Research Note

Green Onions: Potential Mechanism for Hepatitis A Contamination

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ABSTRACT

The largest documented foodborne hepatitis A outbreak in U.S. history occurred in November 2003. The source of that outbreak was green onions from a farm in Mexico. Two biomarkers were used to determine ways in which hepatitis A virus (HAV) can contaminate onions. Fluorescent microspheres (1.0 to 10 μm) and HAV vaccine were placed on the soil and in the liquid medium of hydroponically cultivated onions. Reverse transcription PCR (RT-PCR) was used to identify HAV RNA. Microspheres were found on the outside and inside of the pot-grown onions for up to 60 days. RT-PCR revealed HAV RNA from the vaccine in well-washed green onions. In the hydroponically grown onions, microspheres were found throughout the onion after only 1 day. RT-PCR also revealed HAV RNA inside the hydroponically grown onions. Both biomarkers support the hypothesis that HAV can contaminate the inside of the growing onion and can be taken up intracellularly through the roots. Once inside, the particles are impossible to remove by cleaning.

Hepatitis A virus (HAV) is a pathogen that can cause serious liver disease and in some rare cases death in humans. This picornavirus is found only in primates and replicates in the primate liver (1). Hepatitis A is a relatively self-limiting infection and accounts for around 100 deaths annually in the United States (11). The virus is usually transmitted directly from person to person but also can be transmitted through contaminated food and water. Foodborne cases account for only a small number of the hepatitis A cases reported in the United States yearly (2 to 5%) (3, 5). Between 1995 and 2003, 105 outbreaks of foodborne hepatitis A, with a total of 3,582 illnesses, were reported (3). This number of cases probably is an underestimate because many foodborne illnesses go unreported or unidentified (4). The majority of foodborne outbreaks of hepatitis A are associated with improper sanitation by food handlers or the use of contaminated water for cleaning food products (6). However, the movement of HAV into the system of produce from contaminated groundwater or irrigation sources has become a topic of increasing interest.

The contamination of food products, such as produce, with HAV can occur at multiple steps throughout the farm-to-table food product chain, including cultivation, harvest, processing, and handling (6). The early steps in the food line are even more important with produce because these products are usually only minimally processed. Contamination of produce in the field is one possible cause of the multiple outbreaks of hepatitis A associated with fresh produce. An outbreak of hepatitis A in 1997 was associated with contaminated frozen strawberries and resulted in 262 cases in five states. The source of the strawberries was a farm in Mexico. Another large outbreak occurred in 1988 and involved iceberg lettuce used at three restaurants in Kentucky. The source was also believed to be a farm in Mexico (6, 12). This outbreak was attributed to contamination of the lettuce irrigation water (12). The largest documented foodborne outbreak of hepatitis A in the United States occurred in 2003 in Pennsylvania and resulted in more than 600 cases and three deaths (13). This outbreak was traced to green onions that came from a farm in Mexico. Through extensive investigation after this outbreak, food handler contamination was ruled out as a cause. The likely source of the HAV appeared to be the raw onions themselves, which had been contaminated at the point of cultivation. Because the contaminated onions were used as an ingredient in several dishes served at the restaurant, they were chopped and stored in bulk, providing the opportunity for cross-contamination from the contaminated onions to clean onions and other ingredients and probably contributing to the size of this outbreak (13).

The objective of this experiment was to evaluate methods of internal HAV contamination in green onions using two biomarkers (fluorescent beads and HAV vaccine).

MATERIALS AND METHODS

Biomarkers. Onions were grown in the presence of two traceable biomarkers: fluorescent microspheres and an attenuated HAV vaccine. The fluorescent microspheres (Molecular Probes, Eugene, Oreg.) were readily visible with a fluorescent microscope.
and ranged from 1.0 to 10 μm in diameter. The HAV vaccine (Merck Inc., Whitehouse Station, N.J.) was derived from HAV grown in cell culture in human MRC-5 diploid fibroblasts and contained whole inactivated virus particles. The virus was inactivated in formalin and then adsorbed onto amorphous aluminum hydroxypophosphate sulfate. Each milliliter of the vaccine contained approximately 50 U of HAV antigen, which was purified and formulated without a preservative. The 50-U dose of HAV contained less than 0.1 μg of nonviral protein, less than 4 × 10⁻⁶ μg of DNA, less than 10⁻⁴ μg of bovine albumin, and less than 0.8 μg of formaldehyde (7).

