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Detection of Noroviruses in Foods: A Study on Virus Extraction Procedures in Foods Implicated in Outbreaks of Human Gastroenteritis

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ABSTRACT

Disease outbreaks in which foods are epidemiologically implicated as the common source are frequently reported. Noroviruses and enteric hepatitis A viruses are among the most prevalent causative agents of foodborne diseases. However, the detection of these viruses in foods other than shellfish is often time-consuming and unsuccessful. In this study, three virus concentration methods were compared: polyethylene glycol (PEG) plus NaCl, ultracentrifugation, and ultrafiltration. Two RNA extraction methods, TRIzol and RNeasy Mini Kit (Qiagen), were compared for detection of viruses in whipped cream and lettuce (as representatives of the dairy and vegetable-fruit food groups, respectively). A seeding experiment with canine calicivirus was conducted to determine the efficiency of each virus extraction procedure. The PEG-NaCl-TRIzol method was most efficient for the detection of viruses in whipped cream and the ultracentrifugation–RNeasy–Mini Kit procedure was best for detection on lettuce. Based on the seeding experiments, food items implicated in norovirus-associated gastroenteritis outbreaks were subjected to the optimal procedure for a specific composition and matrix. No noroviruses were detected in the implicated food items, possibly because the concentration of virus on the food item was too low or because of the presence of inhibitory factors. For each food group, a specific procedure is optimal. Inhibitory factors should be controlled in these procedures because they influence virus detection in food.

Norovirus and hepatitis A are the most common causes of foodborne viral gastroenteritis and hepatitis worldwide *(24, 25).* To a lesser extent, rotaviruses, enteroviruses, astroviruses, and hepatitis E virus also are important *(33, 44–46, 52).* Noroviruses, previously known as Norwalk-like viruses, are a genetically diverse group of RNA viruses belonging to the family *Caliciviridae.* Genetically, noroviruses can be divided into five genogroups (GGI, GGII, GGIII, GGIV, and GGV), which consist of different genotypes. A prototype virus represents each genotype: GGI includes Norwalk virus (GGI-1) and Southampton virus (GGI-2), GGII includes Hawaii virus (GGII-1) and others *(1, 51),* GGIII includes Jena virus, GGIV includes Alphatron, and GGV is found in mice. Norovirus infections can cause vomiting, diarrhea, and nausea within 24 to 48 h after exposure *(34).* Hepatitis A virus causes an acute infection of the liver, with fever, nausea, headache, and jaundice *(7).* Outbreaks of norovirus and hepatitis A virus infections occur throughout the world. In the United States, England, Wales, and The Netherlands, 60 to 70% of the reported gastroenteritis outbreaks are caused by noroviruses *(14, 32, 48, 50).* These outbreaks have occurred in various settings such as nursing homes *(5),* hospitals *(50),* cruise ships *(21),* schools and universities *(23),* and restaurants and events with catered meals *(36).* Transmission has occurred by person-to-person contact *(6)* and through contaminated water *(10, 19)* and food *(20, 36, 37).* Most of the food items implicated in these outbreaks are raw or uncooked, such as oysters, mussels, fruits, vegetables, sandwiches, dairy products, baked products, and salads, which are associated with high risks of infection *(3, 9, 18, 20, 27, 39, 40).* These foods are most likely contaminated through sewage-contaminated surface water or by infected food handlers during harvesting, packaging, or food preparation *(9, 35).* Epidemiological investigations of outbreaks have been hindered by a lack of animal models or cell culture systems for the detection of noroviruses and hepatitis A virus in foods other than shellfish. However, hepatitis A virus and noroviruses can be detected with PCR-based methods *(3, 50).*

A few procedures have been developed for the detection of viruses in foods such as fruits and vegetables *(11),* fresh produce *(8),* ham, turkey, and roast beef *(43),* and lettuce and hamburger *(42).* The efficacy of these procedures for virus detection on lettuce was compared in a round-robin study in France *(29).* The aim of the current study was to develop procedures for the concentration and extraction of human pathogenic viruses from various types of food. The efficacy of the selected procedures for detecting viruses in naturally contaminated food items associated with gastroenteritis outbreaks was determined.

