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# Detecting the presence of infectious hepatitis A virus in molluscs positive to RT-nested-PCR

**D. De Medici, L. Croci, S. Di Pasquale, A. Fiore and L. Toti**

*Laboratorio Alimenti, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

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**Aims:** The objective of this study was to determine the presence of infectious hepatitis A virus (HAV) in molluscs naturally contaminated with viral HAV-RNA.

**Methods and Results:** One hundred and forty-two mollusc samples were analysed for the presence of viral HAV-RNA using RT-nested-PCR; positive samples were then analysed with an integrated method, cell-culture RT-PCR, to detect infectious virus. Viral HAV-RNA was detected in 34·5% of the samples while 12·7% of the total samples were positive for the presence of infectious virus.

**Conclusions:** The results demonstrate the validity of the screening method (RT-nested-PCR) and the necessity of applying a method that is capable of detecting the presence of infectious HAV.

**Significance and Impact of the Study:** The study demonstrates that in any case, to determine the safety for human consumption, the results of RT-nested-PCR must be confirmed with an integrated cell-culture PCR method.

## INTRODUCTION

Hepatitis A virus (HAV) is responsible for a large proportion of the annual number of reported cases of hepatitis infection worldwide (Graff *et al*. 1993; Anon 1998). One of the most clearly implicated foods in the transmission of HAV has been bivalve molluscs, mainly because they are often eaten raw or only slightly cooked (Wanke and Guerrant 1987; Gerba 1988). Worldwide, approximately 7% of the reported cases of HAV infection have been associated with mollusc consumption (Gerba and Goyal 1978; Cliver *et al*. 1983), and in 1988, the consumption of contaminated clams was reported as the cause of more than 300 000 cases of HAV infection in Shanghai, China (Xu *et al*. 1992).

In Italy, according to the Italian National Epidemiological Surveillance System for Acute Hepatitis Viruses (SEIEVA, 'Sistema Epidemiologico Integrato per le Epatiti Virali Acute'), in the period from 1995 to 1997, 71% of the notified cases of acute viral hepatitis infection were cases of HAV infection (Ciccozzi *et al*. 1999), and the consumption of mussels was implicated in 72% of the cases in southern Italy (Ciccozzi *et al*. 1999).

*Correspondence to: Dario De Medici, Laboratorio Alimenti, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy (e-mail: dario.demedici@iss.it).*

In the European Union, the directive that regulates the sale of molluscs (European Community 1991) only specifies bacteriological parameters (e.g. *Salmonella* and *Escherichia coli*) for determining the suitability of molluscs for consumption. However, the absence of bacteria does not necessarily signify the absence of viruses (Goyan *et al*. 1979; Wait *et al*. 1983; Croci *et al*. 1998) and even the detection of bacteriophage, whose use has been proposed by various authors (Havelaar and Hogeboom 1984; Lucena *et al*. 1994), has proven to be relatively ineffective as an indicator of the presence of infectious viruses (Croci *et al*. 2000).

In the past 10 years, molecular biological techniques such as the polymerase chain reaction (PCR), have emerged as effective methods for detecting enteric viruses in mussels (Atmar *et al*. 1993; Graff *et al*. 1993; Lees *et al*. 1994; Le Guyader *et al*. 1996; Cromeans *et al*. 1997; Fiore *et al*. 2000). However, these techniques have certain limitations (Richards 1999); most importantly, it is not known whether they are able to distinguish between infectious and non-infectious viruses. In fact, RT-PCR has a positive reaction even when applied to RNA viruses that are no longer infectious (Ma *et al*. 1994; Ojen *et al*. 1995; Hilfenhaus *et al*. 1997), and in a recent study, HAV-RNA was amplified from sterile seawater for 232 d, though it was infectious in cell cultures for only 35 d (Arnal *et al*. 1998).

The objective of the present study was to determine the presence of infectious HAV, applying the integrated

cell-culture RT-PCR method to molluscs naturally contaminated with viral RNA, as determined by RT-nested-PCR.

