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Detection of Norwalk Virus in Stool by Polymerase Chain Reaction

XI JIANG,[1] JIANXIANG WANG,[1] DAVID Y. GRAHAM,[1,2,3] AND MARY K. ESTES[1,2]*

Division of Molecular Virology[1] and Department of Medicine,[2] Baylor College of Medicine, and Veterans Administration Medical Center,[3] Houston, Texas 77030

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A method of reverse transcription (RT) and polymerase chain reaction (PCR) for the detection of Norwalk virus in human stools was developed. A cationic detergent, cetyltrimethylammonium bromide, was found to effectively remove from stool extracts factors that inhibit the RT-PCR assay. The specificities of the tests were shown by hybridization of the amplified DNA with Norwalk virus-specific cDNA probes and a consistent correlation between virus detection in stools and infection of volunteers. RT-PCR detected virus in stool samples diluted 10^{-4} and was about 100 times more sensitive than dot blot hybridization. In serial stool samples collected before and at different times after inoculation of 10 volunteers with Norwalk virus, 37 of 55 were positive by RT-PCR, but only 27 were positive by dot blot hybridization ($\chi^2 = 22.96$; $P < 0.001$). Further application of this method should allow detection of Norwalk virus in food or environmental samples such as shellfish and shellfish waters.

Norwalk virus or Norwalk virus-like agents are important pathogens that cause acute epidemic gastroenteritis in humans (11). These viruses are spread by waterborne, person-to-person, and food-borne routes. It has been estimated that up to 42% of outbreaks of acute nonbacterial gastroenteritis in adults in the United States may be due to Norwalk virus or Norwalk virus-like agents (12). Currently, diagnosis of Norwalk virus infection relies on immunologic tests, including immune electron microscopy, radioimmunoassays (RIAs), immune adherence hemagglutination assays, and enzyme immunoassays. Because these tests use human convalescent-phase serum from patients, they are of relatively low sensitivity because of the low affinity of the antibodies for the viruses. In addition, sources of serum are limited. Recently, we and others have successfully cloned the Norwalk virus genome (7, 15). Using Norwalk virus-specific cDNAs, we showed that Norwalk virus contains a polyadenylated single-stranded RNA genome of about 7.7 kb. Based on this knowledge, we developed a reverse transcription (RT) and polymerase chain reaction (PCR) method, originally described by Saiki et al. (16), for the detection of Norwalk virus in human stools.

MATERIALS AND METHODS

Human volunteer studies. Adult volunteers were students or staff working in the Texas Medical Center. The Norwalk virus inoculum (8FIIa) used in the volunteer studies was kindly supplied by A. Kapikian (Laboratory of Allergy and Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.). The virus was administered to the volunteers as described previously (7). All stools were collected in plastic containers, and bulk stools and aliquots were stored at −70°C for later use.

Extraction of viral RNA from stools. Stool samples were suspended in water (10 to 50% [wt/vol]) and extracted once with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113; also referred to as genetron; Dupont Co., Wilmington, Del.). Virus in the aqueous phase was precipitated with 8% polyethylene glycol 6000 (BDH Chemicals, Gallard-Schlesinger, Carle Place, N.Y.) in the presence of 0.4 M NaCl. The pellet was suspended in proteinase K buffer (0.1 M Tris-HCl [pH 7.5], 12.5 mM EDTA, 0.15 M NaCl, 1% [wt/vol] sodium dodecyl sulfate, and 400 μg of proteinase K per ml) and incubated at 37°C for 30 min. Ten percent cetyltrimethylammonium bromide (CTAB; Sigma Chemical Co., St. Louis, Mo.) and 4 M NaCl were added separately to give final concentrations of 1.25% CTAB and 0.45 M NaCl. After incubation at 56°C for 30 min, the mixture was extracted with an equal volume of phenol-chloroform and reextracted with an equal volume of chloroform. The RNA in the sample was then precipitated with 2.5 volumes of ethanol, and the RNA either was used directly for the RT-PCR tests or was stored at −20°C for later use. The RNAs for dot blot hybridization were prepared by a similar protocol, but the CTAB steps (addition of CTAB-NaCl and incubation at 56°C for 30 min) were omitted.

