Journal of Virological Methods xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Comparison of two extraction methods for the detection of hepatitis A virus in lettuces using the murine norovirus as a process control

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Article history:
Received 3 August 2012
Received in revised form 3 April 2013
Accepted 13 May 2013
Available online xxx

Keywords:
RT-qPCR
Hepatitis A virus
Murine norovirus
Process control
Lettuces

A B S T R A C T

Enteric viruses are important agents of foodborne diseases. In recent years, raw fruits and vegetables have frequently been involved in foodborne transmission of enteric viruses to humans, particularly noroviruses and hepatitis A virus (HAV). Although viral contamination can occur at any stage of food processing, primary production is a critical stage in which prevention measures are essential to minimise the risk of infection to consumers. Due to the low infectious doses and low concentrations of enteric viruses in food samples, an efficient and rapid virus concentration method is required for routine control and risk assessment. In this study, the virus concentration reference method proposed by the CEN/TC275/WG6/TAG4 working group for samples of soft fruits and salad vegetables was compared with a method including a filtration step in order to recover hepatitis A virus (HAV) on lettuces. Murine norovirus (MNV-1) was used as a process control and detected simultaneously with HAV in a one-step duplex RT-qPCR following both procedures. The HAV LOD ranged from 10 to 100 PFU/25 g of lettuce in the presence or absence of MNV-1, regardless of method used. In conclusion, MNV-1 offers a very reliable and simple way to monitor the quality of the detection procedures. Although it has been found that both methods achieved an identical limit of detection, the method including a filtration step requires less processing and could be proposed as an alternative method.

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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 transmission or by ingestion of contaminated water and food, particularly shellfish, soft fruits and vegetables (Beuchat, 2006; Butot et al., 2007). HAV infection is common throughout the developing world where infections are most frequently acquired during early childhood and are usually asymptomatic or mild, resulting in a high proportion of adults immune to HAV. In developed countries, HAV infections are less common and low vaccine coverage has led to a high proportion of susceptible individuals, this creates a potential for extended hepatitis A outbreaks when contaminated products are widely distributed (Mohd Hanafiah et al., 2011). Different vegetables and fruits including different types of salads, onions, berries and, more recently, semi-dried tomatoes have been associated with HAV outbreaks (Calder et al., 2003; Carvalho et al., 2012; Gallot et al., 2011; Hernández et al., 1997; Petrignani et al., 2010a, 2010b; Rosenblum et al., 1990; Wheeler et al., 2005). Contamination may occur during growth in the field as well as during processing, storage, distribution and final preparation. 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). A rapid technique for detecting the presence of HAV in contaminated food samples is therefore essential to enable potential health risks to be assessed (Sánchez et al., 2007). 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. 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. The European Committee for Standardization (CEN) has asked a technical advisory group (TAG) to develop a standard method (qualitative/quantitative) for detection of norovirus and HAV in foodstuffs. The CEN/ISO/TS 15216 is due for publication in the first half of year 2013 and within a year these proposed protocols will be validated and then published as ISO or CEN standard methods. The method includes classical PCR controls and a process control. The latter measures virus recovery during the whole extraction and test procedure using a heterologous non-enveloped positive-sense ssRNA virus spiked into the test sample and assayed in parallel with the target viruses (Lees and CEN WG6 TAG4, 2010).

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0166-0934/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jviromet.2013.05.003

Please cite this article in press as: Coudray, C., et al., Comparison of two extraction methods for the detection of hepatitis A virus in lettuces using the murine norovirus as a process control. J. Virol. Methods (2013), http://dx.doi.org/10.1016/j.jviromet.2013.05.003

Although the TAG recommended the MC_0 strain of the Mengo virus (Costafreda et al., 2006; Le Guyader et al., 2009), the method allows freedom in the choice of the process control virus. The selected virus should exhibit morphological and physicochemical properties and environmental persistence similar to the target viruses, thus providing comparable extraction efficiency (Lees and CEN WG6 TAG4, 2010). Ideally, process control should be unlikely to contaminate the tested food sample naturally (Baert et al., 2011). Murine norovirus (MNV) is morphologically and genetically similar to human noroviruses, and shows considerable promise as a human norovirus surrogate (Karst et al., 2003; Wobus et al., 2006). Recently, MNV-1 has been successfully tested as a process control when detecting NoV and HAV in some food samples (Martin-Latil et al., 2012a; Stals et al., 2011a, 2011b) and HEV in water (Martin-Latil et al., 2012b). 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 lettuce by a one-step duplex RT-qPCR and to compare the efficiency of the CEN procedure with that of a method based on filtration using a positively charged membrane.

