Applied and Environmental Microbiology, Feb. 2009, p. 618–624 Vol. 75, No. 3
0099-2240/09/$08.00+0 doi:10.1128/AEM.01507-08

Detection and Quantification of Noroviruses in Shellfish[▽]

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Received 3 July 2008/Accepted 23 November 2008

Noroviruses (NoVs) are the most common viral agents of acute gastroenteritis in humans, and high concentrations of NoVs are discharged into the environment. As these viruses are very resistant to inactivation, the sanitary consequences are contamination of food, including molluscan shellfish. There are four major problems with NoV detection in shellfish samples: low levels of virus contamination, the difficulty of efficient virus extraction, the presence of interfering substances that inhibit molecular detection, and NoV genetic variability. The aims of this study were to adapt a kit for use with a method previously shown to be efficient for detection of NoV in shellfish and to use a one step real-time reverse transcription-PCR method with addition of an external viral control. Comparisons of the two methods using bioaccumulated oysters showed that the methods reproducibly detected similar levels of virus in oyster samples. Validation studies using naturally contaminated samples also showed that there was a good correlation between the results of the two methods, and the variability was more attributable to the level of sample contamination. Magnetic silica very efficiently eliminated inhibitors, and use of extraction and amplification controls increased quality assurance. These controls increased the confidence in estimates of NoV concentrations in shellfish samples and strongly supported the conclusion that the results of the method described here reflected the levels of virus contamination in oysters. This approach is important for food safety and is under evaluationfor European regulation.

Noroviruses (NoVs) are the most common viral agents of acute gastroenteritis in humans. These viruses are nonenveloped, icosahedral viruses with a single-stranded, positive-sense RNA genome and constitute a genus in the family *Caliciviridae* (4). NoVs are genetically and antigenically diverse. As reproducible methods for cultivation of NoVs have not been developed, genetic characterization based on complete capsid gene analysis has been used to classify them into five distinct genetic groups (or genogroups). Three genogroups contain human strains (genogroups I, II, and IV), and the other two genogroups (genogroups III and V) contain strains that infect only animals (4). Genogroup II NoVs (more precisely, genogroup II.4 NoVs) are the predominant cause of NoV infections, but they cause NoV infections within a larger population of cocirculating genotypes (4, 24, 37). The majority of infections occur during winter months, but sporadic cases also occur throughout the year (4, 24). Thus, a large variety of NoVs are discharged into sewage and the environment. NoVs are very resistant to inactivation and have been detected in wastewater treatment plant effluents and in surface waters (10, 26, 38, 40). The sanitary consequences include contamination of drinking water, of foods such as vegetables, and of mollusks (20, 21, 32, 35, 39). Bivalve molluscan shellfish, such as oysters, can filter large volumes of water as part of their feeding activities and are able to accumulate and concentrate different types of pathogens resulting from fecal human pollution. The adoption of regulations that specify acceptable levels of bacterial enteric pathogens in shellfish tissues (European regulation 91/492/EC) or in shellfish-growing water (United States National Shellfish Sanitation Program) has significantly decreased the impact of bacteria as causes of shellfish-associated disease outbreaks (8). However, these regulations have failed to prevent many outbreaks of viral origin, and there have been many examples of gastroenteritis and hepatitis outbreaks in different parts of the world (8, 20, 32).

To protect the consumer, it is important to have sensitive and rapid methods for directly detecting the viral pathogen of concern in shellfish. A number of methods to do this have been described over the past 15 years, demonstrating that detection of viruses in shellfish is possible. However, there are four major problems for detection of NoVs in shellfish samples: low levels of virus contamination, variability in virus or nucleic acid extraction, the presence of interfering substances that inhibit molecular detection, and NoV genetic variability.

The aims of this study were to adapt the Nuclisens kit (BioMerieux), which is a paramagnetic silica-based guanidium extraction technique, for use with a method previously shown to be efficient for NoV detection both in field studies and in outbreak investigations, to validate the modified method using bioaccumulated or naturally contaminated oyster samples, and to estimate the concentrations of NoV in naturally contaminated oysters using real-time reverse transcription-PCR (rRT-PCR) and quality controls.

