GIV noroviruses and other enteric viruses in bivalves: a preliminary study

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INTRODUCTION

Current water treatment practices are unable to provide virus-free wastewater effluents, consequently human pathogenic viruses are routinely introduced into marine and estuarine waters (Bosch A. & Le Guyader S. 2010). Bivalves filter large volumes of water as part of their feeding activities, and accumulate and concentrate different pathogens from human fecal pollution. Thus consuming raw or improperly cooked shellfish is a major risk factor for foodborne disease. In an effort to control shellfish-borne infections, the European Union Council has proposed several control measures for shellfish safety (Mesquita et al. 2011). However, periodic emergence of viral outbreaks associated with shellfish consumption continues to pose a real public health concern. Moreover, outbreaks have occurred as a result of the consumption of molluscs with accepted values of coliform standards (Brooks et al. 2005; Le Guyader et al. 2008a).

The aim of the study was to evaluate the presence of six different groups of waterborne enteric viruses in molluscs: norovirus (NoV) genogroups I, II, and IV, adenovirus (AdV), enterovirus (EV), astrovirus (AsV), hepatitis A virus (HAV), and hepatitis E virus (HEV).

The advent of molecular techniques, and particularly procedures based on nucleic acid amplification through the polymerase chain reaction (PCR) provided tools for the specific and sensitive monitoring of health significant enteric viruses in shellfish, thus enabling a safer evaluation of shellfish virological quality.

MATERIALS AND METHODS

Fresh and frozen mussels and clams were collected during official control monitoring programs, from harvesting areas, restaurants, fish markets and shellfish markets in the South of Italy (Sicily) (Table 2). Collected bivalves yielded a total of 11 hepatopancreas samples for testing: nine originating from mussels and two from...
clams. Each sample consisted of 25 g of hepatopancreas homogenized with 0.05 M glycine buffer pH 9.2, processed with a double PEG8000 precipitation followed by high speed centrifugation (10000x g) (Croci et al., 1999; De Medici et al., 2001). A parallel extraction, using proteinase K (0.1 mg/ml) in aqueous solution was performed on the hepatopancreas samples.

This second extraction, requiring 2 g of hepatopancreas, was feasible only in 7 of the 11 samples due to insufficient material. From our 11 samples, we thus obtained 18 extractions (11 using glycine and 7 using proteinase K), as shown in Table 2.

The final pellet was resuspended in 3 ml of PBS (Phosphate Buffered Saline) with antibiotics and antifungals (Croci et al. 1999), and 2 ml were subjected to nucleic acid extraction using commercial kits based on the selective binding of nucleic acids to a silica membrane (NucliSENS miniMAG extraction, bioMÉrieux Italia S.p.A., Rome, Italy). Viral genomes were eluted from the

