Research Note

Survival of Hepatitis A Virus in Spinach during Low Temperature Storage

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ABSTRACT

Spinach leaves are frequently consumed raw and have been involved with past foodborne outbreaks. In this study, we examined the survival of hepatitis A virus (HAV) on fresh spinach leaves in moisture- and gas-permeable packages that were stored at 5.4 ± 1.2°C for up to 42 days. Different eluents including phosphate-buffered saline (PBS), pH 7.5 (with and without 2% serum), and 3% beef extract (pH 7.5 and 8) were compared for how efficiently they recovered viruses from spinach by using a simple elution procedure (<1 h). The recoveries were compared and determined by a plaque assay with FRhK-4 cells. Culture grade PBS containing 2% serum was found to be appropriate for HAV elution from spinach leaves, with an average recovery of 45% ± 10%. Over 4 weeks of storage at 5.4 ± 1.2°C, HAV in spinach decreased slightly more than 1 log, with 6.75% of the original titer remaining. HAV survived under refrigerated temperatures on spinach leaves with a D-value of 28.6 days (equivalent to an inactivation rate of −0.035 log of HAV per day, r² = 0.88). In comparison, HAV in PBS containing 2% serum under the same storage conditions remained constant throughout 7 weeks. The inactivation rate of −0.035 log each day for HAV on spinach leaves was possibly due to the interaction of the virus and the leaf.

Seventy-six million foodborne illnesses are estimated to occur each year in the United States (13). Surveillance of the foodborne diseases occurring from 1998 to 2002 revealed that one-third of the U.S. foodborne outbreaks had known etiology (10). Among the identified etiological agents, viruses were responsible for 33% of the outbreaks and 41% of the illnesses (10). Hepatitis A virus (HAV) has been implicated in produce-borne outbreaks and illnesses in the United States via contaminated green onions (5, 20), lettuce (15), and strawberries (7, 14). Fresh fruits and vegetables that are consumed raw may become vehicles for transmission if viral contamination occurs anywhere from farm to table. In recent years, an increase of produce-borne infections could be attributed to increased consumption of minimally processed produce, increased international trade, and changes in agronomic and processing practices (1).

HAV, like many other enteric viruses, contains single-stranded genomic RNA. HAV is known to be relatively stable and resistant to environmental factors such as acidity (16), cold (3), and freezing temperatures (14). Knowledge of the survival rates of HAV in fresh produce during common storage conditions could assist in risk assessment of produce-borne viral diseases. Survival of HAV in lettuce, fennel, carrot, and bell pepper was reported previously (4, 19), and HAV was found to survive better than feline calicivirus and many bacterial pathogens on produce surfaces at 18 to 31°C (19). Variability in HAV survival was observed among lettuce, fennel, and carrot stored at 4°C for 9 days (4). In this study, we estimated the inactivation or reduction rate of HAV on inoculated fresh spinach leaves during extended cold storage up to 42 days. Since illness-implicated produce is recalled after hepatitis symptoms appear, the study’s time frame was designed to approximate the incubation period required for the symptoms to appear after consumption (4 ± 1 weeks average). Viral survival in contaminated produce is an important factor in successfully identifying viral etiology in produce. The main objectives of this study were to compare HAV recovery from spinach by different eluents and to estimate HAV survival in spinach during extended storage.

MATERIALS AND METHODS

Virus and mammalian cell culture. Fetal rhesus kidney (FRhK-4) cells were grown in Eagle’s minimum essential medium supplemented with 15% fetal calf serum, 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.11% sodium bicarbonate, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 0.1 mg/ml kanamycin, and 0.1 mg/ml gentamycin (Gibco/Invitrogen, Carlsbad, CA). The cytopathic strain of HAV HM175 was propagated in FRhK-4 cells. The HAV-infected cells were freeze-thawed three times and then extracted with an equal volume of chloroform. The mixture of HAV-infected cell lysate and chloroform was centrifuged at 2,000 × g for 15 min at 4°C. The supernatant containing viruses was collected after centrifugation and used for inoculation.

