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Broadly Reactive and Highly Sensitive Assay for Norwalk-Like Viruses Based on Real-Time Quantitative Reverse Transcription-PCR

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We have developed an assay for the detection of Norwalk-like viruses (NLVs) based on reverse transcription-PCR (RT-PCR) that is highly sensitive to a broad range of NLVs. We isolated virus from 71 NLV-positive stool specimens from 37 outbreaks of nonbacterial acute gastroenteritis and sequenced the open reading frame 1 (ORF1)-ORF2 junction region, the most conserved region of the NLV genome. The data were subjected to multiple-sequence alignment analysis and similarity plot analysis. We used the most conserved sequences that react with diverse NLVs to design primers and TaqMan probes for the respective genogroups of NLV, GI and GII, for use in a real-time quantitative RT-PCR assay. Our method detected NLV in 99% (80 of 81) of the stool specimens that were positive by electron microscopy, a better detection rate than with the two available RT-PCR methods. Furthermore, our new method also detected NLV in 20 of 28 stool specimens from the same NLV-related outbreaks that were negative for virus by electron microscopy. Our new assay is free from carryover DNA contamination and detects low copy numbers of NLV RNA. It can be used as a routine assay for diagnosis as well as for elucidation of the epidemiology of NLV infections.

The Norwalk-like viruses (NLVs), members of the family *Caliciviridae*, are major causes of acute nonbacterial gastroenteritis and a major public health concern. NLV infections often result from ingesting contaminated food (14, 30), such as oysters (4, 8) and water (3, 33), or by person-to-person transmission in semiclosed communities, such as hospitals (35), schools (20), nursing homes (18), and cruise ships (12).

The major obstacle in the laboratory diagnosis of NLV infection is the lack of a tissue culture system for propagating the viruses. Therefore, electron microscopy (EM) has been routinely used (5) to detect NLV particles in stool specimens. However, the sensitivity of EM detection is low, requiring at least 10^6 viral particles per ml of stool.

Reverse transcription-PCR (RT-PCR) has been increasingly used for detection of viruses and would be an attractive alternative for NLV detection. The key to an efficient RT-PCR assay is finding conserved sequences to use as PCR primers. The great sequence diversity of the NLV genome has made this a challenging undertaking. Primers for previous assays have been taken from open reading frame 1 (ORF1) of the RNA-dependent RNA polymerase (RdRp) gene (1, 2, 9, 13, 15, 26, 27, 31, 37) or from ORF2 of the capsid protein gene (6, 10, 11, 13, 28, 38, 39).

Recently, we reported genogroup-specific primer sequences in the capsid gene (21). With those primers, our assay had a higher detection rate and allowed further differentiation of strains when the PCR products were sequenced. However, not all EM-positive stool specimens were positive by this RT-PCR.

In this study, we sought to improve the primers. We explored sequences at the ORF1-ORF2 junction, the most conserved region in the NLV genome (19), and established an NLV detection assay for routine use with real-time quantitative RT-PCR.

MATERIALS AND METHODS

Stool specimens and examination by EM. Stool specimens were obtained from 210 symptomatic individuals from 37 outbreaks of nonbacterial acute gastroenteritis in Saitama Prefecture, Japan, between January 1997 and March 2000. The outbreaks occurred in a variety of settings, including restaurants, nursery schools, hotels, schools, dormitories, residences, and a nursing home. All stool specimens were examined by EM. NLVs were identified as round granular particles with a diameter of ~35 nm, a ragged edge, and an amorphous surface appearance, according to described criteria (5). Screening by EM revealed NLV particles in 110 stool specimens from the 210 patients.

RNA extraction from stool specimens. A 10% (wt/vol) stool suspension was prepared with distilled sterile water and clarified by centrifugation at 3,000 × *g* for 20 min. Viral RNA was extracted from 140 μl of a 10% stool suspension with a QIAamp viral RNA kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. RNAs were eluted with 60 μl of diethyl pyrocarbonate-treated water and kept at −80°C until used in RT.

PlotSimilarity analysis. We used the complete genome sequences of 14 NLVs. Five were previously described, including Nowalk/68 virus (16), Southampton virus (22), strain BS5 (32), Camberwell virus (34), and Lordsdale virus (7). Nine others resulted from our own work, including the NLV genogroup I (GI) strain SzUGI and the NLV GII strains Saitama U1, U3, U4, U16, U17, U18, U201, and U25 (19). These full-length sequences were analyzed to generate similarity plots with the PlotSimilarity program of the Wisconsin Sequence Analysis Package version 9 (Genetics Computer Group, Madison, Wis.).

Sequencing of the ORF1-ORF2 junction regions of NLVs. To amplify the NLV GI ORF1-ORF2 junction region, PCR was carried out with a mixture of three forward primers, G1FF (5′-ATHGAACGYCAAATYTTCTGGAC-3′, 5′-ATHGAAAGACAAATCTACTGGAC-3′, and 5′-ATHGARAGRCARCTNTGGTGGAC-3′, corresponding to nucleotides [nt] 5075 to 5097 in Norwalk/68), and a reverse primer, G1SKR (21). To amplify the NLV GII ORF1-ORF2 junction region, PCR amplification was also performed with a mixture of three forward primers, G2FB (5′-GGHCCMBMDTTYTACAGCAA-3′, 5′-GGHCCMBMDTTYTACAAGAA-3′, and 5′-GGHCCMBMDTTYTACARNAA-3′, correspond-

