Temperature-Dependent Survival of Hepatitis A Virus during Storage of Contaminated Onions

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Pre- or postharvest contamination of green onions by hepatitis A virus (HAV) has been linked to large numbers of food-borne illnesses. Understanding HAV survival in onions would assist in projecting the risk of the disease associated with their consumption. This study defined HAV inactivation rates in contaminated green onions contained in air-permeable, moisture-retaining high-density polyethylene packages that were stored at 3, 10, 14, 20, 21, 22, and 23°C. A protocol was established to recover HAV from whole green onions, with 31% as the average recovery by infectivity assay. Viruses in eluates were primarily analyzed by a 6-well plaque assay on FRhK-4 cells. Eight storage trials, including two trials at 3°C, were conducted, with 3 to 7 onion samples per sampling and 4 to 7 samplings per trial. Linear regression correlation ($r^2 = 0.80$ to 0.98) was observed between HAV survival and storage time for each of the 8 trials, held at specific temperatures. Increases in the storage temperature resulted in greater HAV inactivation rates, e.g., a reduction of $0.033 \log \text{ PFU/day}$ at $3.4 \pm 0.3^\circ\text{C}$ versus $0.185 \log \text{ PFU/day}$ at $23.4 \pm 0.7^\circ\text{C}$. Thus, decimal reduction time (D) values of 30, 14, 11, and 5 days, respectively, were obtained for HAV in onions stored at 3, 10, 14, and 23°C . Further regression analysis determined that 1 degree Celsius increase would increase inactivation of HAV by $0.007 \log \text{ PFU/day}$ in onions ($r^2 = 0.97$). The data suggest that natural degradation of HAV in contaminated fresh produce is minimal and that a preventive strategy is critical to produce safety. The results are useful in predicting the risks associated with HAV contamination in fresh produce.

Thirty-one pathogens have been identified as the causative agents for food-borne illness outbreaks in the United States (21). Among these etiological agents, four or five enteric viruses contributed to approximately half (21) of those illnesses for which the etiology was identified. For example, among 479 food-borne outbreaks with a laboratory-confirmed single etiology in 2008, enteric viruses were responsible for 49% of the outbreaks and 47% of the illnesses (8). Although hepatitis A virus (HAV) has not been implicated in the majority of viral illnesses, hepatitis A is a critical food-borne viral disease due to the persistent survival of HAV, the severity of the disease, and longer incubation (15 to 50 days) than norovirus infection (approximately 36 h).

More than 2,500 hepatitis A cases from 85 food-borne outbreaks have been documented in the United States since 1997, as summarized in Table 1. Among these cases of food-borne hepatitis A, 61.4% (1,098/2,580) of the illnesses were attributed to contaminated green onions, the top-ranked food item associated with food-borne hepatitis A in the United States for the years between 1997 and 2009. Outbreak investigations have also indicated that imported green onions might be associated with the outbreaks in 1998 and 2003 (2, 14, 27). The etiological agents were identified in patient specimens but not in the implicated green onions. Possible reasons for the failure include the long incubation period of the disease, which might have resulted in (i) unavailability of the incriminated food items or (ii) virus degradation.

Routes of virus contamination in fresh produce could be via irrigation water, fertilizers, soils, or hand handling. HAV has been found to survive longer than poliovirus and echovirus at 25°C in primary effluents with and without resuspended soils (24). In addition, these survival differences between HAV and other viruses became less significant as the quality of water improved. In human and animal waste mixtures, HAV has survived longer at 25°C (decimal reduction time [D] value, 8.1 days) than poliovirus (D value, 6.8 days) (12, 13). HAV has shown lower inactivation rates

than poliovirus in mineral water at 4°C and 23°C in long-term storage (6). On porous and nonporous surfaces, such as aluminum, china, and latex, that are commonly found in kitchen or postharvest processing plants, the survival of HAV has been found to be significantly longer than that of poliovirus and adenovirus (1). There have been a few limited studies that reported HAV survival on fresh produce. HAV was reported to survive during prolonged storage of spinach leaves at 5.4°C , with a D value of 28.6 days (23). Sixty-two percent of inoculated HAV survived on romaine lettuce leaves that were stored for 12 days in a sealed-atmosphere plastic package at 4°C (5). HAV also showed greater resistance than feline calicivirus, a possible surrogate for human norovirus, on lettuce and bell pepper when stored at room temperatures (25). Therefore, consumption of HAV-contaminated foods results in greater risks than consumption of foods contaminated by other enteric viruses. This is not only due to the severity of hepatitis A, but also to the environment-resistant nature of HAV.

