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## Research Note

# Identification and Sequence Analysis of Hepatitis A Virus Detected in Market and Environmental Bivalve Molluscs

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## ABSTRACT

In Italy in 1998, hepatitis A virus (HAV) was responsible for an infectious disease transmitted by contaminated bivalve molluscs. To determine the presence of HAV in the bivalves collected during a 1-year follow-up study, hepatitis A RNA was extracted and amplified by a nested reverse transcriptase–PCR method overlapping the VP1/2A region. The HAV genome was detected in 24 (14.1%) of 170 samples: 19 clams (*Tapes decussates* and *Tapes semidecussatus*), 1 oyster (*Crossostea gigas*), and 4 mussels (*Mytillus galloprovincialis*). Eleven positive samples were collected from marketing areas, and 13 positive samples were collected from growing areas. Seventeen of the 24 positive samples had been taken from domestic products, and 7 had been imported. Sequence analysis showed the presence of genotypes IA and IB. Our results suggest significant presence of HAV in bivalves from both marketing (public consumption) and environmental (growing) areas.

Hepatitis A virus (HAV) belongs to the *Picornaviridae* family *(24)*; it is known to cause acute (but not chronic) hepatitis. HAV can be transmitted by the oral–fecal route and can thus be spread in the aquatic environment as well as through the contamination of food, such as bivalves *(11, 22, 31)*.

HAV has a single serotype, and infection with HAV causes lifelong immunity. HAV is endemic to Italy and the other Mediterranean countries, with an average annual incidence of two to three cases per 100,000 persons. During the Apulia outbreak in 1998, however, the incidence of HAV increased to 10 to 12 cases per 100,000 persons. Epidemiological analyses performed during this outbreak indicated that the consumption of raw seafood was the major risk factor *(7, 13, 29)*.

In developing countries, the infection is acquired in childhood and usually causes an asymptomatic disease. In industrialized countries, however, improvements in social and hygienic conditions have led to a decline in the incidence of the disease and a subsequent shift toward the disease being observed in the adult population for the first time, accompanied by an increase in cases requiring hospitalization. Bivalves, including mussels, clams, and oysters, are filter-feeding molluscs and, for this reason, have been implicated as vectors of human pathogens *(9, 10)*. In Italy, according to data from the Surveillance System for Acute Viral Hepatitis *(29)*, between 50 and 60% of clinical HAV infections are caused by the consumption of raw or partially cooked bivalves *(21)*. European and Italian laws that are used to classify growing and harvesting areas for bivalves are divided into three areas that meet the following microbiological standards: (i) the category A standard encompasses areas from which fecal coliforms in molluscs are present in <300 most probable numbers (MPN) per 100 g of mollusc flesh and intravalvular liquid, and *Escherichia coli* is present in <230 MPN/100 g of mollusc flesh and intravalvular liquid; (ii) the category B standard encompasses areas from which fecal coliforms in molluscs are present in levels that are <6,000 organisms per 100 g, and *E. coli* is present in levels that are <4,600/100 g of mollusc flesh and intravalvular fluid; and (iii) the category C standard encompasses areas from which fecal coliforms in molluscs are present in levels that are <60,000 organisms per 100 g. If the molluscs are collected from marketing areas (human consumption), they must meet the category A standards; however, these molluscs must not contain any detectable *Salmonella* in excess 25 g of mollusc flesh. The three parameters described above are the only parameters used to evaluate the hygienic quality of bivalves and their growing areas *(1–3, 14, 15)*. They do not address the presence of human enteric viruses *(26, 30)*.

A nucleic acid–based method, such as reverse transcriptase–PCR (RT-PCR), is highly specific and can detect very small amounts of viral particles in mussel homogenates *(18, 20, 23)*. In recent years, RT-PCR has been applied to detect both cultivable and noncultivable viruses *(17, 28)*. However, the successful application of molecular methods to identify enteric viruses has been limited by the presence of RT-PCR inhibitors in shellfish bodies. Several methods for the extraction of viral genomes have been re-

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ported in the scientific literature *(5, 20)* that avoid the carryover of nonspecific inhibitors. Dissections of the digestive tract and diverticula (hepatopancreas) appear to reduce the presence of inhibitors and to increase the sensitivity of such molecular methods *(4)*.

To evaluate the presence of HAV in central Italy, we collected and tested 170 samples of clams (*Tapes decussates* and *Tapes semidecussatus*), mussels (*Mytillus galloprovincialis*), and oysters (*Crossostea gigas*) by RT-PCR to detect the presence of viral genomes. The amplified product of each positive sample was sequenced to perform genomic evaluation.

## MATERIALS AND METHODS

**Sample processing for virological analysis.** Clams (*T. decussates* and *T. semidecussatus*), mussels (*M. galloprovincialis*), and oysters (*C. gigas*) were collected by the IZSLT (''Zooprofilattico'' Institute of Latium and Tuscany Regions) and immediately frozen.