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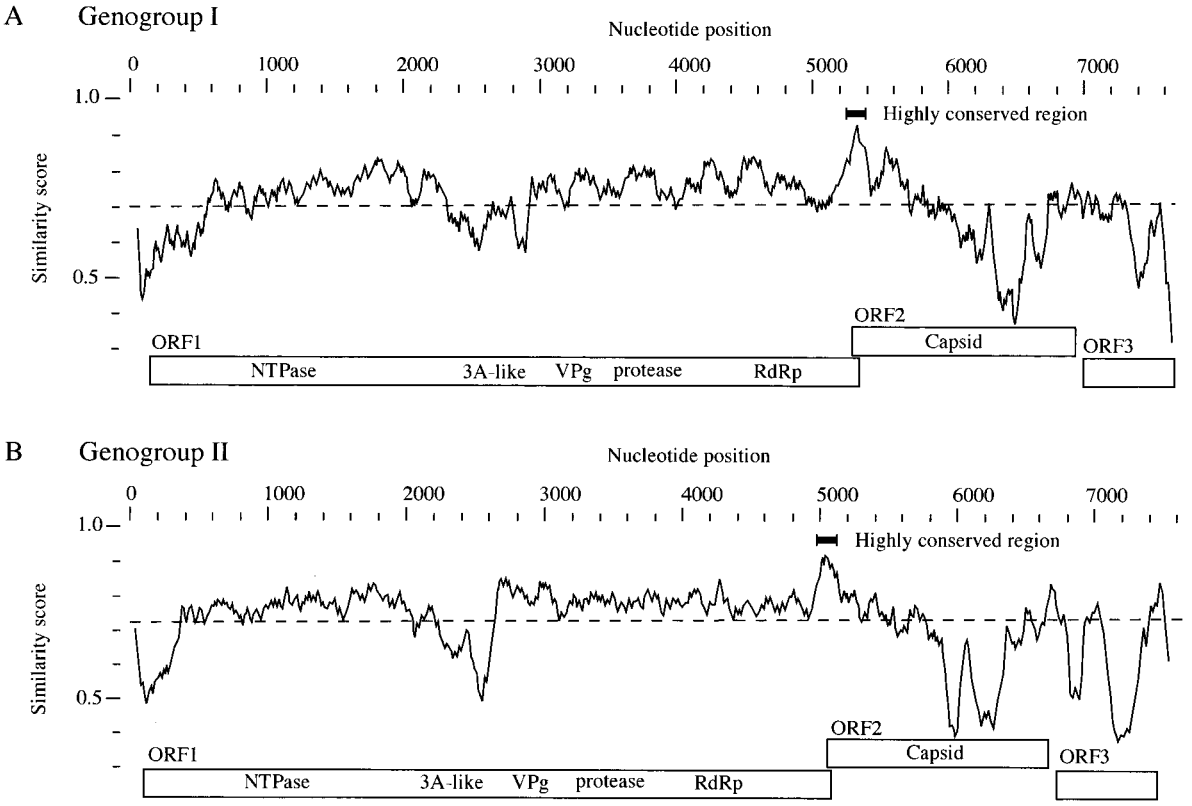


FIG. 1. Nucleotide sequences of full-length NLV genomes were analyzed with the PlotSimilarity program of Genetics Computer Group software. Similarity scores of a 150-nt sliding window were plotted. Four NLV GI strains (SzUGI, Norwalk/68, Southampton, and BS5) (A) and 10 NLV GII strains (U1, U3, U4, U16, U17, U18, U201, U25, Lordsdale, and Camberwell) (B) were compared. The locations of the ORFs are noted. Thick lines depict the most conserved regions. The average similarity score within each genogroup is represented as a dotted line.

ing to nt 4922 to 4941 in the Camberwell virus) and a reverse primer, G2SKR (21). The forward primers were designed by aligning the full-length NLV GI or GII sequences. RT was carried out as described previously (21). Ten microliters of cDNA was added to 40 μl of PCR mixture containing 5 μl of 10× Ex *Taq* buffer; 2.5 mM $MgCl_2$; 200 μM (each) dATP, dGTP, dTTP, and dCTP; 20 pmol of primers; and 2.5 U of Ex *Taq* DNA polymerase (Takara Shuzou, Kyoto, Japan). Conditions for PCR on the GeneAmp PCR system 9600 (Perkin-Elmer, Wellesley, Mass.) were as follows: initial denaturation at 95°C for 10 min; 40 amplification cycles with denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 2 min; and a final incubation at 72°C for 7 min. The 597-bp NLV GI PCR product corresponded to nt 5075 to 5671 in Norwalk/68, and the 468-bp NLV GII PCR product corresponded to nt 4922 to 5389 in Camberwell. The PCR product was cloned into TA-cloning vector pT7 Blue (Novagen, Madison, Wis.). The DNA sequence was determined from at least three clones with the BigDye terminator cycle sequence kit and ABI 377A sequencer (Applied Biosystems, Foster City, Calif.).

Conventional RT-PCR assays. A part of the RdRp gene was amplified with primers MR3 and MR4 for the first PCR (23) and with Yuri22F and Yuri22R (31) for the nested PCR. The capsid N-terminal/shell (N/S) domain was amplified with genogroup-specific primer pairs (21). RT-PCR was carried out as described previously (21). The PCR products were separated by electrophoresis in 3% agarose gels and visualized by ethidium bromide staining.

