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Environmental Detection of Genogroup I, II, and IV Noroviruses by Using a Generic Real-Time Reverse Transcription-PCR Assay

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Norovirus is the most common agent implicated in food-borne outbreaks and is frequently detected in environmental samples. These viruses are highly diverse, and three genogroups (genogroup I [GI], GII, and GIV) infect humans. Being noncultivable viruses, real-time reverse transcription-PCR (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 assays has been challenging due to sequence diversity and has 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 on a qualitative basis using a one-step real-time RT-PCR assay. 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 levels of fluorescence to be added and may increase assay sensitivity when multiple strains from different genogroups are present. When it was applied to sewage sample extracts, this generic assay successfully detected norovirus in all samples found to be positive by the genogroup-specific RT-PCRs. The generic assay also identified all norovirus-positive samples among 157 archived nucleic acid shellfish extracts, including samples contaminated by all three genogroups.

Viral contamination of water samples and foodstuffs is increasingly recognized through outbreak investigations, epidemiological surveys, and sample analysis. Among the great 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 (genogroup I [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 is reported in clinical cases. Infection with many strains is dependent on histo-blood group antigen (HBGA) expression, as HBGAs serve as attachment factors necessary to initiate virus infection (3). NoVs are the major cause of nonbacterial gastroenteritis worldwide and have been identified as the predominant cause of food-borne 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 a strategy to prevent virus-associated outbreaks. The achievement of sensitive methods and real-time reverse transcription (RT)-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) on a qualitative basis using a one-step rRT-PCR assay. The generic assay, developed and optimized on the basis of previously reported primers and probes, showed a sensitivity comparable to that of genogroup-specific assays. The newly developed assay was used to analyze naturally contaminated samples, such as raw and treated sewage and shellfish samples, and the results were compared to those of genogroup-specific real-time RT-PCR (spe-rRT-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 strains, nine GII strains, and one GIV strain (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 and poliovirus (PV) type 3 (both from B.-M. Marcillé, CHU Nantes), hepatitis A virus (HAV) HM175 and mengovirus (MgV) strain vMC_0 (both from A. Bosch, University of Barcelona, Barcelona, Spain), and rotavirus (RV) RF (D. Poncet, INRA-CNRS, Gif sur Yvette). For hepatitis E virus (HEV), a porcine stool isolate characterized as genotype 3 was included (N. Pavio, ANSES, Paris, France).

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

Archived NA samples. Archived NAs from 157 naturally contaminated shellfish samples (oyster, mussel, and clam) (12–14; unpublished data), 8 bioaccumulated oyster samples (11), and 16 untreated and 12 treated wastewater samples (7) were included in the study.

Primers and probes. For the 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

Received 26 June 2013 Accepted 13 August 2013
Published ahead of print 16 August 2013
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doi:10.1128/AEM.02112-13

