Use of TaqMan Real-Time Reverse Transcription-PCR for Rapid Detection, Quantification, and Typing of Norovirus

A. Angelica Trujillo,^{1,2*} Karen A. McCaustland,¹ Du-Ping Zheng,¹ Leslie A. Hadley,¹ George Vaughn,³ Susan M. Adams,^{1,2} Tamie Ando,¹ Roger I. Glass,¹ and Stephan S. Monroe¹

National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia¹; Atlanta Research and Education Foundation, Decatur, Georgia²; and National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia³

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Noroviruses (NoVs) are the most commonly identified cause of outbreaks and sporadic cases of acute gastroenteritis. We evaluated and optimized NoV-specific TaqMan real-time reverse transcription (RT)-PCR assays for the rapid detection and typing of NoV strains belonging to genogroups GI and GII and adapted them to the LightCycler platform. We expanded the detection ability of the assays by developing an assay that detects the GIV NoV strain. The assays were validated with 92 clinical samples and 33 water samples from confirmed NoV outbreaks and suspected NoV contamination cases. The assays detected NoV RNA in all of the clinical specimens previously confirmed positive by conventional RT-PCR and sequencing. Additionally, the TaqMan assays successfully detected NoV RNA in water samples containing low viral concentrations and inhibitors of RT and/or PCR, whereas the conventional method with region B primers required dilution of the inhibitors. By means of serially diluted NoV T7 RNA transcripts, a potential detection limit of <10 transcript copies per reaction mixture was observed with the GII assay and a potential detection limit of <100 transcript copies per reaction mixture was observed with the GI assay. These results and the ability to detect virus in water that was negative by RT-PCR demonstrate the higher sensitivity of the TaqMan assay compared with that of a conventional RT-PCR assay. The TaqMan methods dramatically decrease the turnaround time by eliminating post-PCR processing. These assays have proven useful in assisting scientists in public health and diagnostic laboratories report findings quickly to outbreak management teams.

Noroviruses (NoVs) are a group of noncultivable, genetically diverse single-stranded RNA viruses belonging to the family *Caliciviridae*. These viruses are responsible for the majority of outbreaks of acute gastroenteritis in industrialized countries (11, 12, 15, 20, 23, 28). In the United States alone, an estimated 23 million cases of NoV illnesses are reported each year, and the economic consequences of these cases are likely to be substantial (21, 23, 24). Outbreaks of NoV have been caused by contaminated food and/or drinking water, person-to-person virus transmission, and airborne droplets of infected vomitus (14, 21, 22, 23). Contaminated water poses an especially serious health risk since results from human volunteer studies indicate that the minimum infectious dose of NoV may be as low as 10 to 100 PCR units (C. Moe, Emory University, personal communication). Waterborne outbreaks have been caused by contaminated surface water, ground water, drinking water, and mineral water (1, 6, 18, 19, 35). NoV outbreaks are difficult to control and present a major public health challenge; thus, rapid diagnosis can be critical for the control of outbreaks.

NoVs are separated into five genogroups on the basis of sequence comparison of the RNA polymerase and capsid region of the genome. Genogroups I, II, and IV are associated with infections in humans. To date, 29 genetic clusters (8 from GI; 17 from GII; 2 from GIII; and 1 each from GIV and GV)