While these shellfish were frozen, 15 g of digestive apparatus (hepatopancreas) was removed from several of these bivalves by dissection. A total of 75 ml (ratio = 1:5) of 0.05 M glycine, pH 9.5 (Merck, Milan, Italy), was added to the hepatopancreas, and the sample was homogenized at maximum speed for 5 min in a Waring blender. The homogenate was immediately purified by centrifugation (10,000 $\times$ *g* for 15 min at 4°C); then, the supernatant fluid was collected, the pH was adjusted to 7.2 to 7.4, and the supernatant was concentrated by precipitation with the final addition of 10% (wt/vol) polyethylene glycol 6000 (Merck) and 0.3 mol/liter NaCl (Merck). The precipitated viruses were recovered by centrifugation at 10,000 $\times$ *g* for 45 min, and the pellet was resuspended in the smallest volume of sterile phosphate-buffered saline (PBS; Gibco, Milan, Italy). After a one-part sample: two-parts chloroform extraction, the virus in its aqueous phase was further purified by ultracentrifugation at 60,000 $\times$ *g* for 1 h at 4°C in a Beckman Optima TL-100 ultracentrifuge with a TLA 100.4 rotor (Beckman Instruments, Fullerton, Calif.). The pellet was collected in sterile PBS (Gibco) and divided into two equal aliquots.

**Viruses and cell cultures.** A positive HAV virus control was prepared from infected cells using a laboratory strain. HAV (obtained from A. Bosch, University of Barcelona, Spain), cytopathic strain HM175, was propagated in an FrhK cell line. The cell lysate was clarified by centrifugation at 2,500 $\times$ *g* for 15 min at 4°C, and the raw supernatant was used as a positive control.

**Bacteriological analyses.** All of the bacteriological analyses of fecal coliforms, *E. coli*, and *Salmonella* were performed according to the method accepted by the International Organization for Standardization *(14, 15)* and Italian law *(1–3)*, as well as in accordance with the standard required by the National System for Accreditation of Laboratories (SINAL) in conformity with the European ISO standard UNI CEI EN ISO/IEC 17025.

**Molecular methods.** One of the two aliquots of the ultracentrifuged virus was extracted by the QIAmp viral RNA kit (Qiagen, Milan, Italy). One fifth of the final elution volume, corresponding to 1.5 g, was used for the RT-PCR test.

The primers that were used to amplify the 317-nucleotide fragment of the overlapping VP1/2A junction region of HAV have previously been described *(12)*. All of the enzymes were obtained from Promega (Milan, Italy).

TABLE 1. *Distribution of HAV-positive samples by RT-PCR*[a]

| | | Samples | HAV positive (%) |
|---|---|---|---|
| Samples | Clam | 73 | 19 (26.0)[b] |
| | Mussel | 75 | 4 (5.3) |
| | Oyster | 22 | 1 (4.5) |
| Total | | 170 | 24 (14.1) |
| Origin | Domestic | 120 | 17 (14.1) |
| | Imported | 41 | 7 (17.5) |
| Total | | 161 | 24 (14.9) |
| Source | Market | 88 | 11 (12.3)[c] |
| | Area A | 34 | 4 (11.7) |
| | Area B | 27 | 6 (22.2) |
| | Monitoring | 72 | 13 (17.8)[d] |
| | Area A | 67 | 11 (16.4) |
| | Area B | 3 | 0 (0) |
| Total | | 160 | 24 (15.0) |
| Bacteriological limits | Less than | 99 | 10 (10.1) |
| | Greater than | 30 | 8 (26.6) |
| Total | | 129 | 18 (13.9) |

[a] Some data were missed.
[b] The statistical analysis shows significant value: $P < 0.01$.
[c] Four samples were collected from area A, and six were collected from area B; for one sample the data were not available.
[d] Eleven samples were collected from area A; for two samples, the data were not available.

**Sequence analysis of the VP1/2A junction region of HAV.** The amplified products were purified with the QIAgen PCR purification kit and sequenced by the Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 2.0, using 0.8 ng of sequencing mix per bp. The sequencing was performed in an ABI Prism DNA Sequencer (Perkin-Elmer, Norwalk, Conn.).

## RESULTS

A total of 170 samples of bivalves were collected from areas that fit the above-described legal classification system with respect to marketing and environmental areas.

Overall, in our study, 24 (14.1%) of the 170 samples were scored HAV positive: 19 (26.0%) clams (*T. decussates* and *T. semidecussatus*), 4 (5.3%) mussels (*M. galloprovincialis*), and 1 (4.5%) oyster (*C. gigas*). Statistical analysis as performed by the chi-square test showed a significant association between HAV presence and clams ($P < 0.01$); however, a 1-year study with respect to the temporal distribution of HAV-positive samples did not show a seasonal variation. A total of 17 (14.1%) samples were collected from domestic products, and 7 (17.5%) samples were collected from products that had been imported from different European countries. Thirteen positive samples (17.8%) were collected from environmental (growing) areas, whereas 11 (12.3%) were collected from marketing areas. HAV was detected in 15 of the 24 samples from areas previously classified as category A, and 6 of the 24 samples were collected from areas classified as category B. For the other three positive samples, no data were available about A- or B-harvesting area classification (Table 1).