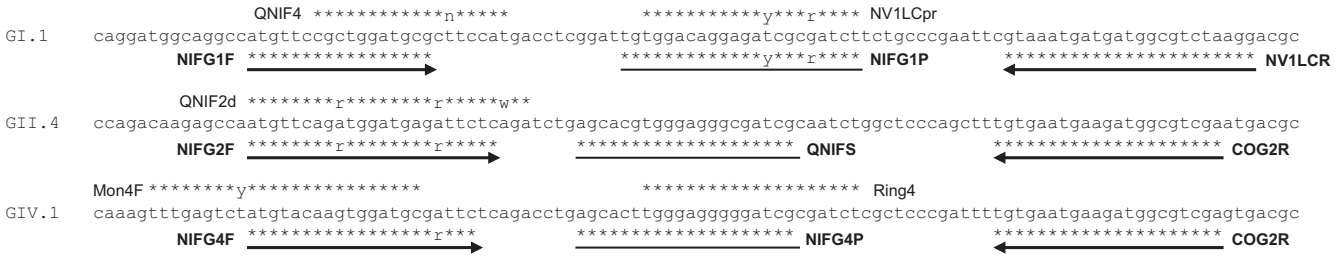

FIG 1 Oligonucleotide primers and probes. The sequences of reference strains GI.1 Norwalk NoV (GenBank accession no. M87661, nt 5271 to 5380), GII.4 Lordsdale NoV (GenBank accession no. X86557, nt 4998 to 5107), and GIV.1 Saint-Cloud NoV (GenBank accession no. AF414427, nt 681 to 790) are presented. The primers and probes used in the generic real-time RT-PCR assay developed are presented below the reference sequences, and the original ones are presented above. Asterisks, unmodified bases; letters, mixed bases (n, any nucleotide; y, C or T; r, A or G); arrows, primer orientation; all probes were positive sense.

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) (Fig. 1). For AiV, AstV, HAV, HEV, MgV, PV, RV, and sapovirus (SaV), primers and probes described previously were used (19–27).

rRT-PCR. All amplifications were performed with an Mx3000P quantitative PCR (qPCR) system (Agilent Technologies, France) using an RNA UltraSense One-Step quantitative RT-PCR system (Life Technologies, France) and 5 μl of extracted sample per well (final volume, 25 μl). The thermal conditions consisted of the RT reaction for 15 min at 55°C, inactivation of reverse transcriptase, and activation of *Taq* polymerase for 5 min at 95°C, followed by 45 cycles of denaturing for 15 s at 95°C, annealing for 1 min at 55°C, and extension for 1 min at 65°C. For the spe-rRT-PCR, conditions were as previously described: a final concentration of 900 nM for the reverse primer (for GI, primer NV1LCR; for GII/GIV, primer COG2R), a final concentration of 500 nM for the forward primer (for GI, primer QNIF4; for GII, primer QNIF2d; for GIV, primer Mon4F), and a final concentration of 250 nM for the probe (for GI, probe NV1LCpr; for GII, probe QNIFS; for GIV, probe Ring4) (22). For the gen-rRT-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) (Fig. 1) at a final concentration of 400 nM for each primer and a final concentration of 200 nM for each probe. All probes were labeled using the same fluorescent dye and quencher (6-carboxyfluorescein and black hole quencher). These conditions were applied in preliminary studies (Table 1) with the primers and probes currently used in our laboratory (i.e., QNIF4, NV1LCR, NV1LCpr, QNIF2d, COG2R, QNIFS, Mon4F, COG2R, and 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 under the conditions used for the spe-rRT-PCR (see above).

The cycle threshold (C_T) 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 C_T ± standard deviation, calculated using the results for triplicate wells. For naturally contaminated samples, screening was first performed using a single well to determine the C_T for all 3 spe-rRT-PCRs and gen-rRT-PCR, as limited amounts of extracts were available. When a discordant result was obtained for one genogroup, triplicate wells were used to determine whether the sample was 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).

TABLE 1 Comparison of spe-rRT-PCR and gen-rRT-PCR with three NoV reference strains[a]

NoV	Concn	Mean C_T value ± SD		
		Spe-rRT-PCR		Gen-rRT-PCR, mixed
		Separate	Mixed	
GI.1	High	25.6 ± 0.2	29.7 ± 0.2	25.6 ± 0.3
	Medium	31.3 ± 0.3	35.2 ± 1.3	31.0 ± 0.1
	Low	37.8 ± 0.8	No C_T	38.2 ± 0.9
GII.3	High	23.9 ± 0.1	28.5 ± 0.3	24.7 ± 0.3
	Medium	30.3 ± 0.2	35.4 ± 0.1	31.5 ± 0.1
	Low	37.0 ± 1.3	No C_T	36.7 ± 1.2
GIV.1	High	28.2 ± 0.2	28.2 ± 0.1	24.7 ± 0.2
	Medium	35.0 ± 0.4	34.7 ± 0.1	31.4 ± 0.2
	Low	No C_T	No C_T	36.7 ± 0.3

[a] Each NA was amplified at three concentrations by spe-rRT-PCR using primers and probes separately (GI, GII, or GIV) or mixed together and by the gen-rRT-PCR.

