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Application of PCR To Detect Norwalk Virus in Fecal Specimens from Outbreaks of Gastroenteritis

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Norwalk virus (NV) and other small round-structured viruses (SRSVs) are frequent causes of gastroenteritis outbreaks. The recent cloning and sequencing of the NV genome has made it possible to detect NV and Norwalk-related viruses from fecal specimens by reverse transcription (RT)-PCR. We applied this technique to the examination of a total of 139 fecal specimens from 19 outbreaks characterized by NV serology, including 56 samples from 7 NV outbreaks, 36 from 6 Norwalk-related virus outbreaks, and 47 from 6 outbreaks with SRSVs visualized by electron microscopy that were serologically unrelated to NV. Three primer pairs were evaluated: two pairs in the polymerase region of NV and one pair near the 3′ end of the genome. When one set of primers (primer pair 51-3) from the polymerase region was used, 40% of all samples were positive by RT-PCR and specimens from the NV outbreaks were more likely to be positive (64%) than those from outbreaks associated with Norwalk-related viruses (44%) or SRSVs (8%). To determine the relationship of the outbreak strains to NV, we compared the sequences of a 145-base portion of the polymerase gene from 10 specimens obtained from five different outbreaks characterized as NV by serology. No two outbreak strains had the same sequence in this 145-base portion of the polymerase gene, and the identities of the nucleotide and amino acid sequences of these products compared with the sequences of the corresponding region of NV ranged from 62 to 79% and 69 to 90%, respectively. Because of sequence diversity in the polymerase region, the successful application of RT-PCR to investigations of outbreaks of suspected NV-associated gastroenteritis will depend on the use of either multiple primer pairs or primers made against regions of the genome that are more conserved.

Norwalk virus, a 27-nm, small round-structured virus (SRSV), was discovered in fecal specimens from patients involved in an outbreak of gastroenteritis that occurred in Norwalk, Ohio, in 1968 (1, 16). As the first SRSV recognized, Norwalk virus is the prototype of a group of SRSVs that includes two additional serotypes in the United States (the Hawaii and Snow Mountain agents), four serotypes in the United Kingdom (UK1 to UK4) (18, 19), and nine serotypes in Japan (24). SRSVs are recognized as important etiologic agents of acute, nonbacterial gastroenteritis and have been implicated in numerous foodborne and waterborne outbreaks. However, investigations of SRSV outbreaks and the characterization of this group of viruses have been limited by the lack of adequate laboratory techniques that detect these agents. Norwalk virus has not been grown in cell culture, no suitable animal models are available, and diagnosis has relied on the observation of 27- to 33-nm structured viruses by electron microscopy (EM) or the detection of seroconversion in paired serum specimens by an enzyme immunoassay (EIA) with reagents from infected volunteers. The use of radioimmunoassays, antigen immunoassays, and Western immunoblotting has been limited because of their relatively low sensitivities and dependence on reagents from human subjects. Detection of Norwalk virus antigen in fecal specimens is further complicated by the short duration of fecal shedding and the low concentration of virus in stool samples (16).

Recent cloning and sequencing of the Norwalk virus genome (12, 21) have led to its classification in the family *Caliciviridae* (14, 15) and to the development of a reverse transcription (RT)-PCR method that detects Norwalk virus in fecal specimens (4, 13), a new EIA for serum antibodies that uses recombinant Norwalk virus capsid protein, and a new EIA for antigen that uses hyperimmune animal serum made against the recombinant protein (10, 14, 22). The molecular characterization of SRSVs has been further aided by recent sequence information about two additional strains, the Southampton agent isolated from a family outbreak in 1991 (17) and a Japanese virus (SA-1283) isolated from a child with diarrhea in 1984 (24, 27).