MATERIALS AND METHODS

Virus strains and RNA extraction. Fecal samples containing genogroup I.1 NoV (Norwalk virus strain 8FIIa-containing stool collected from an infected volunteer at the Baylor College of Medicine) or genogroup II.4 NoV (stool collected from a symptomatic patient, kindly provided by P. Pothier, CHU Dijon) were used for bioaccumulation experiments. Viral RNAs were extracted

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▽ Published ahead of print on 1 December 2008.

from 10% suspensions of stools using a Nuclisens kit (BioMerieux) as recommended by the manufacturer, were eluted in 100 μl of RNase-free water, and were kept at −20°C until they were used. For some bioaccumulation experiments, virus titers in stools were determined by rRT-PCR as described below. Mengovirus strain vMC_0 was propagated in HeLa cells, and the virus titer was determined as described previously (25).

Oyster samples. (i) Bioaccumulated oysters. Natural seawater freshly collected from a clean area was used for bioaccumulation experiments. Live oysters were purchased directly from a producer and then immersed on the same day and incubated for 24 h in large tanks of seawater at the laboratory. Seawater (25 to 50 liters) was artificially contaminated with fecal samples containing genogroup I.1 or II.4 NoV and mengovirus. For some experiments a stool was titrated before seeding, and 1 g (approximately 10^{10} RNA copies) of the genogroup I.1 stool or 5 g (approximately 10^9 RNA copies) or 0.05 g (approximately 10^7 RNA copies) of the genogroup II.4 stool was used in separate bioaccumulation experiments. For the experiment that included mengovirus bioaccumulation, 10^6 50% tissue culture-infective doses of mengovirus was added to the stool dilution before it was added to seawater. The seawater was continuously aerated to maintain adequate oxygenation, and the room temperature was controlled (about 12°C), as experiments were conducted at different times of the year. Following 24 h of bioaccumulation, the oysters were dissected, digestive tissues (DT) were recovered, and 1.5-g portions were frozen. The weights of recovered DT were recorded.

(ii) Naturally contaminated samples. Shellfish samples were collected from different areas of France between March 2001 and January 2008, and DT (1.5 g) were kept frozen until they were used.

Shellfish processing. DT were homogenized, extracted by vortexing with an equal volume of chloroform-butanol for 30 s, and treated with Cat-Floc T (Calgon, Ellwood City, PA) for 5 min on a bench before centrifugation for 15 min at 13,500 × *g*. The resulting suspension was precipitated with polyethylene glycol 6000 (PEG 6000) (Sigma, St. Quentin, France) for 1 h at 4°C and centrifuged for 20 min at 11,000 × *g* at 4°C (2). For some extractions, approximately 10^6 50% tissue culture-infective doses of mengovirus was added to dissected tissues before the homogenization step.

Nucleic acid extraction and purification. (i) Method A. Viral nucleic acids were purified from concentrated virus as previously described (2). Briefly, the PEG 6000 precipitate was digested with 0.2 mg of proteinase K (Amresco, Solon, OH) for 30 min at 56°C and then was extracted with an equal volume of phenol-chloroform (Applied Biosystems, Foster City, CA) and precipitated first with ethanol, then with 1.4% (wt/vol) cetyltrimethylammonium bromide (Sigma), and again with ethanol to concentrate the RNA. The pellet was suspended in 100 μl of RNase-free water with 20 U of RNase inhibitor (Invitrogen) and kept frozen (−80°C).

(ii) Method B. The Nuclisens extraction kit (BioMerieux, Lyon, France) was used according to the manufacturer's instructions, with minor modifications. The PEG 6000 pellet was suspended in 1 ml of RNase-free H_2O, mixed with the lysis buffer (2 ml), and incubated for 30 min at 56°C. After a brief centrifugation to eliminate particles (if needed), 50 μl of paramagnetic silica was added and incubated for 10 min at room temperature. All washes were performed using the magnetic ramp, and nucleic acids were recovered in 100 μl of elution buffer (BioMerieux, Lyon, France). Twenty units of RNase inhibitor (Invitrogen) was added, and nucleic acids were kept frozen (−80°C) until they were used.