have been identified, demonstrating a high degree of genomic diversity among NoVs (2, 13, 41, 42). NoV traditionally has been detected by conventional reverse transcription (RT)-PCR, a method that requires confirmation by probe or sequence analysis (3, 9). Human NoVs cannot be cultivated, and immunoassays developed to date have not been adequately sensitive for detecting sporadic cases. Additionally, because NoVs are so diverse, designing one set of primers to detect all strains with equal efficiency is difficult. More-sensitive techniques are required to detect NoV in food and water samples, in which viral loads are typically much lower than those found in clinical samples. To date, several methods have been developed to increase the detection rate of NoV, including PCR and probe hybridization with multiple genotype-specific oligonucleotides and SYBR green analysis (3, 4, 9, 25, 26, 29, 31, 40). However, these assays have limitations. Conventional RT-PCR is time-consuming, and SYBR green analysis uses an intercalating dye that binds to all double-stranded DNA, including primers-dimers and nonspecific products. Additionally, detection of NoV in water requires methods for concentrating the virus from a large volume of water in order to increase sensitivity, a process that can coconcentrate RT-PCR inhibitors and/or erroneous targets (32). Unlike SYBR green-based quantitative methods, probe-based quantitative RT-PCR uses a fluorescently labeled, target-specific probe that results in increased specificity and sensitivity. In addition to their utility with clinical samples, probe-based quantitative methods can be especially useful in the detection of virus in samples with low viral concentrations.

To improve the detection of NoV, we developed, expanded,

* Corresponding author. Mailing address: 1600 Clifton Road N.E., Mail Stop G-04, Atlanta, GA 30333. Phone: (404) 639-4256. Fax: (404) 639-3645. E-mail: Atrujillo@cdc.gov.

and evaluated real-time RT-PCR methods based on TaqMan probe hydrolysis technology, which was previously described (17). These TaqMan assays provide sensitive, specific, and quantitative results in NoV diagnostic assays and have been successfully applied to clinical and environmental samples containing NoVs in low copy numbers. These assays rapidly detect and identify NoVs belonging to genogroups I, II, and IV and do not require any post-PCR processing. This straightforward, sensitive, and specific method will assist scientists in public health and diagnostic laboratories reduce the time needed for routine diagnosis of NoV infection and will aid in the management of outbreaks.

MATERIALS AND METHODS

Clinical specimens tested. The TaqMan assay was optimized for the LightCycler platform and validated by using an archived panel of 65 NoV-positive stool samples collected as part of the investigation of sporadic cases and outbreaks of acute gastroenteritis that occurred in the United States between June 1999 and June 2004. Because NoVs are genetically diverse, specimens were chosen to include a good representation of NoV clusters that have been reported thus far (2, 43; Fig. 1). In addition, we tested 20 clinical samples from NoV outbreaks that were confirmed negative when tested with primers for region A (the RdRp gene located in open reading frame 1 [ORF1]), region B (the 3' end of ORF1), region C (a short stretch close to the 5' end of ORF2), and RNA polymerase region 5 (primer pair 289 and 290) (2, 16). Finally, seven stool samples from NoV-negative but rotavirus ($n = 4$), astrovirus ($n = 2$), and sapovirus ($n = 1$)-positive outbreaks were also tested.

Specimens were determined to be positive for NoV by conventional duplex RT-PCR with individual primer sets GI, GII, and GIV, and they were confirmed positive and classified into genogroups on the basis of analysis of the nucleotide sequences of the amplified products. Samples were drawn from two to four outbreaks each from three clusters of GI strains, eight clusters of GII strains, and one GIV strain.

Stool sample preparation and extraction of total nucleic acids. Fecal samples were prepared as previously described, with some modifications (38). In brief, 0.1 g of formed stool or 0.1 ml of watery stool was suspended in 1 ml of diethyl pyrocarbonate-treated water (Ambion, Austin, TX), yielding a 10% suspension. To clarify the stool suspension, 1 ml of 2,3-dihydrodecafluoropentane (Vertrel, New Britain, CT) was added and the mixture was vortexed for 1 min and clarified by centrifugation at $2,060 \times g$ for 10 min at 4°C. The suspension (0.2 ml) was mixed with NucliSens lysis buffer (0.9 ml; BioMérieux, Durham, NC), and total nucleic acid was extracted by the method of Boom et al. (8), with the NucliSens extractor (BioMérieux, Durham, NC), as directed for small sample volumes. RNA samples were stored at -70°C until ready for use.

