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

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SUMMARY

We evaluated the presence of the enteric viruses: norovirus, adenovirus, enterovirus, astrovirus, hepatitis A virus, and hepatitis E virus in bivalves using nested PCR methods and cell culture assays. Noroviruses GII.4 and GIV.1, adenoviruses types 1 and 2, hepatitis A, and echovirus type 7 were detected in the shellfish tested, which were often co-infected. 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 the first to document the presence of GIV NoV in shellfish.

KEY WORDS: Enteric viruses, Shellfish, PCR assays, GIV norovirus

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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

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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, bioMÈrieux 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	ATACCACTATGATGCAGAYTA	1422	TCATCATCACCATAGAAIGAG	327	NoV GI
446	1423	TCNGAAATGGATGTTGG	1422	TCATCATCACCATAGAAIGAG	188	
475	1449	GGGACTCWACACAAAATAGACAAA	1448	ACATCACCGGGGGTATTRTTTGG	1013	
476	1423	TCNGAAATGGATGTTGG	1316	TCCTTAGACGCCATCATCAT	687	
443	1421	ATACCACTATGATGCAGAYTA	1422	TCATCATCACCATAGAAIGAG	327	NoV GII
444	1421	ATACCACTATGATGCAGAYTA	1424	AGCCAGTGGGCGATGGAATTC	237	
437	1356	AGCCNTNGAAATNATGGT	1367	CCACCWGCATAACCATTRTACAT	1048	
438	1364	YTCYTTCTATGGYGATGATGA	1319	TCGACGCCATCTTCATTCACA	516	
603	1531	GCACTCGGCATCATGACAAAATTCA	1565	CCAACCCARCCATTRTACATTT	1526	NoV GIV
612	1531	GCACTCGGCATCATGACAAAATTCA	1532	GTTTGGGTCCCAATTCCAA	995	
546	1522	TCATCATCACCATAGAAIGAG	1525	TGCAGCAACAGTGATGTAACC	1311	
542	1523	TCNGAAATGGATGTTGG	1524	AGCCAGTGGGCGATGGAATTC	839	
653	1661	TTAYGGKGATGCCTTTGATGACACC	1662	TRATAACGGCCATRTTCCAGACAGTATTCC	302	HEV
654	1663	TGTTTGAGAATGACTTTTCTGAGTTTGAYT	1664	TTCCAAAACCCTCGCAGAGAC	175	
666	1679	CCAYCAGTTYATHAAGGCTCC	1680	TACCAVCGCTGRACRTC	348	
667	1681	CTCCTGGCRTYACWACTGC	1682	GGRTGRTTCCAIARVACYTC	172	
711	1722	CAAGGHTGGCGYTCKGTTGAGAC	1723	CCCTTRTCCTGCTGAGCRTTCTC	506	
712	1724	GYTCKGTTGAGACCWCBGGBGT	1725	TTMACWGTCRGCTCGCCATTGGC	457	
675	1693	AAGGWATBTTYCAGACYGTTGGKAGTGG	1694	AACTTGWATYTCAATKCCAAAYCTWGCRTA	389	HAV
676	1695	CTTGACCACATYCTGTCTYTGGC	1696	TTVACMACATCMARYTTDGCHACYTCATG	278	
582	1551	TICTTTGACATICGIGGIGTICTIGA	1553	CTGTCIACIGCCTGRTTCCACA	720-850	AdV
583	1554	GGYCCYAGYTTYAARCCCTAYTC	1555	GGTTCTGTCICCCAGAGARTCIAGCA	688-821	
461	1246	CGGTACCTTTGTACGCCTGT	1247	ATTGTCACCATAAGCAGCCA	545 EV	EV
601	1246	CGGTACCTTTGTACGCCTGT	1249	GAAACACGGACACCCAAAGTA	117	
696	1706	CGHTCACAATCTAGRGGBCKR	1707	ARCACCAYTCTGAAGCAAGYTC	688	AsV
697	1705	CAACTCAGGAAACAGGGTGT	1704	TCAGATGCATTGTCRTTGGT	449	
650	1654	CCCGTGGAGAAGGTTAGTGA	1655	CTTGTCAACCCGAGTTGGTT	630 FCV	FCV
651	1656	CAAGGTATTTGCGGTCGATT	1657	ACCATCATCCCCGTAAGTCA	340	

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 2 - *PCR results obtained from mussel samples.*

Sample ID		*Collection site*													
			PCR ID												
			NoV GI		*NoV GII*		*NoV GIV*		*HEV*			*HAV*	*AdV*	*AsV*	*EV*	*FCV*
			446	*476*	*444*	*438*	*612*	*542*	*654*	*667*	*712*	*676*	*583*	*697*	*601*	*651*
44097	G	Restaurant	-	-	-	-	-	-	-	-	-	-	-	-	-	+
64821	G	Store	-	-	-		-	-	-	-	-	-	-	-	-	+
77829	G	Store	-	-	-		-	-	-	-	-	+	-	-	-	+
64818	G	Store	-	-	-	-	-	-	-	-	-	+	-	-	-	+
7790	PK	Fish market	-	-	+	-	-	-	-	-	-	+	-	-	-	+
	G		-	-	+	-	-	-	-	-	-	-	+	-	-	+
6916	PK	Fish canning	-	-	-	-	-	-	-	-	-	-	+	-	-	+
	G	industry	-	-	+	-	-	-	-	-	-	+	+	-	+	+
85089	PK	Harvesting area	-	-	+	-	-	-	-	-	-	-	-	-	+	+
	G		-	-	-	+	+	-	-	-	-	+	-	-	-	+
85087	PK	Harvesting area	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	G		-	-	+	-	-	-	-	-	-	+	+	-	-	+
86337	PK	Store	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	G		-	-	-	-	-	-	-	-	-	+	+	-	-	+
2346	PK	Store frozen	-	-	-	-	-	-	-	-	-	-	+	-	-	+
	G		-	-	+	-	-	-	-	-	-	-	-	-	-	+
45849	PK	Restaurant	-	-	-	-	-	-	-	-	-	+	-	-	-	+
	G		-	-	+	-	-	-	-	-	-	+	-	-	-	+

PK = proteinase K extraction; G = glycine extraction

PCR products were analysed in 1% agarose gels by electrophoresis, stained with 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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