## MATERIALS AND METHODS

### Samples

Blue mussels and other bivalve molluscs (i.e. clams and cockles) were collected directly from the markets of cities in areas of southern Italy with a high incidence of HAV infection.

### Virus Extraction

Mussels were rinsed with sterile distilled water, the body and liquor removed and homogenized in a blender (Osterizer Pulse Magic 16, Milwaukee, WI, USA; 30 s at maximum speed). Seventy-five grams of homogenate were diluted 1 : 1 in glycine buffer (0·05 M, pH 9·2), stirred for 30 min, and centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant was collected and the pH adjusted to 7·2. Polyethylene glycol (PEG 8000, Sigma, MO, USA), 50% (w/v) in 7·5% NaCl (PEG-NaCl solution), was added (1 : 4) to obtain a final concentration of 12·5%. The mixture was stirred overnight at 4°C and then centrifuged at 10 000 *g* for 1 h at 4°C. The pellet was resuspended in 10 ml of 10X Dulbecco's phosphate buffered saline (PBS) (Euroclone Ltd., Wetherby, UK). The mixture was stirred for 10 min at room temperature and centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant was collected, and PEG-NaCl solution was added (1 : 4). The mixture was stirred overnight at 4°C and then centrifuged at 10 000 *g* for 45 min at 4°C. The pellet was resuspended in 3 ml of PBS and centrifuged at 10 000 *g* for 10 min at 4°C. One and a half millilitres of the solution were extracted with 30% chloroform and centrifuged at 3000 *g* for 5 min. The aqueous phase was transferred to a new vial, whereas the remaining phase was extracted with 500 $\mu$l of cell-culture medium [Eagle minimum essential medium (EMEM) with Earle's Salts (Euroclone)] and centrifuged at 3000 *g* for 5 min. The extracts were then combined, extracted with 30% chloroform, and centrifuged at 3000 *g* for 5 min. The supernatant was stored at −20°C prior to use.

### RT-nested-PCR

RT-nested-PCR was performed as described previously (Croci *et al*. 1999), [30 PCR cycles, each consisting of 25 s at 95°C, 30 s at 49°C, and 1 min at 70°C, used for both the first and second (nested) PCR]. The primers were selected on the basis of regions conserved among the HAV strains, HM 175 (GenBank accension no. M14707; Cohen *et al*. 1987), L.A. (K02990; Najarian *et al*. 1985), MBB (M20273; Paul *et al*. 1987), GBM (X75214; Graff *et al*. 1994) and FG (X83302; Beneduce *et al*. 1995). The primers (Table 1) were checked for cross-reactivity with other enteric viruses.

### Integrated method (cell-culture RT-PCR)

Samples found to be positive for the presence of viral RNA were subjected to cell culture RT-PCR using FRhK-4-derived Frp3 cells (Venuti *et al*. 1985). Cultures of Frp3 cells were grown with EMEM (Imperial, UK) supplemented with 10% Fetal Bovine Serum (FBS) (Imperial) at 37°C and in 5% $CO_2$ in 25-$cm^2$ flasks for 3 d. To one ml of mussel extract, 100× antibiotics – antimycotic (Imperial) solution (1 : 100 v/v) was added and stored at 4°C overnight. The same amount of antibiotics – antimycotic (Euroclone Ltd.) solution was then added, and the sample was maintained at 37°C for 2 h. This solution was then used to inoculate the cell monolayer, leaving it in contact for one hour at 37°C and 5% $CO_2$. The monolayer was then washed three times with 2 ml of EMEM to eliminate all of the virus not infecting the cells; 5 ml of EMEM, supplemented with 2% FBS and 1 mmol $l^{-1}$ guanidine-HCl (Siegl and Eggers 1982), was then added and incubation was performed at 37°C and in 5% $CO_2$. After 15 d, the monolayer was subjected to three cycles of freezing thawing; after centrifugation at low speed (2000 *g* for 5 min), supernatants were used for RT-PCR to confirm the presence of infectious virus. RT-PCR was performed using primers 1 and 2 (Table 1) and the above-described amplification conditions.

