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Use of Heat Release and an Internal RNA Standard Control in Reverse Transcription-PCR Detection of Norwalk Virus from Stool Samples

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Norwalk virus (NV) and the Norwalk-like viruses are important human pathogens that cause epidemic acute viral gastroenteritis. Current techniques used to recover NV from clinical samples involve multistep viral extraction and elution procedures with subsequent viral detection by reverse transcription-PCR (RT-PCR). In this study, a simple method using heat to recover viral RNA from 45 stool samples was compared to a conventional viral RNA extraction technique, with subsequent analysis by RT-PCR. In addition, we used an internal RNA standard for the detection of inhibitors present in processed samples. Our results indicate that the use of heat to recover NV RNA from stool samples has a sensitivity for the detection of NV RNA that is similar to the more labor-intensive, time-consuming, conventional RNA extraction technique. The use of an RNA internal standard permits the detection of inhibitors present in processed samples, allowing the identification of false negatives. The standard we developed has the advantage of allowing differential detection between wild-type viral RNA and standard using internal oligoprobe hybridization.

Norwalk virus (NV) and the Norwalk-like viruses are important human pathogens that cause epidemic acute viral gastroenteritis. Because of the fastidious nature of these viruses and their inability to be propagated in cell culture, detection has focused on nucleotide sequence and antigenic characteristics (9, 13–16). The molecular technique of reverse transcription-PCR (RT-PCR) is extremely sensitive and specific and has enabled epidemiological studies to identify NV and related viruses as the causative agents in outbreaks of gastroenteritis (1, 7, 10, 12, 18, 19, 21, 24). Although sensitive and specific, RT-PCR requires the removal or inactivation of potential inhibitors present in samples (4, 17, 25). Most methods developed to recover viral RNA from clinical or environmental samples involve multistep extraction and elution procedures using a combination of several reagents such as guanidinium thiocyanate, cetyltrimethylammonium bromide (CTAB), polyethylene glycol, silica, Sephadex, phenol-chloroform, and sodium acetate. Each of these methods is used to concentrate and purify viruses and viral RNA as well as to remove inhibitors from samples for subsequent RT-PCR detection (4, 5, 14, 20, 22, 23). While generally effective, these methods are time consuming and create the potential for viral loss during each processing step. In addition, unless an internal RT-PCR-positive control is run with each sample, it is not possible to determine if a negative result is due to the absence of virus in the sample or the presence of inhibitors (2, 4, 6, 8).

In an effort to reduce sample processing time and effort, 45 stool samples from a human volunteer study (9, 14) were examined by a simple single-step method using heat to recover NV RNA. This method was compared to a conventional viral extraction method for recovery of NV RNA for subsequent RT-PCR. Results from both of these extraction methods were then compared to those of an antigen enzyme-linked immunosorbent assay (Ag ELISA). In addition, we expanded upon our previously reported use of a RNA internal standard to identify inhibitors present in processed samples (4).

Stool samples were suspended in phosphate-buffered saline (10% [wt/vol] suspension) and extracted with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon) (Dupont Co., Wilmington, Del.). After centrifugation for 10 min at 3,000 × *g* and 4°C, the aqueous phase was retained and stored at 4°C until tested. One-hundred-microliter aliquots of freon-extracted stool were then processed by a previously described conventional RNA extraction method (14). Briefly, freon-extracted samples were precipitated with 8% polyethylene glycol 6000–0.4 M NaCl, digested with 400 μg of proteinase K per ml, extracted with CTAB-NaCl, phenol-chloroform, and chloroform in succession, and precipitated in ethanol. The nucleic acid pellet was suspended in 100 μl of MilliQ (Millipore, Bedford, Mass.) H_2O and stored at −20°C until use. For the heat release method, Freon-extracted stool samples were diluted 10- and 100-fold in MilliQ H_2O, heated to 95°C for 5 min, chilled on ice for 2 min, and assayed immediately for NV by RT-PCR.

The NV oligonucleotide primers used for RT-PCR have been described previously (3, 4). Primers amplified the polymerase gene region of the virus, and the sequences were as follows: downstream primer (NVp35), 5′-CTTGTTGGTTTG AGGCCATAT-3′; upstream primer (NVp36), 5′-ATAAAAG TTGGCATGAACA-3′. Hybridization studies were done with primers homologous to regions between the upstream and downstream primers; for combined NV and internal standard detection, the hybridization oligonucleotide (NVp69) sequence was 5′-GGCCTGCCATCTGGATTGCC-3′; for NV-specific detection (within a deleted region of the internal RNA standard), the hybridization oligonucleotide (SR65d) sequence was ACATCAGGTGATAAGCCAGT (1).

