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An Evaluation of Hepatitis E Virus Molecular Typing Methods

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BACKGROUND: Hepatitis E virus (HEV) is a major cause of acute viral hepatitis. Better understanding of HEV subtypes involved in hepatitis E infections is essential. Investigation of sources and routes of transmission and the identification of potential clusters/outbreaks rely upon molecular typing of viral strains. A study was carried out to evaluate the ability of laboratories to undertake molecular typing with genotype and subtype determination.

METHODS: A blinded panel of 11 different *Orthohepevirus* A strains was distributed to 28 laboratories performing HEV sequence analysis. Laboratories used their routine HEV sequencing and genotyping methods.

RESULTS: Results were returned by 25 laboratories. Overall, 93% samples were assigned to the correct genotype and 81% were assigned to the correct subtype. Fragments amplified for typing ranged in size and the sequencing assays targeted both the structural and non-structural protein-coding regions. There was good agreement between the reported sequences where methods targeted overlapping fragments. In some cases, incorrect genotypes/subtypes were reported, including those not contained in the panel, and in one case, a genotype was reported for a blinded control sample containing Zika virus; collectively these data indicate contamination problems.

CONCLUSIONS: In general, identification of genotypes was good; however, in a small number of cases, there was a failure to generate sequences from some of the samples. There was generally broad agreement between the use of online typing tools such as the one provided by HEVnet and curated lists of published HEV reference sequences; however, going forward harmonization between these resources is essential.

Introduction

Globally, hepatitis E virus (HEV) is a major cause of acute viral hepatitis. Hepatitis E virus belongs to the *Hepeviridae*, a diverse family of viruses infecting a wide range of animal hosts including mammals, birds as well as fish. The *Orthohepevirus* genus is divided into four species (A to D) and includes HEV strains that infect humans (1). The vast majority of human cases of hepatitis E are caused by strains within species A, which comprises 8 genotypes and 36 subtypes (2, 3). Subtype diversity is complex and is updated periodically when new subtypes are reported (3), although some remain unassigned (4). Two genotypes, namely 1 and 2, are restricted to humans being spread by fecal–oral route. In contrast, HEV genotypes 3 and 4 are endemic in animal species including pigs and wild boar and may cause zoonotic infections in humans primarily by consumption of contaminated meat and meat products. Genotype 3 HEV, the main driver of hepatitis E infections in Europe (5), is particularly diverse at the molecular level and includes related viruses found in rabbits with evidence of occasional infection in humans.

Consequences of HEV infection may be particularly severe for pregnant women (genotype 1) and individuals with underlying liver disease. More recently chronic infection has emerged as an issue for immunosuppressed patients caused almost exclusively by HEV genotype 3.

The World Health Organization's (WHO) European Action plan for viral hepatitis includes recommendations for harmonized surveillance throughout the region (6). In response to this, the European Centre for Disease Prevention and Control (ECDC) hepatitis E working group has developed a framework for hepatitis E surveillance in the European Union/European Economic Area (EU/EEA) with the focus on the epidemiology of acute and chronic infections (7). To this

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end, a better understanding of HEV subtypes involved in hepatitis E infections is essential. Investigation of routes of transmission as well as the identification of potential clusters/outbreaks relies upon molecular analysis of viral strains. The HEVnet network and database, recently established by the Dutch National Institute for Public Health and the Environment (RIVM), is a new resource helping to underpin molecular epidemiological studies (4) reflecting the One Health approach to HEV infections.

During the first HEVnet meeting held at the RIVM in October 2017, there was interest in evaluating Sanger sequencing protocols to identify which methods might be the most robust and broadly applicable to a wide range of genotypes. This was discussed again at the third ECDC Hepatitis E virus expert group meeting in November 2018 and it was agreed that such a study would be a useful exercise which would also fulfil part of the mandate of the ECDC "*to foster sufficient capacity within the EU for detection, identification and characterisation of infectious agents which threaten public health*" (Regulation 851/2004, Article 5-3). The ECDC supports and commissions external quality assessment activities in public health microbiology laboratories in EU member states in order to verify the quality and comparability of surveillance data. Consequently, a study, coordinated by the Paul-Ehrlich-Institut (PEI—a member of ECDC Hepatitis E virus expert group as well as HEVnet) was carried out to understand challenges and assess capabilities as well as overall performance of molecular typing of HEV strains.