Primers and probes. Previously described primers and probes were used. For genogroup I QNIF4 (5′-CGCTGGATGCGNTTCCAT-3′, where N is A, C, G, or T), NV1LCR (5′-CCTTAGACGCCATCATCATTTAC-3′), and NV1LCpr (5′-TGGACAGGAGAYCGCRATCT-3′, where Y is C or T and R is A or G) and for genogroup II QNIF2d (5′-ATGTTCAGRTGGATGAGRTTCTCWGA-3′, where R is A or G and W is A or T), COG2R (5′-TCGACGCCATCTTCA TTCACA-3′), and QNIFs (5′-AGCACGTGGGAGGGCGATCG-3′) were used for amplification in rRT-PCR assays (10, 13, 23, 37). The two probes were labeled with 6-carboxyfluorescein at the 5′ end and with 6-carboxytetramethylrhodamine at the 3′ end. Mengovirus was detected using the method described by Costafreda et al. (9).

rRT-PCR assay. The rRT-PCR was carried out using the Platinum quantitative RT-PCR Thermoscript one-step system (Invitrogen, France). After optimization of reagents and cycling conditions, the following final parameters described by Costafreda et al. (9) were used: 20 μl of reaction mixture containing 900 nM downstream primer, 500 nM upstream primer, 250 nM Taqman probe, and buffer and enzymes at concentrations recommended by the manufacturer. The Rox concentration was adapted to the apparatus (1× for the ABI Prism 7000 or 7300 apparatus and 0.1× for the MX3000P apparatus). Five microliters of nucleic acid extract or control was added per well, and the final total volume in each well was 25 μl. All samples were analyzed at least in duplicate undiluted and after 10-fold dilution. Endpoint dilutions were amplified to compare the sensitivities of the two methods for bioaccumulation experiments. The temperature and time parameters were as follows: RT for 30 min at 55°C and for 5 min at 95°C and 45 cycles of 15 s at 95°C, 1 min at 60°C, and 1 min at 65°C.

Construction of RNA ECs. The genomic sequence amplified by rRT-PCR was identified using GenBank data for Norwalk virus (genogroup I.1) and Lordsdale virus (genogroup II.4), and the sequences were modified by inserting a BamHI site in order to distinguish the external control (EC) from the viral amplimer. For genogroup I the EC sequence was 5′-CGCTGGATGCGCTTCCATGACCTCG GATTGTGGACAGGAGATCGCGATCTTCTGCGGATCCGAATTCGTAA ATGATGATGGCGTCTAAGG, and for genogroup II the EC sequence was 5′-ATGTTCAGATGGATGAGATTCTCAGATCTGAGCACGTGGGAGGG CGATCGCAATCTGGCTCGGATCCCCAGCTTTGTGAATGAAGATGGC GTCGA-3′ (the BamHI sites are underlined).

These two sequences (90 bases for the genogroup I EC and 95 bases for the genogroup II EC) were obtained as purified oligonucleotides (Sigma-Proligo, France), amplified using Pfu *Taq* DNA polymerase (Promega, Charbonnier les Bains, France), and then cloned into pGEM-3Zf(+). Then both vectors were transformed in *Escherichia coli*, and transformant clones were screened. Plasmids were extracted, linearized, and transcribed in vitro using the Promega Riboprobe system. After DNase treatment, RNA standards were purified and quantified by using the optical density at 260 nm and by endpoint rRT-PCR detection.