NoV detection and genetic characterization by region B conventional duplex RT-PCR. Partial NoV sequences for region B were amplified by conventional RT-PCR with primers Mon 431 and Mon 433 for genogroup I and primers Mon 432 and Mon 434 for genogroup II (Table 1). These primers amplify a small region within the 3' end of the ORF1 portion of the genome. The product length is 213 bp, with a unique sequence length of 172 bp. In cases where region B primers produced a negative or indeterminate result, primers for region C that are specific only for amplifying GII strains were used (7). The region B RT-PCR mixture consisted of 1 µl of RNA and a final volume of 49 µl of a reaction mixture containing 19.3 µl of diethyl pyrocarbonate-treated water; 2.5 µl of 20 mM dithiothreitol (Invitrogen); 0.1 µl of Triton X-100; 0.07 µl of 1.44 M β-mercaptoethanol; 0.3 µM each primers Mon 431, Mon 432, Mon 433, and Mon 434; 25 µl of Master-Amp G 2X PCR Pre-Mix (Epicenter); 0.5 µl of 40 U/µl Protector RNase inhibitor (Roche Inc., Indianapolis, IN); 0.09 µl of 20 U/µl Super Reverse Transcriptase (Molecular Genetic Resources, Tampa, FL); and 0.25 µl of 5 U/µl AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). The capsid region RT-PCR mixture consisted of 1 µl of RNA and a final volume of 49 µl of a reaction mixture containing 0.6 µM each capsid primer (Mon 381, Mon 383), 33.8 µl of H₂O from the QIAGEN One Step RT-PCR kit (QIAGEN Inc., Valencia, CA), 10 µl of QIAGEN One Step RT-PCR 5× buffer, 2 µl of a deoxynucleoside triphosphate mixture (10 mM each), and 2 µl of One Step enzyme mix (QIAGEN Inc., Valencia, CA). The thermocycling program for the one-step conventional region B RT-PCR consisted of RT for 10 min at 42°C; denaturation for 3 min at 94°C; 40 PCR cycles consisting of 94°C for

FIG. 1. Phylogenetic dendrogram of strains belonging to clusters of human NoVs. Underlined clusters denote GI, GII, and GIV strains tested by real-time RT-PCR (43).

30 s, 50°C for 90 s, and 60°C for 30 s; and then 72°C for 7 min. The thermocycling conditions for the capsid L region RT-PCR consisted of linearizing the RNA by heating 1 µl of RNA with 0.6 µM each capsid primer at 94°C for 3 min, followed by addition of the cocktail. The RNA was then reverse transcribed for 60 min at 42°C and then subjected to activation for 15 min at 95°C; 40 PCR cycles consisting of 94°C for 60 s, 50°C for 90 s, and 60°C for 120 s; and 72°C for 7 min.

PCR products were purified with the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA). Fluorescent dideoxy-chain terminators (Applied Biosystems, Foster City, CA) were used to sequence both strands with an automated sequencer (model 3100; Applied Biosystems). All strains tested were previously found to be NoV positive, and this was confirmed through sequencing.

Primers and probes for real-time RT-PCR. For detecting NoV GI and GII, we used primers and TaqMan probes previously described (17; Table 1). These primers and probes target NoV sequences at the ORF1-ORF2 junction, a highly conserved region of the NoV genome. The primers and probes for GI were chosen from the corresponding Norwalk/68 virus (GenBank accession no. M87661), and those for GII were chosen from the corresponding Camberwell virus (GenBank accession no. AF145896). Primers and TaqMan probes for GIV NoV detection were developed at the Centers for Disease Control and Prevention (CDC) and used the corresponding

TABLE 1. Primer and probe oligonucleotides used for real-time quantitative RT-PCR for genogroups I, II, and IV and primers for region B

