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Research Note

Human Norovirus RNA Persists in Seawater under Simulated Winter Conditions but Does Not Bioaccumulate Efficiently in Pacific Oysters (*Crassostrea gigas*)

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ABSTRACT

Norovirus (NoV) is the principal agent of bivalve molluscan shellfish–associated gastroenteric illness worldwide. Currently, noncultivable human NoVs can be detected in bivalve molluscan shellfish by using molecular methods such as real-time reverse transcription PCR assays (qRT-PCR). In addition to infectious viruses, this methodology may also detect noninfectious NoV, including fragments of the NoV genome. This study addresses, in part, the implications of qRT-PCR results for the detection of NoV in shellfish in the absence of an infectivity assay. To evaluate environmental persistence, the stability of a short fragment of the NoV genome, spanning the qRT-PCR target in the open reading frame 1/2 junction, was assessed in seawater under artificial environmental conditions simulating winter in the United Kingdom (1 mW/cm^2 UV irradiation, 8°C) during a 4-week period. Detectable RNA levels decreased exponentially (T_{90} of approximately 141 h); however, sequences were still detectable for up to 2 weeks. The ability of Pacific oysters (*Crassostrea gigas*) to bioaccumulate NoV particles (from human feces) and RNA fragments was also compared using qRT-PCR. Oysters exposed to NoV particles subsequently were positive for NoV by qRT-PCR at levels several orders of magnitude in excess of the theoretical limit of detection, whereas oysters exposed to similar quantities of NoV RNA were either negative or positive at significantly lower levels. Therefore, although noninfectious fragments of NoV RNA may persist in the environment under winter conditions, this type of material will not be efficiently bioaccumulated by Pacific oysters and should not significantly contribute to positive qRT-PCR results.

Human norovirus (NoV) is the most common cause of nonbacterial gastroenteritis globally, with high attack rates in both children and adults *(1, 37)*. NoV can be found in high levels in the feces of infected individuals, raw sewage, rivers, and estuarine and marine waters *(5)*. Transmission can be person to person via the fecal-oral route or from consumption of contaminated water or foodstuffs. Filter-feeding bivalve molluscan shellfish have frequently been linked to outbreaks of NoV infection *(18),* particularly during winter months *(9)*.

NoV cannot currently be routinely cultured in the laboratory. Therefore, existing methods, such as real-time reverse transcription PCR assays (qRT-PCR) for detection of NoV in food and environmental samples are based upon detection of a fragment of the viral genome. However, it is not clear whether detection of such fragments is correlated with the presence of infectious virus and thus corresponds to a health risk. If free or particle-associated NoV RNA can be concentrated in bivalve molluscan shellfish tissues, use of qRT-PCR may produce NoV-positive results that do not reflect consumer risk.

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NoV particles are stable both inside the human host and in the environment and may survive in seawater or in bivalve molluscan shellfish and consequently remain a risk to public health for extended periods *(18)*. Several authors have reported that most viruses and pathogenic bacteria occur in the environment as particle-associated organisms *(2, 26, 29)*. Various factors affect virus survival in the environment, including water temperature and the extent of UV irradiation. Many enteric viruses can survive longer during low-temperature periods in the winter than during summer months *(13, 21, 22)*. The higher temperatures may damage both viral capsids and nucleic acids, possibly preventing binding of the virus to host receptors or causing inactivation of enzymes required for replication by damaging genes encoding these enzymes *(3)*. The germicidal and inactivation properties of UV radiation are well documented *(11, 16)*. Several authors have suggested that because NoV possesses a single-stranded RNA (ssRNA) genome, it could be less resistant to UV radiation than double-stranded DNA or RNA viruses *(12, 34)*. The survival of free RNA in the environment is thought to be transient *(31)*.

In this study, we assessed the persistence of NoV RNA in seawater under artificial winter conditions and examined the potential for the uptake of particulate-adsorbed NoV

genomic RNA fragments in Pacific oysters (*Crassostrea gigas*) to provide a better understanding of the significance of qRT-PCR results in bivalve molluscan shellfish with respect to consumer health risks.

