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Hepatitis A Virus

Molecular Detection and Typing

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1. Introduction

Hepatitis A virus (HAV) infection is the leading cause of viral hepatitis throughout the world ***(1)***. HAV infection is mainly propagated via the fecal-oral route ***(2)***, and waterborne ***(3)*** and foodborne ***(4–8)*** outbreaks of the disease have been reported.

HAV, the prototype of the genus *Hepatovirus*, belongs to the family Picornaviridae. Its 7.5-kb single-stranded RNA genome bears different distinct regions: the 5' and 3' noncoding regions (NCR), the P1 region, which encodes the structural proteins VP1, VP2, VP3, and a putative VP4, and the P2 and P3 regions encoding nonstructural proteins associated with replication ***(9)***. A single HAV serotype has been described, although seven genotypes have been defined ***(9)***.

Since environmental samples usually contain low numbers of viral particles, sensitive methods such as molecular techniques based on nucleic acid amplification are required for their detection. However, even with the adoption of these techniques, the choice of the most adequate target is of relevant importance. The target region should be highly conserved, to increase the chance of detection, and should have an appropriate structure and length to allow sensitivity high enough for these kind of samples. As a target region, we have chosen a fragment of the 5'NCR flanked by highly conserved sequences that have been used for the primer design (forward primer from position 68 to position 85; reverse primer from position 222 to position 240 in the HM175 strain of HAV; GenBank accession number M14707) ***(4)***. The internal part of this region, however, may present a certain degree of variation mainly owing to insertions and/or deletions, causing a variable size of the amplimer obtained, i.e., the wild-type HM175 strain gives a size of 174 bp whereas the cell-adapted pHM175 strain gives a size of 186 bp. For this reason it is extremely important to include a confirmative method such as Southern blot hybridization with an internal probe from a region not affected by the insertions/deletions ***(4)***.

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When genotyping is the objective of the study, analysis of the sequence corresponding to the VP1X2A junction is the method of choice ***(10,11)***, allowing HAV isolates to be differentiated into seven genotypes ***(10)***. It should be pointed out that although the sequence employed aligns with the VP1X2A junction region used for genotyping purposes in other picornaviruses, such as poliovirus ***(10)***, in the case of HAV (position 3024–3191 of the HM175 strain) it actually represents a 2A sequence, since it contains only 1 codon of VP1 and 55 of 2A ***(12,13)***. This method consists of the amplification of a 360-bp fragment (from position 2949 to position 3308) that includes the previously mentioned genotyping region. The size of the amplimer (360 bp) induces a loss of detection sensitivity, which is partially overcome by the application of nested polymerase chain reaction (PCR) procedures. However, the use of nested PCR techniques may introduce important cross-contamination problems since the first amplimer product should be further manipulated into a second reaction. Consequently, there is a growing tendency to avoid nested reactions as much as possible in diagnostic laboratories. This is the reason why we have adopted amplification of a shorter fragment of the 5'NCR for the generic detection of HAV. HAV genotyping is subsequently performed on those samples that are clearly positive, by the 5'NCR method, in a single PCR reaction using the previously mentioned 360-bp fragment.

All nucleic acid polymerasing reactions are susceptible to inhibitors, and reverse transcriptase (RT) is especially sensitive to inhibitory substances, which may be found in water samples. In environmental studies, a water concentration step is frequently required to reduce the sampled volume to an amount able to be analyzed in the laboratory. Unfortunately, substances inhibitory to RT-PCR are concentrated along with the viruses. Preconditioning of the sample should then be performed prior to the molecular amplification. In the present work, the method of choice for this purpose has been lyophilization, which efficiently removes several volatile inhibitors and at the same time allows viral concentration ***(14)***.

