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

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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 as 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), 1b (2 strains), 2a, 3a, 4a, and 5a.

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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

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1. Background

Worldwide, hepatitis E virus (HEV, species Orthohepeivirus 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].

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 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 preparation of the genotype 3 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

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**Table 1**

<table>
<thead>
<tr>
<th>Code</th>
<th>Genotype</th>
<th>Strain</th>
<th>Origin</th>
<th>Sample Type</th>
<th>Anti-HEV - IgM/IgG</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>8567/13</td>
<td>1a</td>
<td>India</td>
<td>Plasma</td>
<td>n.d.</td>
<td>n.a. n.a.</td>
<td></td>
</tr>
<tr>
<td>8568/13s</td>
<td>1a</td>
<td>Kol-15</td>
<td>India</td>
<td>Plasma</td>
<td>+/-</td>
<td>AB630971</td>
</tr>
<tr>
<td>8569/13</td>
<td>1e</td>
<td>Sudan</td>
<td>Plasma</td>
<td>+/-</td>
<td>n.a. n.a.</td>
<td></td>
</tr>
<tr>
<td>8570/13</td>
<td>3b</td>
<td>JRC-HE3</td>
<td>Japan</td>
<td>Plasma +/−</td>
<td>JN995s69#</td>
<td></td>
</tr>
<tr>
<td>8571/13</td>
<td>3c</td>
<td>Oct 8</td>
<td>Sweden</td>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8572/13</td>
<td>3e</td>
<td>Oct 3</td>
<td>Germany</td>
<td>Plasma</td>
<td>JN995s64#</td>
<td></td>
</tr>
<tr>
<td>8573/13</td>
<td>3f</td>
<td>Oct 12</td>
<td>Sweden</td>
<td>Plasma</td>
<td>JN995s73#</td>
<td></td>
</tr>
<tr>
<td>8574/13s</td>
<td>3 (rabbit)</td>
<td>France</td>
<td>Stool</td>
<td>n.a.</td>
<td>M5211750</td>
<td></td>
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<tr>
<td>8575/13</td>
<td>4c</td>
<td>HRC-HE15</td>
<td>Japan</td>
<td>Plasma</td>
<td>LC387631</td>
<td></td>
</tr>
<tr>
<td>8576/13</td>
<td>4g</td>
<td>HRC-HE58</td>
<td>Japan</td>
<td>Plasma</td>
<td>LC387632</td>
<td></td>
</tr>
<tr>
<td>8577/13</td>
<td>2</td>
<td>Mex 14</td>
<td>Mexico</td>
<td>Stool n.a.</td>
<td>K578717</td>
<td></td>
</tr>
<tr>
<td>8577/13s</td>
<td>2</td>
<td>Mex 14</td>
<td>Mexico</td>
<td>Stool n.a.</td>
<td>K578717</td>
<td></td>
</tr>
</tbody>
</table>

n.d. not determined
n.a. not applicable

N.B. XXXX/13 s, denotes panel members derived from stool samples

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.
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® TagScreen 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® TagScreen 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. 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 the samples that were tested was expected to be positive) was chosen as the end-point.

For qualitative assays, participants were requested to assay each sample alongside the IS. In the first assay, a series of one log₁₀ dilution steps, were used to obtain estimates of end-points. Half-log₁₀ 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. Cₚ 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 log₁₀ 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 EGC63 (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 Cₚ 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
reported inhibition of some of the undiluted samples, including some of the stool-derived materials; however this was not consistent across all laboratories using this system. Laboratory 6 used the NucliSENS easyMag extraction platform. Laboratory 7 reported inhibition of some of the undiluted samples, including some of the stool-derived materials; however this was not consistent across all laboratories using this system.

### 3.3. Qualitative assay results

The individual laboratory relative potency estimates (log_{10} IU/ml) for the candidate panel samples are shown in Table S2. The respective overall mean estimates were also evaluated by analysis of C_{7} 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 overall mean estimates for all laboratories for the qualitative assays are shown in Table S2.

#### 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_{7} 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 [6].
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 (genotypes 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 °C). 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

![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.](image-url)
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.
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. In contrast, the potencies reported for the genotype 3 strains were less variable and reflect the availability of a large number of sequences. In contrast, the potencies reported for the genotype 3 strains were less variable and reflect the availability of a large number of sequences. In contrast, the potencies reported for the genotype 3 strains were less variable and reflect the availability of a large number of sequences.

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). 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 [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 log10 loss of titre after storage at +20°C, compared to the normal storage temperature of −20°C.

Stability studies demonstrated 0-0.3 log10 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