We applied RT-PCR to the detection of Norwalk virus and related SRSVs in fecal specimens from patients involved in outbreaks of acute gastroenteritis previously shown to be associated with this group of viruses by serology and EM. Three primer pairs were evaluated: two from the RNA-dependent RNA polymerase gene, a region we hypothesized might be conserved among both Norwalk and Norwalk-related viruses, and one from the third open reading frame near the 3′ end, a region which may be serotype-specific. Because of the low numbers of PCR-positive samples detected in some outbreaks, we sequenced polymerase gene PCR products from

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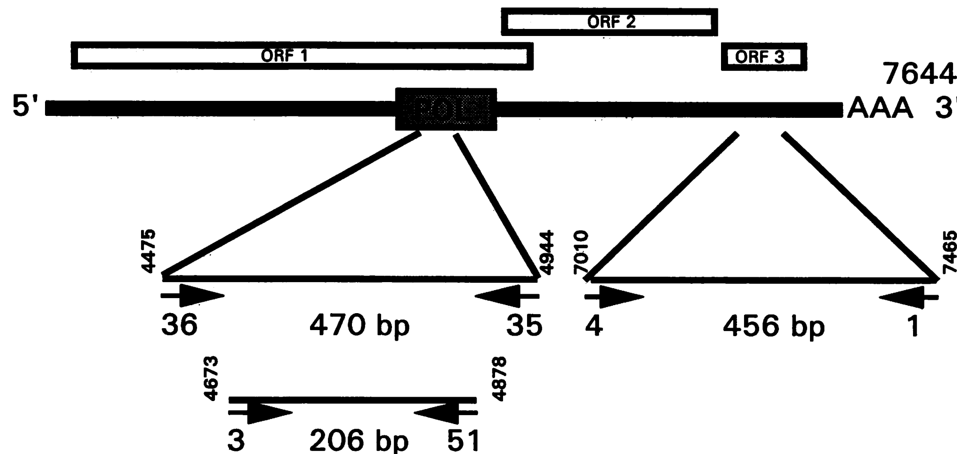

FIG. 1. Norwalk virus genome and locations of primer sets. The positions of the three primer pairs and the sizes of the resulting PCR products are indicated (in base pairs). Primer pairs 35-36 and 51-3 are located in the RNA-dependent RNA polymerase gene in the first open reading frame (ORF1). Primer pair 1-4 is located near the 3′ end in the third open reading frame (ORF3).

viruses involved in several outbreaks which were serologically classified as being caused by Norwalk virus to determine whether this low frequency of detection could be attributed in part to a genetic divergence of recent Norwalk virus strains from the prototype Norwalk virus isolated in 1968 from which our primers were designed.

MATERIALS AND METHODS

Fecal specimens. Fecal specimens from patients with acute gastroenteritis were collected during outbreak investigations conducted in Australia (Oysters 1978 [23], Oysters 1990, Airline 1991 [25], Daycare 1988 [11], Daycare 1991) and the United States between 1978 and 1992 and were stored at 4 or −20°C. Sera from these outbreaks were routinely screened either by a biotin-avidin EIA (7) or by a recombinant Norwalk virus capsid protein EIA (14, 22) which became available in 1991, and fecal specimens were examined for SRSVs by EM. The results of serologic tests of six or more paired serum specimens were used to classify outbreaks as associated with Norwalk virus when 50% or more of the paired serum specimens demonstrated a fourfold or greater rise in titer and as associated with Norwalk-related viruses when 10 to 50% of the paired serum specimens showed seroconversion (8). Outbreaks were classified as associated with SRSVs when fewer than 10% of serum specimens showed seroconversions but the fecal samples contained SRSVs visible by EM. Two outbreaks from which no sera were collected were also classified as associated with SRSVs because SRSVs were visible in the fecal samples. Fecal specimens from patients involved in representative outbreaks from each of the three categories described above were examined under code. When possible, EM results were used to identify fecal specimens with visible SRSVs that we hoped would be good candidates for RT-PCR testing. In addition, we tested 27 fecal specimens from two child-care center outbreaks that were attributed to human calicivirus by EM and solid-phase immune EM (11). Prior to the classification of Norwalk virus in the *Caliciviridae* family, human caliciviruses were believed to be serologically distinct from the Norwalk group of viruses (3, 20) and associated with gastroenteritis in children.