2. Materials

2.1. Sample Preconditioning

1. Plastic containers.
2. Autoclaved (121°C for 45 min) molecular biology grade distilled water (conductance 2×10^{-6} ohm^{-1} cm^{-1}; Panreac, Barcelona, Spain; *see* **Notes 1** and **2**).
3. Freeze Drying Bench Top 3 from Virtis (Gardiner, NY) or similar.

2.2. RNA Extraction (see Note 3)

1. Washing buffer (L2): add 120 g of guanidinium isothiocyanate (Applichem, Darmstadt, Germany) to 100 mL of 0.1 *M* Tris-HCl, pH 6.4. Heat at 56°C to dissolve. Store in aliquots in the dark at room temperature. This solution is stable for 3 wk.
2. Lysis buffer (L6): add to 200 mL of L2 washing buffer 22 mL of 0.2 *M* EDTA, pH 8.0, and 2.44 mL Triton X-100. Store in aliquots in the dark at room temperature. This solution is stable for 3 wk.
3. Silica solution:
   a. Add 60 g of silicon dioxide (Sigma, St. Louis, MO) to 500 mL of autoclaved (121°C for 45 min) distilled H_2O in a 500 mL RNase-free bottle and keep it for 24 h at room temperature.

b. Aspirate 430 mL of the supernatant and resuspend the pellet in 500 mL of autoclaved (121°C for 45 min) distilled H_2O.
c. Allow to stand for 5 h at room temperature and aspirate 450 mL of the supernatant.
d. Add 600 µL of 8.7 *M* HCl to this supernatant and distribute it in aliquots of 0.5 mL before autoclaving at 121°C for 20 min.
e. Store in the dark at room temperature.
f. This solution is stable for 6 mo.

4. 70% Ethanol (*see* **Note 4**).
5. 100% Acetone.
6. TE buffer, pH 8: 10 m*M* Tris-HCl and 1 m*M* EDTA. Adjust the pH to 8.0. Aliquots are distributed in 1.5-mL tubes and autoclaved at 121°C for 45 min.
7. Vortex.
8. Microcentrifuge.
9. Heating block.
10. 1.5-mL Microcentrifuge tubes.
11. Tips with filter.
12. Gloves.

2.3. RT

1. Murine Moloney leukemia virus (M-MLV) reverse transcriptase RNase H Minus (Promega, Madison, WI) supplied with 5X RT buffer (250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$, 50 m*M* dithiothreitol).
2. Deoxynucleotide triphosphates (dNTPs) (Promega).
3. Reverse primers (HAV240: 5'-241GGAGAGCCCTGGAAGAAAGA222-3' and VP1-3285: 5'-3285AGTCACACCTCTCCAGGAAAACTT3308-3').
4. Autoclaved (121°C for 45 min) distilled H_2O.
5. Thermocycler AB 2700 (Applied Biosystems, Foster City, CA) or similar.
6. Micropipets and filter tips.
7. 0.2-mL PCR tubes.

2.4. PCR

1. Expand High Fidelity PCR enzyme (Roche, Mannheim, Germany) supplied with 10X Expand High Fidelity buffer and supplemented with 15 m*M* $MgCl_2$.
2. Deoxynucleotide triphosphates (dNTPs).
3. Reverse (HAV240: 5'-241GGAGAGCCCTGGAAGAAAGA222-3' and VP1-3285: 5'-3285AGTCACACCTCTCCAGGAAAACTT3308-3') and forward (HAV68: 5'-68TCACCGCCGTTTGCCTAG85-3' and VP1-2949: 5'-2949TATTTGTCTGTCA CAGAACAATCAG2973-3') primers.
4. Autoclaved (121°C for 45 min) distilled H_2O.
5. Thermocycler AB 2700 or similar.
6. Micropipets and filter tips.
7. 0.2-mL PCR tubes.

2.5. Electrophoresis

1. 10X TBE buffer: 0.9 *M* Tris-HCl, pH 8.3, 0.9 *M* boric acid, 0.02 *M* EDTA. Stable for long periods.
2. 10X TAE buffer: 0.4 *M* Tris-HCl, pH 8.3, 0.11 *M* acetic acid, 0.01M EDTA. Stable for long periods.