There is limited information on HAV survival in contaminated fresh produce, and specifically in green onions. In the current research, we planned to elucidate HAV inactivation rates on inoculated green onions contained in air-permeable, moisture-retaining packages stored at different temperatures simulating proper and abused temperatures for common storage and shipping of fresh green onions postharvest. In order to report accurate survival rates of HAV on onions, the possible effect of onion freshness

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TABLE 1 Food-borne hepatitis A outbreaks in the United States, 1997–2009, for which the vehicle was identified

Food group ^a	Total no. of illnesses ^b	No. of outbreaks ^c	Reference(s)
Produce			
Green onion	1,098 (601, 333, 73, 43, 32, 16) ^d	6	2, 9, 14, 27
Berries	253 (213, 29, 8, 3)	4	7, 9, 16
Tomato	23	1	9
Romaine lettuce	22	1	9
Unspecified vegetables	21 (16, 5)	2	9
Fruits	21	1	7
Ready-to-eat foods			
Salad and sandwich	105 (40, 35, 20, 4, 4, 2)	6	9
Coleslaw	16	1	9
Deli meat	10	1	9
French fries	7	1	9
Salsa	4	1	9
Sushi	4	1	9
Ice and tap water			
Ice	48 (40, 8)	2	9
Tap water	14	1	9
Shellfish			
Oyster	39	1	3, 9, 22
Crab	7	1	9
Crab dishes	6	1	9
Dairy products			
Ice cream and yogurt	15	1	9
Milkshake	8	1	9
Multiple foods	66 (43, 8, 12, 3)	4	7, 9
Unidentifiable foods ^e	793	47	7, 9
Total	2,580	85	

^a Outbreak-incriminated foods were identified and described on CDC websites (<http://www.cdc.gov/foodborneoutbreaks> and http://www.cdc.gov/outbreaknet/pdf/surveillance/1997_linelist.pdf) and in individual publications.

^b Total number of hepatitis A cases between 1997 and 2009 for all outbreaks. For a food group causing more than one outbreak, the numbers of illnesses per outbreak are listed within parentheses.

^c Total numbers of all food-borne hepatitis A outbreaks reported between 1997 and 2009.

^d Of the 6 outbreaks, 4 occurred in 2003, 1 occurred in 2000, and 1 occurred in 1998.

^e The specific incriminated food item(s) was not identified in the outbreak.

on HAV recovery was evaluated, as well. It is believed that adequate knowledge of HAV survival on contaminated produce during prolonged storage may be useful to facilitate outbreak investigations and to better predict and control risks associated with HAV-contaminated foods and thus to improve the health and safety of consumers.

MATERIALS AND METHODS

HAV and mammalian cells. Fetal rhesus monkey kidney (FRhK-4) cells, host cells to propagate HAV, were grown in Eagle's minimum essential medium (MEM) supplemented with 15% fetal bovine serum, 15 mM HEPES, 0.11% sodium bicarbonate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mg/ml kanamycin, and 0.1 mg/ml gentamicin (Gibco/Invitrogen, Carlsbad, CA). The cytopathic strain HAV HM175

was propagated in FRhK-4 cells. The HAV-infected cells were treated by three freeze-thaw cycles, extracted with an equal volume of chloroform, and centrifuged at $2,000 \times g$ for 15 min at 4°C. The HAV-containing supernatant was collected after centrifugation and then used for inoculation. To increase the inoculum titer, thawed HAV stock was concentrated by ultracentrifugation with a TLA-110 rotor at 75,000 rpm ($234,000 \times g$) for 2 h at 4°C in an Optima TLX Ultracentrifuge (Beckman Coulter, Fullerton, CA). The supernatant was removed, and pellets were reconstituted in the original stock solution and maintained on ice. This resulted in a 10-fold or higher concentration.

Inoculation, storage, and recovery of HAV from green onions. Fresh whole green onions were purchased from local food stores a day prior to the experiments. Based on preliminary experiments, outer leaves of green onions frequently withered throughout the storage time, especially at up to 29 days of storage. The outer leaves sometimes became moldy after weeks under refrigeration or days at room temperature. Each inoculation of 20 to 30 μ l of HAV was conducted by gently spreading the inoculum along an inner (the 3rd) green leaf. Each inoculated whole green onion was individually packed in an 11-in-long clear polyethylene terephthalate (PET) herb container with holes (Cool-Pak, Oxnard, CA) to avoid cross-contact between samples. After the inocula were air dried for 20 min in a biosafety level II cabinet, 5 to 7 containers were placed and sealed in a high-density polyethylene (HDPE) produce bag that was stored in one of the 3 selected storage formats (refrigerator, temperature-controlled incubator, or laboratory shelf) in order to produce storage temperatures of 3, 10, 14, 20, 21, 22, and 23°C. A rapid-extraction protocol was developed (Fig. 1) to elute HAV from whole green onions by folding the inoculated whole onion once and placing it in a smaller Whirl-Pak bag (7-in width) filled with 15 to 30 ml eluent (1% beef extract-100 mM Tris-50 mM glycine, pH 8) sealed by a heat sealer. This sealed bag was double bagged