A new RNA internal standard for NV was constructed as previously described (4), with modifications to provide greater separation between virus and internal standard PCR products during gel electrophoresis and to permit differential detection between virus and standard by internal oligoprobe hybridization. A new NV primer (NVp152) which contained the se-

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quence of the downstream primer (NVp35) followed by a 51-base deletion, 20 bases of virus-specific sequence, an additional 72-base deletion, and an additional 19 bases of virus-specific sequence was made. The sequence was 5′-CTTGTTG GTTTGAGGCCATATACAATCTCATCATCACCATAAA TTATCCAGTGATTTATGC-3′. The NVp152 primer was used in a RT-PCR mixture with NVp36, with NV 8FIIa RNA as a template, to generate an amplicon containing a 123-bp deletion. The resulting amplicon was ligated into the TA cloning vector pCRII and transformed into competent *Escherichia coli* One Shot cells. Clones with the proper insert were identified, and orientation was determined by restriction enzyme digestion. After linearization with *Spe*1 (Promega, Madison, Wis.), RNA was synthesized with T7 polymerase as previously described (4). Confirmation that the correct NV sequence had been ligated into the cloning vector was obtained by sequencing the inserted DNA with M13 forward and reverse primers complementary to the plasmid sequence upstream and downstream of the insert (data not shown). Plasmid DNA was removed by digestion with 20 μ of RNase-free DNase (Promega) for 30 min at 37°C followed by extraction with phenol-chloroform and then with chloroform. After precipitation with ammonium acetate, a second digestion was performed with 20 U of RNase-free DNase for 30 min at 37°C to remove remaining DNA. This exhaustive digestion was used after we found that the standard method of 2 U of RNase-free DNase for 15 min at 37°C was insufficient to adequately remove plasmid DNA from the synthesized internal RNA transcripts (data not shown). The RNA was then precipitated with 2 M lithium chloride, with subsequent ethanol precipitation. The RNA pellet was suspended in water and quantitated by spectroscopy at a wavelength of 260 nm, and aliquots were stored at −70°C. Ninety-six copies of internal standard RNA were added to RT-PCR preparations to monitor for inhibitors.

Viral nucleic acids obtained by both conventional RNA extraction and heat release were used in RT-PCR mixtures as previously described (4), with modifications. Thin-walled RT-PCR tubes were used, and reactions were run in a PTC-100 thermal cycler with a hot bonnet (MJ Research, Inc., Cambridge, Mass.). Following 1 h of RT at 43°C and heat denaturation of the enzyme at 95°C for 5 min, the cDNA was amplified by the following cycling conditions: initial heat denaturation at 94°C for 4 min, 40 cycles of template denaturation at 92°C for 30 s, primer annealing at 55°C for 50 s, primer extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Amplified products were 470 and 347 bp in length for NV and internal RNA standard, respectively, and were detected by agarose gel electrophoresis and ethidium bromide staining. Hybridization and detection by slot blots were performed as previously described (4).

To calculate an end point for the generated internal standard RNA and for contaminating plasmid DNA (containing the inserted NV standard sequence) used to generate the internal standard, a series of 10-fold dilutions of NV internal RNA standard was amplified by RT-PCR and PCR with the NV-specific primers. The RT-PCR end point detection was 20 μl of a 10^{-12} dilution (10 copies of standard). The PCR end point detection was 20 μl of a 10^{-6} dilution, demonstrating a 10^6-fold difference between internal standard RNA and contaminating DNA (data not shown).

The same end point detection (10^{-5}) was observed for samples prepared by heat release and conventional RNA extraction of NV from stool (Fig. 1). Absence of either NV or internal RNA standard RT-PCR product in the 10^{-1} dilution of heat release (lane 2) and undiluted RNA extraction (lane 9) indicated inhibitors were present at these dilutions. These results suggested that RT-PCR amplification of samples prepared by heat release and RNA extraction at a 100-fold dilution should provide adequate dilution of inhibitors while still allowing the detection of viral RNA in samples with low levels of NV as determined by Ag ELISA (see below).

