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Evaluation and Validation of Real-Time Reverse Transcription-PCR Assay Using the LightCycler System for Detection and Quantitation of Norovirus

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We developed an assay for the detection and quantitation of norovirus with the LightCycler SYBR Green-based real-time reverse transcription-PCR (real-time LC RT-PCR) and previously published primers in the capsid and the polymerase gene. One hundred thirty-two stool specimens from the Provincial Laboratory for Public Health (Microbiology), Alberta, Canada, and the Centers for Disease Control and Prevention, Atlanta, Ga., were used to validate the new assay. The samples were collected from patients involved in outbreaks of acute gastroenteritis or children who presented with sporadic gastroenteritis. The real-time LC RT-PCR assay detected norovirus strains from three genogroup I (G-I) clusters (G-I/1, G-I/2, and G-I/3) and 10 genogroup II (G-II) clusters (G-II/1, G-II/2, G-II/3, G-II/4, G-II/6, G-II/7, G-II/10, G-II/12, G-II/15, and G-II/16). There was 100% concordance with the results from 58 stool specimens which tested positive by conventional RT-PCR assays. By dilution analysis, the real-time LC RT-PCR was 10,000 times more sensitive than the conventional RT-PCR. The new assay increased the number of samples in which noroviruses were detected by 19%. The real-time LC RT-PCR had a wide dynamic range, detecting from 5 to 5×10^6 copies of RNA per reaction, resulting in a theoretical lower limit of detection of 25,000 copies of RNA per g of stool. No cross-reactions were found with specimens containing sapovirus, rotavirus, astrovirus, and adenovirus. Because of the high sensitivity and specificity of the assay with a relatively rapid and simple procedure, the real-time LC RT-PCR will be useful as a routine assay for the clinical diagnosis of norovirus infection.

Noroviruses are one of the most common etiological agents of outbreaks of acute gastroenteritis and have a significant public health impact worldwide (5). They have also been reported as a relatively common cause of acute gastroenteritis in children (3, 16, 17, 20, 22). Noroviruses are a genetically diverse group of viruses belonging to the *Norovirus* genus in the family *Caliciviridae* (21). The virion contains a single positive-strand RNA genome of approximately 7.6 kb. Based on comparisons of the genetic sequences of the viral RNA-dependent RNA polymerase and the capsid protein, noroviruses are subdivided into five genogroups (G-I to GV). Genogroup I (G-I), with seven clusters, and genogroup II (G-II), with 16 clusters, contain most of the strains that infect humans.

Historically, diagnosis of norovirus was made by electron microscopic examination of stool samples. The limit of detection with electron microscopy is estimated to be 10^6 viral particles per g of stool (4, 7). Due to the genetic and antigenic diversity of norovirus strains, most attempts to develop diagnostic enzyme immunoassays have resulted in tests that are unable to detect widely different strains (2). Cloning and sequencing of Norwalk virus and other norovirus strains in the 1990s allowed the development of broadly reactive molecular detection assays (10). The reverse transcription-PCR (RT-PCR) assay has become the principal diagnostic tool for noroviruses (2). However, conventional RT-PCR assays for norovirus are cumbersome and cannot easily quantitate the viral load. Real-time quantitative RT-PCR systems are slowly replacing conventional RT-PCR assays for the detection and quantification of RNA viruses in research and diagnostic laboratories. Two fluorescent detection systems, SYBR Green I intercalating dye and specific hybridization probes, have been employed. SYBR Green I dye binds to double-stranded nucleic acids generated during the amplification, providing sensitive detection of products in real time. Because a signal will be generated by any amplified DNA, this approach is relatively less specific than those with specific hybridization probes (18). To date, there have been limited reports on real-time RT-PCR assays for the detection and quantitation of noroviruses in stool specimens (11, 14).

The present study evaluated, optimized, and validated a real-time LightCycler (LC) RT-PCR assay for detecting and quantifying noroviruses with archived stool samples. Various nucleic acid extracting methods, primer pairs, and amplification conditions for RT-PCR assays were compared. The optimal assay uses different primer pairs for genogroups G-I and G-II and is sensitive and specific for detecting a broad range of norovirus strains.

MATERIALS AND METHODS

Clinical specimens. Archived stool specimens were obtained from the Provincial Laboratory for Public Health (PLPH), Alberta, Canada, and the Centers for Disease Control and Prevention (CDC), Atlanta, Ga. A total of 132 stool specimens were used in the study. Eighty-eight stool specimens were from PLPH: 27 specimens from investigations of outbreaks of acute gastroenteritis that had tested positive for norovirus by conventional RT-PCR (1) and 61 specimens were from sporadic gastroenteritis in children less than 6 years of age. For those 61

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pediatric samples, electron microscopy and viral culture had been performed according to the standardized PLPH algorithm. Then these 61 samples were tested side by side with real-time LC RT-PCR as well as conventional RT-PCR for norovirus. The CDC provided 44 stool specimens previously tested by conventional RT-PCR and included 4 positive for norovirus G-I (cluster designations: G-I/1, G-I/2, and G-I/3), 18 positive for norovirus G-II (cluster designations: G-II/1, G-II/2, G-II/3, G-II/4, G-II/6, G-II/7, G-II/10, G-II/12, G-II/15, and G-II/16), two positive for sapovirus, and 20 specimens with no identifiable enteric viruses (1, 5, 6).