I. Virus-inoculation and onion-packaging:

Select 3-leaf whole onions and trim each to fit a PET produce container, 11" long

Spread inocula on inner green leaves, air-dry 20 min in BSL II hood

Package each onion per PET container, seal 3-7 containers in a

HDPE-produce bag

Store packed onions at 3 to 23°C ambient temperatures

II. Elution:

Unpack onions at specific sampling time

Fold each onion within a Whirl-Pak bag using sterile forceps

Add 15-30 ml elution buffer to the sample, and pulsify 90 sec. (3 x 30 sec.)

Syringe-filter via 0.2 μ m, 33 mm-diam. filters

III. Virus analysis:

Titer viruses in filtrates by infectivity assay

RT-PCR-analyze the filtrates reserved for the recovery comparison

FIG 1 Inoculation, elution, and analysis of HAV in green onions.

TABLE 2 Virus recoveries from inoculated fresh and aged onions after 20 min of air drying followed by inoculation

Trial	Virus inoculum (PFU/onion)	Viruses recovered from:		Virus recovery ^b (%)	Recovery ratio of aged to fresh onions ^c
		Fresh onions	Aged onions ^a		
HAV recovery trials ^d					
1109	$(5.3 \pm 0.8) \times 10^3$	$(2.0 \pm 0.4) \times 10^3$	— ^e	38	
1123	$(5.7 \pm 1.7) \times 10^3$	$(1.7 \pm 0.5) \times 10^3$		29	
216	$(1.2 \pm 0.2) \times 10^5$	$(3.2 \pm 0.8) \times 10^4$		27	
Onion age-related HAV recovery trials ^f					
502	—	$(3.3 \pm 1.5) \times 10^3$	$(4.5 \pm 2.0) \times 10^3$	—	1.4
516	—	$(4.0 \pm 2.0) \times 10^3$	$(4.0 \pm 1.7) \times 10^3$	—	1.0
406	—	$(3.3 \pm 0.7) \times 10^6$ ($C_T = 34.1 \pm 0.3$)	$(2.4 \pm 1.1) \times 10^6$ ($C_T = 34.7 \pm 0.7$)	—	0.7
411	—	$(1.1 \pm 0.5) \times 10^6$ ($C_T = 35.9 \pm 0.7$)	$(1.6 \pm 0.5) \times 10^6$ ($C_T = 35.2 \pm 0.5$)	—	1.5

^a Aged onions were under refrigeration for ≥ 4 weeks before inoculation.

^b Recoveries were calculated based upon each trial inoculum as 100%. Average, $31\% \pm 6\%$.

^c HAV recovered from fresh onions was considered 1. Averages: 502 and 516, 1.2 ± 0.3 ; 406 and 411, 1.1 ± 0.6 .

^d The onion sample number for each trial was 3 to 5. Therefore, 6 to 10 data were averaged with duplicate plating.

^e —, no data.

^f Fresh and aged onions were coinoculated with the same inocula of HAV simultaneously. HAV levels in trials 502 and 516 are expressed in PFU per onion. HAV levels in trials 406 and 411 are expressed in RT-PCR units per onion with threshold cycle (C_T) values. Onion sample numbers ranged from 3 to 5 in each trial.

with a stomacher bag (Seward, Bohemia, NY). The PUL100 pulsifier (Microgen Bioproducts, Surrey, United Kingdom) was used to elute the virus from the onion. The sample bag (onion and eluent) was pulsed or vibrated vigorously for a total of 90 s with 30-s intervals to change the sample bag to different orientations. The eluent was filtered through a 0.2- μ m syringe filter (Millipore, Billerica, MA) before plaque assay.

Concentration of HAV in eluates. When eluted HAV levels were suspected to be low, eluate samples were concentrated approximately 10-fold by placing a 5-ml sample in an Amicon Ultra-15 centrifuge unit (Millipore, Billerica, MA) and centrifuging the sample at $3,000 \times g$ for 20 min at 4°C in a Sorvall Legend RT centrifuge (Thermo Scientific, Asheville, NC) to a final sample volume of 500 μ l or less.