Primers. Norwalk virus-specific primers (primer pairs 1 and 4, 8 and 9, and 16 and 17) were purchased from either the Midland Certified Reagent Company, Midland, Texas, or from the Department of Cell Biology, Baylor College of Medicine. The sequences of these primers were selected randomly from different regions of the nucleotide sequence of the viral genome (8) and were as follows: primer 1, 5′-ATTGAGAGCCTCCGCGTG-3′; primer 4, 5′-GGTGGC GAAGCGGCCCTC-3′; primer 8, 5′-CATATCTATAACT GCTGA-3′; primer 9, 5′-ATGCTATATACATAGGTC-3′; primer 16, 5′-CAACAGGTACTACGTGAC-3′; and primer 17, 5′-AGCAAATCTTGGGCCACA-3′. The primers were purified by high-pressure liquid chromatography (Midland Certified Reagent Company) or by removal from the resin by using ammonium hydroxide and then ethanol precipitation (Baylor College of Medicine). Random primers (hexamers) were purchased from Promega (Biological Research Products, Madison, Wis.).

RT-PCR assay. Both RT and PCRs were carried out under the conditions recommended by the manufacturers. The reverse transcriptase (from avian myeloblastosis virus) was purchased from Life Sciences Inc. (St. Petersburg, Fla.), and AmpliTaq was purchased from Perkin-Elmer Cetus

* Corresponding author.

(Norwalk, Conn.). Negative reagent controls were run in each experiment. For RT, a 30-μl reaction mixture was made. It contained 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl), 3.3 mM deoxynucleoside triphosphates, 1.0 μM primers or 10 μM random primers, 40 U of RNasin, 10 U of reverse transcriptase, and purified viral RNA. Each reaction was carried out for 1 h at 42°C. For PCR following RT with random primers, 70 μl of 1× PCR buffer containing the two Norwalk virus-specific primers (1.0 μM each) and *Taq* polymerase (5 U) were added to the RT mixture and put into the thermal cycler (Programmable Thermal Controller; MJ Research Inc., Cambridge, Mass.). For PCR following RT with a Norwalk virus-specific primer, 1.0 μM the second Norwalk virus-specific primer and 5 U of *Taq* polymerase were added to the RT mixture and put into the thermal cycler. The amplification cycle program included denaturation for 1 min at 94°C, 30 to 40 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min 20 s at 49°C, and primer extension for 1 min at 72°C. A final extension was then performed for 15 min at 72°C. The amplified DNA products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized after illumination with UV light. In some experiments, PCR-amplified DNA in agarose gels was hybridized in a dried gel as described previously (6).

Dot blot hybridization. The method of dot blot hybridization was adapted from our previously reported technique of detection of hepatitis A virus from environmental samples (21). Norwalk virus-specific probes were prepared from Norwalk virus cDNAs, covering the same regions of the Norwalk virus genome as the PCR-amplified products. Insert cDNAs were isolated by restriction enzyme digestion and sized on a SeaPlaque agarose (FMC BioProducts, Rockland, Maine) gel. The DNA fragments then were labeled in the agarose with [^{32}P]dCTP by the random primer technique and conditions described by Promega. Both prehybridization and hybridization were carried out in a solution without formamide at 65°C, as described previously (5).

RIA. Pre- and postinfection serum samples from one volunteer (volunteer 505) given Norwalk virus were used as capture antibody at 1:800 dilutions. Stool suspensions were extracted with genetron and diluted 1 to 10 in phosphate-buffered saline containing 1% bovine serum albumin and 10% fetal calf serum. Samples of 50 μl were added to each well. Purified immunoglobulin G from another volunteer (volunteer 519) given Norwalk virus was labeled with ^{125}I and was used as the detector antibody. Positive/negative (P/N) ratios of the counts per minute were calculated for the reactivity of each sample with the post- and preinfection serum samples, respectively. A P/N ratio of ≥2.1 was considered a positive reaction.

RESULTS

Development of RT-PCR to detect Norwalk virus in stool. Three pairs of primers were designed for the RT-PCR test on the basis of the primary sequence of the Norwalk virus genome. The three pairs of primers were located at the 5′ end, the center, and the 3′ end of the virus genome, and their amplified products were 815, 662, and 456 bp, respectively. Figure 1 shows the relative locations of these primer pairs on the Norwalk virus genome and typical results of RT-PCR-amplified products seen in an agarose gel after 30 cycles of amplification by using Norwalk virus RNA as the template. Each pair of primers gave a single major band of the expected size after amplification. To save material, we routinely used only one pair of primers (the 3′ end set called primer pair 1 and 4) for further tests. However, to confirm reactivities, the other two sets of primers were used in some experiments.