The bacteriological analysis of shellfish bodies showed acceptable values, according to the European Community,

in 10 (10.1%) samples; for 8 (26.6%) samples, the values exceeded the European limits; for 6 samples, the bacteriological data were not available.

The presence of HAV was determined by amplification of the VP1/2A region. Twenty-one of 24 amplicons were sequenced, and the nucleotide sequences were aligned by ClustalW software, whereas for the three other amplicons, the sequences were not determined because of the small amount of amplified product. With regard to the genotype classification, 4 amplicons were classified as genotype IA, and 17 were classified as genotype IB. Four isolates of the genotype IB were obtained from Greece and showed identical sequences and 100% homology in the VP1/2A region with the isolate IT-CAP-00, identified by Chironna et al. *(7)* in the Apulia region. The alignment of genotype IA sequences showed eight point mutations, but only one caused an amino acid (AA) substitution in position 25 of the 56 AAs of the VP1/2A region. In contrast, the genotype IB sequences presented several point mutations, which caused 6 AA substitutions in positions 2, 3, 4, 15, 25, and 51 in the VP1/2A region. The AA substitution in position 3 was present in 14 of 17 amplicons.

## DISCUSSION

Detection of viral pathogens can be performed by several methods, but cell culture, at present, represents the only method for isolating infectious viruses *(19).* However, cell culture is not a feasible method because of the absence of a susceptible cell line; moreover, the method is time-consuming, and immunofluorescence, enzyme-linked immunosorbent assays, or molecular tests are needed to confirm the presence of noncytopathic viruses.

Because, in Italy, there is no surveillance system to correlate the presence of enteric viruses with human transmission as reported by Sanchez et al. *(27),* we can only speculate on the influence of bivalves during episodes of gastroenteritis. The Surveillance System for Acute Viral Hepatitis *(29)* gives epidemiological confirmation that 50 to 60% of HAV diseases are linked to the consumption of mussels. HAV was identified in 14.1% of the tested samples (Table 1). This value is similar to the values reported in the scientific literature. LeGuyader et al. *(20)* reported that 13% of the samples in highly fecal-polluted seawater were positive but that no sample was positive in occasionally contaminated seawater. Lee et al. *(16)* and Chung et al. *(8)* reported 12 and 15% positive samples, respectively, whereas other authors have reported the detection of HAV in marine sediments or seawaters but not in bivalves. In the Apulia region, Chironna et al. *(7)* recorded 20% in nondepurated mussels, 10% in depurated mussels, and 23% in marketable mussels. HAV is endemic in the Apulia region and caused a large outbreak in 1997 to 1998, with >7,000 cases, but the highest value in marketable mussels can be caused by the popular method of ''refreshing the product'' in the marketplace using polluted seawater. HAV has been identified in 19 of 24 clams as opposed to 5 positive samples in mussels and oysters ($P < 0.01$). We have no particular hypothesis to explain this result, except for the different local situation (central Italy) or sample collection.

The most recently reported outbreak of HAV in Spain was associated with imported clams from South America *(26).* The samples were collected both for the classification of seawater growing area and for public consumption (Table 1). Samples collected in an area classified as B must be depurated before consumption, according to European Union regulations, whereas from growing area A, bivalves can be directly marketed. In this study, 15 samples, both for monitoring and market from area A, were HAV positive, with 11.7% of the samples collected for public consumption (Table 1). Depending on the international market of the food, there is a possible international circulation of enteric viruses, as described by Sanchez et al. *(27)*; in fact, the genomic analysis of four samples obtained from Greece showed a homology identical to the Italian strain IT-COL-OO *(6).* This Italian strain was isolated from a human, whose the only risk factor was related to the consumption of raw seafood. An analysis of the sample source, market, and monitoring was performed on 15 positive samples from area A; as 8 positive samples exceeded the bacteriological limits according to European law, this clearly confirms the inadequacy of these parameters for evaluating the hygienic quality of seafood.

HAV shows that a unique serotype and a genomic analysis of the VP1/2A region led to a classification of the virus as seven distinct genotypes and that a possible correlation existed among the different isolates. Few data are available on the HAV genotypes present in Italy, but all genotypes are connected to specific outbreaks *(6, 25).* A genomic analysis of the amplified products confirms the findings of previous studies and shows the presence of genotype IA (4 samples) and genotype IB (17 samples), which differs from the findings of Chironna et al. *(6),* who reported a large circulation of genotype IA (84%) versus genotype IB (16%). The RT-PCR test does not yield the infectivity of the enteric viruses, but it is the only method for evaluating the presence of enteric viruses in a short amount of time. This study confirms the feasibility of the molecular methods according to LeGuyader et al. *(19, 20).*

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