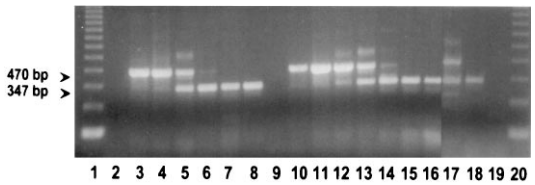

FIG. 1. End point detection of NV in stool using a 10-fold dilution series, heat release versus RNA extraction, with 96 copies of internal standard per RT-PCR mixture. Equal volumes of Freon-extracted stool sample were processed by either heat release or RNA extraction with subsequent $\log_{10}$ dilutions of the sample. Eighteen microliters of each dilution was assayed by RT-PCR. Lanes: 1 and 20, 123-bp molecular weight markers; 2, 10^{-1} dilution, heat release; 3, 10^{-2} dilution, heat release; 4, 10^{-3} dilution, heat release; 5, 10^{-4} dilution, heat release; 6, 10^{-5} dilution, heat release; 7, 10^{-6} dilution, heat release; 8, 10^{-7} dilution, heat release; 9, undiluted sample, RNA extraction; 10, 10^{-1} dilution, RNA extraction; 11, 10^{-2} dilution, RNA extraction; 12, 10^{-3} dilution, RNA extraction; 13, 10^{-4} dilution, RNA extraction; 14, 10^{-5} dilution, RNA extraction; 15, 10^{-6} dilution, RNA extraction; 16, 10^{-7} dilution, RNA extraction; 17, 10^{-2} dilution, NV RNA positive control; 18, MilliQ H_2O, RT-PCR internal standard positive control; 19, 20 μl of MilliQ H_2O RT-PCR negative control.

We next compared detection of NV RNA in 45 stool samples processed by both conventional RNA extraction and heat release. The recovered viral RNA was assayed for NV in RT-PCR mixtures containing the internal RNA standard. NV-specific and internal standard PCR products were detected by gel electrophoresis and by hybridization with NVp69 and SR65d probes. A representative subset is shown in Fig. 2. RT-PCR results were compared to Ag ELISA results previously obtained for all samples tested (9) (Table 1). At a 10^{-2} dilution, samples positive or equivocal for NV by Ag ELISA were positive by both RNA extraction and heat release. For

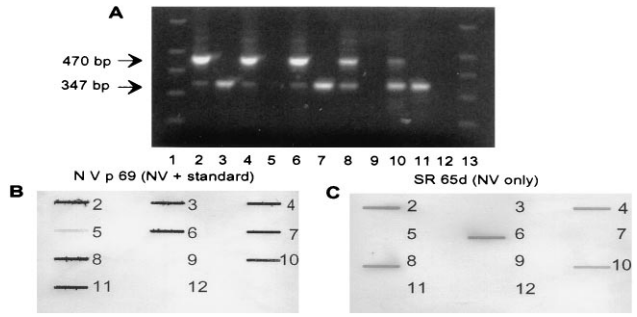

FIG. 2. RT-PCR with 96 copies of internal RNA standard amplified and subjected to slot blot detection using 18 μl of 10^{-2} dilutions (lane 9, 10^{-1} dilution) of a representative subset of stool samples processed by both heat release and RNA extraction. (A) Lanes (numbers in parentheses are Ag ELISA A_{414} readings): 1 and 13, 123-bp molecular weight markers; 2, heat release of sample 538 05 (0.986); 3, heat release of sample 508 05 (0.001); 4, heat release of sample 551 13 (0.044); 5, heat release of sample 507A05 (0.001); 6, RNA extraction of sample 538 05 (0.986); 7, RNA extraction of sample 508 05 (0.001); 8, RNA extraction of sample 551 13 (0.044); 9, RNA extraction of sample 507A05 (0.001); 10, 18 μl of a 10^{-2} dilution of NV RNA, positive control; 11, 18 μl of MilliQ H_2O, RT-PCR internal standard positive control; 12, 20 μl of MilliQ H_2O, RT-PCR negative control. (B) Slot blot detection of PCR products after hybridization with NVp69, a probe designed to identify both NV RNA and internal RNA standard amplified by RT-PCR. Slot blot wells correspond to lanes in panel A. (C) Slot blot detection of PCR products after hybridization with SR65d, a probe designed to identify only NV RNA amplified by RT-PCR (probe sequence in deleted region of internal RNA standard). Slot blot wells correspond to lanes in panel A.

