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Improved detection of human enteric viruses in foods by RT-PCR

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Abstract

Human enteric viruses (including hepatitis A virus (HAV) and Norwalk-like viruses (NLVs)) are now recognized as common causes of foodborne disease. While methods to detect these agents in clinical specimens have improved significantly over the last 10 years, applications to food samples have progressed more slowly. In an effort to improve the sensitivity and speed of virus detection from non-shellfish food commodities by reverse transcription-polymerase chain reaction (RT-PCR), we (i) evaluated multiple RNA extraction methods; (ii) compared alternative NLV primer sets; and (iii) developed a one-step RT-PCR method. Hamburger and lettuce samples, processed for virus concentration using a previously reported filtration–extraction–precipitation procedure, were inoculated with HAV or NV. Several RNA extraction methods (guanidinium isothiocyanate, microspin column, QIAshredder™ Homogenizer, and TRIzol) and primer pairs were compared for overall RNA yield (μg/ml), purity (A_{260}/A_{280}), and RT-PCR limits of detection. The use of TRIzol with the QIAshredder™ Homogenizer (TRIzol/Shred) yielded the best RT-PCR detection limits (<1 RT-PCR amplifiable units/reaction for NV), and the NVp110/NVp36 primer set was the most efficient for detecting NV from seeded food samples. A one-step RT-PCR protocol using the TRIzol/Shred extraction method and the NVp110/NVp36 or HAV3/HAV5 primer sets demonstrated improved sensitivity (>10-fold) over the routinely used two-step method. HAV RNA was detected by RT-PCR at initial inoculum levels corresponding to <10 and <100 PFU per 300 μl sample concentrate (corresponding to 6 g food sample) for hamburger and lettuce, respectively. NV RNA was detected by RT-PCR at initial inoculum levels <5 and <50 RT-PCR amplifiable units per 300 μl concentrate (corresponding to 6 g food sample) for hamburger and lettuce, respectively. Residual RT-PCR inhibitors were effectively removed as evidenced by the ability to detect viral RNA in food concentrates without prior dilution. The methods reported here show promise for rapid, sensitive detection of human enteric viruses in foods.

Keywords: Norwalk virus; Hepatitis A virus; RNA purity; RT-PCR; Foods

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1. Introduction

The consumption of fecally contaminated foods is now recognized as a predominant mode for the transmission of human enteric viruses, which are increasingly recognized as a significant public health concern. Data from the US Centers for Disease Control and Prevention (CDC) ranks hepatitis A virus (HAV) as fourth among identified causes of foodborne disease (Bean et al., 1996) and recent epidemiological projections indicate that the Norwalk-like viruses (NLVs) may account for over 60% of all foodborne disease in the US (Mead et al., 1999). Both virus groups are shed in the feces of infected humans and are transmitted via the fecal–oral route. The major routes of contamination of foods with human enteric viruses include: (1) shellfish harvested from fecally contaminated estuaries; (2) fruits or vegetables irrigated or washed with fecally contaminated water; and (3) foods that become contaminated during preparation through contact with fecally contaminated surfaces or hands of infected food handlers (Jaykus, 2000a). In addition, the NLVs have been shown to be spread by aerosolization of vomitus which likely contributes to a high degree of secondary spread (Marks et al., 2000; Patterson et al., 1997). Outbreaks of HAV have been associated with raw oysters and clams (Desenclos et al., 1991; Halliday et al., 1991), frozen strawberries and raspberries (Hutin et al., 1999; Niu et al., 1992; Reid and Robinson, 1987) and lettuce (Rosenblum et al., 1990). The NLVs have been associated with many outbreaks of foodborne disease, including the consumption of contaminated bivalve mollusks (e.g. clams, cockles, mussels and oysters), commercial ice, sandwiches, bakery products and salads (reviewed by Jaykus, 2000a). Previous outbreaks have demonstrated that virtually any food is at-risk for viral contamination if in contact with human fecal matter.

There are many reasons why viral contamination of foods is difficult to identify, but the fact remains that such detection is rarely done. Since the levels of contamination are likely to be low (< 10 infectious units per g food) and large sample sizes of the implicated foods need to be tested, it is necessary to separate and concentrate the viruses from the food matrix prior to the application of detection methods such as cell culture infectivity assay, immunological, and molecular techniques. Nucleic acid amplification methods, such as reverse transcription-polymerase chain reaction (RT-PCR), have shown great promise for the detection of these agents in clinical specimens (Ando et al., 1995; Jiang et al., 1992; Moe et al., 1994), but there remain many obstacles to the routine detection of viruses in foods by RT-PCR, including the need for further sample concentration, the presence of residual food-related inhibitors, and the choice of broadly reactive RT-PCR primers.