HAV plaque assay. Eluate sample dilutions were prepared in duplicate for inoculation onto confluent FRhK-4 cells grown in 6-well plates. The inoculated cells were incubated at 37°C with 5% CO₂ for 90 min with gentle rotation once every 20 min to enhance viral infection. After the 90-min incubation, 4 ml of an overlay mixture was added to each well and allowed to solidify. The overlay mixture was prepared by mixing equal volumes of 1.8% agar (melted and tempered; BD Sparks, MD) and complete MEM (supplemented with 4% serum, not 15%) with the additional ingredients 2% nystatin and 80 mM MgCl₂ (Sigma-Aldrich, St. Louis, MO). On day 7 of incubation at 37°C with 5% CO₂, an additional 4 ml of the same Eagle's MEM agar (as stated above) with additional neutral red (1%; Sigma-Aldrich, St. Louis, MO) was added to each well. Viral plaques (as clear areas) were counted after 3 to 5 days of incubation of cells at 37°C in 5% CO₂, which allowed the neutral red to stain viable cells. Viral plaques in the range of 5 to 75 per well were enumerated and used for calculation.

HAV qRT-PCR assay. Nucleotide sequences of HAV primers and probe within the HAV 5'-untranslated region published by Jothikumar et al. (17) were used in the study. The Taq-Man probe was labeled with 6-carboxyfluorescein and a black hole quencher at the 5' and 3' ends, respectively. The sample eluates collected were 100-fold diluted with DNase/RNase-free water (HyClone, Logan, UT). These served as templates before the quantitative reverse transcription (qRT)-PCR analysis. Each reaction mixture of 20 μ l contained 500 nM each primer (forward and reverse) and 250 nM TaqMan probe. An RNA UltraSense one-step quantitative RT-PCR kit (Invitrogen, Carlsbad, CA) was utilized. Five microliters of 100-fold-diluted eluate was first mixed with both primers and 12 units of RNasin (Promega, Madison, WI), heated to 100°C for 10 min in a thermal cycler (PTC-200 Peltier; MJ Research, Watertown, MA)

to heat-burst virions, and immediately cooled to 4°C to allow the primers to hybridize with viral RNAs. Then, 4 μ l of 5 $\times$ reaction mixture, TaqMan probe, and RNA UltraSense enzymes was added to the mixture of primers and templates. The qRT-PCRs took place in an Opticon (Bio-Rad, Hercules, CA) for 120 min, including a 20-min RT at 50°C, followed by PCR initiation at 95°C for 2 min and 49 PCR cycles (consisting of denaturing at 95°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 15 s). The fluorescence was accumulated and measured at the end of each cycle.

Statistical analysis. The means, standard deviations, regression equations, *t* test, regression analysis, and correlation coefficients were calculated using Microsoft Excel (2003).

RESULTS

HAV recovery from whole green onions. To gain knowledge of virus survival during postharvest shipping and storage, HAV was inoculated on green onions, and the onions were examined periodically for HAV levels under proper and abused temperature storage. Due to the emphasis on viral-RNA extraction and PCR detection, the virus detection method in the FDA *Bacteriological Analytical Manual* was not utilized in the current survival study (26). Instead, a rapid virion extraction protocol (<1 h) was developed and followed by an infectivity assay. Using the rapid-extraction protocol (Fig. 1) developed to elute HAV from whole green onions, the average recovery rate was determined. HAV inocula ranged from 3 to 5 log PFU per onion for 3 independent trials (Table 2); the average HAV recovery rate of 31% by infectivity was derived from inoculated whole green onions using the protocol.

HAV inoculum was spread on the surface of the inner leaf. HAV rates of recovery from fresh onions were compared to the rates for those eluted from freshly inoculated aged onions (Table 2). Recovery comparison trials 502 and 516 were determined by infectivity assay. Trials 406 and 411 were analyzed by qRT-PCR to evaluate if the rates of recovery of HAV RNAs from old and fresh onions differed. Simultaneous inoculations of identical quantities of HAV were carried out between fresh and aged onions for each of the 4 trials. However, each inoculum was not reanalyzed. The results were an average of 6 to 10 replicate data that were derived

TABLE 3 HAV survival in inoculated green onions during cold, intermediate, and room temperature storage