Viral RNA extraction. A 20% (wt/vol) suspension of stool specimen was prepared with phosphate-buffered saline and clarified by centrifugation at 1,400 $\times$ *g* for 15 min. Viral RNA was extracted from 100 μl of the stool suspension (equivalent to 20 mg of stool) with the NucliSens extraction kit (Organon Teknika, Durham, N.C.) for specimens obtained from the CDC and a silica-based in-house method (15) for specimens obtained from the PLPH. The RNA was eluted in 50 μl of elution buffer, aliquoted, and stored at −70°C until testing was performed. An equivalent volume of phosphate-buffered saline and a known norovirus-positive specimen were included in each batch of RNA extraction as negative and positive controls, respectively.

Primers for real-time LC RT-PCR and conventional RT-PCR. For the detection of norovirus G-II, we used primers selected from a conserved region of the norovirus polymerase gene, NVP 110 (reversed), 5′-AC(A/T/G)AT(C/T)TCAT CATCACCATA-3′ (4865 to 4884 in GenBank accession no. X87661) (13), and SR46 (forward), 5′-TGGAATTCCATCGCCCACTGG-3′ (4766 to 4786 in GenBank accession no. X87661) (1). For the detection of norovirus G-I, the primers from the capsid gene of norovirus CapA, (reversed) 5′-GGC(A/T)GTTCCCA CAGGCTT-3′ (6897 to 6914 in GenBank accession no. X87661), and CapB, 5′-TATGTTGACCCTGATAC-3′ (6737 to 6753 in GenBank accession no. X87661), were used (25).

RT reaction. The RT reaction was carried out with SuperScript II RNase H^- reverse transcriptase kit (Invitrogen). A final volume of 20 μl of RT reaction containing 5 μl of 5× first transcript buffer, 5 mM dithiothreitol, 20 U of RNaseOut recombinant RNase inhibitor, 100 U of SuperScript II reverse transcriptase, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 1.25 μM primer NVP110 or primer CapA, and 5 μl of RNA (equivalent to 2 mg of stool) was incubated at 45°C for 1 h and then inactivated at 70°C for 15 min.

Conventional PCR. A final volume of 50 μl of reaction containing 5 μl of cDNA (equivalent to 0.5 mg of stool) from the RT reaction, 5 μl of 10× PCR buffer, 2 mM $MgCl_2$, 0.375 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 μM each of the primers, and 2.5 μl of AmpliTaq polymerase (PE Biosystem) was carried out on a GeneAmp PCR System 9600 (Perkin Elmer). The reaction was preheated at 95°C for 3 min and followed by 40 thermal cycles of 30 s at 94°C, 30 s at 50°C, and 45 s at 72°C and a final extension at 72°C for 5 min. The PCR products were separated in a 3% agarose gel and visualized by ethidium bromide staining.

Quantitative real-time LightCycler RT-PCR. A SYBR Green I system was utilized in the reaction; 20 μl of the PCR mixture containing 2 μl of cDNA (equivalent to 0.2 mg of stool) from the RT reaction, 3 mM $MgCl_2$, 0.5 μM each of primer, and 2 μl of the reagent from an LC-FastStart DNA Master SYBR Green kit (Roche Diagnostics) were added to a capillary tube and loaded into the LightCycler (Roche Diagnostics). The thermal cycling conditions were as follows: an initial denaturation at 95°C for 6 min, followed by 45 cycles of 10 s of denaturing at 95°C, 10 s of annealing at 50°C, and 10 s of extension at 72°C. The data were collected in the single mode with channel setting F1/1 during the annealing phase. To establish external standard curves for the quantification of norovirus, RNA transcripts from strains S5 and S19, corresponding G-I/4 and G-II/12, respectively (provided by T. Ando, CDC), were 10-fold diluted (5 × 10E6 to 5 copies per reaction) and run in real-time LC RT-PCR. To determine potential contamination from untranscribed plasmid DNA in the RNA preparations, the same serial dilutions were tested with real-time LC PCR without the RT reaction.

For data analysis, the melting temperature, fluorescence (d[F1]/dT) under the melting curve window, and C_t, which is defined as the fractional cycle number where the fluorescence passed the fixed threshold, in the quantification window were selected as the evaluating parameters. The readout of the reaction with a melting temperature of 82 to 85°C, fluorescence d[F1]/dT above 1.5, and C_t value below 38.00 against a baseline of fluorescence signal at 2.0 was used to indicate a positive reaction (see Fig. 2).

