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ORIGINAL PAPER

Detection of Potential Infectious Enteric Viruses in Fresh Produce by (RT)-qPCR Preceded by Nuclease Treatment

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Abstract Foodborne illnesses associated with contaminated fresh produce are a common public health problem and there is an upward trend of outbreaks caused by enteric viruses, especially human noroviruses (HNoVs) and hepatitis A virus (HAV). This study aimed to assess the use of DNase and RNase coupled to qPCR and RT-qPCR, respectively, to detect intact particles of human adenoviruses (HAdVs), HNoV GI and GII and HAV in fresh produce. Different concentrations of DNase and RNase were tested to optimize the degradation of free DNA and RNA from inactivated HAdV and murine norovirus (MNV), respectively. Results indicated that 10 µg/ml of RNase was able to degrade more than 4 $\log_{10}$ (99.99%) of free RNA, and 1 U of DNase degraded the range of 0.84–2.5 $\log_{10}$ of free DNA depending on the fresh produce analysed. The treatment with nucleases coupled to (RT)-qPCR was applied to detect potential infectious virus in organic lettuce, green onions and strawberries collected in different seasons. As a result, no intact particles of HNoV GI and GII were detected in the 36 samples analysed, HAdV was found in one sample and HAV was present in 33.3% of the samples, without any reasonable distribution pattern among seasons. In conclusion, RT-qPCR preceded by RNase treatment of eluted samples from fresh produce is a good alternative to detect undamaged RNA viruses and therefore, potential infectious viruses. Moreover, this study provides data about the prevalence of enteric viruses in organic fresh produce from Brazil.

Keywords Enteric viruses · Fresh produce · (RT)-qPCR · DNase · RNase

Introduction

Foodborne illnesses are a common public health problem that can be caused by a great variety of bacteria, viruses, parasites, by their harmful toxins or also by poisonous chemicals present in food. The Centre for Disease Control and Prevention (CDC), which collects data on outbreaks of foodborne disease in the United States, reported that one in six Americans get sick each year after consumption of contaminated foods or beverages (http://www.cdc.gov/foodsafety/foodborne-germs.html). The European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) stated in their summary report on trends and sources of foodborne outbreaks that 9.2% of the total 4362 foodborne outbreaks that occurred in the European Union during 2015 were caused by viruses (EFSA and ECDC 2016). However, viruses were the agents most frequently reported in 2014, accounting for 20.4% of total foodborne outbreaks (EFSA and ECDC 2015). World Health Organization (WHO) also found that there is an upward trend of foodborne outbreaks caused by viruses. Particularly, the latest summary of WHO, reported that human noroviruses (HNoV) were responsible for the largest number of cases of foodborne diseases worldwide, highlighting the global importance of this agent (WHO 2015). In conclusion, national and international organizations agree that the presence of pathogenic viruses in food should be monitored. In this regard, the release of ISO

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15216-1 in 2013 encouraged the implementation of routine analysis to detect foodborne viruses in at-risk foodstuffs (Anonymous 2013).

Leafy green vegetables and fruits were responsible for 30 and 21%, respectively, of HNoV foodborne outbreaks in the USA from 2009 to 2012, which emphasizes the importance of the fresh produce in the transmission of foodborne viruses. International trade can also increase the risk, especially if the produce comes from countries with poor food safety standards (Callejón et al. 2015). However, some studies demonstrated that, while there is a potential for HNoV contamination in pre-harvest stages, most HNoV contaminations occur during food preparation (Hall et al. 2014). Another virus less prevalent but causative of high rates of hospitalization and death in the United States is hepatitis A virus (HAV; Scallan et al. 2011). HAV is transmitted mainly by faecal-oral route (Cook and D'Agostino 2013) and so far, several outbreaks of hepatitis A transmitted by contaminated fresh produce have been reported (Collier et al. 2014; Donnan et al. 2012; Lassen et al. 2013). The prevalence of HAV varies among different regions of the world, being highly prevalent in developing countries where sewage treatment and hygiene practices are poor (Rodríguez-Lázaro et al. 2012). Human adenoviruses (HAdVs), except the types 40 and 41, are not considered as common causative agents of foodborne outbreaks (EFSA and ECDC 2014). However, they are increasingly included in the monitoring studies of foodborne viruses because they have been proposed as indicators for the presence of human faecal contamination (Maunula et al. 2013). HNoV, HAV and HAdV in common are non-enveloped viruses and thus are more resistant in the environment.

