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Detection of hepatitis A virus in mussels from different sources marketed in Puglia region (South Italy)

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Abstract

Hepatitis A virus (HAV) infection is endemic in Puglia (South Italy). Epidemiological studies indicate that shellfish consumption, particularly mussels, is a major risk factor for HAV infection, since these products are eaten raw or slightly cooked. Nested reverse transcriptase-polymerase chain reaction (RT-PCR) has been shown to be a sensitive technique for the detection of HAV in mussels. The aim of the present study was to detect the presence of HAV in a large sample of mussels by nested RT-PCR and to confirm the presence of infectious viral particles in positive samples by cell culture infection and RT-PCR confirmation. Two hundred and ninety samples of mussels from different sources were collected between December 1999 and January 2000. One hundred samples were collected before being subjected to depuration, 90 after depuration, and 100 were sampled in different seafood markets. HAV-RNA was detected in 20 (20.0%) of non-depurated mussels, in 10 (11.1%) of depurated samples, and in 23 (23.0%) of samples collected in the shellfish markets, without any significant difference in the prevalence of positive samples by collection sources ($\chi^2 = 4.79$, $p = 0.09$). Of the 53 samples found positive by nested RT-PCR, 18 (34.0%) resulted positive by cell culture assay. No relationship between viral contamination and bacterial contamination was found ($p = 0.41$). This study confirms the usefulness of molecular techniques in detecting HAV in shellfish and, thus, for the screening of a large sample of naturally contaminated mussels. Improved shellfish depuration methods are needed to obtain virus-safe shellfish and reduce the risk for public human health.

Keywords: HAV; Mussels; Depuration; Nested RT-PCR; Cell culture assay

1. Introduction

Viral hepatitis A represents a significant public health problem in many countries due to the persistent circulation of the virus in the environment and the possible contamination of water and food.

In industrialized countries, the improved hygienic and sanitary conditions, and the application of public health measures have led to a decline in the incidence rate of hepatitis A and to a shift of the infection towards adulthood.

In Italy, in recent years, there has been a marked reduction of the incidence of hepatitis A virus (HAV) infection (Zanetti et al., 1994; Stroffolini et al., 1993;

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Coppola et al., 1998) although several outbreaks have been described (Stroffolini et al., 1990; Divizia et al., 1993; Mele et al., 1994). In southern Italy, and particularly in Puglia, HAV infection still shows an intermediate level of endemicity with annual incidence rates up to 30 cases/100,000 and recurrent outbreaks (Germinario et al., 2000). In particular, in 1996 and 1997, two large outbreaks of hepatitis A occurred in Puglia with 5673 and 5382 cases, respectively, and incidence rates of 138 and 132 cases/100,000 (Malfait et al., 1996; Lopalco et al., 1997).

Epidemiological studies indicate that shellfish consumption in Italy is a major risk factor for HAV infection, since these products are eaten raw or slightly cooked (Mele et al., 1989, 1997). This has contributed to public concern about shellfish safety.

In the European Union, the directive that regulates the sale of molluscs (European Directive of Council 91/492/CEE 1991) (Anonymous, 1991) only specifies bacteriological parameters (i.e., *Salmonella* and *Escherichia coli*) for determining the suitability of molluscs for consumption, yet the absence of bacteria does not necessarily signify the absence of viruses (Goyal et al., 1979; Wait et al., 1983). Even bacteriophage detection, 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 viruses (Gerba, 1979; Jofre, 1992; Croci et al., 2000).

Depuration is the common method used to obtain marketable shellfish. However, this method is inadequate for complete virus elimination, even if performed with advanced systems (De Medici et al., 2001a). In fact, the viruses may persist in the shellfish for several days even after they have been transferred to clean water and fecal coliform bacteria are eliminated (Sobsey et al., 1988).

Therefore, there is a growing interest in indicators of human-specific viral fecal pollution. Nucleic acid-based techniques, especially reverse transcriptase-polymerase chain reaction (RT-PCR), have emerged rapidly as methods of choice for sensitive and specific detection of enteric viruses (RNA viruses) other than traditional virological methods such as cell culture (Le Guyader et al., 1994; Lees et al., 1995; Cromeans et al., 1997; Lopez-Sabater et al., 1997).

Nevertheless, the low concentrations of virus and the presence of inhibitors of amplification reaction render the detection of viral genome in samples of complex composition such as shellfish difficult. In addition, the choice of a suitable method for RNA extraction ensuring an adequate viral recovery and the elimination or inactivation of inhibitory substances is also crucial for a successful detection of viral genomes (Arnal et al., 1999).