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MATERIALS AND METHODS

Viruses. Canine calicivirus (CaCV) was used in the seeding experiments at a concentration of 2.5×10^6 $TCID_{50}$ (50% tissue culture infective dose) per ml. RNA was extracted from a nonseeded food item as a negative control. Human norovirus genogroup II RNA was used as a positive norovirus reverse transcription (RT) PCR control.

Food samples. Whipped cream and leaf lettuce were both obtained from local commercial sources. Naturally contaminated food items were acquired from outbreaks in which foodborne gastroenteritis was implicated *(48).*

Artificial contamination of whipped cream and leaf lettuce. To be able to directly compare different viral RNA extraction procedures for whipped cream and leaf lettuce, 5 g of each food product was seeded with CaCV at either 2.5×10^4 or 2.5×10^5 $TCID_{50}$ during the first washing step of the virus concentration procedure. For the comparison of procedures, the artificially contaminated food items were tested in duplicate.

Virus concentration and RNA extraction procedures. For the extraction of viruses from foods, three types of virus concentration methods that have been previously used for the isolation of enteric viruses from lettuce *(29)* were compared; polyethylene glycol (PEG)–NaCl precipitation, ultracentrifugation, and ultrafiltration. For the extraction of viral RNA, two RNA extraction methods were selected: a TRIzol method and the RNeasy Mini Kit (Qiagen, Hilden, Germany). To determine the viral input, CaCV at 2.5×10^5 $TCID_{50}$ was extracted with each RNA extraction method.

PEG-NaCl precipitation. Five grams of food product was added to 4 ml of phosphate-buffered saline (PBS; pH 7.2) in a 50-ml tube, vortexed, and rotated for 5 min. The supernatant was decanted into a clean 50-ml tube, and the washing step was repeated. Five milliliters of VertrelXF (1,1,1,2,3,4,4,5,5,5-decafluorpentane, Fluka, Steinheim, Switzerland) *(35)* was added to the supernatant, vortexed, and rotated for 5 min. The sample was centrifuged for 10 min at 5,000 $\times$ *g* at 4°C. After centrifugation, the supernatant was added to a final concentration of 10% PEG–0.3 M NaCl in a clean 50-ml tube. This mixture was rotated during incubation for 2 h at 4°C. After centrifugation for 30 min at 9,500 $\times$ *g* at 4°C, the supernatant was decanted, and the pellet was resolved in either 100 μl of PBS followed by RNA extraction using the RNeasy Mini Kit or in 1 ml of TRIzol.

Ultracentrifugation. Five grams of food product was added to 4 ml of PBS (pH 7.2) in a 50-ml tube, vortexed, and rotated for 5 min. The supernatant was decanted into a clean 50-ml tube, and the PBS wash step was repeated. Five milliliters of VertrelXF was added to the supernatant, vortexed, and incubated with rotation for 30 min at room temperature. Then the sample was centrifuged for 20 min at 13,000 $\times$ *g* at 4°C. The supernatant was decanted into an ultracentrifuge tube (Beckman Coulter, Fullerton, Calif.) and centrifuged for 2 h at 120,000 $\times$ *g*. The supernatant was decanted, and the pellet was resuspended in either 100 μl of PBS followed by RNA extraction using the RNeasy Mini Kit or in 1 ml of TRIzol.

Ultrafiltration. Concentration of viruses can also be achieved by ultrafiltration using Centricon Plus-20 filters (100,000 nominal molecular weight limit; Biomax-100, Amicon, Millipore, Etten-Leur, The Netherlands). Five grams of food product was added to 10 ml of glycine buffer (glycine-NaCl, pH 9.5) in a 50-ml tube and shaken for 15 min at room temperature. The pH was adjusted to 9.5. The supernatant was decanted and centrifuged for 10 min at 10,000 $\times$ *g*. This supernatant was transferred to the microconcentrator (Centricon Plus-20, Amicon) and reduced to a volume of 100 to 200 μl by centrifugation at a maximum speed of 4,000 $\times$ *g* at 4°C for 10 to 40 min, depending on the viscosity of the sample. The virus concentrate was stored at 4°C until extraction of viral RNA with TRIzol or with the RNeasy Mini Kit.