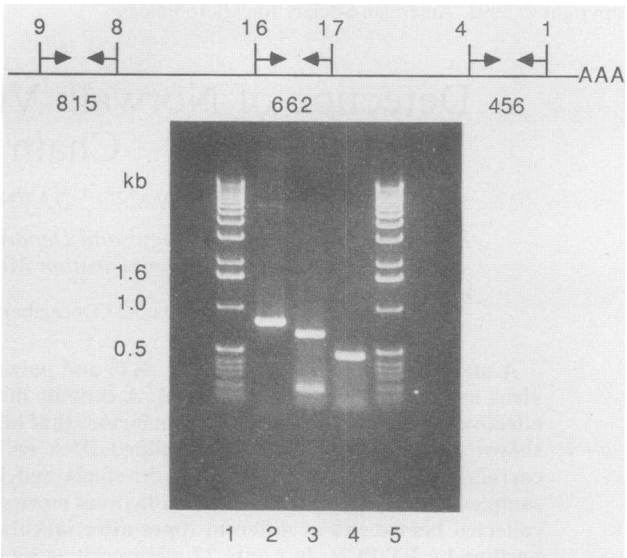

FIG. 1. Detection of Norwalk virus in stool by RT-PCR. RNA was extracted from a stool sample (sample 543-11) by the CTAB technique and amplified by RT-PCR as described in the text. Lanes 1 and 5, 1-kb markers from Bethesda Research Laboratories (the markers that migrated as 1.6, 1.0, and 0.5 kb are labeled); lane 2, PCR with Norwalk virus primers 8 and 9; lane 3, PCR with Norwalk virus primers 16 and 17; lane 5, PCR with Norwalk primers 1 and 4. The amplified products were separated on an agarose gel and visualized with UV light after staining with ethidium bromide. The small product seen in lane 3 was made in variable amounts in different experiments. The positions of the three primer pairs used in this study are given above the autoradiograph. The numbers below the map indicate the sizes (in base pairs) of the RT-PCR product.

In our preliminary experiments, a strong inhibitory factor(s) for both reverse transcriptase and *Taq* polymerase was observed in nucleic acid extracted from the stool samples. Several methods [including dialysis, heating, phenol-chloroform extraction, and oligo(dT) cellulose chromatography of the sample] were evaluated to remove this inhibitory factor(s). Positive results were seen only by using the oligo(dT) method, but the efficiencies of the reactions with material prepared by this method were low (data not shown). Subsequent work showed that inclusion of the cationic detergent CTAB in the RNA extraction steps significantly increased the signals of the reactions. The use of CTAB was based on our previous results of dot blot hybridization for the detection of hepatitis A virus and rotavirus in shellfish (21). CTAB was found to be able to remove inhibitory factor(s) from the stool samples prepared for RT-PCR (Fig. 2).

The use of random primers and specific primers during the first-strand cDNA synthesis step was compared by examining the products made from dilutions of virus-containing stool samples. Specific primers showed slightly higher sensitivities based on the intensities of the products made from each virus dilution. However, the same endpoint dilution of sample was detected by using either type of primer (data not shown). Therefore, on the basis of work performed to date, either type of primer can be used.

The most critical step of RT-PCR was found to be the RT for the first-strand cDNA synthesis. Initially, we performed

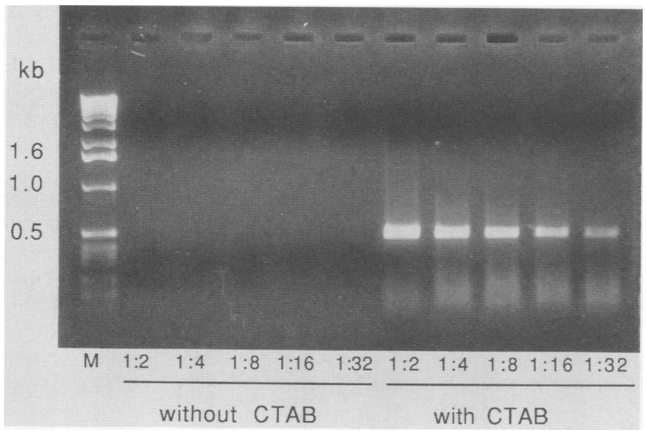

FIG. 2. Comparison of RNA extraction methods with and without CTAB for the RT-PCR assay. Serial dilutions of a stool sample (sample 543-11) were extracted with phenol-chloroform without and with CTAB extraction. The nucleic acid was amplified by RT-PCR by using primers 1 and 4. The amplified products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light. M, 1-kb markers from Bethesda Research Laboratories (see Fig. 1).