| Table 1 - PCRs and primers used in this study. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **PCR** | **Primer fw** | **Sequence 5’-3’** | **Primer rev** | **Sequence 5’-3’** | **Product (bp)** | **Pathogen** |
| 443  | 1421 | ATACCACTATGATGCGAAYTA | 1422 | TCATCATCACATGAAAGAAG | 327 | NoV GI |
| 446  | 1423 | TCNGAAATGGATGTTGG | 1422 | TCATCATCACATGAAAGAAG | 188 | NoV GII |
| 475  | 1449 | GGGACTCAGTATGACAAAAT | 1448 | ACATCACGGGGGTATTTTGG | 1013 | NoV GIV |
| 476  | 1423 | TCNGAAATGGATGTTGG | 1316 | TCCTTGAGCCATCATCAT | 687 | HEV |
| 443  | 1421 | ATACCACTATGATGCGAAYTA | 1422 | TCATCATCACATGAAAGAAG | 327 | EV |
| 444  | 1421 | ATACCACTATGATGCGAAYTA | 1424 | AGCCAGTGGGCGATTGG | 839 | AsV |
| 546  | 1522 | TCATCATCACATGAAAGAAG | 1525 | TGCAAGACAGTGAAGAC | 1311 | FCV |
| 542  | 1523 | TCNGAAATGGATGTTGG | 1524 | AGCCAGTGGGCGATTGG | 839 | HEV |
| 563  | 1661 | TTAACGGCGATCTTCGACGAG | 1662 | TRATTACCAGTTCACGATTCACC | 302 | AdV |
| 564  | 1662 | TTAACGGCGATCTTCGACGAG | 1664 | TTTCAAACCCTCGCAGGAC | 175 | HAV |
| 565  | 1663 | TTAACGGCGATCTTCGACGAG | 1666 | TTTCAAACCCTCGCAGGAC | 175 | \[HEV\] |
| 653  | 1693 | CCACTATAATGACAACTCATTC | 1694 | AACTTGATYTCATKCAAAYCTWGCRTA | 389 | EV |
| 654  | 1695 | CCACTATAATGACAACTCATTC | 1696 | TTTCAACATCATATYTCATKCAAAYCTWGCRTA | 389 | AsV |
| 582  | 1551 | TTCCTGAGAACTTCTAAGG | 1553 | CTGTCGCCCTTTGAGACCTTGC | 720-850 | FCV |
| 583  | 1554 | TTCCTGAGAACTTCTAAGG | 1555 | CTGTCGCCCTTTGAGACCTTGC | 720-850 | FCV |
| 461  | 1246 | CGGTACCTATGAGATGCTTG | 1247 | ATTGTACGATAAGCAGCA | 545 EV | EV |
| 696  | 1705 | CGATGATGAGATGCTTG | 1704 | TACGATGATGAGATGCTTG | 449 | AsV |
| 697  | 1706 | CGATGATGAGATGCTTG | 1707 | TACGATGATGAGATGCTTG | 449 | AsV |
| 650  | 1654 | CCGTGAGGAGATGCTTG | 1655 | TTTCAACCCCGATGTTT | 630 FCV | FCV |
| 651  | 1656 | CCGTGAGGAGATGCTTG | 1657 | ACCATCATCCCGTAAAGTCA | 340 | FCV |
silica in 100 µl elution buffer and stored in aliquots at -80°C until use. A feline calicivirus (FCV) control was added prior to genome extraction to test for the presence of inhibitors. For each extraction, 14 different nested-type molecular tests (published and newly designed) were performed, using the primers outlined in Table 1. A set of positive-control virus RNAs for each of the viruses being tested and negative controls (sterile water used as amplification target) were used to ensure that primers were working and that there was no contamination. Moreover a PCR for the FCV was performed to test for inhibition of amplification reactions. Screening of RNA extracts for NoV GI and GII was based on PCR assays targeting the RNA polymerase (RdRp) gene (PCR 446 and 444) and the capsid gene (PCR 476 and 438) (La Rosa et al. 2007). For GIV NoV, two different assays were used, targeting the ORF1 (PCR 612) and the ORF2 (PCR 542) (La Rosa et al. 2008; La Rosa et al. 2010a). Three sets of specific primers (broad-range and genotype-specific) were used for HEV detection (PCR 654, 667, and 712) (La Rosa et al. 2011b). Finally, broad-range assays were used for HAV (PCR 676) (this study), AdV (PCR 583) (Crawford-Miksza & Schnurr 1996; La Rosa et al. 2011a), EV (PCR 601) (Pina et al. 1998) and AsV (PCR 697) (Noel et al. 1995). Previously described primers (PCR 651) were used for FCV controls (La Rosa et al. 2010b). All

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Collection site</th>
<th>PCR ID</th>
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<tbody>
<tr>
<td></td>
<td>NoV GI NoV GII NoV GIV HEV HAV AdV AsV EV FCV</td>
<td></td>
</tr>
<tr>
<td>44097</td>
<td>G Restaurant</td>
<td>446 476 444 438 612 542 654 667 712 676 583 697 601 651</td>
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<tr>
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<td>64818</td>
<td>G Store</td>
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</tr>
<tr>
<td>7790</td>
<td>PK Fish market</td>
<td>- - + - - - - - - - - -</td>
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<tr>
<td>6916</td>
<td>PK Fish canning industry</td>
<td>- - + - - - - - - - - -</td>
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<tr>
<td>85089</td>
<td>PK Harvesting area</td>
<td>- - + - - - - - - - - -</td>
</tr>
<tr>
<td>85087</td>
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<td>2346</td>
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<tr>
<td>45849</td>
<td>PK Restaurant</td>
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PK = proteinase K extraction; G = glycine extraction
PCR products were analysed in 1% agarose gels by electrophoresis, stained with Gel Red (Biotium) and visualized on a UV light box. Each nested PCR product was designed to migrate at a unique position, enabling virus identification. Electrophoresis results were confirmed by purification, cycle sequencing of both strands, and sequence analysis on an ABI-310 gene analyzer (Applied Biosystems, Foster City, CA, USA). Samples were considered negative if: (i) no PCR product corresponding to a virus was detected and (ii) the internal control product (FCV) was present.

