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

Grant Agreement Number: 101246587

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

Project Acronym: EURL-PH-FWDV

EU4Health Programme

Deliverable: D2.1 – Laboratory protocol for molecular typing of HAV using Sanger sequencing

WP2 – HAV diagnostics and technical support

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Document overview

Project Summary

Project acronym	EURL-PH-FWDV
Grant Agreement	101246587
Project Title	EU Reference Laboratory for public health on Food- and water-borne viruses
Webpage	https://www.fhi.no/en/hd/laboratory-analysis/projects/the-european-reference-laboratory-for-public-health-on-food--and-water-borne-viruses-eurl-ph-fwdv/
Call	EU4H-2024-DGA-MS-IBA-03
Start date	01/01/2026
Duration	84 months
Coordinator	NIPH

Document Control

Work Package	**2**
Deliverable Related No	
Deliverable No	**D2.1**
Deliverable Name	**Laboratory protocol for molecular typing of HAV using Sanger sequencing**
Lead Beneficiary	**RIVM**
Type of document	**Report (R)**
Dissemination level	**Public (PU)**
Due date (DoA)	**31.03.2026**
Delivery date	**27.03.2026**
Version	**1.0**
The deliverable agreed with ECDC *(applicable to deliverables which must be 'agreed with' ECDC, as per GA)*	**Not applicable**

Contributors

Contributors	Name	Organisation	Role in EURL
Lead Beneficiary	Harry Vennema	RIVM	WP2-leader, management team
Contributing author(s)	Sheba Lothe	NIPH	Lab coordinator, management team
Contributing author(s)	Garth Daryl Tylden	NIPH	Senior expert, management team
Reviewer(s)	Kathrine Stene-Johansen	NIPH	Project leader, management team
Reviewer(s)	Jon Bråte	NIPH	WP5-leader, management team

Document Review History

Version	Release Date	Reason for change	Status (Draft/In-review /Submitted)
V1.0			

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1. BACKGROUND

A. Purpose of the document

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

This document provides information on a molecular diagnostic typing protocol for HAV, which is based on the protocols established by the reference laboratory for hepatitis A 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 HAV genotyping.

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 HAV should be conducted in BSL 2 containment conditions with the use of appropriate personal protective equipment (PPE). Personnel should be vaccinated against hepatitis A.

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 a amplification/detection area. The reagent preparation area is the clean area, and the specimen preparation- and DNA amplification areas are successively contaminated areas. The workflow is from clean to contaminated areas.

C. Hepatitis A virus (HAV) and genome

1. Introduction

HAV is a non-enveloped virus that belongs to the genus Hepatovirus, Picornaviridae family. HAV has a positive-polarity, single-stranded RNA that is approximately 7.5 kb in length (Nainan O et al., 2006). The genome of the reference strain with Genbank accession NC_001489 consists of a 5' untranslated region (UTR) of 734 to 740 nucleotides, a coding region of 6,678 to 6,684 nucleotides (regions P1, P2 and P3,) and a 3' untranslated region of 60 nucleotides (excluding the poly-A tail). The P1 region encodes the three major proteins of the viral capsid, VP1, VP2, and VP3. A fourth viral capsid protein (VP4), essential for virion formation, is not detected in mature viral particles. Each of the capsid proteins is cleaved from the precursor polyprotein by the viral protease 3C, encoded in the P3 region. The native conformation of the capsid proteins VP1 and VP3 forms a single, dominant, serologic epitope on the viral capsid and elicits a neutralizing antibody response. Nonstructural proteins encoded in the P2 and P3 regions are involved in host-cell manipulation, RNA synthesis and virion formation. VPg (virion protein, genome linked), also encoded in the P3 region, is covalently linked to the 5' genome terminus and is involved in initiation of RNA synthesis (Nainan O. et al, 2006) (figure 1).

In table 1 are described the length (nt) of each region showed in the figure 1.

