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Duplex real-time qRT-PCR for the detection of hepatitis A virus in water and raspberries using the MS2 bacteriophage as a process control

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A B S T R A C T

Hepatitis A virus (HAV) infection is the leading worldwide cause of acute viral hepatitis. An important aspect of viral control is rapid diagnosis. Epidemiological studies have linked hepatitis A outbreaks to the consumption of drinking water or soft fruits exposed to faecal contamination. Real-time reverse transcriptase PCR (qRT-PCR) is now widely used for detecting RNA viruses in food samples. Efficiency of viral concentration, nucleic acid extraction and the presence of potential inhibitors of the RT-PCR reaction must be monitored to prevent false negative results. In this study, the MS2 bacteriophage used as a process control was detected simultaneously with HAV in a one-step duplex real-time qRT-PCR. The assay was developed for testing water and raspberries. Adding MS2 showed no loss of sensitivity for HAV detection in water and raspberry samples. The limit of detection of HAV with this new approach was 10 PFU for 1.5 L of bottled water, 100 PFU for 1.5 L of tap water, 50 PFU for 25 g of fresh raspberries and 100 PFU for 25 g of frozen raspberries. The data show that the MS2 offers a very reliable and simple way to monitor false-negative results, making it a valuable tool in the routine diagnostics laboratory.

1. Introduction

Hepatitis A virus (HAV) is a positive single-stranded RNA virus classified in the *Hepatovirus* genus of the *Picornaviridae* family. Hepatitis A virus (HAV) is the main cause of human enteric hepatitis. HAV is stable in the environment and is particularly resistant to disinfectants, heating, pressure and low pH (Koopmans et al., 2002). HAV is transmitted mainly via the faecal-oral route, either by person-to-person contact or by ingestion of contaminated water and food, particularly shellfish, soft fruits and vegetables. Numerous epidemiological studies have linked viral hepatitis A infections to the consumption of raw vegetables or drinking water contaminated by faeces (Rosenblum et al., 1990; Hernández et al., 1997; Long et al., 2002). Detection of HAV on the basis of its infectivity is complicated by the absence of a reliable cell culture method and the low contamination levels of food samples. The development of sensitive, reliable techniques for the detection of HAV in food and water samples helps ensure the safety of these products. Many different methods have been described for HAV detection in foods, however only a few of them have been applied in combination with qRT-PCR (Butot et al., 2007; Dubois et al., 2007; Mattison et al., 2009; Sánchez et al., 2007). To date, real-time qRT-PCR has been one of the most promising detection methods due to its sensitivity, specificity and speed. The main obstacles concerning routine HAV detection in food include the low concentrations of virus recovered and the presence of inhibitory substances in the samples. An internal control is thus mandatory for reliable diagnostic PCR assays (Hoorfar et al., 2004).

In order to control viral nucleic acid extraction efficiency in clinical and shellfish samples, Costafreda et al. (2006) have proposed the use of a mutant mengovirus MC_0 strain. Feline calicivirus (FCV) has also been demonstrated as an efficient internal control for monitoring the RNA extraction process and amplification procedure of hepatitis E virus (HEV) in clinical samples using a multiplex HEV/FCV TaqMan assay (Ward et al., 2009). Recently, FCV has been proposed as a process control for HAV detection in food and water samples (Mattison et al., 2009; Di Pasquale et al., 2010). However, FCV does not have the same pH stability profile as the enterically infecting viruses (Cannon et al., 2006). To address this issue, another approach has suggested using the MS2 bacteriophage to monitor the efficiency of the extraction, reverse transcription and amplification steps in control clinical diagnosis (Dreier et al., 2005). The MS2 bacteriophage has also been used as an internal control to monitor the RNA extraction efficiency and to detect inhibition of norovirus detection by real-time PCR assays in faecal samples (Rolfe et al., 2007). The MS2 bacteriophage is non-pathogenic to humans, sta-

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ble, and easily propagated. Thus the MS2 bacteriophage provides an ideal candidate, which could be used as a universal process control in all RNA virus assays.

The aim of this study was to develop an assay using the MS2 as a process control to monitor the efficiency of HAV detection in raspberries and water samples. For this purpose, a duplex real-time qRT-PCR was optimised and the limit of detection (LOD) of HAV with this new approach was determined in spiked tap water, bottled water, fresh raspberries and frozen raspberry samples.

2. Materials and methods

2.1. Viruses, bacteriophages and cells

HAV strain HM175/18f, clone B (VR-1402) was obtained from the American Type Culture Collection (ATCC). This clone replicates rapidly and has cytopathic effects in cell culture (Lemon et al., 1991). HAV stock containing 2×10^6 PFU/mL was produced by propagation in foetal rhesus monkey kidney (FRhK-4) cells (ATCC, CRL-1688) (Cromeans et al., 1987) and titrated by plaque assay (Dubois et al., 2006). Results were expressed in plaque-forming units/mL (PFU/mL).

