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Assessment of human enteric viruses in cultured and wild bivalve molluscs

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Abstract:

Standard and real-time reverse transcription-PCR (rRT-PCR) procedures were used to monitor cultured and wild bivalve molluscs from the Ría de Vigo (NW Spain) for the main human enteric RNA viruses, specifically, norovirus (NoV), hepatitis Avirus (HAV), astrovirus (AsV), rotavirus (RT), enterovirus (EV), and Aichi virus (AiV). The results showed the presence of at least one enteric virus in 63.4% of the 41 samples analyzed. NoV GII was the most prevalent virus, detected in 53.7% of the samples, while NoV GI, AsV, EV, and RV were found at lower percentages (7.3, 12.2, 12.2, and 4.9%, respectively). In general, samples obtained in the wild were more frequently contaminated than those from cultured (70.6 vs. 58.3%) molluscs and were more readily contaminated with more than one virus. However, NoV GI was detected in similar amounts in cultured and wild samples (6.4 × 102 to 3.3 × 103 RNA copies per gram of digestive tissue) while the concentrations of NoV GII were higher in cultured (from 5.6 × 101 to 1.5 × 104 RNA copies per gram of digestivetissue) than in wild (from 1.3 × 102 to 3.4 × 104 RNA copies per gram of digestive tissue) samples.

Keywords: molluscs · enteric viruses · hepatitis A virus · norovirus · viral prevalence · viral quantification · seafood industry

1. Introduction

Bivalve molluscs growing in coastal areas can be contaminated by human sewage, which can contain more than 100 types of viruses. Because of their filter-feeding nature, bivalve molluscs may concentrate these human pathogens and, therefore, may constitute an important vector in the transmission of enteric diseases [3,23]. Viral pathogens have been detected in bivalve molluscs from areas with large amounts of shellfish production or consumption throughout the world [3,19,26]. Viruses persist in molluscs for extended periods and, despite improvements, depuration does not eliminate viral particles [20,23,32]. These facts contribute to a well documented human health risk, especially when bivalve molluscs are consumed raw or lightly cooked [2,28]. The periodic occurrence of bivalve molluscs-transmitted disease outbreaks have contributed to a public confidence problem over shellfish safety, and resulted in important economic losses by the seafood industry [27].

Although only noroviruses (NoVs) and hepatitis A virus (HAV) have been clearly implicated in outbreaks linked to shellfish consumption [19,26,33], other enteric viruses such as enterovirus (EV), astrovirus (AsV), rotavirus (RV) [12,19] and Aichi virus (AiV) [17,37] have been detected in shellfish samples. The detection of enteric viruses relies mainly on the use of reverse transcription-PCR (RT-PCR) assays [10], but the low quantity of virus in environmental samples usually requires a time-consuming hybridization step, which enhances both the sensitivity and specificity of the assays, or the sequencing of the ampliocns obtained. Recently, new techniques of real-time reverse transcription-PCR (rRT-PCR) were developed for the main enteric viruses like NoV [13,18,21], HAV [6], AsV [14], or EV [9]. In this work, these new molecular techniques together with standard RT-PCR protocols were used to monitor the presence of enteric viruses in the Ría de Vigo (Galicia, NW Spain), one of the main bivalve mollusc producing areas in the world.

2. Materials and methods

Bivalve sampling. Sampling was performed monthly, from January to December 2005, in the Ría de Vigo, a large estuary situated in southwestern Galicia (NW Spain), concurrently with the official sampling program carried out by the INTECMAR (Technological Institute for the Marine Monitoring of Galicia). Samples (n = 24) of cultured mussel (*Mytilus galloprovincialis*) were obtained in paralell from two independent floating raft parks. Wild molluscs, including mussel (12 samples), clam (*Ruditapes decussatus*) (3 samples), and cockle (*Cerastoderma edule*) (2 samples) were collected from shore areas close (betwenn 500 and 700 m) to the floating rafts. Harvesting areas were classified, according to the current EC regulation [Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organization of official controls of the products of animal origin for human consumption. Off J Eur Communities L226:83-127], as B (230 to 4,600 *Escherica coli*/100 g mollusc tissue) for cultured mussel, and C (>4,600 *E. coli*/100 g mollusc tissue) for wild molluscs. Each sample consisted of at least 10 mussels or 20 clams/cockles. Molluscs were kept at 4ºC and arrived at the laboratory within 24 h.