HAV inoculation and elution from spinach leaves. Fresh baby spinach leaves in bags and plastic containers from different
producers were purchased from local markets. The steps in inoculation and elution of viruses from spinach are described in Figure 1. Ten microliters of chloroform-extracted HAV stock (described above) was spread on the surface of each leaf using a micropipette tip. Each leaf was placed in a Petri dish and air dried 30 min in a biological safety cabinet. First, a total of eight eluents (elution buffers) were evaluated for their ability to elute and recover viruses from spinach. The eluents used were distilled water, Butterfield phosphate buffer (pH 7.1), phosphate-buffered saline (PBS, pH 7.5), cell culture grade), PBS plus 2% serum, and 3% beef extract plus 0.1% Tween 80 (pH 7.5 and pH 8). The pH was adjusted by 1 N NaOH and measured by a pH meter before eluents were autoclaved. Beef extract has commonly been used to elute viruses from water and other environmental samples, but it inhibits PCR (18). Both desiccated and powdered beef extracts (Becton Dickinson, Franklin Lakes, NJ) were compared for their elution efficiencies. In preparing the beef extract solutions, Tween 80 was first mixed with 20 ml of 3% beef extract, then filtered through a 0.45-µm-pore-size membrane and added to the autoclaved 3% beef extract solution at a final concentration of 0.1% Tween.

Spinach sampling during low temperature storage. Inoculated leaves were placed in Petri dishes and packed in polypropylene clear plastic bags with the inoculated side up. The thickness of the storage bags (0.85 mil [8.5 × 10⁻⁴ in.] permitted air exchange for leaves during storage. Data from three to five leaves were averaged for each sampling during storage. Sampling was conducted at intervals of 1 h, 1 day, 1 week, 2 weeks, 4 weeks, and 6 weeks after virus inoculation (Fig. 2 and Table 2). Two trials were completed: trial 1 for 28 days and trial 2 for 42 days. The inoculation level of HAV (3.5 log eluted from a leaf) in trial 1 was approximately 1 log lower than that (4.7 log inoculated and 4.5 log eluted from a leaf) in trial 2.

HAV plaque assay. Approximately 4 days after the seeding of FRhK-4 cells onto 6-well culture plates (35 mm in diameter per well; Becton Dickinson, Franklin Lakes, NJ), confluent cells were inoculated with three dilutions (mostly in two- or threefold) of a virus sample in duplicate. The inoculated cells were incubated at 37°C with 5% CO₂ for 80 min. A 4-ml overlay of Eagle’s minimum essential medium supplemented with 0.9% agarose was added to each well (allowed to solidify) after 80-min incubation at 37°C. Seven days later, an additional 4 ml of Eagle’s minimum essential medium supplemented with neutral red and 0.9% agarose was added to each well and incubated at 37°C with 5% CO₂ for another 3 to 5 days to allow neutral red to stain viable cells. Viral plaques (clear areas) were then enumerated. For data accuracy, only plaque numbers in the range of 10 to 80 were used for calculation. At the end of 4 weeks of storage in trial 1, HAV in eluates had to be concentrated 10-fold by Centricron 15 (Millipore, Billerica, MA), because plaque numbers per well were fewer than 10. Centricron 15 tubes containing eluates were centrifuged at 4,000 × g for 20 min at 5°C.

Statistical analysis. Means, standard deviations, regression equations, correlation coefficients, and t tests were carried out using Microsoft Excel.

