Environmental detection of GI, GII and GIV norovirus using a generic real-time RT-PCR assay.

Short title: Norovirus generic real-time RT-PCR

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ABSTRACT

Norovirus is the most common agent implicated in foodborne outbreaks and is frequently detected in environmental samples. These viruses are highly diverse and three genogroups infect humans (GI, GII, and GIV). Being non-cultivable viruses, real-time RT-PCR is the only sensitive method available for their detection in food or environmental samples. Selection of consensus sequences for the design of sensitive assay has been challenging due to sequence diversity and led to the development of specific real-time RT-PCR assays for each genogroup. Thus, sample screening can require several replicates for amplification of each genogroup (without considering positive and negative controls or standard curves). This study reports the development of a generic assay that detects all three human norovirus genogroups using a one-step real-time RT-PCR assay on a qualitative basis. The generic assay achieved good specificity and sensitivity for all three genogroups, detected separately or in combination. At variance with multiplex assays, the choice of the same fluorescent dye for all three probes specific to each genogroup allows the fluorescence to be added and may increase assay sensitivity when multiple strains from different genogroups are present. Applied to sewage sample extracts, this generic assay successfully detects all positive samples compared to genogroup-specific RT-PCR. The generic assay also identified all norovirus-positive samples among 157 archived nucleic acid shellfish extracts, including samples contaminated by all three genogroups.

Keywords: human norovirus, genogroups I, II and IV, real-time RT-PCR, generic RT-PCR, shellfish, sewage analysis.
Viral contamination of water samples and foodstuffs is increasingly recognized through outbreak investigations, epidemiological surveys, and sample analysis. Among the large diversity of human enteric viruses discharged into the environment, norovirus (NoV) is the most common pathogen. Belonging to the Caliciviridae family, the Norovirus genus is divided into six genogroups, and three of these (GI, GII, and GIV) infect humans (1, 2). NoVs cause gastroenteritis characterized by vomiting and diarrhea in persons of all ages, and a predominance of GII strains are reported in clinical cases. Infection with many strains is dependent on histo-blood group antigen (HBGA) expression, as HBGAs serve as an attachment factor necessary to initiate virus infection (3). NoVs are the major cause of non-bacterial gastroenteritis worldwide and have been identified as the predominant cause of foodborne outbreaks (4). The large amount of virus shed by infected persons and the high level of resistance to inactivation in the environment are likely factors associated with virus prevalence in environmental waters (5-7). Although food handlers have been implicated as the source of food contamination in some outbreaks, it is clear that foods such as berries, green vegetables and shellfish can be contaminated during production (8-10).

Screening of food or environmental waters such as raw or treated sewage is one approach that can be considered as a strategy to prevent virus-associated outbreaks. The achievement of sensitive methods and in real-time reverse transcription PCR (rRT-PCR) allows controls on food or environmental samples. The aim of this study was to develop a generic assay that can detect all three human NoV genogroups (GI, GII, and GIV) using a one-step rRT-PCR assay on a qualitative basis. The generic assay, developed and optimized based on previously reported primers and probes, showed a sensitivity comparable to genogroup-specific assays. The newly developed assay was used to analyze naturally contaminated samples such as raw and treated sewage and shellfish samples, and compared to genogroup specific-real-time RT-PCR.

MATERIALS AND METHODS
Virus strains, stool samples and reference materials.

To validate the NoV assays in this study, we used human fecal samples containing GI.1 and GII.3, as well as a reference NoV RNA panel containing in vitro transcribed RNA from nine GI, nine GII, and one GIV genotypes (provided by H. Vennema and M. Koopmans, RIVM, Bilthoven, the Netherlands). Other human enteric viruses were obtained from cell cultures: Aichi virus (AiV) genotype A (P. Pothier, CHU Dijon), astrovirus (AstV) type 4, poliovirus (PV) type 3 (both from B-M. Marcillé, CHU Nantes), hepatitis A virus (HAV) HM175, mengovirus (MgV) strain vMC0 (both from A. Bosch, University of Barcelona, Spain), and rotavirus (RV) RF (, D. Poncet, INRA-CNRS, Gif sur Yvette). For hepatitis E virus (HEV), a porcine stool characterized as a genotype 3 was included (N. Pavio, ANSES, Paris, France).