The MS2 bacteriophage strain was obtained from culture collection (ATCC 15597-B1). The MS2 suspension was produced by the Laboratory of Physical Chemistry and Microbiology for the Environment, Nancy University, UMR 7564 CNRS, France, as described by Langlet et al. (2008). The final viral concentrations were about 10^{10} PFU/mL and the suspensions were stored as stock suspension at 4 °C prior to any experiment. Ten-fold serial dilutions of MS2 phage suspension were made in buffered peptone water from 10^{10} to 10^6 PFU/mL.

2.2. Inoculation of water samples

The principal chemical characteristics of the purchased bottled mineral water used to perform the study were: Ca^{2+}, 11.5 mg/L; Mg^{2+}, 8 mg/L; Na^{2+}, 11.6 mg/L; K^+, 6.2 mg/L; Cl^-, 13.5 mg/L; $NO_3{}^-$, 6.3 mg/L; $So_4{}^{2-}$, 8.1 mg/L; SiO_2, 31.7 mg/L, $HCO_3{}^-$, 71.0 mg/L; F^-, 0.2 mg/L. The pH was measured in the laboratory at 7. Tap water (Maisons-Alfort) used in this study was collected into polyethylene flasks. Chlorine residues in 1.5 L of tap water were neutralised with 750 μL of 10% sodium thiosulfate ($Na_2S_2O_3$) solution (Sigma–Aldrich, Saint-Quentin-Fallavier, France) (Méndez et al., 2004) and the tap water was stored overnight at 4 °C before viral inoculation. Bottled mineral water and tap water samples (1.5 L) were spiked with 1–10^4 PFU of HAV. The inocula were 100 μL of a dilution in DEPC treated water (Fisher Bioblock Scientific, Illkirch, France) of the HAV stock suspension. Samples co-inoculated with MS2 as process control were spiked with 10^6 PFU of bacteriophage. The inoculum was 100 μL of a dilution in buffered peptone water (peptone 1 g/L, NaCl 8.5 g/L, pH7) of the MS2 stock suspension. Uninoculated water samples were used as a negative control. The series of steps from the spiking to the RNA extraction were performed three times.

2.3. Inoculation of fruit samples

Two batches of fresh raspberry samples (25 g) were spiked with 5×10^1 to 5×10^3 PFU of HAV. Each inoculum was distributed in about 20 spots on the surface of each 25 g sample of fruit. Each raspberry sample was then placed in a 400 mL polypropylene bag containing a filter compartment (Seward, Norfolk, United Kingdom) and left to dry for 20 min at room temperature, in an air flow cabinet with the bag open. After 10 min of drying, one batch of fresh raspberry samples was co-inoculated with 10^6 PFU of bacteriophage MS2 per 25 g sample, as a process control. The inoculum (100 μL of a dilution in buffered peptone water of the MS2 stock suspension) was distributed in about 20 spots on the surface of the raspberries and left to dry for 10 min in the air flow cabinet. As an alternative, two batches of frozen raspberry samples were obtained by freezing fresh HAV-inoculated raspberry samples for 48 h at −20 °C. One batch of frozen raspberry samples was co-inoculated with 10^6 PFU of bacteriophage MS2 per 25 g and left to dry for 20 min in the air flow cabinet. Uninoculated raspberry samples were used as a negative control. The series of steps from the spiking to the RNA extraction were performed three times.

2.4. Sample processing for recovery of viruses and viral RNA extraction

Viruses were concentrated from inoculated mineral water and tap water by membrane filtration under vacuum using a Zetapor (Cuno Filtration SAS 3M, Cergy Pontoise, France) 47 mm positively-charged membrane of pore size 0.45 μm. The flow rate used during filtration was approx. 1.5 L/17 min. Filters were then incubated directly for 10 min at room temperature in a 60 mm diameter Petri dish containing 3 mL of NucliSens® easyMAG™ lysis buffer (BioMérieux, Marcy l'Etoile, France) as described previously by Perelle et al. (2009). The entire 3 mL lysate was then collected and processed using the NucliSens® easyMAG™ platform (BioMérieux) for total nucleic acid purification by the "off-board Specific A protocol" according to the manufacturer's instructions. Lastly, nucleic acids were eluted in 70 μL of elution buffer and stored at −80 °C.