RT-PCR. For RT-PCR, 20 to 50% stool suspensions were prepared and extracted by the polyethylene glycol-cetyltrimethylammonium bromide (CTAB) method developed by Jiang et al. (13). We evaluated two primer pairs in the polymerase region (primer 35, 5′-CTTGTTGGTTTGAGGCCATAT-3′; primer 36, 5′-ATAAAAGTTGGCATGAACA-3′ [27]; primer 51, 5′-GTTGACACAATCTCATCATC-3′; and primer 3, 5′-GCACCATCTGAGATGGATGT-3′) and one pair near the 3′ end of the genome (primer pair 1-4); primer pair 1-4 has been described previously (13) (Fig. 1).

The RT-PCR method of Jiang et al. (13) was modified by the use of avian myeloblastosis virus Super Reverse Transcriptase (Molecular Genetic Resources, Tampa, Fla.), native *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 0.45 μM primers, and a 42°C primer annealing temperature. All specimens were tested by single-round RT-PCR at least twice. PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light. Positive samples were those with a visible DNA product that comigrated with the PCR product from the stool of a Norwalk virus-infected volunteer (6). Specimens giving weakly positive or ambiguous results were retested with the same extract or with a new extract of the sample. Stool specimens from Norwalk virus-infected volunteers were included as positive controls in each extraction and RT-PCR, and water was included as a negative control.

Sequencing of PCR products. PCR products from primer pair 51-3 were purified for sequencing by extracting with chloroform, precipitating with ethanol, and drying in a Speed Vac Concentrator (Savant Instruments, Hicksville, N.Y.). The DNA was suspended in 100 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), purified by centrifugation through Sephacryl S-400 spun columns (Pharmacia LKB Biotechnology, Piscataway, N.J.), precipitated in ethanol, and suspended in 20 μl of water. The concentration of DNA was estimated by UV absorption at 260 nm, and approximately 100 ng of template DNA was used in each sequencing reaction. Oligonucleotide primers 51-3 were used to determine the sequence in both directions. Template-primer mixtures were heated to 100°C for 3 min, snap-cooled in dry ice-ethanol, and transferred to an ice bath. Sequencing by the Sanger dideoxy nucleotide chain termination method (26) was done with $[^{35}S]$deoxyadenosine 5′-[α-thio]triphosphate (NEN Research Products, Boston, Mass.) and the Sequenase kit (Version 2.0, United States Biochemical Corporation, Cleveland, Ohio)

TABLE 1. Outbreaks of gastroenteritis screened for Norwalk virus by RT-PCR

Classification of outbreak by serology and EM results	Outbreak			Original results (no. of samples positive/ total no. tested)		RT-PCR results		
	Identification no.	Yr	Description	NW serology	EM	No. of fecal samples tested	No. or % of samples positive with primer pair: 35-36	51-3
Norwalk virus	1	1985	Camp	12/12	ND[a]	5	0	1
	2[b]	1992	Cruise ship[c]	10/12[d]	7/19	19	0	16
	3[b]	1978	Oysters[e]	15/20	28/72	5	0	4
	4[b]	1986	Cruise ship	7/10	ND	6	1	5
	5	1989	Restaurant	6/8	1/3	5	0	3
	6[b]	1986	Nursing home	6/9	ND	6	0	3
	7[b]	1990	Cruise ship[f]	7/12[d]	4/23	10	ND	4 (1)[g]
	Subtotal					56	1	36 (64.3)%
Norwalk-related virus	8	1991	Nursing home	2/4[d]	7/9	9	0	5
	9	1988	Camp	4/13	1/2	5	ND	2
	10	1989	Restaurant	2/7	4/7	5	0	3
	11	1991	Airline[h]	2/7[d]	6/38	7	0	1
	12	1991	Tennis camp	2/5[d]	3/6	5	ND	3
	13	1991	Nursing home	1/6[d]	5/15	5	4	2
	Subtotal					36	4	16 (44.4)%
SRSVs	14	1991	Nursing home	1/13[d]	5/12	5	0	1[g]
	15	1989	Cruise ship	0/8	5/21	5	1[g]	3 (1[i])
	16	1990	Oysters	0/7	7/30	5	0	0
	17[j]	1988	Child care[k]	0/7	24/75	3	0	0
	18[j]	1991	Child care		16/24	24	0	0
	19	1991	High school		1/8	5	0	0
	Subtotal					47	1	4 (8.5)%
Total						139	6/125 (4.8)%	56/139 (40.3)%

