Promulgated, Jun 16, 2014
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# Methods of Test for Food Microorganisms- Test of Hepatitis A virus

## 1. Scope

This method is applicable to the detection of hepatitis A virus (HAV) in shellfish, drinking water, vegetables and fruits.

## 2. Method

After HAV concentration and RNA extraction, analytes are determined by reverse transcription polymerase chain reaction (RT-PCR).

**2.1.** Working environment

The working platform needs to be spacious, clean and well-lit. Procedures of sample pretreatment, RT-PCR reagent preparation and PCR assay require separate compartments to avoid cross-contamination.

**2.2.** Equipment (Note 1)

**2.2.1.** Biological safety cabinet (BSC): class II or above.

**2.2.2.** Autoclave.

**2.2.3.** Refrigerator: capable of operating at 5 ± 3°C.

**2.2.4.** Freezer: capable of operating at -30 ± 3°C.

**2.2.5.** Ultra-low temperature freezer: capable of operating at -70 ± 5°C.

**2.2.6.** Homogenizer.

**2.2.7.** Balances: weighing up to 2,000 g with sensitivity of 0.1 g, weighing up to 120 g with sensitivity of 5 mg.

**2.2.8.** Shaker.

**2.2.9.** pH meter.

**2.2.10.** UV light box: UV lamps with wavelengths of 312 nm and 365 nm.

**2.2.11.** Microwave oven or hot plate.

**2.2.12.** Polymerase chain reaction reactor: GeneAmp® PCR System 9700 or an equivalent product.

**2.2.13.** Electrophoresis tank: for DNA electrophoresis.

**2.2.14.** Thermomixer: with temperature control at 55°C and mixing function, and can maintain the internal temperature difference within 0.5°C.

**2.2.15.** Microrefrigerated centrifuge: applicable to various microcentrifuge tubes, with centrifugal force ≥ 20,000 ×g, temperature control at 4°C.

**2.2.16.** Vortex mixer.

**2.2.17.** pump.

**2.2.18.** Glass filter set: 47 mm diameter and sterilizable.

Note 1: The attached brands of equipment are not necessarily the best compared with others, and vice versa.

**2.3.** Chemicals

**2.3.1.** Virus extraction: sodium chloride, potassium chloride, glycine, sodium hydroxide, anhydrous disodium hydrogen phosphate, potassium dihydrogen phosphate, polyethylene glycol 6000 (PEG 6000), polyethylene glycol 8000 (PEG 8000), chloroform, butanol, sulfuric acid, hydrochloric acid, boric acid, magnesium chloride ($MgCl_2 \cdot 6H_2O$), ethylenediaminetetraacetic acid disodium salt ($Na_2$-EDTA) and tris(hydroxymethyl)aminomethane (Tris-base), reagent grade; beef extract powder and peptone, microbiological grade; pectinase from *Aspergillus niger* or *Aspergillus aculeatus*, molecular bioanalytical grade.

**2.3.2.** Viral RNA extraction: commercial kits for viral RNA extraction, with a large volume of test solution (1 mL) for fruit and vegetable samples.

**2.3.3.** Viral RNA processing: Deoxyribonuclease I (DNase I) 5 U/μL.

**2.3.4.** Reverse transcription reaction: commercial kits for viral RNA reverse transcription, including reverse transcriptase, 5x buffer solution, 10 mM deoxyribonucleoside triphosphate (dNTP), random-primer, 0.1 M dithiothreitol (DTT) and RNase inhibitor.

**2.3.5.** Polymerase chain reaction

**2.3.5.1.** DNA polymerase: *Taq* DNA polymerase (5 U/μL), including 10x PCR buffer solution (containing 20 mM magnesium chloride), or equivalent.

**2.3.5.2.** Deoxyribonucleoside triphosphate (dNTP) solution: containing 25 mM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP).