Nucleic acids (NAs) were extracted from 10% stool suspension using NucliSens kit (bioMerieux, Lyon, France) following the manufacturer’s instructions (11) or by 5-min boiling for cell culture strains.

Archived nucleic acid samples.

Archived NAs from 157 naturally contaminated shellfish samples (oyster, mussel and clam) (12-14) (and unpublished data), eight bioaccumulated oyster samples (11), and 16 untreated and 12 treated wastewater samples (7) were included in the study.

Primers and probes.

For the genogroup-specific real-time RT-PCR (spe-rRT-PCR) detecting NoV GI, GII and GIV separately, previously described primers and probes were used (i.e. QNIF4, NV1LCR, and NV1LCpr for GI, QNIF2d, COG2R, and QNIFS for GII, and Mon4F, COG2R, and Ring4 for GIV) (15-18). These primers and probes were modified for the generic rRT-PCR (gen-rRT-PCR) detecting the three genogroups on a qualitative basis (NV1LCR, COG2R, NIFG1F, NIFG2F, NIFG4F, NIFG1P, QNIFS, NIFG4P) (Figure 1). For AiV, AstV, HAV, HEV, MgV, PV, RV, and SaV, primers and probes described previously were used (19-27).
Real-time RT-PCR.

All amplifications were performed with an Mx3000P QPCR System (Agilent Technologies, France) using RNA UltraSense One-Step Quantitative RT-PCR System (Life technologies, France), and 5-μL of extracted sample per well (final volume of 25 μL). The thermal conditions consisted of RT reaction for 15 min at 55 °C, inactivation of RT and activation of Taq polymerase for 5 min at 95 °C, followed by 45 cycles of denaturing for 15 sec at 95 °C, annealing for 1 min at 55 °C, and extension for 1 min at 65 °C. For the spe-rt-PCR, conditions were as previously described, final concentration of 900 nM for the reverse primer (GI: NV1LCR or GII/GIV: COG2R), 500 nM for the forward primer (GI: QNIF4 or GII: QNIF2d or GIV: Mon4F), and 250 nM for the probe (GI: NV1LCpr or GII: QNIFS or GIV: Ring4) (22). For the gen-rt-PCR, the same conditions were used except that each well contained a mixture of two reverse primers (NV1LCR and COG2R), three forward primers (NIFG1F, NIFG2F, and NIFG4F), and three probes (NIFG1P, QNIFS, and NIFG4P) (Figure 1) at a final concentration of 400 nM for each primer and 200 nM for each probe. All probes were labeled using the same fluorescent dye and quencher (FAM, BHQ). These conditions were applied in preliminary studies (Table1) with the primers and probes currently used in our laboratory (i.e. QNIF4, NV1LCR, NV1LCpr, QNIF2d, COG2R, QNIFS, Mon4F, COG2R, Ring4 mixed together). For later experiments (Table 2), newly designed primer and probe sets for GI (NIFG1F, NV1LCR, and NIFG1P), GII (NIFG2F, COG2R, and QNIFS), and GIV (NIFG4F, COG2R, and NIFG4P) were used separately in conditions used for the spe-rt-PCR (see above).

The cycle threshold (Cₜ) was defined as the cycle at which a significant increase in fluorescence occurred. For assays of reference strains and bioaccumulated samples, results are expressed as the mean Cₜ ± standard deviation calculated using triplicate wells. For naturally contaminated samples, screening was first performed using a single well to determine Cₜ for all 3 spe-rt-PCR and gen-rt-PCR, as limited amount of extracts was available. When a discordant result was obtained for one genogroup, triplicate wells were used to determine whether the
sample is positive in both assays for this specific genogroup. The rRT-PCR assays for AiV, AstV, HAV, HEV, MgV, PV, RV, and SaV were performed as described previously (19-27).