Materials and Methods

PARTICIPATING LABORATORIES

The study was open to hepatitis E virus reference laboratories in member states of the EU/EEA (7) as well as to members of HEVnet (4) and to other interested parties via these networks. Participation was on a voluntary basis. Panels were distributed to 28 laboratories, 26 in Europe (25 in the EU/EEA representing 10 member states) and 2 in North America.

PANEL

The panel contained 12 different samples, representing *Orthohepevirus* A genotypes 1-4. Eleven of the samples included in the panel were well-characterized, lyophilized HEV-positive samples from patients and blood donors formulated in pooled human plasma (8–14). The panel also contained one negative control which comprised cell culture-derived, heat-inactivated Zika virus (ZIKV) diluted in pooled human plasma (15). The samples were blinded and coded E1 to E12. The samples included in the panel are listed in Table 1. For some samples, complete genome sequences were available for some strains and partial sequences were available for others (Table 1), in all cases whole genome sequencing had been carried out using next-generation

Table 1. Study samples.[a]

Sample	Code number	Origin	Viral load (log_{10} IU/mL)	HEV subtype	Reference(s)
E1	EDQM BRP	Germany	4.6	3f	Adlhoch et al. (8); Baylis et al. (10)
E2	PEI/WHO 8576/13	Japan	3.7	4g	Baylis et al. (13)
E3	PEI/WHO 8569/13	Sudan	3.3	1e	Baylis et al. (13)
E4	PEI/WHO 8573/13	Sweden	3.7	3f[b]	Baylis et al. (13)
E5	PEI/WHO 11474/16		0	Negative (ZIKV)	Baylis et al. (15)
E6	PEI/WHO 8571/13	Sweden	3.3	3c	Baylis et. al. (13)
E7	PEI/WHO 8572/13	Germany	3.5	3e	Baylis et al. (13)
E8	PEI/WHO 8577/13s	Mexico	5.2	2a	Kaiser et al. (11)
E9	PEI/WHO 8574/13s	France	4.7	3 (rabbit)	Kaiser et al. (12)
E10	PEI/WHO 8568/13s	India	4.3	1a/1f	Baylis et al. (13)
E11	PEI/WHO 8575/13	Japan	4.0	4c	Baylis et al. (13)
E12	PEI/WHO 8570/13	Japan	4.2	3b	Baylis et al. (9)

[a]Phylogenetic analysis was performed for RdRP, Cap and whole genome sequences (8, 9, 13, 14) by comparison with previously reported genotypes in the databases plus reference strains (2, 3). Analysis was consistent between the different genomic regions and across each genome.
[b]E4 was assigned as 3f based on phylogenetic analysis; it has been provisionally assigned 3l by HEVnet.

sequencing (14). Assignment of genotypes/subtypes was performed as previously described (13, 14) by phylogenetic analysis of the strains using reference sequences proposed by Smith et al. (2).

DISPATCH OF PANELS

All panels were shipped at ambient temperature, and participants were requested to store the samples at −20 °C or below until analysis.

STUDY PROTOCOL

Laboratories were requested to use their routine method for HEV sequencing and for determination of genotypes and subtypes. Before testing, samples were reconstituted by participants in 0.5 mL of molecular grade water and agitated gently for 10 minutes and tested without further dilution.

Participants recorded their methods including: the RNA extraction method, the sample volume amplified, the protocol for cDNA synthesis and amplification as well as the primers used, and the region of the HEV genomic targets in the sequencing assay. The methods used by the participants are listed in online Supplemental Table 1. In addition, sequence data for each sample were returned to the study organizers for comparison with the original sequences and prototype reference strains.

EVALUATION OF RESULTS

The genotype and subtype of HEV for each of the samples was reviewed and compared to the expected genotype and subtype determined by the study organizers (Table 1). Consensus reference sequences for each sample were created by regrouping the submitted sequences by sample, aligning them together with the closest reference sequence (see online Supplemental Table 2) using 'clustalo' (16) and resolving them with 'cons' (17). The submitted sequences were then aligned to the matching sample consensus sequence using 'minimap2' (18) using the flag '-k 5' to set the kmer size to 5 and maximize the number of reads that aligned. The resulting alignments were visualized using Integrative Genomic Viewer (19); 'samtools' (20) was used at various points in the pipeline for sorting and indexing the alignments. The sequences were mapped according to the full-length sequence, where available for the samples in the panel (8–14). Where only partial sequences were available, alignments were performed using sequences of HEV reference strains (2, 3) which were used as a template to generate a consensus sequence as described above.