Upon review of the literature regarding the detection of viruses in foods (Jaykus, 2000b), it becomes apparent that there are two major areas for which methodological developments are needed. The first of these is the development of rapid, simple and efficient methods to extract and concentrate viruses from food matrices. The second research need includes refinement of RT-PCR amplification methods to increase assay speed, sensitivity and specificity in food systems. This would include efforts to refine RNA extraction methods to decrease the level of RT-PCR inhibitors co-extracted from complex food matrices (Aggarwal and McCaustland, 1998; Drebot and Lee, 1997; Kok et al., 2000; Shieh et al., 1999), development of amplification protocols that improve detection speed and minimize the potential for cross contamination (Tanzer et al., 1999), and identification of broadly reactive primer sets whose use is specifically optimized to food matrices. As such, the purpose of this study was to address these research needs in an effort to improve the speed and sensitivity of virus detection from non-shellfish food commodities. The specific objectives were to: (i) evaluate multiple RNA extraction methods; (ii) compare primers targeting the RNA dependent RNA polymerase region of Norwalk-like viruses; and (iii) develop a one-step RT-PCR method. In all instances, optimization of assay performance was ultimately done in food systems, as these were the matrices to which these methods will ultimately be applied.

2. Materials and methods

2.1. Viruses

Hepatitis A virus, cytopathic strain HM-175, was grown in FRhK-4 (fetal rhesus monkey-derived) cells (Cromeans et al., 1987). Viruses in infected cell lysates were purified after two rapid freeze-thaw cycles followed by extraction with an equal volume of trichlorotrifluoroethane (Freon) as previously described (Sobsey et al., 1978). The virus pools used were stored as 1 ml aliquots at −80 °C until needed. Norwalk virus was obtained as stool samples from human volunteers experimentally infected with the well-characterized 8FIIA prototype strain (Jiang et al., 1990) of Norwalk inoculum (C.L. Moe, Department of Epidemiology, University of North Carolina at Chapel Hill). The stool specimens diluted to 10 or 20% suspensions in phosphate-buffered saline (PBS), constituted the Norwalk virus inoculum used in all experiments.

2.2. Food sample processing

To simulate the isolation and purification of viral RNA from processed food samples, uninoculated lettuce and hamburger were prepared using a filtration–extraction–precipitation procedure previously described (Leggitt and Jaykus, 2000). Fifty-gram lettuce and hamburger (plain hamburger in sandwich form) samples were purchased from local commercial sources and processed by the addition of 280 ml of 0.05 M glycine/0.14 N saline buffer (pH 9.0), followed by blending at maximum speed for 90 s in a Servall Omni-Mixer (Ivan Sorvall, Inc, Norwalk, CT). Sample homogenates were filtered through three layers of cheesecloth (American Fiber and Finishing Inc., Albermarle, NC). Hamburger samples were further processed by extraction with a 60% volume of Freon (Fisher Scientific, Suwanee, GA), while lettuce samples were processed without Freon extraction. Clarification was achieved by centrifugation at $3500 \times g$ for 10 min. The resulting supernatants were collected and adjusted to pH 7.2–7.3 using 1 M HCl, supplemented with 5 M NaCl to reach a final concentration of 0.3 M, and precipitated with 6% polyethylene glycol 8000 (PEG) by overnight incubation at 4 °C. The samples were centrifuged at $6000 \times g$ for 20 min at 4 °C, and the pellets were resuspended in 50 mM Tris-0.2% Tween 20 (pH 9.0; 25 ml per food sample). Resuspended precipitates were held at room temperature for 60 min and vortexed every 5 min, followed by centrifugation at $14\,000 \times g$ for 15 min at room temperature. The secondary PEG precipitation step was done by adjusting the pH of the eluant to 7.2–7.3 and 0.3 M NaCl, supplementing with 12% PEG 8000, and incubating at 4 °C for 2–3 h. The precipitates were recovered by centrifugation at $7500 \times g$ for 15 min at 4 °C and resuspended in 50 mM Tris-0.2% Tween 20 (pH 8.0; 2.5 ml per food sample). In order to evaluate RNA extraction and RT-PCR amplification efficiency apart from virus extraction efficiency, these secondary PEG resuspensions were inoculated with either HAV or NV and then assayed by the test RNA extraction and RT-PCR protocols.

2.3. RNA extraction methods

Several RNA extraction methods were compared for overall RNA yield (μg/ml) and purity (A_{260}/A_{280}) and values were statistically analyzed by ANOVA using the Statistical Analysis System software (SAS Institute, Cary, NC) at $P \leq 0.05$. In addition, the efficiency of each RNA extraction method for subsequent nucleic acid amplification was evaluated by serial dilution RT-PCR. Initial experiments were done using 4×10^5 plaque forming units (PFU) of HAV from infected cell culture lysates or 1×10^4 RT-PCR-amplifiable units (RT-PCRU) of NV in 10% fecal specimens. HAV RNA was extracted using three different methods, while four methods were evaluated for NV fecal samples. Final RNA pellets were resuspended in 80 μl of diethyl pyrocarbonate (DEPC)-treated water and stored at −80 °C prior to RT-PCR amplification. Total RNA yield and purity were analyzed spectrophotometrically (Shimadzu UV-260 spectrophotometer, Kyoto, Japan) as previously reported (Sambrook and Russell, 2001).