**Specificity and reactivity of the gen-rRT-PCR.**

NA extracts from AiV, AstV, HAV, HEV, MgV, PV, RV, and SaV, and the reference NoV RNA panel were diluted and adjusted to get C_T values of ~30 by rRT-PCR using the respective pathogen-specific primers and probe, and amplified by the gen-rRT-PCR.

**Sensitivity of the gen-rRT-PCR.**

Reference GI.1, GII.3, and GIV.1 NA extracts were 10-fold serially diluted (upon a range of C_T values from 24 to 38 as determined by spe-rRT-PCR), and amplified by gen-rRT-PCR. To mimic multiple contaminations, these NA extracts adjusted to C_T values of ~36 were mixed (2 µL each) in different combinations (2 or 3 genogroups) and amplified.

**RESULTS**

**Primer and probe design.**

In preliminary studies, the previously developed GI-, GII-, and GIV-specific primers and probes were mixed to amplify high, medium, and low concentrations of three NoV strains (GI.1, GII.3, or GIV.1). An increase of at least 4 C_T units was observed compared to the spe-rRT-PCR (i.e., using primers and probes separately), with low concentrations not being detected (Table 1). The spe-rRT-PCR assays target a short conserved region from the junction between open reading frames 1 (ORF1) and 2 (ORF2) that allows efficient detection of strains from the different genogroups. This area was also selected for development of the gen-rRT-PCR assay. Based on reference strain sequence analysis, a number of different primers and probes were evaluated (data not shown), and the best combination is described in Figure 1. For GI NoV, the forward primer (NIFG1F: atgttccgctggatgcg) was moved 6 bases upstream from QNIF4, the probe (NIFGIP: tgtggacaggagaycgcratct with y: c or t and r: a or g) was made longer than NV1LCpr by adding 2
bases to the 5’end, and the reverse primer (NV1LCR: ccttagccatcatcatttac) was not modified. The reverse primer (COG2R: tcgacgccatcttcattcaca) and probe (QNIFS: agcaagtgggagggcgatcg) for GII were not changed but the forward primer QNIF2d was shortened 3 bases at the 3’end (NIFG2F: atgttcagrtgatagtttct with r: a or g). For GIV NoV, the GII reverse primer COG2R, which matches perfectly with available GIV NoV sequences, was selected, the forward primer (NIFG4F: atgtacaagtggatgcgrttc with r: a or g) was moved 9 bases downstream, and the probe (NIFG4P: agcacttgggagggggatcg) 6 bases upstream from Mon4F and Ring4, respectively. The amplified fragments for each of the three genogroups are approximately 90 nucleotides in length (92 for GI, 89 for GII, and 89 for GIV). The absence of hairpin-dimer formation and probe-probe, primer-primer and probe-primer interactions were verified (data not shown). The Tm for all reverse primers is 56°C while for the forward primers Tm are ~55°C (with a maximum of 58°C for NIFG2F). All three probes are positive sense and with Tm ~70 °C. As a consequence, the extracts amplified with the new primers and probes combinations were detected at comparable C_T values compared to the spe-rRT-PCR, with a better sensitivity for the GIV strain (Table 1).

Gen-rRT-PCR specificity.

The modified sequences were checked for non-specific annealing by Blast, and none was found. Next, NA extracts of other human enteric viruses that can be detected in food or environmental samples were adjusted to a comparable level as estimated by pathogen-specific rRT-PCR assays, and these samples were amplified by the gen-rRT-PCR assay in triplicate. None of them [AiV (C_T 30.6 ± 0.2), AsV (C_T 31.1 ± 0.6), HAV (C_T 30.3 ± 0.7), HEV (C_T 29.4 ± 0.1), PV (C_T 31.7 ± 0.9), RV (C_T 29.0 ± 0.2), SaV (C_T 30.1 ± 0.1)] was detected by the gen-rRT-PCR. The gen-rRT-PCR did not amplify MgV (C_T 31.8 ± 0.2) used in our laboratory as an extraction control.

Gen-rRT-PCR reactivity.