Real-time quantitative RT-PCR. To prevent carryover contamination by NLV cDNA and to reduce nonspecific amplification, viral RNA extracted with a QIAamp viral RNA kit was treated with DNase I before RT. Viral RNA (12.5 μl) was added to a reaction mixture (2.5 μl) containing DNase I buffer (150 mM Tris-HCl [pH 8.3], 225 mM KCl, 9 mM $MgCl_2$) and 1 U of RQ1 DNase (Promega Madison, Wis.). The reaction mixture was incubated at 37°C for 30 min to digest DNA and then at 75°C for 5 min to inactivate the enzyme. DNase I-treated RNA (15 μl) was added to 15 μl of another mixture containing 100 mM Tris-HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, a 1 mM concentration of each deoxynucleoside triphosphate, 10 mM dithiothreitol, 75 pmol of random hexamers (pdN6; Amersham Pharmacia Biotech, Piscataway, N.J.), 30 U of RNasin (Promega), and 200 U of SuperScript II RNase H (−) reverse transcriptase (Gibco BRL, Gaithersburg, Md.). RT was performed at 42°C for 2 h, and the enzyme was inactivated at 70°C for 15 min. cDNA solutions were stored at −20°C.

The real-time quantitative RT-PCR was carried out in 50 μl of a reaction mixture containing 5 μl of cDNA, 25 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) containing dUTP and uracyl *N*-glycosylase (UNG), a 400 nM concentration of each primer, and either 15 pmol of RING1(a)-TaqMan Probe (TP) and 5 pmol of RING1(b)-TP fluorogenic probes for NLV GI detection or 5 pmol of RING2-TP fluorogenic probe for NLV GII detection. PCR amplification was performed with an ABI Prism 7700 sequence detector (Applied Biosystems) under the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation at 95°C for 10 min, and then 45 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 56°C for 1 min. Amplification data were collected and analyzed with Sequence Detector software version 1.6 (Applied Biosystems).

In each operation, an NLV GI- or GII-specific standard curve was generated by a 10-fold serial dilution (10^7 to 10^1 copies) of purified NLV GI or GII cDNA plasmids. Plasmid standards containing PCR products of the ORF1-ORF2 junction were prepared with strains SzUG1 and U201 with primer sets G1FF-G1SKR and G2FB-G2SKR, respectively.

Genome sequences. The following partial and complete genome sequences were also used in this study: Norwalk/68, GenBank accession no. M87661; Southampton, L07418; Camberwell, AF145896; Lordsdale, X86557; BS5, AF093797; KY-89, L23828; Desert Shield, U04469; Chiba, AB042808; Melksham, X81879; Hawaii, U07611; Toronto, U02030; OTH-25, L23830; Arg320, AF190817; Bristol, X76716; Mexico, U22498; Queensarms, AJ313030; and OC98008, AF315812.

Nucleotide sequence accession numbers. The complete nucleotide sequences of the nine strains (19) were deposited in DDBJ with the following accession numbers: Saitama SzUG1 (SzUG1), AB039774; Saitama U1 (U1), AB039775;

TABLE 1. Detection of NLVs by using conventional RT-PCRs and real-time quantitative RT-PCR with 81 EM-positive stool specimens from 36 outbreaks of nonbacterial acute gastroenteritis

Outbreak			Stool code	Conventional RT-PCR		Real-time RT-PCR[b]
No.	Mo-yr	Place		For RdRp[a] (nested)	For capsid[b,c]	
1	Jan.-97	Restaurant	U1[d]	+	GII	GII
			U2[d]	+	GII	GII
2	Feb.-97	Food product	U3[d]	−	GII	GII
			U4[d]	−	GII	GII
3	Oct.-97	Nursery school	U5[d]	+	GII	GII
			U6[d]	+	GII	GII
			U27	+	GII	GII
			U28	+	GII	GII
4	Nov.-97	Nursing home	U7[d]	+	GII	GII
			U8[d]	+	GII	GII
5	Nov.-97	Food product	U9[d]	+	GII	GII
6	Dec.-97	Restaurant	U29	+	GII	GII
7	Dec.-97	Restaurant	U10[d]	−	GII	GII
			U11[d]	+	GII	GII
8	Dec.-97	Restaurant	U12[d]	+	GII	GII
			U13[d]	+	GII	GII
9	Jan.-98	Restaurant	U30	+	GII	GII
			U31	+	GII	GI + GII
10	Jan.-98	Restaurant	U32[d]	−	−	−
11	Jan.-98	Hotel	U201[d]	+	GII	GII
			U15[d]	+	GII	GII
12	Feb.-98	Dormitory	U16[d]	+	GII	GII
			U17[d]	+	GII	GII
13	May-98	School	U18[d]	+	GII	GII
			U19[d]	+	GII	GII
			U20[d]	+	GII	GII
			U21[d]	+	GII	GII
14	Dec.-98	Restaurant	U22[d]	+	GII	GII
			U23[d]	+	GII	GII
			U24[d]	+	GII	GII
15	Dec.-98	Restaurant	U25[d]	−	−	GII
			U26[d]	−	−	GII
16	Mar.-99	Hotel	KU4[d]	+	GI	GI
			KU6[d]	+	GI	GI
			KU7[d]	+	GI	GI
17	Apr.-99	Restaurant	KU8[d]	+	GI	GI
			KU9[d]	+	GII	GII
18	Apr.-99	Restaurant	KU10[d]	−	GI	GI
19	May-99	School	KU17[d]	−	GII	GII
20	May-99	School	KU24[d]	−	GI	GI
			KU25	−	GI	GI
21	Jun.-99	Hotel	KU34[d]	+	GII	GII
22	Jun.-99	Food product	KU44[d]	+	GII	GII