TABLE 1. Comparison of RT-PCR detection of NV (using both the heat release and RNA extraction methods) with Ag ELISA[a]

Ag ELISA results[b]	RT-PCR detection (no. of samples) of NV obtained by[c]:											
	Heat release at dilution:						RNA extraction at dilution:					
	10^{-1}			10^{-2}			10^{-1}			10^{-2}		
	Pos	Neg	Inhib	Pos	Neg	Inhib	Pos	Neg	Inhib	Pos	Neg	Inhib
Positive	6	0	12	18	0	0	15	0	3	18	0	0
Equivocal	0	0	3	3	0	0	3	0	0	3	0	0
Negative	2	6	16	6	17	1	6	15	3	5	19	0
Total	8	6	31	27	17	1	24	15	6	26	19	0

[a] RT-PCR results were confirmed by internal oligoprobing with SR65d and NVp116. Total number of samples, 45.
[b] Positive, $A_{414} > 0.1$; equivocal, $A_{414} = 0.05$ to 0.1; negative, $A_{414} < 0.05$.
[c] Pos, positive; Neg, negative; Inhib, inhibited.

stools negative by Ag ELISA, 5 of 24 RNA extraction and 6 of 24 heat release samples were positive by RT-PCR at a 10^{-2} dilution. For RNA extraction at a 10^{-1} dilution, one additional sample negative by Ag ELISA became positive by RT-PCR. The same antigen-negative samples were positive by heat release (10^{-2}) and RNA extraction (10^{-1}). However, for RT-PCR detection at 10^{-1} dilutions, 31 (69%) of the heat release samples and 6 (13%) of the RNA-extracted samples contained inhibitors. Following most RNA extraction procedures, the recovered RNA has been assayed directly without dilution of the resuspended RNA. Without the addition of an internal RNA standard control, 18 of the heat release and 3 of the RNA-extracted samples inhibited at the 10^{-1} dilution, which were positive by RT-PCR at the 10^{-2} dilution, would have falsely been considered negative. All RT-PCR-positive samples were from individuals who had a seroconversion as determined previously by an antibody ELISA (9).

ELISAs and RT-PCR for the molecular detection of viruses in clinical and environmental samples from potential gastroenteritis outbreaks have been used in epidemiological studies to determine the incidence of Norwalk virus as a cause of gastroenteritis (1, 7, 10, 12, 18, 19, 21, 24). A major concern in interpreting results has been whether inhibitors remained in these samples. Conventional dogma has assumed that, at least for clinical stool samples, extensive sample processing is necessary to adequately purify a stool sample before RT-PCR detection of viral pathogens. A major cause for these extensive purification procedures has been the inability to determine if inhibitors have been adequately removed from each sample before analysis. Hale et al. (11) recently compared the effectiveness of four RNA extraction methods for the detection of small round-structured viruses in fecal specimens. They determined that a guanidinium thiocyanate-silica-based extraction method was effective in removing inhibitors but that the method was 10-fold less sensitive than methods using the metal chelating agent Chelex-100 or Sephadex G200 column chromatography, both of which require less manipulation of fecal specimens. In their study, RNA extraction using polyethylene glycol precipitation followed by phenol-chloroform extraction with the addition of CTAB was the least sensitive method and did not effectively remove inhibitors from 7 of 36 seeded fecal samples (11). However, this conclusion may be incorrect because partial or complete inhibition of sample amplification was determined entirely by visualization of PCR product band intensity following agarose gel electrophoresis and not by the use of oligoprobe hybridization or an internal standard control.

We now describe a simple method to recover viral RNA from a diluted stool sample by heat release followed by RT-PCR detection. A large number of samples can be processed in a very short period of time without the use of costly reagents or potential viral RNA loss during multiple extraction steps. Heat release has a sensitivity equal to or greater than that of the conventional RNA extraction method for recovery of NV from stools (Fig. 1 and Table 1). In addition, in the RT-PCR mixture of each individual sample, an internal RNA standard is coamplified to determine the presence of potential inhibitors, reducing the risk of false-negative results. The internal standard we developed has the advantage of allowing differential detection between wild-type viral RNA and internal standard by using internal oligoprobe hybridization.