The reactivity of the gen-rRT-PCR was evaluated using the reference NoV RNA from nine GI, nine GII, and one GIV genotypes, all NAs being adjusted to the same C_T values (Table 2) (the C_T
value for GI.5 was adjusted using NIFG1F-NVILCR and NIFG1P since it was not detected by the spe-\(\text{r}\)-RT-PCR assay). Using primers and probes for gen-\(\text{r}\)-RT-PCR separately, no differences in \(C_T\) values were observed except for two strains (GI.5 and GIV.1), for which detection sensitivity was improved compared to spe-\(\text{r}\)-RT-PCR. Using the gen-\(\text{r}\)-RT-PCR among the nine GI strains analyzed, the GI.7 and GI.8 were less efficiently amplified (loss of 2.8 and 1.8 \(C_T\) units, respectively). \(C_T\) values obtained with the NIFG1F-NVILCR and NIFG1P alone were identical to the spe-\(\text{r}\)-RT-PCR, showing that the loss of sensitivity was due to the presence of the other primers and probes rather than sequence mismatches. The GI.5 strain that was not detected by the spe-\(\text{r}\)-RT-PCR assay (three mismatches with the forward primer QNIF4) was successfully detected by the gen-\(\text{r}\)-RT-PCR. The \(C_T\) values for the GII strains were very similar for the two assays (less than 1 \(C_T\) unit difference). Another major improvement of this assay was for the detection of GIV.1, the new primers and probe increasing the sensitivity by more than 3 \(C_T\) units.

Based on GIV.1 sequences available in GenBank and the better fit of primers and probe, similar findings would be anticipated for other GIV.1 strains.

**Gen-\(\text{r}\)-RT-PCR sensitivity.**

The GI.1 and GII.3 NoV NA extracts, and GIV.1 RNA fragment were 10-fold serially diluted and amplified by the gen-\(\text{r}\)-RT-PCR. The sensitivity limits achieved were quite similar to those obtained with the spe-\(\text{r}\)-RT-PCR assays (Figure 2). The variability among replicates was less than 1 \(C_T\) unit for high \(C_T\) values (near the limit of detection). For the GIV detection, the new set of primers and probe lowered the \(C_T\) values (Figure 2).

As contamination with multiple NoV strains can occur in food or environmental samples, NoVs from different genogroups were mixed in different combinations and amplified. When two virus strains were mixed together, the mean \(C_T\) values were lower than when a comparable amount of the single strain was assayed. When GI.1, GII.3 and GIV.1 were amplified separately, \(C_T\) values were 37.1 \(\pm\) 1.1, 36.7 \(\pm\) 0.4 and 35.7 \(\pm\) 0.4, respectively. When two genogroups were mixed, the resulting \(C_T\) values were 35.1 \(\pm\) 0.2 for GI.1 and GII.3, 34.9 \(\pm\) 0.3 for GII.3 and GIV.1,
and 34.7 ± 0.2 for GI.I and GIV.1. The decrease observed CT units demonstrates that fluorescence increased following the mixture of two strains. Similarly, when all three genogroups were mixed, the CT value decreased further (34.1 ± 0.5). The shift of magnitude for this assay was almost 2 CT units, confirming that all strains were amplified, considering variability. If strains were mixed at different ratios, the CT variations were less demonstrative as CT values follow the most concentrated strain (data not shown).

**Gen-rRT-PCR applied to sewage sample extracts.**

NA extracts of 16 untreated and 12 treated wastewater samples known to be positive for at least one NoV genogroup were selected (Figure 3). The CT values obtained with the gen-rRT-PCR were lower than or similar to those obtained with the spe-rRT-PCR, with one exception (arrow 1 Figure 3B), with the GI NoV spe-rRT-PCR giving a lower CT value. On the other hand, arrow 2 showed a sample containing only a GI NoV for which the gen rRT-PCR was more sensitive than the spe-rRT-PCR.

**Gen-rRT-PCR applied to shellfish sample extracts.**

Initial studies on shellfish samples were performed using oyster extracts contaminated in bioaccumulation experiments. NA extracts obtained from different experiments were amplified by the spe-rRT-PCR and gen-rRT-PCR in triplicate (Table 3). CT values were similar but consistently higher in the gen-rRT-PCR assay than the spe-rRT-PCR, with a difference of 0.4 to 1.7 CT units between the two assays. When the new primers and probes for the gen-rRT-PCR were tested separately and compared to the primers and probes used for spe-rRT-PCR, the maximum difference in CT values was 0.5 CT units (observed for a GII sample). For the GI samples the differences observed ranged from 0.1 to 0.3 CT units. These results confirmed the earlier observation that mixing primers and probes can lead to a decrease in sensitivity as measured by CT detection level.