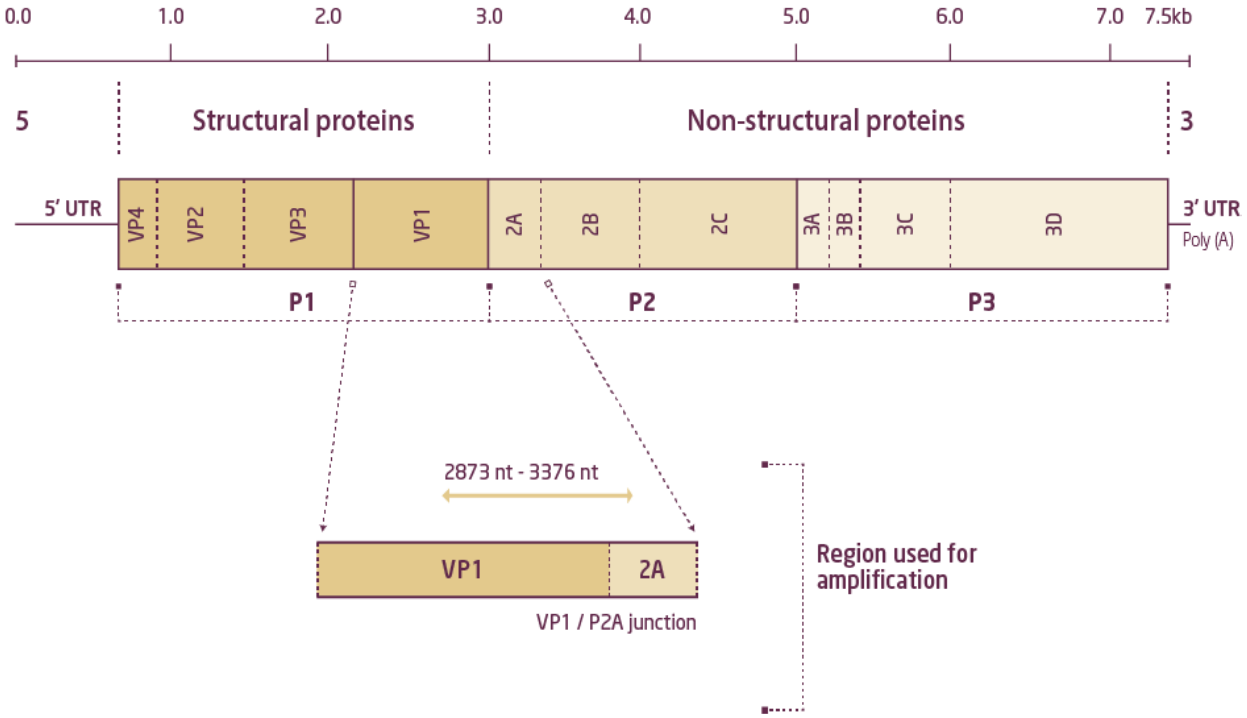

Figure 1. Hepatitis A genome structure. Image source: Nainan et al., Clin Microbiol Rev. 2006 Jan;19(1):63-79.

Table 1: Lengths (nt) of each HAV genomic region

Region		Length	Nucleotides
	5´UTR	734 (741)*	1-734
P1			
	VP4	62 - 68	735 - 803
	VP2	665	804 - 1469
	VP3	737	1470 - 2207
	VP1	900	2208 - 3107
P2			
	2A	567	3108 - 3674
	2B	312	3675.-.3995
	2C	1004	3996 - 5000
P3			
	3A	221	5001 - 5222
	VPg	68	5223 - 5291
	3C	656	5292 - 5948
	3D	1466	5949 - 7415
3’ UTR		62	7416 - 7478

*Downstream AUG codon at nt 741 to 743 preferred for translation initiation.

