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International Journal of Food Microbiology 73 (2002) 29–34

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.com/locate/ijfoodmicro

The survival of hepatitis A virus in fresh produce

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Received 25 March 2001; received in revised form 27 July 2001; accepted 18 September 2001

Abstract

Fresh produce has been repeatedly implicated as the source of human viral infections, including infection with hepatitis A virus (HAV). The objective of the present study was to evaluate the HAV adsorption capacity of the surface of various fresh vegetables that are generally eaten raw and the persistence of the HAV. To this end, the authors experimentally contaminated samples of lettuce, fennel, and carrot by immersing them in sterile distilled water supplemented with an HAV suspension until reaching a concentration of 5 log tissue culture infectious dose ($TCID_{50}$)/ml. After contamination, the samples were stored at 4 °C and analysed at 0, 2, 4, 7, and 9 days. To detect the HAV, RT-nested-PCR was used; positive samples were subjected to the quantitative determination using cell cultures. The three vegetables differed in terms of their adsorption capacity. The highest quantity of virus was consistently detected for lettuce, for which only a slight decrease was observed over time (HAV titre = 4.44 ± 0.22 log $TCID_{50}$/ml at day 0 vs. 2.46 ± 0.17 log $TCID_{50}$/ml at day 9, before washing). The virus remained vital through the last day of storage. For the other two vegetables, a greater decrease was observed, and complete inactivation had occurred at day 4 for carrot and at day 7 for fennel. For all three vegetables, washing does not guarantee a substantial reduction in the viral contamination.

Keywords: Hepatitis A virus; Vegetables; Survival

1. Introduction

The role of food in the transmission of the hepatitis A virus (HAV) has been extensively documented (Cliver, 1997; Mele et al., 1997; Codex Alimentarius Commission, 1999), with the most commonly implicated foods being molluscs (Richards, 1985; Desenclos et al., 1991; Halliday et al., 1991; Lees, 2000) and fresh produce (Beauchat, 1995; Appleton, 2000). With a particular regard to fresh produce, this type of food has been reported to be the source of a fairly large number of cases of HAV infection in various countries (Ramsay and Upton, 1989; Mausezahl et al., 1996; Hernandez et al., 1997; Pebody et al., 1998; Dentinger et al., 2001), not to mention its involvement in an increasing number of viral infections in general, especially gastroenteritis caused by Caliciviruses (White et al., 1986; Ponka et al., 1999). Particularly dangerous are those products with a relatively brief period of growth and which are eaten raw (e.g., green salad) (Rosenblum et al., 1990; Pebody et al., 1998), for which viral contamination in general has often been reported (Monge and Arias, 1996; Hernandez et al., 1997).

The contamination of fresh produce can occur during growth, as a result of the use of contaminated fertilizers or waste-water (Tierney et al., 1977; Badawy

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0168-1605/02/$ - see front matter
PII: S0168-1605(01)00689-4

et al., 1990), and during the preparation and distribution, with the virus being transmitted by infected food-handlers when certain hygienic norms are not followed (Griffin et al., 1982; Rosenblum et al., 1990; Dalton et al., 1996). In fact, experimental studies have shown that approximately 9.2% of the infectious virus can be transferred from the contaminated hands of the handlers to lettuce (Bidawid et al., 2000).

The objective of the present study was to evaluate the adsorption capacity of the surface of various fresh vegetables and the persistence of the HAV. To this end, samples of experimentally contaminated lettuce, fennel, and carrots were stored at 4 °C and analysed at selected intervals for the presence of the HAV using RT-nested-PCR before and after washing. The positive samples were subjected to the quantitative determination on cell cultures, both to quantify the presence of infectious virus and to evaluate the eventual decreases in viral concentration.

2. Materials and methods

2.1. Virus

HAV was adapted and titrated, as previously described, in Frp-3 cells (Venuti et al., 1985) derived from FRhK-4. The virus had been isolated from the stool of a person with acute HAV infection and was kindly provided by Professor A. Panà (Tor Vergata University, Rome).

2.2. Samples

Samples of lettuce (*Lactuca sativa*), fennel (*Foeniculum vulgare*), and carrot (*Daucus carota*), were taken directly from produce markets. The samples were cut into small pieces (on the average of not larger than 9 cm^2) and experimentally contaminated with HAV, as described below.