To detect enteric viruses in spiked raspberries, a slightly improved form of the elution-concentration method described previously by Dubois et al. (2007) was used. Each inoculated fruit sample was placed in a 400 mL polypropylene bag containing a filter compartment and was soaked in 40 mL of elution buffer (Tris–HCl 100 mM, glycine 50 mM, 1% beef extract, pH 9.5), supplemented with 180 units of pectinase (Sigma–Aldrich, Saint-Quentin-Fallavier, France) for 20 min at room temperature with constant shaking. The rinse fluid was removed via the filter compartment of the bag and was centrifuged at 10,000 × *g* for 30 min at 4 °C to pellet the fruit particles. The pH of the decanted supernatant was adjusted to 7.2 ± 0.2 by the addition of 5N HCl while the fluid was swirled constantly. The neutralised supernatant was supplemented with 10% (w/v) polyethylene glycol (PEG) 6000 (Promega, Madison, CA, USA), and 0.3 M NaCl, and was then incubated for at least 2 h at 4 °C. Viruses were concentrated by centrifugation of the solution at 10,000 × *g* for 30 min at 4 °C. The pellet was resuspended in 500 μL of phosphate buffered saline and then vortexed with 5 mL of chloroform: butanol, 1:1 (v/v). The suspension was held for 5 min at room temperature, and then centrifuged at 6000 × *g* for 15 min at 4 °C. The upper aqueous phase containing viruses was recovered, supplemented by NucliSens® easyMAG™ lysis buffer (BioMérieux) up to 3 mL and subjected to the NucliSens® easyMAG™ platform for total nucleic acid extraction by the "off-board Specific A protocol" according to the manufacturer's instructions.

2.5. Primers and probes

The primers and the 3′-minor groove binder (MGB) TaqMan® probe targeting the non-coding region at the 5′ end (5′-NCR) of HAV used in this study have been described by Costafreda et al. (2006). The primers and the MGB-TaqMan® probe targeting the A protein (maturation protein) of the MS2 bacteriophage were designed using Primer Express® software (Applied Biosystems, Foster City CA, USA). The probes were labelled with the *6*-FAM (HAV probe) or VIC (MS2 probe) reporter dyes at the 5′-end and an MGB at the 3′-end. The sequence of the primer pairs and the TaqMan probes used was as follows: for HAV, the sense primer (HAV68) was 5′-TCACCGCCGTTTGCCTAG-3′, the antisense primer

(HAV241) was 5′-GGAGAGCCCTGGAAGAAAG-3′ and the TaqMan probe (HAV150−) was 5′-FAM-CCTGAACCTGCAGGAATTAA–MGB-3′. For MS2, the sense primer (MS2-132F) was 5′-GCGAGCTTTTA-GTACCCTTGATAGG-3′, the antisense primer (MS2-206R) was 5′-GTCTCACCGTCCGCGTAAAC-3′ and the TaqMan probe (MS2-162T) was 5′-VIC–CGAGACCTTCGTCCC-MGB-3′. All the primers and probes were purchased from Applera France S.A. (Courtaboeuf, France).

2.6. Real-time qRT-PCR conditions

One-step duplex real-time qRT-PCR amplifications were performed in duplicate on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a 25 μL reaction mixture containing 1× of ThermoScript reaction mix, 1× of ROX reference dye and 0.5 μL of ThermoScript Plus/Platinum Taq enzyme mix, which are components of the Platinum® Quantitative RT-PCR ThermoScript™ One-Step System (Fisher Bioblock Scientific, Illkirch, France), 500 nM of HAV68 forward primer, 900 nM of HAV240 reverse primer, 250 nM of probe HAV150(−), 400 nM of MS2-132F forward primer, 400 nM of MS2-206R reverse primer, 200 nM of probe MS2-162T and 5 μL of RNA extract. Positive controls containing RNA extracted from virus suspensions, and a negative control containing all the reagents except the RNA template were included with each set of reaction mixtures. The one-step qRT-PCR program was 60 min reverse transcription of RNA at 55 °C, followed by a 15 min denaturation step at 95 °C, and finally 40 cycles of 15 s at 95 °C, 1 min at 60 °C and 1 min at 65 °C. The fluorescence was recorded at the end of the elongation steps (1 min at 65 °C) by the apparatus for each amplification cycle. The cycle threshold (Ct) was defined as the cycle number at which a sample's ΔRn fluorescence crossed the set threshold value of 0.2. All samples were characterised by a corresponding Ct value. Negative samples gave no Ct value. A standard curve for each system was generated using 10-fold dilution of viral suspension. The slopes (S) of the regression lines were used to calculate the amplification efficiency (E) of the real-time qRT-PCR reactions, according to the formula: $E = 10^{|1/s|} - 1$ (Tichopad et al., 2003). HAV and MS2 recovery rate percentages in spiked samples were calculated by reference to the corresponding standard curve.