2. HAV genome regions for typing

Different regions of the HAV genome have been used to characterize HAV strains and genotypes such as the C terminus of the VP3 region; the N terminus of the VP1 region; junction of the VP1/P2A regions; VP1- P2B regions and the entire VP1 region. However, no region has proved to be the best region to genotype HAV and to differentiate variants. The region selected (VP1/2A junction) to use in this protocol to type HAV strains was chosen because there is already a significant amount of information regarding sequences typed in this region which will allow us to improve our sequence comparison.

The protocol described here for typing HAV strains amplify a 503 nt fragment spanning the VP1/2A junction region that ranges from nt. 2873 to nt. 3376 (figure 1); Reference the HAV strain, genbank NC_001489. The resulting sequence is 460 nt without the primers.

D. Sequence-based typing methods

1. Type and quantity of human specimens used for molecular HAV detection

Conventionally, HAV diagnosis is performed using sera/plasma specimens, but stools or dried blood spots can also be used (Table 2; Desbois et al., 2009).

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

	Volume required for RNA extraction (Minimum amount)	Transport and maintenance requirements
Serum or plasma samples	140-400 µl	Short term storage (<48 hours): room temperature or refrigerated or frozen. Storage longer than 48 hours: sera should be frozen (- 20ºC). International shipment on dry – ice.
Stools	10% stool suspension of 1 g of stool in 10ml viral transport medium	Refrigerated
Dried Blood spots	25 µl serum spotted on filter paper (1.1 cm diameter)	Room temperature

2. RNA extraction procedure

Viral nucleic acid extraction can be done using an automated system or manual extraction. RNA isolation Kit using automated MagNA Pure LC System (Roche), or manual QIAamp Viral RNA Mini Kit (Qiagen, Germany) are suitable for extractions following the manufacturer's instructions.

3. RT-PCR controls

- Positive control – The positive control used for the PCRs, is a tissue culture stock of 1* 107 TCID 50 /ml or a previously well-defined and characterized patient sample
- Negative control – RNAase free H_2O

4. PCR Primers

Table 3. PCR Primers used in both one-step and two-step RT-PCR protocols

Name	Sequence (5' >3')	Direction	Region	PCR
HAV 6.1 codehop	TATGCYGT5TCWGG5GC5YTRGAYGG	F	VP1-2a	First-outer
HAV 10 codehop	TCYTTCATYTCWGTCCAYTTYTCATCATT	R	VP1-2a	First-outer
HAV 8.2 codehop	GGATTGGTTTCCATTCARATTGCNAAYTA	F	VP1-2a	nested-inner
HAV 11 codehop	CTGCCAGTCAGAACTCCRGCWTCCATYTC	R	VP1-2a	nested-inner

number "5"on sequence oligo´s means inosine (number depends on supplier); Y = C/T; W = A/T;
R = A/G;N = any base

2. HAV 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 and used later if necessary. The one step protocol does not include a separate cDNA synthesis step and therefore requires less time.

2a. HAV genotyping by Two-step RT-PCR

The following protocols are for genotyping HAV 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·
- M DTT, 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 v3.1 (BDT), Applied Biosystems
- Sequence buffer (Big Dye Buffer), Applied Biosystems
- Thermocycler PCR/sequence PCR
- Perkin Elmer PE- 9700 Thermal Cycler

2. Procedure

2.1 cDNA synthesis (RT- step)

1. Prepare cDNA master mixture
2. Add 5 µl of the RNA specimen to the 5 µl cDNA master mix (final volume = 10 µl)
3. Incubate according to thermal conditions (Table 5):

Table 4. Master mix

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
5x RT Buffer	5x	1x	2
dNTps	10 mM	1mM	1
HAV10 codehop primer inner	100 µM	5 µM	0.5
DTT	0.1M	5 mM	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

4. Prepare the PCR master mix while cDNA reactions are incubating OR cDNA can be stored at −20°C for further use.

2.2 Perform conventional PCR reactions

Prepare the PCR master mix (1st reaction)

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
HAV 6.1 codehop 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