TRIzol RNA extraction. For the extraction of viral RNA, the virus pellet was dissolved in TRIzol with a maximum of 10% sample volume per 1 ml of TRIzol (Invitrogen, Live Technologies, Auckland, New Zealand) and incubated for 5 min at room temperature. After incubation, 200 μl of chloroform per 1 ml of TRIzol was added, and the solution was vortexed and centrifuged for 20 min at 16,100 $\times$ *g* at 4°C. The upper aqueous phase was transferred to a clean Eppendorf tube, and 10 μl of silica *(2)* was added. After vortexing, the mixture was incubated for 30 min at room temperature under rotation. After centrifugation for 30 s at 16,100 $\times$ *g,* the supernatant was decanted. The silica pellet was washed three times with 400 μl of 70% ethanol and once with 400 μl of acetone. After removal of the supernatant, the pellet was dried for 5 min at 56°C. RNase-free water (35 μl) was added, and the silica beads were vortexed and incubated for 30 min at a temperature of 56°C. The sample was centrifuged for 1 min at 16,100 $\times$ *g,* and the supernatant was transferred to a clean Eppendorf tube. Viral RNA was stored at −70°C until further testing.

RNeasy Mini Kit RNA extraction. Viral RNA extraction using the RNeasy Mini Kit was mainly performed as described by the manufacturer (plant and fungi protocol). Viral RNA was resuspended in 35 μl of RNase-free water and stored at −70°C until further testing.

Norovirus and CaCV RT-PCR. For the detection of noroviruses, a single round RT-PCR was employed using the specific forward primer JV12Y and the reverse primer JV13I as previously described *(49).* For the detection of norovirus type Birmingham, the specific forward primer JV12BH (5′-GTTTCATTAT GATGCTGACTA-3′) and reverse primer NVP110 (5′-AC(A/T/G)AT(C/T)TCATCATCACCATA-3′) were selected. For the detection of CaCV, the specific reverse primer YGDD (5′-AATCTCATCATCACCATAAGT-3′) and forward primer BR1 (5′-CTGGGGWTGYGAYGTTGG-3′) were used *(12).* The RT-PCR was performed in 0.6-ml tubes in a thermocycler 480 (Perkin Elmer, Norwalk, Conn.). Complementary DNA synthesis was started by incubating a mixture of 1 μl of primer stock (50 pmol/μl), 3 μl of water, and 5 μl of RNA for 2 min at 95°C and then cooling this mixture at 4°C for 2 min. A total volume of 6 μl of the RT mix was prepared (containing 10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$, 1 mM dNTPs, and 5 U/μl avian myeloblastosis virus reverse transcriptase) and added to 9 μl of the RNA mix. Subsequently, cDNA was heated for 5 min at 95°C and then cooled at 4°C for 5 min. A 5-μl volume of cDNA was added to 45 μl of PCR mix containing 10 mM Tris-HCl (pH 9.2), 75 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U/μl *Taq* polymerase, and 15 pmol of each primer. After RT-PCR, the products were visualized by electrophoresis in a 2% agarose gel. RT-PCR amplification was confirmed by Southern blot hybridization using a mix of four probes specific for human genogroups 1 and 2 (UK3, 5′-GTCCCCTGACATCATACAGGCT-3′; JV5, 5′-TCACCAGA GGTTGTCCAAGC-3′; GG1, 5′-ATGGAYGTTGGYGAYTATG T-3′; and GG2, 5′-GAAYTCCATCRCCCAYTG-3′) *(47)* for the detection of norovirus and a CaCV-specific probe (5′-CAGGTA GGGATCAATATGGA-3′) for the detection of CaCV.