RESULTS

Comparison of elution efficiencies. Three eluents were first selected and compared: PBS (cell culture grade, pH 7.5), PBS (Butterfield, pH 7.1), and water. The HAV recovery by culture grade PBS (pH 7.5) was 25% ± 9% and was higher than recovery by the other two eluents (following the elution procedure in Fig. 1). In an attempt to improve HAV recovery and to retain bioactivity during the elution process, bovine serum (2%) was incorporated into PBS, pH 7.5. In the next side-by-side experiments, an

### TABLE 1. Comparison of five eluents’ efficiency in recovery of HAV from spinach leaves

<table>
<thead>
<tr>
<th>Eluent</th>
<th>HAV recovery by cellular infectivity</th>
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<tr>
<td>PBS + 2% serum</td>
<td>(1.96 ± 0.36) × 10³</td>
</tr>
<tr>
<td>3% beef extract P, pH 7.5</td>
<td>(1.88 ± 0.62) × 10³</td>
</tr>
<tr>
<td>3% beef extract P, pH 8.0</td>
<td>(1.90 ± 0.48) × 10³</td>
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<tr>
<td>3% beef extract D, pH 7.5</td>
<td>(1.66 ± 0.79) × 10³</td>
</tr>
<tr>
<td>3% beef extract D, pH 8.0</td>
<td>(1.63 ± 0.46) × 10³</td>
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* Values (mean ± standard deviations) were averaged from seven to eight samples, collected from two independent experiments.
* The differences in recovery were insignificant between the simple eluent, PBS plus 2% serum, and any of the complex eluents (by t test with P of 0.05).
* P, powdered beef extract plus 0.1% filtered Tween 80.
* D, desiccated beef extract plus 0.1% filtered Tween 80.
improved recovery (39%) by PBS plus 2% serum was observed, significantly (t test, P of 0.05) higher than the 26% recovery by PBS alone. We therefore evaluated complex eluents (Table 1) that might further enhance the recovery or survival of viruses during the elution process (Fig. 1). Powdered beef extracts recovered higher quantities of viruses than desiccated beef extract solutions (Table 1). Of the four complex eluents evaluated, 3% powdered beef extract plus 0.1% Tween 80 (pH 8) recovered the highest quantity of HAV from fresh spinach leaves. However, the simple eluent PBS plus 2% serum (pH 7.5) still recovered a greater quantity of HAV (38.5%) than complex eluents.

**Survival of HAV in fresh spinach leaves stored at 5.4 ± 1.2°C.** PBS containing 2% serum was chosen as the eluent for the survival part of the study: it had better HAV recovery than complex eluents, and it did not inhibit PCR as beef extract possibly would. Viruses were eluted from fresh and stored spinach leaves using the procedure shown in Figure 1. The overall recovery of HAV from spinach leaves by PBS plus 2% serum was approximately 45% of the original inoculation levels.

We observed moderate drops in HAV levels in sampling conducted 24 h after inoculation, with a reduction of 0.3 log in trial 1 (Fig. 2) and 0.59 log in trial 2 (Table 2 and Fig. 2). Interestingly, HAV levels varied little within the first week in both trials. Although the HAV inoculum in trial 1 was approximately 1 log lower than that in trial 2, the overall HAV levels in both trials decreased similarly and slowly over 4 to 6 weeks at refrigerated temperatures (Fig. 2). At the end of 4 weeks of storage in trial 1, HAV in eluates were concentrated 10-fold by Centricon 15 for proper enumeration of viral plaques. When Centricon ultrafiltration was used in our laboratory, an average 29.3% recovery of virus was derived (with three individual recoveries of 18, 34, and 36%); taking this average recovery into account, the survival rate for HAV in spinach leaves in trial 1 was 8.5% after 4 weeks of storage (Fig. 2). To avoid using the concentration step in the later stage of storage in trial 2, a higher level of HAV inoculation was implemented; 5% of the original HAV inoculum survived after 4 weeks of storage (Table 2). Averaging both trials, the overall survival rate for HAV in spinach leaves was 6.75% after 4 weeks under refrigeration, a reduction slightly greater than 1 log. In trial 2, 2% of the HAV original inoculum was found in leaves after 6 weeks at refrigerated temperatures, 5.4 ± 1.2°C.