MATERIALS AND METHODS

Production of NoV RNA. Purified ssRNA of 126 bases (NoV genogroup I [GI]) and 131 bases (NoV genogroup II [GII]) carrying the qRT-PCR target sequence (which spanned the open reading frame [ORF] 1/2 junction of the NoV genome) were transcribed from plasmids (Dr. Soizick LeGuyader, Ifremer, Nantes, France) produced by ligating the target sequences from the Norwalk strain of NoV GI or the Lordsdale strain of NoV GII into vector pGEM-3Zf (+) (Promega, Madison, WI). Before RNA transcription, the plasmids were linearized; 37.5 μl of molecular grade water, 5 μl of reaction buffer (Promega), and 2.5 μl of *Xba*I restriction enzyme (Promega) were added to 5 μl (~500 ng) of each plasmid and incubated at 37°C for 2 h. The linearized plasmid DNA was then purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's instructions. In vitro RNA transcription was carried out using the Riboprobe in vitro transcription system (Promega). A reaction mix containing 20 μl of transcription buffer (5×), 10 μl of 100 mM dithiothreitol, 2.5 μl of RNasin, 20 μl of a mix of 2.5 mM concentrations of rATP, rGTP, rCTP, and rUTP, 3 μl of T7 polymerase, and 39.5 μl of nuclease-free water was added to 5 μl of linearized DNA. The reactions were mixed by pipetting and incubated at 37°C for 2 h. After incubation, 5 μl of RQ1 RNase-free DNase was added to each reaction and incubated at 37°C for 15 min to degrade the remaining DNA template. The RNA transcripts were then purified using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. The RNA preparations were then checked for DNA contamination with qRT-PCR mastermix in which the RT enzyme had been heat inactivated at 95°C. In both cases, there was <0.03% DNA contamination (data not shown). The concentration of RNA in copies per microliter was calculated for each preparation by extrapolation from absorbance at 260 nm as measured using a NanoDrop spectrophotometer (Thermo Scientific, Pittsburgh, PA). RNA transcripts were stored at −20°C until required.

Stability of NoV RNA fragments in seawater. A 500-ml volume of natural seawater was equilibrated to 8 ± 0.5°C and then exposed to UV light at 1 mW/cm^2 (SOL 500 UV transilluminator, Honle, Grafelfing, Germany). The UV radiation intensity was adjusted with plastic filter sheets, simulating average winter conditions in the southern United Kingdom as measured from November 2008 through March 2009 (data not shown). The water was then spiked with approximately 1×10^5 copies of NoV GI RNA transcripts. Duplicate samples of 1 ml were removed after 0 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 1 day, 2 days, 1 week, and weekly thereafter up to 4 weeks. Seawater was mixed by stirring before sample collection. All samples were frozen at −20°C until the end of the sampling period to enable simultaneous testing.

Adsorption of NoV RNA and virus particles to solids from seawater. Suspended solids were sedimented from 400 ml of natural seawater by centrifugation at 4,000 × *g* and then resuspended in 50 μl of natural seawater. NoV RNA or viral particles (see below) were added to this suspension and incubated for 30 min at 22°C to allow adsorption to suspended solids.

Bioaccumulation in Pacific oysters. Thirty liters of artificial seawater, made by adding Seamix (Peacock Salt, Ayr, UK) to distilled water, was added to two aerated 48-liter tanks and equilibrated to 18°C. Thirty Pacific oysters were then added to each tank and left to acclimatize for 96 h to allow resumption of filter-feeding activity. Aliquots of a NoV GII.4.2004–positive fecal sample (99.7% sequence homology in ORF2 to Monastir strain, EU650225) and NoV GII RNA transcripts, each containing approximately 6×10^6 PCR-detectable copies, were allowed to adsorb to the suspended solids. Ten oysters were removed for testing for prior NoV contamination using methods described below, and then adsorbed NoV RNA transcripts and fecal material were added to the tanks. Aliquots of a GI.4-positive fecal sample (96.2% sequence homology in ORF2 to Chiba virus, AB022679) containing approximately 6×10^6 PCR-detectable copies were added to each tank to act as a bioaccumulation control. Oysters were allowed to bioaccumulate for 16 h and then tested for NoV GI and GII as described below. This procedure was repeated on four occasions.

Preparation of oyster homogenate. Oysters were opened aseptically, and the animals were removed from their shells. The peripheral flesh and organs were cut away from the digestive glands (stomach and digestive diverticula), which were pooled and finely chopped with a sterile razor blade. Homogenates were then prepared by treating a 2-g portion of chopped digestive glands with 100 μg/ml Proteinase K solution (30 U/mg; Promega) as described previously *(17)* but modified to include a secondary incubation of 60°C instead of 65°C for 15 min. Homogenates were stored at 4 ± 0.5°C until RNA extraction and qRT-PCR analysis.

RNA extraction. Total RNA was extracted from 500 μl of seawater or shellfish homogenate using a NucliSENS miniMAG extraction machine and NucliSENS magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions (eluting in 100 μl of elution buffer). A negative extraction control (water only) was included with each set of samples extracted. Eluted RNA was stored at −20°C until required.