2.3.1. Extraction 1—GITC (NV and HAV)

Guanidinium isothiocyanate (GITC) extraction was based on the method of Chomczynski and Sacchi (1987). Briefly, extraction solution containing 4 M GITC, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol was combined with the sample at a 1:1 (v/v) ratio and vortexed for 1 min. For further purification, phenol/chloroform (400 μl phenol/250 μl chloroform) was added and the mixture vortexed for 1 min. After centrifugation at $8100 \times g$ for 10 min at room temperature, the RNA-containing upper aqueous phase was re-extracted with 350 μl of chloroform and vortexing for 1 min. After centrifugation again at $8100 \times g$ for 10 min at room temperature, RNA was precipitated with 1 ml of isopropanol supplemented with 40 μl of 2 M sodium acetate (pH 4.0) at −80 °C for 1 h. The RNA was pelleted by centrifugation at $16\,000 \times g$ for 20 min at 4 °C, washed with 1 ml of cold 70% ethanol, and centrifuged again at $16\,000 \times g$ for 15 min at 4 °C. Precipitated RNA was air dried, resuspended in DEPC-treated water, and stored at −80 °C.

2.3.2. Extraction 2—TRIzol (NV and HAV)

The TRIzol® Reagent (Gibco BRL®, Rockville, MD), a commercially available guanidinium–phenol-based solution, was also evaluated. In brief, 1 ml of TRIzol was added to 200 μl of the 10% NV fecal sample, vortexed for 30 s and incubated at room temperature for 5 min. Residual protein was removed by the addition of 200 μl of chloroform, mixing for 30 s, incubation at room temperature for 3 min, and centrifugation for 15 min at $12\,000 \times g$ and 4 °C. The aqueous phase was precipitated in 500 μl of isopropanol by mixing for 15 s, incubation for 10 min at room temperature, and centrifugation for 10 min at $12\,000 \times g$ and 4 °C. The resulting RNA pellet was washed with 1 ml of 75% ethanol and centrifuged for 5 min at $7500 \times g$ and 4 °C. The RNA pellet was air dried, resuspended in 80 μl of DEPC-treated water, and stored at −80 °C.

2.3.3. Extraction 3—spin column (HAV)

RNA isolation by the spin column method was done using the commercially available QIAamp® Viral RNA Mini Kit (QIAGEN®, Valencia, CA) using the procedure described by the manufacturer. In brief, 200 μl of cell culture lysate was mixed with 800 μl of virus lysis buffer, mixed by pulse vortexing for 15 s and allowed to stand at room temperature for 10 min. Absolute ethyl alcohol (800 μl) was added, followed by vortexing for 15 s. The mixture was applied to the spin column in 630 μl aliquots until all of the lysate was loaded. Each application was followed by centrifugation at $5200 \times g$ for 1 min. Prior to eluting the viral RNA, wash buffers were loaded onto the spin column which was then centrifuged at $16\,000 \times g$ for 3 min. The RNA was eluted by addition of two 40 μl aliquots of the proprietary elution buffer, followed by centrifugation at $5200 \times g$ and storage of the eluant at −80 °C.

2.3.4. Extraction 4—shred/spin (NV)

Before extracting NV RNA with the QIAamp® Viral RNA Mini Kit, 200 μl of the 10% NV fecal sample was loaded into a QIAshredder™ Homogenizer (QIAGEN®, Valencia, CA) and centrifuged at $16\,000 \times g$ for 2 min. Approximately 80% (160 μl) of the sample was recovered from the QIAshredder™ Homogenizer. The recovered sample was further processed using Extraction 3 (spin column) procedures as described above, with adjustments made to account for the slightly smaller sample volume.

2.3.5. Extraction 5—TRIzol/shred (NV)

After resuspending the NV RNA pellet from Extraction 2 (TRIzol) in 80 μl of DEPC-treated water, the resuspension was loaded into a QIAshredder™ Homogenizer and centrifuged at $16\,000 \times g$ for 2 min. The resulting RNA sample ($\sim$65 μl) was stored at −80 °C.

2.4. RT-PCR primers and oligoprobes

The oligonucleotide primer and probe sequences used in RT-PCR and hybridization reactions, respectively, are shown in Table 1. For HAV, the genomic region corresponding to the VP1 to VP3 capsid protein interphase was the target for the 192-bp amplicon. For NV, four primer pairs were evaluated within the polymerase

region. The NV polymerase region, shown to be the most conserved among several regions of the NV genome was chosen because it has the advantage of broader detection of different NV strains (Honma et al., 2000).

2.5. RT-PCR

A conventional two-step RT-PCR was performed using the Gene-Amp kit (Applied Biosystems, Foster City, CA) as described in the manufacturer's instructions, except that reaction volumes for RT were increased from 20 to 30 μl to accommodate a 10-μl sample. Total RNA was denatured by heating mixtures at 99 °C for 5 min, followed by the addition of RT (50 U) and RNase inhibitor (20 U). RT was done at 42 °C for 1 h with the corresponding 3′ primer (0.4 μM), followed by heating to 99 °C for 5 min to inactivate the enzymes. After chilling on ice, the tubes were supplemented with 2.5 U of *Taq* DNA polymerase and the corresponding 5′ primer (0.4 μM). For all primer pairs tested, PCR amplifications were performed for 40 cycles with the following amplification parameters: (HAV, 95 °C/1.5 min; 55 °C/1.5 min; and 72 °C/1.5 min); (NV3/51, 40 cycles of 94 °C/0.5 min; 43 °C/0.5 min; and 72 °C/1.5 min; followed by 72 °C/7 min); (JV12/13, 94 °C/1 min; 37 °C/1.5 min; and 74 °C/1 min); (NVp110/NVp36, 40 cycles of 94 °C/1 min; 50 °C/1.3 min; and 72 °C/1 min; followed by 72 °C/15 min); (MR4a/9b, 40 cycles of 95 °C/1 min; 42 °C/1 min; and 72 °C/1.5 min; followed by 72 °C/7 min).