Construction of quantification standards. For genogroup I, parts of the first two open reading frames (nucleotides 146 to 6935) of Norwalk virus (GenBank accession number M87661) were cloned in the pCRII TOPO (Invitrogen) vector. For genogroup II, the sequence between nucleotides 4191 and 5863 of the Houston virus (GenBank accession number EU310927) was cloned into the same vector. Both vectors were transformed in *E. coli*, and transformant clones were screened. The in vitro transcription was performed with linearized plasmid samples using the Promega Riboprobe system. After DNase treatment, RNA was purified and quantified by using the optical density at 260 nm (10).

rRT-PCR controls and quantification. The cycle threshold (C_T) was set manually at 0.1, and it was always on the logarithmic portion of the amplification curve and was distinguishable from the background fluorescence.

(i) Extraction efficiency. After extraction of samples seeded with the mengovirus, undiluted and 10-fold-diluted extracts were subjected to rRT-PCR for mengovirus. The C_T value of the sample was compared to the C_T value of the positive control used in the extraction series and to a standard curve obtained by endpoint dilution. The difference (ΔC_T) was used to determine the extraction efficiency, using $100e^{-0.6978\Delta C_T}$ (9).

(ii) rRT-PCR efficiency. Samples were amplified with the EC for genogroups I and II. Equal volumes (2.5 μl) of an EC and a sample were mixed and amplified as described above. One hundred to 1,000 RNA copies were used to evaluate the presence of amplification inhibitors that might prevent detection of low virus concentrations in shellfish extracts. The ΔC_T value was obtained by subtracting the C_T value obtained for the sample from the C_T value obtained for the uninhibited control (RNA EC mixed with RNA-free water) on the same plate and was used to estimate the amplification efficiency, which was expressed as a percentage. Some NoV-containing samples could not be evaluated as RT-PCR efficiencies greater than 100% were obtained with them; however, no more than partial sample inhibition could have occurred since viral RNA was detected.

(iii) Quantitation. The number of RNA copies present in each positive sample that could be evaluated was estimated by comparing the sample C_T value to standard curves. The final concentration was then adjusted based on the volume of nucleic acids analyzed and was expressed per gram of DT. For some samples, the virus concentration was expressed uncorrected and corrected by taking into account the extraction efficiency, but no adjustment was made for rRT-PCR efficiency.

RESULTS

rRT-PCR optimization. To enhance the sensitivity of the rRT-PCR, the standard conditions recommended by the manufacturer (short cycle) and optimized conditions (long cycle) were compared using viral RNA extracted from stools. The long cycle and optimized concentrations improved the sensitivity for both genogroups from one to three cycles for detection of viral RNA purified from naturally contaminated shellfish (data not shown). The rRT-PCR conditions described by

TABLE 1. Comparison of methods used with bioaccumulated shellfish

Method	Expt	Genogroup I NoV concn[a]	Genogroup II NoV concn[a]	
			High concn	Low concn
A	1	4.3×10^4	2.2×10^6	3.7×10^4
	2	2.2×10^5	3.3×10^6	1.7×10^4
	3	2.8×10^4	6.4×10^5	2.7×10^4
	Mean[b]	6.4×10^4	1.7×10^6	2.0×10^4
B	1	1.5×10^5	5.3×10^6	3.9×10^4
	2	1.0×10^5	2.5×10^6	2.0×10^4
	3	ND[c]	8.5×10^6	7.8×10^4
	Mean[b]	1.2×10^5	4.8×10^6	3.9×10^4

[a] Concentrations were calculated by taking into account the volume of nucleic acid extract used for rRT-PCR and the corresponding standard curve and were expressed as the number of RNA copies/g of DT.
[b] Geometric mean values were calculated for the three experiments.
[c] ND, not done.

Costafreda et al. (9) (long cycle conditions) were used for all the experiments described here.

Comparison of methods using bioaccumulated oysters. Bioaccumulated oysters were analyzed by both methods in triplicate (except for the genogroup I bioaccumulation experiment, as an insufficient amount of DT was recovered and method B was performed only in duplicate) (Table 1). For all experiments, undiluted and 10-fold-diluted nucleic acids were analyzed in duplicate and were used for quantification. The two methods gave the same concentration for genogroup II NoV in bioaccumulated oysters at high concentrations.