Measurements of sensitivity, specificity, and reproducibility. To compare the detection range of the real-time LC RT-PCR with conventional PCR, two norovirus RNA specimens of genotypes G-II/1 and G-II/4 were diluted from neat to 10^{-9} and assayed with both methods. For the study of specificity, nucleic acid extracts from known sapovirus, rotavirus, astrovirus, and adenovirus strains were assayed with the same primer pairs in the LC RT-PCR. The reproducibility of the real-time LC RT-PCR was also evaluated in five consecutive runs of two clinical specimens known to be norovirus positive.

Statistical methods. The sensitivity of detection between the real-time LC and conventional RT-PCR was analyzed by Fisher's exact test. C_t values for the specimens that had tested positive by both the conventional RT-PCR and real-time LC RT-PCR and the specimens that tested positive only by real-time LC RT-PCR were compared by *t* test (Statistica, Tulsa, Okla.). The precision of the LC-PCR for detecting norovirus was expressed by a coefficient of variation. The confidence interval was set at 95%, with a significance at $P < 0.05$.

RESULTS

The detection of norovirus by conventional and real-time LC RT-PCR of all the stool samples is summarized in Table 1. All 44 stool samples (100%) that tested positive at PLPH for norovirus genogroup II (G-II) also tested positive for G-II by the real-time LC RT-PCR. Five of the 36 specimens (14%) that tested negative at PLPH for norovirus tested positive by the real-time LC RT-PCR. For the 18 specimens that had previously tested positive for G-II at CDC, only 12 specimens (66.7%) tested positive with conventional RT-PCR, while 17 of the 18 samples (94%) tested positive by the real-time LC RT-PCR. Moreover, 6 of 20 stool samples (30%) that had tested negative previously at CDC tested positive for G-II with the real-time LC RT-PCR. Only 2 of these 6 positive specimens tested positive by the conventional RT-PCR. Another set of 4 specimens that had tested positive for G-I genogroup by CDC were all confirmed (100%) by the real-time LC RT-PCR.

Norovirus G-I-positive samples occasionally gave positive signals with the G-II primers in real-time LC RT-PCR. There was no cross-reactivity with the 12 stool samples that had tested positive for rotavirus, sapovirus, astrovirus, and adenovirus (Table 1). The mean C_t value of the specimens that were tested positive for norovirus G-II by both the conventional RT-PCR and the real-time LC RT-PCR was 24.38 ± 4.43, while the mean C_t value of specimens that tested positive only by the real-time RT-PCR was 32.56 ± 2.41 ($P < 0.01$, *t* test). The implication is that the conventional RT-PCR cannot detect Norovirus for specimens with a C_t value greater than 31 (Fig. 1). A very good negative regression existed between copies of norovirus equivalents per gram of stool specimen and C_t values of real-time RT-PCRs ($R^2 = 0.94$). Of the 14 samples that were positive by real-time RT-PCR but negative by conventional PCR, 2 samples were sequenced and confirmed as norovirus.

A standard curve was created with 10-fold dilutions of RNA transcripts of S19 plasmid DNA (5×10^6 to 5 genome copies) to quantify norovirus G-II in the stool specimens. Linear regression of the C_t values and the quantity of RNA revealed a good negative linearity ($r = -1$, error = 0.0434, slope = −3.411, and intercept = 36.09). The slopes corresponding to the efficiency of RT-PCR were −3.39 cycles per $\log_{10}$ unit with the RNA standard and −3.33 cycles per $\log_{10}$ unit with the RNA from stool specimens. With 0.2 mg of stool used in each RT-PCR, the lower detection limit of the real-time LC RT-PCR was calculated to be 25,000 copies per gram of stool for norovirus G-II. A total of 15 stool specimens from patients associated with outbreaks of acute gastroenteritis were quantified, and the median of the copy numbers for norovirus G-II was 25,385,000 copies per gram of stool (range, 43,000 to 6,935,000,000). The small amount of norovirus plasmid DNA

TABLE 1. Summary of norovirus detection by conventional and real time RT-PCR in clinical stool specimens from PLPH, Canada, and CDC, Atlanta[a]

Samples	Specimens from CDC						Specimens from PLPH			
	Original result from CDC		Conventional RT-PCR assay for norovirus		LightCycler real-time RT-PCR for norovirus		Original result from PLPH		LightCycler real-time RT-PCR for norovirus	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Positive for norovirus G-II by conventional RT-PCR at CDC or PLPH ($n = 62$)[a]	**18**		**12**	**6**	**17**	**1**	**44**		**44**	**0**
G-II/1	1		1	0	1	0				
G-II/2	2		2	0	2	0				
G-II/3	4		3	1	3	1				
G-II/4	2		2	0	2	0				
G-II/6	2		1	1	2	0				
G-II/7	1		0	1	1	0				
G-II/10	2		1	1	2	0				
G-II/12	1		1	0	1	0				
G-II/15	2		1	1	2	0				
G-II/16	1		0	1	1	0				
Positive for norovirus G-I by conventional RT-PCR at CDC or PLPH ($n = 4$)[b]	**4**		**NT[e]**	**NT**	**4**	**0**				
G-I/1	2		NT	NT	2	0				
G-I/2	1		NT	NT	1	0				
G-I/3	1		NT	NT	1	0				
Negative for norovirus by conventional RT-PCR at CDC or PLPH ($n = 56$)[c]		**20**	**2**	**18**	**6**	**14**		**36**	**5**	**31**
Positive for other enteric viruses ($n = 10$)[d]	**2**		**0**	**2**	**0**	**2**	**8**		**0**	**8**
Sapovirus	2		0	2	0	2	2		0	2
Rotaviruses							2		0	2
Astroviruses							2		0	2
Adenoviruses							2		0	2