[a] ND, not done.
[b] PCR products from these outbreaks were sequenced.
[c] Khan et al. (16a).
[d] Serology by recombinant Norwalk EIA (14).
[e] Murphy et al. (23).
[f] Herwaldt et al. (11a).
[g] PCR band was faint.
[h] Rouch et al. (25).
[i] Size of PCR product was slightly larger than predicted.
[j] Diagnosed as human calicivirus by EM.
[k] Grohmann et al. (11).

according to the manufacturer's recommendations. The labelling mixture was diluted 1:10, and the labelling reaction time was less than 1 min at room temperature and was followed by a 5-min extension time at 37°C. Sequencing reactions were run on an 8% polyacrylamide–6 M urea gel in TBE buffer (89 mM Tris [pH 8.3], 89 mM boric acid, 2 mM EDTA). The gel was vacuum dried and exposed to X-ray film for 12 h to 10 days at room temperature. Nucleotide and deduced amino acid sequences were analyzed with the Genetics Computer Group software package (5).

RESULTS

A total of 139 fecal specimens, including 56 specimens from seven outbreaks associated with Norwalk virus, 36 specimens from six outbreaks associated with Norwalk-related viruses, and 47 specimens from six outbreaks associated with SRSVs, were examined by single-round RT-PCR (Table 1). Using the primer pair 51-3, we found that 40% of all samples were positive by RT-PCR and that specimens from the outbreaks associated with Norwalk virus by serology were more likely to be positive (64%) than those from outbreaks associated with Norwalk-related virus (44%) ($P = 0.06$) and SRSVs (8%) ($P < 0.0001$). Few of the fecal specimens produced positive PCR products with the other two primer pairs. Only 6 (4.8%) of 125 specimens tested with primer pair 35-36 were positive, and 4 (5%) of 80 specimens tested with primer pair 1-4 were positive (data not shown). Three specimens were positive with both primer pairs 35-36 and 51-3, and one specimen was positive with primer pairs 1-4 and 51-3.

Positive PCR products were detected in fecal samples from 15 of the 19 outbreaks examined with primer pair 51-3. Among the fecal samples tested, there were one or more PCR-positive specimens from each of the outbreaks associated with Norwalk virus or Norwalk-related viruses. Fecal samples from two of the six SRSV outbreaks were PCR positive with primer pair 51-3, and a third SRSV outbreak (outbreak 19) had a PCR-positive specimen with primer pair 1-4. There was no correlation between the RT-PCR results and EM observation of a visible SRSV particle for 57 specimens from nine outbreaks for which we had corresponding data.