2.3. Sample contamination

For each vegetable, 200 g was divided into two aliquots of 100 g each and treated as follows:

1. One aliquot (100 g) was immersed in 800 ml of sterile, distilled water supplemented with a suspension of HAV until obtaining a concentration of 5 log tissue culture infectious dose ($TCID_{50}$)/ml (Reed and Muench, 1938).
2. The other aliquot (100 g) was also immersed in 800 ml of sterile distilled water yet without the addition of the virus; this aliquot was used as the control.

The samples were immersed for 20 min, strained, and left out to dry.

Both the contaminated and control aliquots were further divided into five aliquots (20 g each) and stored at 4 °C. At days 0, 2, 4, 7, and 9, one contaminated aliquot (20 g) and one control aliquot (20 g) were collected and further divided into two aliquots each (10 g). One of the 10-g aliquots was analysed without washing; the other was washed with 100 ml of potable water for approximately 5 min, to simulate domestic practices; it was then left out to dry.

For each vegetable, the experiments were repeated three times.

2.4. Virus extraction

2.4.1. Elution

Ten grams of the sample, supplemented with 25-ml 3% Beef Extract (Lab-Lemco Powder—Oxoid) (pH 9.5), was shaken for 20 min. Then, the Beef Extract, filtered with 0.22-μm millipore filters, was divided into two aliquots of 12.5 ml each (equivalent to 5 g of the vegetable) and stored at −80 °C until use. The first aliquot was tested using RT-nested-PCR; if positive for the presence of viral RNA, the second aliquot was used for the quantitative determination on cell cultures.

2.5. RT-nested-PCR

2.5.1. Extraction of viral RNA

The 12.5-ml aliquot of Beef Extract was centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatant was subjected to ultracentrifugation at 200,000 × *g* for 2 h at 4 °C (Beckman L7-55). The pellet was resuspended in 334 μl of sterile tridistilled water and transferred to an Eppendorf vial (1.5 ml) containing 666 μl of Solution D (Afzal and Minor, 1994), vortexing for 30 s. One hundred microliters of CsCl (Baker Analyzed Reagent-Deventer, Holland)

cushion (5.7 M solution of CsCl in 25 mM sodium acetate, pH 5.0, RI = 1.4000) was gently placed at the bottom of the tube by piercing through the liquid. After centrifugation in an Eppendorf microfuge at 13,000 rpm for 20 min at 4 °C, the supernatant was discarded, and the pellet was washed twice with 1 ml of 70% ethanol and then dried.

2.5.2. RT-PCR

The dried pellet was resuspended in 90 μl of RT reaction mixture containing 1 × PCR Buffer II (Perkin Elmer, NJ, USA—Roche AO1658), 2.5 mM $MgCl_2$ Solution (Perkin Elmer—Roche AO1555), 0.25 mM each of deoxynucleoside triphosphate 100 mM (dNTP) (Takara-Shuzo, Japan), 20 U of Rnase Inhibitor (Perkin Elmer—Roche AO2922), 1.25 U of MuLV reverse transcriptase (Perkin Elmer—Roche A00854), and 100 pmol of primer anti-sense (5′ -CAGGGGCATTTAGGTTT-3′ HAV strain FG position 669–685) then, the mixture was incubated at 42 °C for 50 min. The reaction was terminated by heating the mixture at 95 °C for 3 min. One hundred picomoles of primer sense (5′ -CATATGTATGGTATCTCAACAA-3′ HAV strain FG position 1063–1084), 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer—Roche A00782), and DNase–RNase–free water (Sigma, USA W-4502) were added to a final volume of 100 μl. The mixture was subjected to 30 PCR cycles, each consisting of 25 s at 95 °C, 10 s at 49 °C and 1 min at 70 °C. A final extension was carried out for 5 min at 72 °C.

2.5.3. Nested-PCR

Five microliters of the first amplification reaction was further amplified in 95 μl of the reaction mixture containing 1 × PCR Buffer II (Perkin Elmer), 2.5 mM $MgCl_2$ (Perkin Elmer), 0.25 mM each of dNTP (Takara), 100 pmol of primer anti-sense (5′ -TGATAGGACTGCAGTGACT-3′ HAV strain FG position 807–825), 100 pmol of primer sense (5′ -CCAATTTTGCAACTTCATG-3′ HAV strain FG position 1000–1018), and 2.5 U of *Taq* DNA polymerase (Perkin Elmer). The amplification conditions were those described for the first PCR amplification.