To further evaluate the applicability of the gen-rRT-PCR assay, 157 archived oyster,
mussel and clam NA extracts were analyzed using the spe-rRT-PCR and the gen-rRT-PCR. Sixty samples were negative and 97 samples were positive by both the spe-rRT-PCR and gen-rRT-PCR (Figure 4). The greatest variability was observed among the 15 GI positive samples with one sample showing a difference of more than 3 C_T units in favor of the gen-rRT-PCR (arrow 1 Figure 4 A). Surprisingly the gen-rRT-PCR assay improved the C_T values compared to the spe-rRT-PCR, at odds with the results obtained in the strain validation studies (Table 2). Two samples considered as negative by the spe-rRT-PCR assay (C_T values of 41) gave C_T values under 40 in the gen-rRT-PCR, and thus were interpreted as positive, these samples being negative for the other NoV genogroups. Among the 38 GII NoV positive samples similar C_T values were obtained with both assays (Figure 4 A). Additionally, 39 samples positive both for GI and GII NoV were also detected by the gen-rRT-PCR. Measured C_T values resemble the C_T obtained for NoV GII, which was usually the lowest C_T value. In only two samples (arrows 2 and 3 Figure 4 B), NoVs were detected more efficiently by the spe-rRT-PCR, but these samples were still positive by the generic assay. Five samples contaminated by all three NoV genogroups were successfully detected by the gen-rRT-PCR.

DISCUSSION

Development of rRT-PCR allows sensitive and specific detection of NoV for environmental monitoring, food analysis or clinical diagnosis. As there is no cell line or animal model to detect human NoVs (28), rRT-PCR is the standard detection method and has been recognized as an ISO technical specification for NoV detection in food samples (ISO/TS 15216 -1 and 2). The work described here is the development of a gen-rRT-PCR assay able to detect a wide diversity of NoV strains belonging to the three human genogroups over a large concentration range, compatible with environmental analysis including shellfish. By combining detection of all three genogroups in one assay, the cost per reaction is lowered to a third of the original cost and allows the sensitive screening between positive or negative samples.

Since the first demonstration of a conserved area among genetic diversity of these viruses
advantages of probe-based rRT-PCR compared to conventional RT-PCR have been demonstrated in many occasions both in terms of sensitivity, specificity, and simplicity of application. This method is now the most widely used in environmental studies such as sewage or water sample analysis (5-7), food (29-31) or shellfish samples (22, 32-35). Almost all rRT-PCR assays target the ORF1-ORF2 junction region, originally targeted 10 years ago (18). However, some primer or probe modifications have been made to increase sensitivity or to adapt to newly described NoV strains (15, 17, 36-43). It is important to adapt the assay to strain evolution and the assay described here was checked against the sequence of the newly described strains such as the GII.4 2012 variant, (Hu/GII.4/Sydney/NSW0514/2012/AU, JX459908). However, although the developed assay was able to detect all tested genotypes, the ongoing emergence of new variants and the high genetic diversity of NoV may make it necessary to continue to evaluate the performance of these primers or probes in the future.

The recognition that NoVs are highly prevalent and that food plays an important role in their transmission is now clear (9, 44). NoVs have been detected on berries, tomatoes, and shellfish from the European, Canadian, American and Japanese markets with prevalence frequencies ranging from less than 4% to more than 76% (31, 35, 45, 46, 47) A review performed by an expert panel on the biology, epidemiology, diagnosis and public health importance of foodborne viruses identified NoVs (as well as HAV) as the most frequent causes of foodborne illness among all virus/food commodity combinations (8). One recommendation of this expert panel was to obtain more data on NoV food contamination to develop a risk assessment and to evaluate the impact of food on NoV epidemiology. For oysters, for which more data are available, one recommendation is that NoV testing should be considered for food business operators (48).