[a] The 62 G-II-positive samples included 18 outbreak samples and 27 outbreak samples previously tested by conventional RT-PCR at CDC and PLPH, respectively, as well as 17 samples that were collected from children with sporadic gastroenteritis and tested side by side with conventional and real time RT-PCR. For each sample category with columns of lightface numbers, the boldface number at the top of each column represents the total of the lightface numbers listed below it.

[b] The four G-I-positive samples were provided by CDC.

[c] The 56 samples included 20 outbreak samples that previously tested negative by conventional RT-PCR at CDC and 36 samples that were collected from children with sporadic gastroenteritis and tested side by side with conventional and real time RT-PCR. A total of 11 samples tested positive by real time RT-PCR.

[d] The 10 samples were positive for other viruses, including four sapoviruses (two from CDC and two from PLPH), and each two rotaviruses, astroviruses, and adenoviruses (from PLPH).

[e] NT, not tested.

detected in the RNA transcripts was excluded in the calculation because of no noticeable effect on the RNA detection. The dynamic range of the LC RT-PCR with the G-I primers was narrower, with positive detection of 5×10^3 to 5×10^6 genomic copies of the RNA transcripts of the S5 plasmid DNA. The lower detection limit for norovirus G-I was calculated to be 25,000,000 copies per gram of stool.

TABLE 2. Comparison of the detection of norovirus genogroup G-II in 118 clinical stool specimens from the PLPH and CDC by a conventional RT-PCR assay with a real-time LightCycler RT-PCR assay

Conventional assay result	Real-time LC RT-PCR assay result (no.)					
	Specimens from CDC			Specimens from PLPH		
	Positive	Negative	Total	Positive	Negative	Total
Positive	14	0	14	44	0	44
Negative	9	15	24	5	31	36
Total	23	15	38[a]	49	39	80[b]

[a] Excluding four specimens known to be positive for norovirus G-I and two specimens known to be positive for sapovirus.

[b] Excluding eight specimens known to be positive for other viruses: two rotavirus, two adenovirus, two sapovirus, and two astrovirus.

The sensitivity of the real-time LC RT-PCR was compared with the conventional RT-PCR with neat to 10^{-9} dilutions of two stool samples that tested positive for norovirus G-II. The lower detectable range of the samples with real-time LC RT-PCR and with the conventional RT-PCR assay was 10^{-8} to 10^{-6} dilutions and 10^{-4} to 10^{-2} dilutions, respectively. Thus, the real-time LC RT-PCR was 4-log more sensitive than the conventional RT-PCR for detecting norovirus G-II in stool samples (Fig. 2). In the 118 specimens screened with the G-II primers, a total of 14 samples from both laboratories tested positive only by the real-time LC RT-PCR and 58 tested positive by both assays (Table 2). Real-time LC RT-PCR increased the detection rate by 19% (14 of 72). In terms of the precision of the real-time LC RT-PCR assays, the mean coefficients of variation of the C_t value from two norovirus-positive samples were 0.85% (95% confidence interval = 0.251, alpha = 0.05 at 95%) and 1.92% (95% confidence interval = 0.626, alpha = 0.05 at 95%), respectively.