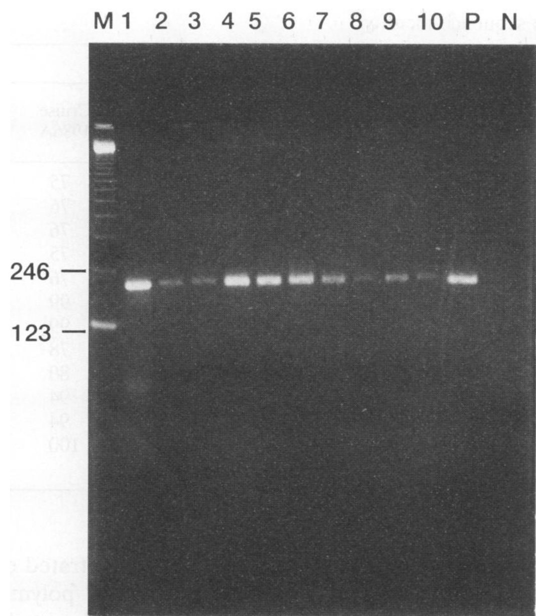

FIG. 2. UV-illuminated agarose gel showing the 206-bp PCR product from 10 fecal specimens from five outbreaks of gastroenteritis (lanes 1, 2, and 3, Cruise 1992; lanes 4 and 5, Cruise 1990; lanes 6 and 7, Cruise 1986; lanes 8 and 9, Nursing Home 1986; lane 10, Oysters 1978), a positive control (lane P; fecal specimen from a Norwalk virus-infected volunteer), and a negative control (lane N; water). The molecular mass marker (lane M) was a 123-bp ladder (GIBCO-BRL, Gaithersburg, Md.). A 3% agarose gel with 0.5 μg of ethidium bromide per ml was run at 140 V for 90 min.

To determine whether the low frequency of Norwalk virus detection by RT-PCR might be explained in part by the sequence diversity of the outbreak strains, we sequenced PCR products made with primer pair 51-3 from 10 specimens obtained from five Norwalk virus outbreaks (Fig. 2) and compared the sequence of a 145-base region of the polymerase gene with the sequences from the Norwalk virus (12) and two other SRSVs, the Southampton virus (17) and the Japanese virus (27) (Fig. 3). The sequence of each outbreak strain was unique and demonstrated some genetic divergence from the reference strains. When we compared the sequences of the outbreak strains with that of the prototype 8FIIA Norwalk strain and with each other, the sequence of the Japanese virus was most similar to that of the prototype Norwalk virus and had 20 base substitutions. The strains detected in Norwalk virus outbreaks had 26 to 51 base substitutions, and the Southampton virus had 37 base substitutions when its sequence was compared with that of the prototype Norwalk strain. Similar nucleotide substitution patterns were observed among isolates from several different outbreaks.

Among the 10 strains from outbreaks associated with Norwalk virus, nucleotide identity to the Norwalk virus sequence ranged from 62 to 79% (Table 2) and was similar to the identity seen between the Norwalk virus and Southampton virus sequences in this region of the polymerase gene (74%). For four outbreaks, we sequenced the virus isolates from several fecal samples and found that in three of these outbreaks (Cruise 1992, Nursing Home 1986, and Cruise 1990), the different specimens had virus isolates with identical sequences. However, two separate strains (A and B) from a fourth outbreak (Cruise 1986) shared only 66% sequence identity, indicating either that more than one strain may have been involved in this outbreak or that one of the patients may

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                    1                                                                         70
Norwalk             AGGTGATTAT GTCATCAGGG TCAAAGAGGG GCTGCCATCT GGATTCCCAT GTACTTCCCA GGTGAACAGC
Japan               g-----c--- --t--a---- -------a-- c--a-----a -----t--c- -c-----t-- a--a--t---
Cruise 1992 A       t--c------ --g-----t- -t-----t-- t--------- --c--t---- -c-----a-- ------t---
Cruise 1992 B       *****----- --g-----t- -t-----t-- t--------- --c--t---- -c-----a-- ------t---
Cruise 1992 C       t--c------ --g-----t- -t-----t-- t--------- --c--t---- -c-----a-- ------t---
Nursing Home 1986 A t--c--c--c a-----c-t- -------a-- ct-------a --------t- -c--g--a-- ---a-----t
Nursing Home 1986 B t--c--c--c a-----c-t- -------a-- ct-------a --------t- -c--g--a-- ---a-----t
Cruise 1986 A       t--c--c--c a-----c-t- -------a-- ct-------a --------t- -c--g--a-- ---a-----t
Oyster 1978         t--a--c--- -----a--t- -------at- ct-------a -at--t---- -c--a--a-- ---t------
Cruise 1990 A       t-----c--c a----tc-t- -a-----a-- ct-a-----a -----t--t- -c--g--a-- ---a--t--t
Cruise 1990 B       t-----c--c a----tc-t- -a-----a-- ct-a-----a -----t--t- -c--g--a-- ---a--t--t
Cruise 1986 B       g-----c-tc aag--atcaa ----c----- t--c------ --gg-g--t- -c--c--g-- atg----tc-
Southampton         t--c--c--c -----a--a- -g--g--a-- c--c------ --t------- -c--a--a-- a--t--t--t