**2.3.5.3.** Hepatitis A virus

(target region: 5-terminal untranslated region)

Primer F: HAV68 5'- TCACCGCCGTTTGCCTAG -3'

Primer R: HAV240 5'- GGAGAGCCCTGGAAGAAAG-3'

PCR amplicon size 173 bp

(target region: protein coat structure gene VP1/P2A)

Primer F: VP1-4 5- CGTTGCTTCCCATGTCAGAG -3'

Primer R: VP1-5 5- GACCTTCCCATAAACTTGTAG -3'

PCR amplicon size 369 bp

**2.3.6.** Electrophoresis: $Na_2$-EDTA, bromophenol blue, xylene cyanol FF, ethidium bromide, Tris-base, glycerol, boric acid and agarose, molecular bioanalytical grade. DNA molecular weight marker (100 bp DNA ladder marker).

**2.3.7.** Control substance: hepatitis A virus.

**2.4.** Apparatus and materials (Note 2)

**2.4.1.** Adjustable microdispenser: 10 μL, 20 μL, 200 μL or 1000 μL.

**2.4.2.** Pipette tips: 10 μL, 20 μL, 200 μL or 1000 μL.

**2.4.3.** Pipette or automatic pipette/pipette tip: sterile, 1mL pipette with scale of 0.01 mL, 5 mL and 10 mL pipettes with scale of 0.1 mL.

**2.4.4.** Glass or plastic bottle: 50 mL, 100 mL, 250 mL, 500 mL, 1000 mL and 2000 mL.

**2.4.5.** Microcentrifuge tubes: 200 μL, 1.5 mL, 2 mL.

**2.4.6.** Centrifuge tube: 50 mL, PP material.

**2.4.7.** Centrifugal filter tube: 15 mL, screening for substances with molecular weight greater than $10^5$ Daltons.

**2.4.8.** Sterile bag, sterile bag with filter (400 mL).

**2.4.9.** Medicine spoons, scissors, knives and tweezers: sterilizable.

**2.4.10.** Sterile filter membrane: cellulose acetate membrane, hydrophilic, 0.22 μm pore size.

**2.4.11.** PCR reaction tubes: 200 μL, 500 μL and 96-well reaction plate.

**2.4.12.** Electrophoresis gel casting tray: including comb.

Note 2: The plastic or glassware used are DNase-free and RNase-free.

**2.5.** Reagents preparation

**2.5.1.** Phosphate buffered saline (PBS)

Dissolve 76.5 g of sodium chloride, 7.2 g of anhydrous disodium hydrogen phosphate and 2.1 g of potassium dihydrogen phosphate with deionized water to 1000 mL, as a 10-fold PBS buffer solution. Take 100 mL of 10x PBS buffer solution, add deionized water to 1000 mL, sterilize at 121°C for 15 min, the final pH is 7.4.

**2.5.2.** PEG 6000- NaCl Solution

Dissolve 26.4 g of sodium chloride with deionized water to 380 mL, and then add 120 g of PEG 6000, mix well, sterilize at 121°C for 15 min.

**2.5.3.** Chloroform-butanol solution

Mix chloroform and butanol at the ratio of 1:1 (v/v) in a brown bottle.

**2.5.4.** 50 mM sulfuric acid solution

Add 1.39 mL of sulfuric acid slowly into 200 mL of sterile deionized water, and mix well. After cooling, dilute with sterile deionized water to 500 mL.

**2.5.5.** 0.5 mM sulfuric acid solution

Dilute 50 mM sulfuric acid solution to 0.5 mM with deionized water.

**2.5.6.** 1 mM sodium hydroxide solution

Dissolve 4 g of sodium hydroxide with sterile deionized water to 100 mL, and then dilute 1 mL with sterile deionized water to 1000 mL.

**2.5.7.** 6 N hydrochloric acid solution

Add 50 mL of hydrochloric acid slowly into 80 mL of sterile deionized water, and mix well. After cooling, dilute with deionized water to 100 mL.

**2.5.8.** 100-fold Tris-EDTA solution

Dissolve 12.1 g of Tris-base and 2.9 g of EDTA with 80 mL of deionized water, adjust pH to 8.0 with 6 N hydrochloric acid solution, and dilute with deionized water to 100 mL. Sterilize at 121°C for 15 min. (commercially available)

**2.5.9.** 0.5 M EDTA solution

Dissolve 186.1 g of Na2-EDTA with 800 mL of deionized water, then add 20 g of sodium hydroxide, adjust pH to 8.0, dilute with deionized water to 1000 mL.