TABLE 2 gen-rRT-PCR reactivity among different NoV strains[a]

NoV	Mean C_T value ± SD		
	Spe-rRT-PCR, separate	Gen-rRT-PCR	
		Separate	Mixed
GI.1	28.3 ± 0.1	29.1 ± 0.1	27.9 ± 0.6
GI.2	30.0 ± 0.1	29.1 ± 0.2	30.5 ± 0.3
GI.2	31.3 ± 0.3	30.4 ± 0.4	31.3 ± 0.5
GI.3	30.3 ± 0.1	29.2 ± 0.1	30.3 ± 0.3
GI.4	29.4 ± 0.4	28.6 ± 0.4	30.4 ± 0.8
GI.5	No C_T	28.3 ± 0.4	30.9 ± 0.6
GI.6	28.5 ± 0.1	27.3 ± 0.4	29.1 ± 0.1
GI.7	31.3 ± 0.3	31.1 ± 0.1	34.1 ± 0.7
GI.8	30.8 ± 0.2	30.8 ± 0.2	32.6 ± 0.7
GII.1	30.5 ± 0.2	30.4 ± 0.3	30.7 ± 0.2
GII.2	29.2 ± 0.5	29.4 ± 0.4	29.4 ± 0.6
GII.3	31.4 ± 0.2	31.6 ± 0.8	31.8 ± 0.1
GII.4	29.3 ± 0.2	29.3 ± 0.1	29.4 ± 0.1
GII.6	30.1 ± 0.1	30.2 ± 0.1	30.2 ± 0.2
GII.7	30.8 ± 0.1	30.4 ± 0.5	30.2 ± 0.1
GII.10	31.3 ± 0.1	31.4 ± 0.2	31.9 ± 0.2
GIIb	31.8 ± 0.3	31.7 ± 0.2	32.2 ± 0.4
GIIc	30.2 ± 0.2	29.9 ± 0.1	30.6 ± 0.3
GIV.1	34.1 ± 0.1	30.5 ± 0.5	30.7 ± 0.3

[a] NoV RNA fragments were diluted to obtain a C_T of ~30 using the spe-rRT-PCR assay (except for GI.5 RNA adjusted with the modified GI primers and probe) and amplified by the gen-rRT-PCR using primers and probes separately (GI, GII, or GIV) or as a mixture, as described in the Materials and Methods section.

Specificity and reactivity of gen-rRT-PCR. NA extracts from AiV, AstV, HAV, HEV, MgV, PV, RV, and SaV and the reference NoV RNA panel were diluted, 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 gen-rRT-PCR. Reference GI.1, GII.3, and GIV.1 NA extracts were 10-fold serially diluted (over 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, and GIV.1). An increase of at least 4 C_T units compared to the results of the spe-rRT-PCR (i.e., using primers and probes separately) was observed, with low concentrations not being detected (Table 1). The spe-rRT-PCR assays target a short conserved region from the junction between open reading frame 1 (ORF1) and ORF2, which allows efficient detection of strains from the different genogroups. This area was also selected for development of the gen-rRT-PCR assay. On the basis of the results of sequence analysis of the reference strain, a number of different primers and probes were evaluated (data not shown), and the best combination is described in Fig. 1. For GI NoV, the forward primer (NIFG1F [ATGTTCCGCTGGATGCG]) was moved 6 bases upstream from QNIF4, the probe (NIFGIP [TGTGGACAGGAGAYCGCRATCT, with Y being C or T and R being 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 [AGCACGTGGGAGGGCGATCG]) for GII were not changed, but the forward primer QNIF2d was shortened by 3 bases at the 3′ end (NIFG2F [ATGTTCAGRTGGATGAGRTTCTC, with R being A or G]). For GIV NoV, the GII reverse primer COG2R, whose sequence matches perfectly the available GIV NoV sequences, was selected; the forward primer (NIFG4F [ATGTACAAGTGGATGCGRTTC, with R being A or G]) was moved 9 bases downstream and the probe (NIFG4P [AGCACTTGGGAGGGGGATCG]) was moved 6 bases upstream from Mon4F and Ring4, respectively. The amplified fragments for each of the three genogroups are approximately 90 nucleotides (nt) in length (92 nt for GI, 89 nt for GII, and 89 nt for GIV). The absence of hairpin-dimer formation and probe-probe, primer-primer, and probe-primer interactions were verified (data not shown). The melting temperature (T_m) for all reverse primers was 56°C, while for the forward primers the T_m was ~55°C (with a maximum of 58°C for NIFG2F). All three probes were positive sense and had T_ms of ~70°C. As a consequence, the extracts amplified with the new primer and probe combinations were detected at C_T values comparable to those for the spe-rRT-PCR, with a better sensitivity for the GIV strain (Table 1).