Recently, a sensitive and specific method allowing the identification of HAV in mussels at a concentration as low as 1 $TCID_{50}$/10 g of mollusc has been reported (Croci et al., 1999a; Fiore et al., 2000).

A limit of the molecular approach in detecting HAV in shellfish is that PCR is not able to discriminate between infectious and noninfectious viral particles (Richards, 1999). Therefore, it could be important to evaluate the presence of infectious virus in shellfish to assess the real risk of infection after shellfish consumption.

The aim of the present study was to determine the presence of HAV in a large sample of naturally polluted mussels from different sources collected in Puglia (South Italy) by nested RT-PCR and to confirm the presence of infectious viral particles in positive samples by cell culture infection and RT-PCR confirmation.

2. Materials and methods

2.1. Collection and storage of the samples

Naturally contaminated mussels (*Mytilus galloprovincialis*) were collected between December 1999 and January 2000. This period was chosen since there is a wider consumption of mussels in Puglia during these 2 months, particularly during Christmas holidays, and most of them are imported from other European countries (principally Greece and Spain). In addition, epidemiological data suggest that in this period, consumption of raw seafood is the most implicated risk factor in HAV infection since most of the cases are notified at the beginning of the year with a peak of incidence in February (Malfait et al., 1996). A total of 290 samples of mussels, each consisting of about 500 g, were collected. One hundred samples were obtained from commercial producers before being subjected to depuration. In particular, 70 samples of these were from Greece, 20 from Spain, and 10 from a shellfish-

growing site in Puglia (Taranto). Ninety samples were collected soon after depuration before being sold for human consumption (70 of them were from Greece and 20 from North Italy). The samples, before depuration, were not the same taken after depuration. The remaining 100 samples were collected in different shellfish markets of the region but no information about the provenience were available.

The samples were stored at 4 °C and transported to the laboratory. They were immediately processed and bacteriological analysis was performed within 24 h. Processed samples were stored at −20 °C and later used for HAV testing. The mussels were washed and scrubbed thoroughly in running water and opened aseptically. The bodies and intervalve water, collected from 500 g of mussels, were homogenized in a Waring blender at maximum speed for 5 min. Samples were then divided into 10 g aliquots and stored at −20 °C until use. An aliquot of fresh homogenate was used for bacteriological analysis.

2.2. Bacteriological analysis

Analysis of fecal coliforms (FCs) and *E. coli* in mussels was performed by the most probable number (MPN) method in liquid broth, and values obtained according to published tables (Anonymous, 1995). Values above acceptable levels (300 FCs/100 g and 230 *E. coli*/100 g) were considered too contaminated for human consumption (Anonymous, 1993). In addition, *Salmonella* was detected using the technique indicated in the previously specified procedures.

2.3. Virus extraction and concentration

Ten grams of homogenate were thawed at room temperature and then diluted (1:2, w/v) in glycine buffer (Sigma, St. Louis, MO) 0.05 M at pH 9.2. The mixture was stirred for 30 min at room temperature, centrifuged for 15 min at 10,000 × *g* at 4 °C, and the aqueous phase was harvested. Polyethylene glycol (PEG 8000, Sigma) as 50% (w/v) solution in 7.5% NaCl was added to a final concentration of 12.5% to the aqueous phase. The mixture was stirred overnight at 4 °C and then centrifuged at 10,000 × *g* for 1 h at 4 °C. The PEG-containing supernatant was discarded, the pellet was suspended in 5 ml of phosphate-buffered saline (Gibco BRL/Life Technologies, Rockville) and then centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was collected and PEG–NaCl solution was added to a final concentration of 12.5%. The mixture was stirred overnight at 4 °C and then centrifuged at 10,000 × *g* for 45 min at 4 °C. The supernatant was discarded, the pellet was accurately suspended in 1.5 ml of phosphate-buffered saline, and then stirred for 10 min at room temperature. After centrifugation at 10,000 × *g* at 4 °C for 15 min, the supernatant was harvested and stored at −20 °C until testing for RT-nested PCR and if positive at the RNA-HAV presence for integrated method.

2.4. Viral RNA extraction and purification

Viral RNA was extracted from supernatant by a GITC-silica gel-based method using RNeasy kit (Qiagen, Hilden, Germany) according to the protocol supplied with the kit with minor modifications. Briefly, 1 ml of a GITC lysis buffer was added to 250 μl of supernatant and incubated at room temperature for 10 min. The silica gel–RNA complexes were then washed, in turn, with GITC buffer and 75% ethanol. RNA was eluted with 45 μl sterile RNAse-free water.