A one-step RT-PCR for HAV and NV detection, combining the cDNA synthesis and PCR in

Table 1
Sequence of RT-PCR oligonucleotide primers and probes

Primer and probe sets	Sequence (5′→3′) (polarity)	Location (bp)	Amplicon size (bp)	Viruses	Reference
Primers					
HAV3	CTCCAGAATCATCTCCAAC (−)	2208–2226	192	HAV	Cohen et al., 1987
HAV5	CAGCACATCAGAAAGGTGA G (+)	2035–2054			Schwab et al., 1995
NV51	GTTGACACAATCTCATCATC (−)	4871–4890	206	NV, SMA, TV	Moe et al., 1994
NV3	GCACCATCTGAGATGGATG T (+)	4685–4704			
JV12	ATACCACTATGATGCAGATT A (+)	4552–4572	327	UK1, 2, 3, 4	Vinje and Koopmans, 1996
JV13	TCATCATCACCATAGAAAGA G (−)	4858–4878		NET/MX	
NVp110	AC(A/T/G)AT(C/T) TCATCATCACCATA (-)	4865–4884	398	UK1, 2, 3, 4	Le Guyader et al., 1996
NVp36	ATAAAAGTTGGCATGAACA (+)	4487–4505			
MR4a	TACCACTATGATGCAGATTA (+)	4553–4572	328	HV, MDV6	Lew et al., 1994
MR9b	ACTATCTCATCATCACCATA (−)	4865–4884		G2 SRSV	
Probes					
NV int	ATGTCATCAGGGTCAAAGA GG (+)	4713–4733			De Leon et al., 1992
HAV int	TGCTCCTCTTTATCATGCTA TG (+)	2171–2192			Cohen et al., 1987 Tsai et al., 1994

a single reaction, was developed. One μl of viral RNA was added to a final reaction volume of 50 μl consisting of 25 μl of MasterAmp 2X PCR Premix A (Epicentre Technologies, Madison, WI), 1 mM dithiothreitol, 0.2% Triton X-100, 2 mM 2-mercaptoethanol, 0.5 μM each of the upstream and downstream primer, 20 U RNase inhibitor, 2.25 U AMV Super Reverse Transcriptase, and 1.25 U *Taq* DNA polymerase. For the one-step RT-PCR, the RT step (42 °C/60 min) was followed by an initial denaturation at 94 °C/3 min and the PCR amplification ensued (HAV—95 °C/1.5 min, 55 °C/1.5 min, and 72 °C/1.5 min; NVp110/NVp36—40 cycles of 94 °C/1 min, 50 °C/1.3 min, and 72 °C/1 min, followed by 72 °C/15 min). In all cases, a 15 μl portion of RT-PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and amplicons visualized with UV light.

2.6. Southern hybridization and immunological detection

RT-PCR products separated on 2% agarose gels were transferred to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN) by the method of Southern (Sambrook and Russell, 2001). The DNA was bound by cross-linking with UV light (Ultraviolet Products, Inc, San Gabriel, CA) for 3–5 min at a distance of 15 cm. Using the DIG Oligonucleotide Tailing Kit (Roche Diagnostics Corporation) oligonucleotide probes for target viruses were 3′-end-labeled with digoxigenin-dUTP as per the manufacturer's instructions. Hybridization was completed using the ExpressHyb™ Hybridization Solution (CLONTECH Laboratories, Inc., Palo Alto, CA). Briefly, membranes were prehybridized for 30 min at 55 °C and then hybridized in the same solution with 75 pmol of labeled probe for 1 h at 55 °C. After hybridization, each filter was washed twice with 2× SSC, twice with 0.1× SSC and five times with 6× SSC at 55 °C and then processed for an enzyme-catalyzed colorimetric reaction using the DIG Nucleic Acid Detection Kit (Roche Diagnostics Corporation) according to the manufacturer's instructions. Immunological detection of RT-PCR-oligoprobe hybrids was performed using an anti-digoxigenin alkaline phosphatase antibody conjugate and enzyme-catalyzed colorimetric reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) as substrates.

3. Results and discussion

3.1. RNA extraction efficiency and purity

For sensitive detection of viruses from foods by RT-PCR, nucleic acid extracts from the food matrix must be as pure as possible. Wilde et al. (1990) reported high susceptibility of reverse transcriptase to interfering or inhibitory substances when detecting target RNA from complex samples, and hence nucleic acid purification is particularly important when detecting RNA viruses in a food matrix. We evaluated several RNA extraction methods with respect to their efficacy in removing RT-PCR inhibitors. Initial experiments were done using HAV cell culture lysate and fecal suspensions of NV. The efficacy of the RNA extraction techniques was evaluated by the concentration and purity of RNA recovered, and the RT-PCR limit of detection as evaluated by serial dilution RT-PCR. For HAV, the yield and purity, 92.7 μg/ml and 1.95, respectively, of RNA extracted from cell culture lysates using the QIAamp® Viral RNA spin column were significantly greater ($P \leq 0.05$) than with the TRIzol and GITC methods (Table 2). Using serial dilutions of a previously quantified HAV cell culture lysate, detection using a 2-step RT-PCR amplification was possible at a 10^{-4} dilution, corresponding to approximately 5 PFU of HAV (data not shown).