REFERENCES

1. **Ando, T., S. S. Monroe, J. R. Gentsch, Q. Jin, D. C. Lewis, and R. I. Glass.** 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridization. J. Clin. Microbiol. **33:**64–71.
2. **Arola, A., J. Santti, O. Ruuskanen, P. Halonen, and T. Hyypia.** 1996. Identification of enteroviruses in clinical specimens by competitive PCR followed by genetic typing using sequence analysis. J. Clin. Microbiol. **34:**313–318.
3. **Atmar, R. L., T. G. Metcalf, F. H. Neill, and M. K. Estes.** 1993. Detection of enteric viruses in oysters by using the polymerase chain reaction. Appl. Environ. Microbiol. **59:**631–635.
4. **Atmar, R. L., F. H. Neill, J. L. Romalde, F. Le Guyader, C. M. Woodley, T. G. Metcalf, and M. K. Estes.** 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. Appl. Environ. Microbiol. **61:**3014–3018.
5. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
6. **Cone, R. W., A. C. Hobson, and M. L. Huang.** 1992. Coamplified positive control detects inhibition of polymerase chain reactions. J. Clin. Microbiol. **30:**3185–3189.
7. **Dowell, S. F., C. Groves, K. B. Kirkland, H. G. Cicirello, T. Ando, Q. Jin, J. R. Gentsch, S. S. Monroe, C. D. Humphrey, C. Slemp, D. M. Dwyer, R. A. Meriwether, and R. I. Glass.** 1995. A multistate outbreak of oyster-associated gastroenteritis: implications for interstate tracing of contaminated seafood. J. Infect. Dis. **171:**1497–1503.
8. **Goswami, B. B., W. H. Koch, and T. A. Cebula.** 1994. Competitor template RNA for detection and quantitation of hepatitis A virus by PCR. BioTechniques **16:**114–121.
9. **Graham, D. Y., X. Jiang, T. Tanaka, A. R. Opekun, H. P. Madore, and M. K. Estes.** 1994. Norwalk virus infection of volunteers: new insights based on improved assays. J. Infect. Dis. **170:**34–43.
10. **Green, J., J. P. Norcott, D. Lewis, C. Arnold, and W. G. Brown.** 1993. Norwalk-like viruses: demonstration of genomic diversity by polymerase chain reaction. J. Clin. Microbiol. **31:**3007–3012.
11. **Hale, A. D., J. Green, and W. G. Brown.** 1996. Comparison of four RNA extraction methods for the detection of small round structured viruses in faecal specimens. J. Virol. Methods **57:**195–201.
12. **Hyams, K. C., J. D. Malone, A. Z. Kapikian, M. K. Estes, X. Jiang, A. L. Bourgeois, S. Paparello, R. E. Hawkins, and K. Y. Green.** 1996. Norwalk virus infection among Desert Storm troops. J. Infect. Dis. **167:**986–987.
13. **Jiang, X., D. Y. Graham, K. N. Wang, and M. K. Estes.** 1990. Norwalk virus genome cloning and characterization. Science **250:**1580–1583.
14. **Jiang, X., J. Wang, D. Y. Graham, and M. K. Estes.** 1992. Detection of Norwalk virus in stool by polymerase chain reaction. J. Clin. Microbiol. **30:**2529–2534.

15. **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.
16. **Kaplan, J. E., G. W. Gary, R. C. Baron, N. Singh, L. B. Schonberger, R. Feldman, and H. B. Greenberg.** 1982. Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. Ann. Intern. Med. **96:**756–761.
17. **Kreader, C. A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. **62:**1102–1106.
18. **Le Guyader, F., F. H. Neill, M. K. Estes, S. S. Monroe, T. Ando, and R. L. Atmar.** 1996. Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. Appl. Environ. Microbiol. **62:**4268–4272.
19. **Moe, C. L., J. Gentsch, G. Grohmann, T. Ando, S. S. Monroe, X. Jiang, J. Wang, M. K. Estes, Y. Seto, C. Humphrey, S. Stine, and R. I. Glass.** 1994. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. J. Clin. Microbiol. **32:**642–648.
20. **Schwab, K. J., R. De Leon, and M. D. Sobsey.** 1995. Concentration and purification of beef extract mock eluates from water samples for the detection of enteroviruses, hepatitis A virus, and Norwalk virus by reverse transcription-PCR. Appl. Environ. Microbiol. **61:**531–537.
21. **Sharp, T. W., K. C. Hyams, D. Watts, A. F. Trofa, G. J. Martin, A. Z. Kapikian, K. Y. Green, X. Jiang, M. K. Estes, M. Waack, et al.** 1995. Epidemiology of Norwalk virus during an outbreak of acute gastroenteritis aboard a US aircraft carrier. J. Med. Virol. **45:**61–67.
22. **Shieh, Y.-S. C., D. Wait, L. Tai, and M. D. Sobsey.** 1995. Methods to remove inhibitors in sewage and other fecal wastes for enterovirus detection by the polymerase chain reaction. J. Virol. Methods **54:**51–66.
23. **van der Hoek, L., R. Boom, J. Goudsmit, F. Snijders, and C. J. Sol.** 1995. Isolation of human immunodeficiency virus type 1 (HIV-1) RNA from feces by a simple method and difference between HIV-1 subpopulations in feces and serum. J. Clin. Microbiol. **33:**581–588.
24. **Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, K. Y. Green, and M. K. Estes.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J. Virol. **68:**5982–5990.
25. **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.