2.5. RT-PCR assay

RT-PCR primers were derived from HAV conserved sequence as previously described (De Medici et al., 2001b). The 22 nucleotide primer 1: 5′ -CAT ATG TAT GGT ATC TCA ACA A-3′ (1084–1063) [position refers to the FG HAV strain; Beneduce et al., 1995] and the 17 nucleotide primer 2: 5′ -CAG GGG CAT TTA GGT TT-3′ (669–685), yielded a predicted 415 bp RT-PCR product.

The superscript one step RT-PCR system (Gibco BRL/Life Technologies) was used for the detection of HAV. RT-PCR was carried out in a volume of 50 μl containing 23 μl of total RNA extract from the mussel tissue, 1X PCR buffer, 0.1 μM each of primers 1 and 2, 1.5 mM $MgSO_4$, 10 U RNaseout, 200 μM of each dNTP, 1 μl of enzyme blend superscript II reverse transcriptase and Taq DNA polymerase. Amplification conditions were provided by 30 min reverse transcription at 50 °C, denaturation for 2 min at 94 °C, and then 35 amplification cycles (denaturation at 94 °C for 15 s, annealing at 49 °C for 30 s, and

extension at 70 °C for 45 s). Finally, a 5-min extension cycle at 70 °C was performed on a 2400 Perkin Elmer thermal cycler. Two negative and one positive controls were employed in each batch of experiments. As positive control, an aliquot of the supernatant obtained from mussel tissues seeded with HAV at a concentration of 10 $TCID_{50}$/10 g of homogenate was used during the RNA extraction phase and amplification to confirm the absence of inhibitors for PCR.

2.6. Nested PCR

For the nested PCR amplification, the 17 nucleotide primer 3: 5′ -CCA ATT TTG CAA CTT CAT G-3′ (1029–1047) and the 19 nucleotide primer 4: 5′ -TGA TAG GAC TGC AGT GAC T-3′ (836–854) were used.

Amplified DNA (2.5 μl) from the first PCR reaction was added to a new batch of 50 μl of reaction mixture containing 1X PCR buffer (Gibco BRL/Life Technologies), 1.0 mM $MgSO_4$, 200 μM of each dNTP (Boehringer Mannheim, Indianapolis, USA), 0.1 μM each of primers 3 and 4, and 1.25 U of Platinum Pfx DNA polymerase (Gibco BRL/Life Technologies). The reaction mixture was then subjected to a denaturation step of 2 min at 94 °C and to 35 PCR cycles each consisting of 15 s at 94 °C, 30 s at 49 °C, and 45 s at 68 °C, followed by a final extension of 5 min at 68 °C. This amplification step yielded a 211 bp product.

In order to reduce the possibility of sample contamination by DNA amplified molecules, standard precautions were applied in all the manipulations. Separate areas were used for reagents, samples, and amplified samples.

2.7. Detection of amplified products

Amplified products were analysed by electrophoresis on a 1.2% agarose gel (Invitrogen BV, Groningen, NH) and visualized under ultraviolet rays.

2.8. Integrated method (cell-culture/RT-PCR)

The samples found to be positive for the presence of viral RNA were subjected to cell culture RT-PCR using Frp3-cell culture as previously described (De Medici et al., 2001a). Frp3-cell culture was grown with EMEM (Imperial) supplemented with 10% Fetal Bovine Serum (FBS) (Imperial) at 37 °C for 3 days. To 1 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) solution was added, and the sample was maintained at 37 °C for 2 h. This solution was used to inoculate the cell monolayer, leaving it in contact for 1 h at 37 °C and 5% CO_2. The monolayer was washed three times with 2 ml of EMEM to eliminate all of the virus not infecting the cells. Five ml of EMEM, supplemented with 2% FBS and 1 mmol l^{-1} guanidine–HCl (Siegl and Eggers, 1982) was added and incubation was performed at 37 °C and in 5% CO_2. After 15 days, 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 and the above described amplification conditions.

2.9. Statistical analysis

Data were processed using the software package Epi.Info (version 6.04c). Statistical evaluation was performed using χ^2-test and Fisher's exact test where appropriate. If a *p*-value was below 0.05, the difference between proportions was considered statistically significant.