**2.5.10.** Buffer peptone water (BPW)

Dissolve 10 g of peptone, 5 g of sodium chloride, 3.5 g of anhydrous disodium hydrogen phosphate and 1.5 g of potassium dihydrogen phosphate with deionized water to 1000 mL, divide into diluting containers, and sterilize at 121 °C for 15 min, final pH is 7.2 ± 0.2.

**2.5.11.** Tris-glycine-beef extract buffer (TGBE)

Dissolve 12.1 g of Tris-base, 3.8 g of glycine and 10 g of beef extract with deionized water to 1000 mL, sterilize at 121°C for 15 min, final pH is 9.5 ± 0.2.

**2.5.12.** TGBE buffer solution containing pectinase

Take 100 mL of TGBE buffer solution, add pectinase *Aspergillus niger* 75 unit or *Aspergillus aculeatus* 2850 unit, prepare prior to use.

**2.5.13.** 0.5x TBE (Tris-borate-EDTA) buffer solution

Dissolve 54 g of Tris-base and 27.5 g of boric acid with deionized water, add 20 mL of 0.5M EDTA solution, and dilute with deionized water to 1000 mL, as 5x TBE buffer solution. (commercially available) Dilute the 5x TBE buffer solution to 0.5x with deionized water, prepare prior to use.

**2.5.14.** 6x gel loading buffer

Dissolve 0.25 g of bromophenol blue and 0.25 g of xylene cyanol FF with sterile deionized water, add 30 mL of glycerol, and dilute with sterile deionized water to 100 mL. (commercially available) Store at 4°C.

**2.5.15.** 2.5% agarose gel

Weigh 2.5 g of agar, add 100 mL of 0.5x TBE buffer solution, heat and stir until the agar is completely dissolved. Let agarose solution cool down to about 50 °C, pour it into the electrophoresis gel casting tray with the well comb, sit until the gel solidify.

**2.5.16.** Gel staining solution

Dissolve 0.1 g of ethidium bromide with 10 mL of deionized water as a stock solution (10 mg/mL). Dilute to 1 μg/mL with deionized water before use. Ethidium bromide is a known carcinogen, should pay attention to safety when operating.

**2.6.** Virus concentration

**2.6.1.** Shellfish

**2.6.1.1.** Treatment of shellfish

Open the shells of the shellfish with a sterilized knife or forceps, take out the flesh part and remove the mantle and the white tissue. Dissect out the digestive gland. (Figure 1)