Reaction set-up

1. Prepare the master mix.
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:

1st reaction thermal cycler program

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	∞

Prepare the PCR master mix for nested-PCR (2nd reaction)

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
HAV 8.2 codehop primer	10 µM	0.8 µM	4
HAV 11 codehop 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

Reaction set-up

1. Prepare the nested- PCR master mix.
2. Add 1µl of the PCR product (1st reaction) to the 49 µl nested-PCR master mix.
3. Mix and briefly centrifuge the tubes.
1. Run the thermal cycler program:

2nd reaction thermal cycler program

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
Cooling	4	∞

2b. HAV genotyping 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 RT-PCR Reaction setup (1st reaction)

Reaction set-up

1. Prepare RT-PCR master mixture
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
HAV 6.1 codehop primer	25 µM	0,5 µM	0,5
HAV 10 codehop primer	25 µM	0,5 µM	0,5
SuperScript III RT Platinum Taq polymerase	100x	1x	1
H_2O (RNAase and DNAase free)			5.5
Final mix volume			20

Table 11. Thermal cycler program 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)

Prepare the nested PCR master mix

Reaction set-up

1. Prepare the nested- PCR master mix.
2. Add 1µl of the PCR product (1st reaction) to the 24 µl nested-PCR master mix.
3. Mix and briefly centrifuge the tubes.
4. Incubate according to thermal conditions shown below (Table 14)

Table 12: Nested PCR master mix

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
2.5X MasterMix (Nested PCR) (seeTable 13)	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

Table 13: 2.5X MasterMix (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
HAV 11 Codehop primer	25 µM	2 µM	0.8
HAV 8.2 Codehop primer	25 µM	2 µM	0.8
H_2O (RNAse and DNAase free)			4,45
Final mix volume			10

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 10 µl each sample on 1.5% agarose gel made up with1X TBE buffer and containing GelRed dye according to manufacturer's instructions. Expected product size is 520 bp (VP1/2a), same size as the positive HAV control.

1. Product clean-up

It is necessary to remove RT-PCR component reagents prior to gene sequencing. Use the ExoSAP- IT Kit and follow the manufacturer's instructions.

2. Gene Sequencing

Table 15. Set of primers for sequencing

Name	Sequence (5' >3')	Direction	Region	PCR
HAV 8.2 sequence	GGATTGGTTTCCATTCA	F	VP1-2a	sequence
HAV 11 sequence	CTGCCAGTCAGAACTCC	R	VP1-2a	sequence

2.1 Prepare the master mix for sequencing

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 1 or Primer 2	3.2 µM	1
Final mix volume		18

2.2 Reaction set-up

1. Prepare the master mix using ABI BigDye Terminator v1.1 or v3.1 Cycle Sequencing kits, Applied Biosystems.
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. Thermal cycle program

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

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

Alignment

Each Forward sequence should be aligned with correspondent Reverse sequence using appropriate software.

Submission

Before submission to the database you should remove the codehop primers sequence in both ends of the fragment from the consensus sequence (final sequence 460 bp).

4. References:

HAV Genome characterization

Cohen,J.I., Ticehurst,J.R., Purcell,R.H., Buckler-White,A. and Baroudy,B.M. Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. J. Virol. 61 (1), 50-59 (1987).

Nainan OV, Xia G, Vaughan G, Margolis HS. Diagnosis of hepatitis a virus infection: a molecular approach. Clin Microbiol Rev. 2006 Jan;19(1):63-79.

Test specimens

Desbois D, Roque-Afonso AM, Lebraud P, Dussaix E.Use of dried serum spots for serological and molecular detection of hepatitis a virus. J Clin Microbiol. 2009 May;47(5):1536-42. doi: 10.1128/JCM.02191-08. Epub 2009 Mar 25.

Typing PCR

Stene-Johansen K, Tjon G, Schreier E, Bremer V, et al. Molecular epidemiological studies show that hepatitis A virus is endemic among active homosexual men in Europe.J Med Virol. 2007 Apr;79(4):356-65.