Gen-rRT-PCR specificity. The modified sequences were checked for nonspecific annealing by BLAST analysis, and none was found. Next, NA extracts of other human enteric viruses that can be detected in food or environmental samples were adjusted so that their C_Ts were comparable, 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.

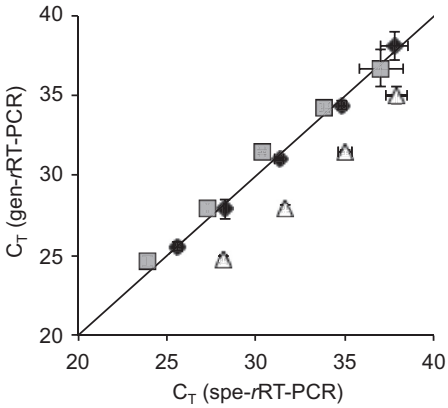

FIG 2 Comparison of C_T values obtained with the gen-rRT-PCR and the spe-rRT-PCR assays. Serial dilutions of the three reference strains were amplified using both assays, and the respective C_T values are reported. Black diamonds, GI.1; gray squares, GII.3; white triangles, GIV.1. The error bars represent the standard deviations of triplicate measurements.

Gen-rRT-PCR reactivity. The reactivity of the gen-rRT-PCR was evaluated using the reference NoV RNA from nine GI strains, nine GII strains, and one GIV strain, with 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 NIFGIP, since it was not detected by the spe-rRT-PCR assay). Using primers and probes for gen-rRT-PCR separately, no differences in C_T values were observed, except for those for two strains (GI.5 and GIV.1), for which the detection sensitivity was improved compared to that of the spe-rRT-PCR. Using the gen-rRT-PCR with the nine GI strains analyzed, the GI.7 and GI.8 strains were less efficiently amplified (loss of 2.8 and 1.8 C_T units, respectively). The C_T values obtained with NIFG1F-NV1LCR and NIFG1P alone were identical to those obtained by the spe-rRT-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-rRT-PCR assay (three mismatches were found with the forward primer QNIF4), but it was successfully detected by the gen-rRT-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, with the new primers and probe increasing the sensitivity by more than 3 C_T units. On the basis of 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-rRT-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-rRT-PCR. The sensitivity limits achieved were quite similar to those obtained with the spe-rRT-PCR assays (Fig. 2). The variability among replicates was less than 1 C_T unit for high C_T values (near the limit of detection). For GIV detection, the new set of primers and probe lowered the C_T values (Fig. 2).

