International Journal of Food Microbiology 243 (2017) 36–45

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Digital RT-PCR method for hepatitis A virus and norovirus quantification in soft berries

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ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form 18 November 2016
Accepted 21 November 2016
Available online 23 November 2016

Keywords:
Hepatitis A virus
Norovirus
Digital RT-PCR
RT-qPCR
Soft berries
PCR inhibition

ABSTRACT

Raw fruits may harbour many pathogens of public health concern including enteric viruses, which are the leading cause of foodborne outbreaks. Recently, consumption of soft berries has been associated with increasing reports of norovirus and hepatitis A virus outbreaks in Europe. Due to their low infectious doses and low concentrations in food samples, an efficient and sensitive analytical method is required for virus detection. In this study we explored two different ways to improve the reference method for the detection of enteric viruses in soft fruits (ISO/TS 15216-1; 15216-2): an additional purification step after RNA extraction; and the detection of enteric viral genome by an absolute quantification method (microfluidic digital RT-PCR). Both of these approaches led to an improvement of enteric virus detection in soft berries by greatly lowering PCR inhibition, raising viral extraction efficiencies and enabling validation of controls using pure RNA extracts. The PCR inhibitor removal step can be easily included in the routine method. Absolute quantification by digital RT-PCR may be a relevant alternative method to standardize quantification of enteric viruses in foodstuffs.

1. Introduction

Among the enteric viruses implicated in foodborne outbreaks, human noroviruses (NoV) belonging to genogroups I (GI) and II (GII) and hepatitis A virus (HAV) are the leading causative agents (EFSA, 2013; Gould et al., 2013; Hall et al., 2012). HAV and NoV are small non-enveloped viruses and have a positive-sense, single-stranded RNA genome. HAV is classified in the Hepatovirus genus of the *Picornaviridae* family and NoV belongs to the *Caliciviridae* family. They are mainly transmitted *via* the faecal-oral route either by person-to-person contact or by ingestion of contaminated water and food (Kotwal and Cannon, 2014; Matthews et al., 2012; Nainan et al., 2005). Contamination may occur during growth in the field as well as during processing, storage, distribution or meal preparation (Baert et al., 2008; Koopmans and Duizer, 2004; Parashar et al., 1998). HAV and NoV are highly stable in the environment: in cold temperatures, they can survive for several months (Carter, 2005; Rzezutka and Cook, 2004; Seitz et al., 2011) and in high temperatures, NoV and HAV can withstand temperatures of up to 60 °C for 30 min and 68 °C for 20 min respectively (Cannon et al., 2006; Coudray-Meunier et al., 2013; Tuladhar et al., 2012). They may also be resistant to some disinfectants, pressure and low pH (Cannon et al., 2006; Kingsley et al., 2014; Koopmans and Duizer, 2004; Li and Chen, 2015). Nevertheless, HAV and NoV are destroyed by boiling rapidly, so the greatest risk for foodborne norovirus infection is *via* uncooked foodstuffs such as shellfish, berries and vegetables (Kotwal and Cannon, 2014; Matthews et al., 2012; Nainan et al., 2005).

Most berries are consumed without any process *e.g.* washing step or virus inactivation treatment (EFSA, 2014). In many foodborne outbreaks, they are the probable or confirmed causative agents. Recently, consumption of berries has been associated with increasing reports of NoV and HAV outbreaks in Europe, particularly since 2009 (Tavoschi et al., 2015). NoV and HAV outbreaks linked to raspberries (Maunula et al., 2009; Müller et al., 2015; Niskanen Taina et al., 2011; RASFF, 2012, 2013, 2014; Sarvikivi et al., 2012) and strawberries (Bernard et al., 2014; Gillesberg Lassen et al., 2013; Mäde et al., 2013; Nordic outbreak investigation team, 2013; RASFF, 2012, 2013, 2014) and HAV outbreaks linked to a mix of frozen berries (RASFF, 2014; Rizzo et al., 2013; Severi et al., 2015) and frozen redcurrants (Terio et al., 2015) have been reported.