Results

DATA RETURNED

Of the 28 laboratories that received the panel, 25 (89%) returned results. All participating laboratories performed Sanger sequencing targeting defined regions of the HEV genome; a single laboratory returned data using next generation sequencing (NGS) as well. Methods included initial cDNA transcription using random primers with subsequent DNA amplification using HEV-specific primers. Some methods were based upon one-step reverse transcription polymerase chain reaction (RT-PCR), whilst others employed initial reverse transcription using HEV-specific antisense primers with either hemi-nested or nested amplification protocols (see online Supplemental Table 1). The sequencing methods targeted open reading frame (ORF2) encoding the capsid (Cap) protein with a smaller number of methods targeting different regions of ORF1, including the RNA-directed (dependent) RNA polymerase (RdRp) and the methyltransferase (Met). Overall, 23 methods targeted Cap, 5 RdRp and 3 Met. Some laboratories used more than one method and reported consensus HEV genotypes and subtypes for the panel of samples (see online Supplemental Table 1). In general, shorter sequences were generated using methods targeting Met and RdRp (about 240 bp to 280/360 bp, respectively), while longer sequences were generated using protocols targeting ORF2 (about 490–990 bp). Laboratory 2 and laboratory 18 generated the longest sequences; however, this was only for a subset of samples; the 5' region of these longer sequences covered the overlapping region with ORF3 which is more conserved. Because of the of the new coronavirus disease (COVID-19) pandemic, laboratory 16 stated that they usually implement 2 methods, however, under the circumstances this was not possible.

RESULTS OF GENOTYPE AND SUBTYPE ANALYSIS

Overall, from the samples tested, 279 of 300 (93%) were assigned to the correct genotype and 242 of the 300 samples (81%) were assigned to the correct subtype. Seventeen laboratories (68%) correctly identified the genotype of all HEV strains included in the panel; subtyping was more variable between laboratories. Laboratory 19, using a method to amplify a 989 bp fragment of ORF2, was unable to sequence any HEV-containing samples in the panel; this laboratory was able to amplify all HEV-samples using real time PCR, showing adequate RNA recovery and suggesting an issue with the amplification/sequencing method. With the exception of laboratory 23, laboratories were able to confirm the absence of HEV in the negative control sample (E5). Laboratory 23 detected HEV 2a sequences in sample E5; this was most likely due to cross contamination with the genotype 2a sample included in the panel (sample E8). There were further instances of contamination. For example, laboratory 6 reported detecting HEV subtype 3a in samples E2, E3 and E4, however, genotype 3a was not included in the panel.

Further analysis of the three sequences, for samples E2 to E4, reported by laboratory 6 revealed they were all identical and shared 100% nucleotide identity with HEV strains P1 and P6, which are widely used for cell culture of HEV being originally derived from the Kernow virus (21). It seems reasonable to assume that contamination was either due to virus, plasmid, or amplified material present in laboratory 6.

In some cases, analysis was more challenging. For sample E4 (HEV subtype 3f), 5 laboratories reported that E4 was an HEV subtype 3f strain, while 14 laboratories classified E4 as a 3l strain, a provisional subtype in HEVnet. Laboratory 12, method b (Cap) reported E4 as an HEV 3i strain.

In the case of sample E10, 92% of laboratories correctly identified this strain as genotype 1, with 9 laboratories reporting this as subtype 1f and 3 laboratories reporting it as subtype 1a. The remaining laboratories simply classified the sample as genotype 1. Previous phylogenetic analysis of RdRp and Cap had suggested that E10 was a subtype 1a strain although the strain clustered closely with 1f as well (13) and whole genome sequencing suggested the strain was 1f. These observations reflect the close relationship of subtypes 1a and 1f, both of which fall within the same clade (2, 3). Consequently, it is not unexpected that the data returned by the participants is varied. Based on the NGS data returned by laboratory 21, both subtype 1a and subtype 1f sequences were identified suggesting that the sample could contain more than one HEV strain as has been observed recently following transfusion transmission (22).

Laboratory 21, reported that sample E5 was negative for HEV sequence, but using NGS identified that the sample was positive for Zika virus (data not shown) and, in addition, human pegiviruses (*Flaviviridae*). This is not unexpected since the sample was diluted in pooled human plasma and such contaminants, part of the normal human virome, have been reported previously (23).