                    71                                                                              145
Norwalk             ATAAATCACT GGATAATTAC TCTCTGTGCA CTGTCTGAGG CCACTGGTTT ATCACCTGAT GTGGTGCAAT CCATG
Japan               ---------- -------c-- c--t------ ---------- -t-------- ---------- --------g- -----
Cruise 1992 A       --c--c---- ----cc-a-- ---a------ t----a--a- t--------- ---c------ ---a-a---- --c--
Cruise 1992 B       --c--c---- ----cc-a-- ---a------ t----a--a- t--------- ---c------ ---a-a---- -****
Cruise 1992 C       --c--c---- ----cc-a-- ---a------ t----a--a- t--------- ---c------ ---a-a---- --c--
Nursing Home 1986 A -----c--t- --c----c-- ct-g-----c a-------a- t---a---c- ------c--- --ca-a---- --c**
Nursing Home 1986 B -----c--t- --c----c-- ct-g-----c a-------a- t---a---c- ------c--- --ca-a---- --c**
Cruise 1986 A       -----c--t- --c----c-- ct-g-----c a-------a- t---a---c- ---------- --ta-a---- --c**
Oyster 1978         --c--c--t- ----cc-c-- c--------- --a--a--a- t--------- ---c------ --ta-a--g- --c--
Cruise 1990 A       -----c--t- --c----c-- ct-g-----c a-------a- t---a---c- ------c--- --ca-a---- --c--
Cruise 1990 B       -----c--t- --c----c-- ct-g-----c a-------a- t---a---c- ------c--- --ca-a---- --c--
Cruise 1986 B       --cgcc---- --c-cc-c-- c--t------ --t-----a- t---gaa-c- ---c-----c a-ca-a---g --***
Southampton         -----c--t- --t----a-- ---g--c--c --t-----a- tg------c- ---g--a--c --ta-c---- -----
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FIG. 3. Nucleotide sequences of Norwalk virus, Southampton virus, Japan virus, and PCR products from 10 fecal specimens from 5 Norwalk virus outbreaks. The sequence alignment was prepared by sequentially using the University of Wisconsin Genetics Computer Group sequence analysis software programs (5) Lineup, Pileup, and Pretty. Only nucleotides which were different from those of the Norwalk virus reference strain (8FIIA) are shown. Hyphens denote identical nucleotides; asterisks denote areas near the primers which could not be determined. A, B, and C denote different fecal specimens from the same outbreak.

TABLE 2. Comparison of nucleotide sequence identity

Virus	% Identity												
	Norwalk virus	Japan	Cruise 1992A	Cruise 1992B	Cruise 1992C	Nursing home 1986A	Nursing home 1986B	Southampton	Oysters 1978	Cruise 1990A	Cruise 1990B	Cruise 1986A	Cruise 1986B
Norwalk virus	100	86	79	79	79	75	75	74	73	72	72	75	62
Japan		100	77	77	77	76	76	74	76	77	77	76	64
Cruise 1992A			100	100	100	76	76	75	84	74	74	76	68
Cruise 1992B				100	100	76	76	75	84	74	74	75	68
Cruise 1992C					100	76	76	75	84	74	74	76	68
Nursing home 1986A						100	100	79	80	96	96	99	67
Nursing home 1986B							100	79	80	96	96	99	67
Southampton								100	75	77	77	78	65
Oysters 1978									100	77	77	80	66
Cruise 1990A										100	100	94	65
Cruise 1990B											100	94	65
Cruise 1986A												100	66
Cruise 1986B													100

