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Detection and Quantification of Noroviruses in Shellfish

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Abstract:

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 have not been developed, genetic characterization based on the complete capsid gene analysis has been used to classify them into five distinct genetic groups (or genogroups). Three genogroups (G) contain human strains (I, II and IV) and the other two (III and V) contain strains that only infect animals (4). GII NoVs, and more precisely GII.4, are the predominate cause of NoV infections, but they do so within a larger population of co-circulating genotypes (4, 25, 37). The majority of infections occur during winter months but sporadic cases also occur throughout the year (4, 25). Thus, a large variety of NoVs is 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, 27, 38, 40). The sanitary consequences include contamination of drinking water, of foods such as vegetables, and of mollusks (21, 22, 32, 35, 39). Bivalve molluscan shellfish, such as oysters, can filter large volume of waters as part of their feeding activities and are able to accumulate and concentrate different types of pathogens from fecal human pollution. The institution of regulations to specify acceptable levels of bacterial enteric pathogens in shellfish tissues (European regulation, 91/492/EC) or in shellfish growing waters (United States National Shellfish Sanitation Program) has decreased significantly 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 are many examples of gastroenteritis and hepatitis outbreaks occurring in different parts of the world (8, 21, 32).

To protect the consumer, it is important to have sensitive and rapid methods to directly detect the viral pathogen of concern in shellfish. A number of methods have been described over the

1 past 15 years demonstrating that virus detection in shellfish is possible. However, NoV
2 detection in such samples presents four major problems: low levels of virus contamination,
3 variability in virus / nucleic acid extraction, the presence of interfering substances that inhibit
4 molecular detection, and NoV genetic variability.

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

11 MATERIALS AND METHODS.

12 Virus strains and RNA extractions.

13 Fecal samples containing GI.1 (Norwalk virus strain 8FIIa stool collected from an infected
14 volunteer, Baylor College of Medicine), or GII.4 NoVs (stool collected from a symptomatic
15 patient, kindly provided by P. Pothier, CHU Dijon) were used for bio-accumulation
16 experiments. Viral RNAs were extracted from 10% suspensions of stool using the Nuclisens
17 kit (BioMerieux) as recommended by the manufacturer and was eluted in 100 µl of RNAse-
18 free water and kept at –20°C until used. For some bioaccumulation experiments, virus titers in
19 stool were measured by real-time RT-PCR as described below. The Mengovirus (strain
20 vMC_0) was propagated in HeLa cells and virus titer was determined as described (26).

21 Oyster samples.

22 *Bioaccumulated oysters:* Natural seawater freshly collected from a clean area was used for
23 bioaccumulation experiments. Live oysters were purchased directly from a producer and
24 immersed the same day for 24 hours in large tanks of seawater at the laboratory. Seawater (25

to 50 l) was artificially contaminated with fecal samples containing GI.1 or GII.4 NoV and mengovirus. For some experiments stool was titrated before seeding and one g (approximately 10^{10} copies) of the GI.1 stool, 5g (approximately 10^{9} copies) or 0.05g (approximately 10^{7} copies) of the GII.4 stool were used in separate bioaccumulation experiments. For the experiment including mengovirus bioaccumulation, 10^{6} $TCID_{50}$ mengovirus were added to the stool dilution before addition into seawater. Seawater was continuously aerated to maintain adequate oxygenation and the room temperature was controlled (about 12°C), as several experiments were conducted at different periods of the year. Following 24 hours of bioaccumulation, the oysters were dissected and digestive tissues (DT) were recovered and frozen in 1.5 g portions. The weights of recovered DT were recorded.

Naturally contaminated samples: Shellfish samples were collected from different areas in France between March 2001 to January 2008 and were kept frozen as DT (1.5 g) until used.

Shellfish processing.

Digestive tissues were homogenized, extracted by vortexing with an equal volume of chloroform:butanol for 30 sec, and treated with Cat-Floc T(Calgon, Ellwood City, USA) for 5 min on the bench before centrifugation for 15 min at 13,500 x*g*. The resulting suspension was precipitated with polyethylene glycol 6000 (Sigma, StQuentin, France) for one hour at 4°C and centrifuged for 20 min at 11,000 xg at 4°C (2). For some extractions, approximately 10^{6} $TCID_{50}$ mengovirus was added in dissected tissues before the homogenization step.