The results of the genotyping/subtyping are shown in Tables 2 and 3 and described in greater detail for each sample in the online Supplemental Results.

GRAPHICAL REPRESENTATION OF THE HEV SEQUENCE REGIONS ANALYZED BY PARTICIPANTS

Alignments of the sequences reported by the participants for the samples E1 (one of the best reported samples), E3 (the most challenging sample), and E9 (containing the most viral quasispecies) in the panel are shown in Fig. 1, A–C; the remaining samples are included in online Supplemental Fig. 1. Each figure shows the location of the fragments sequenced in relation to the major ORFs. There is very clear overlap of the methods sequencing Met, RdRp and Cap. The sequence data returned by the participating laboratories have been aligned against the full-length sequences where available or to the closest reference strains as described above; the associated sequence alignments are available at https://figshare.com/articles/dataset/HEV_Sequence_Files/14161247. In all cases, there was evidence of polymorphisms in the samples (indicated by vertical lines), most marked for E9 obtained from a chronically infected patient. The presence of quasispecies is not unexpected in RNA virus populations in natural infections. In some cases, polymorphisms may be a consequence of sequencing methods where proofreading enzymes are not employed.

While laboratory 4 correctly identified the subtype of sample E4, inspection of the alignment (Supplemental Fig. 1 – Sample E4) revealed that the submitted sequences were clearly outlying with a large number of polymorphisms. A similar phenomenon was observed with sample E6 for laboratory 4 (Supplemental Fig. 1 – Sample E6). The reason for this is unclear; laboratory 4 confirmed that sequence data for these two samples was restricted to a single strand. It is possible that raw data, corresponding to other unrelated samples were submitted in error, since these sequences shared only approximately 95% nucleotide identity with HEV sequences available in GenBank.

In the case of sample E9, containing the HEV strain similar to viruses identified in rabbits, the highest number of polymorphisms were observed for this sample when data were compared across laboratories. Sample E9 was the only sample in the panel obtained from a chronically, rather than an acutely, infected individual allowing time for the accumulation of greater numbers of quasispecies. The patient was immunosuppressed and the stool sample was obtained approximately 7 months after initial identification of HEV in the serum of the infected patient (12).

Discussion

Better understanding of HEV subtypes involved in hepatitis E infections is essential. Investigation of sources and routes of transmission as well as the identification of potential clusters/outbreaks relies upon molecular typing of viral strains. This is the first practical study evaluating performance of both sequencing protocols and tools used to assign genotypes and subtypes. Inclusion of all 4 major *Orthohepevirus* A genotypes infecting humans in the study was important to evaluate the robustness of different subtyping methods employed by participating laboratories. There was good identification of HEV genotypes, including epidemic strains (genotypes 1 and 2) as well as zoonotic strains (genotypes 3 and 4), however, greater differences were observed at the subtype level.

Table 2. Hepatitis E virus genotype and subtype assignment of samples by participating laboratories.[a]