Bivalve processing for virus concentration and RNA extraction. On arrival, molluscs were shucked and the stomach and digestive diverticula were dissected, and mixed to make 1.5 g portions, that were kept frozen (–20ºC) until analysis. Previous studies have demonstrated that most enteric viruses are localized in these tissues

[29,32], and the sample processing is easier than using the whole mollusc body. For analysis, tissues were thawed on ice, homogenized with glycine buffer pH 9.5, extracted with chloroform-butanol and with Cat-floc (Calgon, Ellwood City, PA, USA). The resulting suspension was then precipitated with polyethylene glycol 6000 (Sigma) [1,16,21]. Viral nucleic acid was extracted and purified from the suspended polyethylene glycol pellet using Nuclisens MiniMAG (BioMérieux, France), a semiautomated extraction procedure based in magnetic particles [18], and then suspended in 100 µl of RNase-free water and kept frozen (–80ºC).

Standard RT-PCR. Samples were analyzed to detect RV, AsV and AiV by standard RT-PCR. For AiV, a one-step nested PCR step was necessary to increase the assay sensitivity. RT was performed with 20 µl mixture containing 2 µl of nucleic acid (NA) extract, 1X buffer II (Applied Biosystems), 5 mM $MgCl_2$, 1 mM dNTP, 2 U of RNase Inhibitor (Applied Biosystems), 1.25 µM downstream primer (Table 1) and 50 U of the MuLV reverse transcriptase (Applied Biosystems) as previously described [16]. Briefly, after a RT step of 30 min at 42ºC (37ºC for AiV) and a denaturation step for 5 min at 95ºC, PCR mix was added containing, at final concentrations, 1X buffer II (Applied Biosystems), 1.25 mM $MgCl_2$, 0.5 µM forward primer (Table 1) and 2.5 U of *Taq* polymerase (Applied Biosystems). Amplification was performed for 40 cycles (94ºC for 30 s, 50ºC for 30 s, and 72ºC for 30 s) with final extension at 72ºC for 7 min in a thermocycler (9600 or 2400, Applied Biosystems). Nested PCR for AiV was performed using 2 µl of amplification product and 0.5 µM of each primer (Table 1) in the same conditions than in the PCR above. Analysis of the amplification product was performed by 9% polyacrilamide gel electrophoresis, and the product was confirmed as a distinct good size band after ethidium bromide staining [15].

TABLE 1

Samples were considered as positive only if the amplicons were detected by hybridization using specific biotin labelled probes (Table 1) with the commercial kit Hybridowell universal (Argene, France) following manufacturer´s instructions. A negative control, containing no nucleic acid, was introduced in each run, as well as a specific positive control for each viral pathogen (RNA from viral stocks).

Real-time reverse transcription-PCR. For NoV, HAV and EV, rRT-PCR were carried out using the Platinum quantitative RT-PCR Thermoscript one-step system (Invitrogen, France) in a 25 µl of a reaction mixture containing 5 µl of extracted RNA, 1 X of Thermoscript reaction buffer, 0.9 µM of reverse primer, 0.5 µM of forward primer, 0.45 µM of probe, 0.5 µl of ROX and 0.5 µl of Thermoscript Plus/Platinum *Taq* enzyme mix (Invitrogen). ROX was employed as a passive internal reference for the normalization of the reporter dye signal. Table 1 shows the sequences of primers and probes used. The rRT-PCR was performed with an ABI Prism 7000 SDS detector (Applied Biosystems) or with a Mx3000P QPCR System (Stratagene) in a 96-well format under the following conditions [6,17]: reverse transcription at 55ºC for 1 h, denaturation at 95ºC for 5 min, and then 45 cycles of amplification with a denaturation at 95ºC for 15 s, annealing at 60ºC for 1 min, and extension at 65ºC for 1 min. Samples showing cycle thresholds (Ct) values ≤41, with no evidence of amplification in the negative controls were considered as positive.

