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# Comparison of two extraction methods for the detection of hepatitis A virus in semi-dried tomatoes and murine norovirus as a process control by duplex RT-qPCR

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ABSTRACT

Enteric viruses are important agents of foodborne diseases. Due to their low infectious doses and low concentrations in food samples, an efficient and rapid virus concentration method is required for routine control. Because of the absence of a reliable cell culture method for most of the enteric viruses involved in outbreaks, reverse transcription quantitative real-time PCR (RT-qPCR) is now widely used for the detection of RNA viruses in food samples. One of the general requirements for viral diagnosis concerns the use of a process control to monitor the efficiency of viral particle concentration, nucleic acid extraction and the presence of potential inhibitors of the RT-PCR reaction. Recent epidemiological studies have linked hepatitis A outbreaks to the consumption of semi-dried tomatoes (SDT) in Australia, the Netherlands and France. In this study, the virus concentration reference method proposed by the CEN/TC275/WG6/TAG4 working group for samples of soft fruit and salad vegetables was compared to a method including an ultracentrifugation step to recover hepatitis A virus (HAV) in SDT. Murine norovirus (MNV-1) was used as a process control and detected simultaneously with HAV in a one-step duplex RT-qPCR in both procedures. The LOD of HAV was 10 PFU and 1 PFU of HAV/25 g of SDT in the presence or absence of MNV-1 respectively, whatever the method used.

We conclude that both methods achieved an identical limit of detection and that the MNV-1 offers a very reliable and simple way to monitor the quality of the extraction procedures and the presence of RT-qPCR inhibitors.

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## 1. Introduction

Hepatitis A virus (HAV) is a positive single-stranded RNA virus classified in the *Hepatovirus* genus of the Picornaviridae family. HAV infection is the leading worldwide cause of acute viral hepatitis (Koopmans and Duizer, 2004). 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 (Beuchat, 2006; Butot et al., 2007). The food and water-borne route account for 2–5% of the total disease burden. Numerous epidemiological studies have linked viral hepatitis A infections to the consumption of raw vegetables or drinking water contaminated by faeces (Hernández et al., 1997; Rosenblum et al., 1990). HAV is stable in the environment and is particularly resistant to disinfectants, heating, pressure and low pH (Koopmans et al., 2002; Koopmans and Duizer, 2004). Contamination may occur during growth in the field as well as during processing, storage, distribution or final preparation. Recently, different epidemiological studies reported a link between hepatitis A outbreaks and the consumption of semi-dried tomatoes in Australia, the Netherlands and France, (Anonymous, 2009; Gallot et al., 2011; Lacey et al., 2009; Petrignani et al., 2010a, 2010b). All outbreaks were caused by highly similar IB strains, although the French outbreak strain differed very slightly from the Australian and Dutch strains. The strains clustered with viruses known to circulate in the geographic region that includes Turkey, and the outbreak investigation identified semi-dried tomatoes (SDT) imported from Turkey as the most likely vehicle of transmission (Gallot et al., 2011).

The development of sensitive, reliable techniques for the detection of HAV in food and water samples is helpful in order to ensure the safety of these products (Sánchez et al., 2007). Detection of HAV on the basis of its infectivity is complicated by the absence

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of a reliable cell culture method and the low contamination levels of food samples. To date, RT-qPCR has been one of the most promising detection methods due to its sensitivity, specificity, speed and ability to deliver quantitative data. In 2004, the European Committee for Standardisation (CEN) tasked a technical advisory group (TAG4) with the development of standard methods (qualitative/quantitative) for the detection of norovirus and HAV in foodstuffs. Even if standard methods have recently been developed for a range of risk food including soft fruits and vegetables, they need to be further validated before publication as ISO or CEN standard methods. All these methods are based on a final detection of virus genome using reverse transcription quantitative real-time PCR (RT-qPCR), and the efficiency of viral concentration, nucleic acid extraction as well as the presence of potential inhibitors of the RT-qPCR reaction must be monitored by using a process control to identify false negative results. Although the TAG proposed the $MC_0$ strain of Mengo virus (Costafreda et al., 2006; Le Guyader et al., 2009), there is as yet no consensus on the choice of process control. The selected virus should exhibit similar morphological and physicochemical properties and environmental persistence to the target viruses, thus providing comparable extraction efficiency (Lees and CEN WG6 TAG4, 2010). Ideally, the process control should be unlikely to naturally contaminate the tested food sample (Baert et al., 2011). The MS2 bacteriophage has already been used as a process control for HAV detection in spiked food samples (Blaise-Boisseau et al., 2010) and as an internal control to monitor the RNA extraction efficiency and the presence of inhibitors for norovirus detection by RT-qPCR assays in faecal samples (Rolfe et al., 2007). Feline calicivirus (FCV) has also been shown to be 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). FCV has also been proposed as a process control for HAV detection in food and water samples (Di Pasquale et al., 2010a, 2010b; Mattison et al., 2009). However, FCV does not exhibit the same pH stability profile as the enterically infecting viruses (Cannon et al., 2006). Recently, the first murine norovirus (MNV) has been characterized and adapted to cell culture on murine macrophage-related cells (Karst et al., 2003; Wobus et al., 2004). MNV is morphologically and genetically similar to human noroviruses, and shows considerable promise as a human norovirus surrogate (Wobus et al., 2006). Recently, MNV-1 has been successfully tested as process control when detecting NoV in bottled water and fresh produce (Stals et al., 2011a, 2011c).