	Code	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12		
	(Sub)Type	3f	4g	1e	3f	neg.	3c	3e	2a	3 rab.	1a/1f	4c	3b	Correct genotype n=12 (%)	Correct subtype n=12 (%)
Laboratory	Target														
1	Cap	3f	4g	1e	3l (p)	neg.	3c	3e	2a	3 rab.	1f	4c	3b	12 (100%)	12 (100%)
2	Cap	3f	4g	neg.	3f	neg.	3c	3e	2a	3 rab.	1a	4c	3b	11 (92%)	11 (92%)
3a	Met	3f	4g	1e	neg.	neg.	3c	3e	2a	3 rab.	1f	4	3b		
3b	RdRp	3f	4	1e	3l (p)	neg.	neg.	neg.	2a	3 rab.	1	4c	3b		
3c	Cap	3f	4g	1e	3l (p)	neg.	3c	3e	2a	3 rab.	1	4f	3		
3 cons.		3f	4g	1e	3l (p)	neg.	3c	3e	2a	3 rab.	1	4f	3	12 (100%)	10 (83%)
4	Cap	3f	4g	1e	3f	neg.	3c	3e	2a	3 rab.	1f	4f	3b	12 (100%)	11 (92%)
5a	RdRp	3f	neg.	neg.	short	neg.	3c	3e	2a	3 rab.	1	neg.	3b		
5b	Cap	3f	4g	neg.	3f	neg.	3c	3e	2a	3 rab.	1	4f	3b		
5 cons.		3f	4g	neg.	3f	neg.	3c	3e	2a	3 rab.	1	4f	3b	11 (92%)	9 (75%)
6	Met	3f	3a	3a	3a	neg.	3c	3e	2a	3 rab.	1	4	3b	10 (83%)	7 (58%)
7	Cap	3f	4g	1e	3l (p)	neg.	3c	3	2a	3 rab.	1	4c	3	12 (100%)	9 (75%)
8	Cap	3f	4g	1e	3l (p)	neg.	3c	3e	2a	3 rab.	1f	4c	3b	12 (100%)	12 (100%)
9	Cap	3f	4g	1e	3f	neg.	3c	3e	2a	3 rab.	1f	4c	3b	12 (100%)	12 (100%)
10	Cap	3f	4g	1e	3l	neg.	3c	3	2a	3 rab	1	4c	3	12 (100%)	9 (75%)
11	Cap	3f	4g	1e	3l	neg.	3c	3	2a	3 rab.	1	4c	3	12 (100%)	9 (75%)
12a	RdRp	neg.	4g	neg.	3i	neg.	3c	3e	neg.	3 rab.	1f	neg.	3b		
12b	Cap	3f	neg.	neg.	neg.	neg.	neg.	neg.	2a	neg.	neg.	4c	neg.		
12 cons.		3f	4g	neg.	3i	neg.	3c	3e	2a	3 rab.	1f	4c	3b	11 (92%)	10 (83%)
13	Cap	3f	4g	neg.	3f	neg.	3c	3e	2a	3 rab.	neg.	4	3b	10 (83%)	9 (75%)
14a	Met	neg.	4g	1e	neg.	neg.	neg.	3e	2a	3 rab.	neg.	neg.	3b		

Continued

Table 2 Continued

14b	RdRp	neg.	neg.	1e	3l	neg.	3c	3e	2a	3 rab.	1f	4c	3b		
14c	Cap	3f	4g	1e	neg.	neg.	neg.	neg.	neg.	3 rab.	neg.	neg.	3b		
14 cons.		3f	4g	1e	3l (p)	neg.	3c	3e	2a	3 rab.	1f	4c	3b	12 (100%)	12 (100%)
15	RdRp	3f	4g	1e	3	neg.	3c	3e	2a	3	1	4c	3b	12 (100%)	9 (75%)
16	Cap	3f	4g	1e	3l (p)	neg.	3c	3	2a	3 rab.	1	4c	3	12 (100%)	9 (75%)
17	Cap	3f	4g	neg.	3l (p)	neg.	3c	3e	2a	3 rab.	1a	4c	3b	11 (92%)	11 (92%)
18	Cap	3f	4g	1e	3l (p)	neg.	3c	3e	2a	3 rab.	1f	4c	3b	12 (100%)	12 (100%)
19	Cap	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	1 (8%)	1 (8%)
20	Cap	3f	4g	1e	3	neg.	3c	3	2a	3 rab.	1a	4c	3	12 (100%)	9 (75%)
21	Cap	3f	4g	1e	3l (p)	neg.	3c	3	2a	3 rab.	1	4c	3	12 (100%)	9 (75%)
21	NGS	3f	4g	1e	3l (p)	neg.	3c	3e	2a	3 rab.	1a/1f	4c/4f	3b	12 (100%)	12 (100%)
22	Cap	3f	4g	neg.	3l (p)	neg.	3c	3e	2a	3 rab.	1f	4c	3b	11 (92%)	11 (92%)
23	Cap	3f	4g	3	3l (p)	2a	3c	3	2a	3 rab.	1	4c	3	10 (83%)	7 (58%)
24	Cap	3f	4g	1e	3l (p)	neg.	3c	3	2a	3 rab.	1f	4c	3b	12 (100%)	11 (92%)
25	Cap	3f	4g	1e	3l (p)	neg.	3c	3e	2a	3 rab.	1	4c	3b	12 (100%)	11 (92%)

[a]Blue boxes indicate HEV positive samples reported as negative by participants. Green boxes (of differing shades) indicate expected HEV genotypes and subtypes. White boxes indicate expected HEV genotypes, although the correct subtype has not been reported. Orange boxes correspond to reporting of the incorrect HEV subtype. Red boxes correspond to false positive results.
[b]Scoring for sample E11–see online Supplemental Results.
[c]Abbreviations: neg., negative; 3 rab., rabbit; 3l (p), provisional assignment of subtype by the HEVnet tool; short, short sequence reported with no genotyping; cons., consensus sequence from more than one method reported.