Nucleic acid extraction and purification.

Method A: Viral nucleic acids were purified from concentrated virus as previously described (2). Briefly, the PEG 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 in ethanol, then with 1.4%

(wt./vol.) cetyltrimethylammonium bromide (Sigma), and again with ethanol to concentrate the RNA. The pellet was suspended in 100µl of RNase-free water with 20 units of RNAse inhibitor (Invitrogen) and kept frozen (-80°C).

Method B: The Nuclisens Extraction kit (BioMerieux, Lyon, France) was used following manufacturer's instructions with minor modifications. The PEG pellet was suspended in one 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 were 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) were added and nucleic acids were kept frozen (-80°C) until used.

Primers and probes.

Previously described primers and probes were used. For GI, QNIF4 (5'-CGCTGGATGCGNTTCCAT-3' with n: a/c/g/t), NV1LCR (5'-CCTTAGACGCCATCATCATTTAC-3') and NV1LCpr (5'-TGGACAGGAGAYCGCRATCT-3' with y: c/t and r: a/g) and for GII, QNIF2d (5'-ATGTTCAGRTGGATGAGRTTCTCWGA-3' with r: a/g and w: a/t)), COG2R (5'-TCGACGCCATCTTCATTCACA-3'), QNIFs (5'-AGCACGTGGGAGGGCGATCG-3') were selected for the amplification by real-time RT-PCR assay (10, 14, 24, 37). The two probes were labeled with 6-carboxy fluorescein (FAM) at the 5' end and 6-carboxy-tetramethylrhodamine (TAMRA) at the 3' end. Mengovirus detection was performed using the method described by Costafreda et al. (9).

Real-time RT-PCR assay.

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

Construction of RNA external controls.

The genomic sequence amplified by rRT-PCR was identified from GenBank for Norwalk virus (GI.1) and Lordsdale virus (GII.4) and the sequences were modified by inserting a BamHI site in order to distinguish the external control (EC) from the viral amplimer. For GI the EC sequence was : 5'-
cgctggatgcgcttccatgacctcggattgtggacaggagatcgcgatcttctgc*ggatcc*gaattcgtaaatgatgatggcgtctaagg
and for GII: 5'-
atgttcagatggatgagattctcagatctgagcacgtgggagggcgatcgcaatctggctc*ggatcc*ccagctttgtgaatgaagatgg
cgtcga-3' (italic letters represent the BamHI site).

These two sequences (90 bases for GI EC and 95 bases for GII EC) were ordered as purified oligonucleotides (Sigma-Proligo, France), amplified using pfu *Taq* DNA polymerase (Promega, Charbonnier les Bains, France) and then cloned into the pGEM-3Zf(+).Then both

vectors were transformed in *E. coli* and transformant clones were screened. Plasmids were extracted, linearized, and *in vitro* transcribed using the Promega riboprobe system. After DNAse treatment, RNA standards were purified and quantified by optical density at 260 nm and end point real-time RT-PCR detection.

Construction of quantification standards.

For GI, parts of the two first open reading frames (nt 146-6935) of the Norwalk virus (Genbank M87661) were cloned in the pCRII TOPO (Invitrogen) vector. For GII, the sequence between nucleotide 4191 and 5863 of the Houston virus (Genbank 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 on linearized plasmid samples using the Promega riboprobe system. After DNAse treatment, RNA was purified and quantified by optical density at 260 nm (OD_{260}) (10).

***r*RT-PCR controls and quantification.**

The Cycle threshold (C_T) was set manually at 0.1 and was always on the logarithmic portion of the amplification curve and was distinguishable from the background fluorescence.

