

# Rapid and Quantitative Detection of Hepatitis A Virus from Green Onion and Strawberry Rinses by Use of Real-Time Reverse Transcription-PCR

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**In this study, an immunomagnetic capture method and a real-time reverse transcription-PCR assay were used to quantify hepatitis A virus (HAV) in green onion and strawberry rinses. This combined protocol detected as low as 0.5 PFU HAV in produce rinses and concentrated HAV levels up to 20-fold.**

Hepatitis A virus (HAV) is responsible for acute infectious hepatitis, which is endemic worldwide. Recent studies suggested that HAV is one of the most common causes of illness through oral-fecal infection (16). The major routes of food contamination include shellfish, fruits, and vegetables, which often become contaminated through contaminated water in their growing area or during preparation through contact with fecally contaminated surfaces or infected food handlers (12). Recent food-borne outbreaks of hepatitis A have been associated with strawberries (2, 11, 19) and green onions (9). Poor growth of most wild-type HAV strains *in vitro* makes cell culture propagation of HAV difficult to perform (10). Real-time PCR is one of the most promising detection methods due to its sensitivity, specificity, speed, and possibility to deliver quantitative data. However, its use has thus far been limited to clinical and environmental samples (1, 5, 22). The main obstacles concerning routine detection of HAV in food include the presence of inhibitory substances in the samples and the low concentration of virus recovered. Immunomagnetic separation (IMS) is one of the sample treatment methods that can address these limitations. It is the purpose of this study to apply a real-time reverse transcription-PCR (RT-PCR) assay combined with IMS pretreatment for quantification of HAV in fresh produce.

Fresh produce was purchased locally. Twenty-five grams of sample was vigorously shaken with 250 ml phosphate-buffered saline (PBS; pH 7.4) for 2 min. After centrifugation at  $1,000 \times g$  for 15 min to remove heavy materials, the rinse was stored at  $-20^{\circ}\text{C}$ . In all experiments, nonseeded samples were used as negative controls. FRhK-4 (fetal rhesus monkey kidney-derived) cells and cytopathic HAV strain HM-175 24A were used (provided by T. Cromeans, Centers for Disease Control and Prevention, Atlanta, Ga.). HAV was propagated as described by Cromeans et al. (6), and the virus was enumerated by plaque assay following the method of Mullendore et al. (18). Viral RNA was extracted using TRIzol reagent (Invitrogen, Rockville, MD) according to the manufacturer's instructions. A real-time RT-PCR assay was performed using the Light-

Cycler HAV quantification kit (catalog no. 3246795; Roche Diagnostics, Germany) and LightCycler equipment (Roche Diagnostics) according to the manufacturer's instructions. Monoclonal antibodies against HAV (anti-HAV 1009; Argene, New York) were coupled with streptavidin-coated Dynabeads M-280 (2.8  $\mu\text{m}$  in diameter; 10 mg/ml) and Dynabeads MyOne (1.05  $\mu\text{m}$  in diameter; 10 mg/ml) (Dyna, Great Neck, NY). Antibodies were first biotinylated using the EZ-Link Sulfo-NHS-LC biotinylation kit (Pierce Biotechnology, Rockford, IL) and then attached to Dynabeads according to the manufacturer's instructions. To optimize the IMS conditions, different quantities of Dynabeads (12.5, 25, 37.5, 50, 67.5, 75, and 100  $\mu\text{l}$ ) were added to 1 ml PBS seeded with  $10^5$  PFU HAV. After incubation at room temperature for 1.5 h the beads were collected and washed three times with 1 ml PBS. Finally, the collected beads were resuspended in 50  $\mu\text{l}$  PBS, boiled for 5 min, and chilled on ice. The beads were harvested by centrifugation at  $12,000 \times g$  for 1 min. The supernatant was analyzed using real-time PCR. The same procedure was used to compare the antigen capture abilities of both bead types.

In this study, both PFU and RNA copy number were used as the measures to indicate the amount of HAV in the real-time RT-PCR assay. In the present study PFU as a detection unit was used to compare our data with data from previous studies (14), but since its use is not applicable for noncytopathic wild-type HAV strains and also because there is a variation in viral RNA copies per PFU in different HAV strains, the use of

TABLE 1. Detection of HAV RNA standards in serial 10-fold dilutions using real-time RT-PCR

Log dilution	No. of copies		CV (%) <sup>a</sup>
	Mean	SD	
0 <sup>b</sup>	900,333	25,423	2.8
1	82,500	5,703	6.9
2	8,110	690	8.5
3	1,237	122	9.9
4	110	6	5.5
5	6	2	40.0
6	0.2	0.4	173.2

<sup>a</sup> The CV of a set of data was determined by dividing the standard deviation by the arithmetic mean of the measured values.

<sup>b</sup> HAV RNA standard was  $9.8 \times 10^5$  copies.

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TABLE 2. HAV RNA copies detected in seeded buffer and samples using real-time RT-PCR

Sample	No. of RNA copies at HAV dilution (PFU/mL) of:			
	10 <sup>5</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>
PBS	2,028 <sup>Aa</sup>	365 <sup>A</sup>	27 <sup>Ac</sup>	3 <sup>A</sup>
Green onion <sup>b</sup>	1,800 <sup>A</sup>	146 <sup>B</sup>	8 <sup>B</sup>	1 <sup>A</sup>
Strawberry <sup>b</sup>	1,879 <sup>A</sup>	152 <sup>B</sup>	10 <sup>B</sup>	1 <sup>A</sup>

<sup>a</sup> Means in the same column followed by different superscript capital letters are significantly different by Fisher's protected LSD test ( $P \leq 0.05$ ).