To ensure food safety, the development of sensitive, reliable and quantitative methods for detecting enteric viruses in soft berry samples is needed. Detection of these enteric viruses on the basis of their infectivity is complicated by the absence of a reliable cell culture method and the low levels of contamination of food samples (DiCaprio et al., 2013; Sánchez et al., 2007; Stals et al., 2013). In recent decades, RT-qPCR has been one of the most promising detection methods due to its sensitivity, specificity, reproducibility, speed, and minimal risk of carryover contamination as specific amplicons are detected in a closed tube. The ISO/TS 15216-1 and 15216-2 standards using real-time RT-

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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.11.022
0168-1605/

PCR for both quantitative determination and qualitative detection of NoV and HAV in foodstuffs (including soft fruits) were published in 2013 (ISO/TS 15216-1, 2013; ISO/TS 15216-2, 2013). However, this approach based on relative genomic quantification requires calibrated standards (DNA, RNA) and has serious drawbacks due to RT-qPCR inhibition. (Bustin and Nolan, 2004; Cao et al., 2015). In order to monitor the performance of the analysis, two types of controls have been included in the ISO/TS. A process control virus, morphologically and genetically similar to pathogenic viruses (classically mengovirus, murine norovirus (MNV) or Tulane virus), is inoculated before sample processing in order to verify that the extraction (from virus elution and concentration to nucleic acid isolation) was performed correctly. The second control is an external amplification control (RNA carrying the target sequence of interest) added prior to the RT-PCR detection step to monitor for RT-PCR inhibition in samples.

Digital PCR technology (dPCR) has the potential to overcome some limitations of qPCR. It is an end-point, sensitive and accurate absolute quantification approach that enables the determination of target copy numbers without external quantitative standards. These improvements are achieved by partitioning the sample (on the microlitre scale) prior to PCR amplification on microfluidic chips or micro-droplets into thousands to millions of individual reactions, so that each reaction contains one or no copies of the nucleic acid target. At the end of the amplification by PCR with the same primers and dye-labelled probes as qPCR, the ratio of positive to total partitions enables determination of the absolute number of targeted nucleic acids in the original sample by using binomial Poisson statistics (Dube et al., 2008; Pinheiro et al., 2012). Moreover, being an end-point approach, dPCR is robust to variations in amplification efficiency (Hindson et al., 2013). This could explain why the impact of inhibitors linked to matrix-type components is reduced in food or environmental samples (Cao et al., 2015; Coudray-Meunier et al., 2015; Rački et al., 2014a).

The aim of this study was to improve the analytical procedure described in the ISO/TS to detect enteric viruses (NoV (GI, GII) and HAV) in various types of berries. We evaluated two different approaches, (1) the addition of a purification step after RNA extraction to reduce RT-PCR inhibition; and (2) the detection of enteric viruses by microfluidic RT-dPCR compared with RT-qPCR in terms of sensitivity and susceptibility to inhibition. MNV was used as a control process virus for monitoring the recovery of the viral extraction methods.

2. Materials and methods

2.1. Viruses and cells

NoV GI and NoV GII were obtained from stool samples (E5569 and E1077 respectively) of infected humans provided by the French National Reference Centre for Enteric Viruses in Dijon, France. The faecal samples were suspended in 1× Phosphate Buffered Saline (PBS), pH 7, to obtain a final 10% suspension (*w*/*v*), vortexed and centrifuged at 3000*g* for 30 min at 4 °C. Aliquots of supernatant (100 μL) were kept frozen at −80 °C for later use. The titres of the clarified faecal suspensions were determined in NoV GI and NoV GII genomic copies with a RT-qPCR standard curve obtained with the ten-fold diluted *in vitro* RNA transcripts (see Section 2.2). NoV GI and NoV GII stocks had titres of approximately 5.0E + 07 and 2.0E + 08 genome copies/mL respectively.

