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EU Reference Laboratory for Public Health on Food- and Water-borne Viruses

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Document overview

Project Summary

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1. BACKGROUND

A. Purpose of the document

For hepatitis E virus (HEV), the main objective of the EURL-PH-FWDV is to harmonise genetic typing of HEV in the EU/EEA, laying the groundwork for sharing molecular information on HEV-positive samples in EU/EEA and in different geographic regions of the globe. In order to enhance the molecular surveillance of HEV for outbreak detection, it is important to improve the comparability of sequences by using standardized protocols for sequencing of HEV. It is essential to have a minimum sequence length in the same selected genome region that will allow robust comparison of HEV sequences.

This document provides information on a molecular diagnostic typing protocol for HEV, which is based on the protocols established by the reference laboratory for HEV at the Dutch National Institute of Public Health and the Environment (RIVM). The protocol is further evaluated and supported by the EURL-PH-FWDV. As part of this, the protocol is now extended with two options for the cDNA and PCR step, a) the original protocol with a two- step approach with separate cDNA and PCR step or b) a one-step RT-PCR approach for HEV genotyping. Both these options utilize the HEVNET primers.

All laboratories wishing to implement these protocols, using similar commercial kits/reagents, should always optimize and validate the test to ensure adequate specificity and sensitivity.

For any questions, feedback or additional information please contact EURL-PH-FWDV@fhi.no.

B. Laboratory practical considerations

Molecular diagnostic techniques are rapid and sensitive methods used for clinical diagnostics; however, the validation of results and biosafety of laboratory personnel depend on several practical considerations:

a) Training of personnel:

Laboratory personnel should be experienced in molecular biology techniques and familiar with protocols.

b) Biosafety:

Diagnostic laboratory work on clinical specimens from patients who are suspected of being infected with HEV should be conducted in BSL 2 containment conditions with the use of appropriate personal protective equipment (PPE).

c) Good laboratory practices:

Ensuring that the recommended reagents are used and handled properly is critical, as reactions are complex, and problems with a single reagent can have significant effect on the results.

d) Facilities and handling areas:

Specimen and reagent handling facilities with appropriate room separation for various steps of RT-PCR must be in place to prevent carryover contamination. Separate rooms include a reagent preparation area, a specimen preparation area, and an amplification/detection area. The reagent preparation area is the clean area. The specimen preparation and DNA amplification areas are successively contaminated areas. The workflow is from clean to contaminated areas.

C. Hepatitis E virus (HEV) and genome

1. Introduction

HEV is quasi-enveloped in blood and non-enveloped in faeces and belongs to the species *Paslahepevirus balayani* (genus *Paslahepevirus*, family *Hepeviridae*). HEV has a positive-polarity, single-stranded RNA that is approximately 7.2 kb in length. The genome of the reference strain used is Genbank accession NC_001434 (Table 1).

In Table 1 are described the length (nt) of the regions shown in Figure 1.

Table 1. Lengths (nt) of each HEV genomic region of the reference strain with Genbank accession NC_001434 and a total length of 7176 nt.