In order to be able to extend the use of a single process control for the detection of the main enteric viruses, the aim of the present study was to investigate the use of MNV-1 as a process control for detecting HAV on semi-dried tomatoes by a one-step duplex RT-qPCR and compare the efficiency of the CEN procedure to that of a method including ultracentrifugation.

## 2. Materials and methods

### 2.1. Viruses 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 $10^7$ 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).

MNV-1 (CW1 strain) was provided by Dr H. Virgin from Washington University, USA to the ANSES, Fougères Laboratory, France and was propagated in mouse leukemic monocyte macrophage (RAW 264.7, ATCC TIB-71) cell line (Cannon et al., 2006). RAW 264.7 was grown at 37 °C in an atmosphere containing 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% L-Glutamine, 1% non-essential amino acids, 10% foetal bovine serum (HyClone, Invitrogen) and 0.5% penicillin-streptomycin. MNV-1 stock containing $10^{6,75}$ $TCID_{50}$/mL (50% tissue culture infective dose/mL) was produced by ANSES, Fougères Laboratory, France as previously described (Wobus et al., 2004).

### 2.2. Inoculation of semi-dried tomatoes (SDT)

Semi-dried tomatoes (SDT) were purchased from a local market. Four batches of SDT samples (25 g) were spiked with $10^{-1}$ to $10^3$ PFU of HAV. Each inoculum of 100 μL was distributed in about 20 spots on the surface of each 25 g sample of fruit and left to dry overnight at 4 °C to increase the numbers of viral particles adhering as previously described by Fraisse et al. (2011) and Stals et al. (2011a). After the drying step, each spiked tomato sample was placed in a 400 mL polypropylene bag containing a filter compartment (Seward, Norfolk, United Kingdom). Two batches of tomato samples were co-inoculated with $5 \times 10^2$ $TCID_{50}$ of murine norovirus per 25 g sample, as a process control, just before adding elution buffer, which corresponds to the earliest opportunity prior to virus extraction to control for extraction efficiency (particularly if naturally contaminated samples are to be analysed). Uninoculated tomato samples were used as a negative control. All experiment steps from the spiking to the RNA extraction were performed three times.

### 2.3. Sample processing for recovery of viruses

#### 2.3.1. Method A

To detect enteric viruses in semi-dried tomatoes, the elution-concentration method described for soft fruits in the "CEN/TC275/WG6/TAG4 viruses in foods" draft document was used. Briefly, each inoculated tomato sample placed in a 400 mL polypropylene bag containing a filter compartment was soaked in 40 mL of elution buffer (Tris–HCl 100 mM, glycine 50 mM, 1% beef extract, pH 9.5) covering the sample, 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 5 N HCl while the fluid was swirled constantly. The neutralised supernatant was supplemented with 10% (wt/vol) polyethylene glycol (PEG) 6000 (Sigma–Aldrich, Saint-Quentin Fallavier, France), and 0.3 M NaCl, and was then incubated overnight at 4 °C. Viruses were concentrated by centrifugation of the solution at 10,000 *g* for 30 min at 4 °C. The supernatant was discarded and an additional centrifugation was performed at 10,000 *g* for 5 min at 4 °C to highly compact the pellet. The pellet was resuspended in 1 mL of ultra-pure RNAse-free water and vortexed with 1 mL of chloroform: butanol, 1:1 (v/v). The suspension was then incubated for 5 min at room temperature, and centrifuged at 8000 *g* for 15 min at 4 °C. The upper aqueous phase containing viruses was directly processed by the nucleic acid extraction procedure.

#### 2.3.2. Method B

We used method B to compare the purification of the viruses with chloroform: butanol *versus* ultracentrifugation after virus concentration by PEG. Method B consists in following method A until the stage of centrifugation at 10,000 *g* for 30 min at 4 °C, carried out after the precipitation by 10% (wt/vol) polyethylene

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