All survival data listed above were calculated on a baseline: the HAV titers eluted from spinach leaves shortly after inoculation. Two regression equations were fit for each trial (Fig. 2). Both regression lines presented reasonable correlation coefficients, \( r^2 = 0.89 \) and 0.87 for trials 1 and 2, respectively (an average of \( r^2 = 0.88 \)). Thus, the log changes of HAV titer were linearly correlated to the storage time. Interestingly, similar HAV inactivation or reduction rates were observed in the two trials (with slopes of \(-0.036 \) and \(-0.034 \) for trials 1 and 2, respectively), despite different levels of inoculation (1-log difference) and different sampling and storage times. Overall, HAV survived under refrigerated temperatures on spinach leaves with a D-value of 28.6 days. One of the background controls (HAV levels in PBS plus 2% serum alone) illustrated that HAV levels remained fairly constant, 21 to 29%, from the first to the seventh weeks during the same refrigeration storage (Table 2).

**DISCUSSION**

Consumption of contaminated fruits and vegetables is the probable cause of many foodborne viral outbreaks and illnesses, but the viral etiological agents are not always identifiable in implicated fresh produce. In this study, we examined the survival of HAV in fresh spinach under refrigeration storage for 28 to 42 days, the approximate time required for symptoms to appear after consumption.

Inactivation of viruses may have occurred during their exposure to different environments, including spinach leaf contact, dehydration from air drying, temperature changes, and physical forces such as shaking or mixing. Experiments comparing PBS with and without 2% serum showed that HAV infectivity was better preserved by the addition of serum. In fact, our molecular assay (not shown in this report) illustrated that both solutions eluted the same quantity of viral genomic RNA. Although PBS plus 2% serum was appropriate for virus elution from spinach, preliminary experiments showed that this eluent may not be appropriate for other produce; lower recovery of HAV from green onions was observed when this simple eluent was applied.
On the surfaces of produce, the survival rates of HAV have been found to be closer to those of Clostridium perfringens and phage PRD1 than to those of bacteria and feline calicivirus (19). The loss of 2 log or more of HAV on lettuce, fennel, and carrot was reported after 9 days’ storage at 4°C (4). On the contrary, our study demonstrated that a 1-log reduction of HAV on spinach leaves under refrigeration took 28.6 days. Interestingly, the reduction rate of HAV on spinach leaves was similar to the inactivation rate of −0.030 log of HAV reported in seawater at 5°C (3) and close to that in groundwater as well (8). HAV inactivation rates in groundwater were similar to those of coxsackievirus and phage PRD1 (reduced at 0.02 to 0.04 log/day) (8). HAV survived longer than poliovirus in mineral water (2). As shown by one of our control experiments in this study, no apparent inactivation of HAV occurred when HAV was inoculated into the eluent and stored 7 weeks under the same conditions. Chemical constituents of spinach leaf possibly accelerated the inactivation rate or die-off of HAV under refrigeration. Antiviral activity could be one explanation; antiviral activity was reported in apple pulp and skin when poliovirus type 1 and coxsackievirus B5 were tested (9). Since moisture droplets were visible inside of the storage bags (possibly via condensation), the HAV inactivation might not be attributed to the dehydration of the leaves. In addition, HAV in marinade (pH 3.75) did not show significant reduction over 4 weeks, while HAV in marinated mussels was reduced by 1.7 log (6). Furthermore, up to 30% of HAV remained infectious on volunteers’ finger pads 4 h after application (11). HAV in dried feces was still infectious after a month at 25°C (12). In general, HAV survived relatively well in environments such as soil, wastewater, coastal water, and shellfish (6, 17). The knowledge gained from studies of the inactivation or reduction rate of viruses defined in food matrices could be useful in future for assessing risks associated with viral contamination in foods.

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REFERENCES