TABLE 1. *Virus concentration methods followed by RNA extraction using either TRIzol or RNeasy Mini Kit*

Method	Elution step	Concentration step	RNA extraction step
PEG-NaCl precipitation	PBS Vertrel XF	PEG-NaCl	TRIzol or RNeasy Mini Kit (Qiagen)
Ultracentrifugation	PBS + Vertrel XF	Ultracentrifugation	TRIzol or RNeasy Mini Kit
Ultrafiltration	Glycine buffer, adjust pH to ≥9.5	Ultrafiltration	TRIzol or RNeasy Mini Kit

Hybridization. For Southern blotting, the CaCV and norovirus RT-PCR products in the agarose gel were transferred to a positively charged nylon membrane (Boehringer, Almere, The Netherlands) by vacuum blotting (Millipore) in a blot buffer containing 0.5 M NaOH and 0.6 M NaCl. Southern blot hybridization of norovirus and CaCV were performed at 42°C. After hybridization, the bound probe was visualized by enhanced chemiluminescence detection (Roche Diagnostics, Almere, The Netherlands).

Sequencing. Positive RT-PCR fragments were processed by purifying the PCR products with a PCR purification kit (Qiagen) and sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction. Nucleotide sequences were edited with Seqman II and aligned by Bionumerics (version 2.0, Applied Maths, Kortrijk, Belgium) using the unweighted pair group method with arithmetic mean (UPGMA) after multiple sequence alignment of a 145-nucleotide segment of the polymerase gene.

RESULTS

Seeding experiments. The most sensitive and standardized procedure for the detection of pathogenic viruses in food items was established by performing seeding experiments. Lettuce and whipped cream were seeded with CaCV at 2.5×10^5 $TCID_{50}$/ml during the first washing step. Virus concentration methods were PEG-NaCl precipitation, ultracentrifugation, or ultrafiltration. A summary of the three virus concentration methods is given in Table 1. RNA was extracted with TRIzol or with the RNeasy Mini Kit. The loss of virus particles during virus concentration and viral RNA extraction procedures was estimated in 10-fold serial endpoint dilutions by CaCV RT-PCR and hybridization. Virus recovery results for lettuce and whipped cream are given in Table 2. For seeded lettuce, a loss of 10 to 1,000 PCR-detectable CaCV units was observed depending on the method, and the ultracentrifugation–RNeasy Mini Kit procedure was the most efficient method. For seeded whipped cream, the same range of virus loss was found. However, the most efficient procedure for this dairy product was achieved by the combination PEG-NaCl precipitation and TRIzol.

Food items implicated in outbreaks. In 2002, food was collected during seven norovirus-associated gastroenteritis outbreaks in a 1-year intensive study of outbreaks of gastroenteritis in The Netherlands (Table 3) *(48).* Of these seven norovirus outbreaks, two were associated with GGII.4 Grimsby viruses and two were associated with GGI.3 Birmingham viruses. In the remaining three outbreaks, noroviruses were not specified (Table 3). A total of 42 food items that might have been involved in the outbreaks were collected. Evidence for involvement of the implicated food item was mainly based on descriptive information and not on analytical case-control or cohort studies.

The food items were categorized: 9 were dairy, 20 were meat, 8 were fruits or vegetables, and 5 were grains (Table 4). Primers JV12Y and JV13I were used for detection of noroviruses in food items associated with outbreaks 1 and 4 through 7, in which GGII.4 Grimsby or unspecified noroviruses were detected in the fecal samples of affected individuals. In outbreaks 2 and 3, norovirus strain GGI.3 Birmingham was implicated as the causative viral agent, which is less well-amplified with these primers and therefore the alternative primer set JV12BH and NVP110 was used. The number of food items implicated in one outbreak ranged from 2 to 17 (Table 4). Those fruit-vegetable or dairy food items that were collected separately were tested using the ultracentrifugation–RNeasy Mini Kit or PEG-NaCl precipitation–TRIzol procedure, respectively, with minor changes (Table 5). For the five mixed-food items containing a dairy component (applesauce, tomato, ginger, pickled ice, and pickled salad), the PEG-NaCl precipitation–TRIzol procedure was applied. For all tested food items, 93% were tested with the PEG-NaCl precipitation–TRIzol procedure and 7% were tested with the ultracentrifugation–RNeasy Mini Kit procedure. Noroviruses were not detected in any of the 42 samples. One food sample produced a PCR fragment of the expected size for norovirus after gel electrophoreses, but this positive signal could not be confirmed by specific hybridization. Sequencing of this

TABLE 2. *RT-PCR results of different CaCV extraction procedures in virus-seeded lettuce and whipped cream*[a]