2.7. Statistical analysis

The Excel spreadsheet function LINEST was used for complete linear least squares curve fitting. The slope and intercept were reported with a confidence interval. The confidence intervals are computed as follows: $CI = t(\alpha,df) \cdot SE$ where $t(\alpha,df)$ is the critical value of the t statistic corresponding to the desired probability level (α) and the degrees of freedom (df) available for the estimation of slope and intercept and where SE is the standard error. The SE values for slope and intercept and $t(\alpha,df)$ values were, respectively, obtained from the LINEST and TINV functions of Excel. When sample size per group was low, the Mann–Whitney test was used to test for significant difference, for example for testing for difference between groups with different inocula. This test is a non-parametric test that uses the ranks of data to test the hypothesis that two samples come from the same population. When sample size was large, Student's t-test was used.

3. Results

3.1. Standard curves

In each spiking experiment set, standard curves relating to either HAV or MS2 were plotted by testing serial dilutions from 10 to 10^4 PFU of HAV and from 10^4 to 10^8 PFU of MS2 suspensions. The quantities of viruses in the dilutions were expressed by reference to the theoretical PFU used for viral RNA extraction. The standard curves obtained with the real-time qRT-PCR reactions were used to estimate the quantities of viruses detected in water and fruit samples, respectively. Taking into account 24 Ct values generated in the whole spiking experiment set, the mean parameters of the standard curves were as follows: standard curves obtained showed efficiency of 99%, a regression coefficient of 0.9923, a slope of −3.3395 and an intercept of 35.812 with HAV; and efficiency of 91%, a regression coefficient of 0.9999, a slope of −3.5581 and an intercept of 47.523 with MS2.

3.2. Duplex real-time qRT-PCR for the detection of HAV in water and raspberry samples using MS2 as a process control

3.2.1. Experimental detection of HAV and MS2 in water and raspberries

Frozen raspberry samples were inoculated with the MS2 bacteriophage (from 10^9 to 10^5 PFU/25 g) and processed to recover viruses and to extract RNA. The MS2 genome was then detected using one-step simplex real-time qRT-PCR to determine the optimal input of process control (MS2) per extraction. Optimisation experiments showed that 10^6 PFU of bacteriophage MS2 was the most reliable input per sample for reproducible MS2 detection and non-interference with target HAV amplification (data not shown).

Three runs of experiments with bottled water samples spiked with ten-fold dilutions of HAV and MS2 showed that the process control was detected consistently in RNA extracts. RNA extracts issued from each experiment set were tested in duplicate and the mean Ct values corresponding to the MS2 process control were calculated. In bottled water, the recovery rate of the MS2 process control ranged from 26 ± 2 to 64 ± 10%. The MS2 recovery rate calculated for other spiked samples (tap water and raspberries) was slightly lower, although the process control was still detected consistently in the RNA extracts. In these cases, the MS2 recovery rate ranged from 8 ± 6 to 26 ± 4%. As expected, no viral RNA was detected in the uninoculated fruit or water samples (Table 1).

The parameters for the curves from the spiked samples with either different concentration sets of HAV, or different concentration sets of HAV plus 10^6 PFU of the MS2 process control, are reported in Fig. 1. The confidence intervals of the slopes and intercepts of the regression lines obtained for the different sample type overlap. It showed that the presence of MS2 or sample type did not affect qRT-PCR amplification.

The mean HAV recovery rates for the different seeding levels and each product type are detailed in Table 1. The HAV recovery percentage varied widely from a low of 3% (frozen raspberries) to a high of 81% (bottled water), with an overall mean of about 58% in bottled water, 24% in tap water, 16% in fresh raspberries and 11% in frozen raspberries spiked with the process control, whereas it was around 45% in bottled water, 26% in tap water, 12% in fresh raspberries and 6% in frozen raspberries in the absence of the process control. The Mann–Whitney test revealed that there was no significant difference in recovery for different inocula whatever the sample type. Therefore percentages of recovery obtained for different inocula were considered together for each sample type. Difference between percentages of recovery of fresh versus frozen raspberry and of bottled water versus tap water were evaluated with the Student's t-test. It revealed that percentages of recovery are significantly different at $p < 0.01$ for bottled and tap waters (with or without MS2) and for fresh and frozen raspberry (with or without MS2).

3.2.2. HAV detection limit in spiked samples

The LOD, which was defined as the lowest amount of HAV in a test sample that has been detected reproducibly in the three exper-

Table 1
Percentage recovery calculated for HAV and MS2 in spiked bottled water, tap water (Panel A) and fresh raspberries, frozen raspberries (Panel B).