For shellfish exposed to low concentrations of genogroup II NoV, triplicate nucleic acid extracts that were not diluted and diluted 10-fold were analyzed in triplicate (resulting in 18 C_T values). Both methods gave reproducible results, as demonstrated by the mean C_T values and standard deviations, although method A was less likely to completely remove substances that interfered with target amplification (data not shown). The virus concentrations in oyster tissues were similar when they were calculated using mean C_T values obtained with undiluted nucleic acids by method B and mean C_T values obtained with 10-fold-diluted nucleic acids by method A (to eliminate the impact of inhibitors) (Table 1).

Endpoint dilution was performed with extracts from oysters with bioaccumulated genogroup I or II NoVs to compare the detection limits of the two methods. For genogroup I NoVs, both methods were able to detect ~100 copies/g of DT (five positive C_T values for 10 method A replicates and six positive C_T values for 10 method B replicates). For genogroup II as few as 10 copies/g of DT were detected (two positive C_T values for 10 method A replicates and four positive C_T values for 10 method B replicates).

Comparison of methods using naturally contaminated samples. Sixty-three samples collected from the field were analyzed using both methods. The removal of inhibitors was evaluated using EC RNAs for both genogroups I and GII with undiluted and 10-fold-diluted nucleic acids. The amplification efficiencies of both methods were more than 91% (data not shown). For NoV detection, identical results were obtained for 39 (62%) of the samples. Seventeen samples were positive for NoV (genogroup I and/or genogroup II), and 22 samples were negative by both methods. Genogroup I NoVs were found in 12 (41%) samples with method A and in 25 (86%) samples using method B. Genogroup II NoVs were identified in 14 (58%) samples with method A and in 19 (79%) samples with method B. The geometric mean genogroup I NoV concentrations for the 12 samples identified by method A and the 25 samples identified by method B were similar (3.9×10^2 and 1.9×10^2 RNA copies/g DT, respectively). The geometric mean genogroup II NoV concentration for the 14 samples identified using method A was higher than the geometric mean genogroup II NoV concentration for the 19 samples identified using method B (1.2×10^2 and 1.9×10^1 RNA copies/g DT, respectively).

Reproducibility of quantitation with naturally contaminated samples. To evaluate whether the observed differences between the two methods might be explained by uneven distribution of the virus within a sample or by sample inhibition, six naturally contaminated samples were analyzed in triplicate in separate extraction experiments (Table 2). None of the samples showed significant evidence of inhibition (the RT-PCR efficiency varied from 89 to 99.6% for genogroup I and from 92.17 to 103.7% for genogroup II). No genogroup I NoVs were detected in any of the three replicates for two samples, while one or two of the replicates were positive for the other four

TABLE 2. Reproducibility of method B for NoV detection for six naturally contaminated samples, each extracted three times

Sample	Genogroup I NoV				Genogroup II NoV			
	rRT-PCR efficiency (%)[a]	Concn[b] (RNA copies/g DT)			rRT-PCR efficiency (%)[a]	Concn[b] (RNA copies/g DT)		
		Assay 1	Assay 2	Assay 3		Assay 1	Assay 2	Assay 3
1	97.0 ± 1.7	330	+DL[c]		100.7 ± 0.6	16	43	38
2	95.6 ± 2.5				100.9 ± 2.6			+DL
3	94.8 ± 4.3				98.2 ± 5.2			
4	96.7 ± 1.9			250	102.4 ± 0.3	110	+DL	88
5	97.6 ± 1.7	840			99.7 ± 3.9	190	58	
6	94.8 ± 5.0	910			100.1 ± 1.8	110	+DL	53

[a] rRT-PCR efficiency was calculated based on coamplification of genogroup I and II RNA external controls with pure and 10-fold-diluted nucleic acid extract.
[b] The genogroup I or II NoV concentration was calculated based on C_T values obtained for pure and 10-fold-diluted nucleic acid extract and the corresponding standard curve.
[c] +DL, positive sample, but the level was too close to the limit of detection for quantification.