Extraction efficiency: after extraction of samples seeded with the mengovirus, undiluted and ten-fold diluted extracts were subjected to *r*RT-PCR for mengovirus. The C_T value of the sample was compared to the Ct value of the positive control used in the extraction series, and to a standard curve made by end point dilution. This difference (ΔC_T) was used to determine the extraction efficiency, using the equation $100e^{-0.6978\Delta CT}$ (9).

rRT-PCR efficiency: samples were amplified with the EC for GI and GII. Equal volumes (2.5 µl) of the EC and the sample were mixed and amplified as described. One hundred to one thousand RNA copies were used to evaluate for 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 to the C_T value of he uninhibited control (RNA EC mixed with RNA-free water) on the same plate and used to estimate the amplification efficiency expressed in %. Some NoV-containing samples could not be evaluated as they yielded RT-PCR efficiencies greater than 100%; however, no more than partial sample inhibition could be present since viral RNA was detected.

Quantitation: the number of RNA copies present in each positive evaluable sample 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 reported per g of DT. For some samples, virus concentration was expressed uncorrected or corrected by taking into account the extraction efficiency, but no adjustment was made for *r*RT-PCR efficiency.

RESULTS

*r*RT-PCR optimization.

To enhance sensitivity of the *r*RT-PCR, standard conditions (short cycle) as recommended by the manufacturer and optimized conditions (long cycle) were compared using viral RNA extracted from stool. The long cycle and optimized concentrations improved the sensitivity for both genogroups from one to three C_T for detection of viral RNA purified from naturally contaminated shellfish (data not shown). The long cycle conditions were used for all the experiments presented here.

Methods comparison using bioaccumulated oysters.

Bioaccumulated oysters were analyzed by both methods in triplicate (except for the GI bioaccumulation experiment, as an insufficient amount of digestive tissues were recovered and method B was only performed in duplicate) (Table 1). For all experiments, undiluted and ten-fold diluted nucleic acids were analyzed in duplicate and were used for quantification.

Both methods gave the same concentration for GI or GII NoV bioaccumulated oysters at high concentrations.

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

End point dilutions were performed on extracts from oysters bioaccumulated with GI or GII NoVs to compare detection limits of the two methods. For GI NoVs, both methods were able to detect ~100 copies/g of DT (five positive Ct values among 10 replicates for method A and six among 10 for method B). For GII as few as ten copies / g of DT were detected: two positive Ct values among 10 replicates for method A and four positive Ct values among 10 replicates for method B.

Methods comparison using naturally contaminated samples.

Sixty-three samples collected from the field were analyzed using both methods. Removal of inhibitors was evaluated using RNAs EC both for GI and GII with undiluted and ten-fold diluted nucleic acids. Both methods had amplification efficiencies of more than 91% (data not shown). For NoV detection, identical results were found for 39 (62%) of the samples. Seventeen samples were positive for NoV (GI and/or GII) and 22 were negative by both methods. GI NoVs were found in 12 (41%) samples with method A and in 25 (86%) samples using method B. GII NoVs were identified in 14 (58%) samples with method A and in19 (79%) samples with method B. The geometric mean GI NoV concentrations for the 12

samples identified by method A and the 25 samples identified by method B were similar (3.9 x10^2 RNA copies /g DT and 1.9 x 10^2 RNA copies /g DT, respectively). The geometric mean GII NoV concentration for the 14 samples identified using method A were higher than for the 19 samples identified using method B (1.2x10^2 RNA copies /g DT and 1.9x 10^1 RNA copies /g DT, respectively).

Reproducibility of quantitation on naturally contaminated samples.

To evaluate whether the observed differences between the two methods might be explained by uneven distribution of the virus within the 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 (% of RT-PCR efficiency varied from 89 to 99.6 for GI and 92.17 to 103.7 for GII). No GI 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 samples. In contrast, all replicates gave the same result for four samples (one negative and three positive) in the GII NoV assay, and the other two samples had one or two replicates positive. These results suggest that either there is a heterogeneous distribution of virus within naturally contaminated samples or that the variability is due to the presence of very low concentrations of virus, as demonstrated by the detection of positive samples that were too close to the limit of detection for quantification.

Validation of Mengovirus extraction control.