Since many of the enteric viruses are fastidious, quantitative PCR (qPCR) is usually used to detect and identify virus contamination in food (Rodríguez-Lázaro et al. 2012). However, the main drawback of qPCR is that it cannot discriminate between infectious and non-infectious viral particles, which is extremely important in terms of public health. Therefore, adaptations of qPCR consisting on the measurement of nucleic acids derived from undamaged viruses have been developed to assess virus infectivity (Knight et al. 2012). In this context, a strategy consisting in the enzymatic treatment of the samples with DNase or RNase nucleases prior to nucleic acid extraction can increase the probability of detecting nucleic acids from undamaged viral particles. This strategy was already successfully employed in our laboratory to detect undamaged DNA viruses in samples from swine manure treatment systems (Viancelli et al. 2012) and in water samples (Fongaro et al. 2013). RNase combined with a proteinase K treatment was firstly employed to discriminate intact HAV, feline calicivirus and poliovirus from those inactivated by UV, hypochlorite and high-temperature (Nuanualsuwan and Cliver 2003). Therefore, further studies need to be performed to prove that this could be a proper method to predict the infectivity of enteric viruses in fresh produce samples. Considering the above, the present work aimed to assess the use of DNase coupled to qPCR to detect undamaged particles of HAdV and RNase coupled to reverse transcription qPCR (RT-qPCR), to detect undamaged particles of HNoV GI, HNoV GII and HAV in fresh produce samples.

Materials and Methods

Fresh Produce Sampling

Fresh lettuce, strawberries and green onions with organic certification (Ecocert, IBD and Ecovida) were purchased from a local store and kept at 4 °C prior to analysis, when necessary. In total, 36 food samples were collected in October (spring), January (summer), April (autumn) and July (winter) during 2015 and 2016. Three replicates of each product representing 9 samples per season were tested. Fresh produce was processed following guideline ISO/TS 15216-1 (Anonymous 2013), weighting 25 g of each fresh produce and slicing in small pieces when necessary. Samples were inoculated with an internal process control virus to allow the calculation of viral recovery efficiency. Briefly, 100 µl of a murine norovirus type 1 (MNV-1) suspension at a concentration of 10^6 Plaque Forming Units/ml (PFU/ml) was uniformly distributed as small drops onto the sample surface. Then, samples were placed in a biosecurity cabinet for approximately 1 h, until the drops dried.

Preparation of Viral Stocks

MNV-1 was propagated in RAW 264.7 cells (a macrophage-like Abelson murine leukaemia virus-transformed cell line). These cells were cultured in Dulbecco Eagle's Minimum Essential Medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS), 1.5% HEPES, 1% non-essential amino acids and 1% L-glutamine and incubated at 37 °C with 5% CO_2. The cells were infected with MNV-1 and, after 48 h of incubation, they were subjected to three freezing and thawing cycles to lyse the cells. The cell lysates were harvested and centrifuged at $1600\times g$ for 5 min to separate the viral particles from the cell debris. The obtained viral suspension was titrated, aliquoted and stored at −80 °C prior to use.

HAdV-2 stocks were produced by infecting A549 cells (permissive cells derived from human lung carcinoma cells). A549 cells were cultured in Eagle's Minimum

Essential Medium (MEM; Gibco, Carlsbad, CA, USA) supplemented with 5% FBS and 1 mM sodium pyruvate and incubated at 37 °C with 5% CO_2. Infection of the cells and virus harvesting were performed following the same procedures as those detailed for MNV-1.