As contamination with multiple NoV strains can occur in food

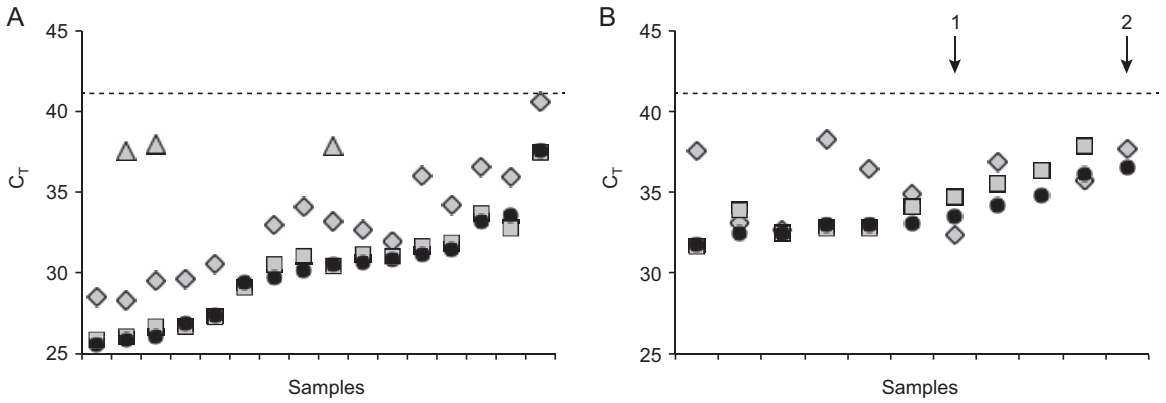

FIG 3 Analysis of sewage sample extracts. Archived nucleic acids from raw sewage (A) and treated sewage (B) samples were analyzed by both assays. Gray symbols, spe-rRT-PCR (diamonds, GI; squares, GII; triangles, GIV); black circles, gen-rRT-PCR; dashed line, limit of acceptable C_T values; arrows, samples discussed in the text.

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 those obtained 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 ± 1.1, 36.7 ± 0.4, and 35.7 ± 0.4, respectively. When two genogroups were mixed, the resulting C_T values were 35.1 ± 0.2 for GI.1 and GII.3, 34.9 ± 0.3 for GII.3 and GIV.1, and 34.7 ± 0.2 for GI.I and GIV.1. The decrease in the number of C_T units observed demonstrates that fluorescence increased when strains of two genogroups were mixed. Similarly, when all three genogroups were mixed, the C_T value decreased further (34.1 ± 0.5). The shift of magnitude for this assay was almost 2 C_T units, confirming that all strains were amplified, when the variability of the rRT-PCR is considered. If strains were mixed at different ratios, the C_T variations were less demonstrative, as the C_T values followed the value for 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 (Fig. 3). The C_T values obtained with the gen-rRT-PCR were lower than or similar to those obtained with the spe-rRT-PCR, with one exception (Fig. 3B, arrow 1), with the GI NoV spe-rRT-PCR giving a lower C_T value. On the other hand, arrow 2 in Fig. 3B shows the results for a sample containing only a GI NoV isolate 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). C_T values were similar but consistently higher in the gen-rRT-PCR assay than the spe-rRT-PCR assay, with a difference of 0.4 to 1.7 C_T units between the two assays. When the new primers and probes for the gen-rRT-PCR were tested separately and the results were compared to those for the primers and probes used for the spe-rRT-PCR, the maximum difference in C_T values was 0.5 C_T unit (observed for a GII sample). For the GI samples, the differences observed ranged from 0.1 to 0.3 C_T unit. These results confirmed the earlier observation that mixing primers and probes can lead to a decrease in sensitivity, as measured by the C_T 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 the gen-rRT-PCR (Fig. 4). The greatest variability was observed among the 15 GI-positive samples, with 1 sample showing a difference of more than 3 C_T units in favor of the gen-rRT-PCR (Fig. 4A, arrow 1). Surprisingly the gen-rRT-PCR assay improved the C_T values compared to those obtained by the spe-rRT-PCR, a finding that is at odds with the results obtained in the strain validation studies (Table 2). Two samples considered negative by the spe-rRT-PCR assay (C_T value, 41) gave C_T values under 40 in the gen-rRT-PCR and were thus interpreted to be positive; these samples were negative for the other NoV genogroups. Among the 38 GII NoV-positive samples, similar C_T values were obtained with both assays (Fig. 4A). Additionally, 39 samples positive for both GI and GII NoV were also detected by the gen-rRT-PCR. The measured C_T values resembled the C_T values obtained for NoV GII, which was usually the lowest C_T value. In only two samples (Fig. 4B, arrows 2 and 3), 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.