Screening environmental and food samples requires attention be given to the presence of potential inhibitors of the RT or PCR enzymes. Strategies to assess the presence of inhibitors and to evaluate extraction efficiency include testing undiluted and diluted NA extracts with and without internal controls (20, 22, 45, 46). This can lead to the need for at least two wells per genogroup and eventually one well per genogroup with internal control so at least 6 to 9 separate
amplifications, without considering positive and negative controls or standard curves. The time spent for analysis and the costs are an issue, especially if other viral or bacterial pathogens need to be screened and the frequency of positive results is <10% (45). The development of generic assays for virus identification in clinical samples is one strategy to address this problem (49, 50). To date, such an approach for NoVs has not been feasible due to their genetic diversity. This study describes the successful development of a generic RT-PCR assay allowing a sensitive detection of GI, GII, and GIV NoVs, based on a model previously developed for sapovirus (27).

One characteristic of NoV epidemiology, besides high genetic diversity, is the co-circulation of strains in the human population (1). NoV GII strains are more frequently detected in clinical cases, but GI strains are relatively more frequently found associated with foodborne outbreaks (10, 51), making their detection important for environmental or food samples screening. It may also be important to look for GIV NoVs as they have been detected in sewage (7, 52, 53) and shellfish samples (unpublished data)(14, 54). Improving primer and probe design for GIV strains may help to determine their circulation in the environment and to prevent further distribution among human population.

Multiplex rRT-PCR assays for GI and GII NoVs, based upon mixtures of previously published primers and probes, have been successfully applied in the analysis of clinical samples (55-57). However, the ratio of concentrations of the 2 genogroups can have an impact on assay sensitivity, as noted in the current studies. When different concentrations were mixed together, a mutually competitive effect was observed compared to individual GI and GII reactions (58). The loss in sensitivity was not considered to be a major disadvantage in clinical diagnosis using fecal samples, as viral loads in feces are high (59). A concern is that this may not be the case when analyzing environmental samples that contain lower virus concentrations. A recently described duplex assay had no loss of sensitivity compared to GI and GII monoplex assays, and when applied to surface and ground water samples this assay was more efficient that conventional RT-PCR (60). Only one other study targeting all three human NoV genogroups in a multiplex assay based on GI and GII primers/probes described previously and newly designed primers and probe
for GIV has been described (18, 61). As noted by these authors, the sensitivity of the developed multiplex real-time assay was lower than those of its corresponding monoplex assays due to interactions of primers and probes, confirmed by the failure of NoV detection in three of seven food or environmental samples. In contrast, although our generic assay amplified two GI genotypes less efficiently than monoplex assays, all water and shellfish samples were still positive when evaluated by the new developed assay. Use of the same fluorescent dye for all three TaqMan probes allows the fluorescence to be added. This may increase assay sensitivity when several genogroups are mixed, but presents the disadvantage of no discrimination between genogroups. However, the sensitivity achieved is quite similar to the spe-rRT-PCR on NA extracts from stool or bioaccumulated shellfish samples, when only qualitative determination are made. This study also demonstrates the importance of validation using naturally-contaminated samples as the GI detection is efficient using the gen-rRT-PCR, despite the lost of sensitivity observed on the reference NoV RNA panel. Even if precautions are taken to purify NA from environmental samples, various inhibitors may be present depending on the sample matrix. Additionally, other NAs may be present in extracts, possibly interfering with the amplification. The other challenge for environmental samples is to achieve adequate sensitivity (62). This is critical as low concentrations of NoV may constitute a health risk (63, 64). The validation of this assay on sewage or shellfish extracts, two types of challenging samples based on inhibitors or low contamination, demonstrates that it is efficient. Two shellfish samples negative in the spe-rRT-PCR ($C_T \sim 41$) detected positive with the gen-rRT-PCR could raise a specificity question although it is plausible that these detections were due to improved assay sensitivity. No false negative or positive results were observed and differences in $C_T$ values were always minimal, making us confident in its application for shellfish sample analysis based on our experience.