Phase 1: plants grown in soil. In phase 1, the biomarkers were added to the green onions by spraying them on leaves and soil surrounding the plant. Green onions take approximately 80 days to grow from seed to a mature plant. We planted green onion seeds (Gerry-Morse Seed, Fulton, Ky.) in potting soil in 16 pots (12 in. [30.5 cm] diameter and 12 in. deep). All seeds were planted on 1 June 2004 (week 0) and harvested on 24 August 2004 (week 12). Four pots were used for control, four were used for fluorescent microspheres, and the remaining eight pots were used for HAV experiments. At week 4, fluorescent microspheres were sprayed on the outside skin of the plant and soil. Each pot was examined weekly. At week 9 (3 August 2004) and week 11 (17 August 2004), the HAV vaccine was added at the base (two pots at week 9 and two pots at week 11) or to the leaves (two pots at week 9 and two pots at week 11) of the plants.

Phase 2: hydroponic plants. We also grew green onions from seeds (Gerry-Morse Seed) hydroponically. The hydroponic system was set up indoors at 18 to 22°C. In a 10-liter hydroponic tank, nutrient water was circulated daily between 8 and 10 a.m. and between 3 and 5 p.m. (Baby Bloom System, American Hydroponics, Arcata, Calif.). Light for the plants was provided by 400-watt high-pressure sodium wide-spectrum bulbs (photosynthetic photo flux density of 400 to 700 nm; American Hydroponics, Arcata, Calif.), with 8 h of light daily. The nutrient solution was added at 7.5 ml/liter at the beginning of the experiment and weekly thereafter. The pH of the solution was monitored weekly and stayed between 5.5 to 6.5.

For the hydroponic experiments, 1 ml of fluorescent microspheres was placed in the circulating nutrient solution only, and the plants were assessed after 1, 2, 7, and 21 days. HAV was placed in the circulating solution after 30 days, and the plants were assessed after 7 days.

Harvest. Great care was used during harvest to avoid manipulation of the plant. To avoid contamination from the growth medium, the plants in pots were cut off above the soil level and the hydroponic plants were cut off above the level of the lava rocks. The plants were carefully and thoroughly washed individually. The outermost layer of each plant was removed for analysis. For conventional plants, the inside layers were analyzed separately, and the outermost three layers were carefully peeled off. Hydroponic plants did not grow as well, and the layers were not separated. All specimens were kept separately in new plastic bags.

The onions were sectioned with a cryotome (Microm, Walldorf, Germany), and 20-μm-thick slices were examined under ×40 magnification with a BX 51 microscope (Olympus, Tokyo, Japan). Fluorescence was quantified in six high-power fields from the top, middle, and bottom of five separate green onions at each time point (n = 30 per group) with an Adobe Photoshop 7.0 Image intensity pixel histogram (Adobe Systems, San Jose, Calif.). Negative controls were green onions not exposed to fluorescent microspheres.

RNA extraction. Total RNA from green onions was extracted with Trizol reagent (Invitrogen Life Technologies, Carlsbad, Calif.) following the manufacturer’s instructions. Green onion tissue (100 mg) was homogenized with a Polytron in 1 ml of Trizol, and 0.2 ml of chloroform was added to this homogenate, which was then centrifuged at 12,000 × g for 15 min at 4°C. The clear aqueous phase was transferred to a new tube, and the same volume of isopropanol was added to precipitate the RNA. After centrifugation at 12,000 × g for 10 min at 4°C, the RNA pellet was washed with 75% ethanol, centrifuged at 7,500 × g for 5 min at 4°C, air dried, and resuspended in 20 μl of nuclease-free water.

For HAV vaccine, 800 μl of Trizol was added to 100 μl of the vaccine plus 20 μg of glycogen and 200 μl of chloroform. The mixture was vortexed for 2 min, incubated at room temperature for 20 min, and centrifuged at 12,000 × g for 20 min at 4°C. The upper phase was precipitated with the same volume of isopropanol overnight at −20°C. After centrifugation, the pellet was washed with 70% ethanol, air dried, and resuspended in 20 μl of nuclease-free water.