TABLE 3 Sensitivity of the gen-rRT-PCR assay to NoV RNA extracted from oyster samples contaminated in bioaccumulation experiments[a]

NoV	Concn[b]	Mean C_T value ± SD	
		Spe-rRT-PCR	Gen-rRT-PCR
GI.1	Medium	28.2 ± 0.2	29.4 ± 0.1
		30.2 ± 0.1	31.4 ± 0.1
	Low	33.9 ± 0.4	35.3 ± 0.4
		34.1 ± 0.2	35.8 ± 1.0
GII.3	Medium	28.2 ± 0.1	28.7 ± 0.2
		28.4 ± 0.2	28.8 ± 0.2
	Low	33.8 ± 0.1	34.6 ± 0.6
		35.6 ± 1.0	36.9 ± 0.1

[a] Bioaccumulated oyster NA extracts were amplified using the two rRT-PCR assays.
[b] For each concentration, two different samples were analyzed.

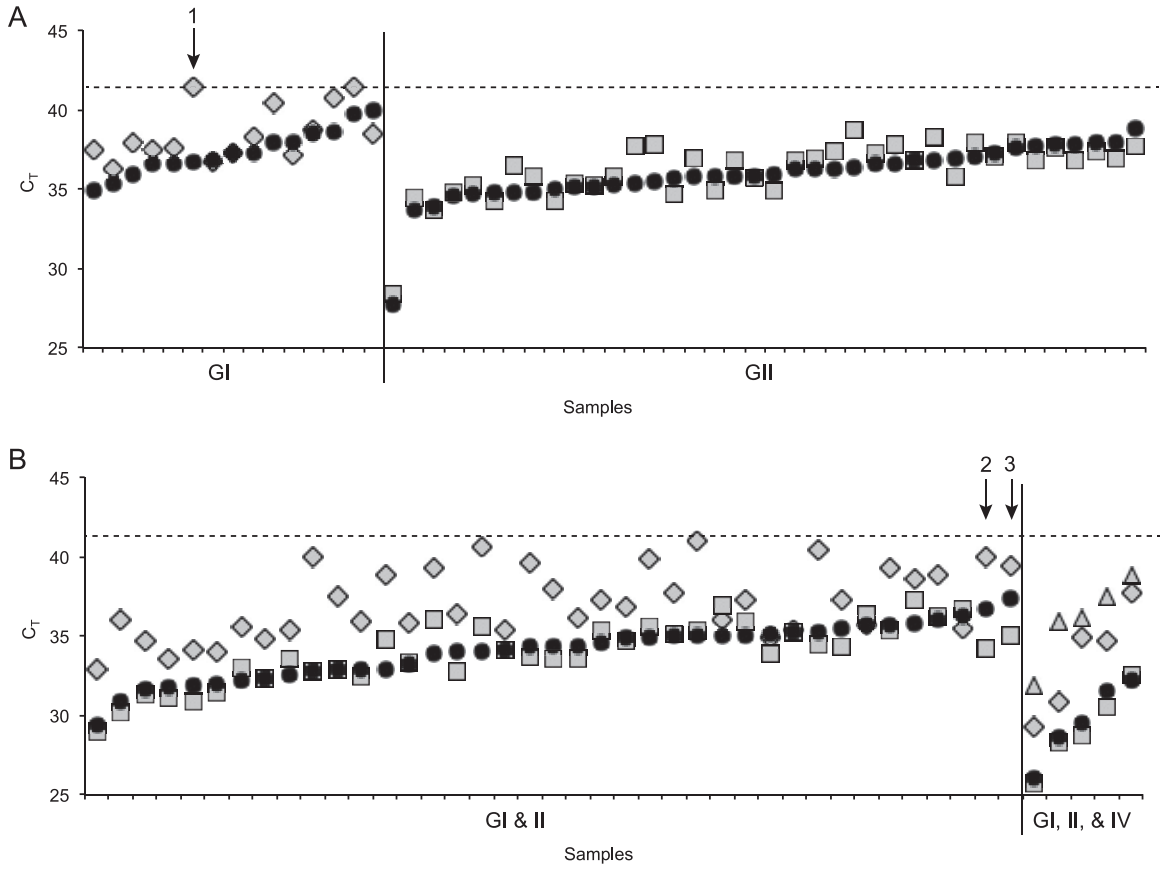

FIG 4 Analysis of shellfish sample extracts. Archived nucleic acids from naturally contaminated shellfish samples were analyzed by both assays. Gray symbols, spe-rRT-PCR (diamonds, GI; squares, GII; triangles, GIV); black circles, gen-rRT-PCR; dashed line, limit of acceptable C_T values; arrows, samples discussed in the text.