TABLE 3. Quantitation of viruses in bioaccumulated shellfish[a]

Bioaccumulation expt	Extraction efficiency (%)	NoV concn			Mengovirus concn	
		Uncorrected	Corrected	Expected	Uncorrected	Expected
Genogroup I NoV only	20.48 ± 14.7	9.58×10^6	5.46×10^7	2.0×10^8		
Genogroup II NoV only	33.64 ± 5.3	5.08×10^6	1.36×10^7	4.0×10^8		
Genogroup I NoV + mengovirus		1.12×10^7		1.5×10^8	3.93×10^4	1.2×10^5
Genogroup II NoV + mengovirus		8.12×10^6		4.4×10^8	2.29×10^3	1.4×10^4

[a] Arithmetic means for extraction efficiency and geometric means for virus concentrations were calculated by using three replicates. The concentrations (geometric means of three replicates) are expressed as the number of RNA copies/g of DT calculated without taking the extraction efficiency into account (Uncorrected) or taking the extraction efficiency into account (Corrected). The expected concentration was calculated based on the amount of virus seeded into seawater and the weight of DT obtained, assuming that the oyster concentrated 100% of the virus input in the DT.

samples. In contrast, all replicates gave the same result for four samples (one negative and three positive) in the genogroup II NoV assay, and for the other two samples one or two replicates were positive. These results suggest that either the distribution of virus within naturally contaminated samples was heterogeneous or that the variability was due to the presence of very low concentrations of virus, as demonstrated by the detection of positive samples in which the levels were too close to the limit of detection for quantification.

Validation of mengovirus extraction control. Oysters were bioaccumulated with known amounts of genogroup I or II NoV with or without mengovirus. Extraction was then performed using method B (PEG 6000 and Nuclisens kit), and mengovirus was added when it had not been added during bioaccumulation. After extraction, a mengovirus rRT-PCR was performed to evaluate the extraction efficiency (Table 3). The average extraction efficiencies were 20% (range, 10 to 37%) for the genogroup I NoV bioaccumulation studies and 34% (range, 30 to 37%) for the genogroup II NoV bioaccumulation studies. Thus, the corrected NoV concentrations were three- to fivefold higher than the measured values. Even after correction for extraction efficiency, only 23 to 28% of the NoV placed in the bioaccumulation tank was present in the DT harvested from the oysters.

Mengovirus was bioaccumulated with genogroup I or II strains to evaluate its behavior compared to that of NoV. These experiments were performed together with the genogroup I or II NoV bioaccumulation experiments using the same oysters, and hence the extraction efficiencies could not be calculated as described above. The uncorrected mengovirus concentrations were 16 to 32% of the concentrations expected based on the virus input, and the majority of the input virus could be accounted for if the same extraction efficiency correction factors were used. In contrast, only 2 to 7% (not corrected for extraction efficiency) of the input NoV placed in the bioaccumulation tank was present in the DT harvested from the oysters (Table 3).

Analysis of naturally contaminated samples and quantification. One hundred oyster samples that were collected from different areas in France and were suspected to be contaminated were analyzed by using method B and adding mengovirus to evaluate the extraction efficiency. The extraction was repeated for samples for which the mengovirus extraction efficiency was less than 10%. The average extraction efficiency was 37.1% (range, 10.1 to 124%). Fifty-five of the samples were negative and 45 of the samples were positive for NoV (genogroup I and/or II). The average extraction efficiency for positive samples was 38.6% (range, 10.8 to 95.9%). Ten samples were positive for both genogroup I and II NoVs, 9 samples were positive only for genogroup I NoVs, and 26 samples were positive only for genogroup GII NoVs (Table 4). The geometric mean concentrations of genogroup I and II NoVs, corrected for extraction efficiency, were 1,300 and 525 RNA copies/g of DT, respectively.