		TRIzol			RNeasy Mini Kit		
Food	Method	Viral input	Virus recovery	Loss of input virus	Viral input	Virus recovery	Loss of input virus
Lettuce	PEG-NaCl	10^5	10^3	100	10^6	10^4	100
	Ultracentrifugation	10^5	10^3	100	10^5	10^4	10
	Ultrafiltration	10^5	10^2	1,000	10^5	10^3	100
Cream	PEG-NaCl	10^5	10^4	10	10^6	10^4	100
	Ultrafiltration	10^5	10^3	100	10^6	10^3	1,000

[a] Duplicate 10-fold dilutions were tested.

TABLE 3. *Seven norovirus-associated foodborne gastroenteritis outbreaks in 2002 and food items implicated in each outbreak*

Outbreak no.	Outbreak date	Implicated norovirus
1	January 2002	Not specified
2	November 2002	Birmingham
3	September 2002	Birmingham
4	October 2002	Grimsby
5	November 2002	Grimsby
6	November 2002	Not specified
7	November 2002	Not specified

PCR-positive product revealed no norovirus-specific sequence.

To control for possible inhibition in the samples, 40 of the 42 food items tested were seeded with CaCV at 2.5×10^4 $TCID_{50}$. The loss of virus input was determined by analysis of 10-fold serial dilutions of the RNA by RT-PCR followed by hybridization of the RT-PCR products. Virus loss ranged from 1 to 5 log units; 38% of the food items had a 1-log virus loss, 33% had a 2-log loss, 20% had a 3-log loss, 5% had a 4-log loss, and 5% had a 5-log loss (Table 4). Of all food items, the meat products produced the least inhibition with the PEG-NaCl precipitation–TRIzol procedure; from 25% of the items with 1-log virus loss to 3% with a 5-log loss. For grain products, more inhibition was obtained with the same procedure.

Food items with a more than 2-log virus loss (30%) were tested with an alternative procedure. Eleven food items were retested, 10 complex food items with the ultracentrifugation–RNeasy Mini Kit procedure and one homogeneous food item with the ultrafiltration–RNeasy Mini Kit procedure. The virus loss in these retested food items ranged from 1 to 5 log units. One product had a 4-log increase in virus recovery in the second procedure, three food items had a 3-log increase, two food items had a 2-log increase, three food items had a 1-log increase, and only two food items showed no improvement. Spiced and nonspiced food items such as applesauce with cinnamon and pepper pâté had a 5-log virus loss with the PEG-NaCl precipitation–TRIzol procedure. Both food items were retested with the alternative procedure ultracentrifugation–RNeasy Mini Kit, which resulted in a significant improvement in recovery of seeded virus from pepper pâté but not from applesauce with cinnamon. Some food items had low pH, e.g., pickled salad (pH 4.9) and pickled ice (pH 6.2). By adjusting the pH to 9.5 with 1 M NaOH, a 1-log (ice) or 2-log (salad) improvement in virus recovery was achieved. Although virus loss was highly reduced in some cases, no noroviruses were detected in the food items.

DISCUSSION

Several virus concentration and RNA extraction methods were applied to different food items suspected to be causes of various disease outbreaks. Seeding experiments revealed that for food groups, such as dairy and fruits or vegetables, virus concentration methods and RNA extraction methods differed in the efficiency of virus recovery. These differences may be explained either by the way the food item was prepared, e.g., cooking or mashing, the composition of the food item, e.g., the degree of fats, proteins, or polysaccharides, or the matrix of the food item, e.g., solid or liquid. The virus concentration methods compared in this study were precipitation, ultracentrifugation, and ultrafiltration. However, not all of these methods were appropriate for all kinds of food. For instance, a virus concentration procedure based on filtration can be more successfully used on food items with a solid matrix, which produce a clear eluate after the virus is washed from the food item. Products with high amounts of fat are not ultracentrifuged because excessive fat components will accumulate on top of the supernatant, making it more difficult to remove the supernatant and subsequently extract viral RNA. We found that the optimal method for concentration of whipped cream (a dairy product) was PEG precipitation, because filters get clogged and centrifugation yields a voluminous pellet. The ultracentrifugation concentration method was optimal for lettuce, a food item with a solid matrix that may be washed off and whose eluate can be concentrated with ultracentrifugation. RNA extraction from lettuce eluate was more efficient with the RNeasy Mini Kit than with TRIzol probably because inhibitors are less well-extracted with the silica beads. However, RNA extraction by use of TRIzol was preferred over the RNeasy Mini Kit to remove fat components and proteins, which are present in meat and dairy products. RNA extraction by use of the RNeasy Mini Kit is specialized for removal of polysaccharides, which are present in food items such as vegetables and shellfish.