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 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) and HAV stock containing 1.55×10^8 PFU/mL. The correlation with the genomic quantity was 1 PFU = 10^5 genome copies HAV by measuring absorbance at 260 nm of RNA extracts.

MNV-1 (CW1 strain) was provided to the ANSES Fougères Laboratory in France by Dr H. Virgin from Washington University in the USA and was propagated in a mouse leukemic monocyte macrophage cell line (RAW 264.7, ATCC TIB-71) (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 5.62×10^6 $TCID_{50}$/mL (50% tissue culture infective dose/mL) was produced by the ANSES Fougères Laboratory (France) as previously described (Wobus et al., 2004). The correlation with the genomic quantity was 1 $TCID_{50}$ = 2×10^6 genome copies MNV-1 by measuring absorbance at 260 nm of RNA extracts.

2.2. Inoculation of lettuces

Lettuces were purchased from a local market. For each of four repetitions of experiments, 24 lettuce samples (25 g) were placed in 400 mL polypropylene bags containing a filter compartment (Seward, Norfolk, United Kingdom). Four samples were contaminated with each of the 6 following amounts of HAV: 0, 1, 10, 10^2, 10^3, 10^4 PFU. Each 100 μL HAV inoculum was distributed to about 20 spots on the surface of each 25 g sample of lettuce and left to dry overnight at 4 °C to increase the number of adhering viral particles. Uninoculated lettuce samples were used as a negative control. For each HAV contamination level, half of the samples (2/4) was also contaminated with 560 $TCID_{50}$ of murine norovirus, used as a process control and added just before the elution step, which corresponds to the earliest opportunity prior to virus extraction to monitor the extraction yield. For each HAV contamination level (including 4 lettuce samples), one sample without MNV-1 and one sample with MNV-1 were analysed by both methods (A and B) (Fig. 1).

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

2.3.1. Initial processing

Each inoculated lettuce sample which was 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 (TGBE), pH 9.5) covering the sample, 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 8500 × *g* for 30 min at 4 °C to pellet the food sample particles.

2.3.2. Method A

After the initial processing, 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 for 2 h at 4 °C. Viruses were concentrated by centrifugation of the solution at 8500 × *g* for 30 min at 4 °C. The supernatant was discarded and additional centrifugation was carried out at 8500 × *g* for 5 min at 4 °C to compact the pellet. The method A consists of the elution-concentration method described for vegetables in the "CEN/TC275/WG6/TAG4 viruses in foods" draft document with minor modifications (Fig. 1).

2.3.3. Method B

Method B was used to compare the concentration by PEG *versus* filtration after virus elution. After the initial processing, viruses were concentrated from supernatant by membrane filtration under vacuum using a Zetapor (Cuno Filtration SAS 3M, Cergy Pontoise, France) 47 mm positive-charged membrane of pore size 0.45 μm. The flow rate used during filtration was approx. 40 mL/6–7 min (Fig. 1).

2.3.4. RNA extraction

The pellet obtained with the method A was resuspended in 3 mL of NucliSens® easyMAG™ lysis buffer (BioMérieux, Marcy l'Etoile, France) for 10 min at room temperature. The filters obtained with the method B were placed in a 60 mm diameter Petri dish and directly incubated with 3 mL of NucliSens® easyMAG™ lysis buffer (BioMérieux) for 10 min at room temperature as described previously by Perelle et al. (2009). The whole 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. Nucleic acids were finally eluted in 80 μL of elution buffer and stored at −80 °C.

2.4. RT-qPCR

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 TaqMan® probe targeting the ORF1 polyprotein of the murine norovirus which were designed using Beacon Designer software (Bio-Rad, Marnes-la-Coquette, France) were previously used by Martin-Latil et al. (2012a). The HAV probe and MNV-1 probe were respectively labelled with the 6-FAM or Cy5 reporter dyes at the 5′-end, and an MGB or BHQ2 at the 3′-end. All

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