One-step qRT-PCR. For both NoV genogroup-specific qRT-PCR primer and probe sets (Table 1), duplicate or triplicate aliquots (depending on the particular experiment) of 5 μl of sample or extraction control RNA were added to adjacent wells of a 96-well optical reaction plate, and the volume was increased to 25 μl with one-step reaction mix prepared using the RNA Ultrasense one-step qRT-PCR system (Invitrogen, Carlsbad, CA) (final concentrations of 1× reaction mix, 500 nM forward and 900 nM reverse primers, and 250 nM probe, plus 0.5 μl of Rox and 1.25 μl of enzyme mix per reaction). Wells containing nuclease-free water and the same one-step reaction mixes also were included on each plate as negative controls. To enable quantification of each sample RNA in copies per microliter, log dilution series of the GI and GII plasmids (range: 1×10^4 to 1×10^0 copies per μl) were included on each qRT-PCR plate. The plate was incubated at 55°C for 60 min, 95°C for 5 min, and then 45 cycles of 95°C for 15 s, 60°C for 1 min, and 65°C for 1 min on an SDS7000 real-time PCR machine (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Stability of NoV RNA fragments in seawater. NoV GI RNA spiked into seawater and held under conditions similar to those of a northern European winter (8°C, 1 mW/cm^2 UV radiation) was detected by qRT-PCR for up to 14 days postcontamination. Decay of detectable RNA was exponen-

TABLE 1. *Sequences of norovirus primers and probes used in this study*

Primer or probe	Sequence	Reference
Norovirus GI assay		
QNIF4	CGC TGG ATG CGN TTC CAT	*7*
NV1LCR	CCT TAG ACG CCA TCA TCA TTT AC	*33*
TM9	FAM-TGG ACA GGA GAT CGC-MGB	Adapted from *15*
Norovirus GII assay		
QNIF2	ATG TTC AGR TGG ATG AGR TTC TCW GA	*23*
COG2R	TCG ACG CCA TCT TCA TTC ACA	*24*
QNIFS	FAM-AGC ACG TGG GAG GGC GAT CG-BHQ-1	Adapted from *23*

tial ($r^2 = 0.966, P < 0.001$) with a T_{90} (time needed for 90% of the PCR signal to disappear) of 141 h (Fig. 1). This finding is in accordance with those of Tsai et al. *(36),* who found that naked poliovirus 1 RNA was detectable in filtered seawater at 4°C for up to 21 days. In previous studies, poliovirus 1 and F-specific RNA bacteriophage genome fragments persisted to some extent in solution after exposure to high levels of germicidal UV radiation *(31).* Tsai et al. suggested that survival was a function of fragment size, with preferential survival of small fragments (<145 bases). In the present study, NoV RNA transcripts (126 to 131 bases) complementary to the ORF1-2 region primer-probe binding site of the NoV were used. In previous studies, viral RNA has been more stable in artificial, filtered, or sterile seawater than in natural seawater because of the antiviral activity of natural seawater, e.g., from RNases (derived from natural marine microflora) *(10, 36).* Because double-stranded RNA viruses have been reported to be more resistant to the effects of UV radiation *(14),* the potential for secondary structure formation from NoV GI RNA sequences used in this study was checked using RNAfold software (University of Vienna RNA Web server: http://rna.tbi.univie.ac.at). The results indicated that formation of RNA complexes was theoretically possible (a minimum free energy of −27.60 kcal/mol was determined for the optimal secondary structure), potentially reducing the rate of degradation due to UV irradiation. Regardless of this possibility, detection of NoV RNA fragments for extended periods revealed the potential for naked RNA to persist in the marine environment.