Continued on following page

TABLE 1—*Continued*

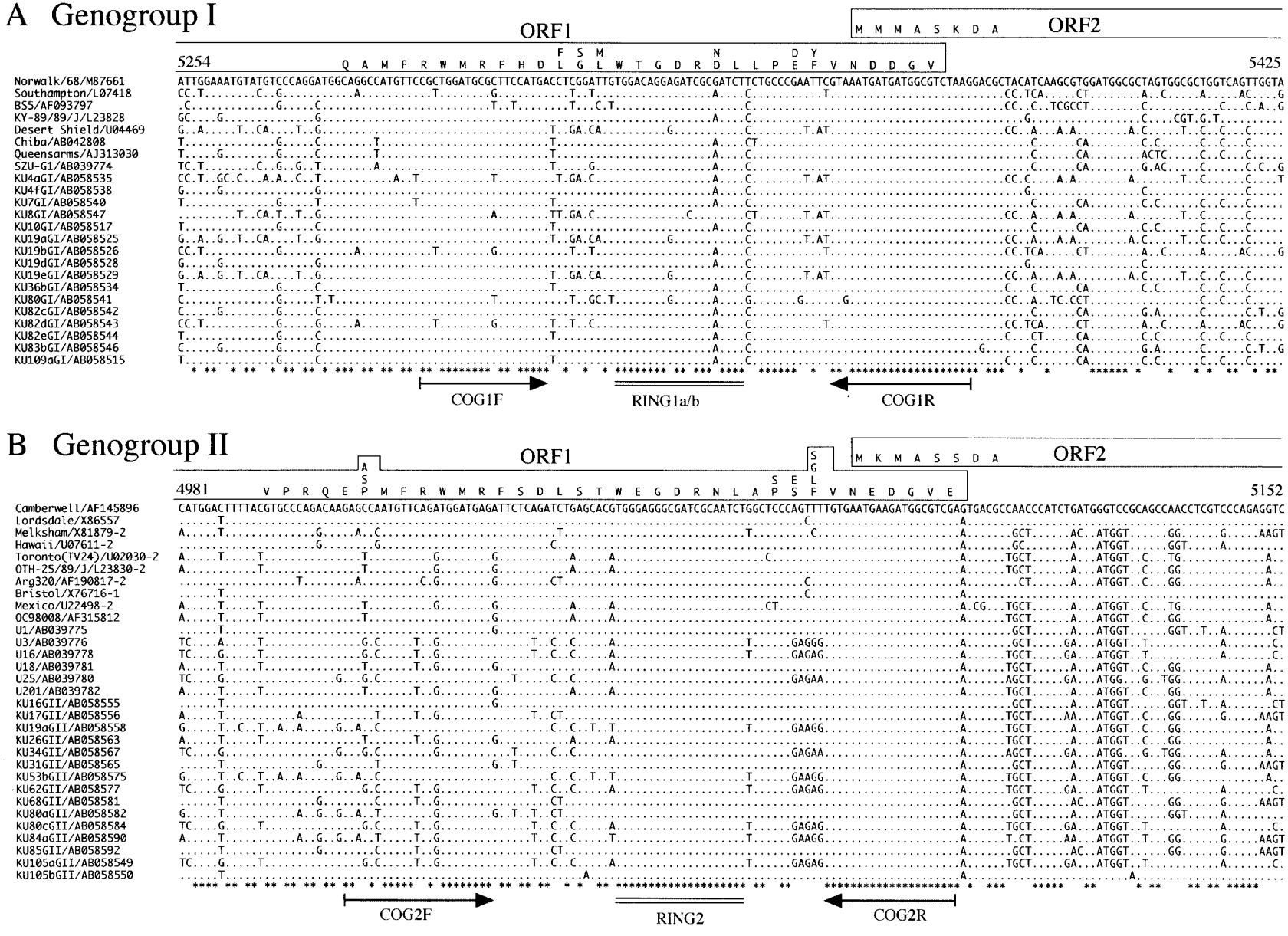
Outbreak			Stool code	Conventional RT-PCR		Real-time RT-PCR[c]
No.	Mo-yr	Place		For RdRp[a] (nested)	For capsid[b,c]	
23	Oct.-99	Restaurant	KU45	−	−	GII
24	Oct.-99	Nursery school	KU62[d]	+	−	GII
			KU63[d]	−	−	GII
			KU64[d]	−	−	GII
			KU66[d]	+	−	GII
25	Nov.-99	School	KU68[d]	+	GII	GII
26	Nov.-99	Restaurant[e]	KU80[d]	+	GI + GII	GI + GII
			KU82[d]	+	GI + GII	GI + GII
			KU83[d]	+	GI + GII	GI + GII
			KU84[d]	+	GII	GII
27	Dec.-99	School party	KU85[d]	−	GII	GII
			KU88[d]	+	GII	GII
			KU89[d]	+	−	GII
			KU90	+	GII	GII
			KU91	+	GII	GII
28	Dec.-99	School	KU93[d]	+	GII	GII
29	Dec.-99	Restaurant	KU98[d]	+	GII	GII
			KU99[d]	−		GII
			KU101[d]	+	GII	GII
30	Dec.-99	Restaurant	KU105[d]	+	GI + GII	GI + GII
			KU106	+	GI + GII	GI + GII
			KU109[d]	+	GI + GII	GI + GII
			KU111[d]	−	GI + GII	GI + GII
			KU112[d]	−	GI	GI
			KU115[d]	−	GI	GI
31	Jan.-00	Food product	KU5[d]	+	−	GII
32	Jan.-00	Family	KU16[d]	+	GII	GII
33	Jan.-00	Restaurant[e]	KU18[d]	+	GII	GII
			KU19[d]	+	GI + GII	GI + GII
			KU26[d]	+	GII	GII
			KU27[d]	−	GII	GII
34	Mar.-00	Restaurant	KU31[d]	+	−	GII
			KU32[d]	+	−	GII
35	Mar.-00	Restaurant[e]	KU35[d]	+	GII	GII
			KU36[d]	+	GI + GII	GI + GII
			KU37[d]	+	−	GII
36	Mar.-00	Hotel	KU49[d]	+	GII	GII
			KU53[d]	+	GII	GII
Total			81	62	67	80
Detection rate (%)				77	83	99