DISCUSSION

The low virus concentrations present in shellfish require the use of methodologies that efficiently recover viruses from shellfish tissues and that yield purified nucleic acid preparations that do not contain inhibitors of RT-PCR. In shellfish, the greatest concentrations of human enteric viruses are found in the stomach and digestive diverticula (34). Therefore, we decided to specifically target these tissues for analysis (2). This approach has several advantages compared with testing whole shellfish; it is less time-consuming, it results in increased test sensitivity, and it is associated with a decrease in the sample-associated interference with RT-PCR. Based on these obser-

TABLE 4. Analysis of naturally contaminated samples

NoV(s) identified	No. of samples	Avg extraction efficiency (%)	Genogroup I NoV geometric mean concn (RNA copies per g of DT)		Genogroup II NoV geometric mean concn (RNA copies per g of DT)	
			Uncorrected	Corrected	Uncorrected	Corrected
None	55	35.8				
Genogroup I NoV only	9	42.7	296	966		
Genogroup II NoV only	26	36.1			150	590
Genogroups I and II NoVs	10	41.5	594	1,690	134	388
Total	100	38.6	427	1,300	145	525

vations, we developed a sensitive method in which virus is concentrated using PEG precipitation and nucleic acids are extracted using proteinase K digestion, and this method has been successfully used in collaborative trials, environmental studies, and investigations of outbreaks (3, 16–18, 22, 32). An important limitation of this method has been its large number of steps, especially for nucleic acid purification, which makes it labor-intensive and time-consuming. We developed a streamlined method that uses a commercial kit for nucleic acid detection that performs at least as well for virus detection as our previous method when it is used for shellfish contaminated with NoVs naturally or by bioaccumulation.

The nucleic acid extraction kit used is based on a modification of the method of Boom et al. (7) and allows good recovery of viral nucleic acids and efficient removal of inhibitors. Several other studies that utilized this approach for analysis of shellfish, food, and environmental waters have been described (9, 10a, 31, 33, 36). One major advantage of this kit is that it allows analysis of up to 1 ml of shellfish concentrate. Besides this large volume, the use of paramagnetic silica facilitates washes, the extraction is fast, and the availability of premixed reagents is convenient for reproducibility. We compared different paramagnetic silica preparations available in the market using our own reagents or Nuclisens kit reagents and found that there were no differences in recovery (data not shown). The only modification that we made to the manufacturer's protocol was to increase the length of the first incubation step to 30 min in a water bath at 56°C instead of 10 min at room temperature, as this increased the recovery of nucleic acids. Overall, not including the costs associated with the dissection step and any personnel costs, we estimated the cost for the different steps of both methods, taking into account the tubes, tips, and reagents. The first part of the procedure up to when the PEG pellet was obtained took about 2.5 h, and the cost for six samples was approximately 7 euros. Then method A took about 5 h and cost approximately 12 euros, whereas method B took about 1.5 h and cost 42 euros.

No differences in assay performance were observed when we compared our old method to the new modified version when bioaccumulated shellfish were evaluated. In shellfish that were contaminated with NoV by bioaccumulation, virus contamination appeared to be homogeneous (there was no variability between replicates). However, this was not the case for naturally contaminated oyster samples. There was some variability between samples for detection of NoV contamination. Also, the kit-based method detected more NoV-contaminated samples than the older method. During bioaccumulation all oysters were exposed to contaminated water for 24 h under controlled conditions. The variability seen in oysters collected from the field, especially following accidental contamination, may be explained in part by shorter exposure times and varying conditions that affect oyster feeding, so that that the exposure of individual shellfish to virus is more heterogeneous. Another possibility is that the variability observed was due to low levels of virus contamination that were close to the limit of detection. To address the latter possibility, tissues from several oysters can be analyzed simultaneously. The 1.5 g of DT analyzed by this method represents approximately three to four oysters. A possible future improvement could be increasing the sample size to up to six oysters, which is the number of oysters served in restaurants.