Cell culture assays on FRhk-4 (Fetal Rhesus monkey Kidney), A549 (Human Lung Carcinoma) and BGM (Buffalo Green Monkey kidney) were performed to test for the infectivity of viruses.

RESULTS

Bivalves appeared to be significantly contaminated. Only two of the 18 extracts were found negative for viral RNA (FCV controls were positive, attesting to the lack of inhibition). Negative and positive controls yielded the expected results. A total of 27 PCR products were sequenced (Table 1).

Four out of six classes of enteric viruses (norovirus, adenovirus, enterovirus and hepatitis A) were detected in the shellfish tested, which were often co-infected (with up to four different viral groups in a single sample).

NoV was the most common virus detected, followed by HAV, AdV, and EV. Specifically, we detected NoVs GII.4 (N=8) and GIV.1 (N=1), AdVs types 1 (N=5) and 2 (N=1), HAV (N=9), and echovirus type 7 (N=3). The nucleotide sequence data obtained in this study have been submitted to GenBank and assigned accession numbers HE573250 to HE573272. No viruses were isolated from cell cultures.

DISCUSSION

Epidemiological evidence suggests that enteric viruses are the most common etiological agents transmitted by bivalve shellfish. Noroviruses GI and GII, recognized as the leading cause of gastroenteritis in humans, have been detected in wastewater treatment plant effluents, in surface waters and in shellfish worldwide (Glass et al. 2009; Mattison 2011; Suffredini et al. 2011). In a review by Baert and collaborators, bivalve shellfish accounted for 17.5% (7/40) of internationally reported foodborne norovirus outbreaks in 2000-2007 (Baert et al. 2009).

In this study, we detected GII.4 (8 positivities, 6 samples) but no GI NoVs. Different studies report a predominance of NoV GII in shellfish (mostly GII.4) over GI strains (Loisy et al. 2005; Suffredini et al. 2008), in line with the worldwide dominance of this genogroup. Interestingly, in this study we also detected a strain of NoV belonging to the genogroup GIV, in a mussel sample (ID 85089) collected in a harvesting area in Syracuse. The sequence showed 100% identity with GIV sequences detected in 2009 in sewage samples in Italy (La Rosa et al. 2010a). The sample in question was co-infected with NoV GII.4, HAV and echovirus type 7.

While genetic and epidemiological data on the more common human norovirus genogroups (I and II) are increasingly available, little is known about human genogroup IV. The numerous environmental and clinical studies on NoVs published so far include only very few reports on GIV NoV (Fankhauser et al. 2002; Iritani et al. 2002; La Rosa et al. 2008; Lindell et al. 2005; Vinje & Koopmans 2000), none addressing GIV NoV in shellfish. Few entries exist in public databases and the complete genome of GIV norovirus is not available. The result is a lack of information on its genetic diversity, diffusion, clinical significance and pathogenesis.

In previous studies, our group detected GIV NoV in sewage samples collected at wastewater treatment plants in Italy and in a small collection of clinical samples taken from patients with severe symptoms of gastroenteritis (La Rosa et al. 2008; La Rosa et al. 2010a). Similar results were obtained in a recent study investigating GIV NoV in water environments in Japan (Kitajima et al. 2010; Kitajima et al. 2011).

To the best of our knowledge, our results are the first to attest the presence of GIV NoV in seafood, suggesting that this genogroup could be implicated in NoV-related foodborne gastroenteritis, along with GI and GII.
Our results also show a significant presence of HAV genome in mussels (9 positivities, 8 samples). This virus has been successfully isolated from oysters, mussels and clams in different parts of the world and is a well-known agent of foodborne infections (Croci et al. 1999; Croci et al. 2000; Di Pasquale et al. 2010; Kittigul et al. 2010; Manso et al. 2010). HAV infection is the most serious viral infection linked to shellfish consumption. The first documented shellfish-borne outbreak occurred in Sweden in 1955, involving 629 cases associated with raw oyster consumption (ROOS 1956).