Storage time (days)	Avg HAV level (% survival) for trial no. ^a :							
	Cold storage		Storage at 10.3°C ≤ temp ≤ 23.4°C					
	1123	824	1102	1007	910	620	1214	216
0	3.26 ± 0.21	4.50 ± 0.10	4.57 ± 0.08	4.50 ± 0.05	4.32 ± 0.08	3.83 ± 0.11	3.60 ± 0.26	4.50 ± 0.13
1	2.95 ± 0.25 (49)	3.94 ± 0.12 (28)	— ^b	—	3.76 ± 0.20 (28)	3.46 ± 0.50 (43)	3.25 ± 0.10 (44)	4.11 ± 0.13 (41)
2	—	—	4.28 ± 0.06 (52)	4.36 ± 0.15 (72)	—	—	—	—
3	—	—	—	—	—	3.20 ± 0.14 (23)	3.06 ± 0.29 (28)	3.82 ± 0.39 (21)
4	—	—	—	4.22 ± 0.15 (53)	3.39 ± 0.18 (12)	—	—	—
5	—	—	4.07 ± 0.10 (32)	—	—	3.10 ± 0.28 (19)	—	—
6	—	—	—	4.33 ± 0.17 (68)	3.34 ± 0.14 (11)	—	—	3.45 ± 0.47 (9)
7	—	4.08 ± 0.36 (38)	—	—	—	—	2.23 ± 0.09 (4)	—
8	—	—	3.76 ± 0.07 (16)	3.76 ± 0.09 (18)	—	—	—	2.85 ± 0.30 (2)
9	—	—	—	—	2.73 ± 0.16 (3)	—	2.09 ± 0.32 (3)	—
10	—	—	—	3.64 ± 0.07 (14)	—	—	—	—
11	—	—	3.64 ± 0.09 (12)	—	—	—	—	—
12	—	—	—	3.49 ± 0.07 (10)	—	—	—	—
14	2.79 ± 0.17 (34)	—	3.50 ± 0.14 (9)	—	—	—	—	—
15	—	3.63 ± 0.25 (14)	—	—	—	—	—	—
16	—	—	3.42 ± 0.15 (7)	—	—	—	—	—
21	—	3.67 ± 0.43 (15)	—	—	—	—	—	—
22	2.45 ± 0.05 (15)	—	—	—	—	—	—	—
28	—	3.29 ± 0.20 (6)	—	—	—	—	—	—
29	2.09 ± 0.10 (7)	—	—	—	—	—	—	—

^a HAV survival was calculated based upon day 0 as 100%. Each HAV level (log PFU per onion ± SD) was averaged from 6 to 14 data points (3 to 7 onion samples). The last data in each column represent the last sampling of each trial.

^b —, no data.

from either fresh or aged onion samples. The HAV levels from aged onions did not appear to decrease in trials 502 and 516. In fact, an identical level in trial 516 and higher levels of HAV were recovered from aged onions in trial 502. Thus, the average recovery ratio was 1.2 for those from aged onions versus those from fresh onions (Table 2). When RT-PCR assays were utilized in trial 406, HAV recovery was 70% for aged onions if the recovery of fresh onions was considered 100%. In contrast, in trial 411, the average HAV recovery from aged onions was greater than that from fresh onions. The ratio of recovered HAV RNA from inoculated aged onions to that from fresh onions was 1.1, as determined by qRT-PCR (Table 2). It was observed that HAV rates of recovery from inoculated fresh and aged onions were not significantly different (*t* test; *P* > 0.05), although larger standard deviations were observed in the recovery trials by qRT-PCR. It was concluded that HAV levels analyzed in this study at all samplings (up to 29 days) were not affected significantly by onion age or freshness. Therefore, the HAV level decreases throughout storage were due mainly to HAV survival, not the difference in recovery from old or fresh onions.

HAV survival in onions stored at cold, intermediate, and room temperatures. A total of 8 independent storage trials were conducted with inocula ranging from 3.26 to 4.57 log PFU of HAV per onion sample (Table 3). Each HAV level listed in Table 3 (all determined by infectivity) was an average of 6 to 14 data points derived from 3 to 7 inoculated onions. The HAV survival percentage was calculated based upon the eluted HAV after inoculation and 20 min of air drying on day 0 as 100%.

The storage temperatures in all eight trials were recorded continuously, with the data collection intervals ranging between 1 and 6 h (Table 4). Trials 1123 and 824 were designated cold-temperature storage; trials 1102 and 1007 were intermediate-temperature trials, with average temperatures of 10°C and 14°C, respectively. The remaining four trials (910, 620, 1214, and 216) in Table 4 were designated room temperature trials. Overall, the average temperatures of two trials (1123 and 824) conducted in a refrigerator were both 3°C, with one having a wider standard deviation than the other. The average temperatures of the four trials at ambient temperatures were between 20.2 and 23.4°C. The temperatures of

TABLE 4 Cold, intermediate, and room temperature storage temperatures

Parameter	Temp recorded for trial no.:							
	1123	824	1102	1007	910	620	1214	216
Mean (°C)	3.1 ± 1.8	3.4 ± 0.3	10.3 ± 1.4	14.2 ± 0.3	20.2 ± 2.1	20.7 ± 1.5	22.4 ± 0.8	23.4 ± 0.7
Temp range (°C)	0.4–11	2.7–4.2	9.9–26.4 ^a	13.8–15.8	16.8–25.7	18.9–23.9	21.4–24.4	22.2–26.4
Total no. of recordings (interval [h])	695 (1) ^b	151 ^c (3)	191 (2)	144 (2)	216 (1)	48 ^d (2)	36 (6)	192 (1)

^a Refrigeration broke down during this trial. Onions might have been under room temperatures for maximum 5 out of 382 h.