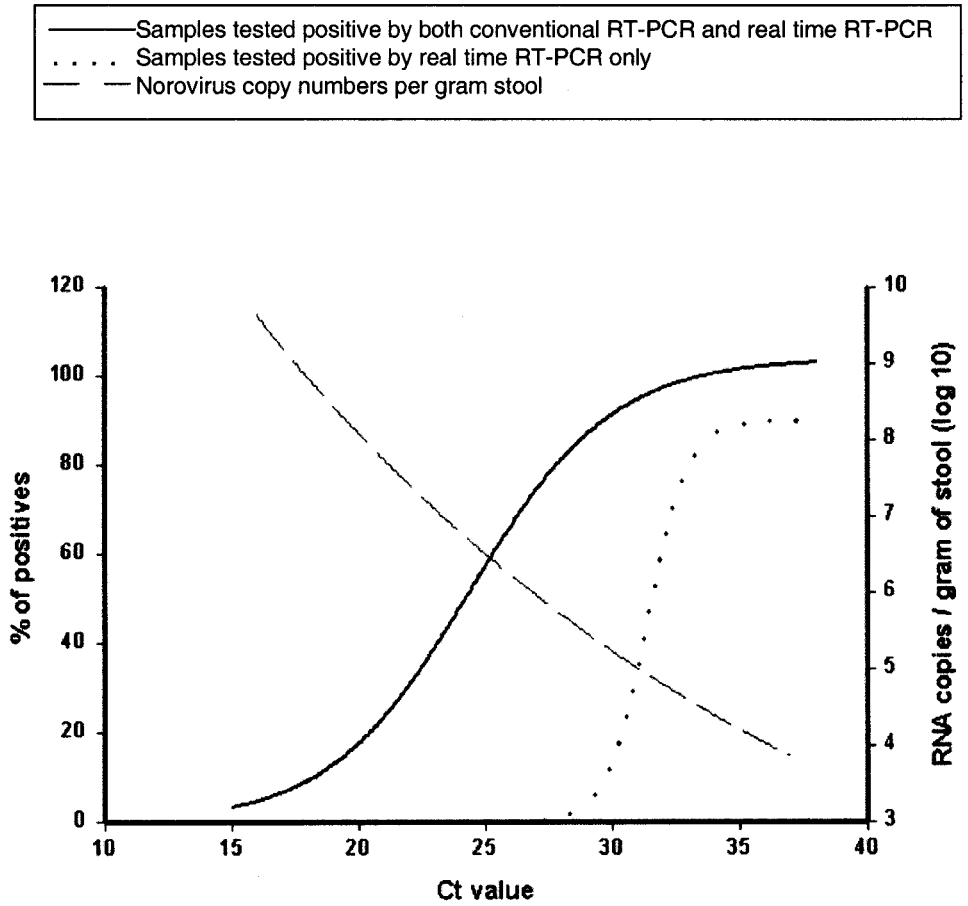


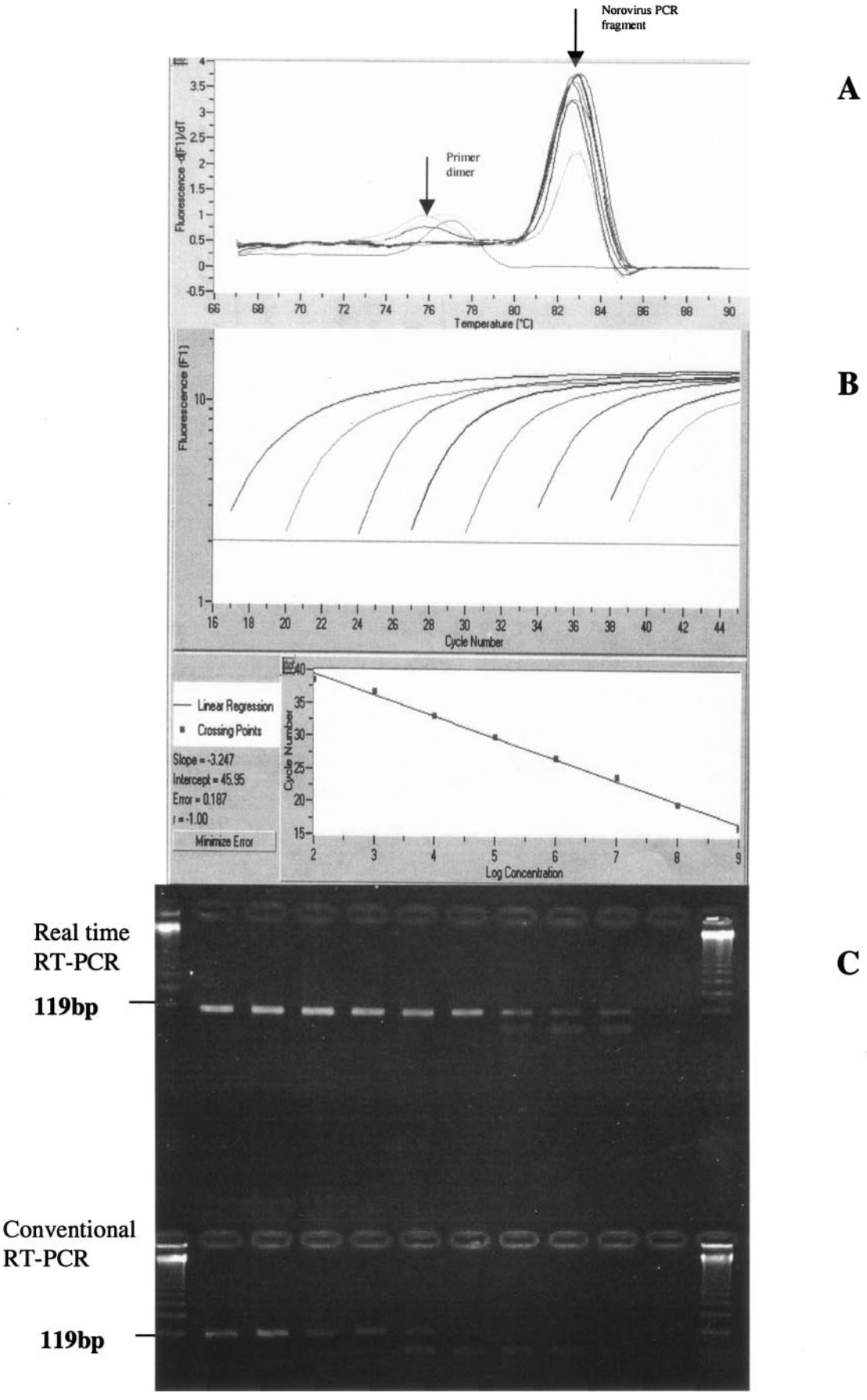
FIG. 1. Two curves fitted by plotting the percentage of specimens that tested positive for norovirus by both the real-time LC RT-PCR and conventional RT-PCR assays against the C_t (threshold cycle) of the samples and by plotting the percentage of specimens that tested positive only by the real-time RT-PCR against the C_t values (the sigmoid curves). The C_t values were also plotted against the RNA copies per gram of stool (dashed line). Specimens detectable by both RT-PCR assays contained high concentration of norovirus RNA (median, $10^{6.5}$ at the cross point), and the specimens that tested positive only by the real-time LC RT-PCR contained low copy number of the norovirus RNA (median, 10^5 at the cross point). When the norovirus RNA copy number per gram of stool is $\leq 10^5$ and the C_t value is >31, the specimen will only test positive by the real-time LC RT-PCR assay.