RT and PCR in one tube into which all the reagents for both reactions were added simultaneously. Subsequent experiments showed that these conditions, although convenient, were not optimal for RT. Instead, we found that if the RTs and PCRs were separated by adding the PCR reagents to the tube after the first RT reaction had been completed, the test sensitivity was increased 10 to 100 times (data not shown).

The number of cycles required for virus detection was examined by using serial dilutions of one stool sample. Increasing signals were observed until 40 cycles of amplification. In the stool with a high concentration of virus, 25 to 30 cycles were sufficient for virus detection, while in stools with a low concentration of virus, more cycles were required (Fig. 3). For subsequent studies, we used 40 cycles of amplification to obtain maximum sensitivity of virus detection.

Sensitivity of RT-PCR for detecting Norwalk virus in stool. The sensitivity of RT-PCR for Norwalk virus detection first was tested by using plasmid DNA as the template. A detection level of 0.01 fg of DNA (approximately 10 genome copies) was obtained. Because of the difficulty of quantitating viral RNA in stool samples (due to the low concentration of virus in stool and the lack of a culture system), we used dilutions of stools to compare the sensitivities between RT-PCR and hybridization tests for the detection of Norwalk virus. Serial dilutions of stool were made and processed for virus detection by both methods. Norwalk virus was detected by RT-PCR in a 10^{-4} dilution of the stool sample, equivalent to 0.15 to 0.012 mg of stool, while 125 mg of the same stool sample was required to show a positive result by dot blot hybridization (Table 1).

A series of stool samples collected from 10 Norwalk virus-inoculated volunteers at different times after infection also were tested for the presence of Norwalk virus by RT-PCR and hybridization. Nine of these 10 volunteers had symptomatic infections. A total of 55 stool samples were tested. Thirty-seven were positive by PCR, while only 27 were positive by hybridization (Table 2). All samples positive by hybridization were also positive by PCR. The increased sensitivity of the PCR detection method was most striking for stool samples collected between 25 and 48 h after infection of volunteers. At these times, PCR detected virus in 91% of the stool samples, while hybridization detected virus in only 57% of the samples. Only three samples from the one volunteer who had an asymptomatic infection were tested. Only the sample from the latest time point (at 68 h postinoculation) was positive by RT-PCR.

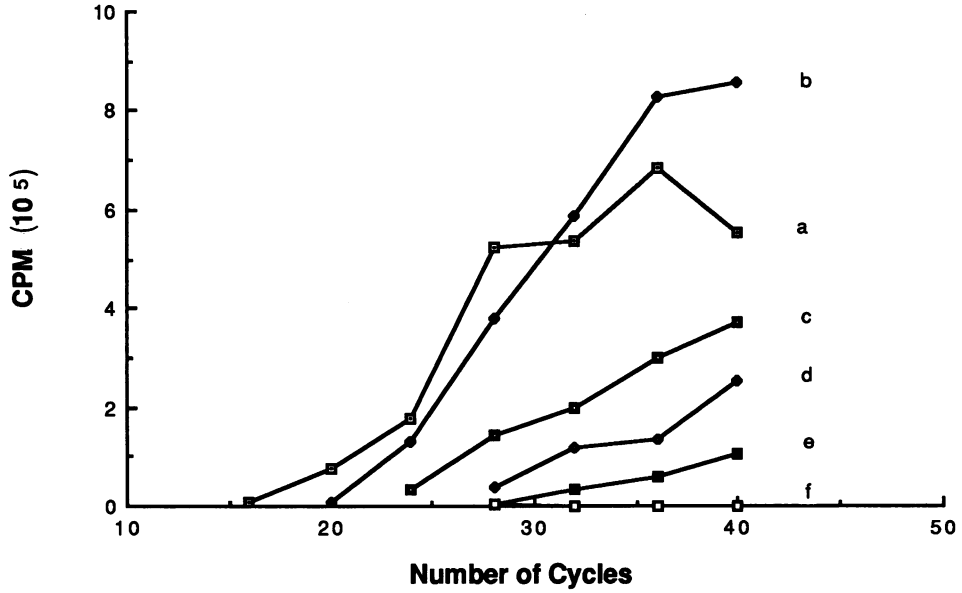

FIG. 3. Evaluation of the number of cycles of amplification required for virus detection by RT-PCR. Serial 10-fold dilutions (from a to e) of one stool sample were tested for Norwalk virus by RT-PCR. Sample f was a negative control which contained all the reagents but which lacked viral RNA. [^{32}P]dCTP was included during the amplification reaction, and aliquots of the reactions were taken out after different numbers of amplification cycles. After gel electrophoresis of each aliquot, the band of amplified product in a dried gel was cut out and its radioactivity was determined by counting in a scintillation spectrometer.