### Sequencing

Those samples that were shown to be positive for viral RNA at screening, but in which infectious virus was not observed,

**Table 1** HAV primer sequences used for PCR and nested PCR

| Oligonucleotide* | Sequence | Position† |
|---|---|---|
| Primer 1 | 5′-CATATGTATGGTATCTCAACAA-3′ | 1092–1113 |
| Primer 2 | 5′-CAGGGGCATTTAGGTTT-3′ | 698–714 |
| Primer 3 | 5′-CCAATTTTGCAACTTCATG-3′ | 1029–1047 |
| Primer 4 | 5′-TGATAGGACTGCAGTGACT-3′ | 836–854 |

*Primers 1 and 2 used for PCR; primers 3 and 4 used for nested PCR.
†Position refers to the HM-175 HAV strain (Cohen *et al*. 1987).

**Table 2** Number of samples positive to RT-nested-PCR and cell-culture RT-PCR

| Sample | Sample size | Positive to RT-nested-PCR | | Confirmed with integrated method | |
|---|---|---|---|---|---|
| | | N° | % | N° | % |
| Mussels | 89 | 30* | 33·7 | 12 | 13·5 |
| Other molluscs | 53 | 19 | 35·8 | 6 | 11·3 |
| Total | 142 | 49 | 34·5 | 18 | 12·7 |

*One sample was positive to RT-PCR (one amplification cycle), but it was not positive for the presence of infectious virus.

were subjected to sequencing in order to exclude false positives. The samples were cloned into vector PCR II (TA-Cloning System Invitrogen Co., Carlsbad, California, USA) and sequenced (Sanger *et al*. 1977) using the Sequenase PCR Product kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden) to confirm their identity.

## RESULTS

The results obtained using RT-nested-PCR and cell-culture RT-PCR are shown in Table 2. Of the 142 samples analysed by RT-nested-PCR (89 mussels and 53 other bivalve molluscs), 49 (34·5%) were positive for HAV-RNA and 93 (65·5%) were negative. Of the positive samples, 18 (12·7% of the total samples) showed the presence of infectious virus when examined using the integrated method. HAV-RNA was detected after the nested phase in all but one of the positive samples, in which the quantity of viral RNA was high enough, was detected after a single amplification cycle (RT-PCR) and no infectious virus was detected in this sample.

## DISCUSSION

The results of this study demonstrate that in molluscs, as previously shown in artificial seawater (Arnal *et al*. 1998), HAV-RNA is more persistent than infectious virus. These results, when considered together with those of studies that have shown a high fragility of free genomic RNA (Tsai *et al*. 1995), suggest that the detected viral RNA originated from viral particles whose capsids were partially denaturated yet nonetheless capable of protecting the RNA from degradation. However, this denaturation apparently does not allow the virus to continue to be infectious.

The results also confirm that only those methods for which a second, nested amplification can be performed are sensitive enough to detect viral RNA in molluscs, where enteric viruses are expected to be present in lower numbers (De Medici *et al*. 1998). Even concentrations of viral RNA sufficiently high to be revealed after the first phase of amplification (i.e. RT-PCR) are not proof of the presence of infectious virus. Although the presence of viral RNA does not necessarily indicate the presence of an immediate health-risk for the consumer, it does indicate that the molluscs originated from a contaminated environment. Thus all of the necessary measures for determining and controlling the cause of contamination or for intensifying the control of lots of mussels from the same origin should be adopted.

To determine the safety for human consumption, the results of RT-nested-PCR must be confirmed with an integrated cell-culture PCR method. Only the capacity to grow on cell cultures provides the necessary evidence that viruses are competent to perform many of the cyto-infective processes required to cause human infection (Richards 1999; Lees 2000). Efforts should thus be made to render this method as rapid as possible, as proposed for detecting enteroviruses in water (Reynolds *et al*. 1996). With regard to the use of this method for detecting other enteric viruses, this is not yet possible for certain enteric viruses. For example, human caliciviruses cannot be cultivated (Green 1997) and further studies will be needed to overcome this limitation.

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