3. Seakem LE agarose (BioWhittaker, Walkersville, MD).
4. GelStar Nucleic Acid Gel Stain (BioWhittaker).
5. 6X loading buffer: 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue (Sigma) in distilled water. Store at 4ºC. Stable for long periods.
6. Prepare a working solution of the DNA molecular weight marker IX (72–1353 bp) (Roche) by mixing 10 µL of marker with 73.40 µL of TBE or TAE, and 16.6 µL of 6X loading buffer.
7. Horizontal gel tank, gel mold, gel combs (Bio-Rad, Hercules, CA) and power pack (Amersham Pharmacia Biotech Europe, Freiburg, Germany), or similar.
8. ImageMaster VDS gel imaging system analyzer (broad band UV 260–400 nm, peak at 312 nm; Amersham Pharmacia Biotech Europe) or similar.

2.6. Southern Blot

1. Positively charged nylon membrane (Roche).
2. Whatman 3MM paper.
3. Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) or similar.
4. TBE buffer.
5. 150 m*M* NaOH. Stable for long periods.
6. 20% sodium dodecyl sulfate (SDS). Stable for long periods.
7. 20X SSC buffer: 3 *M* NaCl, 300 m*M* sodium citrate, pH 7.0. Autoclave at 121ºC for 20 min (*see* **Note 5**). Stable for long periods.
8. Digoxigenin (DIG)-labeled probe (5'-150TTAATTCCTGCAGGTTCAGG169-3').
9. Standard solution: 5X SSC buffer supplemented with 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent (Roche). Heat to 60°C to dissolve the detergents and the blocking reagent. Dispense in 10-mL aliquots and store at –20°C.
10. Prehybridization solution: standard solution supplemented with 100 µg/mL salmon sperm DNA. To denature the salmon DNA, boil a 100X solution before being added to the standard solution (*see* **Note 6**).
11. Hybridization solution: DIG-labeled probe diluted in prehybridization solution.
12. Buffer I: 100 m*M* maleic acid, 150 m*M* NaCl, pH 7.5 (*see* **Note 7**). Autoclave at 121°C for 20 min. Stable for long periods.
13. Blocking solution: add 1 g of blocking reagent to 100 mL of buffer I. Autoclave at 121°C for 20 min, store at 4°C, and open it carefully in sterile conditions. Stable for 2 mo.
14. Anti-DIG alkaline phosphatase-labeled antibody (anti-DIG-AP) (0.75 U/µL; Roche).
15. Washing buffer: add 0.3 mL of Tween-20 to 100 mL of buffer I. Store at 4°C. Stable for long periods.
16. Phosphatase buffer: 100 m*M* Tris-HCl and 100 m*M* NaCl, pH 9.5. Store at 4°C. Stable for long periods.
17. 25 m*M* CSPD solution (Roche). Store in the dark.
18. Fixative solution: dilute 1:5 the fixative G350 (AGFA-Gevaert, Mortsel, Belgium). Store in the dark. Stable for several months.
19. Developer solution: dilute 1:5 the developer G150 (AGFA). Store in the dark. Stable for several months.
20. Film: AGFA curix RP2 100NIF 13 × 18.
21. Autoradiographic cassette (Gevamatic AGFA 18 × 24).
22. Plastic bags.
23. Trays.
24. Forceps.

25. Aluminum paper.
26. Plastic bag sealer.
27. UV lamp.
28. Water bath: Certomat (22–100 ± 0.5°C; Braun Biotech, Melsungen, Germany) or similar.
29. Vacuum oven.
30. Belly Dancer.
31. Dark room.

2.7. DNA Purification

2.7.1. Purification of an Amplimer From Single Band-Containing Solutions

1. High Pure PCR Product Purification Kit (Roche).
2. 96° Ethanol.
3. Standard tabletop centrifuge.
4. Microcentrifuge tubes.