3. Results

Table 1 shows the virological and bacteriological results of 290 mussel samples collected in Puglia. Fifty-three (18.2%) of the samples resulted positive by nested RT-PCR and 34.0% (18/53) of these were positive for the presence of live and infectious virus. HAV-RNA was found in 20 out of 100 non-depurated samples (20.0%), 35.0% (7/20) of positive samples, and 7.0% of the non-depurated samples were confirmed to have infectious virus by cell culture assay. The majority of non-depurated samples (70) were imported from Greece and 17 (24.2%) tested positive for HAV by nested RT-PCR. The 29.4% (5/17) of contaminated mussels equal to 7.1% of all non-depurated samples from Greece also showed infectious virus. None of the samples from Spain was found to be contaminated with

Table 1
Survey of HAV in 290 mussel samples from different sources marketed in Puglia (South Italy)

Samples (*from*)	Total number	Positive by nested RT-PCR		Positive by cell culture assay		FCs[a] > 300/100 g		*E. coli* > 230/100 g	
		No.	%	No.	%	No.	%	No.	%
Non-depurated	100	20	20.0	7	7.0	2	2.0	2	2.0
Greece	*70*	*17*	*24.2*	*5*	*7.1*	*1*	*1.4*	*1*	*1.4*
Spain	*20*	*0*	*0.0*	*0*	*0.0*	*0*	*0.0*	*0*	*0.0*
Italy (Taranto)	*10*	*3*	*30.0*	*2*	*20.0*	*1*	*10.0*	*1*	*10.0*
Depurated	90	10	11.1	4	4.4	3	3.3	0	0.0
Greece	*80*	*8*	*10.0*	*3*	*3.7*	*2*	*2.5*	*0*	*0.0*
Italy (North)	*10*	*2*	*20.0*	*1*	*10.0*	*1*	*10.0*	*0*	*0.0*
Collected in seafood markets	100	23	23.0	7	7.0	4	4.0	0	0.0
Total	290	53	18.2	18	6.2	9	3.1	2	0.7

[a] Fecal coliforms.

HAV, whereas 3 out of 10 samples (30%) from Taranto (Puglia) showed positive for HAV viral RNA and two (20%) of all samples from Taranto were positive by cell culture. Of the 90 samples collected after depuration, 10 (11.1%) contained viral RNA. The 40.0% (4/10) of mussels resulted positive by nested RT-PCR, equal to 4.4% of all depurated samples, showed infectious virus. Ten percent (8/80) of depurated samples from Greece and 20.0% (2/10) of the samples from North Italy were positive by nested RT-PCR. The 37.5% (3/8) of Greek depurated mussels positive for HAV-RNA showed infectious virus (3.7% of total number), while the prevalence of infectious virus among depurated samples from North Italy was 10.0%. Twenty-three percent of mussel samples collected in seafood markets were positive for HAV and 30.4% (7/23) of these were positive by cell culture assay (7.0% of all samples from seafood markets). No significant difference was found in the prevalence of viral RNA in mussel samples by source (non-depurated, depurated, and collected in seafood markets; $\chi^2 = 4.79$, $p = 0.091$). Nevertheless, an increasing prevalence of positive samples for HAV was observed in mussels collected in seafood markets in comparison with those collected after depuration. In addition, a statistically significant difference ($\chi^2 = 5.49$, $p = 0.019$) in the prevalence of positive samples by nested RT-PCR emerged between samples from Greece before and after depuration, although the samples taken before and after depuration were not the same. No difference emerged when considering the prevalence of positive samples with respect to sources (non-depurated, depurated, and taken from the markets) by cell culture assays ($p = 0.926$).

The data from bacteriological analyses show that only 3.1% and 0.7% of the total mussel samples had fecal coliforms and *E. coli*, respectively, above the limits established by European directive (300 FCs/100 g and 230 *E. coli*/100 g). Bacterial contamination was lower than the standard for 52 (98.1%) of 53 samples contaminated with HAV. No significant difference in the prevalence of bacterial contamination, above the standards, with respect to the collection site of the samples was detected ($p = 0.708$). None of the mussel samples tested showed the presence of *Salmonella*.

4. Discussion

Detection of viral pathogens epidemiologically linked to shellfish-associated viral diseases is of primary importance to assess the potential hazard for public health. A variety of methods have already been reported regarding shellfish processing for detection of viral RNA (Le Guyader et al., 1994; Atmar et al., 1995; Lopez-Sabater et al., 1997; Croci et al., 1999a). Several studies have demonstrated the successful application of PCR to the detection of the virus in shellfish artificially contaminated in the laboratory. However, few studies have been reported on the application of molecular methods for detection of HAV in naturally polluted shellfish (Desenclos et al., 1991; Le Guyader et al., 1994, 2000; Croci et al.,

1999a; Lee et al., 1999), the majority of them consisting of small surveys.