Panel A					
Inocula		Samples			
HAV (PFU)	MS2 (PFU)	Bottled water (1.5 L)		Tap water (1.5 L)	
		HAV recovery % (positive Ct data/ 6)	MS2 recovery %	HAV recovery % (positive Ct data/ 6)	MS2 recovery %
0	0	nd (0/6)	nd	nd (0/6)	nd
1	0	nd (0/6)	nd	nd (0/6)	nd
10	0	*50 ± 11* (6/6)	nd	19 ± 12 (5/6)	nd
10^2	0	47 ± 8 (6/6)	nd	*28 ± 4* (6/6)	nd
10^3	0	40 ± 6 (6/6)	nd	22 ± 4 (6/6)	nd
10^4	0	46 ± 5 (6/6)	nd	29 ± 11 (6/6)	nd
0	10^6	nd (0/6)	54 ± 16	nd (0/6)	17 ± 8
1	10^6	12 ± nd (1/6)	48 ± 8	nd (0/6)	19 ± 2
10	10^6	*81 ± 24* (6/6)	64 ± 10	10 ± 5 (3/6)	18 ± 6
10^2	10^6	63 ± 13 (6/6)	52 ± 10	*29 ± 11* (6/6)	15 ± 5
10^3	10^6	44 ± 6 (6/6)	47 ± 10	15 ± 5 (6/6)	8 ± 6
10^4	10^6	47 ± 3 (6/6)	26 ± 2	28 ± 7 (6/6)	12 ± 9
Panel B					
Inocula		Samples			
HAV (PFU)	MS2 (PFU)	Fresh raspberries (25 g)		Frozen raspberries (25 g)	
		HAV recovery % (positive Ct data/ 6)	MS2 recovery %	HAV recovery % (positive Ct data/ 6)	MS2 recovery %
0	0	nd (0/6)	nd	nd (0/6)	nd
50	0	17 ± 9 (5/6)	nd	7 ± 3 (4/6)	nd
10^2	0	*16 ± 4* (6/6)	nd	*3 ± 2* (6/6)	nd
5×10^2	0	12 ± 2 (6/6)	nd	5 ± 4 (6/6)	nd
10^3	0	8 ± 3 (6/6)	nd	9 ± 4 (6/6)	nd
5×10^3	0	14 ± 5 (6/6)	nd	9 ± 2 (6/6)	nd
0	10^6	nd (0/6)	12 ± 4	nd (0/6)	29 ± 5
50	10^6	*25 ± 13* (6/6)	21 ± 2	14 ± 9 (3/6)	26 ± 12
10^2	10^6	14 ± 5 (5/6)	14 ± 6	*8 ± 6* (6/6)	21 ± 4
5×10^2	10^6	14 ± 10 (6/6)	15 ± 9	14 ± 3 (6/6)	26 ± 4
10^3	10^6	16 ± 9(6/6)	12 ± 3	11 ± 3 (6/6)	16 ± 3
5×10^3	10^6	14 ± 8 (6/6)	11 ± 4	11 ± 2 (6/6)	17 ± 4

Results are expressed as means ± standard errors. For each inoculation level, three experiments were performed and RNA extracts were tested twice, resulting in six Ct determinations for each sample type. The number of positive Ct determinations is mentioned for HAV. The mean percentage recovery corresponding to the LOD for each sample type has been italicised. nd: not determined.

iment sets, was determined with and without addition of the MS2 bacteriophage. The results showed that the addition of the MS2 bacteriophage had little influence on the limit of detection (LOD) of HAV in spiked samples. The LOD of the assay was 10 PFU of HAV/1.5 L of bottled water *vs.* 100 PFU of HAV/1.5 L of tap water (Table 1, Panel A). In frozen raspberries, the LOD was 100 PFU of HAV/25 g. The LOD of the assay was 50 PFU of HAV/25 g of fresh raspberries when the MS2 was added, whereas it was only 100 PFU of HAV/25 g without the MS2 process control (Table 1, Panel B).