DISCUSSION

Real-time nucleic acid amplification systems are excellent diagnostic tools with high sensitivity and specificity and fast turnaround time. There are only two published studies of real-time RT-PCR assays for the detection of norovirus. One of the studies also used the SYBR Green method (14), and the other used a hybridization probe method in the TaqMan system (11). While Kageyama et al. presented encouraging results with their local stool specimens with the TaqMan system, our study has the unique advantage of having the real-time assay validated with various norovirus strains from both Alberta and the United States. With the two primer sets, our LC RT-PCR assay detected all but 1 of the 66 noroviruses previously identified by the two reference laboratories.

The main difficulty in developing a molecular assay for norovirus is the design and construction of the primers because of the genetic diversity of the genus. Heterogeneities existed even in the conserved regions of the viral genome. For example, only a 64% homology has been reported in highly conserved regions within a genogroup (23), and nucleotide homology could be as low as 36% in the 2C helicase to 64% in the 3D polymerase among different genogroups (2). These observa-

FIG. 2. Comparison of sensitivity of norovirus detection by real-time LC RT-PCR and conventional RT-PCR in a series of 10-fold dilutions of norovirus RNA (neat to 10^{-9}). A. Melting curve analysis of norovirus PCR fragments and primer-dimers. The melting temperature (T_m) for the norovirus PCR fragment is 82.50°C, and the T_m for the primer-dimmer is less than 78°C. B. Amplification profile of real-time LC RT-PCR. SYBR Green dye intensity was plotted against the cycle number (top), and the linear regression is shown (bottom). C. The RT-PCR products by real-time (top) and conventional (bottom) RT-PCR were separated on a 3% agarose gel stained with ethidium bromide. Lanes 2 to 11 correspond to dilutions from neat to 10^{-9}, respectively.


Norovirus PCR fragment
A
Primer dimer
Fluorescence -d(F1)/dT
Temperature (°C)
B
Fluorescence (F1)
Cycle Number
Linear Regression
Crossing Points
Slope = -3.247
Intercept = 45.95
Error = 0.187
r = -1.00
Minimize Error
Cycle Number
Log Concentration
C
Real time RT-PCR
119bp
Conventional RT-PCR
119bp

tions implied that it would be a challenge to use a single set of primer for detection. However, Kageyama et al. recently reported that the nucleotide homology between genogroups could reach from 86 to 100% in the ORF1-ORF2 junction of 71 norovirus strains (11). Based on those observations, we decided to use a single conserved and degenerate primer pair in our real-time LC RT-PCR to detect the various genotypes. Our strategy was to use the relatively less specific SYBR Green system to maximize the detection of the different noroviruses within a genogroup and use two primer sets, one for each genogroup, to maximize the sensitivity of the assay. We compensated for the relatively lower specificity of the SYBR Green system with a combined criterion of C_t values and melt curves as the detection threshold as previously suggested by Ririe et al. (18).