have had a sporadic case of illness caused by another strain. Partial sequence data (95 bases) compiled independently in the Houston laboratory for a third specimen from the Cruise 1986 outbreak was identical to the sequence of the Cruise 1986A strain. Of note, the sequences of the Cruise 1986A, Cruise 1990 A and B, and Nursing Home 1986 A and B strains were more closely related to each other (94 to 99%) than they were to the sequences of any of the reference strains.

Since 75% of all the base substitutions occurred at the third positions of codons, the 49 amino acids predicted from the nucleotide sequences were less diverse than the nucleotide sequences, and amino acid sequence identity to Norwalk virus ranged from 100% for the Japanese strain to 69% for the Cruise 1986B strain (Fig. 4). The Southampton virus had a 94% identity to Norwalk virus. Three outbreak strains (Cruise 1986A, Nursing Home 1986, and Cruise 1990) had amino acid sequences that were identical to each other and had a 90% sequence identity to Norwalk virus.

DISCUSSION

Using RT-PCR, we were able to detect Norwalk and Norwalk-related viruses in 40% of all fecal specimens tested and from 15 (79%) of the 19 gastroenteritis outbreaks screened. The observation that most of the PCR-positive specimens came from outbreaks associated with Norwalk virus and fewer came from outbreaks associated with Norwalk-related viruses and SRSVs suggests a correlation between the results of Norwalk virus serology and the results of RT-PCR with specific Norwalk virus primers. At the same time, sequence analysis of the PCR products from 10 specimens obtained from five outbreaks associated with Norwalk virus demonstrated diversity in the region of the RNA-dependent RNA polymerase gene between the Norwalk viruses that could be detected with our primers and the sequence of the reference strain. The nucleotide sequences of viruses from several chronologically and geographically separate outbreaks were closely related to each other and were uniformly dissimilar to those of Norwalk virus and Southampton virus, the latter of which is reported to be of the same serotype as the Snow Mountain agent (17).

The efficiency with which we detected Norwalk and Norwalk-related viruses was dependent on the set of primers used. Primer pair 35-36, designed from homologous regions in the polymerase genes of Norwalk virus and feline calicivirus, was used with the expectation that it would be broadly reactive (13). However, we found that under our experimental conditions these primers were less sensitive in single-round RT-PCR than the other polymerase primer set (primer pair 51-3). The primers from the 3′ end of the genome (primer pair 1-4) also had poor sensitivity, confirming the observation of Jiang et al. (13).

Primer pair 51-3 was designed from the prototype Norwalk virus sequence to be applied to a collection of specimens of epidemiologic interest and was able to detect positive PCR products in one or more specimens from all of the outbreaks associated with Norwalk virus or Norwalk-related viruses. When sequence information became available for the Japan and Southampton viruses, we found that primer pair 51-3 was completely homologous to the Japan virus and that 18 of 20 bases in each primer were homologous with the Southampton virus sequence. Primer pair 35-36 also was completely homol-