The use of nested RT-PCR for HAV detection on mussel extracts was suggested because of its sensitivity, since low concentrations of virus are expected in mussels (Severini et al., 1993; Hafliger et al., 1997; Muniain-Mujika et al., 2000).

In our study, the screening of 290 naturally polluted mussel samples for HAV, with the nested RT-PCR, revealed that the level of contamination found (18.2%) seems to be slightly higher than that previously reported in other studies (Chung et al., 1996; Lee et al., 1999; Le Guyader et al., 2000). All positive samples, except one, met the microbiological standard of less than 300 FCs or 230 *E. coli* in 100 g of shellfish. Therefore, our results confirm previous laboratory findings indicating a lack of correlation between fecal contamination and the presence of viral pathogens in shellfish (Gerba, 1979; Richards, 1985; Jofre, 1992; Lee et al., 1999; Croci et al., 2000). A relevant finding was the presence of hepatitis A viral RNA in 24.2% of samples imported from Greece before tank-based depuration, indicating that these mussel samples came from virus-polluted harvesting areas, but only one sample showed unacceptable fecal contamination indicators, in compliance with the European Directive. On the contrary, none of the samples coming from Spain (although a small number) showed viral contamination while the rest of the samples obtained from local commercial producer (Taranto, Puglia) were heavily polluted (30.0%). Concerning the purified mussel samples, a remarkable reduction in the percentage of contaminated mussels, especially those from Greece, was observed after the depuration treatment. It has been found that depuration decreases the viral load but may still fail to fully remove HAV (De Medici et al., 2001a). Another notable point regards the increased prevalence (23.0%) of HAV-RNA found in mussels collected in seafood markets in comparison with depurated samples. A possible explanation of this result could be related to the habit of seafood dealers of keeping seafood alive in seawater taken from probable contaminated sources, although this practice is prohibited. This aspect has already emerged in previous studies (Malfait et al., 1996; Lopalco et al., 1997). In addition, since there is a greater demand for seafood during Christmas holidays in Puglia, it is probable that the depuration period is shortened and, thus, inadequate or that non-depurated shellfish are sold in seafood markets in this period.

Nevertheless, it has been pointed out that the use of RT-PCR does not differentiate between infectious and noninfectious viruses since amplified nucleic acid could originate either from viable virus or damaged noninfectious virus and require additional confirmation (Reynolds et al., 1996; Richards, 1999; Lees, 2000). Hence, we investigated the presence of live virus in mussels, subjecting all samples that tested positive by PCR screening to a cell culture assay and detecting the presence of HAV in culture supernatant by RT-PCR. This integrated method was applied since even virus not capable of producing the cytopathic effect, because present in very low concentration, can be detected. In our study, about one-third of mussels positive for the presence of HAV-RNA showed to be contaminated by live virus (6.2% of all samples). Previous reports have shown a correlation between the detection of viral RNA by PCR and the detection of infectious particle (Kopecka et al., 1993; Graff et al., 1993).

The detection of HAV in mussels marketed in Puglia is of some concern. Our findings strongly support the hypothesis that such products are an important source for hepatitis A cases in Puglia as emerged in case control studies (Malfait et al., 1996) and can explain the advent of the large epidemics, which periodically occur in this region (Germinario et al., 2000). In fact, shellfish are largely consumed especially during Christmas holidays in this region and they are commonly eaten raw or slightly cooked. Only a drastic heat treatment can assure a complete inactivation of virus (Croci et al., 1999b) and only thorough cooking of shellfish can reduce the risk to human health. On the other hand, the presence of HAV-RNA and live virus in purified mussels demonstrates that depuration as currently practised cannot guarantee consumer protection. Therefore, continuous surveillance of seafood products in the markets is advisable. However, in order to prevent shellfish-associated viral diseases, better systems for the monitoring and control of viral contamination on a routine basis are needed.

Current molecular techniques allow the detection of HAV in the environment and in food. Our findings confirm that the use of an integrated nested RT-PCR screening cell culture assay procedure is useful to detect mussel samples containing live viruses. Further

advances in the development of more standardized and less expansive methods, also for quantitative viral detection in shellfish, are still necessary.

Finally, molecular epidemiological investigations should clarify the relationship between mussel HAV strains and viral strains isolated from human cases from this region.

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