2.7.2. Purification of an Amplimer From Mixed Band-Containing Solutions

1. Electrophoresis material.
2. Scalpel.
3. Heating block.
4. High Pure PCR Product Purification Kit (Roche).
5. 96° Ethanol.
6. Isopropanol.
7. Standard tabletop centrifuge.
8. Microcentrifuge tubes.

2.8. DNA Sequencing

1. ABI PRISM BigDye Terminator Cycle Sequencing ready Reaction Kit (Applied Biosystems).
2. Primer VP1-2949 (5'-2949TATTTGTCTGTCACAGAACAATCAG2973-3')
3. Autoclaved (121°C for 45 min) distilled H_2O.
4. 95° Ethanol ACS for analysis (Carlo Erba Reagenti, Italy).
5. 70% Ethanol.
6. Thermocycler AB 2700 (Applied Biosystems) or similar.
7. ABI PRISM 3700 DNA Analyzer (Applied Biosystems) or similar.
8. 0.2-mL Tubes.
9. Vortex.
10. Microcentrifuge.

3. Methods

3.1. Sample Preconditioning (see Note 8)

1. Collect 50 mL of sewage or wastewater in a plastic container.
2. Lyophilize for 48 h at –70ºC temperature and 30–40 mtorr pressure.
3. Resuspend the solid pellet in 500 µL of water.

3.2. RNA Extraction Procedure Based on the Boom Method (15) (see Note 9)

1. Add 40 µL of silica solution to 900 µL of L6 buffer in a 1.5-mL microcentrifuge tube (*see* **Notes 10** and **11**).

2. Add 50 µL of sample.
3. Vortex the mixture for 5 s, keep it at room temperature for 10 min, and mix again for 5 s.
4. Centrifuge at 12,000*g* for 15 s and discard the supernatant (*see* **Notes 10** and **13**).
5. Wash the silica pellet twice with 900 µL of L2 buffer, twice with 900 µL of 70% ethanol, and once with 900 µL of acetone. Centrifuge for 15 s at 12,000*g* after each wash and discard the supernatants (*see* **Notes 10**, **12**, and **13**).
6. Place the tube, with the lid open, at 56°C in a dry heating block for 10 min.
7. Add 50 µL of TE buffer, vortex, and incubate for 10 min at 56°C with the lid closed (*see* **Notes 10** and **12**).
8. Vortex and centrifuge the tube at 12,000*g* for 2 min.
9. Recover the nucleic acid-containing supernatant in a new tube (*see* **Notes 13** and **14**) and store it at 4°C (*see* **Note 15**).

3.3. Two-Step RT-PCR for the Amplification of a Fragment of the 5′NCR and Confirmation by Southern Blot for the Generic HAV Detection (see Note 16)

3.3.1. RT

1. Add 10 µL of the extracted RNA to a 0.2-mL PCR tube.
2. Incubate the tube at 99°C for 5 min to denature the RNA.
3. Chill the tube on ice.
4. Prepare the RT mix (volume for one sample): 5.000 µL 5X RT buffer; 0.125 µL 100 µ*M* primer HAV 240; 2.500 µL 2 m*M* dNTPs; 0.040 µL M-MLV reverse trascriptase (200 U/µL); 7.300 µL Distilled H_2O; for a total of 15.000 µL.
5. Add the 15 µL of the RT mix to each tube containing denatured RNA.
6. Incubate the tubes at 45°C for 1 h.
7. Keep the tubes at 4°C.