Food screening for NoV is likely to become more frequent in coming years or even mandatory. Depending on samples analyzed, season (summer compared to winter), or climatic event, a large number of samples may be negative. Additionally, in some situations, the qualitative information, as provided by the assay developed in this work, may be sufficient to
take sanitary decision like recall from the market or prevent trading of contaminated foods, at least until molecular assays are developed that allow an assessment of virus infectivity (65,66). For samples needing more precise data such as genogroup identification and virus quantification, genogroup specific RT-qPCR then can be performed, including controls to allow virus quantification.

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**FIGURE LEGENDS**

**Figure 1**: Oligonucleotide primers and probes. The sequences of reference strains GI.1 Norwalk virus (GenBank accession n° M87661, nt 5271 to 5380), GII.4 Lordsdale virus (GenBank accession n° X86557, nt 4998 to 5107), and GIV.1 Saint-Cloud virus (GenBank accession n° AF414427, nt 681-790) are represented. The primers and probes used in the developed generic real-time RT-PCR assay are represented below the reference sequences, and the original ones are above. Stars represent un-modified bases and letters mixed bases (n: any; y: c or t; r: a or g), the arrows indicate the primer orientation, with all the probes being a positive sense.

**Figure 2**: Comparison of C_Τ_ values obtained with the gen-rRT-PCR and the spe-rRT-PCR assays. Serial dilutions of the three reference strains were amplified using both assays, and the respective C_Τ_ values are reported. Black diamond: GI.1, grey square: GII.3, and white triangle: GIV.1. The error bars represent the standard deviations of triplicate measurements.

**Figure 3**: Analysis of sewage sample extracts. Archived nucleic acids from raw sewage (A) and treated sewage (B) samples were analyzed by both assays. Grey: spe-rRT-PCR (diamond: GI, square: GII, and triangle: GIV). Black circle: gen-rRT-PCR. Dashed line: limit of acceptable C_Τ_ value. Arrows indicate samples discussed in the text.

**Figure 4**: Analysis of shellfish sample extracts. Archived nucleic acids from naturally contaminated shellfish samples were analyzed by both
Figure 1
Figure 2
Figure 4

A

B

Samples

GI

GII

GI & II

GI, II, & IV

C

T

25

30

35

40

45

25

30

35

40

45
### TABLE 1. Comparison of spe-\(\varepsilon\)-RT-PCR and gen-\(\varepsilon\)-RT-PCR on three NoV reference strains.

<table>
<thead>
<tr>
<th>NoV</th>
<th>Concentration</th>
<th>spe-(\varepsilon)-RT-PCR</th>
<th>gen-(\varepsilon)-RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Separate</td>
<td>Mix</td>
</tr>
<tr>
<td>GI.1</td>
<td>High</td>
<td>25.6 ± 0.2</td>
<td>29.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>31.3 ± 0.3</td>
<td>35.2 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>37.8 ± 0.8</td>
<td>no C(T)</td>
</tr>
<tr>
<td>GII.3</td>
<td>High</td>
<td>23.9 ± 0.1</td>
<td>28.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>30.3 ± 0.2</td>
<td>35.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>37.0 ± 1.3</td>
<td>no C(T)</td>
</tr>
<tr>
<td>GIV.1</td>
<td>High</td>
<td>28.2 ± 0.2</td>
<td>28.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>35.0 ± 0.4</td>
<td>34.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>no C(T)</td>
<td>no C(T)</td>
</tr>
</tbody>
</table>

Each NA was amplified at three levels of concentrations by the spe-\(\varepsilon\)-RT-PCR using primers and probes separately (GI, GII, or GIV) or mix together, and by the gen-\(\varepsilon\)-RT-PCR. The results are expressed as mean C\(T\) value ± standard deviation.