TABLE 1. Comparative sensitivities of detecting Norwalk virus in stools by PCR and dot blot hybridization

Stool sample no.	Test method	Test result with the following dilution of stool[a]:					
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
502A-03	PCR	NT	+	−	−	−	−
	Hyb[b]	±	−	−	−	−	NT
505A-05	PCR	NT	+	+	+	+	−
	Hyb	+	+	±	−	−	NT
544A-04	PCR	NT	+	+	−	−	−
	Hyb	−	−	−	−	−	NT
544A-05	PCR	NT	+	+	+	−	−
	Hyb	−	−	−	−	−	NT

[a] NT, not tested; +, strong signal seen; −, no signal seen; ±, weak but clear signal seen.
[b] Hyb, dot blot hybridization.

Specificity of RT-PCR detection of Norwalk virus in stools. The specificities of the RT-PCR tests were first determined from the unique sizes of the amplified DNA products on agarose gels. No extra bands were seen in the 55 stool samples tested by RT-PCR with the 1-4 primer pair. The specificities of the tests were also confirmed by hybridization of the amplified DNA products with Norwalk virus-specific probes after gel electrophoresis (data not shown).

To confirm further the specificity of the RT-PCR test for Norwalk virus detection, an additional 55 stool samples from well persons (preinfection stools from volunteers and other individuals without any clinical symptoms of gastroenteritis) or from Norwalk virus-infected volunteers were coded and tested. All 25 normal or preinfection stool samples were negative, and all 30 stool samples from the Norwalk virus-infected individuals were positive for virus by RT-PCR. The infection status of the volunteers was determined by the presence of clinical symptoms (vomiting, diarrhea, fever, nausea, or malaise) and by demonstration of seroresponses measured by a new enzyme-linked immunosorbent assay (9). The 30 positive stool samples were from 12 volunteers who each showed a fourfold or greater increase in antibody titers against Norwalk virus (data not shown). The RT-PCR results were also compared with virus detection by dot blot hybridization and RIA. Figure 4 shows a typical result of Norwalk virus detection in the stool from a patient with severe illness. Virus detection by RT-PCR generally agreed with the results obtained by dot blot hybridization and RIA. However, the RT-PCR was more sensitive than the other two tests. The specificity of the test with the 1-4 primer pair also was shown by a lack of obtaining any RT-PCR products from stool samples containing human calicivirus (one stool sample tested), hepatitis A virus (two stool samples tested), rotaviruses (two stool samples tested), poliovirus (two stool samples tested), and other small round non-Norwalk viruses (nine stool samples tested).

TABLE 2. Comparison of RT-PCR and dot blot hybridization for detection of Norwalk viruses in 55 stool samples from human volunteers

Assay	No. of positive stools/total no. of stools collected from volunteers at the following time (h)[a]:							
	<0	0–12	13–24	25–48	49–72	73–96	97–120	Total
PCR	0/3	0/4	1/8	21/23	9/9	4/5	2/3	37/55[b]
Hyb[c]	0/3	0/4	1/8	13/23	7/9	4/5	2/3	27/55[b]

[a] A total of 55 stools were tested from 10 volunteers who were found to be infected on the basis of antibody seroconversion measured by using a new enzyme-linked immunosorbent assay with purified recombinant Norwalk virus particles produced by using the baculovirus expression system (9). Nine of these 10 volunteers had symptomatic infections.
[b] For the stool samples tested, 27 were positive and 18 were negative by both RT-PCR and dot blot hybridization. The remaining 10 samples were positive by PCR but not by dot blot hybridization. This indicated that the PCR assay is significantly more sensitive ($\chi^2 = 22.96$; $P < 0.001$) than the dot blot hybridization test. All the RT-PCR-positive and dot blot hybridization-negative samples recently were tested with a new antigen enzyme-linked immunosorbent assay by using hyperimmune antiserum made to recombinant Norwalk virus particles (data not shown), and they all were positive, indicating that the RT-PCR results were correct.
[c] Hyb, dot blot hybridization.

