–735.
[4] S.A. Baylis, P. Wallace, E. McCulloch, H.G.M. Niesters, C.M. Nübling, Standardization of nucleic acid amplification tests: the approach of the World Health Organization, J. Clin. Microbiol. 57 (2019) pii: e01056-18.
[5] M.A. Purdy, T.J. Harrison, S. Jameel, X.J. Meng, H. Okamoto, WHM Van der Poel WHM, DB Smith DB, ICTV Report Consortium, ICTV Virus Taxonomy Profile: Hepeviridae, J. Gen. Virol. 98 (2017) 2645–2646.
[6] C. Adlhoch, M. Kaiser, G. Pauli, J. Koch, H. Meisel, Indigenous hepatitis E virus infection of a plasma donor in Germany, Vox Sang. 97 (2009) 303–308.
[7] S.A. Baylis, E. Terao, J. Blümel, K.O. Hanschmann, Collaborative study for the establishment of the Ph. Eur. Hepatitis E virus RNA for NAT testing biological reference preparation batch 1, Pharmeur. Bio. Sci. Notes 2017 (2017) 12–28.
[8] J.F. Drexler, A. Seelen, V.M. Corman, A. Fumie Tateno, V. Cottontail, R. Melim Zerbinati, F. Gloza-Rausch, S.M. Klose, Y. Adu-Sarkodie, S.K. Oppong, E.K. Kalko, A. Osterman, A. Rasche, A. Adam, M.A. Müller, R.G. Ulrich, E.M. Leroy, A.N. Lukashev, C. Drosten, Bats worldwide carry hepatitis E virus-related viruses that form a putative novel genus within the family Hepeviridae, J. Virol. 86 (2012) 9134–9147.
[9] J.J. Wenzel, J. Preiss, M. Schemmerer, B. Huber, A. Plentz, W. Jilg, Detection of hepatitis E virus (HEV) from porcine livers in Southeastern Germany and high sequence homology to human HEV isolates, J. Clin. Virol. 52 (2011) 50–54.
[10] K. Matsubayashi, J.H. Kang, H. Sakata, K. Takahashi, M. Shindo, M. Kato, S. Sato, T. Kato, H. Nishimori, K. Tsuji, H. Maguchi, J. Yoshida, H. Maekubo, S. Mishiro, H. Ikeda, A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route, Transfusion 48 (2008) 1368–1375.
[11] T. Tanaka, T. Masaharu, E. Kusano, H. Okamoto, Development and evaluation of an efficient cell-culture system for Hepatitis E virus, J. Gen. Virol. 88 (2007) 903–911.
[12] F. Abravanel, K. Sandres-Saune, S. Lhomme, M. Dubois, J.M. Mansuy, J. Izopet, Genotype 3 diversity and quantification of hepatitis E virus RNA, J. Clin. Microbiol. 50 (2012) 897–902.
[13] N. Jothikumar, T.L. Cromeans, B.H. Robertson, X.J. Meng, V.R. Hill, A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus, J. Virol. Met. 131 (2006) 65–71.
[14] J.A. Garson, R.B. Ferns, P.R. Grant, S. Ijaz, E. Nastouli, R. Szypulska, R.S. Tedder,

Minor groove binder modification of widely used TaqMan probe for hepatitis E virus reduces risk of false negative real-time PCR results, J. Virol. Methods. 186 (2012) 157–160.
[15] C. Zhao, Z. Li, B. Yan, T.J. Harrison, X. Guo, F. Zhang, J. Yin, Y. Yan, Y. Wang, Comparison of real-time fluorescent RT-PCR and conventional RT-PCR for the detection of hepatitis E virus genotypes prevalent in China, J. Med. Virol. 79 (2007) 1966–1973.
[16] D.B. Smith, P. Simmonds, J. Izopet, E.F. Oliveira-Filho, R.G. Ulrich, R. Johne, M. Koenig, S. Jameel, T.J. Harrison, X.J. Meng, H. Okamoto, W.H. Van der Poel, M.A. Purdy, Proposed reference sequences for hepatitis E virus subtypes, J. Gen. Virol. 97 (2016) 537–542.
[17] M. Takahashi, T. Tanaka, H. Takahashi, Y. Hoshino, S. Nagashima, Jirintai, H. Mizuo, Y. Yazaki, T. Takagi, M. Azuma, E. Kusano, N. Isoda, K. Sugano, H. Okamoto, Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation, J. Clin. Microbiol. 48 (2010) 1112–1125.