2.5.4. Electrophoresis

Ten microliters of PCR and nested-PCR mixture were analysed by agarose gel electrophoresis (2% agarose; Kodak, New Haven, CT, USA—IB70040).

2.6. Quantitative detection of HAV

If the first aliquot was positive for the presence of viral RNA, the second 12.5-ml aliquot of the extract was subjected to ultracentrifugation as described above, and the pellet was resuspended in 1 ml of Eagle minimum essential medium (EMEM) (Imperial, UK), supplemented with 100 × antibiotics–antimycotic (Imperial) solution (1:100 v/v), and stored at 4 °C overnight.

The assay was conducted as previously described (Franco et al., 1990), using 24-well tissue culture plates and Frp-3 cell culture grown with EMEM

Table 1
Result of RT-PCR, RT-nested-PCR and quantitative determination on cell culture (before and after washing) for samples of lettuce experimentally contaminated with HAV and stored at 4 °C, by the number of days since contamination

Time (in days)	Qualitative determination				Quantitative determination	
	RT-PCR		RT-nested PCR		Log $TCID_{50}$/ml ± SD[a]	
	Not washed	Washed	Not washed	Washed	Not washed	Washed
0	+	+	+	+	4.48 ± 0.22	4.38 ± 0.14
2	+	+	+	+	4.44 ± 0.19	3.82 ± 0.89
4	+	+	+	+	3.48 ± 0.15	2.23 ± 0.15
7	+	+	+	+	2.45 ± 0.10	2.29 ± 0.20
9	+	+/−[b]	+	+	2.46 ± 0.17	2.41 ± 0.81

Concentration of HAV in the contaminated water: 5 log $TCID_{50}$/ml.
All samples used as control showed negative results.
[a] Mean of three determinations ± standard deviation.
[b] Two of the three determinations showed positive results.

Table 2
Result of RT-PCR, RT-nested-PCR and quantitative determination on cell culture (before and after washing) for samples of fennel experimentally contaminated with HAV and stored at 4 °C by the number of days since contamination

Time (in days)	Qualitative determination				Quantitative determination	
	RT-PCR		RT-nested PCR		Log $TCID_{50}$/ml ± SD[a]	
	Not washed	Washed	Not washed	Washed	Not washed	Washed
0	+	+	+	+	4.32 ± 0.18	3.37 ± 0.28
2	+	+	+	+	3.58 ± 0.07	2.51 ± 0.13
4	+	−	+	+	2.37 ± 0.11	1.56 ± 0.21
7	−	−	+	−	<1	n.d.
9	−	−	−	−	n.d.	n.d.

n.d.: not determined.
Concentration of HAV in the contaminated water: 5 log $TCID_{50}$/ml.
All samples used as control showed negative results.
[a] Mean of three determinations ± standard deviation.

(Imperial) supplemented with 10% Foetal Bovine Serum (FBS) (Imperial) at 37 °C and in 5% CO_2 at 37 °C for 3 days. The cell monolayer was inoculated with sample extract (100 μl/well) and left in contact for 1 h at 37 °C and 5% CO_2. The monolayer was then washed three times with 2 ml of EMEM at 2% FBS, in order to eliminate all the virus not infecting the cells. After adding 5 ml of EMEM at 2% FBS, the monolayer was incubated at 37 °C and in 5% CO_2 for 15 days. Four replicates were prepared for each dilution. The cytopathic effect was confirmed by RT-PCR, carried out as described above using the PCR primers. The viral titre was calculated using the method of Reed and Muench (1938) and was expressed in $TCID_{50}$/ml.