DISCUSSION

Cloning of the Norwalk virus genome provided the first step in the development of new, sensitive, specific, and widely available assays for the detection of this virus. We have described the development of RT-PCR (14, 16) for the detection of the Norwalk virus genome. This method should be of interest for both clinical and basic studies of Norwalk virus and possibly of other related viruses. Several possible applications include the following. First, RT-PCR should be useful in monitoring attempts to adapt virus for replication in cell culture. Previous attempts at cultivating Norwalk virus may have stopped prematurely because of the lack of sensitive methods to monitor even abortive virus replication. Second, RT-PCR may be useful for establishing a clinical diagnosis of diarrhea caused by Norwalk virus infection. This method might be expected to be of particular use for Norwalk virus detection, because very low levels of virus are thought to be excreted, and such low virus levels might be missed by antigen-based assays. Third, use of RT-PCR to localize the site of virus replication in the gastrointestinal tract and to monitor the duration and patterns of virus shedding in stool or other clinical samples should further our understanding of the pathogenesis of Norwalk virus infections. Fourth, this technique offers the opportunity for further development of methods for the detection of virus in environmental samples. There is particular interest in the development of methods that can detect Norwalk virus in food, water, and shellfish for public health safety reasons and to protect the shellfish industry. Finally, RT-PCR can be expected to be a powerful tool for molecular epidemiologic studies of Norwalk and related viruses. By combining RT-PCR, cloning, and sequencing methods, it will be of interest to determine and compare the nucleotide sequences of Norwalk virus and Norwalk-like viruses and other agents associated with gastroenteritis (small round-structured viruses) collected from different geographic regions (2, 11). This information will help us to understand the similarities and differences between these viruses at a molecular level, which will be useful for classification and understanding the importance and epidemiology of these individual agents that cause human disease.

Because RT-PCR depends on enzyme amplification of nucleic acid, the quality of the RNA used for the assay is very important for obtaining positive results. In this study, we found that two steps (polyethylene glycol precipitation and CTAB extraction) were critical for processing virus from stool samples to obtain high-quality RNA for successful RT-PCRs. Polyethylene glycol precipitation of viruses not only concentrated the virus but it also removed a significant