Primer or probe	Polarity ^c	Sequence (5'→3') ^a	Position ^b
Real-time RT-PCR primers and probes			
Cog 1F (GI)	+	cgY tgg atg cgl ttY cat ga	5291
Cog 1R (GI)	—	ctt aga cgc cat cat cat tYa c	5375
Ring 1A (GI)	—	FAM ^c -aga tYg cga tcY cct gtc ca-BHQ ^d	5340
Ring 1B (GI)	—	FAM-aga tcg cgg tct cct gtc ca-BHQ	5340
Cog 2F (GII)	+	caR gaR BcN atg tty agR tgg atg ag	5003
Cog 2R (GII)	—	tcg acg cca tct tca ttc aca	5100
Ring 2 (GII)	+	FAM-tgg gag ggc gat cgc aat ct-BHQ	5048
Mon 4F (GIV)	+	ttt gag tcY atg tac aag tgg atg c	718
Mon 4R (GIV)	—	tcg acg cca tct tca ttc aca	815
Ring 4 (GIV)	+	FAM-tgg gag ggg gat cgc gat ct-BHQ	763
Conventional RT-PCR region B			
NoV431 GI	+	tgg acI agR ggl ccY aaY ca	5093–5305
NoV433 GI	—	gaa Yct cat cca Yct gaa cat	5093–5305
NoV432 GII	+	tgg acI cgY ggI ccY aaY ca	5093–5305
NoV434 GII	—	gaa Rcg cat cca Rcg gaa cat	5093–5305

^a R = A or G, Y = C or T, N = any.^b Nucleotide positions for conventional RT-PCR were taken from reference NoV strains in genogroups GI (Norwalk virus 68 [Genbank accession no. M87661]), GII (Hawaii virus [GenBank accession no. U07611]), and GIV (Saint Cloud virus [GenBank accession no. AF414426]). Nucleotide positions for real-time RT-PCR were taken from reference NoV strains in genogroups GI (Norwalk virus 68 [Genbank accession no. M87661]) and GII (Camberwell [Genbank accession no. AF145896]).^c FAM, 6-carboxyfluorescein reporter dye.^d BHQ, black hole quencher dye.^e +, virus sense; —, antiviral sense.

Saint Cloud virus (GenBank accession no. AF414427) (Table 1). These primers target the ORF1-ORF2 junction of the genome.

Preparation of in vitro RNA transcripts. The 3-kb RT-PCR products obtained in a prior study were used as the source of DNA for preparation of in vitro RNA transcripts (4). The amplification products were cloned into either the ClaI/NotI or the ClaI/XbaI sites of the pBluescript II SK (+) plasmid vector (Stratagene, La Jolla, CA) after reamplification with the original primers modified to contain the restriction enzyme sites. After the cloned plasmid DNA was purified by use of the QIAfilter plasmid Mega kit (QIAGEN Inc., Valencia, CA), the DNA insert was cut out with the restriction enzymes, gel purified, and used as the template for in vitro transcription with T7 RNA polymerase with the MEGA script kit (Ambion Inc., Austin, TX). After digestion of the template DNA with RNase-free DNase I (Ambion Inc.) at 37°C for 30 min and phenol-chloroform extraction, followed by ethanol precipitation, the RNA transcripts were dissolved in RNase-free water and the concentrations were calculated after measuring the absorbance at 260 nm. The RNA transcript solutions were diluted with RNA Storage Solution (Ambion Inc., Austin, TX), aliquoted, and kept at –70°C for long-term storage. The genetic groups (2) (GenBank accession numbers) of the 3-kb products that served as the templates for the RNA transcripts were GI/3 (Honolulu 219/1992/US, S4 [accession no. AF414403]) and GII/4 (Burwash Landing 331/1995/US, S24 [accession no. AF414425]). The 3-kb products were reamplified with three primers, SR48ad (forward primer for GI; 5'-CCA TCG ATA CTA GTG AAC AGC ATA AA-3'), SR46ad (forward primer for GII; 5'-C CAT CGA TAC TAG TCC ATC GCC CAC TGG-3'), and VNad (reverse primer for both GI and GII; 5'-A AGG ATC CGC GGC CGC TCT AGA TTT TTT-3').

