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Short communication

# Detection of hepatitis A virus in shellfish by nested reverse transcription-PCR

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**Abstract**

A method for the detection of HAV in shellfish, based on the use of guanidinium isothiocyanate-containing solution for RNA extraction and purification steps, followed by nested PCR, is hereby proposed. Tests were carried out on mollusc samples spiked with HAV strain FG. Results showed that in samples subjected only to one round of PCR it was possible to detect HAV at concentrations of $10^3$–$10^4$ $TCID_{50}$/10 g of mollusc. The use of the nested PCR renders the system more sensitive and specific enabling the identification of HAV concentrations as low as 1 $TCID_{50}$/10 g of mollusc. Furthermore thus method, in addition to allowing the avoidance of confirming tests, such as hybridization, proved to be inexpensive and simple to perform. 

*Keywords:* HAV; Nested-RT-PCR; Mussels

## 1. Introduction

The hepatitis A virus (HAV) causes a high percentage of the infectious hepatitis cases reported each year world-wide (Gerety, 1984; Cliver, 1994). In Italy, infectious hepatitis represents an important public health problem. Data from SEIEVA (Integrated Epidemiological System for Acute Viral Hepatitis-ISS) report thousands of cases per year (Mele et al., 1995). Among the most clearly implicated food in the transmission of HAV are the bivalve molluscs, mainly as they are often eaten raw or slightly cooked (Gerba, 1988; Wanke and Guerrant, 1987). Worldwide, approximately 7% of reported hepatitis A cases have been associated with shellfish consumption (Gerba and Goyal, 1978; Cliver et al., 1983), and in Italy it accounts for 43% of cases (Mele and Cialdea, 1994). After replicating in the human gastrointestinal tract, the viruses are excreted into sewage and may be dispersed into the environment because they can survive standard treatments (Gerba et al., 1975). They can also survive in seawater for periods of time ranging from a few days to several weeks (Metcalf and Stiles, 1967). During their natural feeding process, molluscs extract and concentrate into their body particulate matter, includ-

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ing viruses, from very large volumes of water, which may be polluted with sewage. Traditionally, coliform bacteria have been used as indicators of the sanitary quality of shellfish. However, it has been clearly established that bacterial standards do not always reveal the presence of viruses and that conditions used for the removal of bacteria before commercialization are not effective for eliminating viruses (Franco et al., 1990; Croci et al., 1992). It is therefore necessary to develop rapid, reliable and sensitive approaches for the direct detection of HAV in molluscs, in order to guarantee their wholesomeness. The methods based on the infection of cell cultures, though being the only ones that reveal the infectiousness of viral particles, are expensive and very long, in fact several days are needed to allow the HAV replication (Cliver et al., 1983; Croci et al., 1992) Recently, the polymerase chain reaction (PCR) technique has been used to detect enteroviruses and HAV in environmental samples and in shellfish (Goswami et al., 1993; Le Guyader et al., 1994; Jaykus et al., 1995), offering the advantage of a cost reduction and rapidity in obtaining results. However, generally the proposed methods employ complex preliminary steps, and furthermore, the use of only one amplification phase (PCR) has been shown to provide a low sensitivity (Severini et al., 1993).

In this study we describe a method for the detection of HAV in molluscs, that, taking advantage of using a guanidinium isothiocyanate-containing solution (D solution) (Afzal and Minor, 1994) for RNA extraction and purification steps and of a nested-RT-PCR, showed to be sensitive and simple to perform. The experiments were carried out using HAV experimentally contaminated and natural mussel samples.

## 2. Materials and methods

### *2.1. Samples*

Samples used were molluscs (*Mytilus galloprovincialis*) obtained from a local seafood market, that were experimentally contaminated, and a limited number of mussel samples ($n = 15$) collected from areas of the Adriatic sea particularly exposed to contamination risk. The bodies and intervalve water were collected and homogenized in warring blenders (Osterizer Pulse Magic 16) at maximum speed for 30 s. Samples were then divided into 10 g aliquots and stored at $-20$°C prior to use. Each experiment was repeated three times.

### *2.2. Virus production and sample contamination*

HAV strain FG (Venuti et al., 1985), kindly provided by Prof. Panà (Università di Tor Vergata, Roma), was grown and titrated in Frp/3 cell culture (Franco et al., 1990; Croci et al., 1992). Serial dilutions of viral suspension were added to aliquots of 10 g of mollusc homogenate to obtain final concentrations of $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, 10 and 1 $TCID_{50}$ HAV/10 g of mollusc. As positive control, aliquots spiked with $10^7$ $TCID_{50}$ HAV/10 g were used and as negative controls aliquots not spiked with HAV were used.

### *2.3. Virus extraction*

Ten g of homogenate were diluted 1:2 in glycine buffer (0.05 M, pH 9.2) and homogenized in a warring blender (30 s at maximum speed). After stirring for 30 min, the sample was centrifuged at $8000 \times g$ (Beckman L-55) for 15 min at 4°C to separate the phases. The supernatant was collected and the pH adjusted to 7.2. Polyethylene glycol (PEG 8000, SIGMA) as 50% w/v solution in 7.5% NaCl was added to a final concentration of 10%. The mixture was stirred overnight at 4°C, and then centrifuged at $8000 \times g$ for 1 h at 4°C. The pellet was resuspended in 1 ml of phosphate buffer saline (PBS). The mixture was stirred for 10 min at room temperature and centrifuged at $8000 \times g$ for 15 min at 4°C. The supernatant was stored at $-20$°C prior to use.