Extraction and rRT-PCR efficiencies. Mutant non-virulent infective strain vMC_0, of Mengovirus kindly provided by A. Bosch (University of Barcelona), was employed as a control for the process of nucleic acid extraction as described in [6]. Prior to the viral RNA extraction for the mollusc homogenates, these were spiked with a known amount ($\approx 10^4$ pfu) of vMC_0. Viral RNA extracted from molluscs were tested undiluted and ten-fold diluted to evaluate the effect of RT-PCR inhibitors. Extraction efficiency values

were evaluated by comparing the Ct value for the vMC_0-positive amplification control with the Ct value for the tested virus, and was classified as poor (<1%), acceptable (1 to 10%), or good (>10%) [8]. To test the presence of RT-PCR inhibitors and calculate the rRT-PCR efficiency, co-amplifications of 2.5 µl of each extracted RNA with 2.5 µl containing 10^3 copies of internal controls for the respective virus type, were evaluated in separate experiments [6,17]. To calculate the rRT-PCR efficiency, the Ct value of a sample mixed with internal controls was compared to the Ct value of the internal control alone. Efficiency values were classified in the same three categories than extraction efficiency (poor, acceptable and good) [8]. The numbers of viral RNA copies present in positive samples were estimated using standard curves generated from RNA transcripts as previously described [8,17,18].

3. Results

Extraction and rRT-PCR efficiencies. Extraction efficiency ranged between 2.3 % and 37.9%. According to the classification mentioned above, a total of 28 samples (68.3%) showed a good extraction efficiency (>10%) and 13 samples (31.7%) rendered acceptable extraction values (1–10%). Co-amplifications with internal controls indicated that only partial inhibition was originated by the components of the samples, being the extracted RNA suitable to test the viral presence without false negative results. Moreover, rRT-PCR efficiencies were always good or acceptable. For NoV GI, 37 out of 41 (90.3%) samples tested showed good efficiency (>10%), while only 4 samples (9,8%) presented an acceptable rRT-PCR efficiency (1-10%). For NoV GII, all but one samples (97.6%) rendered a good rRT-PCR efficiency.

Viral results. Of the 41 samples examined, 63.4% contained at least one of the virus studied. In fact, 41.5% of the samples showed the presence of one type of virus, whereas in 17.1% and 4.8% of the samples two and three enteric viruses, respectively, were detected. NoV GII was the most prevalent virus, it being detected in 22 samples (53.7%) (Table 2).

TABLE 2

In cultured mussels (n = 24), most of the positive samples presented only one type of virus (45.8%), although there were samples where two or three different types of enteric viruses were detected (4.1% and 8.3%, respectively)(Table 2). In wild molluscs, 12 out of the 17 samples contained one (6 samples) or two (6 samples) types of enteric viruses (Table 2).

By mollusc species, 6 of 12 wild mussels samples contained NoV GII; 3 samples were positive for EV; 2 for AsV; and only one sample contained NoV GI. Presence of more than one viral type was detected in 5 wild mussel samples. In addition, all three clam samples were contaminated, one with NoV GII and two with RV, whereas both cockle samples were positive for NoV GII, one of them showing also the presence of AsV.

TABLE 3

Norovirus quantification. Quantification (number of viral genomes per gram of mollusc digestive tissue) was carried out for the NoV positive samples, using the standard curves and, taking into account the extraction and rRT-PCR efficiencies. Levels of contamination with both NoV genogroups are shown in Table 3. In general, cultured samples showed lower contamination levels than wild samples. Three cultured samples rendered positive results but with levels too close to the detection limit of the techniques that accurate quantification was not possible.

For NoV GI no differences were observed in the levels achieved in cultured and wild samples (less than 1 log-unit). On the other hand, although similar highest levels for NoV GII were observed for wild and cultures samples, 10 out of 13 cultured samples positive for NoV GII showed levels lower than 500 RNA copies/g tissue whereas 7 out of 9 of the wild samples showed levels exceding this value.

4. Discussion

Current EU regulations establish the use of bacteriological monitoring programmes, based on *Escherichia coli* as indicator, to determine the sanitary quality of molluscs and the classification of their harvesting areas [Regulation (EC) No 854/2004]. Several studies have shown that although such controls have been effective at reducing the risk of bacteriological illness to minimal levels, bivalve molluscs meeting the *E. coli* standards may contain enteric viruses, and therefore, act as vectors of human viral diseases [3,19,26,31]. Other proposed indicators of viral contamination, such as F-specific RNA (FRNA) bacteriophages, have been studied with contradictory results [35]. Therefore, most research approaches were developed towards direct viral detection.