Four methods were compared for extraction of NV RNA from fecal specimens. The amount of amplifiable NV RNA was estimated by RT-PCR such that one RT-PCRU was defined as the last dilution of a stool suspension from which NV RNA could be amplified. Therefore, the titer of NV RNA in a sample was estimated as the reciprocal of that dilution. The QIAamp® Viral RNA spin column was evaluated for use in initial studies, however the complexes of fecal material

Table 2
Comparison of viral RNA concentration and purity from five extraction protocols

Virus	Extraction method	RNA (μg/ml)[a]	Purity (A_{260}/A_{280})[a]
HAV	Spin column	92.7 (9.9)^{m}	1.95 (0.20)^{x}
	TRIzol	64.0 (17.4)^{n}	1.47 (0.11)^{y}
	GITC	25.3 (13.3)^{o}	1.48 (0.23)^{y}
NV	TRIzol/shredder	445.9 (106.1)^{m}	1.45 (0.16)^{x}
	TRIzol	375.3 (48.0)^{m}	1.30 (0.02)^{xy}
	GITC	232.3 (41.4)^{n}	1.25 (0.02)^{y}
	Shredder/spin column	52.7 (13.0)^{o}	2.08 (0.10)^{z}

[a] Average values (mean ± S.D.) for RNA yield or RNA purity of three replicate samples. Different superscript letters identify statistically significant differences ($P \leq 0.05$), as analyzed by ANOVA, in mean RNA yield ($^{m, n, o}$) and RNA purity ($^{x, y, z}$) using different RNA extraction and purification methods.

blocked the flow of sample through the column, even at high centrifugation speeds (16 000 × *g*) or when pre-treated with the QIAshredder™ Homogenizer. Although a low volume of NV RNA was able to pass through the column and had a greater ($P < 0.05$) A_{260}/A_{280} ratio than the three other methods (Table 2), the concentration of viral RNA (52.7 μg/ml) was the lowest out of the four methods evaluated and detection was possible only when using 10 μl of NV RNA, which would be equivalent to approximately 10^3 RT-PCRU (Fig. 1). The yield of NV RNA extracted with TRIzol was significantly greater than with the GITC method ($P < 0.05$), but the RNA purities did not differ. Furthermore, 10-fold dilution of NV RNA obtained from both TRIzol and GITC extraction methods was necessary before the RNA could be amplified by RT-PCR, indicating the presence of residual inhibitors (Fig. 1). An additional complication when using the TRIzol or GITC methods was the inability to completely resuspend the NV RNA pellet. In attempts to overcome that issue, the 80 μl RNA sample from the TRIzol extraction was subsequently passed through the QIAshredder™ Homogenizer, which effectively facilitated RNA resuspension in DEPC-water and decreased the level of inhibitors in the final resuspension. The resulting A_{260}/A_{280} ratio (1.45) was better than that resulting from the GITC method, and no RT-PCR inhibition was evident as seen by the ability to amplify NV RNA in undiluted samples (Fig. 1). Furthermore, RT-PCR detection limits after RNA extraction using the TRIzol/Shred method were better than all other RNA extraction protocols, with detection by 2-step RT-PCR amplification at 10^{-3} dilution, corresponding to 1.25 RT-PCRU of NV. Since fecal specimens may be similar to food matrices with respect to inhibitors and microbial flora, the TRIzol/Shred RNA extraction method was chosen for use in subsequent experiments aimed at detection of viral RNA in food matrices.

3.2. RT-PCR primer evaluation

Our group has used routinely the NV3/51

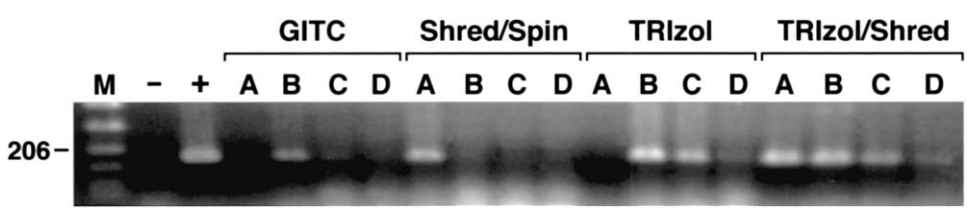

Fig. 1. Comparison of RT-PCR detection limits for Norwalk virus from stool specimens using four different RNA extraction methods: (i) guanidinium isothiocyanate (GITC); (ii) QIAshredder™ Homogenizer followed by QIAamp® Viral RNA Spin Column (shred/spin); (iii) TRIzol® Reagent (TRIzol), and (iv) TRIzol® Reagent followed by QIAshredder™ Homogenizer (TRIzol/shred). Lanes: M, marker; (−), complete reaction cocktail without virus; (+), positive virus control; A, reactions with 10 μl of NV RNA; B, C, and D, reactions with 10 μl of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of each sample treatment, respectively.