Oysters were bioaccumulated with known amounts of GI or GII NoV and with or without mengovirus also added. Extraction was then performed using method B (PEG/Nuclisens), adding mengovirus when it had not been added during bioaccumulation. After extraction, mengovirus rRT-PCR was performed to evaluate the extraction efficiency (Table 3). Extraction efficiency averaged 20% (range 10-37%) for the GI NoV bioaccumulation studies and averaged 34% (range 30-37%) for the GII NoV bioaccumulation studies. Thus, corrected

NoV concentrations were three- to five-fold higher than the measured values. Even after correcting for extraction efficiency, only 23-28% of the NoV placed in the bioaccumulation tank was present in the digestive tissues harvested from the oysters.

Mengovirus was bio-accumulated with GI or GII strains to evaluate its behavior compared to NoV. These experiments were performed together with the GI or GII NoV bioaccumulation experiments on the same oysters and hence the extraction efficiencies could not be calculated as before. Uncorrected mengovirus concentrations were 16-32% of that expected based upon virus input, and the majority of the input virus could be accounted for if the same extraction efficiency correction factors were applicable. In contrast, only 2-7% (not corrected for extraction efficiency) of the input NoV placed in the bioaccumulation tank was present in the digestive tissues harvested from the oysters (Table 3).

Analysis of naturally contaminated sample and quantification.

One hundred oyster samples collected from different areas in France, suspected to be contaminated, were analyzed using method B and adding mengovirus to evaluate extraction efficiency. The extraction was repeated for samples in which the mengovirus extraction efficiency was lower than 10%. The average extraction efficiency was 37.1%, varying from 10.1 to 124%. Fifty-five of these samples were negative and 45 were positive for NoV (GI and/or GII). The average extraction efficiency for positive samples was 38.6%, varying from 10.8 to 95.9%. Ten samples were positive for both GI and GII NoVs, nine for GI NoVs only and 26 for GII NoVs only (Table 4). The geometric mean concentrations for GI and GII NoVs, corrected for extraction efficiency, were 1300 and 525 RNA copies /g of DT, respectively.

DISCUSSION

The low virus concentrations present in shellfish requires the use of methodologies that efficiently recover viruses from shellfish tissues and that yield purified nucleic acid preparations that are devoid of 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 presents several advantages in comparison with testing whole shellfish: it is a less time-consuming procedure, it results in increased test sensitivity, and it is associated with a decrease in the sample-associated interference with RT-PCR. Based on these observations we developed a sensitive method in which virus is concentrated using polyethylene glycol precipitation and nucleic acids are extracted using a proteinase K digestion, and this methods has been successfully applied in collaborative trials, environmental studies and outbreak investigations (3, 17-19, 23, 32). An important limitation of this method has been its large number of steps, especially for the nucleic acid purification, making this approach 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 applied to shellfish contaminated with noroviruses naturally or by bioaccumulation.

The nucleic acid extraction kit is a modification of the Boom method (7) and allows good recovery of viral nucleic acids and efficient removal of inhibitors. Several other studies have been reported that utilize this approach for analysis of shellfish, food and environmental waters (9, 11, 31, 33, 36). One major advantage of this kit is that it allows the analysis of up to one ml of shellfish concentrate. Beside this large volume, the use of paramagnetic silica facilitates washes, the extraction is fast and the availability of pre-mixed reagents is convenient for reproducibility. We compared different paramagnetic silica available on the market using our own reagents or Nuclisens kit reagents and found no difference in recovery

(data not shown). The only modification we made to the manufacturer's protocol was to prolong the first incubation step to 30 min in a water bath at 56°C instead of 10 min. at room temperature, as it increased the nucleic acid recovery. Overall, without including the costs associated with the dissection step, and any consideration for personnel costs, we estimated the cost for the different steps of both methods taking into account the tubes, tips, reagents. The first part of the procedure up to the PEG pellet took about 2.5 hours and the cost for six samples is approximately 7 euros. Then, method A took about 5 hours and cost approximately 12 euros, whereas method B took about 1.5 hours and cost 42 euros.