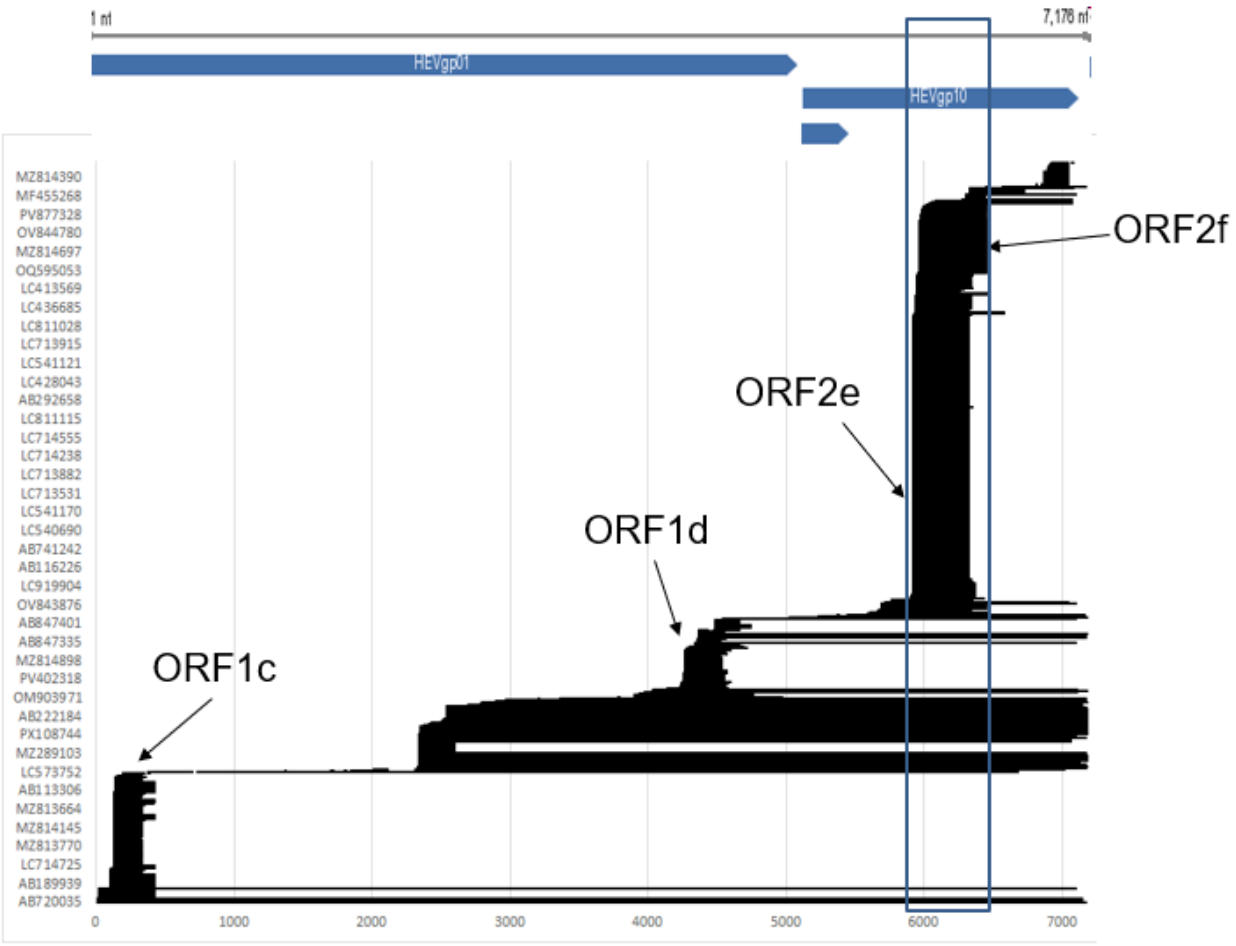
Region	Begin	End	Length
ORF1	4	5085	5082
ORF2	5123	7105	1983
ORF3	5109	5453	345

2. HEV genome regions for typing

Different regions of the HEV genome have been used to characterize HEV strains and genotypes such as ORF1c, ORF1d, ORF2e and ORF2f (see Figure 1). The latter two were used in most cases. In the current protocol, we propose to amplify and sequence a fragment containing the region ORF2e + ORF2f from nt 5885 to 6510 of the reference HEV strain with genbank NC_001434. The resulting sequence is 493 nt (between the nested primers nt 5962 to 6454).

Figure 1. Paslahepevirus balayani sequences were downloaded from Genbank on 9th April, 2026.

9739 sequences longer than 100 nucleotides were mapped to the reference sequence genome NC_001434. Query length and target start were plotted as bars.



D. Sequence-based typing methods

1. Type and quantity of human specimens use for molecular HEV detection

Conventionally, HEV diagnosis is performed using serum or plasma specimens, and for PCR alternatively stool samples can be used.

