Survival and Transfer of Murine Norovirus 1, a Surrogate for Human Noroviruses, during the Production Process of Deep-Frozen Onions and Spinach

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ABSTRACT

The reduction of murine norovirus 1 (MNV-1) on onions and spinach by washing was investigated as was the risk of contamination during the washing procedure. To decontaminate wash water, the industrial sanitizer peracetic acid (PAA) was added to the water, and the survival of MNV-1 was determined. In contrast to onions, spinach undergoes a heat treatment before freezing. Therefore, the resistance of MNV-1 to blanching of spinach was examined. MNV-1 genomic copies were detected with a real-time reverse transcription PCR assay in PAA-treated water and blanched spinach, and PFUs (representing infectious MNV-1 units) were determined with a plaque assay. A ≤1-log reduction in MNV-1 PFUs was achieved by washing onion bulbs and spinach leaves. More than 3 log PFU of MNV-1 was transmitted to onion bulbs and spinach leaves when these vegetables were washed in water containing approximately 5 log PFU/ml. No decline of MNV-1 occurred in used industrial spinach wash water after 6 days at room temperature. A concentration of 20 ppm of PAA in demineralized water (pH 4.13) and in potable water (pH 7.70) resulted in reductions of 2.88 ± 0.25 and 2.41 ± 0.18 log PFU, respectively, after 5 min of exposure, but no decrease in number of genomic copies was observed. No reduction of MNV-1 PFUs was observed on frozen onions or spinach during storage for 6 months. Blanching spinach (80°C for 1 min) resulted in at least 2.44-log reductions of infectious MNV-1, but many genomic copies were still present.

Outbreaks due to norovirus (NoV) infection occur frequently. Cruise ships (45), schools (16), and hospitals (32) are common venues for large-scale NoV outbreaks. Epidemiological researchers have successfully confirmed in many cases NoV in stool samples (23, 40). However, the primary source of infection remains mostly unclear (44). In several cases, food or water was assumed to be the most probable cause of the outbreak while the high secondary infection rate is responsible for most outbreaks in semi-closed settings and institutions (4). Foodborne transmission is associated with fresh produce and ready-to-eat foods (i) through improper handling by symptomatic or asymptomatic infected food service workers (14, 29) and (ii) through contact with feces-contaminated water in the field or during the production process. The incidence of NoV infection outbreaks due to fresh produce is low but probably underestimated (22). Nevertheless, outbreaks associated with coleslaw, tossed salad (48), green salads (25), green onions (47), fresh-cut fruit (26), frozen red fruits (17, 24, 30), and potato salad (36) have been reported. The frequent occurrence and the extent of outbreaks are indications of the stability and persistence of NoV particles in the environment. Resistance of NoVs to environmental disinfectants has been reported (20, 27). However, less information is available on NoV survival and transmission during industrial food processing activities. In the current study, critical steps, e.g., washing, blanching, and peracetic acid (PAA) rinse, used to decontaminate vegetables in an industrial plant that produces deep-frozen onions and spinach were tested. Deep-frozen onions were chosen because onions have been implicated in viral disease outbreaks (47). No heat treatment is involved during onion processing, and the final product can be eaten raw by the consumer by adding thawed shredded onions to a salad. Deep-frozen spinach was studied as a representative green leafy vegetable that is, in contrast to onions, heated before deep-freezing.

Because human NoVs are not culturable in a cell culturing system, no quantitative data could be obtained. A three-dimensional cell culture system reported for human NoVs could be further evaluated in the future as a tool for NoV inactivation studies (43). Murine norovirus 1 (MNV-1) was used as a NoV surrogate in the present study because it is highly similar to human NoVs; it has a similar size, shape, buoyant density, and genomic organization (50). MNV-1 persistence in water and stability under acid conditions makes MNV-1 a better surrogate than feline calicivirus, which is frequently used as a NoV surrogate (5, 15).