[a] +, positive; −, negative. Method described in reference 31.
[b] Method described in reference 21.
[c] GI, GI detected; GII, GII detected; −, negative.
[d] Used for sequence analysis.
[e] From shellfish-related outbreak.

Saitama U3 (U3), AB039776; Saitama U4 (U4), AB039777; Saitama U16 (U16), AB039778; Saitama U17 (U17), AB039779; Saitama U18 (U18), AB039781; Saitama U201 (U201), AB039782; and Saitama U25 (U25), AB039780. The nucleotide sequences of the ORF1-ORF2 junctions were submitted to DDBJ; the accession numbers were AB058511 to AB058529 and AB058534 to AB058598.

RESULTS

PlotSimilarity analysis. Our first goal was to identify a stable region suitable for designing new primers and probes for RT-PCR amplification. To identify the region with highest similar-


A Genogroup I
ORF1
ORF2
M M M A S K D A
5254
5425
Norwalk/68/M87661
Southampton/L07418
BS5/AF093797
KY-89/89/J/L23828
Desert Shield/U04469
Chiba/AB042808
Queensarms/AJ313030
SZU-G1/AB039774
KU4aGI/AB058535
KU4fGI/AB058538
KU7GI/AB058540
KU8GI/AB058547
KU10GI/AB058517
KU19aGI/AB058525
KU19bGI/AB058526
KU19dGI/AB058528
KU19eGI/AB058529
KU36bGI/AB058534
KU80GI/AB058541
KU82cGI/AB058542
KU82dGI/AB058543
KU82eGI/AB058544
KU83bGI/AB058546
KU109aGI/AB058515
COG1F
RING1a/b
COG1R
B Genogroup II
ORF1
ORF2
M K M A S S D A
4981
5152
Camberwell/AF145896
Lordsdale/X86557
Melksham/X81879-2
Hawaii/U07611-2
Toronto(TV24)/U02030-2
OTH-25/89/J/L23830-2
Arg320/AF190817-2
Bristol/X76716-1
Mexico/U22498-2
OC98008/AF315812
U1/AB039775
U3/AB039776
U16/AB039778
U18/AB039781
U25/AB039780
U201/AB039782
KU16GII/AB058555
KU17GII/AB058556
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KU26GII/AB058563
KU34GII/AB058567
KU31GII/AB058565
KU53bGII/AB058575
KU62GII/AB058577
KU68GII/AB058581
KU80aGII/AB058582
KU80cGII/AB058584
KU84aGII/AB058590
KU85GII/AB058592
KU105aGII/AB058549
KU105bGII/AB058550
COG2F
RING2
COG2R

FIG. 2. Alignment of nucleotide sequences and partial predicted amino acid sequences of the ORF1-ORF2 junctions of NLV GI (A) and GII (B). The nucleotide sequences of NLV GI were aligned from nt 5254 to 5425 in Norwalk/68, and those of NLV GII were aligned from nt 4981 to 5152 in Camberwell. The strain name and accession number are shown beside its sequence; a lowercase letter in the strain name indicates a different clone from the same stool specimen. Some strains with identical sequence are omitted from the figure. The conserved amino acid sequences of NLV GI ORF1 are indicated above the sequences between nt 5279 and 5371 in Norwalk/68, and those of ORF2 are indicated between nt 5358 and 5381. The conserved amino acid sequences of NLV GII ORF1 are indicated above the nucleotide sequences between nt 4988 and 5101 in Camberwell, and those of ORF2 are indicated between nt 5085 and 5108. The asterisks below the alignment show consensus nucleotide sequences. Arrows and double lines show locations of newly designed primers and probes, respectively, used in this study.

ity, PlotSimilarity was used to analyze full-length NLV GI sequences, including those of SzUGI, Norwalk/68, Southampton, and BS5 (Fig. 1A), and NLV GII sequences, including those of U1, U3, U4, U16, U17, U18, U201, U25, Lordsdale, and Camberwell (Fig. 1B). The highest nucleotide similarity score was found in the ORF1-ORF2 junction region (the C-terminal RdRp to the N-terminal capsid) (19). We selected this region, corresponding to nt 5250 to 5530 in Norwalk/68 (GI) and to nt 4980 to 5260 in Camberwell (GII), as a promising candidate for the design of real-time quantitative RT-PCR primers and probes.

Nucleotide sequences of ORF1-ORF2 junction regions from 71 strains. To confirm that it is highly conserved within each genogroup, we sequenced the ORF1-ORF2 junction regions of NLVs isolated from 71 stool specimens (Table 1). We used RT-PCR and primer sets G1FF-G1SKR and G2FB-G2SKR to amplify the NLV GI and GII ORF1-ORF2 junction regions, respectively. All sequences of the ORF1-ORF2 junction region were determined from at least three clones. The cDNA sequences for 70 samples were submitted to DDBJ (see Materials and Methods). Only one stool specimen, stool code U32, was not amplified by this RT-PCR.