Table 2. Type and quantity of human specimens use for molecular tests

	Volume need to extract RNA (Minimum amount)	**Transport and maintenance requirements**
Serum or plasma samples	140 - 400 µl	Short term storage (<48 hours): room temperature, refrigerated or frozen. Storage longer than 48 hours: sera should be frozen (- 20°C). International shipment on dry ice.
Stool samples	0.2 ml of 10% stool suspension (0.1 g of stool in 1 ml viral transport medium)	Refrigerated

2. RNA extraction procedure

Viral nucleic acid extraction can be done using an automated system or manual extraction. For example automated MagNA Pure LC System (Roche) with Kit XY and EZ1 Advanced XL (Qiagen) with EZ1&2 Virus Mini Kit v2.0, or manual isolation with QIAamp Viral RNA Mini Kit (Qiagen) are suitable for extractions following the manufacturer's instructions.

3. RT- PCR controls

- Positive control – The positive control used for the PCRs is a sample with a reasonable amount of a virus with a previously determined unique sequence (to be able to distinguish it from test samples)
- Negative control – RNAase free H_2O

4. PCR primers

Table 3. Set of Primers for PCR (1st reaction and nested-PCR)

Name	Sequence (5' >3')	Direction	Region	PCR
HEV-orf2-fo-ch	AAYCARGGiTGGCGYTCiGTiGARAC	F	ORF2	forward-outer
HEV-orf2-ro-ch	GARAAiGGRCGiGAiGGRGCiGG	R	ORF2	reverse-outer
HEV-orf2-fi-ch	GAGGAGGAAGCTACCTCYGGYYTiGTiATGCTYTGYAT	F	ORF2	forward-inner
HEV-orf2-ri-ch	GGAGAAGGAGTTGGTCGRTCYTGYTCRTGYTGRTT	R	ORF2	reverse-inner

i = inosine; Y = C and T; R = A and G; W = A and T; S = C and G; M = A and C; K = G and T; N = A, C, G, and T

2. HEV genotyping protocols

Below are two alternative protocols; a Two-step RT-PCR or a One-step RT-PCR approach. The Two-step protocol includes a separate step for cDNA synthesis, which can be stored for later use if necessary. The one step protocol does not include a separate cDNA synthesis step and therefore requires less time.

2a. HEV amplification by Two-step RT-PCR

The following protocols are for genotyping HEV using a conventional RT-PCR, and sequencing.

1. Reagents and equipment:

- 5X RT buffer Superscript III, Invitrogen
- PCR Nucleotide Mix 10mM, Roche
- Set of Primers for PCRs
- Superscript III RT 10000U (200U/µl), Invitrogen
- RNaseOUT 5000U (40 U/µl), Invitrogen·
- MDTT, Invitrogen
- Taq polymerase buffer (1271318) Roche
- 10X Faststart Taq polymerase buffer (2161567) Roche·
- Taq Polymerase 5 U/µL
- Faststart Taq Polymerase 5 U/µL·

- Agarose
- GelRed dye
- 5x TBE
- ExoSAP-IT (USB)Set of Primers for sequencing
- Big Dye Terminator v1.1 or 3.1 (BDT), Applied Biosystems
- Sequence buffer (Big Dye Buffer), Applied Biosystems
- Thermocycler PCR/sequence PCR
- Perkin Elmer PE- 9700 Thermal Cycler

1. Procedure

2.1 cDNA synthesis (RT- step)

1. Prepare cDNA master mixture (Table 4)
2. Add 5 µl of the RNA specimen to the 5 µl cDNA master mix (final volume = 10 µl)
3. Incubate according thermal conditions (Table 5)
4. Prepare the PCR master mix while cDNA reactions are incubating OR cDNA can be stored at −20°C for future use.