The purpose of the present study was to determine the prevalence of the main enteric viruses in cultured and wild molluscs collected from the Ría de Vigo (NW Spain), one of the most important European mollusc harvesting areas, during a one-year period. Despite the importance of this area for mollusc production, few studies have been performed on this site [30], and none of them included all the enteric viruses analyzed here. The results obtained showed a high number of samples (63.4%) contaminated with at least one of the enteric viruses studied. This percentage increased to 70.6% if only wild samples were considered. These values are similar to those reported in such type of contaminated area in France [15].

Reports of viral contamination in molluscs or harvesting areas have been published [7,19,24,26], but few of them presented results of a regular monitoring program [15,22,30]. Therefore little is known about the occurrence of viruses in molluscan beds. In the present study, the most prevalent virus was NoV GII and, to a lesser extent EV, AsV, NoV GI and RV. Similar results were obtained in a three years study in France [15] for NoV, EV and AsV prevalences, and also in mollusc samples obtained from a commercial producer before depuration in the UK [22]. The decrease of the prevalence of this virus during warm months is well known [15,16]. In addition to a more rapid degradation of viral particles at high temperatures and by the sunlight [23], the lower circulation of these viruses during summer months may be an important factor [34].

Contrarily to the results obtained in a former study in the same geographical area (Ría de Vigo) [30], HAV was not detected in the samples analyzed. This fact can be due to the different experimental approaches employed in the present work, to an increase of sanitary conditions or to a lower endemicity of the viruses in local population. Nor did we detect AiV, which has been first recognized as the cause of oyster-associated gastroenteritis in Japan in 1989 [37], and has been recently detected in oysters implicated in an outbreak in France [17]. In our study, mixed contaminations with more than one enteric virus were observed. Simultaneous detection of different enteric viruses have been also reported in previous studies performed in different countries [11,19,26,30], usually in bivalve molluscs associated with illness outbreaks. It has been suggested that coinfection with multiple viruses could cause more severe symptoms [17].

In general, and accordingly to data previously reported [30], the results achieved in the present study indicated that viral contamination was greater in wild than in the cultured bivalves analyzed. Higher contamination levels in wild molluscs may be related with the proximity of the sampling points to contamination sources. In fact,

these molluscs were harvested at the shoreline, where urban impact is more evident [4,5]. In addition, other factors including decreased shellfish activity at lower temperatures or differential retention of viruses by distinct mollusc species can not be ruled out [19,26]. Note that, although cultured samples from class B areas have to be depurated before they can be placed in the market, the effectiveness of depuration to eliminate viral contamination is limited [17,20,32], and therefore they can constitute a potential public health hazard.

Quantification of noroviruses in molluscs is a complex procedure because it is subjected to problems with inhibition of the RT-PCR reaction by mollusc tissue components, which can cause false negative results [17]. Using in the extraction step a known quantity of an external added virus as control, such as Mengovirus, which does not interfere in the final result of the quantification, as well as internal specific controls to calculate the extraction and rRT-PCR efficiencies, a more real approach of the mollusc viral charge is possible [5]. In the present study, no RT-PCR inhibition was observed in the molluscs samples. However, in a recent study by da Silva et al. [7] it has been shown that only one NoV genogroup was inhibited in some samples, confirming that inhibitors do not affect the different primers and probes equivalently, although the difference between rRT-PCR efficiencies for GI and GII was not statistically significant. The use of these new approaches for quantification increase, in any case, the meaning of the results, and make it possible a better monitoring of harvesting areas.