<sup>b</sup> Green onion and strawberry rinses were seeded with a known titer of HAV.

<sup>c</sup> Data below 100 copies were semiquantitative and were calculated by extrapolation from a standard curve.

RNA copy number as the detection unit was chosen as an alternative way to quantify the amount of HAV. Statistical significance was tested by one-way analysis of variance and Fisher's protected least significant differences (LSD) test.

**Quantification with the real-time RT-PCR assay.** To evaluate the quantitative real-time PCR assay for HAV, serial 10-fold dilutions of the HAV external standards were amplified by real-time RT-PCR to generate a standard curve. The standard curve was linear over the range from 10<sup>6</sup> to 10<sup>1</sup> copies, with a slope of -3.41 ( $R^2 = 0.996$ ). The real-time RT-PCR assay could distinguish as few as 100 copies of the HAV RNA with a high degree of confidence, as indicated by the low coefficient of variation (CV; <10%) (Table 1). Since the CV increased to 173% at low standard RNA copy number, the linear slope between 10<sup>2</sup> and 10<sup>6</sup> RNA copies was used for quantification of HAV. The detection limit of the assay was 10 copies of viral RNA per reaction mixture, as this was the lowest dilution that could be consistently detected. However, a single copy of viral RNA was also occasionally detected. These results were similar to or better than the lowest HAV detection limits reported so far (4, 5, 8, 13, 20).

**Effects of food components on real-time RT-PCR assay.** The numbers of viral RNA copies detected in green onion rinse and strawberry rinse seeded with 10<sup>4</sup> and 10<sup>3</sup> PFU/ml of HAV were significantly lower than those in PBS controls (Table 2). End point detection results showed that real-time RT-PCR could detect 100 PFU/ml HAV (0.5 PFU per PCR) in all seeded green onion and strawberry rinses, but only one-third of samples seeded with 10 PFU/ml HAV were detectable, suggesting a 10-fold-lower sensitivity than that in PBS (Table 3). These results confirm the need for additional sample treatment prior to quantification to remove PCR inhibitors (21).

**Detection of HAV in seeded green onion and strawberry**

TABLE 3. Detection of HAV seeded in samples by real-time RT-PCR

Sample	No. positive/no. samples tested									
	Real-time RT-PCR at log virus titer (PFU/ml) of:					IMS/real-time RT-PCR at log virus titer (PFU/ml) of:				
	5	4	3	2	1	5	4	3	2	1
PBS	7/7	7/7	7/7	7/7	6/7	2/2	2/2	2/2	2/2	2/2
Green onion <sup>a</sup>	5/5	2/2	2/2	5/5	1/3	5/5	2/2	2/2	5/5	2/2
Strawberry <sup>a</sup>	5/5	2/2	2/2	5/5	1/3	5/5	2/2	2/2	5/5	3/3

<sup>a</sup> Green onion and strawberry rinses were seeded with a known titer of HAV.

TABLE 4. Quantification of HAV RNA in green onion and strawberry rinses by direct real-time RT-PCR and IMS/real-time RT-PCR

Sample	Virus titer (PFU/ml)	No. of HAV RNA copies		Ratio of IMS/PCR to PCR
		Direct real-time RT-PCR	IMS/real-time RT-PCR	
Green onion rinse <sup>a</sup>	10 <sup>2</sup>	2 <sup>b</sup>	3	1.5
	10 <sup>5</sup>	2,836 <sup>Ac</sup>	7,053 <sup>B</sup>	2.5
Strawberry rinse	10 <sup>2</sup>	0.2	2	10
	10 <sup>5</sup>	1,879 <sup>A</sup>	38,540 <sup>B</sup>	20.5

<sup>a</sup> Green onion and strawberry rinses were seeded with a known titer of HAV.

<sup>b</sup> Means in the same row followed by different superscript capital letters are significantly different by Fisher's protected LSD test ( $P \leq 0.05$ ).

<sup>c</sup> Data below 100 copies were semiquantitative and were calculated by extrapolation from a standard curve.

**rinses using IMS/real-time RT-PCR.** During the development of the IMS methods, the optimal amount of beads for binding virus in 1-ml samples was found to be 250 µg for both bead types: Dynabeads M280 and MyOne (data not shown). Data from repeated experiments showed that the amount of HAV captured by both beads was not significantly different (up to 21% for M280 and up to 27% for MyOne). Bidawid et al. (4) reported that the IMS system using M280 beads coated with K3-2F2 antibodies specifically captured 40.6% of the virus suspended in PBS (10<sup>3</sup> PFU/ml). Differences between the present study and others may be a result of a number of factors, such as the type of antibodies, the way that antibodies were coated on the beads, and the components in the samples (15, 17).

The ability of IMS to remove PCR-inhibitory materials from the samples and concentrate virus for PCR analysis and, thus, improve the sensitivity of detection has been reported in previous studies (1, 3, 4, 7, 13). In the present study, the sensitivity of the IMS/real-time PCR assay on food samples was determined in fresh produce rinses seeded with HAV. In both green onion and strawberry samples, the sensitivity attained with IMS treatment was 10 PFU/ml, which was 10-fold higher than in samples without IMS treatment (Table 3). Using quantification, in 1 ml strawberry rinse seeded with 10<sup>5</sup> PFU of HAV, IMS-treated samples captured more than 20 times more HAV particles than those without IMS treatments (Table 4).

In summary, this study demonstrated for the first time the application of IMS combined with real-time RT-PCR for quantification of HAV in food rinses. This procedure can be completed within 6 hours and has the potential to be applied for routine surveillance of HAV in fresh produce and environmental samples.

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