Decontamination by washing of MNV-1–inoculated onion bulbs was examined and compared with that obtained by washing MNV-1–inoculated spinach leaves. Contamination of vegetables during the washing step by contact
with infected wash water was also tested. The persistence of MNV-1 was followed in potable water (PW) and used industrial spinach wash water that was stored for 1 week at room temperature. The survival of MNV-1 on frozen shredded onions and chopped spinach was followed for 6 months. The effect of PAA in water on the inactivation of MNV-1 and the survival of MNV-1 after blanching of spinach for 1 min at 80 and 90°C were also determined. Infectious MNV-1 was detected with a plaque assay. In addition, the number of MNV-1 genomic copies was determined from blanched spinach samples and PAA-treated water samples. The objective of this study was to extend our knowledge of the effect on MNV-1, a surrogate for human NoV, of decontamination and inactivation methods used during processing of vegetables.

MATERIALS AND METHODS

Cell culture. Cells of the murine macrophage cell line RAW 264.7 (ATCC TIB-71; kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, St. Louis, Mo.) were maintained in Dulbecco’s minimal essential medium (Cellgro, Mediatech, Herndon, Va.) containing 10% fetal bovine serum (HyClone, Logan, Utah), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, Calif.), 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES; Invitrogen), and 2 mM l-glutamine (Invitrogen) and grown at 37°C under a 5% CO₂ atmosphere.

MNV-1 lysate. RAW 264.7 cells were infected with MNV-1.CW1, passage 5 at a multiplicity of infection of 0.05 (MNV-1:cells) for 2 days. After two freeze-thaw cycles, low-speed centrifugation was used to remove cellular debris from the virus lysate, as described by Wobus et al. (49). The supernatant (i.e., MNV-1 lysate) was stored in aliquots at −75°C.

MNV-1 plaque assay. The number of MNV-1 PFU per milliliter was determined with a plaque assay, as described by Wobus et al. (49).

Inoculation of onion bulbs and spinach leaves. Onion bulbs of the variety Hyskin (The Netherlands) were bought in a local supermarket. One sample contained two peeled onion bulbs in a stomacher bag. Onion bulbs with a diameter of 3.9 to 4.7 cm were selected to maintain a similar size and surface area for exposure to MNV-1. Each sample was inoculated with 2 ml of MNV-1 lysate that was diluted (1:5) in phosphate-buffered saline (PBS; Cellgro), to obtain a final concentration of approximately 6.3 log PFU/ml. The stomacher bags were closed and placed on a shaking platform (rotating at 120 rpm; HS 260, Janke & Kunkel & Co., IKA Labortechnik, Staufen, Germany) overnight at 4°C.

In a similar way, 10 or 50 g of spinach leaves was transferred to stomacher bags to which 1 or 2.5 ml, respectively, of MNV-1 lysate (1:5 dilution in PBS for a final concentration of approximately 6.3 log PFU/ml) was added by drops. The inoculated bags were kept overnight at 4°C.

Virus extraction. MNV-1 was extracted according to a previously described method with some modifications (6). Inoculated spinach or onion bulbs were washed with 100 ml of elution buffer (0.1 M Tris-HCl [Sigma, St. Louis, Mo.], 3% beef extract powder [Sigma], and 0.05 M glycine [Sigma]; pH 9.5) in a stomacher bag with a filter compartment (full filter blender bag, 190 by 300 mm, FBAG-04; Novolab, Geraardsbergen, Belgium) on a shaking platform (rotating at 120 rpm) for 20 min. The filtrate was centrifuged at 10,000 × g for 15 min at 4°C, and the pH of the supernatant was adjusted to 7.2 to 7.4. Polyethylene glycol (PEG) 6000 and NaCl were added to obtain a final concentration of 10% PEG and 0.3 M NaCl. The samples were placed on a shaking platform (rotating at 120 rpm) overnight at 4°C. The next day, the samples were centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was discarded, and the pellet was dissolved in 1 ml of PBS. Virus extracts were stored at −75°C while the number of PFU were determined with the MNV-1 plaque assay. The results obtained with the plaque assay as PFU per milliliter actually represent PFU per two onion bulbs or per 10 g of spinach leaves after the washing procedure. The blanching procedure was tested on 50 g of spinach; thus, the final level is number of PFU per 50 g of spinach.