Where disparities were observed, these were related to sequencing protocols, different approaches used for subtype reporting, and the challenge of contamination. The accuracy of HEV typing relies on successful amplification/sequencing protocols targeting regions allowing sufficient discrimination, used in combination with tools for genotype/subtype assignment. The latter should be comprehensive enough and up-to-date with appropriate thresholds to allow assignment of query sequences and be able to identify new subtypes, although borderline samples may still be challenging. Although the methods used by the participants varied in design, the most critical components in an assay are the virus-specific oligonucleotide primers with sequencing of common regions allowing more meaningful comparisons of data.

While some laboratories used several different amplification/sequencing methods, reporting consensus results, this was not always better at determining genotypes/subtypes compared to some laboratories that used a single method routinely. Although the participants represented a mixture of clinical laboratories as well as ones with a focus on veterinary health, food safety, and environmental surveillance, performance in detection of both human and zoonotic genotypes was quite

Table 3. Summary of hepatitisE virus genotype and subtype assignment of samples by participating laboratories[a]

Code	E1	E2	E3	E4b	E5	E6	E7	E8	E9	E10	E11	E12
Viral load (log_{10} IU/mL)	4.6	3.7	3.3	3.7	0	3.3	3.5	5.2	4.7	4.3	4.0	4.2
(Sub)Type	3f	4g	1e	3f/l	neg.[c]	3c	3e	2a	3 rab.[c]	1a/1f	4c	3b
Correct genotype n = 25: n (%)	24 (96%)	23 (92%)	16 (64%)	24 (96%)	24 (96%)	24 (96%)	24 (96%)	24 (96%)	24 (96%)	23 (92%)	24 (96%)	24 (96%)
Correct subtype n = 25: n (%)	24 (96%)	23 (92%)	16 (64%)	20 (80%)	24 (96%)	24 (96%)	16 (64%)	24 (96%)	23 (92%)	12 (48%)	19 (76%)	17(68%)

[a]Where consensus values have been reported, these have been used to calculate the number of correctly assigned HEV genotypes and subtypes.
[b]In the case of laboratory 6, sample E4 was correctly identified as genotype 3, however, the genotype reflects contamination with a genotype 3a laboratory strain.
[c]Abbreviations: neg., negative; 3 rab, rabbit.

consistent. The use of common methods between different disciplines, reflects the One Health concept which applies to HEV genotypes 3 and 4.

In some cases, for example with sample E3 containing a genotype 1 HEV strain, the viral load was quite low, i.e., 3.3 log_{10} IU/mL, and the least number of positive results were returned for this sample. This might reflect issues due to low viral load, however, sample E6—containing HEV subtype 3c—had an equivalent titer and was less problematic for the participating laboratories. Such viral loads are not uncommon and it is still desirable to be able to obtain sequencing data from such samples to better understand the molecular epidemiology of hepatitis E infections. Surveillance guidance recommends testing and sequencing as soon as possible following onset of hepatitis E symptoms (7) which would correspond to the range of viral loads of the study samples.

One laboratory returned results using NGS/whole genome sequencing (24), while more classical sequencing protocols were used in the other participating laboratories. The benefit of NGS was clear in the amplification of minor variants as well as the identification of the different pathogen (ZIKV) in the HEV negative control and viral sequences associated with the plasma virome. The NGS method, in combination with the HEVnet tool, was very proficient in subtyping even at lower viral loads, and this and similar methods, can be expected to be implemented more widely in the future.

An HEV sequencing method elaborated by the RIVM (25), circulated among members of the HEVnet community was widely used by participants. This method performed very well in most laboratories where it was used, however, in the case of laboratory 14 (method 14c), for example, sequences were only generated for about half of the samples, suggesting issues with implementation of this method in that particular laboratory. Although used by a small number of laboratories, methods targeting the RdRp were not always robustly implemented; only a single laboratory correctly identified all the respective genotypes. It has been reported previously, that while HEV genotyping with shorter sequences is possible, subtyping with shorter fragments can be problematic (26). It is up to laboratories to decide which methods to implement and when optimization is necessary.