In this study, food items were categorized into four groups: dairy (9 items), meat (20 items), fruit or vegetable (8 items), and grain (5 items). A fifth category not analyzed in this study is beverages. Seeding experiments were done to develop efficient procedures for virus detection in lettuce and whipped cream as representatives of the fruits and vegetables and the dairy products, respectively. In the viral disease outbreaks, however, many other food items were implicated, and the samples were often mixed during collection. The efficiency of the procedures for the other food groups (meat, grains, and beverages) was not tested in seeding experiments. However, meat processed by the PEG-NaCl precipitation–TRIzol procedure had the least inhibition compared with the other food items, such as grains. A rapid standardized procedure was developed previously for shellfish *(31).* In the present study, inhibition of virus detection in foods was determined by adding external virus to the food item. A better way to control for inhibition would be the addition of an internal RNA control *(38).* Such an approach was applied for the development of internal controls for norovirus and rotavirus in water samples by amplifying a β-globine fragment with β-globine primers coupled with norovirus or rotavirus primers *(30).* The primer–β-globine DNA products were cloned, and RNA was synthesized with T7 RNA polymerase. These internal controls could be very useful for future optimization of virus concentration and RNA extraction procedures and for investigating gastroenteritis outbreaks.

Because food items are not expected to be contaminated with high concentrations of pathogenic viruses, in-

TABLE 4. *Food items implicated in norovirus-associated gastroenteritis outbreaks*

Procedure 1	Food group[a]	Food item	Outbreak no.[b]	Virus loss	Procedure 2[c]	Virus loss
PEG-TRIzol	D	Cheese	3	10	NT	NT
		Whipped cream	1	10	NT	NT
		Chocolate mousse	4	10	NT	NT
		Cream cheese	5	100	NT	NT
		Vanilla ice	3	1,000	UC/RNeasy	10
		Yogurt + applesauce	4	1,000	UC/RNeasy	100
		Strawberry ice	3	1,000	UC/RNeasy	1,000
		Whipped cream	2	NS[d]	NS	NS
		Strawberry + whipped cream	2	NS	NS	NS
	G	Macaroni	4	100	NT	NT
		Pancake	5	100	NT	NT
		Rye bread	5	100	NT	NT
		White bread	3	100	NT	NT
		Potatoes	3	1,000	UC/RNeasy	10
	M	Meat with gravy	4	10	NT	NT
		Codfish	6	10	NT	NT
		Salmon salad	7	10	NT	NT
		Meat + breadcrumbs	4	10	NT	NT
		Meat slices	3	10	NT	NT
		Meat slices	5	10	NT	NT
		Chicken stew	6	10	NT	NT
		Duck mousse	7	10	NT	NT
		Eel	1	10	NT	NT
		Salad (Russian)	3	10	NT	NT
		Boiled egg	3	100	NT	NT
		Boiled egg	5	100	NT	NT
		Meatball + sauce	5	100	NT	NT
		Smoked salmon	7	100	NT	NT
		Salmon + cheese	5	100	NT	NT
		Salmon	3	1,000	UC/RNeasy	0
		Sausage	3	1,000	UC/RNeasy	0
		Crab salad	7	1,000	UC/RNeasy	100
		Pork	3	1,000	UC/RNeasy	100
		Pepper paté	3	100,000	UC/RNeasy	10
	V	Tomato	3	10	NT	NT
		Ginger	3	10	NT	NT
		Pickled ice, pH 6.2	3	100	NT	NT
		Pickled salad, pH 4.9	3	10,000	NT	NT
		Applesauce + cinnamon	3	100,000	UC/RNeasy	100,000
Ultracentrifugation-RNeasy	V	Vegetable soup	4	100	NT	NT
		Cauliflower	4	100	NT	NT
		Leek	4	10,000	UF/RNeasy	10