All the cells and viruses used in this study were kindly donated by Prof. Rosina Gironès from the University of Barcelona, Spain.

Virus Elution and Concentration

Enteric viruses were recovered from fresh produce samples following a method described in ISO/TS 15216-1 (Anonymous 2013). Briefly, the samples were placed into a sterile plastic bag together with 40 ml of Tris–glycine buffer (TGBE; 100 mM Tris–HCl, 50 mM glycine and 1% beef extract, pH 9.5) and incubated at room temperature for 20 min with constant rocking (approximately 60 oscillations/min). For the strawberries, 30 U of pectinase from *Aspergillus niger* (Sigma-Aldrich, St. Louis, MO, USA) was added to prevent jelly formation in the eluate. Then, the TGBE was recovered by pipetting and centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant was transferred to a clean tube; its pH was adjusted to 7.0 (±0.5) with 1 N HCl; and 0.25 volume of 50% (w/v) polyethylene glycol (PEG) 8000/1.5 M NaCl solution was added. These tubes were incubated with gentle rocking at 4 °C for 60 min and then centrifuged at 10,000×*g* for 30 min at 4 °C. The resulting pellet was resuspended in 500 µl of PBS. Finally, 1 M KCl was added to the resuspended sample, incubated on ice for 30 min and centrifuged at 12,000×*g*, for 10 min at 4 °C. This additional step was included in order to separate the PEG from viruses and obtain a supernatant with less potential inhibitors for further reactions (Colombet et al. 2007).

RNase and DNase Treatment of Viruses Eluted from Fresh Produce

To confirm the ability of RNase to degrade free RNA genomes derived from viruses, inactivated viruses were submitted to enzymatic treatment. First, three concentrations of RNase A, that specifically degrades single-stranded RNA, were tested (1, 10 and 50 µg/ml; Thermo Fisher Scientific, Carlsbad, USA). Every concentration was used to degrade RNA from 10^5 PFU of MNV-1, which were heat-inactivated at 95 °C for 1 h followed by 30 min exposure to UVC radiation (254 nm, 100 µJ/cm^2). RNase and inactivated viruses were added to 200 µl of a sample pool of produce eluates and also to a nuclease-free water sample in order to check potential RNase inhibitors present in fresh produce eluates. The reaction mixture was incubated at room temperature for 10 min and RNase activity was stopped by the addition of 1:20 volume of 10% SDS together with the addition of Proteinase K (Pasloske 2001). Negative controls with undamaged MNV-1, treated and non-treated with RNase, were also included to quantify the real amount of virus added in each sample and also to ensure that RNase A was fully inactivated before nucleic acid extraction. These experiments allowed us to choose the 10 µg/ml RNase A as the suitable concentration to treat sample eluates (see "RNase Treatment of Artificially Contaminated Samples" section), and further experiments were repeated in triplicates in lettuce, green onion and strawberry sample eluates.

DNase I (Invitrogen, Carlsbad, USA) activity was also tested separately following the protocol described by Viancelli et al. 2012. Briefly, 10^6 PFU of HAdV-2 was heat-inactivated at 95 °C for 1 h followed by 30 min exposure to UVC radiation (254 nm, 100 µJ/cm^2) and added to sample eluates and to nuclease-free water. Subsequently, samples were treated with 1 U of DNase. The reactions were incubated at room temperature for 15 min, according to manufacturer instructions. The enzyme activity was stopped by the addition of 25 mM EDTA followed by incubation for 10 min at 65 °C. Negative controls with non-inactivated HAdV-2 were performed following the same protocol described for RNase tests. All the experiments were performed in triplicate for lettuce, strawberry and green onion sample eluates.