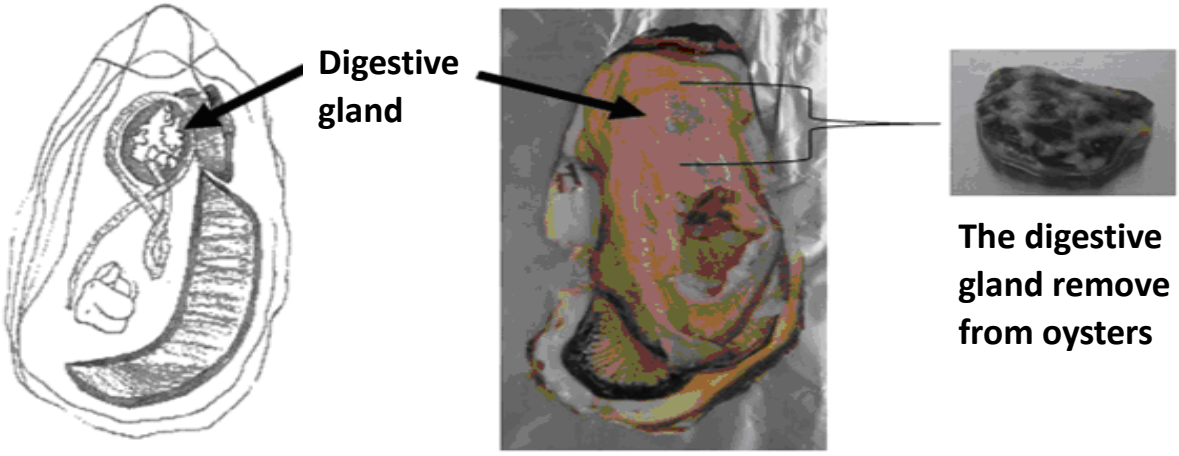

Figure 1. The relative position of the digestive gland in oysters

**2.6.1.2.** Pretreatment of digestive gland

Take 1.5 g of digestive gland into a 50 mL centrifuge tube, add 10 mL of PBS, place the centrifuge tube on ice, and grind with a homogenizing rod in 2 stages, each stage for 30 seconds. Add 6 mL of chloroform-butanol solution, homogenize for 30 seconds, and then rinse the residue from the homogenizing rod with 3 mL of PBS. Rotate and mix the grinded specimen at 4°C for 1 hour, centrifuge at 12,000 xg for 20 min, and collect the supernatant.

**2.6.1.3.** Concentration

Add 10.5 mL of PEG 6000- NaCl solution to the supernatant of section 2.6.1.2, mix well, and continuously rotate overnight at 4°C. Centrifuge the mixture at 12,000 xg for 20 min at 4°C, discard the supernatant, and then extract viral RNA with a commercial kit.

**2.6.2.** Drinking water

**2.6.2.1.** Large volumes of water

Take 100-1000 mL of the sample, add magnesium chloride (final concentration 25 mM), filter through a sterile membrane by filter device (Figure 2). Flush the membrane with 200 mL of 0.5 mM sulfuric acid solution, discard the flushing solution, replace the suction bottle to the specimen collection device (Figure 3). Wash the membrane with 10 mL of 1 mM sodium hydroxide solution, and collect the washing solution to the sterile centrifuge tube in the specimen collection device. The centrifuge tube was pre-filled with 0.1 mL of 50 mM sulfuric acid solution and 0.1 mL of 100-fold Tris-EDTA solution. Take out the centrifuge tube and pour the washing solution into Ultra Centrifugal Filters, centrifuge at 3,000 ×g for 20-30 min at 4°C, concentrate to less than 0.5 mL, transfer the concentrate into a 1.5 mL microcentrifuge tube for RNA extraction.

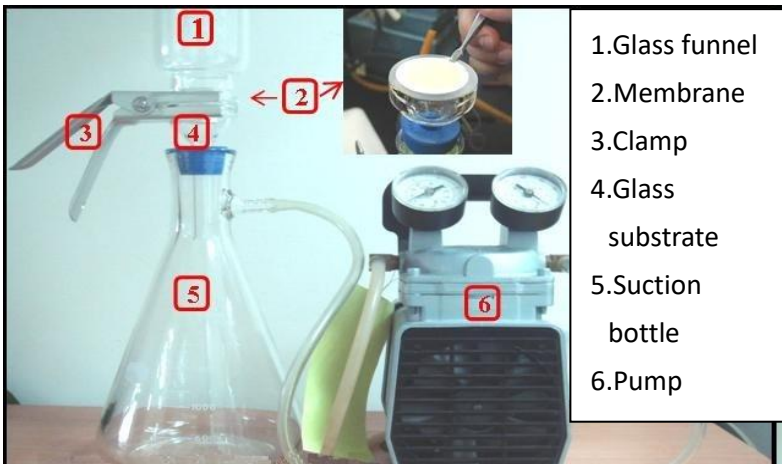


Figure 2. Specimen filter device Figure 3. Specimen collection device

**2.6.2.2.** Volume of water less than 100 mL

Pour sample in batches into Ultra Centrifugal Filters, centrifuge at 3,000 ×g for 20-30 min at 4°C, concentrate to less than 0.5 mL, transfer the concentrate into a 1.5 mL microcentrifuge tube for RNA extraction.