Multiple alignment of the ORF1-ORF2 junction region was performed on each genogroup and with available sequences in the database (Fig. 2). Some stool specimens appeared to contain both NLV GI and GII sequences (e.g., KU80 and KU19) (Fig. 2). Four stool specimens (i.e., KU4, KU80, KU105, and KU19) contained two or more NLVs with sequence variants belonging to the same genogroup (Fig. 2). Multiple alignment of the ORF1-ORF2 junction region revealed that the most highly conserved nucleotide sequences were nt 5279 to 5381 (102 bases) with respect to Norwalk/68 virus (M87661) among NLV GI and nt 4988 to 5108 (120 bases) with respect to Camberwell virus (AF145896) among NLV GII. In this region, the nucleotide identities within the genogroups were 88 to 100% and 86 to 100%, respectively. Interestingly, 20 nt (nt 5357 to 5376 in Norwalk/68) or 21 nt (nt 5080 to 5100 in Camberwell) overlapped by ORF1 and ORF2 exhibited a perfect match within NLV GI or GII, respectively.

Development of real-time quantitative RT-PCR. We designed genogroup-specific primer sets and fluorescent probes in the NLV ORF1-ORF2 junction region for use in real-time quantitative RT-PCR. The primer pair COG1F-COG1R and a mixture of fluorescent probes, RING1(a)-TP and RING1(b)-TP, were used to detect NLV GI. The primer pair COG2F-COG2R and the fluorescent probe RING2-TP were used to detect GII NLVs. The locations of the primers and fluorescent probes are illustrated in Fig. 2, and the nucleotide sequences are shown in Table 2.

To determine the dynamic range of the real-time quantitative RT-PCR, we generated standard curves with 10-fold serial dilutions of NLV GI and GII standard plasmids (from 10^7 to 10^1 copies) (Fig. 3). The amplification curves shifted to the right as the initial amount of the plasmid was reduced (Fig. 3A and B). We constructed standard curves of the threshold cycle (Ct) of the NLV GI and GII standard plasmid against the amount of plasmid (Fig. 3C and D). The correlation between the Ct and the amount of target template was good between 10^7 and 10^1 copies. Cross-reactivity between GI and GII was not observed when the plasmid standards were used.

TABLE 2. Primer and probe oligonucleotides used for real-time quantitative RT-PCR

Genogroup	Primer or probe	Sequence (5′ → 3′)[a]	Polarity[b]	Location
GI	Primer COG1F	CGYTGGATGCGNTTYCATGA	+	5291[e]
	Primer COG1R	CTTAGACGCCATCATCATTYAC	−	5375[e]
	Probe[c] RING1(a)-TP	FAM-AGATYGCGATCYCCTGTCCA-TAMRA[d]	−	5340[e]
	RING1(b)-TP	FAM-AGATCGCGGTCTCCTGTCCA-TAMRA	−	5340[e]
GII	Primer COG2F	CARGARBCNATGTTYAGRTGGATGAG	+	5003[f]
	Primer COG2R	TCGACGCCATCTTCATTCACA	−	5100[f]
	Probe RING2-TP	FAM-TGGGAGGGCGATCGCAATCT-TAMRA	+	5048[f]

[a] Mixed bases in degenerate primers and probes are as follows: Y, C or T; R, A or G; B, not A; N, any.
[b] +, virus sense; −, anti-virus sense.
[c] Mixed probes are used for the GI NLVs.
[d] 6-Carboxyfluorescein (FAM) as the reporter dye is coupled in the 5′ end of the oligonucleotide, and 6-carboxy-tetramethylrhodamine (TAMRA) as the quencher dye is coupled in the 3′ end of the oligonucleotide.
[e] Corresponding nucleotide position of Norwalk/68 virus (accession no. M87661) of the 5′ end.
[f] Corresponding nucleotide position of Camberwell virus (accession no. AF145896) of the 5′ end.

Quantitative detection of NLV RNA. Real-time quantitative RT-PCR was used to detect NLV RNAs from 81 EM-positive stool specimens for evaluation (Table 1). NLV RNA was detected in 80 (99%) of 81 stool specimens, whereas conventional RT-PCR detected NLV in 62 of 81 stool specimens (77%) for the RdRp and in 67 of 81 (83%) for the capsid N/S region (Table 1). All three RT-PCR methods failed to detect NLV RNA from stool code U32.

Of 80 real-time quantitative RT-PCR-positive and EM-positive stool specimens, nine contained NLV GI viruses and 61 contained NLV GII viruses. Interestingly, 10 stool specimens (U31, KU80, KU82, KU83, KU105, KU106, KU109, KU111,