### TABLE 2. Gen-\(\varepsilon\)-RT-PCR reactivity among different NoV strains.

<table>
<thead>
<tr>
<th>NoV</th>
<th>spe-(\varepsilon)-RT-PCR</th>
<th>gen-(\varepsilon)-RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Separate</td>
<td>Mix</td>
</tr>
<tr>
<td>GI.1</td>
<td>28.3 ± 0.1</td>
<td>29.1 ± 0.1</td>
</tr>
<tr>
<td>.2</td>
<td>30.0 ± 0.1</td>
<td>29.1 ± 0.2</td>
</tr>
<tr>
<td>.2</td>
<td>31.3 ± 0.3</td>
<td>30.4 ± 0.4</td>
</tr>
<tr>
<td>.3</td>
<td>30.3 ± 0.1</td>
<td>29.2 ± 0.1</td>
</tr>
<tr>
<td>.4</td>
<td>29.4 ± 0.4</td>
<td>28.6 ± 0.4</td>
</tr>
<tr>
<td>.5</td>
<td>no C(T)</td>
<td>28.3 ± 0.4</td>
</tr>
<tr>
<td>.6</td>
<td>28.5 ± 0.1</td>
<td>27.3 ± 0.4</td>
</tr>
<tr>
<td>.7</td>
<td>31.3 ± 0.3</td>
<td>31.1 ± 0.1</td>
</tr>
<tr>
<td>.8</td>
<td>30.8 ± 0.2</td>
<td>30.8 ± 0.2</td>
</tr>
<tr>
<td>GII.1</td>
<td>30.5 ± 0.2</td>
<td>30.4 ± 0.3</td>
</tr>
<tr>
<td>.2</td>
<td>29.2 ± 0.5</td>
<td>29.4 ± 0.4</td>
</tr>
<tr>
<td>.3</td>
<td>31.4 ± 0.2</td>
<td>31.6 ± 0.8</td>
</tr>
<tr>
<td>.4</td>
<td>29.3 ± 0.2</td>
<td>29.3 ± 0.1</td>
</tr>
<tr>
<td>.6</td>
<td>30.1 ± 0.1</td>
<td>30.2 ± 0.1</td>
</tr>
<tr>
<td>.7</td>
<td>30.8 ± 0.1</td>
<td>30.4 ± 0.5</td>
</tr>
<tr>
<td>.10</td>
<td>31.3 ± 0.1</td>
<td>31.4 ± 0.2</td>
</tr>
<tr>
<td>.b</td>
<td>31.8 ± 0.3</td>
<td>31.7 ± 0.2</td>
</tr>
<tr>
<td>.c</td>
<td>30.2 ± 0.2</td>
<td>29.9 ± 0.1</td>
</tr>
<tr>
<td>GIV.1</td>
<td>34.1 ± 0.1</td>
<td>30.5 ± 0.5</td>
</tr>
</tbody>
</table>

NoV RNA fragments were diluted to obtain a C\(T\) ~30 using spe-\(\varepsilon\)-RT-PCR assay (except for GI.5 RNA adjusted with the modified GI primers and probe) and amplified by the gen-\(\varepsilon\)-RT-PCR using primers and probes separately (GI, GII, or GIV) or by the mixture as described in the method section. The results are expressed as mean C\(T\) value ± standard deviation.
TABLE 3. Sensitivity of the gen-rtRT-PCR assay to NoV RNA extracted from oyster samples contaminated in bioaccumulation experiments.

<table>
<thead>
<tr>
<th>NoV</th>
<th>Concentration*</th>
<th>Spe-rtRT-PCR</th>
<th>Gen-rtRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI.1</td>
<td>Medium</td>
<td>28.2 ± 0.2</td>
<td>29.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>30.2 ± 0.1</td>
<td>31.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33.9 ± 0.4</td>
<td>35.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34.1 ± 0.2</td>
<td>35.8 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>28.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.4 ± 0.2</td>
<td>28.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33.8 ± 0.1</td>
<td>34.6 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35.6 ± 1.0</td>
<td>36.9 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

GII.3  
| Medium         | 28.2 ± 0.1   |
| 28.4 ± 0.2     | 28.8 ± 0.2   |
| 33.8 ± 0.1     | 34.6 ± 0.6   |
| 35.6 ± 1.0     | 36.9 ± 0.1   |

Bioaccumulated oyster NA extracts were amplified using the two rtRT-PCR assays and results are expressed as the mean CT value ± standard deviation, * for each concentration two different samples were analyzed.