Table 4. RT-master mix for cDNA

Reagent	Stock	Final concentration	Volume of reagent add per reaction(µl)
5x RT Buffer	5x	1x	2
dNTPs	10 mM	1mM	1
HEV-orf2-ro-ch primer	100 µM	5µM	0.5
DTT	0.1M	5mM	0.5
RNaseOUT	40 U/ ul	2 U/ ul	0.5
Superscript III RT	200 U/ ul	10 U/ ul	0.5
Final mix volume			5

Table 5. Thermal conditions

Temperature (ºC)	Time (minute)
Room Temperature = 22	10
42	60
95	5

2.2 Perform conventional PCR reactions

Reaction set-up:

1. Prepare the master mix (1st reaction) as in Table 6.
2. Add 40 µl of master mix to the cDNA tube (10 µl).
3. Mix and briefly centrifuge the tubes.
4. Run the thermal cycler program (Table 7):

Table 6. PCR master mixture (1st reaction)

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
10x PCR Buffer (Taq pol 271318)	10x	1x	5
dNTPs	10 mM	0.2 mM	1
HEV-orf2-fo-ch primer	10 µM	1 µM	5
Taq polymerase	5 U/µl	0.05 U/ µl	0.5
H_2O (RNase and DNase free)			28.5
Final mix volume			40

Table 7. Thermal cycler program 1st reaction

Step	Temperature (ºC)	Time (min/sec)
Hold	95	6 min
PCR (35 cycles)	95	30
	42	30
	60	45
Cooling	4	∞

Note we use the "9600" configuration on the Perkin Elmer PE- 9700 Thermal Cycler
Cooling and or heating rate may need to be adjusted dependent on the apparatus.
Too slow may reduce specificity and cause byproducts, too fast may reduce sensitivity.

Prepare the master mixture for Nested-PCR (2nd reaction)

Reaction set-up:

1. Prepare the nested- PCR master mix (Table 8). This may be prepared in a larger batch that can be aliquoted and frozen at -20 °C for later use.
2. Add 1 µl of the PCR product (1st reaction) to the 49 µl nested-PCR master mix.
3. Mix and briefly centrifuge the tubes.
4. Run the thermal cycler program (table 9):

Table 8. Nested-PCR master mixture (2nd reaction)

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
10x PCR Buffer (FS Taq 2161567)	10x	1x	5
dNTPs	10 mM	0.2 mM	1
HEV-orf2-fi-ch primer	10 µM	0.8 µM	4
HEV-orf2-ri-ch primer	10 µM	0.8 µM	4
FS Taq polymerase	5 U/µl	0.05 U/ µl	0.5
H_2O (RNase and DNase free)			34.5
Final mix volume			49

Table 9. Thermal cycler program 2nd reaction

Step	Temperature (ºC)	Time (min/sec)
Hold	95	6 min
PCR (40 cycles)	95	30 sec
	60	20 sec
	72	15 sec
Final extension	72	7 min
Cooling	4	∞

2b. HEV amplification by One-step RT-PCR

1. Reagents and equipment

- SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen), included in the kit
  - 2X RT-mix
- Platinum Taq HiFi, Invitrogen
  - Platinum Taq 10X PCR buffer,
  - 25mM dNTP
  - 50mM $MgSO_4$
- Set of Primers for PCRs (see Table 3)
- 0.2 ml PCR tubes
- Mini centrifuge
- Vortex
- Thermocycler PCR machine

2. Procedure

2.1 One-step RT-PCR reaction setup (1st reaction)

Reaction set-up

1. Prepare One-step RT-PCR master mixture (Table 10)
2. Add 5µl of the RNA specimen to the 20 µl RT-PCR master mix (final volume =25µl)
3. Incubate according to the thermal conditions (Table 11)

Table 10. One-step RT-PCR master mix

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
2x RT-mix	2x	1x	12,5
HEV-orf2-fo-ch	25 µM	0,5 µM	0,5
HEV-orf2-ro-ch	25 µM	0,5 µM	0,5
SuperScript III RT Platinum Taq polymerase	100x	1x	1
H_2O (RNAase and DNase free)			5.5
Final mix volume			20