Washing of onion bulbs. Decontamination from washing was tested with samples that each contained two inoculated onion bulbs. MNV-1–inoculated onion bulbs were aseptically removed from the stomacher bag and transferred to 100 ml of PW for 25 s on a shaking platform. The rinsed onion bulbs were then subjected directly to virus extraction. Three replicates were included, and experiments were repeated three times. Three inoculated samples without a washing step were included as positive controls, and noninoculated samples were included as negative controls.

Contamination of onion bulbs during washing was examined by inoculating 100 ml of PW with 1 ml of MNV-1 lysate (containing approximately 7.0 log PFU/ml). Samples of two onion bulbs (noninoculated) were washed for 25 s and directly subjected to virus extraction. The titer of the infected wash water used for the washing of onion bulbs was directly determined with the MNV-1 plaque assay without a prior virus extraction step.

Washing of spinach. Washing procedures of one or three steps were compared. Inoculated spinach leaves (10 g) were transferred to an empty disinfected container, 350 ml of PW was added, and the spinach leaves were washed for 2 min. The leaves were spun for 1 min in a salad spinner to remove the excess water and then subjected to the virus extraction procedure.

This procedure was repeated three times for the three-step washing procedure. Disinfected working materials were used between every washing and drying step. Washed noninoculated spinach leaves were used as negative controls, and inoculated spinach leaves without a washing procedure were used as positive controls. Three replicates of the samples and controls were analyzed in three independent experiments.

The risk of contamination during washing of spinach in contaminated water was investigated by inoculating 350 ml of PW with 3.5 ml of MNV-1 lysate (containing approximately 7.0 log PFU/ml). Noninoculated spinach leaves (10 g) were washed for 2 min in the infected water, dried, and subjected to the virus extraction procedure. The titer of the infected water used for washing the spinach was directly determined by an MNV-1 plaque assay without a virus extraction step.

PAA. PAA solutions were freshly prepared for each experiment, and the stock solution of PAA (PERADES 150, Tensio, Beveren, Belgium) was titrated before every experiment. The percentage of H₂O₂ was determined by titrating with 0.1 N Ce(SO₄)₂·4H₂O (Sigma) and a ferroin indicator, which consisted of 6.96 mg/ml FeSO₄·7H₂O (Sigma) and 14.8 mg/ml o-phenanthroline monohydrate (Acros Chimica, Geel, Belgium). Potassium iodide (Sigma) and starch (2 g/liter; BD, Le Pont de Claux, France) were then added. The actual concentration of PAA was titrated with 0.1 N Na₂S₂O₃ (Sigma). PAA concentrations of 20, 50, and 150 ppm were prepared in demineralized water (DW) and PW; the PW was obtained from an industrial plant in Belgium. Both
DW (pH 5.62) and PW (pH 8.18) were investigated to test the influence of pH on the reactivity of PAA with MNV-1. Ten milliliters of each concentration was mixed with 1 ml of MNV-1 lysate (containing approximately 7.0 log PFU/ml). After 2, 5, 10, and 15 min, the reaction was stopped by adding the same molar concentration of l-cysteine hydrochloride monohydrate (Sigma, Steinhamm, Switzerland) in a 1:1 ratio. After 2, 5, 10, and 15 min, 0.5 ml of sample was transferred to a microcentrifuge tube, and 0.5 ml of l-cysteine hydrochloride monohydrate at 0.346, 0.115, and 0.046 g/liter was added to samples treated with 150, 50, and 20 ppm PAA, respectively. Two independent experiments were performed, each with two replicates.

Blanching of spinach. Fifty grams of inoculated spinach leaves was added to 300 ml of PW at 80 or 90°C. After 1 min, the hot water was poured off and the spinach was immediately cooled in ice water (4°C) for 1 min. The blanched leaves were dried in a salad spinner (1 min) and directly subjected to the virus extraction procedure. Fifty grams of either inoculated spinach leaves (positive control) or noninoculated spinach leaves (negative control) was added to 300 ml of PW at room temperature. After 1 min, the water was poured off and the spinach was immediately cooled in ice water (4°C) for 1 min. The blanched leaves were dried in a salad spinner and directly subjected to the virus extraction procedure. Experiments were repeated three times with three replicates of samples and controls.