^b A total of 695 temperature data points were recorded at 1-h intervals, with only one recording of 11°C, possibly due to improper shutting of the refrigerator door.

^c Temperatures were recorded from day 0 to day 18.

^d Temperatures were recorded from day 2 to day 5.

TABLE 5 HAV inactivation rates and *D* values for green onions stored at various temperatures

Trial	Temp (°C)	Linear regression equation ^a	Correlation coefficient	<i>D</i> value (days)	
				Exptl ^b	Calculated ^c
1123	3.1	$Y = -0.034X + 3.158$	0.93	29.3	38.4
824	3.4	$Y = -0.033X + 4.251$	0.80	30.0	35.5
1102	10.3	$Y = -0.070X + 4.448$	0.96	14.4	13.0
1007	14.2	$Y = -0.088X + 4.571$	0.89	11.4	9.5
910	20.2	$Y = -0.152X + 4.118$	0.92	6.6	6.8
620	20.7	$Y = -0.137X + 3.705$	0.87	7.3	6.6
1214	22.4	$Y = -0.168X + 3.517$	0.98	6.0	6.1
216	23.4	$Y = -0.185X + 4.412$	0.97	5.4	5.9

^a *Y* and *X*, respectively, represent the HAV level (log PFU) per onion and the storage time in days.
^b The *D* value was derived directly from data collected from each trial.
^c The *D* value was calculated from the regression equation between HAV inactivation rates and temperatures as follows: Y (log HAV reduction per day) = 0.007 times X (storage temperature [°C]) + 0.004.

the other two trials were very close to the temperatures of the incubator originally set at 10 and 14°C.

The general trend was that HAV was inactivated over the storage time on surfaces of green onions in moisture-rich (the water activity [*a_w*] values of onions stored for 21 and 28 days at 3°C were ≥0.99) and air-permeable (O₂ permeability, 279 nmol/m · s · GPa) packages during prolonged storage regardless of temperature. In one of two cold-storage trials (1123), sample eluates on day 14 and after were ultrafiltered and concentrated approximately 10-fold with an Amicon Ultra-15 before plaque assay, due to insufficient plaque numbers per well for counting in the assay. Amicon concentration recovery (about 30%) was taken into consideration in all calculations. For trial 824, sample eluates were not concentrated because of their slightly higher initial HAV titer (4.5 log units). Either with or without concentration, trials 1123 and 824 both reached 6 to 7% HAV survival (Table 3) at the end of 28 to 29 days of storage. Intermediate-temperature trials (1102 and 1007) were conducted with HAV titers of 4.57 and 4.50 log units per onion and maintained in a temperature-controllable incubator. A steady drop in the HAV titer was found in trial 1102, with 7% HAV remaining at the last sampling on day 16. Similarly, a log reduction (10% remaining) occurred on day 12 in trial 1007 despite the fact that titer fluctuations were observed on days 4 and 6 (Table 3). The 7% survival occurred on day 16 in trial 1102 at a storage temperature of 10°C; however, similar HAV survival (6 to 7%) was seen on the 28th and 29th days of cold storage.

Rapid HAV reduction on onion samples was observed during room temperature storage. At the last sampling on day 8 in trial 216, only 2% of inoculated HAV had survived. In trials 910 and 1214, the last sampling date was day 9, and both trials had 3% of the HAV remaining. HAV in eluates of trial 1214 (with lower inocula) on days 7 and 9 was concentrated before the plaque assay, but HAV in trial 910 was not concentrated. Additionally, in trial 620, 19% of the inoculated HAV survived on day 5. This agreed with trials 1214 and 216. The overall conclusion from these 4 trials was that a log reduction of HAV occurred within the first week during room temperature storage regardless of initial contamination levels.

Kinetics of HAV inactivation upon onion storage temperature. For each of the 8 survival trials, a regression analysis was conducted to correlate HAV survival (log PFU per onion) and storage time (days) initially. The analyses of all 8 trials are listed in