Several published primer sets for conventional RT-PCR were evaluated in our study. For G-I, NVp110/p69 (13, 26), SR33/SR48/SR50/SR52 (1) and CapIA/CapIB and for G-II NVp110/NI (8, 13), NVp110/p36 (13, 26), NVp110/SR46 (1, 13), SR33/SR46 (1) and CapIIA/CapIIB (25) (data not shown) were used. The primer set CapIIA/CapIIA for G-I and the combined primer set NVp110/SR46 for G-II met our criteria for sensitivity and specificity. Other primer sets either failed or demonstrated lower sensitivity for the detection of norovirus in the real-time LC RT-PCR system despite previous sensitivity in conventional RT-PCR. Recently, Vinje et al. (24) reported an international collaborative study and compared several RT-PCR assays for the detection and genotyping of noroviruses. Discordance was observed among different molecular assays and laboratories despites each laboratory's attempt to identify the best primers and optimize the methods. We have conducted further experiments with the JV12/JV13 primers (24) with the LC system and found our primers superior to JV12/JV13 (data not shown). The reason for the lack of correlated efficiency of those primers in conventional and real-time RT-PCRs remains unclear.

The limitation of the comparison between conventional and real-time LC RT-PCR is the absence of a gold standard. There were 14 samples that tested negative by conventional RT-PCR with our primers but positive by LC-RT-PCR with the same set of primers. All the LC RT-PCR products were visualized by gel electrophoresis. Two randomly selected discordant samples were also confirmed by sequencing and two other samples had been tested as positive for norovirus by nucleic acid sequence-based amplifications at a different laboratory (data not shown). Moreover, with serially diluted positive sample, we confirmed that the real-time LC RT-PCR assay was 4-log more sensitive than a conventional RT-PCR with the same primer sets. Another approach to increase the sensitivity of conventional RT-PCR is to perform nested RT-PCR, which has been reported to be 10 to 1,000 times more sensitive than single-round RT-PCR. However, a major disadvantage of nested PCR is the high risk of carryover and cross-contamination (2, 9). Since the LC RT-PCR uses a completely sealed capillary system, the risk of contamination is much lower.

The pathogenesis of norovirus remains largely unknown. The particles of infectious norovirus in patients with diarrhea can reach 10^6 to 10^8 per gram of stool during the acute phase of viral gastroenteritis (12), and norovirus is continually shed in stool specimens up to 20 days after infection (15, 19). Viral shedding has also been reported in more than 50% of norovirus-infected volunteers without any clinical symptoms (15). One of the questions is whether there is a direct relationship between viral load and the severity and transmissibility of the gastroenteritis. The ability to quantify viral loads with the real-time LC RT-PCR assay is a valuable tool to further our understanding of norovirus infection.

For norovirus G-II strains, we observed a good negative linearity ($r^2 = 1$, $P < 0.0001$) with 10-fold dilutions of RNA that was reverse transcribed from the DNA plasmid containing the ORF1 and ORF2 sequences. The good regression slope of our quantitative experiments confirmed that there was no interference of the LC RT-PCRs of the clinical specimens in the presence of the standard RNA. With an external standard curve, we accurately quantified viral RNA loads from 4.3×10^4 to 7×10^9 copies per gram of stool in 15 genotypes of G-II norovirus associated with outbreaks of acute gastroenteritis. Our lower detection limit for norovirus G-II was 25,000 copies per gram of stool. Our result was comparable to a recent study where a plasmid DNA standard was used and the lower detection limit was 20,000 copies per gram of stool (11). The advantage of using an RNA instead of DNA standard is the ability to perform identical amplification conditions for both the clinical nucleic extracts and the RNA standard.

However, our data on the quantification of norovirus G-I is limited by the small number of G-I-positive specimens. With 10-fold dilutions of RNA that was reverse transcribed from the G-I DNA plasmid containing ORF1 and ORF2, we could only detect a 3-log range of norovius G-I (from 5×10^6 to 5×10^3). Although the reaction efficiency of RT-PCR could reach almost 100% (slope, -3.32 cycles/$\log_{10}$ unit), the lower detection limit of norovirus G-I was 2.5×10^7 copies per gram of stool, which is similar to the sensitivity by electron microscopy (10^6 particles/ml of stool) (12). The C_t values of PCR in the four specimens were 27.01, 21.33, 27.41, and 22.12. The difference between sensitivity for G-I in comparison to G-II can be due to many factors, for example, primer design and PCR conditions. It is also possible that the copy number of the standard RNA was not accurate because the RNA had been stored at -70°C for more than 3 years.

We conclude that the real-time LC RT-PCR assay developed in our laboratory is sensitive and specific and provides an accurate quantification of all genotypes of norovirus G-II. This real-time LC RT-PCR assay will be useful as a routine diagnostic assay for clinical specimens and will be used to further our study of norovirus.

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REFERENCES

1. **Ando, T., S. S. Monroe, J. R Gentsch,., Q. Jin, D. C. Lewis, and R. I. Glass.** 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and southern hybridization. J. Clin. Microbiol. **33:**64–71.