4. Discussion

A rapid technique to detect the presence of HAV in contaminated food and water samples is essential in the routine diagnostic laboratory and to allow potential health risks to be assessed. The sensitivity of PCR detection assays can be affected by the quality of extracted RNA, or by qRT-PCR inhibitors in food and water samples. The partial recovery of viruses and the presence of qRT-PCR reaction inhibitors influence the method's sensitivity. The extraction, concentration and purification of viral RNA from the samples are the critical steps for virus detection by molecular methods and the use of a process control is extremely useful to monitor the quality of the extraction procedure and the presence of qRT-PCR inhibitors. In this work, the bacteriophage MS2 was used as such a process control. MS2 is an ideal candidate for use as a process control because it is available commercially from international collections, simple to produce at high yields, non-pathogenic to humans and stable. In this study, the fruit and water samples were spiked with a known amount of MS2 bacteriophage to monitor the efficiency of HAV detection. MS2 is an exogenous process control included at a uniform PFU number in each assay. It is a "non-competitive" internal control because it uses a sequence entirely unrelated to the viral target. The automated nucleic acids extraction method based on the commercially-available NucliSens® easyMAG™ system was used to extract both MS2 and HAV viral nucleic acids. It provides a number of potential benefits, in particular a high degree of standardization and reproducibility, providing reliable results in a shorter time. Different studies have recently evaluated the sensitivity of new procedures based on automated nucleic extraction in clinical and water samples (Chan et al., 2008; Loens et al., 2007, 2008; Rutjes et al., 2005; Stevens et al., 2007; Perelle et al., 2009) but none of them have been evaluated to test raspberries. Adaptation of the fruit sample treatment in an automated RNA extraction system is a further development of the method and reduces hands-on time significantly. The detection of HAV and MS2 is based on a duplex real-time qRT-PCR assay. Thus, more attention should be paid to the primer pairs and probe design and the optimisation of the master mix. The one-step duplex real-time qRT-PCR assay shows the advantage of amplifying both the viral target and the process control in the same tube, resulting in a cost reduction. Incorporation of the process control allows calculation of the recovery rate of the method and could provide useful parameters to validate the analysis data. However, given that the recovery rate is sample type dependent, it should be determined for every category of food sample. Taking into account the study, a 25% recovery of the MS2 used as a process control would make it possible to validate the results

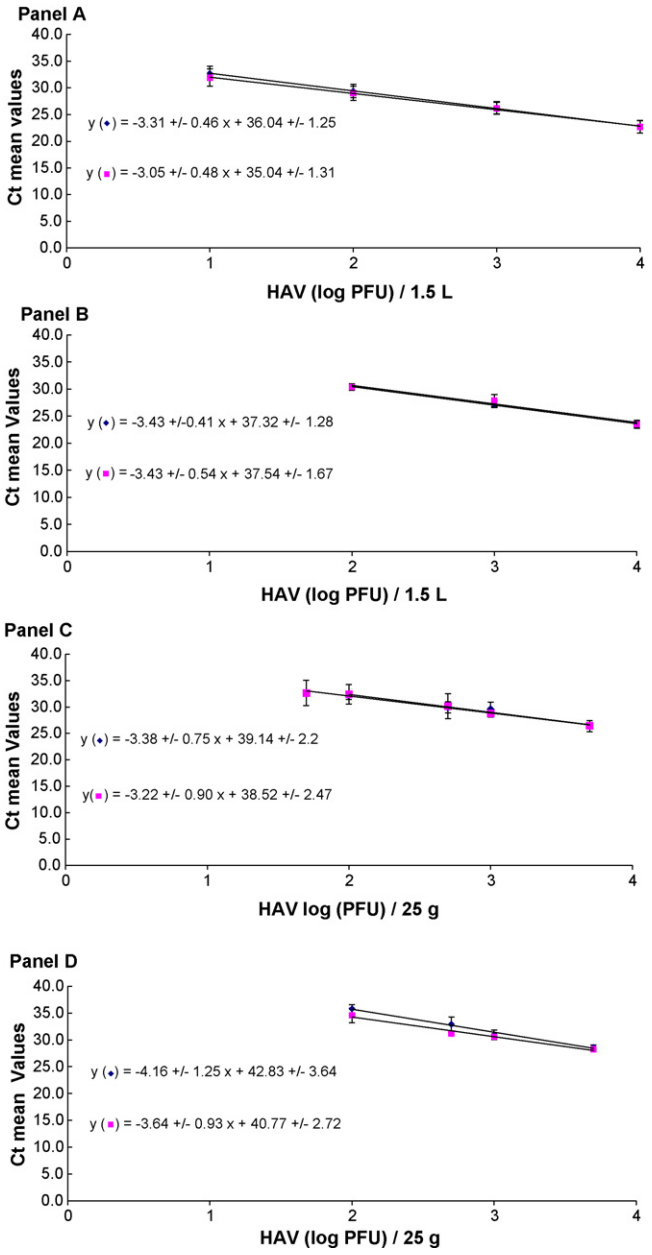

Fig. 1. Real time qRT-PCR amplification curves obtained for HAV detection with the addition of MS2 (■) and without the addition of MS2 (♦) in spiked bottled water (Panel A), tap water (Panel B), fresh raspberries (Panel C), and frozen raspberries (Panel D). Bottled water and tap water (1.5 L) were spiked with 0, 1, 10, 10^2, 10^3 and 10^4 PFU of HAV. Fresh and frozen raspberries were spiked with 0, 50, 10^2, 5×10^2, 10^3 and 5×10^3 PFU of HAV. All experiments were performed three times and RNA extracts were tested twice. Results are expressed in Ct mean values calculated with six positive Ct determinations against viral loading expressed in log PFU.

of the analysis in bottled water samples. In tap water and fruit samples, a 10% recovery of MS2 would be required to validate the results of the analysis.