After testing RNase and DNase activities in artificially contaminated samples with MNV-1 and HAdV-2, enzymatic treatments coupled to (RT)-qPCR were applied to detect undamaged particles of HAdV, HNoV GI, HNoV GII and HAV in naturally contaminated samples.

Nucleic Acid Extraction and Virus Quantification

Nucleic acids were extracted from 200 µl of sample eluates using the QIAamp Viral Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Nucleic acids were eluted in a final volume of 60 µl and stored at −80 °C prior to the quantification assays. Nucleic acid extracts were diluted (1:10) to reduce the inhibitor concentration and enhance PCR efficiency.

Genomic copies of undamaged viral particles of HAdV-2 and MNV-1 from artificially contaminated samples were determined by (RT)-qPCR in a StepOne Plus® Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The same equipment was used to quantify undamaged viral particles of HAdV, HNoV GI, HNoV GII and HAV from naturally contaminated samples. Primers sets and thermal cycling conditions used are described in Table 1. HAdV was quantified in a 25-µl reaction with the 1X PCR Master Mix (Applied Biosystems) containing 5 µl of template, 0.9 µM of each primer and 0.225 µM of TaqMan probe.

Table 1 Primers, probes and thermal cycling conditions used to amplify MNV-1, HAdV, HAV, HNoV GI and HNoV GII

Virus	Primers and probes	Thermal cycling conditions	Source of reference
MNV-1	MNV-F MNV-R MNV-P	45 °C for 10 min; 95 °C for 5 min; 95 °C for 5 s and 60 °C for 45 s (40 cycles)	Baert et al. (2008)
HAdV	HAdV-F HAdV-R ADP1	50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 s and 60 °C for 1 min (45 cycles)	Hernroth et al. (2002)
HAV	HAV-F HAV-R HAV-P	45 °C for 10 min; 95 °C for 5 min; 95 °C for 5 s and 55 °C for 45 s (45 cycles)	Jothikumar et al. (2005)
HNoV GI	COF COR RingA RingB	45 °C for 10 min; 95 °C for 5 min; 95 °C for 5 s and 55 °C for 45 s (45 cycles)	Kageyama et al. (2003)
HNoV GII	G2F G2R G2P	45 °C for 10 min; 95 °C for 5 min; 95 °C for 5 s and 55 °C for 45 s (45 cycles)	Kageyama et al. (2003)

For the RNA viruses (MNV-1, HNoV GI, HNoV GII and HAV), RT-qPCR was performed in a reaction mixture of 20 µl containing 5 µl of template, 0.8 µM of each primer, 0.2 µM of TaqMan probe and 1 × QuantiNova Probe RT-PCR Master Mix (Qiagen, Valencia, CA, USA). All amplifications were done in duplicate. For each assay, standards were generated by cloning the amplicon from positive control into a plasmid (pBR22 for HAdV, pGEM®-T Easy Vector (Promega) for MNV-1 and HAV strain HM175; and pCR2.1-TOPO vector for HNoV GI and GII), and the corresponding copy number was calculated. Ten-fold serial dilutions of plasmids were run in parallel with the samples to obtain standard curves. Non-template controls (NTC) consisting of DEPC water were included in each assay.

Results

RNase Treatment of Artificially Contaminated Samples

As observed in Table 2, 10^5 PFU of MNV-1 added to any matrix, either to sample eluates or water, corresponded to approximately 10^9 genomic copies. When this virus was inactivated and treated with RNase, it was observed that 10 and 50 µg/ml of this enzyme were able to degrade 6 and 7 $\log_{10}$ of RNA from inactivated MNV-1, respectively, both in water and in eluted sample. Results from negative controls consisting of non-inactivated MNV-1 treated with

Table 2 Genome quantification of MNV-1 inoculated on nuclease-free water and sample eluates