Table 5, along with the *D* values, calculated as the number of days required to inactivate 90% of inoculated HAV at the average temperature of each storage trial. HAV level changes in three trials, 824, 1102, and 1214, representing cold-, intermediate-, and room temperature storage, respectively, are shown in Fig. 2, with average HAV levels and standard deviations throughout storage. The slopes of the regression lines that represent virus inactivation rates show greater HAV inactivation (about 5-fold higher) at 22.4°C than at 3.4°C (0.168 and 0.033 log PFU HAV/day projected for 22.4°C and 3.4°C storage, respectively). In general, a trend was observed in which higher storage temperatures produced higher inactivation rates (with greater absolute values of slopes and flatter regression lines indicating die off). Clearly, the HAV inactivation rate of 0.07 log unit per day at 10.3°C was between that for 22.4°C and that for 3.4°C. Table 5 shows that high regression correlations were found in all 8 trials (*r*² ≥ 0.80) between HAV levels in onions and storage time at each specific average temperature (for each trial). When the *D* values were calculated from all experimental data, the *D* values of HAV on inoculated green onions in mois-

FIG 2 Regression analyses of HAV levels in inoculated green onions stored at 22.4°C, 10.3°C, and 3.4°C. The error bars indicate standard deviations.

FIG 3 Linear regression relationship between the HAV inactivation rate (y) and onion storage temperature (x).

ture-retaining, air-permeable packages ranged from 5 to 30 days under storage temperatures from 23 to 3°C (Table 5).

Because good correlations were derived between HAV levels in onions and storage time (in days) regardless of the temperature, we further investigated if the constant HAV inactivation rate from each regression formula in Table 5 had any relation to the average storage temperature used (Fig. 3). Further analysis allowed us to conclude that the HAV inactivation rate was linearly correlated with the storage temperature (Fig. 3). The correlation coefficient of 0.97 indicated that the log reduction of HAV per day per onion was highly correlated with the average temperature for onion storage (Fig. 3). Therefore, we recalculated D values from the regression equation shown in Fig. 3. The calculated D values are listed side by side with the D values derived from the 8 trials in Table 5. The formula in Fig. 3 allowed the prediction that every degree Celsius increase of temperature would increase the inactivation of HAV in onions by 0.007 log PFU per day.

DISCUSSION

Human enteric viruses contribute significantly to food-borne outbreaks and illnesses in the United States and worldwide. HAV persists in environments and causes severe hepatitis disease. Contaminated produce has ranked at the top among all food groups implicated in the food-borne disease hepatitis A in the United States. For the past 14 years, green onions have contributed the highest number of the illnesses among the food items listed in Table 1, although contact with an infected individual, leading to secondary infection, is critical to the etiology of the disease. A few outbreak investigations have implied that green onions could be contaminated in the field or during harvest packaging prior to their importation to the United States.

In the current study, HAV contamination of whole onions was performed to mimic surface contamination that might occur during harvest or postharvest via handling or sprinkle irrigation with contaminated water. In our experimental design, we packed only one onion in an 11-in.-long PET herb container with small holes to avoid any cross-contamination via onion contact during storage. It is known that HAV can be transferred easily between surfaces via direct contact. Mbithi et al. reported that 22% of inoculated HAV were transferred within 10 s via contact from

contaminated stainless steel to clean finger pads after the inoculum was dried for 20 min, and 27% reverse transfer was found (20). Nine percent of HAV migrated from contaminated fingertips to clean lettuce during a 10-s contact (4). In the current study, we chose a moisture-retaining, air-permeable HDPE bag to cover PET containers. The HDPE bag has a thickness of 2.0 mil and O_2 permeability of 279 nmol/m · s · GPa. This simulated some commercial packaging of green onions in sealed and moisture-retaining produce bags. The average recovery of 31% of HAV from onions, using the virion extraction protocol (Fig. 1) followed by an infectivity assay, was similar to the HAV recovery from inoculated spinach leaves, 37.3% by using eluent 3% beef extract, pH 8 (23). The virion extraction protocol was proven to be compatible with RT-PCR detection (Table 2). However, all the survival data in the current study had to strictly rely on the infectivity assay. Higher HAV recovery of 75.8% from lettuce was reported by Bidawid et al. (4). The difference was expected because Bidawid used eluted HAV from lettuce immediately after inoculation as 100%, whereas our calculation was based on the titer of the original inocula as 100% (with reduced virus recovery from any inoculated matrix frequently being observed). On the other hand, to calculate the survival percentage in our study, the 100% baseline was the HAV level on day 0 after inoculation, 20 min of air drying, and elution of HAV from onions.

There were a few missing temperature points in trials 620 and 824 due to temperature recorder malfunctions. A large standard deviation was observed in trial 1123, with an average temperature of 3.1°C and a maximum temperature of 11°C, which was recorded once. This could be due to the refrigerator door not being shut properly, but the next temperature recorded was 4.5°C an hour later. In trial 1102, the incubator experienced a power failure for a short period during storage, leaving two temperature points, 20.6°C and 26.4°C. With the 2-h interval of recording, the incubator was out of order for approximately 4 to 5 h. The overall temperature measurement in the current study was intended to be as accurate as possible.