Table 11. Thermal cycler program for One-step PCR

Step	Temperature (ºC)	Time (min/sec)
Pre-incubation	22	10 min
RT-reaction	42	60 min
Denaturation	94	2 min
PCR (35 cycles)	94	15 sec
	42	30 sec
	68	30 sec
Final extension	68	5 min
Cooling	4	∞

2.2 Nested PCR (2nd reaction)

Reaction set-up

1. Prepare the nested- PCR 2.5x master mix (Tabel 12). This may be prepared in a larger batch and frozen at -20C for later use.
2. Prepare the PCR-reaction mix (Table 13)
3. Add 1µl of the PCR product (1st reaction) to the 24 µl nested-PCR rection mix.
4. Mix and briefly centrifuge the tubes.
5. Incubate according to thermal conditions shown below (Table 14)

Table 12: 2.5X Master Mix (Nested PCR)

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
Platimun Taq 10X PCR buffer	10X		2.5
dNTP	25 mM	0.5 mM	0.2
$MgSO_4$	50 mM	6.25 mM	1.25
HEV-orf2-fi-ch	25 µM	2 µM	0.8
HEV-orf2-ri-ch	25 µM	2 µM	0.8
H_2O (RNAse and DNAase free)			4,45
Final mix volume			10

Table 13: Nested PCR master mix

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
2.5X MasterMix (Nested PCR)	2.5	1x	10
Platinum Taq HiFi	5 U/ µl	0,02 IU/ul	0.1
H_2O (RNAase and DNAase free)			13.9
Final mix volume			24

2nd reaction thermal cycler program

Table 14: Thermal cycle program (Nested PCR)

Step	Temperature (ºC)	Time (sec)
Denaturation	94	120
PCR (40 cycles)	94	15
	60	15
	68	30
Final extension	68	120
Cooling	4	∞

3. Product analysis

Run the PCR product for each sample on 1.5% agarose gel.

Expected product size is 566 bp (ORF2), same size as the positive HEV control.

1. Product clean-up

It is necessary to remove RT-PCR component reagents prior to gene sequencing. Use the ExoSAP- IT Kit or NucleoFast® 96 PCR Clean-up kit (Macherey-Nagel) and follow the manufacturer's instructions.

2. Gene Sequencing

Table 15. Set of primers for sequencing

Name	Sequence (5' >3')	Direction	Region	PCR
HEV-orf2-fs	GAGGAGGAAGCTACCTC	F	ORF2	sequence
HEV-orf2-rs	GGAGAAGGAGTTGGTCG	R	ORF2	sequence

2.1 Reaction set-up for sequencing

1. Prepare the master mix using ABI BigDye Terminator v1.1 or 3.1 Cycle Sequencing kits, Applied Biosystems (Table 16), two for each sample using either forward or reverse primer.
2. Add 2 µl of clean PCR product to master mix.
3. Mix and briefly centrifuge the tubes.
4. Run the thermal cycler program (Table 17)

Table 16. Sequencing master mix

Reagent	Final amount	Volume of reagent add per reaction (µl)
H_2O (RNase and DNase free)		8.75
Big Dye Buffer		7
Big Dye		1.25
Primer F or Primer R	3.2 µM	1
Final mix volume		18

Table 17. Thermal cycle program

Step	Temperature (C)	Time (sec)
Denaturation	95	10
PCR (25 cycles)	95	10
	50	5
	60	240
Cooling	4	∞

1.2 Purify the sequencing reaction before capillary electrophoresis and do sequence analysis with bionumerics or other software.

3. Sequence analysis

Each Forward sequence should be aligned with correspondent Reverse sequence using appropriate software and use HEV-typing tool for typing. Before submission to the database, the codehop primer (HEV-orf2-fi-ch and HEV-orf2-ri-ch) sequences at both ends of the fragment from the consensus sequence must be removed (final sequence 493 bp).