TaqMan real-time RT-PCR. We performed quantitative real-time RT-PCR assays with the LightCycler RNA amplification kit hybridization probes (Roche Diagnostics, Alameda, CA). The final reaction mixture (20 µl) consisted of 1 µl of RNA, 4 µl of the LightCycler RT-PCR mixture, 0.4 µl of the LightCycler RT-PCR enzyme mixture, and 5 mM MgCl₂. In the final optimized format, the concentrations of the primers and probes were as follows: for the GI assay, 0.2 µM each GI probe with 0.4 µM each primer; for the GII assay, 0.4 µM probe and 0.4 µM each primer; and for the GIV assay, 0.4 µM probe and 0.3 µM each primer. The thermal cycling conditions consisted of RT for 30 min at 55°C, followed by denaturation at 95°C for 30 s, amplification for 45 cycles, followed by denaturation at 95°C for 0 min, and annealing-extension at 60°C for 60 s.

Fluorescence measurements were taken, and the crossing point (cycle number [CN]) for each sample was calculated by the fit points method. The noise band was set to a minimum value of 3 standard deviations above the background fluorescence (34). The algorithm to minimize error for the LightCycler platform was used to set the crossing point for analysis. The threshold for a positive value was set at greater than three times the background fluorescence. A test result was considered positive

if the genomic target showed positive results (CN) at less than 40 cycles and all positive and negative control reactions gave expected values.

NoV RNA concentration from water samples. In the fall of 2003, the CDC Vessel Sanitation Program collected 12 water samples from cruise ship A, which had repeated outbreaks of NoV gastroenteritis suspected to be linked to its water supply, although the exact source had not been determined. Potable-water samples (10 to 20 liters) were collected from various sites aboard the ship. Prefilters (AP 2504700; Millipore, Bedford, MA) were used to prevent clogging by large-particulate matter. The samples were filtered through a positively charged membrane (0.45-µm pore size; Zetapore; AMF-Cuno, Meriden, CT). After samples were collected, the pre-filter and filters were packaged by a sterile technique, placed at 4°C, and transported to the CDC, where they were processed within 24 h. The prefilter was discarded, and the membrane was transferred to a 200-ml beaker. Sterile 0.5 M lysine (15 ml), pH 8.5, was added, and the filter was shaken for 20 min at room temperature. Fifteen milliliters of sterile 0.5 M arginine, pH 8.5, was added to the beaker and vigorously shaken for an additional 20 min. A sufficient volume of 1 M HCl was added to the supernatant to decrease the pH to 7.3. The solution was vigorously shaken for an additional 20 min at room temperature. The virus was concentrated by adding 15 ml of sterile 30% polyethylene glycol and 0.9 M NaCl and shaken vigorously for an additional 20 min at room temperature. The mixture was transferred into a 50-ml conical tube and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 ml of H₂O and mixed by inverting the tube. The mixture was washed by adding 0.5 ml of a 30% polyethylene glycol–0.9 M NaCl solution and then centrifuged at 10,000 × g for 30 min at 4°C. This process was repeated twice. The retained material was adjusted to 250 µl with sterile H₂O. Total nucleic acid was extracted with the automated Nuclisens Extractor (BioMérieux, Durham, NC) as directed for small sample volumes. RNA samples were stored at –70°C until ready for use. Five microliters of RNA, representing 10% of the water concentrate, was used for NoV detection.

In a separate case of outbreaks of NoV among river rafters, 21 water samples were collected by filtration as described above. Since the river was suspected as the source of NoV contamination, water samples from different locations on the river were collected. Due to the remote location and the lack of refrigeration, the samples were stored at room temperature for 1 week. Upon arrival at the CDC, the samples were refrigerated and processed within 12 h. RNA samples were stored at –70°C until ready for use.