The linear reduction of HAV in onions was correlated with the storage time at any specific temperature ($r^2 = 0.8$ to 0.98). Therefore, we concluded that the inactivation rate of HAV was constant at each storage temperature. During cold storage at 3.1 and 3.4°C, the D values were 29.3 and 30.0 days, respectively. The finding was consistent with the D value of 28.6 days of HAV survival at 5.4°C on spinach leaves (23). Among all inactivation rates for HAV stored at different temperatures, greater inactivation rates were observed in contaminated green onions stored at warmer temperatures (Fig. 2 and Table 5). Stine et al. (25) reported that the HAV inactivation rates on lettuce and bell peppers were 0.29 and 0.18 log PFU/day, respectively, at 25°C average temperature and 86% relative humidity. Since the humid conditions reported by Stine were closer to our moisture-retaining packages in the current study, we applied 25°C to the formula Y (HAV inactivation/day) = $0.0071 \times (\text{temperature in } ^\circ\text{C}) + 0.004$ log unit (Fig. 3) and derived 0.1815 log unit/day of HAV inactivation. Therefore, we concluded that the inactivation rate of HAV on green onions was similar to that on bell peppers stored at room temperature, 0.1815 versus 0.18 log PFU/day of HAV. Also, the HAV survival rates were comparable between onions (in the current study) and spinach leaves (23) at cold temperatures, with D values of about 1 month.

Overall, HAV survival rates on lettuce have been reported differently from one publication to another. A greater inactivation

rate of 0.29 log PFU/day (25) of HAV on lettuce at 24.6°C was reported than was reported in onions in the current study. In addition, a 2-log-unit reduction of HAV on lettuce was reported by day 9 under cold storage (10). Since the lettuce-packing environment was not described in the study, comparison of the data with ours is impossible. On the other hand, a lower inactivation rate of HAV on lettuce was reported by Bidawid et al. (5): 61.7% of HAV remained after 12 days at 4°C under atmospheric conditions.

Higher temperatures also increased the inactivation rate of HAV on other matrices, e.g., stainless steel. HAV inoculated on stainless steel at 35 and 20°C had higher inactivation rates than at 5°C (19). It was also found that the inactivation rates of HAV accelerated even further with increased relative humidity. Higher storage temperatures were more effective in inactivating MS2 coliphage on produce (11), which was in agreement with what we found with HAV in this study. In addition, it was previously reported that with low-heat dehydration of contaminated onions at 47.8°C, 20 h were required to inactivate 1 log HAV on green onions (18). The current study showed that 5.4 days was needed at 23.4°C to achieve 1 log reduction of HAV. However, it should be remembered that the produce dehydration temperature is not the only factor; the reduced water activity of produce also affected the survival of pathogens. The inactivation of HAV on stainless steel surfaces increased as the humidity of the surrounding environment increased when experiments were conducted between 5 and 35°C (19). Importantly, the inactivation rate of HAV on stainless steel at 80% relative humidity at 5°C (19) was almost four times higher than that in onions at 3.4°C found in our study (-0.1344 versus -0.033 log PFU/day). This indicates that produce can provide a more suitable environment for HAV to survive than non-porous inanimate surfaces. In another, totally different matrix, Hewitt and Greening (15) reported that HAV was inactivated by 1.7 log units at 4°C in marinated mussels during 4 weeks of storage. The acidic marinade sauce might have accelerated the inactivation of HAV. Regardless of similar cold temperatures used in the current study, approximately 30 days was needed to inactivate HAV by 1 log unit on contaminated green onions.

The rapid molecular assay, qRT-PCR, used in this study was found to be useful for the estimation and comparison of recovery rates (Table 2) but not practical for estimating the true HAV levels or survival in produce (data not shown). Green onions are frequently stored at refrigeration temperature to ensure freshness and quality. The current study suggests that 1 month is required to reduce HAV by 1 log unit during common cold storage. Only an additional 0.007 log unit of HAV per day can be inactivated by increasing the temperature of the surrounding environment by 1°C. Therefore, once fresh produce is contaminated by HAV at any stage from farm to table, it is difficult to expect natural degradation or elimination of HAV to a safe level during prolonged storage and shipping. The conclusion is that the prevention of viral contamination of fresh produce pre- and postharvest is more effective to ensure food safety than strategies to remove viral contaminants. Good agricultural practices, such as using clean water for food production, training field workers, and maintaining good environmental hygiene, are critical to control the hazard. Farm-to-table responsibility for safe food is mandated by the U.S. Food Safety Modernization Act, which prescribes comprehensive preventive controls for all food producers, including producers of fresh produce and suppliers of imported foods. Implementation

of the preventive controls in every part of the food production chain is important to achieve the ultimate goal of producing safe food for consumers.

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