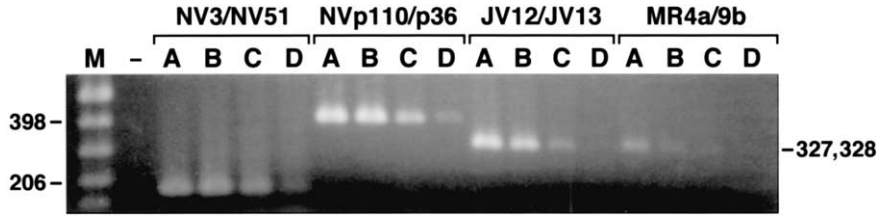

Fig. 2. Comparison of RT-PCR primer pairs for the detection of NV from fecal specimens. The identity of each of the four primer pairs evaluated is given above the figure. RT-PCR products were separated by agarose gel electrophoresis. Lanes: M, marker; (−), complete reaction cocktail without virus; (A) reactions with 10 μl of NV RNA; B, C and D reactions with 10 μl of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of NV RNA, respectively.

primer set during the development of methods to detect NV from foods (Dix and Jaykus, 1998; D'Souza et al., 2000; Leggitt and Jaykus, 2000). Typically, the RNA-dependent RNA polymerase (RDRP) region is the target RT-PCR amplification region because it is the most conserved area of the NLV genome (Green et al., 1994; Wang et al., 1994). After a thorough review of the literature to identify candidate RT-PCR primers targeting the RDRP region (Ando et al., 1995; De Leon et al., 1992; Green et al., 1995a,b; Jiang et al., 1995; Le Guyader et al., 1996; Lew et al., 1994; Moe et al., 1994; Vinje and Koopmans, 1996), we selected four primer pairs (NV3/51; MR4a/9b; NVp110/NVp36; JV12/13) to evaluate in our studies. The criteria for primer selection included a reasonably high annealing temperature, relative lack of primer sequence degeneracy, and broad reactivity. This criteria is essential for application to food systems where high stringency and primer specificity are necessary to prevent nonspecific amplification. While all four primer sets were able to detect NV, the best limit of detection (10^{-3}, i.e. 1.25 RT-PCRU) was seen with the NVp110/NVp36 primer set (Fig. 2). While no single primer pair is able to detect all NLV strains, Honma et al. (2000) recently found the NVp110/NVp36 primer set (Le Guyader et al., 1996) to be the most efficient for broadly detecting NLVs without the need to do separate amplifications as required by the GI and GII primer sets (Ando et al., 1995). An additional benefit of the NVp110/NVp36 primer set is that only one oligonucleotide probe has to be used for subsequent confirmation. Based on these results, the NVp110/NVp36 primer set was used in all subsequent studies.

3.3. RT-PCR protocol evaluation

In our final objective, we sought to develop a one-step RT-PCR protocol to further improve our ability to detect human enteric viruses from foods. This type of method has significant advantages over more traditional two-step RT-PCR protocols, namely, the reduced risk of carry-over contamination between samples, the ability to run the RT reaction at elevated temperatures with the enzyme AMV-reverse transcriptase, reduced labor, and faster detection, particularly when combined with a more rapid (1 h) hybridization protocols as reported in this paper. After optimization of the one-step RT-PCR (data not shown), the detection limits between the one-step and two-step methods were compared, taking advantage of our improvements in RNA extraction cited above. Three-hundred (300) μl volumes of processed hamburger or lettuce concentrates seeded with 10^4 PFU of HAV or 5×10^2 RT-PCRU of NV were extracted for RNA isolation using the TRIzol/shred method, followed by RT-PCR detection, and confirmation of amplicon identity by oligoprobe hybridization. Similar detection limits were achieved with the two-step and one-step RT-PCR methods for HAV seeded into hamburger and lettuce samples (Fig. 3A), however, the target bands obtained with the one-step method were of greater intensity, particularly for lettuce. Likewise, the one-step RT-PCR achieved better detection limits than the two-step method for hamburger and lettuce samples inoculated with NV (Fig. 3B). In this case, an even greater discrepancy was observed with the lettuce samples

seeded with NV, as there was only minimal detection using the two-step method. Overall, detection limits by the one-step RT-PCR method approximated 1.25 PFU per 6 g food sample for HAV, and 0.62 RT-PCRU per 6 g food sample for NV, regardless of food type.