RT-PCR. Reverse transcription (RT) of green onion RNA was performed with 2 μg of the rRNA, oligo (dT) 15 primer (Promega, Madison, Wis.), RNAsin, mouse mammary leukemia virus reverse transcriptase, and dNTP mix (10 mM each). The cDNA templates were prepared from the HAV vaccine RNA with random primers, dithiothreitol, RNAsin, and SuperScript II reverse transcriptase and incubated at 42°C for 1 h. The reaction was inactivated by heat at 70°C for 2 min. For amplification of virus-specific sequences, each PCR contained 2 μl of RT reaction, 40 pmol sense and 40 pmol antisense primers, 200 μmol dNTPs, 2 mM MgCl₂, and 0.4 U of Taq polymerase (Promega). Primers HAV-FW (GGTTTGCTCCTCTTTATCATGCTATG) and HAV-RV (GGAAATGTCTCAGGTACTTTCTTTG) were used to amplify a 245-bp fragment (nucleotides 2,169 to 2,413, accession no. M14707) (10). A 259-bp fragment of the mRNA of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal positive control. Primers for GAPDH were FW (CGTGGAAGTCTCAGGTTCTTCCAC) and RV (GATGCGATGAGCTGTTGCTCATGAGC). PCR amplification was carried out in a Mastercycler (Eppendorf AG, Hamburg, Germany) under the following conditions: initial heat denaturation at 94°C for 5 min, 40 cycles of 94°C for 1 min, 48°C for 80 s, and 72°C for 1 min, and a final extension at 72°C for 15 min. PCR products were loaded onto a 1.5% agarose gel, electrophoresed, and visualized by ethidium bromide staining and UV transillumination.

Statistical analysis. All values are presented as mean ± standard error of the mean. Comparisons among groups were conducted with an analysis of variance with post hoc analysis using Epinfo statistical software (Centers for Disease Control and Prevention, Atlanta, Ga.). Differences were considered significant at P < 0.05.

RESULTS

Phase 1. All the onions grew in the planted pots without difficulty, and the plants were exposed to 12 raining days. The green onions added layers starting underground during the entire 60-day life cycle from seed to mature plant. Both biomarkers persisted despite exposure to multiple raining days.

A 20-μm-thick cross section of onion was observed under low- and high-power microscopy with fluorescent filters. Microspheres placed on the plant surface were visible
FIGURE 1. Microscopic view of soil-grown onion treated with fluorescent microspheres. Normal onion without microspheres (A), and 0.1 ml of fluorescent microspheres placed on onion after 1 week (B), 3 weeks (C), and 8 weeks (D).

inside the onion at each weekly time point for up to 8 weeks after the initial microsphere application at 1 month after seeding, even after the outermost three layers had been carefully removed (Fig. 1).

The onions for the RT-PCR assay were kept in a freezer for a total of three nights. The onions themselves were washed twice: once after picking and once before the assay. The conditions under which the onions were assayed mimicked the conditions of actual green onion shipping and handling.

HAV vaccine placed on either the soil or the plant itself was revealed by RT-PCR assay inside the growing green onion but not on the outside after 1 and 3 weeks. Attenuated HAV vaccine was used to inoculate the plants, either by spraying the vaccine on the plant stem or onto the soil surrounding the plant. Of 10 onions harvested from inoculated plants, all (100%) were positive for HAV RNA on the inside of the plant, but none (0%) had HAV RNA on the outside of the plant (Fig. 2).

Phase 2. Mean fluorescence for six high-power fields of five individual control and treated onions (top, middle, and bottom) 1, 2, 7, and 21 days after fluorescent microsphere application is given in Table 1. Uptake of microspheres into the bottom, middle, and top of the plant 2 days after microsphere application is shown in Figure 3. More fluorescence was observed at the bottom than at the middle or top of each plant at day 1 only (Table 1) ($P < 0.001$). Fluorescence nearly doubled between day 1 and 2 and reached a plateau at day 7, which was maintained until day 21.

HAV vaccine placed in the hydroponic nutrient solution was revealed by the RT-PCR assay inside the growing green onion after 7 days. Of 10 onions harvested from inoculated plants, all (100%) were positive for HAV RNA (Fig. 4).