The most significant outbreak of HAV infection occurred in China in 1988 in which almost 300,000 cases were caused by consumption of clams harvested from a sewage-polluted area (Halliday et al. 1991).

As for AdVs, we detected 5 positive shellfish samples. Studies on AdVs in bivalves are few (Choo & Kim 2006; Hansman et al. 2008; Karamoko et al. 2005; Serracca et al. 2010), and the majority of these are limited to enteric adenoviruses types 40 and 41 (F species). Interestingly, in the present study, we found types 1 and 2, belonging to species C. This species is known to infect more than 80% of the human population early in life (Garnett et al. 2002). AdV-C serotypes are associated with a wide range of illnesses in immunocompromised patients as well as in healthy adults (La Rosa et al. 2011a; Sivan et al. 2007; Smith et al. 1983).

The results of this study are in line with our previous findings showing that species C AdVs in Italy is prevalent in clinical specimens from hospitalized patients (La Rosa et al., 2011), in sewage samples from wastewater treatment plants (unpublished data), and other environmental samples (Cannella et al. 2010). AdVs have been proposed as indicators of human fecal pathogens in the environment, due to their high environmental stability (Bofill-Mas et al. 2006) and high resistance to disinfection treatments. Further studies are required to ascertain their presence in seafood and their association with foodborne illness. EV was detected in only three samples. A low percentage of EV compared to other enteric viruses was detected in similar studies (Mesquita et al. 2011).

In our samples, we found no Astrovirus or HEV. The first group has rarely been detected in mussels (Gabrieli et al. 2007; Le Guyader et al. 2008b; Romalde et al. 2004). HEV has been identified in two studies on bivalves (Li et al. 2007; Song et al. 2010), and shellfish-associated hepatitis E has been documented (Renou et al. 2008; Sadler et al. 2006). In a previous study by our group aimed at providing preliminary information on the occurrence of HEV through the molecular analysis of urban sewage samples (La Rosa et al. 2010b) we found a high proportion of HEV-positive sewage samples in Italy (16%).

We also detected travel-related and autochthonous cases of HEV in Italy in a clinical study (La Rosa et al. 2011b). Risk factors identified in autochthonous cases included exposure to raw seafood (and also with pork liver sausages and wild boar). Further studies are therefore needed to determine the presence of HEV in the natural environment and in shellfish.

A limitation of the present study is the small number of samples. It does, however, include an in-depth virological analysis of each sample to evaluate the presence of six different groups of waterborne enteric viruses (14 different nested/semi-nested assays for each of the 18 samples.

The results obtained may provide a basis for future research. As far as we know, this is the first study to detect such a high level of viral contamination in Italian mussels (up to four different viral groups in a single sample), and to address and document GIV NoV in shellfish. The latter finding, combined with the relatively high frequency of these viruses recently detected in sewage samples (La Rosa et al. 2010a) highlights the potential role of GIV NoV as an emerging enteric pathogen along with GI and GII NoVs.

The concomitant presence of different genotypes (and genogroups) is of particular interest, since it is believed to facilitate NoV recombination in the human gut. Our results on AdV, a group of viruses not often tested for in shellfish, are also interesting. “Non-enteric” serotypes (types 1 and 2), were found in our samples suggesting that the inclusion of AdV (all species) in the evaluation of the virological quality of shellfish should be considered.

In this study no viruses were isolated from cell cultures. This may be explained either by the low sensitivity of cell culture (and low concentration of viruses expected in mussels), or by the presence of the viral genome but no infectious viral
particles. Similar studies confirmed that viral RNA in mussels is more resistant than the infectious virus (De Medici et al. 2001; Serracca et al. 2010). The presence of viral RNA/DNA in our samples, however, indicates a potential health risk for consuming raw or partially cooked bivalves. The establishment of a large-scale monitoring programme to determine the occurrence and frequency of enteric viral contamination of shellfish is recommended.

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REFERENCE


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