RESULTS

We selected 12 NoV strains that each represented a different genetic cluster to evaluate and validate the three genogroup-

FIG. 2. CNs detected from archived specimens of water and stool. A comparison of water (A) ($n = 33$) and stool (B) ($n = 72$) samples is shown. Specimens with CNs of >40 or not detected were considered negative (Neg).

specific real-time TaqMan RT-PCR assays. These strains belonged to GI (GI/3, GI/4, and GI/2), eight belonged to GII (GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/16, and GII/17), and we developed primers and probes to test the single GIV strain (Fig. 1; Table 1). A total of 92 clinical specimens were tested, 7 of which were negative controls for NoV and 65 of which were positive for NoV (Fig. 2B). Additionally, we tested 20 samples confirmed to be NoV negative in regions A, B, C, and 5. These samples were also negative for rotavirus, astrovirus, and sapovirus. All of the negative samples were negative by the TaqMan assays (data not shown). The NoV-positive clinical samples represented 12 clusters from GI, GII, and GIV (Fig. 1 and 2B). All 65 stool samples (100%) that tested positive for NoV by means of conventional duplex RT-PCR also tested positive by the TaqMan real-time RT-PCR assay (Fig. 2B). The specificity of the assays was tested against seven NoV-negative specimens that were positive for rotavirus ($n = 4$), astrovirus ($n = 2$), and sapovirus ($n = 1$); no cross-reactivity was observed (Fig. 2B).

T7 RNA transcripts representing genogroups I and II were selected as standards to determine the copy detection limits and dynamic range of the optimized TaqMan real-time RT-PCR assay (Fig. 3A and B). No GIV transcript was available at the time

of this study; therefore, a stool standard was used for genogroup GIV (Fig. 3C). Standards were diluted in a 10-fold series ranging from undiluted to 10^0 , and the intensity of fluorescence was plotted against the CN. RNA standards demonstrated excellent negative linearity with a wide dynamic range (Fig. 3A and B). The GI/3 transcript standard had a detection limit of <100 copies per reaction mixture (Fig. 3A). The slope was -3.370 , the y intercept was 43.97 , and the R^2 value was 1.00 . A detection limit of <10 copies per reaction mixture was observed in the GII T7 RNA transcript standards. The slope was -3.324 , the y intercept was 39.20 , and the R^2 value was 1.00 (Fig. 3B). We examined the specificity of the GIV primers and probe with six archived samples collected more than 5 years previously that had tested positive for genogroup IV by conventional RT-PCR. Five of the six specimens previously found positive for GIV were found to be positive by use of the TaqMan assay. The sensitivity of the GIV assay was tested with a serial dilution from undiluted to 10^{-9} to determine the detection limit. The dynamic range of the GIV stool standard was narrower, with positive detection from undiluted to 10^5 . The slope and y intercept were -3.310 and 58.96 , respectively, and the R^2 value was 1.00 (Fig. 3C).

These positive results and the high sensitivity of this method

FIG. 3. Relationship between known copy numbers of T7 RNA (GI and GII) or viral RNA from stool extract (GIV) and the detection threshold. T7 RNA transcripts were made for representative GI and GII strains, and a fecal specimen was used for GIV. (A) Serial dilution of a GI/3 NoV RNA transcript demonstrating a wide dynamic range. The detection limit was 9.836×10^1 copies/ μ l, the slope was -3.370 , the y intercept was 43.97 , and the R^2 value was -1.00 . (B) GII/4 serial dilution of a NoV RNA transcript showing a wide dynamic range. The detection limit was 9.05 copies/ μ l, the slope was -3.324 , the y intercept was 39.20 , and the R^2 value was -1.00 . The mean squared error was 0.195 . (C) (GIV) NoV RNA stool standard serial dilutions. The slope was -3.310 , the y intercept was 58.96 , and the R^2 value was -1.00 . The mean squared error was 0.0763 . The detection limit of the GIV stool standard curve was 8.644×10^5 copies/ μ l. The slope, y intercept, and mean squared error were 3.392 , 59.27 , and 0.0764 , respectively, and the R^2 value was -1.00 .