In an effort to better evaluate the detection limits for the combined TRIzol/shredder RNA extraction and the one-step RT-PCR protocol within the food matrix, 300 μl concentrates of hamburger or lettuce were seeded with 1×10^4, 1×10^3, 1×10^2, and 10 PFU of HAV, and processed as described above. Following RT-PCR amplification of viral genomic RNA using the one-step RT-PCR method, HAV could be detected in artificially contaminated hamburger samples at initial inoculum levels of 10 PFU per 6 g food sample (Fig. 4A). Detection limits in lettuce concentrates were one log higher, with 1×10^2 PFU of HAV per 6 g food sample, the lowest inoculum level detected. In similar experiments with the NV, 300 μl hamburger or lettuce concentrates were seeded with 5×10^2, 50, 5, and 5×10^{-1} RT-PCRU of a 20% NV fecal suspension. In this case, as few as 5 PCRU could be detected in hamburger concentrates, and again detection limits in lettuce were one log higher at 50 PCRU per 300 μl sample (corresponding to 6 g food sample) (Fig. 4B). Notably, RT-PCR amplicons could be detected without additional dilution of TRIzol/Shred RNA concentrates, indicating relatively effective removal of residual RT-PCR inhibitors. While RT-PCR products could be visualized by agarose gel electrophoresis, amplicon bands were frequently faint and non-specific amplification occurred in some instances, an issue that has been reported by numerous other investigators (Jaykus et al., 1996; Schwab et al., 2000; Shieh et al., 1999). However, by following amplification with internal oligoprobe hybridization, confirmation of specific amplification could be achieved. While virus detection in lettuce samples was consistently less sensitive than in hamburger, this may be due in part to residual inhibitory

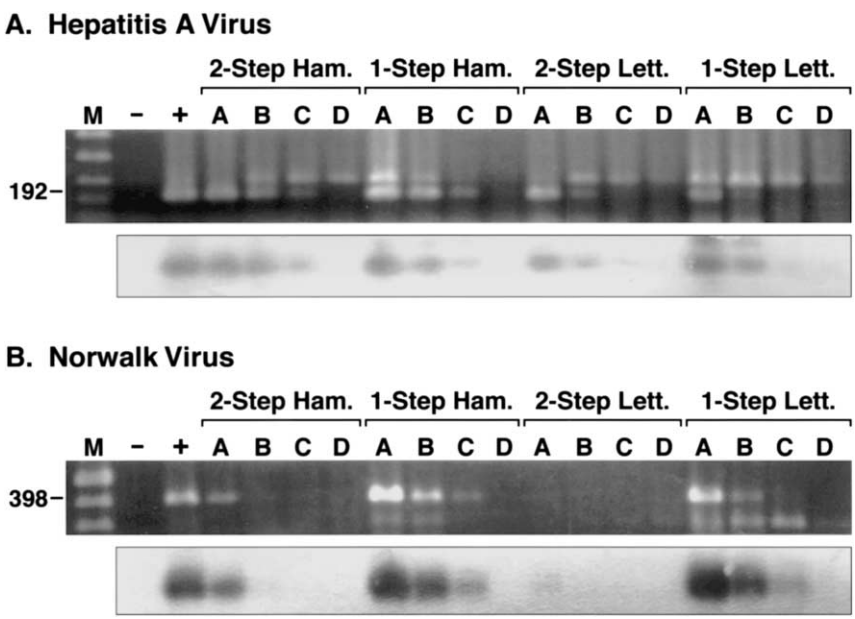

Fig. 3. Comparison of two-step and one-step RT-PCR detection of HAV (Panel A) and NV (Panel B) from hamburger (Ham.) and lettuce (Lett.) samples. RT-PCR products were separated by agarose gel electrophoresis (top) and identity confirmed by Southern transfer and internal oligoprobe hybridization (bottom). Lanes: M, marker; (−), complete reaction cocktail without virus; (+), positive virus control; A, reactions with 10 μl of HAV or NV RNA; B, C, and D, reactions with 10 μl of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of each sample treatment, respectively.

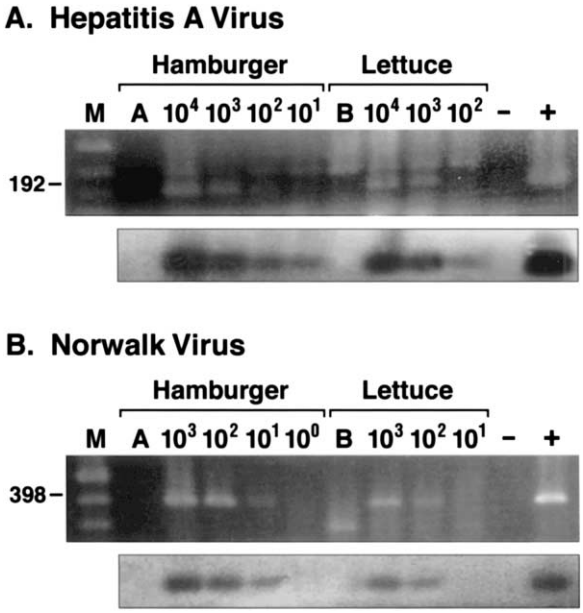

Fig. 4. Detection limits for one-step RT-PCR detection of HAV (Panel A) and NV (Panel B) from processed hamburger and lettuce samples. Three hundred μl of hamburger or lettuce concentrates were inoculated with 10^4, 10^3, 10^2, and 10^1 PFU of HAV or 10^3, 10^2, 10^1, and 10^0 RT-PCRU of NV and further processed by the TRIzol/shredder method to extract total sample RNA followed by one-step RT-PCR. RT-PCR products were separated by agarose gel electrophoresis (top) and confirmed by Southern transfer and internal oligoprobe hybridization (bottom). The corresponding initial inoculum level in processed food samples is given above each lane. Lanes: M, marker; A, uninoculated hamburger sample; B, uninoculated lettuce sample; (−), complete reaction cocktail without virus; (+), positive virus control.

compounds that continue to be associated with plant food matrices.