**2.6.3.** Fruit and vegetable

**2.6.3.1.** Non-soft fruit

Leaf vegetables with small leaves, wrapped leaves and dry beans, take about 10 g, cut in about 2.5 cm pieces; root vegetables, fruit vegetables, take about 25 g, maintain integrity. Divide the samples into two 50 mL centrifuge tubes, add BPW to 50 mL respectively, shake at 80 rpm for 30 min at room temperature. Pipette the eluent into a 50 mL centrifuge tube, centrifuge at 10,000 ×g for 30 min at 4°C, transfer the supernatant to another 50 mL centrifuge tube, add BPW to 45 mL, and then add 5 g of PEG 8000 and 0.176 g of sodium chloride, mix thoroughly, and continuously rotate overnight at 4°C. For larger size sample, use a sterile bag, add 100 mL of BPW, follow the same procedure as before.

**2.6.3.2.** Soft fruit

Take about 25 g of soft fruits (such as strawberries, blueberries, grapes), maintain integrity. Divide the samples into two 50 mL centrifuge tubes, add TGBE buffer solution containing pectinase to 50 mL respectively, shake at 80 rpm for 30 min at room temperature. Pipette the eluent into a 50 mL centrifuge tube, centrifuge at 10,000 ×g for 30 min at 4°C, transfer the supernatant to another 50 mL centrifuge

tube, add TGBE to 45 mL, adjust pH to 7.0, and then add 5 g of PEG 8000 and 0.176 g of sodium chloride, mix thoroughly, and continuously rotate overnight at 4°C. For larger size sample, use a sterile bag, add 100 mL of TGBE buffer solution containing pectinase, follow the same procedure as before.

**2.6.3.3.** Sample solution preparation

Take the mixture from section 2.6.3.1 or 2.6.3.2, two tubes for each sample, centrifuge at 10,000 ×g for 30 min at 4°C. Decant and discard the supernatant, add 3 mL of PBS to the first tube, rinse the inside of the centrifuge tube and the sediment repeatedly, vortex for 20 sec, centrifuge at 10,000 ×g for 1 min at 4°C, transfer the rinse solution into the second tube, repeat the same procedure as before to get about 5-6 mL of PBS washing solution for each sample, add equal volume of chloroform-butanol solution, mix well with a vortex mixer, let stand for 5 min at room temperature, and centrifuge at 10,000 × g for 15 min at 4°C. Take the supernatant for RNA extraction.

**2.7.** Viral RNA extraction

For shellfish samples, take the sediment in section 2.6.1.3 For drinking water samples, take the virus concentrates in sections 2.6.2.1 or 2.6.2.2 Extract viral RNA according to the steps of the commercially available kits, and collect the extracted viral RNA into a sterilized 1.5 mL centrifuge tube as the viral RNA solution. For vegetable and fruit samples, take the sample solution in section 2.6.3.3 to extract viral RNA according to the steps of the commercially available kits for large volume of test solution (1 mL), and use the same nucleic acid affinity column to repeatedly filter and concentrate. Re-dissolved with 100-200 μL of sterile deionized water as the viral RNA solution.

**2.8.** Positive control virus addition

For shellfish samples, add about $10^4$ PCR Units of positive control virus strains into 1.5 g of digestive gland; for drinking water samples, add $10^2$ PCR Units each mL of water samples. For fruit and vegetable samples, before addition of BPW or TGBE buffer solution containing pectinase, add $10^3$ PCR Units of positive control virus strain. Concentrate and extracte viral RNA according to section 2.6 and 2.7.

**2.9.** Treatment of viral RNA solution with DNase I

**2.9.1.** Prepare the mixture according to the following table in a microcentrifuge tube

Viral RNA solution……24.0 μL
10x buffer solution……...3.0 μL
Sterile deionized water……1.0 μL
DNase I (5 U/μL)…… ….2.0 μL
Total volume……..…30.0 μL

**2.9.2.** React at 37°C for 30 min, followed by 5 min at 75°C, and then immediately transfer to an ice bath. The RNA solution treated with DNase I use for the reverse transcription reaction.