### *2.4. RT-nested-PCR*

#### *2.4.1. RNA extraction and purification*

According to Afzal and Minor (1994), 334 μl of supernatant were added to a 1.5 ml Eppendorf tube containing 666 μl of 1.5 × Solution D (Chirgwin et al., 1979), and the tubes were vortexed for 30 s to 1 min.

One hundred microliters of CsCl cushion (5.7 M solution of CsCl in 25 mM sodium acetate pH 5.0;

the refractive index is adjusted to 1.4000, the solution is sterilized, the refractive index is adjusted again and the solution is stored at room temperature) were gently placed into the bottom of the tube by piercing through the liquid.

After centrifugation in an Eppendorf microfuge at 13 000 rpm for 20 min at 4°C, the supernatant was discarded and the pellet was washed twice with 1 ml of 70% ethanol and dried.

*2.4.2. Oligonucleotides for PCR*

The primers were selected from the published sequences of the VP2 and VP4 capsid region on the basis of 100% sequence homology among the HAV strains FG (Beneduce et al., 1995), HM175 (Cohen et al., 1987), L.A. (Najarian et al., 1985) and MBB (Paul et al., 1987). Furthermore, the primers were checked for cross-reactivity with other enteric viruses by computer analysis (NCBI's Advanced Blast program). The primer sequences for PCR (primer pair A, primers 1 and 2) and for nested-PCR (primer pair B, primers 3 and 4) are reported in Table 1. Primer pair A amplified a 385 bp region, primer pair B a 328 bp region.

*2.4.3. Reverse transcriptase-PCR*

The dried pellet was resuspended in 84 μl of reverse transcription (RT) reaction mix containing 20 mM Tris–HCl (pH 8.4), 75 mM KCI, 2.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate (dNTP), and 100 pmoles of primer 2 (antisense). The mixture was heated at 95°C for 3 min. After cooling at 42°C, twenty units of RNasin (Promega) and 1.25 U of AMV reverse transcriptase were added, and the mix was incubated at 42°C for 30 min. The reaction was terminated by heating the mixture at 95°C for 3 min. One hundred pmoles of primer 1 (sense), 2.5 U of Taq DNA polymerase (Promega) and DEPC-treated water to a final volume of 100 μ1 were added. The mixture was subjected to 25 PCR cycles each consisting of 25 s at 95°C, 30 s at 37°C and 1 min at 70°C. A final extension was carried out at 72°C for 5 min.

*2.4.4. Nested PCR*

Five microliters of the first amplification reaction were further amplified in 95 μl of reaction mixture containing 20 mM Tris–HCl (pH 8.4), 75 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphates (dNTP), 100 pmoles of primer 3, 100 pmoles of primer 4 and 2.5 U of Taq DNA polymerase (Promega). Amplification conditions were as described for the first PCR amplification.

*2.4.5. Electrophoresis*

The remaining mixture from the first amplification and the nested-PCR mixture were ethanol precipitated and analyzed by agarose gel electrophoresis (2% agarose).

*2.4.6. Sequencing*

The nested-PCR products from the naturally contaminated samples were cloned into vector PCR II (TA-Cloning System Invitrogen Co) and sequenced (Sanger et al., 1977), using the Sequenase kit (USB Corp) to confirm their identity.

## 3. Results and discussion

The results of the tests performed on mollusc samples experimentally infected with concentrations of HAV ranging from $10^6$ to 1 $TCID_{50}$/10 g of mollusc are shown in Fig. 1. In our experimental conditions the lowest HAV concentration that could be detected by the RT-PCR method was $10^3$ $TCID_{50}$/10 g of mussel (Fig. 1, lane 4). However, this result has been obtained only in 40% of tests, while a clearly visible band was present in 100% of samples when a viral concentration of $10^4$ $TCID_{50}$/

Table 1
HAV primer sequences for the PCR and for nested-PCR

| Oligonucleotide[a] | Sequence | Localization |
|---|---|---|
| Primer 1 | 5′CAGACTGTTGGGAGTGG 3′ | 762–778 |
| Primer 2 | 5′TTTATCTGAACTTGAAT3′ | 1131–1147 |
| Primer 3 | 5′CTTGACCACATCCTGTCT3′ | 780–797 |
| Primer 4 | 5′TGTATGGTATCTCAACAA3′ | 1092–1109 |

[a] Primer 1 and 2 (primer pair A) were used for PCR; primer 3 and 4 (primer pair B) were used for nested-PCR.