2. **Atmar, R. L., and M. K. Estes.** 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clin. Microbiol. Rev. **14:**15–37.
3. **Bon, F., P. Fascia, M. Dauvergne, D. Tenenbaum, H. Planson, A. M. Petion, P. Pothier, and E. Kohli.** 1999. Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France. J. Clin. Microbiol. **37:**3055–3058.
4. **Doane, F. W.** 1994. Electron microscopy for the detection of gastroenteritis viruses, p. 101–130. In A. Z. Kapikian (ed.), Viral infections of the gastrointestinal tract. Marcel Dekker, Inc., New York, N.Y.
5. **Fankhauser, R. L., J. S. Noel, S. S. Monroe, T. Ando, and R. I. Glass.** 1998. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. J Infect. Dis. **178:**1571–1578.
6. **Fankhauser, R. L., S. S. Monroe, J. S. Noel, C. D. Humphrey, J. S. Bresee, U. D. Parashar, T. Ando, and R. I. Glass.** 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. J Infect. Dis. **186:**1–7.
7. **Glass, R. I., J. Noel, T. Ando, R. Fankhauser, G. Belliot, A. Mounts, U. D. Parashar, J. S. Bresee, and S. S. Monroe.** 2000. The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics. J. Infect. Dis. **18(Suppl. 2):**S254–S261.
8. **Green, J., C. Gallimore, J. P. Norcott, D. Lewis, and D. W. Brown.** 1995. Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. J. Med. Virol. **47:**392–398.
9. **Green, J., K. Henshilwood, C. I. Gallimore, D. W. Brown, and D. N. Lees.** 1998. A nested reverse transcriptase PCR assay for detection of small round-structured viruses in environmentally contaminated molluscan shellfish. Appl. Environ. Microbiol. **64:**858–863.
10. **Jiang, X., D. Y. Graham, K. N. Wang, and M. K. Estes.** 1990. Norwalk virus genome cloning and characterization. Science **250:**1580–1583.
11. **Kageyama, T., S. Kojima, M. Shinohara, et al.** 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J. Clin. Microbiol. **41:**1548–1557.
12. **Kapikian, A. Z., M. K. Estes, and R. M. Chanock.** 1996. Norwalk group of viruses, p. 783–810. In B. N. Fields (ed.), Fields virology. Lippincott-Raven, Philadelphia, Pa.
13. **Le Guyader, F., M. K. Estes, M. E. Hardy, F. H. Neill, J. Green, D. W. Brown, and R. L. Atmar.** 1996. Evaluation of a degenerate primer for the PCR detection of human caliciviruses. Arch. Virol. **141:**2225–2235.
14. **Miller, I., R. Gunson, and W. F. Carman.** 2002. Norwalk like virus by light cycler PCR. J. Clin. Virol. **25:**231–232.
15. **Okhuysen, P. C., X. Jiang, L. Ye, P. C. Johnson, and M. K. Estes.** 1995. Viral shedding and fecal IgA response after Norwalk virus infection. J. Infect. Dis. **171:**566–569.
16. **Pang, X. L., S. Honma, S. Nakata, and T. Vesikari.** 2000. Human caliciviruses in acute gastroenteritis of young children in the community. J Infect. Dis. **181**(Suppl. 2)**:**S288–S294.
17. **Parks, C. G., C. L. Moe, D. Rhodes, A. Lima, L. Barrett, F. Tseng, R. Baric, A. Talal, and R. Guerrant.** 1999. Genomic diversity of "Norwalk like viruses" (NLVs): pediatric infections in a Brazilian shantytown. J. Med. Virol. **58:**426–434.
18. **Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer.** 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. **245:**154–160.
19. **Rockx, B., De Wit, M., H. Vennema, J Vinje,., De Bruin, E., Van Duynhoven, Y., and M. Koopmans.** 2002. Natural history of human calicivirus infection: a prospective cohort study. Clin. Infect. Dis. **35:**246–253.
20. **Taylor, M. B., S. Parker, W. O. Grabow, and W. D. Cubitt.** 1996. An epidemiological investigation of Norwalk virus infection in South Africa. Epidemiol. Infect. **116:**203–206.
21. **van Regenmortel M.H., C. M. Fauquet, and D. H. Bishop.** 2002. Virus taxonomy; 7th report of the International Committee on Taxonomy of Viruses. http://www.ictvdb.iacr.ac.uk/Ictv/fr-fs-a.htm. (Online.)
22. **Vinje, J., S. A. Altena, and M. P. Koopmans.** 1997. The incidence and genetic variability of small round-structured viruses in outbreaks of gastroenteritis in The Netherlands. J Infect. Dis. **176:**1374–1378.
23. **Vinje, J., J. Green, D. C. Lewis, C. I. Gallimore, D. W. Brown, and M. P. Koopmans.** 2000. Genetic polymorphism across regions of the three open reading frames of "Norwalk-like viruses". Arch. Virol. **145:**223–241.
24. **Vinje, J., H. Vennema, L. Maunula, et al.** 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. J. Clin. Microbiol. **41:**1423–1433.
25. **Vinje, J., R. A. Hamidjaja, and M. D. Sobsey.** 2004. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. J. Virol. Methods **116:**109–117.
26. **Wang, J., X. Jiang, H. P. Madore, et al.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J. Virol. **68:**5982–5990.