encouraged us to use it to detect NoV in water samples presumed to contain low viral loads. For testing, we chose potable-water samples from a cruise ship (cruise ship A) that had previously reported numerous gastroenteritis outbreaks that were suspected

to be linked to the potable-water supply. Twelve samples were tested twice by conventional RT-PCR and found negative for NoV with region B primers (Fig. 4A; Table 1). The samples were then retested for NoV RNA with the TaqMan assay, and two were positive for low concentrations of NoV, with CNs of 39.63 and 33.42 for samples 1 and 2, respectively (Fig. 4B). The samples were diluted 100-fold and retested with region B primers (Table 1) to rule out the possible presence of RT and/or PCR inhibitors. Faint bands were detected by ethidium bromide staining. Of the two samples, no. 2 was successfully sequenced, confirming it to be a true positive (Fig. 4C).

In addition to the 92 archived stool specimens, we tested 33 water samples from environmental sources suspected to be contaminated with NoVs. Of these, eight were positive for low-level NoV contamination, with a mean CN of 37.911 (Fig. 2A). The TaqMan assay easily and rapidly detected previously identified clusters of GI, GII, and GIV.

DISCUSSION

Newly developed, commercially available nucleic acid amplification systems designed to decrease assay time by monitoring amplification of target sequences in real time by fluorescence resonance energy transfer analysis are widely used for absolute and relative quantification and are a critical tool for basic research, diagnostics, and biotechnology. In this report, we describe the expansion and validation of broadly reactive and semiquantitative TaqMan real-time RT-PCR assays to detect the three NoV genogroups (GI, GII, and GIV) that target the ORF1-ORF2 junction, a highly conserved region of the NoV genome (17, 27). A wide range of NoV strains representing numerous clusters archived at the CDC were tested with the TaqMan assays. The assays detected 64/65 NoV samples that had been previously identified by duplex conventional RT-PCR assay. Viral instability and/or RNA degradation of one specimen may account for the negative result since the samples had been stored at -70°C for more than 6 years and retesting with a conventional RT-PCR assay also yielded a negative result.

NoVs are especially diverse; therefore, designing one set of primers to detect all strains with equal efficiency is difficult. Since NoVs circulating in the community are frequently changing and new clusters are recognized regularly, we entertained the possibility that the primers and probes, over time, would not continue to provide broadly reactive detection of GI, GII, and GIV NoVs. To investigate this possibility, we tested two recently identified NoV strains, GII/16 (accession no. AY502006) and GII/17 (accession no. AY502009) (43). Robust signals were observed with the GII assay, demonstrating continued detection albeit strain evolution (data not shown). The primer and probe sets for GI and GII NoVs designed by Kageyama et al. and our GIV primers and probe set maximize detection over time by using the ORF1-ORF2 region, which contains the highest nucleotide homology (17).

RT-PCR was the first molecular diagnostic method used for the diagnosis of NoV infection in clinical and environmental samples (2, 28, 36). Although this method is powerful, it lacks the sensitivity and specificity needed for samples with low viral loads or samples containing RT-PCR inhibitors. As observed in our study, samples containing low viral concentrations and

FIG. 4. Detection of NoV in water samples from cruise ship A by conventional (A) versus real-time (B) RT-PCR assay. (A) A conventional RT-PCR assay failed to detect NoV RNA from potable water (lanes 2 and 3) compared with a positive control (lane 4, GII strain) and a negative control (lane 5, water). Lanes 1 and 6 were 123-bp ladders in a 3% agarose gel with 0.5 μ g of ethidium bromide run at 120 V for 60 min. (B) A real-time RT-PCR assay identified 2 positive water samples ($\blacktriangle$, $\diamond$) among 12 samples tested. Controls included a GII/4 strain T7 RNA transcript ($\blacksquare$), a GII/4 fecal specimen ($\circ$), and a no-template negative control water sample ($-$). (C) Conventional RT-PCR detected NoV RNA in potable water after samples were diluted 1:100. Lane 1 was a 123-bp ladder in a 3% agarose gel with 0.5 μ g of ethidium bromide run at 120 V for 60 min. Lanes 2 and 5 were positive controls (GII strain); lanes 3 and 7 were water samples 1 and 2, respectively; and lanes 4 and 6 were negative controls.

