Surveillance Study of Hepatitis A Virus RNA on Fig and Date Samples

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A total of 91 fig and 185 date samples were analyzed by reverse transcription (RT) real-time PCR for the presence of hepatitis A virus (HAV) RNA. Two batches of dates tested positive, and the HAV RNA detected was genotyped as IA. These findings warrant further development of methods applicable to food which is consumed untreated and is exported from countries in which HAV is endemic.

Foods implicated in food-borne hepatitis are filter-feeding bivalve molluscs and produce such as leafy vegetables and soft fruits. Produce may become contaminated with viruses during cultivation, harvest, processing, storage, distribution, or final preparation (3, 4). A wide variety of products from regions in which hepatitis A virus (HAV) is endemic are nowadays internationally traded and imported into areas of low endemicity, which may result in a higher likelihood of severe symptomatic illness developing in adults (10). A surveillance study was initiated after the report in 2007 of a Dutch patient with hepatitis A (data not shown) with no risk factors for HAV infection but with a frequent consumption of dates.

Between June 2009 and November 2010, 91 samples of figs and 185 samples of dates from at least 12 exporting countries were collected from retail stores throughout the Netherlands. Samples, each about 250 to 300 g, were transported and stored at room temperature until analyses for the presence of HAV RNA were performed. Of each retail sample, a subsample of 30 g of figs or dates was weighed in a plastic container and inoculated with 2×10^2 50% tissue culture infective doses (TCID₅₀) feline calicivirus (FCV) (10 μ l) (kindly provided by E. Duizer, RIVM, Bilthoven, The Netherlands). FCV was chosen as the sample process control virus (1) because FCV was already in use for detection of norovirus (NoV) RNA and because the use of Mengo MC0, which is more similar to HAV, as a control was not allowed in our laboratory as it is a genetically modified organism (GMO). An equal amount of FCV was added to 90 μ l of H₂O and kept frozen for use as a 100% control sample in downstream analysis.

After an incubation period of 20 min at room temperature, to allow the spike to dry and attach to the matrix, 5 ml of TGBE elution buffer (100 mM Tris, 50 mM glycine, 1% [wt/vol] beef extract, pH 9.5) was added to each container. The surfaces of the fruits were rinsed at least 10 times with the TGBE buffer by repeatedly pipetting. After 15 min, 2 ml of the solution was transferred to an Eppendorf tube and clarified by centrifugation at $10,000 \times g$ at 4°C for 15 min. Subsequently, the supernatant was transferred to a 50-ml tube and used for RNA extraction using the Nuclisens magnetic extraction reagent kit (bioMérieux Benelux, Boxtel, The Netherlands) according to the manufacturer's instructions. The RNA was eluted into 100 μ l of elution buffer included in the kit, and samples were stored at -80°C until further analyses. For virus extraction from fig and date samples, the above-described extraction method was proven to be at least as good in our laboratory as the more established CEN/TAG4 extraction protocol (7) using an elution volume of 40 ml and an overnight virus concentration step of the eluted viruses by polyethylene glycol (PEG) precipitation (Table 1).

Aliquots of 3 μ l of an RNA were analyzed in duplicate using two-step reverse transcription (RT) real-time PCR using primers and probes for HAV and FCV as described earlier (2, 8). A single-stranded RNA (ssRNA) external control HAV (EC-HAV) (2) (kindly provided by A. Bosch) was used as a control for amplification in an external reaction following the CEN/TAG4 proposed approach (7). Samples were considered positive if amplification plots of the real time signals showed an S curve and had a threshold cycle (C_T) value below 43. Results were accepted only when all controls were in place and test samples showed a recovery of FCV higher than 1% and a reverse transcription efficiency higher than 12.5%. For this, the FCV recovery was calculated by subtracting the C_T value of the 100% control sample from the C_T value obtained for a test sample (ΔC_T) and setting the recovery equal to $2^{(-\Delta C_T)} \times 100\%$. Likewise, the efficiency of RT-PCR detection was calculated by subtracting the C_T value of 1 μ l (10 RT-PCR units) ssRNA (EC) HAV standard when added to water from the C_T value for the ssRNA EC-HAV standard when added to a sample ($EC\Delta C_T$) and setting the efficiency equal to $2^{(-EC\Delta C_T)} \times 100\%$.