DISCUSSION

Development of rRT-PCR allows the 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). In the work described here, we developed 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. The assay is compatible with analysis of environmental samples, 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 for positive and negative samples.

Since the first demonstration of a conserved area in the genetically diverse genomes of these viruses (18), the advantages of probe-based rRT-PCR in comparison to conventional RT-PCR have been demonstrated on many occasions in terms of sensitivity, specificity, and simplicity of application. This method is now the most widely used method in environmental studies, such as analysis of sewage or water samples (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 sequences of newly described strains, such as the GII.4 2012 variant (Hu/GII.4/Sydney/NSW0514/2012/AU [GenBank accession no. JX459908]). However, although the assay that was developed was able to detect all genotypes tested, 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.

It is now clearly recognized that NoVs are highly prevalent and that food plays an important role in their transmission (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, 45–47) A review performed by an expert panel on the biology, epidemiology, detection, and public health importance of food-borne viruses identified NoVs (as well as HAV) to be the most frequent causes of food-borne 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 of environmental and food samples requires that

attention be given to the presence of potential inhibitors of the reverse transcriptase 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 an internal control, so at least 6 to 9 separate amplifications, without considering positive and negative controls or standard curves, would be needed. 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 the sensitive detection of GI, GII, and GIV NoVs on the basis of a model previously developed for sapovirus (27). One characteristic of the epidemiology of NoV, besides its high genetic diversity, is the cocirculation 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 to be associated with food-borne outbreaks (10, 51), making their detection important for screening of environmental or food samples. It may also be important to look for GIV NoVs, as they have been detected in sewage (7, 52, 53) and shellfish samples (14, 54; unpublished data). Improving primer and probe design for GIV strains may help to determine their circulation in the environment and to prevent their further distribution in the 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 the 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 when the results were compared to those of individual GI and GII reactions (58). The loss of 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 the sensitivities of GI and GII monoplex assays, and when applied to surface water and groundwater 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 and newly designed primers and probe for GIV has been described previously (18, 61). As noted by these authors, the sensitivity of the multiplex real-time assay that was developed was lower than the sensitivities of the 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 the monoplex assays, all water and shellfish samples were still positive when evaluated by the newly developed assay. Use of the same fluorescent dye for all three TaqMan probes allows the levels of 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 that of the spe-rRT-PCR on NA extracts from stool or bioaccumulated shellfish samples, when only qualitative determinations are made. This study also demonstrates the importance of validation using naturally contaminated samples, as the detection of GI is efficient using the gen-rRT-PCR, despite the loss of sensitivity observed with the reference NoV RNA panel. Even if precautions are taken to purify NAs 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 because of the presence of inhibitors or low levels of contamination, demonstrates that it is efficient. Two shellfish samples negative by the spe-rRT-PCR (C_T, ~41) were found to be positive with the gen-rRT-PCR, and this finding could raise a specificity question, although it is plausible that these detections were due to improved assay sensitivity. No false-negative or false-positive results were observed, and differences in C_T values were always minimal, making us confident in its application for the analysis of shellfish samples on the basis of our experience.

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

ACKNOWLEDGMENTS

This work was supported by the French government (DGAl; 2012-2100762726, program 206-sous action 37), by grant 2010 CESA 010 (HEVECODYN) from the Agence Nationale pour la Recherche (ANR), by the Japan Society for the Promotion of Science (JSPS) through JSPS Research Fellowships for Young Scientists (23-4434), by Public Health Service grant NIH P01 AI057788, by Agriculture and Food Research Initiative competitive grant 2011-68003-30395 from the USDA National Institute of Food and Agriculture, and by the John S. Dunn Research Foundation.

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