A number of investigators have proposed addition of an external virus to a sample as a control to measure the extraction efficiency of molecular virus detection methods (9, 11, 29). Based on the work reported by Costafreda et al. (9), we used mengovirus strain MC_0 as a control for extraction efficiency. Mengovirus, a member of the *Picornaviridae* family, was initially proposed as a control for hepatitis A virus detection methods based on structural characteristics shared with the target virus (9). For NoV, a number of other viruses belonging to the family *Caliciviridae* have been proposed (feline, canine, or murine strains). However, differences in behavior and resistance to inactivation among these viruses make the selection of a control difficult (5, 15). The advantages of mengovirus are that it is unlikely to naturally contaminate shellfish, it is nonpathogenic for humans, and it can be grown in cell culture. The use of a single extraction control for different enteric viruses that may be detected in shellfish or other types of food is also thought to be important for standardization of the method (European working group CEN/Tag4) and for comparisons between different laboratories. To fully evaluate mengovirus behavior compared to NoV behavior, we performed bioaccumulation studies with these viruses. Mengovirus was bioaccumulated like NoV and was as successfully recovered like the genogroup I and II strains. This is a strong argument in favor of using mengovirus as a control for NoV extraction efficiency.

In bioaccumulation experiments, about 10% of the virus added to the tank was detected in oysters. Approximately 0.5 $\log_{10}$ of the virus lost was attributable to the efficiency of the extraction method. The remaining loss may have been due to several other factors. Few data on precise quantification after bioaccumulation are available, but it is likely that some viruses may be present on tank walls or even on shells (6, 27). Also, as shown previously, DT concentrate most but not all viruses (1, 34). The efficiency of virus concentration may also decrease with increasing virus input, as suggested by comparing the levels of mengovirus and NoV recovery. Taking into account all these parameters, we believe that the method described here correctly reflects the amount of viruses present in contaminated oysters.

The modified method (method B), which included mengovirus as a control for extraction efficiency, was used to analyze samples collected from an area suspected to be contaminated (a class B area according to European regulation 91/492/EC) in order to enhance the chance of virus detection. Using this method, we expressed the concentration for one sample as a minimum level and a maximum level (if extraction efficiency was taken into account), which may be a reliable approach to estimate the NoV concentration. Besides the report by Costafreda et al. (9), no other studies have reported the integration of extraction efficiency in quantitative virus estimates. Nishida et al. (29) used echovirus to monitor the efficiency of nucleic acid extraction, but they did not consider it for quantitation. They observed virus concentrations (between 100 and 1,000 copies/g of DT) that were in the same range as the concentrations that we report here and reported in a previous analysis of shellfish implicated in an outbreak (17, 18, 22). Genogroup I NoVs were detected in 19% of the samples, and genogroup II NoVs were detected in 36% of the samples. The

prevalence of genogroup I NoVs is surprising if one considers that most of strains circulating in humans are genogroup II NoVs, with genogroup II.4 predominant (4, 24). In several previous studies workers have observed a similarly high prevalence of genogroup I NoV strains in shellfish and in shellfish-related outbreaks (10a, 12, 13, 17, 18, 29). Genogroup I NoV strains also are detected frequently in treated sewage or surface waters (10, 14, 28). The higher-than-expected prevalence of genogroup I NoVs in the environment may be due to greater resistance of genogroup I strains to inactivation, and in shellfish it may be due to specific binding of genogroup I strains to oyster tissues (10, 19).

Direct detection of viral human pathogens has become the most reliable way to document viral contamination of shellfish. Although previously described methods are sensitive and reproducible, these methods are labor-intensive and take several days to complete. Adaptation of these methods so that readily available reagents can be utilized has become a priority. We show here that a commercially available extraction kit (method B) can be used to detect NoVs in shellfish without a loss of assay sensitivity. The use of a kit that can even be used with an automated apparatus and rRT-PCR is a major step toward standardization. This approach is important for food safety and is under evaluation for European regulation. Moreover, this approach, in conjunction with quality assurance control, should help workers analyze more precisely the level of virus contamination in food and thus should contribute to reductions in shellfish-borne outbreaks.

ACKNOWLEDGMENTS

We thank Joanna Krol, Larissa Haugarreau, and Jean-Claude Le Saux for advice, discussions, and technical assistance. We are grateful to Rosa Pinto for her helpful advice.

This study was supported in part by the European Community SeafoodPlus project (grant Food-CT-2004-506359, Rephepa) and Ifremer.

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