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). The titre of viral production was determined in HAV RNA genomic copies with an RT-qPCR standard curve obtained with the ten-fold diluted *in vitro* RNA transcripts (see Section 2.2). HAV stocks had titres of approximately 4.5E + 10 genome copies/mL.

Dr. H. Virgin from Washington University in the USA provided ANSES's Fougères Laboratory in France with MNV-1 (CW1 strain), which was then propagated in a mouse leukaemic monocyte macrophage (RAW 264.7, ATCC TIB-71) cell line (Cannon et al., 2006). MNV-1 stock was produced as previously described (Wobus et al., 2004). The titre of viral production was determined in MNV-1 RNA genomic copies with an RT-qPCR standard curve obtained with the ten-fold diluted *in vitro* RNA transcripts. MNV-1 stocks had titres of approximately 8.1E + 11 genome copies/mL.

2.2. RNA standards

To summarize, HAV, NoV GI and NoV GII cDNA corresponding respectively to the 39–518, 5257–5413 and 4981–5135 positions of genomic sequence (M59808.1), (M87661) and (X86557) were cloned in pGEM-T Easy vector (Promega, Charbonnières-les-Bains, France). MNV-1 cDNA corresponding to the 3093–3430 positions of genomic sequence (DQ285629) was provided in pBluescript IISK+ vector by Genecust (Dudelange, Luxembourg). Plasmids were then propagated in *E. coli* One Shot® TOP10F′ (Life Technologies, Saint Aubin, France). High quality DNA plasmids containing HAV, NoV or MNV-1 regions were purified using the Qiagen Plasmid kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. The resulting plasmids were then digested with restriction enzymes (Life Technologies) and RNA transcripts were obtained by using the MEGAscript® kit (Life Technologies) according to the manufacturer's protocol. Synthesized RNA samples were treated with Turbo™ DNase (Life Technologies) according to the manufacturer's protocol in order to remove the DNA template following transcription, and purified using the MEGAclear™ kit (Life Technologies). The synthesized RNA samples were confirmed with (RT)-qPCR and quantified by measuring absorbance at 260/280 nm with a NanoDrop ND-1000 (Thermoscientific, Courtaboeuf, France). Aliquots of 10 μL were kept frozen at −80 °C for later use as standards and as external amplification controls to monitor for RT-PCR inhibition in samples. This approach is described in the ISO/TS 15216-1 and 15216-2. An external control (EC) RNA (an RNA species carrying the target sequence of interest) is added to an aliquot of RNA sample and tested using both RT-PCR methods. Comparison of these results with the results of EC RNA in the absence of sample RNA enables the level of RT-PCR inhibition in each sample under test to be determined.

2.3. Inoculation of soft berries samples

Frozen whole raspberries, frozen whole strawberries and frozen red fruit mix (whole raspberries 25%, currants 25%, blueberries 20%, whole blackberries 15%, blackcurrants 15%) were purchased from a local supermarket. Fruits were thawed at 5 °C and strawberries were cut into four pieces before use. Three samples (25 g) of each were spiked in a 400 mL polypropylene bag containing a filter compartment (Seward, Norfolk, United Kingdom) with HAV, NoV GI or NoV GII to obtain inoculation levels per sample of 1.0E + 07, 1.0E + 06 and 1.0E + 05 genome copies (HAV) or 1.0E + 06, 5.0E + 05 and 1.0E + 05 genome copies (NoV). Each 100 μL inoculum was distributed to approximately 15 spots on the surface of each 25 g sample and left to dry overnight at 4 °C to increase the number of adhering viral particles as previously described (Fraisse et al., 2011; Stals et al., 2011a). To control losses of target virus, which can occur at several stages during food sample analysis, samples were spiked just before addition of elution buffer with 1.0E + 08 genome copies of MNV-1. Uninoculated fruit samples (25 g) were used as a negative control. Each experiment set, from spiking to RNA extraction, was performed three times and RNA extracts (pure and diluted ten-fold) were analysed in duplicate by RT-qPCR and RT-dPCR assays.