Peroxidase test. The peroxidase activity was determined using the method described by Ponce et al. (37). The enzyme extract was prepared by mixing spinach (10 g) with 30 ml of water for 3 min with a blender. An additional 20 ml of water was used to rinse the blender. These samples were centrifuged (10,000 x g for 15 min at 4°C), and the supernatant containing the peroxidase enzyme was further used. Peroxidase activity was assessed by mixing 0.1 ml of enzyme extract with 2.9 ml of substrate mixture: 10 ml of 1% guaiacol (Merck, Darmstadt, Germany), 10 ml of 0.3% H2O2 (Sigma), and 100 ml of Na3PO4 (Sigma). Activity was measured at 470 nm with a spectrophotometer (Cary 50 series, Varian, Palo Alto, Calif.).

Persistence of MNV-1 in PW and in used industrial wash water for spinach. PW (100 ml) and used wash water from a industrial spinach production line (100 ml) were inoculated with 1 ml of MNV-1 lysate (containing approximately 7.0 log PFU/ml). Samples were taken two times a day and stored at −75°C until the PFU per milliliter was determined by MNV-1 plaque assay.

Microbiological analysis of used industrial wash water for spinach. Counts for total aerobic bacteria, lactic acid bacteria, and yeasts and molds were determined by standard methods (1–3).

Survival of MNV-1 on deep-frozen shredded onions and chopped spinach. Shredded onions (10 g) and chopped blanched spinach (50 g) obtained from a Belgian industrial plant at the end of the production process but before deep freezing were inoculated with 1 and 2.5 ml, respectively, of MNV-1 lysate diluted in PBS (approximately 6.3 log PFU/ml). The inoculated samples were frozen at −21°C. Three samples of spinach and onions were taken monthly during 6 months and subjected to the virus extraction procedure. Chopped spinach samples were dissolved in 2 ml instead of 1 ml of PBS after the virus extraction step. Therefore, results from the MNV-1 plaque assay were multiplied by 2 to obtain the correct PFU per milliliter.

MNV-1 real-time RT-PCR assay. The number of MNV-1 genomic copies present in blanched spinach samples and PAA-treated water samples was determined. RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations. For spinach, RNA extraction was performed on samples that had been subjected to the virus extraction procedure. For PAA-treated water, RNA extraction was done after adding the neutralizing agent l-cysteine hydrochloride monohydrate (i.e., without the virus extraction step). The cDNA synthesis and real-time reverse transcription PCR (RT-PCR) assay of MNV-1 was carried out as described previously (7).

Statistical analysis. The significance of the differences in the number of MNV-1 particles found on washed and unwashed onion bulbs and washed and unwashed spinach leaves were calculated (t test, \( P < 0.05 \)) with SPSS 15.0, Windows (SPSS Inc., Chicago, IL.). A one-way analysis of variance (Games-Howell, \( P < 0.05 \)) was used to calculate the significance of the reductions in MNV-1 achieved with PAA in DW and PW, by storing shredded onions and chopped spinach in the freezer for up to 6 months, and by blanching spinach.

RESULTS
Decontamination and contamination due to washing of onion bulbs or spinach leaves. Figure 1A illustrates the effect of washing two onion bulbs contaminated with MNV-1 at 4.90 ± 0.26 log PFU. After washing for 25 s, 4.51 ± 0.33 log PFU was recovered from the two onion
FIGURE 2. Contamination from washing onion bulbs and spinach leaves in water containing MNV-1. (A) Water inoculated with MNV-1 (open bar) and washed uninoculated onion bulbs (shaded bar). (B) Water inoculated with MNV-1 (open bar) and washed uninoculated spinach leaves (shaded bar). MNV-1 results are log PFU per milliliter. Error bars are the standard deviations from three experiments with three replicates each.

FIGURE 3. Survival of MNV-1 (log PFU/ml) in potable water (H17005) and used spinach wash water (H12135) during 1 week at room temperature. Error bars are the standard deviations from two experiments with one replicate each.

bulbs, resulting in a mean 0.39-log reduction. Washed onion bulbs had significantly lower numbers of MNV-1 PFU compared with unwashed bulbs (P < 0.05). Figure 1B depicts the effect of washing spinach leaves. Spinach leaves (10 g) contaminated with 4.95 ± 0.38 log PFU before washing contained 3.94 ± 0.41 log PFU/10 g after one washing step of 2 min (i.e., mean 1.01-log reduction), which was significantly lower than the PFU found on unwashed leaves (P < 0.05). For spinach leaves that were washed three times, 3.69 ± 0.18 log PFU/10 g was found by plaque assay (i.e., mean 1.26-log reduction). The reduction obtained with the three-step washing procedure was not significantly different from that obtained with the one-step procedure (P > 0.05).