3. Results

The results of RT-PCR, RT-nested-PCR, and of the quantitative analysis at days 0, 2, 4, 7, and 9 are shown in Tables 1, 2 and 3, for lettuce, fennel, and carrot, respectively. The three vegetables differed in terms of their adsorption capacity. The highest quantity was consistently detected for lettuce, for which a progressive yet a slight decrease was observed over time (HAV titre = 4.44 ± 0.22 log $TCID_{50}$/ml at day 0 vs. 2.46 ± 0.17 log $TCID_{50}$/ml at day 9, before washing). The virus remained vital through the last day of storage (Table 1). For fennel, the quantity of virus at day 0 (4.32 ± 0.18 log $TCID_{50}$/ml before washing) was similar to that of lettuce, though it showed a greater

Table 3
Result of RT-PCR, RT-nested-PCR and quantitative determination on cell culture (before and after washing) for samples of carrot experimentally contaminated with HAV and stored at 4 °C by the number of days since contamination

Time (in days)	Qualitative determination				Quantitative determination	
	RT-PCR		RT-nested PCR		Log $TCID_{50}$/ml ± SD[a]	
	Not washed	Washed	Not washed	Washed	Not washed	Washed
0	+	+	+	+	3.44 ± 0.24	2.51 ± 0.13
2	+/−[b]	−	+	+	2.46 ± 0.64	<1
4	−	−	+	−	<1	n.d.
7	−	−	−	−	n.d.	n.d.
9	−	−	−	−	n.d.	n.d.

n.d.: not determined.
Concentration of HAV in the contaminated water: 5 log $TCID_{50}$/ml.
All samples used as control showed negative results.
[a] Mean of three determinations ± standard deviation.
[b] Two of the three determinations showed positive results.

decrease over time. In fact, at day 7, the virus was not detected by RT-PCR but it could only be detected by RT-nested-PCR and the quantity was lower than the minimum level of detection for the quantitative determination (<1 log $TCID_{50}$/ml) (Table 2). The quantity of the virus detected on the surface of the carrot at day 0 (3.44 ± 0.24 log $TCID_{50}$/ml before washing) was approximately one logarithm lower than the quantities detected for lettuce and fennel, and at day 4 the viral load had decreased to nonquantifiable levels (Table 3). For all three vegetables, washing resulted in a decrease of approximately one logarithm in the quantity of virus detected.

4. Conclusions

The results of this study demonstrate that lettuce, fennel, and carrot maintain a high quantity of HAV on their surface after being immersed in contaminated water, though the quantity varies according to the specific vegetable. Lettuce seems to have the highest adsorption capacity and the most favourable conditions for viral persistence, probably because of the size and the wrinkled texture of its leaves (i.e., a larger amount of exposed surface, a greater protection for the virus). For fennel and carrot, the quantity of virus was not only lower than that for lettuce but it also decreased more rapidly, reaching complete inactivation within several days. For carrot, the low quantity of virus detected immediately after contamination and its rapid decline could be due to the presence of specific substances, which have previously been shown to exert antimicrobic activity (Babic et al., 1994). Furthermore, washing apparently does not guarantee the elimination of the virus. In view of the fact that these products are either eaten raw or used as basic ingredients for different recipes, the vegetables represent a potential hazard for consumers. To prevent the spread of the HAV and of other viral infections, effective information campaigns are fundamental and should target both producers and consumers, with the aim of promoting adequate hygienic measures both in the production and preparation (e.g., the use of potable water for irrigation and in the preparation of foods and the use of clean utensils in preparing foods).

The results of this study also demonstrate the ever-increasing importance of sufficiently rapid and sensitive methods for determining the HAV on produce. RT-nested-PCR seems to satisfy this need, especially for screening. However, it must be taken into account that the virus detected by RT-nested-PCR is not necessarily infectious. In fact, although free viral RNA (i.e., lacking the protection provided by the capside) is known to survive briefly in the environment due to the presence of a high percentage of bacterial endonucleases (Tsay et al., 1995), RT-nested-PCR can reveal RNA originating from viral particles with a capside that is partially denaturated, yet still capable of protecting the RNA from degradation, although the RNA is not infectious (Ma et al., 1994; Hilfenhaus et al., 1997; Arnal et al., 1998). Thus, cell cultures should be used for detecting infectious virus and the consequent risk to consumers (Richards, 1999).

Acknowledgements

The authors gratefully acknowledge Mr. Mark Kanieff for the editorial assistance. This work, was carried out as part of the project ‘‘Biotecnologie’’, was supported by a grant from the National Council of Researches, and of the project ‘‘Foodborne viruses in Europe’’ (EU contract QLK1-CT-1999-00594) supported by EU 5th framework program.

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