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Tables

Table 1. Primer sets and probes used for viral detection in this work

Virus	Primer	Probe	Sequence 5´–3´	Fragment size	Reference
NoV GI	QNIF4		CGCTGGATGCGNTTCCAT	98	[8]
	NV1LCR		CCTTAGACGCCATCATCATTTAC		[34]
		NV1Lpr	6-FAM-TGGACAGGAGAYCGCRATCT-6-TAMRA		[34]
NoV GII	QNIF2d		ATGTTCAGRTGGATGAGRTTCTCWGA	95	[21]
	COG2R		TCGACGCCATCTTCATTCACA		[13]
		QNIFS	6-FAM-AGCACGTGGGAGGGCGATCG-6-TAMRA		[21]
HAV	HAV240		GGAGAGCCCTGGAAGAAAG	174	[6]
	HAV68		TCACCGCCGTTTGCCTAG		[6]
		HAV150	6-FAM-CCTGAACCTGCAGGAATTAA-MGB		[6]
EV	EVR		GGAAACACGGACACCCAAAGTAG	114	[9]
	EVF		TGAATGCGGCTAATCCCAACCTC		[9]
		EVS	6-FAM-TGCGCGTTACGACAGGCCAATCAC-6-TAMRA		[9]
AsV	AV1		CCGAGTAGGATCGAGGGT	90	[14]
	AV2		GCTTCTGATTAAATCAATTTTAA		[14]
		biot-Avs	Biotin- CTTTTCTGTCTCTGTTTAGATTATTTTAATCACC		[14]
RV	VP6.3		GCTTTAAAACGAAGTCTTCAAC	186	[36]
	VP6.4		GGTAAATTACCAATTCCTCCAG		[36]
		biot-RV	Biotin-CAAATGATAGTTACTATGAATGG		[36]
Aichi	6261		ACACTCCCACCTCCCGCCAGTA	342	[37]
	6602		AGGATGGGGTGGATRGGGGCAGAG		[25]
	nested 6309		GTACAAGGACATGCGGCG	160	[25]
	nested 6488		CCTTCGAAGGTCGCGGCRCGGTA		[25]
		biot-Aichi	Biotin-GTACAAGGACATGCGGCG		[25]

Table 2. Number of samples showing the presence of enteric viruses in cultured and wild bivalve molluscs

Virus	Total (n=41)	Cultured (n=24)	Wild (n=17)
NoV GI	1	1	0
NoV GII	13	10	3
HAV	0	0	0
EV	0	0	0
AsV	1	0	1
RV	2	0	2
AiV	0	0	0
NoV GII + EV	3	0	3
NoV GII + NoV GI	2	1	1
NoV GII + AsV	2	0	2
NoV GII + EV + AsV	2	2	0

Table 3. Quantification of NoV genogroups I and II in bivalve molluscs digestive tissue as determined by rRT-PCR

NoV genogroup	Sample	Mollusc	% extraction efficiency	% rRT-PCR efficiency	Ct	RNA copies/g tissue[a]	
						Uncorrected	Corrected
GI	A20	Mussel	21.1	60.9	39.6	148.6	1,155
	C20	Mussel*	25.6	61.8	40.1	101.2	638
	B21	Mussel	14.9	66.7	38.5	327.0	3,293
GII	A11	Mussel	18.5	29.7	36.0	75.3	1,372
	B11	Mussel	12.9	100	35.3	188.0	1,452
	C11	Mussel*	10.4	100	35.3	125.0	1,199
	A12	Mussel	14.9	74.1	36.6	49.0	444
	C12	Mussel*	3.4	100	35.4	112.5	3,248
	A13b	Mussel	17.5	32.7	41.1	+ DL[b]	
	A13m	Mussel	19.8	96.6	41	+ DL	
	B13b	Mussel	14.5	22.8	38.1	16.3	495
	C13	Mussel*	19.5	100	33.2	544.6	2,787
	A14	Mussel	4.5	15.6	35.5	105.6	15,177
	B14	Mussel	23.9	100	35.7	95.6	400
	C14	Mussel*	17	97.9	37.7	22.4	134
	A18	Mussel	32.1	100	41.5	+ DL	
	A19	Mussel	20.8	100	37.5	25.8	124
	B20	Mussel	11.9	66.3	37.8	21.0	266
	C20	Mussel*	25.6	100	36.3	63.6	247
	D20	Clam	7.8	38.7	37.4	28.5	1,021
	E20	Cockle	2.9	70	36.9	40.0	2,782
	A21	Mussel	35.1	100	37.9	20	56
	B21	Mussel	14.9	100	38.2	16.0	107
	E21	Cockle	2.3	71.1	38.3	15.0	1,250
	C22	Mussel*	16.5	100	29.9	5,599.7	33,883

[a]Number of RNA copies calculated without taking the extraction and rRT-PCR efficiencies into account (uncorrected) or taking the extraction and rRT-PCR efficiencies into account (corrected).
[b]+ DL, positive sample but the level was too low for accurate quantification.
*Wild mussel samples.