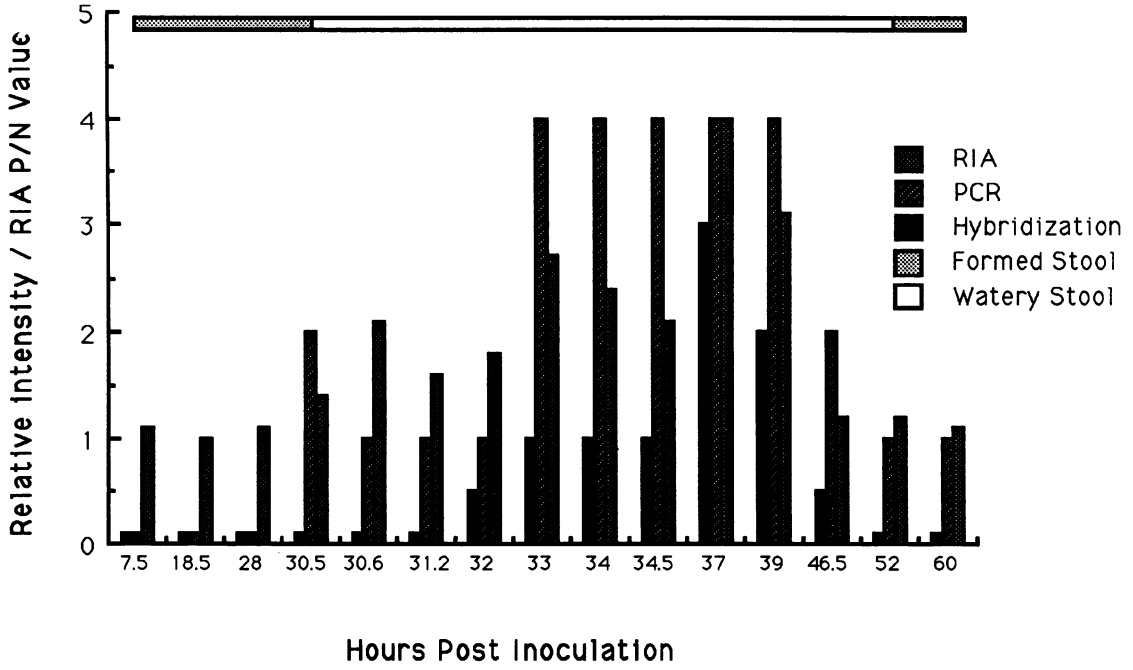

FIG. 4. Detection of Norwalk virus in serial stool samples of one volunteer by RT-PCR, dot blot hybridization, and RIA. A sample size of 0.125 μl of 10% stool suspension from stools collected from one volunteer (volunteer 544) who was administered Norwalk virus was used to extract viral RNA for the dot blot hybridization and PCR, and 0.05 ml of the same sample was used for antigen detection by RIA. The time postinoculation of stool collection from the volunteer and the consistency (formed or watery) of each stool sample are also shown. The PCR and hybridization results were scored as relative intensities on the basis of visual inspection of the hybridization signals on the exposed X-ray film for the hybridization and on the basis of the ethidium bromide-stained bands of the PCR products visualized after electrophoresis on an agarose gel. Maximum staining was scored as 4+, and negative results were scored as 0. A relative intensity of 1 or above is equivalent to a positive result (+) in Table 1. An intensity of 0.5 is equivalent to a plus-or-minus result (±), and an intensity of ≤0.2 is equivalent to a negative result (−) in Table 1. The negative samples were plotted just above the 0 value to indicate that the assay was performed. Six samples (from 30.6 and 33 to 39 h postinoculation) with P/N ratios of ≥2.1 were positive by RIA.

amount of small particles and soluble substances which potentially could inhibit the RT-PCR assay. This step was found to be particularly significant when the virus concentration in a stool sample was very low.

A key question about our results is what minimum number of Norwalk virus particles can be detected by RT-PCR. Unfortunately, it is not possible to quantitate directly the numbers of Norwalk virions in a stool sample because of the very low concentration of virus present. In fact, virus particles can be seen only by performing immune electron microscopy (11). One can estimate that such samples contain approximately 10^5 particles per ml, and if we can detect Norwalk virus RNA in stool samples diluted up to 10^{-4}, this suggests a detection limit of as few as 10 particles and certainly 100 to 1,000 particles. This latter number of particles is consistent with the result that RT-PCR is 100 times more sensitive than hybridization for the detection of Norwalk virus, and previously, we found that the detection limit of hybridization with purified hepatitis A virus particles is 10^4 to 10^5 virions (5).

The CTAB extraction method used in our experiments was modified from that used to extract nucleic acid from shellfish for hybridization assays (21). Specifically, for extraction of virus from stool samples for RT-PCR, we used CTAB in high salt concentrations in conjunction with phenol-chloroform extraction to obtain relatively pure RNA in the aqueous phase. In contrast, for extraction of nucleic acid from shellfish homogenates, we use CTAB in low salt concentrations after phenol-chloroform extraction to obtain a CTAB-RNA precipitate (21). This modification was made by monitoring the recovery of radiolabeled single-stranded RNA added to stool extracts. We found that viral RNA stayed in the aqueous phase, while most of the stool debris separated into the interphase or organic phases when the sample was extracted with phenol-chloroform in the presence of CTAB and a high concentration of salt. Although the precise mechanism by which CTAB effectively purifies the RNA by this procedure is not clear, our results confirm that the salt concentration plays an important role in the behavior of nucleic acid in CTAB solutions (10).

Other techniques (including hydroxyapatite and CF-11 chromatography) have been reported to remove inhibitors from stool samples for PCR testing (4, 20). Although direct comparisons are needed to evaluate the relative efficiencies of these various techniques, a major advantage of our CTAB method over the other techniques is its great simplicity and speed.

While developing this method, we found that contamination of the PCR products was problematic during our early experiments. The major source of contamination was plasmid DNA of Norwalk virus clones which were present in the laboratory. To avoid the problem of contamination, we found it necessary to use a separate room for the preparation of the reagents and samples for testing. This room was physically separated from any rooms used for plasmid work. Special care also had to be taken to avoid cross-contamination between samples during the tests. While these precautions have been noted by others (13, 17, 18) and were

effective in our hands when strictly followed, this problem represents a major difficulty that must be overcome before such methods will be readily useful in clinical laboratories.

The patterns of virus shedding from volunteers tested in the study were similar to those reported previously, except that the RT-PCR method detected more Norwalk virus-positive samples (3). However, the detection of virus shedding was earlier (15 h after virus administration) and lasted longer (at least 6 days [the latest time tested] after virus administration) than reported previously. Further application of these new techniques will allow us to study other important questions about the epidemiology, immunology, and pathogenesis of Norwalk virus infection.