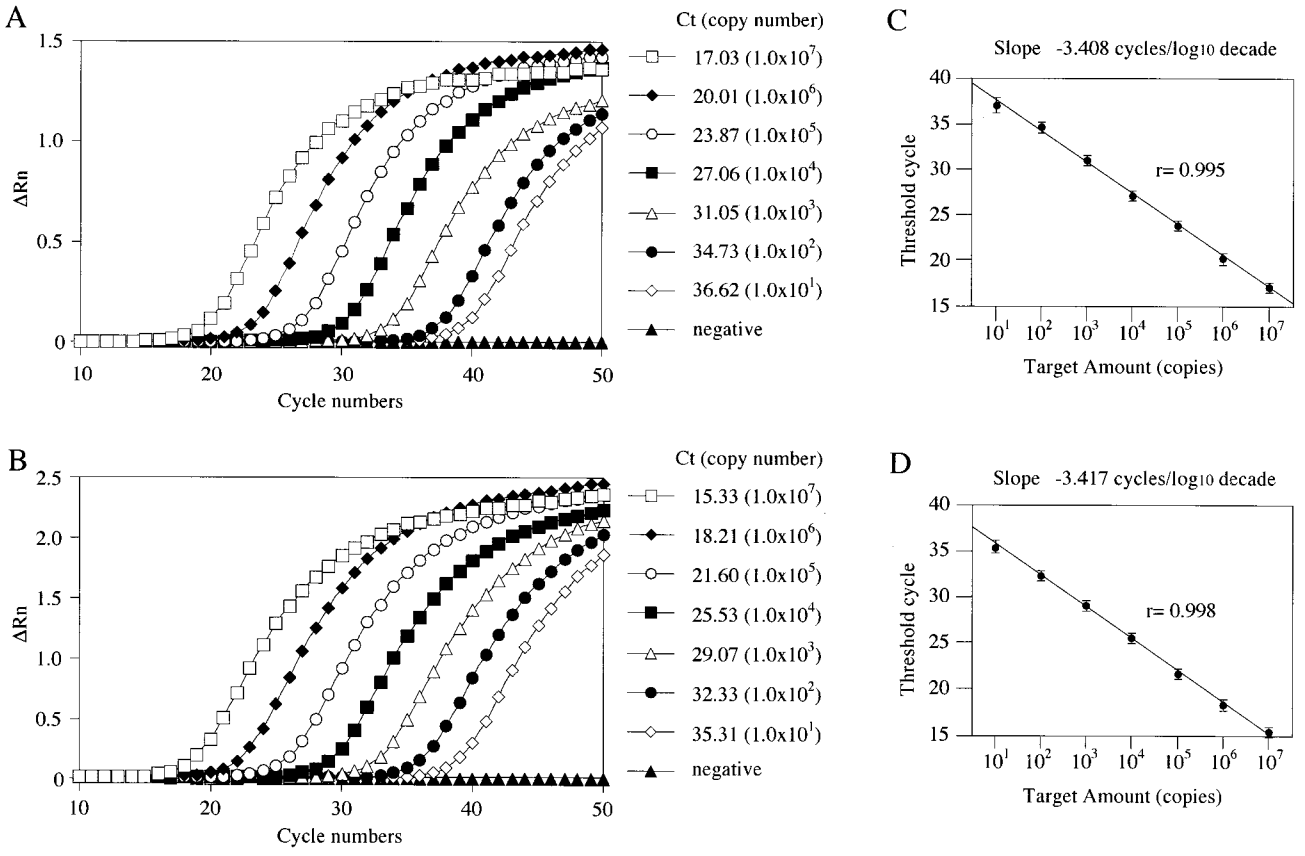

FIG. 3. Real-time RT-PCR quantification of NLV GI and GII standard plasmids. (A and B) Amplification plots of fluorescence intensities (ΔRn) versus the PCR cycle numbers are displayed for serial 10-fold dilutions of standard plasmids (10^7 to 10^1 copy equivalents per reaction) for NLV GI (A) and GII (B). Each plot corresponds to a particular input target quantity marked by a corresponding symbol. Results are the average of those from three reactions. (C and D) Relationship of known numbers of NLV GI (C) and GII (D) standard plasmids to the threshold cycle (Ct). The Ct is directly proportional to the log of the input copy equivalents, as demonstrated by the standard curve. Results are the average of those from three reactions, and error bars indicate standard deviations.

TABLE 3. Detection of NLVs by real-time quantitative RT-PCR against NLV EM-negative stool specimens

Outbreak no.	Detection by TaqMan (no. positive/no. tested)[a]	Genogroup
8	0/2	
9	2/3	GII
14	3/3	GII
16	1/1	GI
19	7/8	GII
27	2/2	GII
28	1/3	GII
33	2/3	GII
34	1/2	GII
37[b]	1/1[c]	GI + GII
Total	20/28	

[a] All stool specimens were NLV negative by EM.
[b] From an oyster-related outbreak (February 1999) not included in Table 1.
[c] Stool code SzU.

KU19, and KU36) from five EM-positive outbreaks (outbreaks 9, 26, 30, 33, and 35) contained a mixture of both NLV GI and GII (Table 1).

The detection limits of NLV GI and GII RNA had Ct values of 36.6 and 35.3, respectively, corresponding to 10 copies per reaction tube. These Ct values corresponded to more than 2.0×10^4 copies of NLV RNA per g of stool specimen. For each gram of stool specimen in EM-positive specimens, Ct values of 17.7 to 30 and 14.1 to 23.8 corresponded to 1.4×10^{10} to 3.2×10^6 and 6.3×10^{10} to 9.2×10^7 copies/g of NLV GI and GII RNAs, respectively.

We also used our method to test EM-negative stool specimens from nine EM-positive outbreaks (outbreaks no. 8, 9, 14, 16, 19, 27, 28, 33 and 34) and one oyster-related but EM-negative outbreak (outbreak 37). Of 28 samples tested, 20 were NLV RNA positive by our method (Table 3). Ct values were 31.9 (9.6×10^5 copies/g) for NLV GI (SzU) and 21.0 to 33.3 (6.1×10^8 to 1.5×10^5 copies/g) for NLV GII.