3.3.2. PCR

1. Add 29.5 µL of distilled H_2O to a 0.2-mL PCR tube.
2. Prepare the PCR mix (volume for one sample): 5.00 µL 10X Expand High Fidelity-$MgCl_2$ buffer; 0.25 µL 100 µ*M* primer HAV 240; 0.25 µL 100 µ*M* primer HAV 68; 5.00 µL 2 m*M* dNTPs; 0.14 µL Expand High Fidelity PCR enzyme (3.5 U/µL); for a total of 10.50 µL.
3. Add the 10.5 µL of the PCR mix to each of the water-containing tubes and 10 µL of the RT product.
4. Transfer the samples to the thermocycler and run the following program: 4 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 55°C, 1 min 30 s at 72°C; and 10 min at 72°C.

3.3.3. Electrophoresis

1. Mix 10 µL of the PCR product with 2 µL of 6X loading buffer and dispense them in a well of a 1.5% TBE-agarose gel. In a parallel well dispense 5 µL of the working solution of the DNA molecular weight marker IX (Roche).
2. Connect the electric power at 70 V and run the samples for approx 2 h in TBE buffer.
3. Stain the DNA-containing agarose gel with a 1:10,000 dilution of GelStar in TBE buffer (*see* **Note 17**).
4. Analyze the DNA amplimers by observation of the stained bands in the gel analyzer using the UV light tray (*see* **Note 18**).

3.3.4. Southern Blot Hybridization

1. Transfer the gel to a glass Petri dish and submerge it in several volumes of 150 m*M* NaOH for 15 min to denature the double-stranded DNA. Keep the glass dish containing the gel on ice with constant shaking.
2. In the mean time, cut the nylon membrane (*see* **Note 19**) and rinse it in 0.5X TBE for 15 min. Wet fiber pads and filter papers with 0.5X TBE.
3. Wash and neutralize the gel by immersion in several volumes of 0.5X TBE. Keep the glass dish containing the gel on ice and shake constantly for 10 min.
4. Prepare the transfer cell as depicted in **Fig 1**. The gel should be in direct contact with the nylon membrane by the open end side of the well (*see* **Note 20**).
5. Close the cassette, and place it in the module.
6. Place 0.5X TBE buffer in the module.
7. Add the frozen Bio-Ice cooling unit.
8. Add a standard magnetic stir bar to maintain the buffer temperature and ion distribution in the tank.
9. Put on the lid, plug the cables into the power supply, and run for 1 h at 60 V.
10. Disassemble the blotting sandwich and remove the membrane.
11. UV-crosslink the wet membrane at 366 nm for 3 min.
12. Bake in a vacuum oven at 120°C for 30 min (*see* **Note 21**).
13. Place the membrane in a bag containing 20 mL of prehybridization solution per 100 cm^2 of membrane surface area.
14. Seal the bag and prehybridize overnight at 40°C.
15. Discard the prehybridization solution and add the hybridization solution containing the DIG-labeled probe at a 5 pmol/mL concentration and at the same volume/surface ratio.
16. Incubate for 2 h at 40°C.
17. Place the membrane in a glass dish and wash it briefly in several volumes of 2X SSC at room temperature with constant shaking.
18. Wash the membrane for 15 min in several volumes of 2X SSC-0.1% SDS at room temperature with constant shaking.
19. Incubate the membrane for 1 min in several volumes of buffer I at room temperature with constant shaking.
20. Block the membrane by gentle agitation in several volumes of blocking solution for 30 min at room temperature (*see* **Note 22**).
21. Dilute the anti-dig-AP 1:10,000 in blocking solution and mix.
22. Discard the blocking solution and incubate the membrane for 30 min in the antibody solution. Use a standard Petri dish for the incubation and the same volume/surface ratio as with the hybridization solution (*see* **Note 23**). Gentle agitation of the dish is required to ensure a constant and complete contact of the antigen-bound membrane and the antibody-containing solution.
23. Discard the antibody solution.
24. Wash the membrane twice (15 min per wash) in several volumes of washing buffer.
25. Equilibrate the membrane in several volumes of phosphatase buffer for two minutes.
26. Prepare the CSPD solution at a 1:100 in phosphatase buffer.
27. Discard the phosphatase buffer, avoiding, however, drying of the filter.
28. Transfer the membrane into a plastic bag with CSPD solution at the same volume/surface ratio as before. Remove any bubble. Protect the bag from the light.
29. Incubate the bag at room temperature for 5 min with constant shaking.