In additional preliminary tests, we used the 1-4 primer pair to screen other samples thought to contain Norwalk virus from several outbreaks of human gastroenteritis from different countries, and we did not find many Norwalk virus-positive samples. This may indicate that the virus which caused these outbreaks was different from the 8FIIa Norwalk virus used as the inoculum for the volunteers. Recently, we designed other primers (primers 35 and 36) which show homology between the RNA polymerase regions of Norwalk virus and feline calicivirus. Testing of such new primers in combination with our previous and other nested primers appears to be useful for the development of tests not only for the detection of Norwalk virus but also for other agents found in stools and currently classified as small round viruses (1, 19).

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REFERENCES

1. **Appleton, H.** 1987. Small round viruses: classification and role in food-borne infections, p. 108–125. *In* Novel diarrhoea viruses. Ciba Foundation Symposium 128. Wiley, Chichester, United Kingdom.
2. **Blacklow, N. R.** 1990. Medical virology of small round gastroenteritis viruses. Med. Virol. **9**:111–128.
3. **Gary, G. W., L. J. Anderson, B. H. Keswick, P. C. Johnson, H. L. DuPont, S. E. Stine, and A. V. Bartlett.** 1987. Norwalk virus antigen and antibody response in an adult volunteer study. J. Clin. Microbiol. **25**:2001–2003.
4. **Gouvea, V., J. R. Allen, R. I. Glass, Z. Y. Fang, M. Bremont, J. Cohen, M. A. McCrae, L. J. Saif, P. Sinarachatanant, and E. O. Caul.** 1991. Detection of group B and C rotaviruses by polymerase chain reaction. J. Clin. Microbiol. **29**:519–523.
5. **Jiang, X., M. K. Estes, and T. G. Metcalf.** 1987. Detection of hepatitis A virus by hybridization with single-stranded RNA probes. Appl. Environ. Microbiol. **53**:2487–2495.
6. **Jiang, X., M. K. Estes, and T. G. Metcalf.** 1989. An in situ hybridization test for quantitative assay of infectious hepatitis A virus. J. Clin. Microbiol. **27**:874–879.
7. **Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes.** 1990. Norwalk virus genome: cloning and characterization. Science **250**:1580–1583.
8. **Jiang, X., K. Wang, M. Wang, and M. K. Estes.** Unpublished data.
9. **Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes.** Submitted for publication.
10. **Jones, A. S.** 1963. Use of alkyltrimethylammonium bromides for the isolation of ribo- and deoxyribo-nucleic acids. Nature (London) **199**:280–282.
11. **Kapikian, A. Z., and R. M. Chanock.** 1990. Norwalk group of viruses, p. 671–693. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, Ltd., New York.
12. **Kaplan, J. E., R. Feldman, D. S. Campbell, C. Lookabaugh, and G. W. Gary.** 1982. The frequency of a Norwalk-like pattern of illness in outbreaks of acute gastroenteritis. Am. J. Public Health **72**:1329–1332.
13. **Kwok, S., and R. Highchi.** 1989. Avoiding false positives with PCR. Nature (London) **339**:237–238.
14. **Kwok, S., and J. J. Sninsky.** 1989. Application of PCR to the detection of human infectious diseases. *In* H. A. Erlich (ed.), PCR technology. Stockton Press, New York.
15. **Matsui, S. M., J. P. Kim, H. B. Greenberg, W. Su, Q. Sun, P. C. Johnson, H. L. DuPont, L. S. Oshiro, and G. R. Reyes.** 1991. The isolation and characterization of a Norwalk virus-specific cDNA. J. Clin. Invest. **87**:1456–1461.
16. **Saiki, P. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.
17. **Saksena, N. K., D. Dwyer, and F. Barre-Sinoussi.** 1991. A "sentinel" technique for monitoring viral aerosol contamination. J. Infect. Dis. **164**:1021–1022.
18. **Sarkar, G., and S. S. Sommer.** 1990. More light on PCR contamination. Nature (London) **347**:340–361.
19. **Seto, Y., T. Ando, K. Haruki, T. Kimura, Y. Hayashi, S. Sekine, S. Okada, K. Ohta, T. Miki, J. Wang, X. Jiang, and M. K. Estes.** Unpublished data.
20. **Wilde, J., J. Eiden, and R. Yolken.** 1990. Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions. J. Clin. Microbiol. **28**:1300–1307.
21. **Zhou, Y.-J., M. K. Estes, X. Jiang, and T. G. Metcalf.** 1991. Concentration and detection of hepatitis A virus and rotavirus from shellfish by hybridization tests. Appl. Environ. Microbiol. **57**:2963–2968.