RT-PCR inhibitors may test falsely negative by conventional RT-PCR methods. However, with the TaqMan assay, sensitivity greater than that of the conventional method was repeatedly observed with samples containing low NoV concentrations or RT-PCR inhibitors. Additionally, the TaqMan assay has the increased specificity of the TaqMan probe, as opposed to previously described SYBR green real-time RT-PCR assays (29). This TaqMan real-time RT-PCR method detects NoV in a genogroup-specific manner, making it possible to determine the genogroup directly without resorting to conventional RT-PCR methods that use degenerate primers and require sequencing for confirmation. By means of serially diluted NoV transcripts, potential detection limits of <10 transcript copies per reaction mixture and <100 transcript copies per reaction mixture were achieved with the GII and GI transcripts, respectively. The assays demonstrated a large dynamic range of at least 6 logs for quantification. The GIV stool had a lower detection limit of $<10,000$ transcript copies per reaction mixture. As opposed to T7 RNA transcripts, the presence of

nucleases and other RT and/or PCR inhibitors in stool may account for the decreased sensitivity of the GIV assay. However, much like the GII transcript standard, a detection limit of <10 copies per reaction mixture was also observed with the GII stool standard (data not shown). The sensitivity difference among the GI, GII, and GIV assays can also be due to many other factors, such as primer design and differences in PCR conditions.

Real-time RT-PCR offers obvious advantages over more traditional RT-PCR formats, but some caution is required when interpreting results. The efficiency of a real-time assay can be estimated by analyzing the exponential phase of the amplification curve (5, 33, 39). Quantitative RT-PCR methods presume that the target and the sample are amplified with similar efficiencies. However, small variations in efficiency reflecting a decline in DNA polymerase activity between standards and unknowns can negatively impact true quantification.

The risk with external standards is that some of the unknown

samples may contain substances that significantly reduce the efficiency of the PCR. When inhibitors are suspected, sample dilution is often effective and the inhibitory factors can be diluted out if the target nucleic acid is present in sufficient quantities. The presence of inhibitors may affect the ability to determine the absolute or true starting copy number in these samples, but for detection and subtyping, semiquantitation or approximation of the concentration is sufficient. Various other approaches have been developed to detect RT and PCR inhibitors in clinical and environmental samples containing NoV. Housekeeping genes such as that for RNase P have been used with fecal samples but have yielded inconsistent results (S. Monroe, personal communication; 10). Internal standard controls of synthetic NoVs can be generated by transcribing NoV plasmids, but this significantly increases the cost of testing (5, 36, 37).

The TaqMan assay is ideal for laboratories handling a large volume of clinical and environmental specimens and provides quantification and immediate typing of GI, GII, and GIV NoV strains. A multiplex real-time RT-PCR assay for the detection of GI and GII NoVs has recently been described (30), but further development to include the GIV primer set is needed. After an initial rapid NoV diagnosis has been made with the TaqMan assays, strain comparison with capsid VP1 (region D) primers can be done since phylogenetic analysis of sequences from this region are consistent with those based on entire capsid sequences (42).

This assay has proven useful for routine diagnostic assays in clinical settings because it eliminates postamplification product processing and thus shortens the turnaround time for reporting results, which can prove critical in an outbreak setting. Finally, the TaqMan assay can assist in determining if NoV contamination is present in samples containing very low viral concentrations.

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