No differences in assay performance were observed when we compared our old method to the newly modified version when bioaccumulated shellfish were evaluated. In shellfish that were contaminated with norovirus by bioaccumulation, virus contamination appeared to be homogenous (no variability between replicates). However, this was not the case for naturally contaminated oyster samples. There was some variability between samples in the detection of NoV-contamination. Also, the kit-based method detected more NoV-contaminated samples than the older method. During bioaccumulation all oysters are exposed to contaminated water for 24 hours and under controlled circumstances. 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 such that the exposure of individual shellfish to virus is more heterogeneous. Another possibility is that the variability observed is due to the low levels of virus contamination that are close to the limit of detection. To address this latter possibility, tissues from several oysters can be analyzed simultaneously. The 1.5 g of digestive tissues analyzed in this method represents approximately three to four oysters. A possible future improvement could be to increase the sample size up to six oysters, which is the number of oysters served in restaurant.

A number of investigators have proposed the addition of an external virus to a sample as a control to measure extraction efficiency of molecular virus detection methods used (9, 12, 30). Based on the work reported by Costafreda et al. (9), we used mengovirus strain MC_0 as a control for extraction efficiency. Mengovirus, a *Picornaviridae* family member, was initially proposed as a control for hepatitis A virus detection methods based on structural characteristics shared by the target virus (9). For NoV, a number of other viruses have been proposed within *Caliciviridae* family (feline, canine or murine strains). However, differences in behavior and resistance to inactivation among these viruses make the selection of a control difficult (5, 16). Advantages of mengovirus are that it is unlikely to naturally contaminate shellfish, it is non-pathogenic 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 type of food is also considered to be important for method standardization (European working group CEN/Tag4) and for comparisons between different laboratories. To fully evaluate mengovirus behavior compared to norovirus we performed bioaccumulation studies with these viruses. Mengovirus was bioaccumulated as norovirus and was successfully recovered as the GI or GII strains. This is a strong argument in favor of mengovirus as a candidate for norovirus extraction efficiency control.

For bio-accumulation experiments, about 10% of the virus added to the tank was detected in oysters. Approximately 0.5 log10 of the virus lost is attributable to the efficiency of the extraction method. The remaining loss may be due to several other factors. Few data are available on precise quantification after bioaccumulation but it is likely that some viruses may be found on tank walls or even on shell (6, 28). Also as shown previously digestive tissues concentrate most viruses but not all (1, 34). The efficiency of virus concentration may also decrease with increasing virus input, as suggested by comparing the mengovirus and

norovirus recoveries. So taking into account all these parameters we believe that the method presented here reflects correctly the amount of viruses present in the contaminated oysters. The modified method (method B), including mengovirus as an extraction efficiency, was used to analyze samples collected from area suspected to be contaminated (class B area according to European regulation, 91/492/EC) to enhance the chance of virus detection. Using this method we expressed the concentration for one sample as a minimum and a maximum level (if extraction efficiency is taken into account), that may constitute a reliable approach to estimate NoV concentration. Beside the report from Costafreda et al. (9), no other studies report the integration of extraction efficiency in quantitative virus estimates. Nishida et al. (30) used echovirus to monitor for efficiency of nucleic acid extraction but they did not consider it for quantitation. They observed virus concentrations (between 100 to 1000 copies/g of digestives tissues) in the same range as what we report here and in a previous analysis of shellfish implicated in an outbreak (18, 19, 23). GI NoVs were detected in 19% of the samples, and GII NoVs in 36%. The prevalence of GI NoVs is surprising if one considers that most of strains circulating in humans are GII NoVs, with GII.4 being predominant (4, 25). Several previous reports have observed a similarly high prevalence of GI NoV strains in shellfish, and in shellfish-related outbreaks (11, 13, 14, 18, 19, 30). GI NoV strains also are detected frequently in treated sewage or surface waters (10, 15, 29). The higher than expected prevalence of GI NoVs in the environment may be due to a greater resistance of GI strains to inactivation, and in shellfish it may be due to specific binding of GI strains to oyster tissues (10, 20).