DISCUSSION

Our results revealed that biomarkers for HAV can be trapped within the growing green onion. The hydroponic system used in this study provided a controlled environment in which to study the possible association of micro-particles and HAV RNA with aerial tissues, with minimal

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<th>Onion section</th>
<th>Luminosity (pixels)</th>
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<tr>
<td></td>
<td>Control Day 1 Day 2 Day 7 Day 21</td>
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<tr>
<td>Top</td>
<td>224 ± 11 5,930 ± 401 13,854 ± 703b 18,543 ± 506b 17,428 ± 745b</td>
</tr>
<tr>
<td>Middle</td>
<td>244 ± 11 7,024 ± 543 15,353 ± 723b 19,249 ± 582b 18,943 ± 626b</td>
</tr>
<tr>
<td>Bottom</td>
<td>250 ± 9 8,324 ± 460 14,531 ± 483b 19,922 ± 716b 18,909 ± 823b</td>
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* Values are mean ± standard error; $n = 30$ plants per group. ANOVA with post hoc analysis.

* $P < 0.001$. Microspheres reached the top of the plant by day 1. Luminosity doubled by day 2 and reached a plateau by day 7. Day 1 value is significantly lower than those for days 2, 7, and 21. Day 2 value is significantly higher than that of day 1 but is lower than those of days 7 and 21.
FIGURE 3. Microscopic view of hydroponically grown onion treated with fluorescent microspheres (arrows). Control plant (A), and onion 2 days after microsphere treatment showing uptake at top (B), middle (C), and bottom (D) of plant.

FIGURE 4. RT-PCR assay of HAV RNA from hydroponically grown onion. A positive band is visible for RNA extracted from the plants treated with HAV vaccine (arrow, lane 4, numbered at the bottom) but not for control plants (lane 3). GAPDH (lanes 5 and 6) is the control for RNA in either extract.

Concerns about environmental contamination or temperature fluctuation. Greater fluorescence was observed at the bottom than at the middle and top of each plant at day 1, and fluorescence nearly doubled between days 1 and 2, reaching a plateau at day 7 that was maintained until day 21.

HAV vaccine placed on either the soil or the plant itself was revealed by RT-PCR assay inside the growing green onion but not on the outside after 1 and 3 weeks. HAV vaccine placed in the hydroponic solution was identified inside the growing green onion after 7 days. All of the onions harvested from inoculated plants were positive for HAV RNA. Thus, HAV RNA and microspheres ranging in size from 1.0 to 10 \( \mu \text{m} \) can be transported from an inoculated nutrient solution to the growing green onion. All layers, outside and inside, were carefully cleaned to remove any vaccine on the outside of the layers. Rain also may have washed the vaccine from the outer layers into the soil for those plants in which the vaccine was sprayed on to the leaves. It is unclear how the vaccine was able to get into the inside layers of the onion (most likely thorough the roots) but not into the outer layer through the roots. The onions were already 2 months old when they were exposed to the HAV vaccine. One speculation is that the outermost layer was no longer viable at that point and was not absorbing nutrients or other elements from the soil.

Our experiments do not definitively prove that live HAV can be taken into the green onion or that the virus would survive. Because the onions did not all grow to marketable size in the hydroponic system, the diminished growing conditions may have contributed to the contamination of the hydroponic onions. In other studies, contamination of produce frequently associated with outbreaks has been examined; Guo and colleagues demonstrated uptake of Salmonella into tomatoes (8, 9).

Contamination of food plants with enteric viruses most likely occurs through irrigation with wastewater, fertilization with sludge, or mismanaged fecal waste near the growing area (11). The last of these three contamination routes is most often associated with poor personal hygiene of workers or lack of control of children in the fields. Viruses can persist in soils for many months when conditions are good, i.e., moist and cool soil or groundwater (2). Viral contamination of groundwater is clearly possible. In a study of well and surrounding groundwater contamination in Wisconsin, Borchardt et al. (2) found that 8% of wells were contaminated with viruses. Two of the viruses found in these contaminated wells were rotavirus and HAV. These two viruses are known foodborne pathogens and can cause foodborne illness when consumed in raw produce.

Because of the harvesting season for certain produce products, a likely source of produce contamination is irrigated large-yield foreign farms. Although practices in the United States usually prevent this type of contamination, practices in countries of origin for certain imported products are not as well monitored. This lack of oversight is a concern for the United States because more than $6 billion in produce was imported into the United States in 2000, and this number is increasing (7). In a recent report, the Centers for Disease Control and Prevention (6) indicated a possible foreign origin for contaminated food involved in six of seven hepatitis A outbreaks associated with predistribution contamination of produce. Thus, foreign trade is a considerable concern for food safety. The possibility of internal green onion contamination by HAV adds urgency to the monitoring of practices on U.S. farms and on farms that export produce to the United States.

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REFERENCES