Contamination was an issue in some laboratories, resulting from "within run" cross-contamination of some of the panel members as well as contamination by HEV-positive material already present the laboratory. Contamination was observed with one-step RT-PCR procedures as well as nested PCR. The use of hemi-nested/nested primers is important in the context of sequencing. One-step RT-PCR methods are more sensitive for detection and quantification of HEV RNA in diagnostic testing

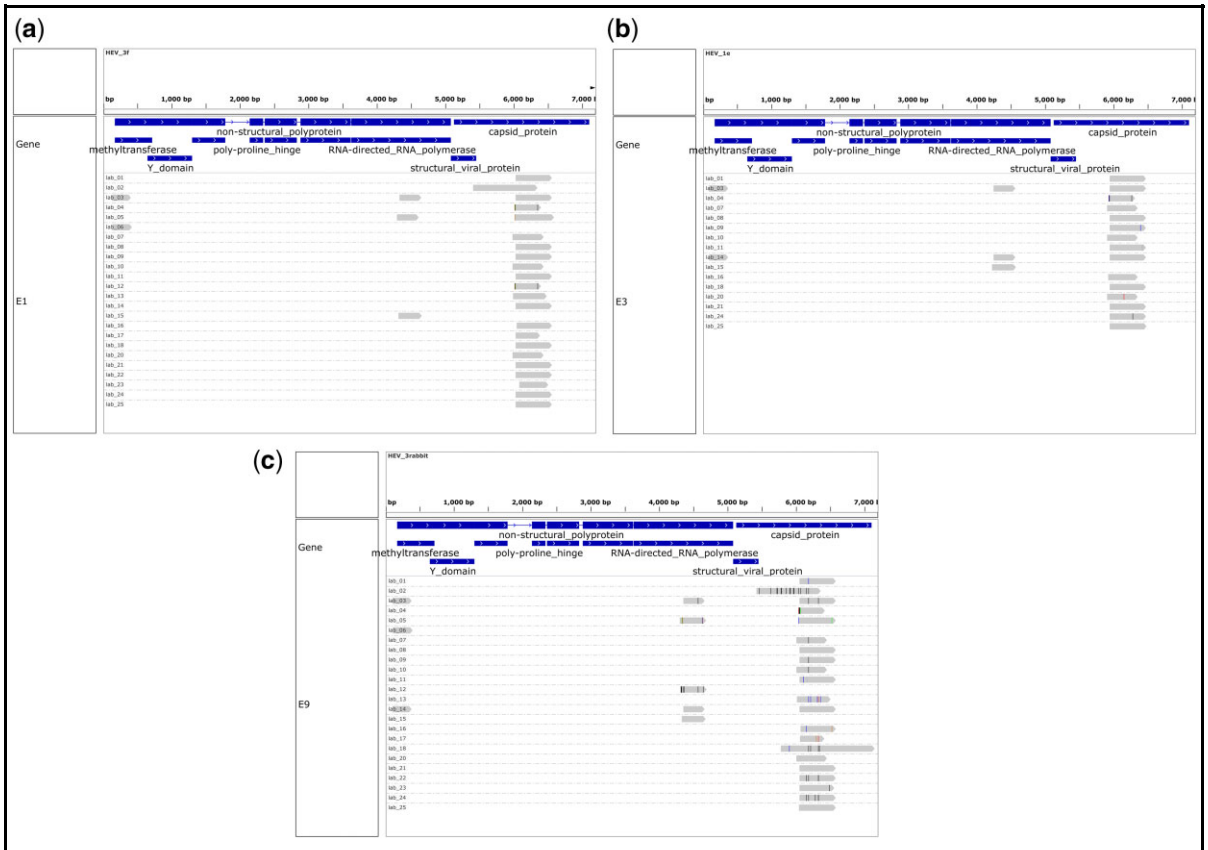

Fig. 1. Graphical representation of the HEV sequence regions analyzed by participants.

(A), (B) and (C), correspond to samples E1, E3, and E9, respectively. The laboratory code numbers are indicated on the left hand side of each figure. The light grey arrows correspond to the regions sequenced by the participating laboratories and differences in sequence compared from the reference/consensus sequences are indicated by vertical colored lines within the arrows. The colors show the identity of nucleotides when there is a mismatch with the reference: A–green; C–blue; G–yellow; T–red; redundant nucleotides (N, Y, R, S, W, K, M, B, D, H, V)–black; insertions–purple. In some cases, laboratories have performed more than one method and sequenced fragment length may vary. At the top of each figure is a summary of the HEV-encoded proteins. NGS data from laboratory 21 is not included in the figure.