Additionally, the effect of washing produce in MNV-1–contaminated water was investigated. Figure 2A shows the results when two uninoculated onion bulbs were washed in MNV-1–contaminated water containing 4.98 ± 0.21 log PFU/ml. In this case, 3.21 ± 0.58 log PFU was transferred to the two onion bulbs. When uninoculated spinach leaves were washed in water infected with MNV-1, 3.74 ± 0.28 log PFU was detected in 10 g of spinach leaves.

Persistence of MNV-1 in PW and in used industrial spinach wash water. PW inoculated with MNV-1 at 4.78 ± 0.12 log PFU/ml was stored at room temperature. No decline in titer was found after 1 week (Fig. 3). The survival of MNV-1 in used industrial spinach wash water was followed in parallel and remained stable until day 6. A 0.79-log decrease was found between days 6 and 7. Microbiological parameters of the used wash water were analyzed on day 0. The counts of total aerobic bacteria, lactic acid bacteria, and yeasts and molds were 5.79, 3.34, and 3.32 log CFU/ml, respectively.

Inactivation of MNV-1 by PAA. The effect of PAA as a function of time and pH is shown in Figure 4 in a three-dimensional graph. No difference in MNV-1 titer (5.96 ± 0.07 and 5.94 ± 0.12 log PFU/ml) was found between DW (pH 5.62) and PW (pH 8.18), respectively, without PAA. At 20 ppm of PAA in DW with a pH of 4.13, a 2.88 ± 0.25-log reduction was obtained after 5 min of contact with DW. However, in PW, the titer reduction was only 1.60 ± 0.42 log PFU/ml after 5 min of contact. Moreover, the pH of PW significantly decreased due to PAA (pH 5.99 without PAA to pH 5.63 with 20 ppm PAA). This suggests that PAA in PW is less effective in inactivating MNV-1 compared to DW.
TABLE 1. MNV-1 in demineralized water treated with 20 and 150 ppm peracetic acid (PAA) as detected with a plaque assay and a real-time RT-PCR assay

<table>
<thead>
<tr>
<th>PAA (ppm)</th>
<th>Time (min)</th>
<th>Plaque assay (log PFU/ml)</th>
<th>RT-PCR (log genomic copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>5.96 ± 0.07</td>
<td>8.20 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.08 ± 0.25</td>
<td>8.61 ± 0.11</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>2.80 ± 0.13</td>
<td>8.57 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.76 ± 0.08</td>
<td>8.64 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.88 ± 0.33</td>
<td>8.53 ± 0.08</td>
</tr>
<tr>
<td>150</td>
<td>10</td>
<td>1.67 ± 0.15</td>
<td>8.36 ± 0.38</td>
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<tr>
<td></td>
<td>15</td>
<td>1.70 ± 0.20</td>
<td>8.50 ± 0.04</td>
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a Values are mean ± standard deviation of two experiments with two replicates each.
b Values are mean ± standard deviation of two experiments with one replicate each.

blanched at the two temperatures (P > 0.05), but a significant difference was found in the number of genomic copies derived from blanched and fresh spinach samples (P < 0.05). The peroxidase activity remaining after the blanching procedure at 80 and 90°C is shown in Table 2 as an indicator of the extent of heating. This measure can be used to compare industrial blanching procedures. The peroxidase activity decreased by a mean of 35.6% when spinach was blanched at 80°C, whereas 78.8% of the activity was lost at 90°C.

Survival of MNV-1 on frozen shredded onions and chopped spinach. No actual decline of log PFU MNV-1 was observed during a 6-month storage period for deep-frozen spinach or onion samples (Fig. 5). The number of MNV-1 determined after 6 months of storage on deep-frozen shredded onions was significantly different from the number for onions stored for 2, 4, and 5 months (P < 0.05) but did not differ from the number obtained after 1 and 3 months of storage (P > 0.05).