Comparative bioaccumulation of NoV particles and NoV RNA fragments by Pacific oysters. Levels of GI and GII NoV (genome copies per gram of digestive glands) in oysters exposed to both human GI NoV–positive fecal material and either human GII NoV–positive fecal material or GII NoV RNA transcripts (containing similar numbers of PCR-detectable copies) are given in Table 2. In previous studies in this and other laboratories *(27, 30),* oysters rapidly concentrated NoV particles from sewage or fecal material under a variety of conditions. Some researchers have suggested that bioaccumulation of viruses in bivalve molluscs may be assisted by the ionic bonding of viral particles to the mucopolysaccharide moiety of shellfish mucus *(8)* and by the presence of human blood group antigen–like structures in oyster gastrointestinal tissues *(35).* NoV GI and GII strains may have different affinities to oyster digestive tissue *(6)* based upon specific carbohydrate structures with a terminal *N*-acetylgalactosamine residue in α linkages *(19).* In this study, Pacific oysters in each set of experiments and in both experimental tanks accumulated GI NoV to similar levels (mean tank-to-tank difference of 0.47 log unit; Table 2), indicating that both sets of oysters were effectively bioaccumulating NoV present in the water. For GII NoV, in two of four experiments detection was possible only from oysters exposed to virus from fecal material, not from oysters exposed to GII NoV RNA transcripts, even though similar numbers of RNA copies (as determined by PCR) were present in both spikes. On the fourth occasion, NoV GII was detected at the limit of detection in oysters allowed to bioaccumulate RNA transcripts; however, oysters from the same batch that had not been bioaccumulating tested positive at the same level, suggesting low level contamination of the harvesting area as opposed to accumulation of transcripts. In experiment 3, bioaccumulation of NoV GII RNA fragments was apparent (oysters were positive at levels well in excess of the limit of detection), however at considerably lower levels (1.74 log units fewer detected) than those found when oysters were exposed to virus from fecal material. On average, there was a 2.9-log difference between NoV GII detected in oysters exposed to virus particles in fecal material and the NoV GII detected in oysters exposed to NoV GII RNA fragments. This difference in bioaccumulation of NoV GII particles and NoV GII RNA fragments was significant (paired *t* test of log copies per gram; $P = 0.007$).

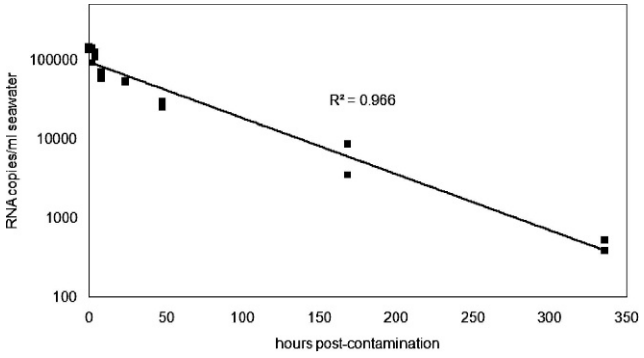

FIGURE 1. *Survival of human NoV GI RNA transcripts in seawater under simulated winter conditions.*

When important risk management decisions are made on the basis of qRT-PCR results, it is important that these results be understood in the context of risk. Several authors have suggested that because qRT-PCR does not distinguish

TABLE 2. *Comparative bioaccumulation of human NoV GII particles and RNA fragments in Pacific oysters*

	NoV (detectable copies/g of digestive gland)[a]					
	Tank 1		Tank 2		Log tank-to-tank difference[b]	
Expt	GI	GII	GI	GII	GI	GII
1	597	10,941	3,137	<LOD	−0.72	2.86
2	705	27,941	977	<LOD	−0.14	3.27
3	2,000	108,444	5,466	1,994	−0.44	1.74
4	19,540	81,217	5,197	≈LOD	0.58	3.73
Mean[c]					0.47	2.90

[a] Tank 1 contained GI and GII fecal material; tank 2 contained GI fecal material and GII RNA transcripts. <LOD, less than the limit of detection (~15 copies per g); ≈LOD, positive at the limit of detection.
[b] Results ≈LOD or <LOD treated as 15 copies per g.
[c] Mean of absolute values regardless of direction of difference.

between infectious viruses and nonviable virus materials (e.g., RNA fragments), a positive sample may be suggestive of contamination but not necessarily of consumer health risk *(20, 28, 32, 38)* and have questioned the use of qRT-PCR in the management of shellfisheries *(4, 25)*. Our findings indicate that short fragments of nonencapsulated NoV RNA can persist in the marine environment for up to 2 weeks and that bioaccumulation of such materials can occur. However, the efficiency of uptake of RNA is significantly lower (mean of 2.9 log units) than that of virus particles, and in three of four repeated experiments NoV GII was detected by qRT-PCR at levels above the environmental background only in oysters exposed to virus particles from fecal material, not in oysters exposed to RNA fragments.

The RNA fragments used in this study may have been too small to efficiently concentrate in the oyster digestive diverticula, the various enzymes and RNases present within the digestive system may have degraded the NoV RNA fragments rapidly rendering the majority undetectable, or NoV accumulation in bivalve molluscs may be dependent predominantly upon interactions between intact virus and receptor sites in the digestive tissues. The outcome of this series of studies indicates that the presence of NoV RNA fragments in the environment is unlikely to make a major contribution to positive qRT-PCR signals. This finding is of significance with respect to interpretation of qRT-PCR results for NoV in Pacific oysters and other shellfish species.

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