[a] D, dairy; M, meat; V, vegetables; G, grains.
[b] For outbreak number, see Table 3.
[c] NT, not tested with the alternative procedure; UC, ultracentrifugation; UF, ultrafiltration.
[d] NS, not tested in the seeding experiment.

hibition or viral RNA loss of 1 to 5 log units indicates insufficient sensitivity. Additional steps are needed for the removal of inhibitors from food extracts to allow detection of these low virus concentrations. Recent advances in method development should be incorporated in the proposed procedure to further improve virus detection in food items. For instance, the recent availability of magnetic silica beads resulted in a significant improvement in removal of inhibitors during extraction of RNA from concentrated water samples *(41)*. Such methods also may lead to improvements in procedures for detection of viruses in foods.

The food items analyzed for the presence of pathogenic viruses were collected from norovirus-associated gastroenteritis outbreaks and therefore had a reasonable chance of containing norovirus. However, all food items tested were negative for the virus. This negative result could be due to coprecipitation of inhibitors during virus concentration and RNA extraction, or the wrong portion of the food item may

TABLE 5. *Adjustments to washing steps 1 and 2 for the three virus concentration methods*

Method	Wash step 1	Wash step 2	Concentration step
PEG-NaCl precipitation	4 ml of PBS, vortex, incubate 10 min at room temp, centrifuge 10 min at 4,000 × *g* (twice)	5 ml of Vertrel, vortex, incubate 10 min at room temp, centrifuge 10 min at 4,300 × *g*	
Ultracentrifugation	4 ml of PBS, vortex, incubate 10 min at room temp, centrifuge 10 min at 4,000 × *g* (twice)	5 ml of Vertrel, vortex, incubate 10 min at room temp, centrifuge 10 min at 4,300 × *g*	Ultracentrifugation 3 h at 120,000 × *g*
Ultrafiltration	Glycine buffer, adjust pH to ≥9.5, incubate 10 min at room temp, centrifuge 10 min at 4,000 × *g* (twice)	Centrifuge 10 min at 4,300 × *g*	

have been tested because of heterogeneous contamination from food handling. Because the infectious dose of norovirus can be as low as a few particles *(16),* these few particles could easily be lost during the multiple virus concentration and extraction steps. Another possible problem is that the true contaminated food item might not have been available for testing. Food items were implicated as causes of outbreaks mainly based on descriptive information and not on analytical case-control or cohort studies. In our study, other generic primer sets were applied if lower sensitivity of detection was expected based on information on norovirus variants detected in fecal samples of affected individuals. However, specific primer sets could be selected and tested, though this requires more time in large outbreaks. Such an approach was successfully employed in an outbreak related to contaminated water *(19)* and raspberries *(28).*

Quantitative detection of viruses has been achieved by various assays such as nucleic acid sequence–based amplification, Lightcycler, and Taqman *(15, 22, 26, 41).* Quantitative virus detection in foods is important for estimation of the risk of infection from food consumption. Dutch legislation concerning pathogens in drinking water requires producers to limit the yearly infection risk to less than 1 in 10,000 exposed consumers. Policy makers should decide on an acceptable risk of infection from consumption of virus-contaminated foods. Because PCR-detectable units do not necessarily represent infectious particles, additional information on the infectivity of the detected virus is needed to prevent overestimation of the infection risk associated with consumption of a specific food item. However, natural hepatitis A virus isolates are not easy to culture in vitro, and noroviruses currently cannot be cultured at all *(13).*

This study was conducted to address the detection of one of the most important foodborne viruses, norovirus. However, other important food- and waterborne viruses causing either serious illness or large disease outbreaks should be considered. For instance, hepatitis A and hepatitis E viruses have been associated previously with serious foodborne diseases *(4, 45).* Recently, enteroviruses caused meningitis in hundreds of bathers in Germany *(17).* In The Netherlands, enteroviruses have been found in surface waters *(41)* and oysters *(31).*

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