There is still very little development of qRT-PCR methods for screening HAV in fruit and other food samples. Among the few methods reported in the literature, Mattison et al. (2009) have shown that HAV could be detected at input levels corresponding to 6×10^3 PFU/25 g of strawberries and to 250 PFU/250 mL of bottled spring water by real-time RT-PCR. Butot et al. (2007) have shown that the average recovery for the different types of berries was ca.14% (from 37.5 to 2.1%) for HAV and the detection limit was 1.2 $TCID_{50}$ (50% tissue culture infective dose) for HAV per 15 g of berries. Papafragkou et al. (2008) have reported a novel method for concentrating HAV from foods prior to application of RT-PCR for detection and have shown that the detection limit varied according to the product, but in most cases, HAV could be detected consistently at input levels corresponding to 10^2 PFU/25 g of food sample. Hence, the procedure reported in the present study provides detection limits that are comparable to those determined by others (Butot et al., 2007; Mattison et al., 2009; Papafragkou et al., 2008).

In summary, a one-step sensitive duplex real-time qRT-PCR assay was developed for the simultaneous detection of HAV and MS2, chosen as a process control, in raspberry and water samples. The methods described in this report demonstrated satisfactory performance and are proposed as appropriate methods for testing foods for HAV contamination. MS2 is a robust option for routine sample process in food virology laboratories because it can provide an indication of the efficiency of the concentration/extraction/detection method as a whole. Use of the MS2 bacteriophage as a process control is also a thorough approach which allows the rapid discrimination of false negative samples from true negative ones in a screening test. In the event of false negatives, more precise information, in particular identifying where the process has failed, might be informative but this requires development of a qRT-PCR internal control that was outside the scope of this study. The European Committee on Normalization (CEN) has an active expert working group (CEN/TC 275/WG6/TAG4) addressing the development of a horizontal EU standard method for the detection of HAV in foodstuffs and bottled water. The results obtained in this study may be an excellent support to the work of the standardization group and further studies should be performed to establish the inter-laboratory reliability of these detection methods.

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References

Butot, S., Putallaz, T., Sánchez, G., 2007. Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. Appl. Environ. Microbiol. 73, 186–192.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., Vinjé, J., 2006. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. J. Food Protect. 69, 2761–2765.

Chan, K.H., Yam, W.C., Pang, C.M., Chan, K.M., Lam, S.Y., Lo, K.F., Poon, L.L., Peiris, M.J., 2008. Comparison of the NucliSens easyMAG and Qiagen BioRobot 9604 nucleic acid extraction systems for diagnosis of RNA and DNA respiratory viruses on nasopharyngeal aspirates. J. Clin. Microbiol. 46, 2195–2199.

Costafreda, M.I., Bosch, A., Pinto, R.M., 2006. Development, evaluation and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl. Environ. Microbiol. 72, 3846–3855.

Cromeans, T., Sobsey, M.D., Fields, H.A., 1987. Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. J. Med. Virol. 22, 45–56.

Di Pasquale, S., Paniconi, M., De Medici, D., Suffredini, E., Croci, L., 2010. Duplex real-time PCR for the detection of hepatitis A virus in shellfish using feline calicivirus as a process control. J. Virol. Methods 163, 96–100.

Dreier, J., Störmer, M., Kleesiek, K., 2005. Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. J. Clin. Microbiol. 43, 4551–4557.

Dubois, E., Hennechart, C., Deboosère, N., Merle, G., Legeay, O., Burger, C., Le Calvé, M., Lombard, B., Ferré, V., Traoré, O., 2006. Intra-laboratory validation of a concentration method adapted for the enumeration of infectious F-specific RNA coliphage, enterovirus, and hepatitis A virus from inoculated leaves of salad vegetables. Int. J. Food Microbiol. 108, 164–171.