The direct detection of viral human pathogens has become the most reliable manner for documenting viral contamination of shellfish. Although previously described methods are sensitive and reproducible, these methods were labor-intensive and took several days to complete. The adaptation of these methods to utilize readily available reagents has become a

priority. We show that use of a commercially available extraction kit (method B) can be applied to the detection of noroviruses in shellfish without loss of assay sensitivity. The use of a kit, that can even be used with an automated apparatus, and *r*RT-PCR constitute a major step for standardization. This approach is important for food safety and is under evaluation for European Regulation. Moreover this approach, in conjunction with quality assurance control will help to analyze more precisely the level of virus contamination in food, and thus will contribute to reduce shellfish borne outbreaks.

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1 Table 1: Methods comparisons on bioaccumulated shellfish.

2

Bioaccumulation		NoV GI[a]	NoV GII[a]	
	Assay		High conc.	Low conc.
Method A	1	4.3 x 10^4	2.2 x 10^6	3.7 x 10^4
	2	2.2 x 10^5	3.3 x 10^6	1.7 x 10^4
	3	2.8 x 10^4	6.4 x 10^5	2.7 x 10^4
	mean[b]	*6.4 x 10^4*	*1.7 x 10^6*	*2.0 x 10^4*
Method B	1	1.5 x 10^5	5.3 x 10^6	3.9 x 10^4
	2	1.0 x 10^5	2.5 x 10^6	2.0 x 10^4
	3	nd	8.5 x 10^6	7.8 x 10^4
	mean[b]	*1.2 x 10^5*	*4.8 x 10^6*	*3.9 x 10^4*

3 [a]: concentration was calculated taking into account the volume of NA used for rRT-PCR the corresponding
4 standard curve and then expressed as number of RNA copies/g of digestive tissues.
5 [b]: geometric mean values calculated for the three experiments. Nd: not done
6

Table 2: Reproducibility of method B for NoV detection in six naturally contaminated samples extracted three times each.

Sample	NoV GI				NoV GII			
	*r*RT-PCR efficiency [a]	Assay (RNA/g DT)			*r*RT-PCR efficiency [a]	Assay(RNA/g DT)		
		1	2	3		1	2	3
1	97.0 ±1.7	330	+DL	-	100.7 ±.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]: *r*RT-PCR efficiency was calculated based on co- amplification of GGI and GGII RNA IC with pure and one log dilution nucleic acid extract, and expressed in %,

[b]: GGI or GGII norovirus concentration was calculated based on Ct values obtained for pure and one log diluted NA and corresponding standard curve.

+DL: positive sample but too close to the limit of detection for quantification.

1 Table 3: Quantitation in bioaccumulated shellfish.

Bioaccumulation experiment	Extraction efficiency	NoV concentration			Mengovirus concentration	
		Uncorrected	Corrected	Expected	Uncorrected	Expected
GI NoV only	20.48±14.7	9.58×10^6	5.46×10^7	2.0×10^8		
GII NoV only	33.64±5.3	5.08×10^6	1.36×10^7	4.0×10^8		
GI NoV + mengovirus		1.12×10^7		1.5×10^8	3.93×10^4	1.2×10^5
GII NoV + mengovirus		8.12×10^6		4.4×10^8	2.29×10^3	1.4×10^4

2 [a]Arithmetic mean for extraction efficiency and geometric mean for virus concentrations calculated from 3
3 replicates.
4 Concentrations, geometric mean of the 3 replicates, are expressed in RNA copies/g of DT calculated without
5 taking into account the extraction efficiency % (without) or with this % (with). The expected concentration was
6 calculated based on the amount of virus seeded into sea water and the weight of digestive tissues obtained,
7 assuming that the oyster concentrated 100% of the virus input into the digestive tissues.
8

1 Table 4: Analysis of naturally contaminated sample.

NoVs Identified	Number of samples	Extraction efficiency average	GI NoV GMC		GII NoV GMC	
			uncorrected	corrected	uncorrected	corrected
none	55	35.8%				
GI NoV only	9	42.7%	296	966	-	-
GII NoV only	26	36.1%	-	-	150	590
GI and GII NoV	10	41.5%	594	1690	134	388
Total	100	38.6%	427	1300	145	525

2 GMC = geometric mean concentration (RNA copies per g of DT)