For spinach, similar numbers of MNV-1 were found after 1, 2, 3, 4, and 6 months of storage (P > 0.05), but...
these numbers differed significantly from those obtained at 5 months ($P < 0.05$).

**DISCUSSION**

Preharvest and postharvest contamination of vegetables is responsible for outbreaks associated with the consumption of fresh produce. The use of waste for fertilizer and unsanitary water for irrigation can spread enteric pathogens into fields and consequently to vegetables (11). Leafy vegetables provide large surfaces for contact with water and for attachment of microorganisms (42). Ward and Irving (46) found that poliovirus survived 13 days on spinach after this crop was irrigated for 2 h with seeded wastewater. The first postharvesting processing steps of spinach remove insects, adhering soil, and dirt from the leaves. However, washing has little effect on microbial contaminants (10, 41). Onions are first manually peeled by a worker. Bidawid et al. (12) reported that feline calicivirus was transferred from soiled finger pads to ham (46%) and lettuce (18%) within 10 s contact time. Therefore, the food handler plays an important role in viral contamination. Data on the effectiveness of washing and disinfectants and processing aids for deactivation of enteric viruses on fresh produce are lacking (41). In the current study, onion bulbs and spinach leaves were decontaminated by washing with PW.

Washing for 2 min resulted in a significant 1.01-log reduction in MNV-1 on spinach leaves. A washing procedure that included three washing steps of each 2 min did not produce a significantly greater reduction of virus. Washing onion bulbs resulted in a significant virus reduction, with a mean of 0.39 log PFU. Croci et al. (18) reported an approximately 1-log decrease in hepatitis A virus by washing cut lettuce, carrots, and fennel. Similar results were obtained by Dawson et al. (19), who found less than 0.4-log reductions of MS2 phage after washing spring onions. The risk of contamination of produce with infectious virus from the wash water is possible. To our knowledge, no previous experiments have been conducted dealing with the possibility of cross-contamination during the washing procedure. More than 3 log PFU MNV-1 was transferred to both spinach leaves and onion bulbs from wash water contaminated with 4.98 log PFU/ml. However, such high levels of viruses are not expected in industrial wash water because microbial contaminants PW should be used, but the data indicated a remarkably fast transfer of MNV-1 (contact times of 2 min and 25 s for spinach and onions, respectively).