Since the real-time quantitative RT-PCR method was designed to distinguish NLV GI and GII, we confirmed the genogroup by sequencing the capsid N/S region of the PCR products from the 70 stool specimens. Using these sequence data, we constructed the phylogenetic dendrogram and found that the genogroups resulting from the phylogenetic analysis matched completely with the results from the real-time RT-PCR method (the dendrograms for the majority of the strains sequenced were shown in our previous studies [19, 21]).

In this real-time quantitative RT-PCR, we observed no cross-reactions in stool specimens containing other enteric viruses, such as rotavirus, enterovirus, adenovirus, and poliovirus, or enteropathogenic bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni*, and *Vibrio parahaemolyticus* (data not shown).

DISCUSSION

We have established a sensitive and broadly reactive NLV RNA detection system that uses real-time quantitative RT-PCR. The primers were based on the ORF1-ORF2 junction region, perhaps the most conserved region of the virus genome. Similarity plots (Fig. 1) and multiple alignment analysis of sequences in this region (Fig. 2) indicated a high degree of conservation in all isolates sequenced. Thus, it seemed an ideal site for designing primers for an RT-PCR-based amplification assay. The system does not require any other detection procedures, such as electrophoresis, Southern hybridization, nested PCR, or sequence analysis. Moreover, to prevent false-positives from carryover contamination, we treated all pre-PCR samples with UNG to destroy any dUTP-containing PCR products (24, 25, 29).

Because our method uses the TaqMan system, we could quantitatively detect NLV cDNA. Linearity was obtained by use of serially diluted standard DNA plasmids (10^7 copies to 10^1 copies) containing the ORF1-ORF2 junction sequence of each genogroup (Fig. 3). With serially diluted standard DNA plasmids, we found a detection limit of 10 copies of cDNA per reaction tube with a cutoff value of 35 to 37 cycles, indicating that our method is highly sensitive. For example, the slopes of the curves of threshold cycle (Ct) values versus serial 10-fold dilutions (range of 10^{10} to 10^1) for KU4 (GI), KU115 (GI), KU98 (GII), and U201 (GII) suspended stool specimen were -3.381, -3.411, -3.422, and -3.412 cycles/$\log_{10}$ unit, respectively. These slopes correlated well with the slopes of the curves of Ct versus serially diluted NLV GI (-3.4075 cycles/$\log_{10}$ unit) and GII (-3.4166 cycles/$\log_{10}$ unit) standard DNA plasmid ($P < 0.01$). We estimated the number of copies of NLV RNA in the original sample. Ct values of 17.0 (GI) and 15.3 (GII) are equivalent to 1×10^7 copies of standard plasmid in the reaction tube and correspond to about 2.0×10^{10} copies of NLV RNA per g of stool specimen. Ct values for stool codes U2, KU44, KU109, and KU111 of 20.1, 14.1, 16.7, and 23.8 corresponded to about 1.1×10^9, 6.3×10^{10}, 1.1×10^{10}, and 9.2×10^7copies of NLV RNA per g of stool specimen, respectively.

The numbers of copies of NLV RNA in positive stool specimens determined by the real-time quantitative RT-PCR were considerably higher than previous estimates of 10^5 to 10^6 copies by EM (17). The higher titers are probably due to the ability of RT-PCR to detect NLV RNAs from additional sources (e.g., from complete and incomplete virus particles and from amplifiable RNA inside the infected intestinal epithelial cells). Our findings are also consistent with those of others who found higher numbers of NLV RNA by PCR methods (2, 13) than by EM.

Given the greater sensitivity of our assay, we wanted to examine more carefully EM-negative samples from gastroenteritis subjects within the same NLV-positive outbreaks. Of 28 EM-negative specimens, 20 were positive for NLV RNA (Table 3). In our assay, EM-negative, RT-PCR-positive stool specimens contained 3 to 30% as much NLV RNA as the EM-positive stool specimens.

The one outlier in this analysis was stool code U32 (outbreak 10), which was positive by EM (i.e., showed an image resembling that of an NLV particle) but negative by all RT-PCR methods. We speculate that the reason might be the presence of an unidentified small round virus that is morphologically similar to NLVs by EM. Conventional RT-PCR methods did not detect other RNA viruses, such as hepatitis A virus, hepatitis E virus, poliovirus, Sapporo-like viruses, and Astro virus (data not shown). Further investigation is needed to explain the results for this sample.

Interestingly, 10 stool specimens from five EM-positive outbreaks (outbreaks 9, 26, 30, 33, and 35) (Table 1), and one stool specimen from outbreak 37 that was negative by EM but positive in our assay (Table 3), showed NLV GI and GII strains. All of these outbreaks occurred in restaurants, and we suspect that they have resulted from ingesting contaminated foods. Four outbreaks (outbreaks 26, 33, 35 and 37) were shellfish related. Similar results of coinfection have been reported previously (36). Oysters, cockles, and mussels have been implicated in food-borne outbreaks. Shellfish are filter-feeding organisms and can concentrate NLV particles from a contaminated habitat. Therefore, coinfection by multiple strains is possible in shellfish-related NLV outbreaks.

The high sensitivity of our method allowed us to detect NLVs that were undetectable by EM or other PCR methods. Our method may have applications in the detection of NLV pathogens in foods, such as oysters, as well as in the monitoring of the levels and seasonal differences of NLV contamination in sewage, river water, and seawater. Furthermore, our primer and probe sequences may be used in other detection methods such as the Light Cycler PCR technique. We believe that this method will be useful for routine diagnosis as well as for clarifying the epidemiology of NLV infections and thus for the public health control of this disease in the future.

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