Fig. 1. Southern blot transfer cell.

30. Transfer the membrane to a new plastic bag (*see* **Note 24**).
31. Incubate for 15 min at 37°C (in the dark).
32. The chemilumininescent signal detection is performed by exposing the photographic film to the membrane-containing bag in the autoradiografic cassette (*see* **Note 25**).
33. Expose for 45 min (*see* **Note 26**).
34. Photographic development: transfer the film into several volumes of developer solution for 3 min. Rinse the film with water, transfer the film to several volumes of fixative solution for 3 min, and finally rinse the film again in water and let dry.

3.4. HAV Genotyping by Two-Step RT-PCR Amplification and Sequencing of a Fragment of the VP1X2A Junction Region

3.4.1. RT

1. Add 10 µL of the extracted RNA to a 0.2-mL PCR tube.
2. Incubate the tubes at 99°C for 5 min to denature the RNA.
3. Chill the tubes on ice.
4. Prepare the RT mix (volume for one sample): 5.000 µL 5X RT buffer; 0.125 µL 100 µM primer VP1-3285; 2.500 µL 2 m*M* dNTPs; 0.040 µL M-MLV reverse trascriptase (200 U/µL); distilled H_2O 7.300 µL; for a total of 15.000 µL
5. Add 15 µL of the RT-mix to each tube containing the denatured RNA.
6. Incubate the tubes at 45°C for 1 h.
7. Keep the tubes at 4°C.

3.4.2. PCR

1. Add 29.5 µL of H_2O in a new 0.2-mL PCR tube.
2. Prepare the PCR mix (*see* **Note 27**) (volume for one sample): 5.00 µL 10X PCR-$MgCl_2$ buffer; 0.25 µL 100 µ*M* primer VP1-2949; 0.25 µL 100 µ*M* primer VP1-3285; 5.00 µL 2 m*M* dNTPs; 0.14 µL Expand High Fidelity PCR enzyme (3.5 U/µL); for a total of 10.50 µL.
3. Add 10.5 µL of the PCR mix to each water-containing tube and 10 µL of RT product.
4. Transfer the samples to the thermocycler and run the following program: 4 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 55°C, 1 min 30 s at 72°C; and 10 min at 72ºC.

3.4.3. Electrophoresis

1. Mix 10 μL of the PCR product with 2 μL of 6X loading buffer and put them over a well of a 0.8% TBE-agarose gel. In a parallel well, dispense 5 μL of the working solution of the DNA molecular weight marker IX (Roche).
2. Connect the electric power up to 70 V and run the samples for approx 2 h in TBE buffer.
3. Stain the DNA-containing agarose gel with a 1/10,000 dilution of GelStar in TBE buffer (*see* **Note 17**).
4. Analyze the DNA amplimers by observation of the stained bands in the ImageMaster, using the UV light tray (*see* **Note 18**).