The survival of MNV-1 was followed in PW, and the level of infectious virus remained stable during 1 week of storage at room temperature. Enríquez et al. (21) reported that poliovirus and hepatitis A virus declined by 4 and 3.5 log PFU after 3 and 50 days, respectively, in tap water at room temperature, whereas adenovirus types 40 and 41 decreased by nearly 2 log PFU after 55 days. No decrease in virus titer was observed until 6 days in industrial used spinach wash water. High counts of bacteria and yeasts and molds were determined on day 0 but apparently did not influence the survival of MNV-1. Bosch et al. (13) suggested that the different inactivation rates of astrovirus at 20°C in tap water and seawater could have been due to antiviral bacteria present in the seawater samples, but such inactivation was not observed in used spinach wash water inoculated with MNV-1. Similarly, Mattison et al. (35) observed lower survival of feline calicivirus on foods stored at room temperature. These authors suggested that food spoilage may have contributed to viral deterioration. The mechanism behind this deterioration is currently not known. Contamination of produce from contact with contaminated wash water could be reduced or prevented by adding a sanitizer to the water. In the current study, PAA (an industrial sanitizer) was investigated. A range of PAA concentrations were prepared in both PW and DW because of the difference in pH, and the resistance of MNV-1 to these conditions was determined. Baldry (8) reported that the effect of PAA is dependent on the pH and stated that PAA is less effective at higher pH values such as 8 (pKₐ 8.2). When MNV-1 was treated with 150 ppm of PAA in PW, pH 6.00, for 2 min, a 3.37-log reduction in virus was found, whereas a 5-min treatment resulted in a 3.40-log reduction. Baldry (8) also reported that treatment with 99 ppm of PAA, pH 6.5, resulted in a 6-log reduction within 1 min for two gram-negative and two gram-positive bacterial strains; 99 ppm of PAA, pH 6.5, also killed several yeast strains (Saccharomyces cerevisiae and Zygossaccharomyces bailii) within 5 min. The results of the current study suggest that MNV-1 is more resistant to PAA than bacteria or yeasts. The concentrations effective against bacteriophages MS2 and φX174 in DW were 15 and 30 ppm, respectively, resulting in greater than 4-log reductions in 5 min (9). Poliovirus required a much higher concentration of PAA, 750 to 1,500 ppm, to produce a 4-log reduction within 15 min (9). It seems that enteric viruses could withstand higher concentrations of PAA compared with the bacteriophages tested. Nevertheless, a concentration as low as 20 ppm of PAA at pH 4.13 and 7.70 resulted in 2.88- and 2.41-log reductions of MNV-1, respectively, after 5 min of exposure. Differences between DM and PW were observed as an influence of the pH on the PAA efficiency, although these differences were in several cases not significant. The effect of 20 and 150 ppm of PAA in DW was also investigated with a real-time RT-PCR assay. No decline of the number of MNV-1 genomic copies was observed at both concentrations tested, not even after the 15-min treatment. However, the real-time RT-PCR assay detected both infectious and noninfectious MNV-1. Sauerbrei et al. (39) tested the resistance of duck hepatitis B virus to PAA. A concentration of 0.005% (0.5 ppm) achieved a 4-log reduction in the median tissue culture infective dose per milliliter after 15 min of treatment. As in the current study, Sauerbrei et al. found high numbers of genomic copies after treatment. The study of Maillard et al. (34) revealed that 100 ppm of PAA (pH 4.5, 10 min) affected the nucleic acid of the *Pseudomonas aeruginosa* bacteriophage F116. However, it was not clear whether PAA affected the nucleic acid within the capsid or released double-stranded DNA from a damaged capsid. The mechanism of PAA action is not yet clear. As an oxidizing agent, it is likely that PAA oxidizes S-H and
N-H groups that modify viral proteins of the envelope and the capsid, resulting in virus inactivation (51).

In contrast to onions, spinach is blanched before being deep frozen. Exposure of spinach to 80 or 90°C for 1 min resulted at least a 2.44-log reduction of MNV-1, but the remaining peroxidase activity was still higher than that after other blanching treatments in vegetables (33, 38). As was shown in a previous study (7), no correlation of real-time RT-PCR assay and plaque assay results was observed after heat treatment, although lower numbers of genomic copies were detected in blanched spinach than were estimated in the heat-treated MNV-1 lysate. Probably the food matrix enhanced the breakdown of inactivated MNV-1 particles.

The stability of MNV-1 on frozen onions and spinach was investigated, but no decline was observed within 6 months. However, variability of the number of MNV-1 PFU among storage months was observed. To our knowledge, no information on the survival of enteric viruses on frozen vegetables has been reported. Kurdziel et al. (31) reported a 90% reduction in poliovirus on frozen strawberries by 8.4 days. After 15 days, a 0.1186-log decline was observed.

In this study, washing of spinach or onions significantly reduced the number of MNV-1 particles but was not sufficient for viral decontamination. In addition, wash water could be implicated as a source of contamination because of the possible transfer of virus particles to vegetables. PAA is a promising sanitizer for washing water; MNV-1 inactivation occurred at rather low PAA concentrations and after short contact times. PAA decomposes to harmless products and releases few or no by-products that are toxic or mutagenic (28). The efficiency of PAA should be further investigated with vegetables to examine the influence of organic material. Blanching is an efficient method of MNV-1 inactivation on spinach, but a lack of correlation between the detection of genomic copies and the infectiousness of the virus should be noted. No corresponding reduction of MNV-1 genomic copies was observed in PAA-treated water with the real-time RT-PCR assay compared with the reduction found based on results of the plaque assay. The RT-PCR assay does not seem to be a proper technique for NoV survival and stability studies. Vegetables that can be eaten raw or unprocessed after thawing are prone to contamination with NoV if exposed to feces. When these viruses are present, wasing is not an effective elimination method. Both decontamination with PAA and a moderate heat treatment may be able to control NoV infections.

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REFERENCES