(27); however, these methods amplify small products and target highly conserved regions, which are only useful for discriminating HEV strains at the genotype level. The use of hemi-nested/nested primers is important in the context of sequencing to increase sensitivity because of the large number of synonymous mutations present in the HEV genome. However, this increase in sensitivity requires strict contamination control.

Different tools (see online Supplemental Table 1) were used to identify HEV genotypes and subtypes; the most frequently used were the Hepatitis E Virus Genotyping Tool—RIVM (HEVnet genotyping tool) (4) and comparison of sequences to HEV reference sequences by phylogenetic analysis (2, 3). In addition, online applications such as HEV GLUE and Genome Detective, and BLAST searching as well as in-house databases were used (see online Supplemental Table 1). The use of HEVnet and comparison with reference strain sequences enabled correct identification of the coded samples; however, there were some slight inconsistencies in results reported when using the HEVnet tool, despite sequencing of common fragments. This observation might be due to more cautious assignment of subtypes by some participants and further work is required to understand the reason for these discrepancies. In the case of sample E7 (HEV subtype 3e), laboratories just using the HEVnet tool and Cap amplification/sequencing protocols for analysis, reported the sample as HEV genotype 3. Correct identification of subtype 3e (sample E7) required the use of HEVnet in combination with further phylogenetic analysis.

Sample E4, a genotype 3f strain derived from a blood donor from Sweden, is most closely related to Swedish HEV strains from pigs including strain swX07-E1 (accession number EU360977). Recently, swX07-E1 was added to the list of subtype 3f HEV reference strains (3). The 3f HEV subtype is extremely diverse, and swX07-E1 had previously been unclassified. Analysis of sample E4 sequences using HEVnet suggests this strain belongs to HEV subtype 3l (p), although it is noted that this is provisional ("p"). However, it should be pointed out that the subtype 3l in the reference sequence annotation is distinct to the provisional 3l (p) designation in HEVnet with the latter being more closely related to 3f strains. The 3l reference strain defined by Smith et al. is more closely related to 3h, 3c and 3i (3). In contrast, in the case of sample E1, another 3f strain, all laboratories were in agreement with the assignment of this subtype. These observations emphasize the value in updating databases and sequence collections and the importance of harmonization nomenclature. A recent study of genotype 3 strains has highlighted that subtype assignment can be ambiguous with outliers being observed for most subtypes (28). This ambiguity is related, in part, to the limited availability of full-length sequences and the diversity of HEV genotype 3. The International Committee on Taxonomy of Viruses (ICTV) ratified proposals for *Hepeviridae* classification at the level of genus, species, and genotypes (1). While members of the ICTV *Hepeviridae* study group have collated lists of reference sequences for *Orthohepevirus* A subtypes, the ICTV does not usually consider classification beyond species. Nevertheless, curation of the proposed reference strains is important for phylogenetic analysis of novel HEV isolates and the list is under constant review. HEVnet is considered to be the platform to exchange the latest sequence data as well as typing and subtyping algorithms. However, it is important that the reference sequences are added to the HEVnet database, particularly when new updates are reported on the ICTV *Hepeviridae* study group website.

Knowledge of different HEV genotypes as well as the emerging identification of minor variants challenges viral subtype determination and exchange of information of latest developments is crucial for laboratories that perform virus characterization on a routine basis. Pathogenesis varies between HEV genotypes and, in the case of HEV genotype 3, hospitalized patients with hepatitis E were more likely to be infected with subtypes 3e and 3f (29, 30).

Conclusion

In this first practical study evaluating the performance of different HEV sequencing methods laboratories performed well and the results are promising. The identified gaps, including optimization of methods and contamination control, need to be considered by the individual participants to improve the standard of performance. Harmonization of curated lists of sequences and those included in online tools and genotyping/subtyping algorithms is essential going forward to improve surveillance efforts of imported and endemic HEV strains.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online

Nonstandard Abbreviations: HEV, hepatitis E virus; ECDC, European Centre for Disease Prevention and Control; RIVM, the Dutch National Institute for Public Health and the Environment; NGS, next-generation sequencing; ORF, open reading frame; Cap, capsid; RdRp, RNA-directed (dependent) RNA polymerase; Met, methyltransferase; ICTV, International Committee on Taxonomy of Viruses.

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