**2.10.** Reverse transcription reaction

**2.10.1.** Prepare the mixture according to the following table in a microcentrifuge tube

Viral RNA solution treated with DNase I……5.0 μL
5x TBE buffer solution…… ……..5.0 μL
10 mM dNTP……..4.0 μL
25 mM magnesium chloride solution……..5.0 μL
Random Primer (3 μg/μL)……1.3 μL
0.1 M DTT……2.5 μL
Ribonuclease Inhibitor (40 U/μL)……1.4 μL
Reverse transcriptase (200 U/μL)……0.8 μL
Total volume……..……25.0 μL

**2.10.2.** Perform reverse transcription reaction according to the following conditions (Note 3)

| Steps | Temperature (°C) | Time (min) |
|---|---|---|
| Reverse transcription | 25 | 10 |
| | 50 | 50 |
| | 85 | 15 |

Transfer to an ice bath immediately after the reaction. This is the cDNA product for polymerase chain reaction.

Note 3: For the same tube of RNA, at least two replicate reverse transcription reactions should be performed.

**2.11.** First polymerase chain reaction (PCR)

**2.11.1.** Prepare the first PCR mixture according to the following table in a

microcentrifuge tube
cDNA product……5.0 μL
10x PCR buffer (containing 20 mM $MgCl_2$)……5.0 μL
2.5 mM dNTP……4.0 μL
10 μM primer F (Note 4)……1.0 μL
10 μM primer R (Note 4)……1.0 μL
DNA polymerase (5 U/μL)……0.5 μL
Sterile deionized water……33.5 μL
Total volume……50.0 μL
Note 4: Use primer pairs HAV68/HAV240 and VP1-4/VP1-5.

**2.11.2.** Perform PCR according to the following conditions

| Steps | Temperature | Time |
|---|---|---|
| 1. Initial denaturation | 95°C | 4 min |
| 2. Denaturation | 95°C | 30 sec |
| 3. Annealing | 50°C | 30 sec |
| 4. Extension | 72°C | 1 min |
| Repeat step 2 to 4 for 40 cycles | | |
| 5. Final extension | 72°C | 7 min |

**2.11.3.** Gel electrophoresis analysis

Take an appropriate amount of 6x loading buffer solution, mix with DNA ladder marker, sterile deionized water (blank group) and PCR products respectively, load into the well of 2.5% gel. Conduct electrophoresis at 50 or 100 volts. After electrophoresis, stain the gel in gel staining buffer for about 10 min, then rinse and fad in water, and transfer to UV transillumination to observe whether there is a clear DNA fragment bright band. When hepatitis A virus is present, the amplicon of the primer HAV68/HAV240 should have an obvious DNA band at size of 173 bp and the primer VP1-4/VP1-5 DNA band at size of 369 bp. When there is no obvious DNA bright band in the first PCR result, the second PCR should be performed. Each reaction should include a positive control group and a blank group. The positive control group is added with hepatitis A virus, and the blank group is sterile deionized water.

**2.12.** Second PCR

**2.12.1.** Prepare the second PCR mixture according to the following table in a microcentrifuge tube

Dilution solution of the first PCR product (Note 5)……..5.0 μL
10x PCR buffer (containing 20 mM $MgCl_2$)……..5.0 μL
2.5 mM dNTP……..4.0 μL
10 μM Primer F (Note 6)…..……1.0 μL
10 μM Primer R (Note 6)…….……1.0 μL
DNA polymerase (5 U/μL)……..0.5 μL
Sterile deionized water…….……33.5 μL
Total volume……50.0 μL

Note 5: The first PCR product is recommended to be diluted with 10 to 20 times with sterile deionized water for use as the DNA template for the second PCR reaction.

Note 6: Use primer pairs HAV68 / HAV240 and VP1-4 / VP1-5.

**2.12.2.** Perform PCR according to Section 2.11.2.

**2.12.3.** Gel electrophoresis analysis and results interpretation according to section 2.11.3.

**2.12.4.** Sequencing and sequence alignment

Perform PCR products sequencing to confirm gel electrophoresis analysis result. Uploaded the sequence data to the NCBI Blast webpage of the U.S. National Institutes of Health, and the sequences were aligned with the GenBank database to confirm the hepatitis A virus. Two repeat tests of the same tube of RNA, if any one of the results is positive, the test result is considered to be positive; when the results of the two repeats are negative, the test result is negative.

**Remark**

The reaction conditions can be adjusted depending on the instruments used if the above reaction conditions are not applicable.

## Inspection flow chart (Shellfish and Water)