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                     1                                                  49
Norwalk              VGDYVIRVKE GLPSGFPCTS QVNSINHWII TLCALSEATG LSPDVVQSM
Japan                ---------- ---------- ---------- ---------- ---------
Nursing Home 1986    ----I----- ---------- --------L- ----M--V-- -----I--*
Cruise 1986 A        ----I----- ---------- --------L- ----M--V-- -----I--*
Cruise 1990          ----I----- ---------- --------L- ----M--V-- -----I--Q
Cruise 1992          ------S--D ---------- ---------L -------V-- -----I--Q
Oysters 1978         ------S--- C---D----- ---------L -------V-- -----I--Q
Cruise 1986 B        ---FK-SIN- -----V---- -W---A--LL -------V-N ----II-A*
Southampton          ---------- ---------- --------L- -------V-- -----I---
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FIG. 4. Deduced amino acid sequences of Norwalk virus, Southampton virus, Japan virus, and virus strains from six outbreaks. The sequence alignment was prepared as described in the legend to Fig. 3. Only amino acids which are different from those of the Norwalk virus reference strain (8FIIA) are shown. Hyphens denote identical amino acids; asterisks denote areas near the primers where the amino acid sequences could not be determined. A and B denote different virus strains from the same outbreak.

ogous to Norwalk virus and the Japan virus, but only 14 of 21 bases in primer 35 and 15 of 21 bases in primer 36 were homologous with the Southampton virus sequence. This may explain in part the greater sensitivity of primer pair 51-3 in comparison with that of primer pair 35-36. Also, the smaller PCR product (206 bp) from primer pair 51-3 may be more readily amplified than the 470-bp product from primer pair 35-36.

The sequence diversity among the Norwalk virus strains involved in these outbreaks and our finding that none of these strains had sequences that were completely homologous with the reference Norwalk virus sequence was surprising and may explain in part the low overall detection frequency of Norwalk virus by RT-PCR. However, comparison of the complete Norwalk and Southampton virus genomes indicates that these viruses share greater homology in the first open reading frame (75%), which contains the polymerase gene, than in the other two open reading frames (69 and 66%, respectively), and that the first open reading frame is therefore the most likely region where broadly reactive primers can be designed.

One limitation of RT-PCR for diagnosing Norwalk virus infection is the low proportion of fecal specimens that were PCR positive. Of the 15 outbreaks in which PCR-positive specimens were detected with primer pair 51-3, only 9 had positive PCR products in 50% or more of the stool samples tested. Although individual specimens from outbreaks associated with Norwalk-related viruses or SRSVs were PCR positive, the effect of sequence divergence on PCR efficiency may be responsible for the lower proportions of PCR-positive results among these groups of specimens. The low detection frequency also may reflect a very low virus concentration in fecal samples, as has been reported previously (13), the presence of PCR inhibitors in stool specimens (13, 28), delayed collection of specimens to a time when the patients were no longer shedding virus, or sample degradation that occurred during storage and handling.

We were able to detect positive PCR products by nested PCR in some samples from the calicivirus outbreaks, suggesting that these specimens may have been single-round PCR negative because of a low degree of homology between the Norwalk virus primers and these calicivirus strains. Improved sensitivity by nested PCR has also been reported for hepatitis C virus and rotavirus in tests in which single-round PCR failure was attributed to the high degree of sequence variability (2, 9). However, nested PCR results can be difficult to interpret because of the increased risk of false-positive results due to contamination, and we decided not to use this method for routine screening.

The present study represents the first time that RT-PCR has been used to detect Norwalk-related viruses in fecal specimens from a series of gastroenteritis outbreaks which were characterized serologically and by EM. Our findings suggest that, although our primer pair 51-3 was broadly reactive, the efficiency of PCR for Norwalk virus may be affected by sequence diversity in a portion of the polymerase region. The successful application of RT-PCR to the investigation of outbreaks may depend on the use of multiple primer pairs or primers made against regions of the genome that are more conserved. Further sequence data from outbreak strains in regions outside of that between primer pair 51-3 may be needed to design better primers. By combining RT-PCR with sequencing, we were able to detect and compare the nucleotide sequences of several Norwalk virus strains associated with gastroenteritis outbreaks in different geographic regions. These results and additional studies of this kind will allow further classification of these viruses at a molecular level and will extend our knowledge of the epidemiology and transmission of these agents.

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