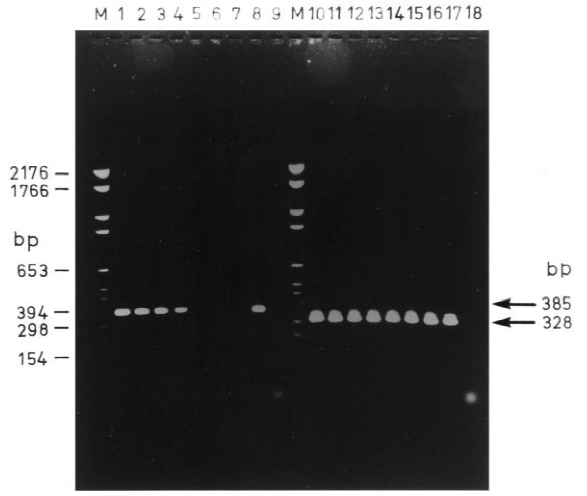


Fig. 1. Sensitivity study of method for HAV detection in shellfish. Lane M: Marker 6 (Boebringer). Lanes 1–7: serial 10-fold dilutions of HAV ($10^6$–$10^0$ $TCID_{50}$/10 g of mussels) subjected to RT-PCR. Lane 8: positive control of RT-PCR. Lane 9: negative control of RT-PCR. Lanes 10–16: serial 10 fold dilutions of HAV ($10^6$ to $10^0$ $TCID_{50}$/10 g of mussels) subjected to RT-nested-PCR. Lane 17: positive control for RT-nested-PCR. Lane 18: negative control for RT-nested-PCR.

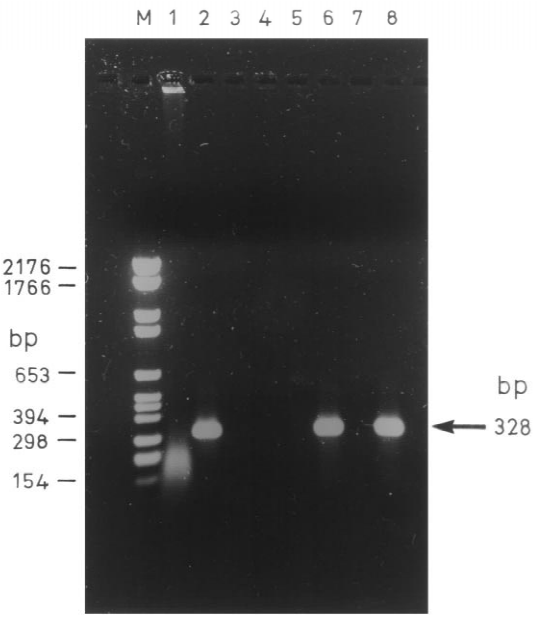


Fig. 2. HAV detection in natural mussel samples by nested-RT-PCR method. Lane M: Marker 6 (Boebringer). Lane 1: negative control. Lane 2: positive control ($10^2$ HAV TCID 50). Lanes 3–8: mussel samples (lanes 6 and 8 positive mussel samples).

10 g of mollusc was used (Fig. 1, lane 3). The addition of a nested phase to RT-PCR significantly raised the level of sensitivity of the assay, revealing HAV concentrations as low as 1 $TCID_{50}$/10 g of mussels in all the tests (Fig. 1, lane 16). Amplified sequences were not detected in the uninfected samples (Fig 1, lanes 9 and 18). The fifteen natural mussel samples resulted all negative after the first amplification (RT-PCR); after the additional phase of nested RT-PCR two samples resulted positive for HAV, while the other thirteen samples were negative. The results of a group of six samples are reported in Fig. 2. Fluorescent bands (Fig. 2 lanes 6 and 8) corresponding to the positive samples can be observed. Sequencing of the amplified products confirmed their identity as HAV.

Thus the proposed method, that combines the use of a guanidinium isothiocyanate-containing solution (solution D) for viral RNA extraction and purification steps and nested-RT-PCR for amplification, results effective and simple to perform. The RNA extraction and the first amplification step can be performed in a single tube, greatly reducing the risk of contaminating the sample. Besides, the combined use of two subsequent amplification steps (nested RT-PCR) it is advisable, as it allows to reach a very high sensitivity without loss of specificity. In fact, clear bands can be detected also from mollusc samples contaminated with HAV concentration as low as 1 $TCID_{50}$/10 g. Because the total particle to infectious particle ratio is about 60/1 for HAV (Le Guyader et al., 1994), this method would allow the detection of less than 60 viral particles/10 g (6 viral particles per g). One complication of introducing a nested step into an amplification technique could be the possibility of contamination with foreign DNA, that can affect an entire experiment. All precautions were taken to avoid carryover of amplification products, including physical separation of pre-amplification and post-amplification procedures, UV irradiation of non-disposable devices, use of aerosol-resistant tips, assembly of master mix solutions to reduce manipulation of samples and the introduction of negative controls in the protocol.

In conclusion, the hereby proposed method, in addition to being fast and inexpensive, reveals a high degree of sensitivity and specificity, especially due to the use of nested RT-PCR, that allows also the

avoidance of confirming tests, such as hybridization, which often requires the use of radioactive materials (Severini et al., 1993; Kammarer et al., 1994). Even though this technique cannot discriminate between infectious and noninfectious particles, it offers a rapid way for screening mussels therefore providing useful indications on the distribution and incidence of HAV in such products.

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