There have been a variety of methods developed to extract viral RNA from complex sample matrices while simultaneously reducing the level of inhibitors. In the case of foods, most of these methods have been tested on shellfish (Atmar et al., 1995; Dix and Jaykus, 1998; Jaykus et al., 1996; Sugieda et al., 1996), and only a few have been reported for other food commodities (Bidawid et al., 2000; Gouvea et al., 1994; Leggitt and Jaykus, 2000; Schwab et al., 2000). Many of the protocols require multiple steps involving the use of reagents such as guanidinium thiocyanate, polyethylene glycol, cetyltrimethylammonium bromide (CTAB), phenol–chloroform, Sephadex, Pro-Cipitate, and Viraffinity (Dix and Jaykus, 1998; Chung et al., 1996; Gouvea et al., 1994; Leggitt and Jaykus, 2000; Shieh et al., 1999). Gouvea et al. (1994) were the first to describe a method to detect NV from representative food commodities such as orange juice, milk, lettuce, melon and shellfish. Their method, which includes adsorption of RNA to hydroxyapatite and sequential precipitation with CTAB and ethanol, is labor-intensive and RT-PCR products, barely visible in the undiluted extracts, could only be identified after nested PCR. Schwab et al. (2000) reported the use of TRIzol to wash deli meats, including samples artificially contaminated with NV and ones implicated in a foodborne outbreak, for subsequent detection by RT-PCR. While the TRIzol method of Schwab et al. (2000) was reasonably simple, and a detection limit of 10–100 RT-PCRU of NV in surface-inoculated deli meats was reported, a major drawback was the presence of residual RT-PCR inhibitors that necessitated 10–100-fold dilution of RNA extracts prior to RT-PCR amplification. Bidawid et al. (2000) used a combination of virosorb filters and immunomagnetic beads, followed by RT-PCR (F-IM-PCR) to capture, concentrate and detect HAV from experimentally contaminated samples of lettuce and strawberries, but again, the presence of residual RT-PCR inhibitors remained problematic. A significant disadvantage of both TRIzol surface wash and F-IM-PCR methods is that they are impractical for products of complex composition (hamburgers, bakery products) or those of more liquid consistencies (mixed salads) that have to undergo homogenization prior to RNA extraction.

Leggitt and Jaykus (2000) developed a method to concentrate human enteric viruses from lettuce and hamburger samples using an elution-concentration approach followed by a guanidinium RNA extraction and detection by RT-PCR. Viral RNA was detected by RT-PCR at initial inoculum levels $\geq 10^3$ PFU per 50-g food sample for HAV and $\geq 1.5 \times 10^3$ RT-PCRU per 50-g food sample for NV. Their method had significant advantages over other reported approaches, as it was applicable to all types of foods and resulted

in virion concentration prior to detection. However, sample RNA concentrates required 10–100-fold dilution prior to RT-PCR to remove residual inhibitors. In an effort to improve the purity of sample RNA extracts obtained from hamburger and lettuce samples, we adopted the use of the TRIzol reagent followed by treatment of the resuspended RNA through the QIAshredder™ Homogenizer. Other investigators have reported improved detection of enteric viruses in shellfish (Shieh et al., 1999) and fecal matter (Hale et al., 1996) by using an additional silica gel membrane purification after guanidinium thiocyanate extraction. We hypothesize that our use of the QIAshredder™ Homogenizer, after resuspension of the TRIzol RNA pellet, filtered out insoluble debris and reduced the viscosity of the solution caused by high molecular weight cellular components. Previous research has shown that decreasing the viscosity of RNA solutions makes the final preparations more suitable for further biochemical analysis and downstream applications, such as RT-PCR (Groppe and Morse, 1993; Krawetz et al., 1986). The improved limits of detection obtained with our TRIzol/shred method were probably due to the combined effects of reduced sample viscosity and removal of extraneous food matrix associated inhibitors, such as proteoglycans, polysaccharides, and lipids (Groppe and Morse, 1993; Richards, 1999). While silica gel-based methods and the TRIzol/shred method may differ in their fundamental approach, both of them were effective at concentrating and purifying RNA from complex sample matrices. This is extremely important because dilution of RNA effectively reduces RT-PCR detection limits several fold, and investigators have reported that the effect of residual RT-PCR inhibitors is greater at low copy numbers of target nucleic acid (Jaykus et al., 1996). In this study, we demonstrated that improved removal of RT-PCR inhibitors did indeed result in improved assay detection limits.

4. Conclusions

The combined RNA extraction and single tube RT-PCR method described in this study demonstrated significant improvements in removal of inhibitors, reduction in sample manipulations, reduced time in detection and confirmation, and improved assay sensitivity for detection of representative human enteric viruses in non-shellfish food commodities. Future studies will seek to combine this method with novel virus extraction/concentration approaches that are also under development in our laboratories. These improved methods will facilitate sensitive detection of enteric viruses in food matrices at levels anticipated in naturally contaminated products and promote the feasibility of virus detection in foods.

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