Dubois, E., Hennechart, C., Merle, G., Burger, C., Hmila, N., Ruelle, S., Perelle, S., Ferré, V., 2007. Detection and quantification by real-time RT-PCR of hepatitis A virus

from inoculated tap waters, salad vegetables, and soft fruits: characterization of the method performances. Int. J. Food Microbiol. 117, 141–149.
Hernández, F., Monge, R., Jiménez, C., Taylor, L., 1997. Rotavirus and hepatitis A virus in market lettuce (*Latuca sativa*) in Costa Rica. Int. J. Food Microbiol. 37, 221–223.
Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., Fach, P., 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J. Clin. Microbiol. 42, 1863–1868 (comment in: J. Clin. Microbiol. 42, 3379–3380).
Koopmans, M., von Bonsdorff, C.H., Vinjé, J., de Medici, D., Monroe, S., 2002. Foodborne viruses. FEMS Microbiol. Rev. 26, 187–205.
Langlet, J., Gaboriaud, F., Duval, J.F., Gantzer, C., 2008. Aggregation and surface properties of F-specific RNA phages: implication for membrane filtration processes. Water Res. 42, 2769–2777.
Lemon, S.M., Murphy, P.C., Shields, P.A., Ping, L.H., Feinstone, S.M., Cromeans, T., Jansen, R.W., 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. J. Virol. 65, 2056–2065.
Loens, K., Bergs, K., Ursi, D., Goossens, H., Ieven, M., 2007. Evaluation of NucliSens easyMAG for automated nucleic acid extraction from various clinical specimens. J. Clin. Microbiol. 45, 421–425.
Loens, K., Ursi, D., Goossens, H., Ieven, M., 2008. Evaluation of the NucliSens miniMAG RNA extraction and real-time NASBA applications for the detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in throat swabs. J. Microbiol. Methods 72, 217–219.
Long, S.M., Adak, G.K., O'Brien, S.J., Gillespie, I.A., 2002. General outbreaks of infectious intestinal disease linked with salad vegetables and fruit, England and Wales, 1992–2000. Commun. Dis. Public Health 5, 101–105.
Mattison, K., Brassard, J., Gagné, M.J., Ward, P., Houde, A., Lessard, L., Simard, C., Shukla, A., Pagotto, F., Jones, T.H., Trottier, Y.L., 2009. The feline calicivirus as a sample process control for the detection of food and waterborne RNA viruses. Int. J. Food Microbiol. 132, 73–77.
Méndez, J., Audicana, A., Isern, A., Llaneza, J., Moreno, B., Tarancón, M.L., Jofre, J., Lucena, F., 2004. Standardised evaluation of the performance of a simple membrane filtration-elution method to concentrate bacteriophages from drinking water. J. Virol. Methods 117, 19–25.
Papafragkou, E., Plante, M., Mattison, K., Bidawid, S., Karthikeyan, K., Farber, J.M., Jaykus, L.A., 2008. Rapid and sensitive detection of hepatitis A virus in representative food matrices. J. Virol. Methods 147, 177–187.
Perelle, S., Cavellini, L., Burger, C., Blaise-Boisseau, S., Hennechart-Collette, C., Merle, G., Fach, P., 2009. Use of a robotic RNA purification protocol based on the NucliSens® easyMAG™ for real-time RT-PCR detection of hepatitis A virus in bottled water. J. Virol. Methods 157, 80–83.
Rolfe, K.J., Parmar, S., Mururi, D., Wreghitt, T.G., Jalal, H., Zhang, H., Curran, M.D., 2007. An internally controlled, one-step, real-time RT-PCR assay for norovirus detection and genogrouping. J. Clin. Virol. 39, 318–321.
Rosenblum, L.S., Mirkin, I.R., Allen, D.T., Safford, S., Hadler, S.C., 1990. A multifocal outbreak of hepatitis A traced to commercially distributed lettuce. Am. J. Public Health 80, 1075–1079.
Rutjes, S.A., Italiaander, R., van den Berg, H.H., Lodder, W.J., de Roda Husman, A.M., 2005. Isolation and detection of enterovirus RNA from large-volume water samples by using the NucliSens miniMAG system and real-time nucleic acid sequence-based amplification. Appl. Environ. Microbiol. 71, 3734–3740.
Sánchez, G., Bosch, A., Pintó, R.M., 2007. Hepatitis A virus detection in food: current and future prospects. Lett. Appl. Microbiol. 45, 1–5.
Stevens, W., Horsfield, P., Scott, L.E., 2007. Evaluation of the performance of the automated NucliSENS easyMAG and EasyQ systems versus the Roche AmpliPrep-AMPLICOR combination for high-throughput monitoring of human immunodeficiency virus load. J. Clin. Microbiol. 45, 1244–1249.
Tichopad, A., Dilger, M., Schwarz, G., Plaffl, M.W., 2003. Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Res. 31 (20), e122 (Erratum in: Nucleic Acids Res. 31 (22) 2003, 6688).
Ward, P., Poitras, E., Leblanc, D., Letellier, A., Brassard, J., Plante, D., Houde, A., 2009. Comparative analysis of different TaqMan real-time RT-PCR assays for the detection of swine Hepatitis E virus and integration of Feline calicivirus as internal control. J. Appl. Microbiol. 106, 1360–1369.