3.4.4. DNA Purification

3.4.4.1. Purification of an Amplimer From Single Band-Containing Solutions

1. Mix the remaining 40 μL of the PCR product with 200 μL of the Binding Buffer provided with the kit.
2. Transfer these 200 μL to the upper reservoir of a filter-tube inserted into a collection tube, both provided with the kit.
3. Centrifuge the combined tubes for 1 min at room temperature at 16,000*g* and discard the flowthrough solution.
4. Add 500 μL of wash buffer to the upper reservoir (1:5 dilution of the 5X wash buffer provided with the kit in 96º ethanol).
5. Centrifuge for 1 min at room temperature at 16,000*g* and discard the flowthrough solution.
6. Add 200 μL of wash buffer.
7. Centrifuge for 1 min at room temperature at 16,000*g* and discard the flowthrough solution.
8. Centrifuge for 5 s at room temperature at 16,000*g*.
9. Insert the filter-tube into a standard microcentrifuge tube.
10. Add 60 μL of the elution buffer (provided with the kit) to the filter-tube, and incubate for 3 min at room temperature.
11. Centrifuge the combined tubes for 1 min at room temperature at 16,000*g* and keep the DNA-containing flowthrough solution.
12. Estimate the quantity of DNA by comparison of its fluorescence intensity with that of the 360-bp band of the DNA Molecular Weight Marker IX (Roche; 1.425 ng/μL) run in parallel in an agarose gel and stained with GelStar.

3.4.4.2. Purification of an Amplimer From Mixed Band-Containing Solutions

1. Run an electrophoresis of the remaining 40 μL of PCR product, as previously described in **Subheading 3.4.3.** but using a 0.8% TAE-agarose gel instead of TBE-agarose. Prepare the agarose gel with a preparative comb.
2. Cut the desired band from the agarose gel with an ethanol-cleaned scalpel (*see* **Note 28**).
3. Weigh a 1.5-mL microcentrifuge tube, add the excised agarose fragment to the tube, and weigh it again to deduce the fragment weight.
4. Add binding buffer at a ratio of 300 μL/100 mg of agarose and mix vigorously.
5. Incubate at 56ºC for a minimum of 10 min to dissolve the agarose. Mix vigorously every 2 min.
6. Add isopropanol at a ratio of 150 μL/100 mg of agarose and mix vigorously.
7. Transfer this solution (up to 700 μL) to the upper reservoir of a filter-tube inserted into a collection tube.

8. Centrifuge the combined tubes for 1 min at room temperature at 16,000*g* and discard the flowthrough solution. Repeat this step with any further volume exceeding 700 µL.
9. Proceed as in the purification of an amplimer from single band-containig solutions from (**Subheading 3.4.4.1.**) **step 4**.

3.4.5. DNA Sequencing

1. Prepare the sequencing mix: 4.00 µL Ready Reaction Mix (*see* **Note 29**); 0.64 µL 5 µ*M* primer VP1-2949 (*see* **Note 30**); x.xx µL 3-10 ng of purified DNA (*see* **Note 31**); x.xx µL distilled water (*see* **Note 31**) for a total of 10.00 µL.
2. Transfer the samples to the thermocycler and run the following program: 25 cycles of 10 s at 96°C; 5 s at 50°C; 4 min at 60°C.
3. Keep the reaction product at 4°C until further processing (*see* **Note 32**).
4. Prepare the precipitation mix: 10.00 µL sequencing product; 64.00 µL 95° ethanol; 26.00 µL distilled water; for a total 100.00 µL.
5. Mix vigorously by vortexing.
6. Allow to stand for 15 min at room temperature.
7. Centrifuge for 30 min at 16,000*g* and discard the supernatant.
8. Add 200 µL of 70% ethanol.
9. Centrifuge for 5 min at 16,000*g* and carefully discard all the supernatant.
10. Keep the tube open at room temperature until the pellet is completely dried.
11. Electrophorese the sequencing product on the ABI Prism Analyzer for 4.5 h according to the manufacturer's instructions.