After analyses of 276 samples, 97.8% of the samples showed a recovery of the sample process control higher than 1%, and 94.6% of the samples had an RT efficiency above 12.5%. In total, 257 (93.1%) of the 276 samples met both inclusion criteria. None of the excluded samples tested positive for HAV RNA. Of the 257 samples that met the inclusion criteria, none of the 88 fig samples tested positive for HAV RNA, but HAV RNA was detected in one of the 169 date samples. The sample was detected at a C_T value of 38.20 in only one of the duplicates, which by extrapolation would correspond to about 15 HAV genome copies per 30 g of dates. Two out of four new subsamples tested also positive (C_T , 36.69 to 38.21). After traceback, HAV was detected in 3 of 14 packages of the same expiry date, with C_T values ranging from 36.83 to 38.80. In the same 6-month period, a hepatitis A patient, whose illness was of unknown source and was finally typed as genotype 1B, had stated that he had eaten dates prior to becoming ill. A total of 38 subsamples from 5 samples from another company were analyzed. Only two subsamples, which had been taken from 2 of the 5 samples, tested positive, with C_T values of 39.18 and 42.0. By extrapolation, these findings would correspond to less than 15 copies per

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TABLE 1 Direct comparison of extraction methods for the detection of HAV RNA on fig and date samples

No. of HAV genome copies inoculated	Result for figs with protocol				Result for dates with protocol			
	TGBE rinse		CEN/TAG4		TGBE rinse		CEN/TAG4	
	<i>n/N</i> ^a	Recovery (%)	<i>n/N</i>	Recovery (%)	<i>n/N</i>	Recovery (%)	<i>n/N</i>	Recovery (%)
1.5×10^5	6/6	52	6/6	61	6/6	56	6/6	56
1.5×10^4	6/6	22	6/6	19	6/6	29	6/6	36
1.5×10^3	5/6	22	4/6	46	5/6	67	6/6	49
1.5×10^2	2/6	109	2/6	72	4/6	89	0/6	0
1.5×10^1	1/6	ND ^b	0/6	0	0/6	0	0/6	0
Total	20/30		18/30		21/30		18/30	

^a The number of samples positive/the number of samples tested.

^b ND, not detected.

30 g of dates, which is far below the limits of detection, which were determined to be 3.2×10^3 and 2.5×10^3 HAV genome copies in 30 g of figs and dates, respectively.

Presumptive positive samples were rerun using a nested classic RT-PCR using primers with slight modifications targeting the VP1-2A region (6, 11). The sequences detected on the dates from the two companies showed similarity for 455 of 460 nucleotides, and were typed as a HAV genotype IA. Finally, no patient with identical sequences has been identified (de dato August 5, 2011) in the Netherlands or in the European Food Borne Virus Network database. However, this could be a result of underreportage or related to the fact that not all human strains are being typed by sequencing following human diagnostics on the basis of serology.

Unfortunately, the method used here is unable to discriminate between RNA from infectious and noninfectious particles. Nevertheless, the demonstration of HAV RNA on dates was considered to pose a serious risk to human health and the dates have therefore been recalled. The findings in this study, and those of recent outbreaks related to semidried tomatoes (5, 9), are suggestive for a broader arsenal of food products that may act as vectors of HAV transmission than previously thought. This makes it necessary to update questionnaires on consumed food items in outbreak investigations and to work on optimized extraction protocols for new food items. Good hygienic practices should be a concern for food business operators in exporting as well as importing countries.

Nucleotide sequence accession numbers. The sequences detected in this study were deposited in GenBank under accession numbers JN873911 and JN873912.

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