4. Notes

1. Unless otherwise stated procedures described molecular biology grade distilled water should be used in all the procedures described.
2. Since the HAV genome is RNA and RNases are ubiquitous and highly resistant to heat inactivation, the plastic material (and whenever possible all solutions) should be autoclaved at 121°C for 45 min.
3. Preparation of these reagents is not necessary when RNA extraction kits such as the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden Germany) are used. The advantages in using this kit are that it is less time-consuming and that the RNA recovery is similar or even better with certain kinds of samples such as shellfish. However, the price is comparatively expensive.
4. All ethanol solutions are prepared from 96° absolute ethanol.
5. The reason to autoclave (121°C for 20 min) most of the Southern blot reagents is not to prevent RNA degradation but rather to avoid bacterial contamination that could interfere with the hybridization reaction as well as immunological detection.
6. If the prehybridization solution is not used immediately, it should be kept on ice to avoid renaturation of the salmon DNA.
7. Since the initial pH of this buffer is around 1.5, the addition of NaOH beads is recommended to increase the pH abruptly to 7.0; at this point, adjust the pH with 1 *N* NaOH.
8. This step refers to a method for the removal of some potential inhibitors of the ulterior molecular techniques ***(14)***. At the same time it allows the viral particles present in the sample to be concentrated, since the initial volume of the sample is 50 mL and the final volume is 500 µL, which corresponds to a 100X concentration.
9. Owing to the crucial problem of cross-contamination in the PCR-based diagnostic procedures, physical separation between each step should be kept at maximum. The nucleic

acid extraction should be performed in a separate and specific room and in a vertical laminar flow hood (P2 level) since HAV is a human pathogen.

10. It is important to autoclave the microcentrifuge tubes with their lids open to avoid potential cross-contamination. For the same reason, use different pipets to add and remove reagents from the tubes, and change the filter tips at each step.
11. Completely resuspend the silica pellet from the stock.
12. Completely resuspend the silica pellet after each wash.
13. Manipulate the guanidinium isothiocyanate wastes very carefully, as well as all the reagents and materials in contact with it, and dispose of them in a container whose one-third part is 10 *N* NaOH.
14. Carefully eliminate all the silica particles. If the first supernatant has silica contamination, repeat the centrifugation step.
15. If further processing is delayed, keep the nucleic acid-containing supernatant frozen at –70ºC.
16. Because of the crucial problem of cross-contamination in the PCR-based diagnostic procedures, physical separation between each step should be kept at maximum. The RT and PCR reaction mix should be prepared in a different room separately from the nucleic acid extraction room. The thermocycler should be located in a third separate facility, and, last but not least, the amplimer-containing tubes should be open and the gels run in a fourth separate room.
17. The DNA bands may also be stained with the traditional ethidium bromide. However, since environmental samples usually present low viral loads, the GelStar stain is suggested to increase the sensitivity (around 0.5–1 log higher).
18. The gel analyzer could be replaced by a UV transilluminator coupled to a Polaroid camera.
19. Avoid touching the nylon membrane with the fingers even when using gloves.
20. Use a tube to roll the air bubbles out gently.
21. The membrane can be used immediately for prehybridization or can be stored dry at 4°C for future use.
22. Longer blocking times may also be performed.
23. To reduce the background, it is preferable to incubate the membrane in Petri dishes rather than plastic bags. It is not useful to increase the incubation time since the background increases correspondingly.
24. To reduce the background, it is important to remove the CSPD reagent completely by changing the plastic bag container.
25. Since it is a photographic development, typical dark room conditions should be observed.
26. Longer exposition times may be used to increase the intensity of the bands. The same membrane may be used for several photographic expositions at different times.
27. Since the genotyping is based on a DNA sequence, it is important to use a DNA polymerase with proofreading activity such as the *Pwo* polymerase included in the Expand enzyme.
28. Avoid excising agarose by cutting the band as accurately as possible.
29. This mix contains all the reagents necessary for the sequencing reaction with the exception of the specific primer and template and should be maintained on ice during preparation of the complete sequencing mix.
30. Since HAV is not very variable, only one strand is sequenced. However, if the quality of the sequence is not good (double sequence, low intensity of peaks, and so on), the second strand is also analyzed by using the VP1-3285 primer. When mutations not described in any database are detected, the second strand is also analyzed to confirm it.

31. The volume necessary to set up a reaction with 3–10 ng of DNA. The remaining amount to a final volume of 10 µL is added as distilled water.
32. It is preferable to continue with the DNA precipitation on the same day.

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