	MNV-1 (Genome copies/ml)	
	Water	Sample eluates
Undamaged MNV-1		
Without RNase	2.01×10^9	2.00×10^9
RNase 10 µg/ml	2.12×10^9	3.00×10^9
RNase 50 µg/ml	2.26×10^9	6.47×10^3
Inactivated MNV-1		
RNase 1 µg/ml	1.10×10^4	2.25×10^8
RNase 10 µg/ml	1.24×10^3	4.74×10^3
RNase 50 µg/ml	2.51×10^2	6.75×10^2

RNase showed that the activity of this enzyme, at a concentration of 10 µg/ml, was not able to degrade the capsid-protected RNA as we could detect 10^9 genomic copies from intact MNV-1 added to water or eluted sample. Contrarily, RNase at a concentration of 50 µg/ml degraded 6 $\log_{10}$ of RNA from negative controls consisting of undamaged MNV-1. In other words, although 50 µg/ml degraded 7 $\log_{10}$ of free RNA from genomes of inactivated viruses, we were not able to inactivate the enzyme at this concentration before extracting genomes from intact viruses. Therefore, the concentration of 10 µg/ml of RNase seemed to be more appropriate to treat viral-eluted samples from fresh produce and was used in further experiments. Table 3 shows the results that confirmed that 10 µg/ml of RNase were able to degrade more than 5 $\log_{10}$ of

Table 3 Genome quantification of MNV-1 inoculated on lettuce, green onion and strawberry sample eluates

	MNV-1 (Genome copies/ml)			
	Without RNase	RNase treatment (10 μg/ml)		
	Undamaged MNV-1	Undamaged MNV-1	Inactivated MNV-1	Log reduction
Lettuce	$3.11\ (\pm1.57) \times 10^9$	$1.71\ (\pm0.86) \times 10^9$	$5.93\ (\pm1.69) \times 10^3$	5.70 ± 0.10
Green onion	$2.51\ (\pm0.68) \times 10^9$	$1.54\ (\pm0.77) \times 10^9$	$6.82\ (\pm9.43) \times 10^4$	5.24 ± 1.50
Strawberry	$3.37\ (\pm0.09) \times 10^9$	$3.00\ (\pm0.68) \times 10^9$	$1.20\ (\pm1.50) \times 10^4$	5.78 ± 0.85

inactivated virus without affecting the RNA from undamaged virus in lettuce, strawberry and green onion sample eluates.

DNase Treatment of Artificially Contaminated Samples

Table 4 shows that 10^6 PFU of HAdV-2 added to sample eluates corresponded to a range of 8.7×10^7–2.4×10^9 genome copies of HAdV, depending on the fresh produce analysed. When these viruses were inactivated and treated with 1U of DNase, the enzyme was able to degrade approximately 2.5 $\log_{10}$ of viral DNA in lettuce samples, 1.4 $\log_{10}$ in strawberry samples and 0.84 $\log_{10}$ in green onion samples. Results from negative controls consisting of undamaged HAdV-2 treated with DNase showed that, in case of lettuce, DNase degraded from 0.08 to 1.11 logs of DNA genomes from intact viruses, demonstrating that the enzyme was not completely inactivated before extracting DNA from viral particles.

Detection of Undamaged Enteric Viruses in Naturally Contaminated Fresh Produce

Undamaged viral particles of HAdV, HAV, HNoV GI and HNoV GII were quantified in naturally contaminated samples after being treated with 10 μg/ml of RNase and with 1U of DNase (Table 5). The recovery efficiency, calculated as the percentage of MNV-1 recovered after virus concentration by PEG precipitation in fresh produce samples, was also determined for each sample analysed (Table 5). High $R2$ values (more than 0.95) and high efficiencies (from 95 to 110%) were obtained in all qPCR assays, indicating the validity of these quantifications (data not shown). Results show that no intact particles of HNoV GI and HNoV GII were detected in the 36 samples analysed. HAdV was found in one sample of strawberry, while intact particles of HAV were present in 6 samples of lettuce, 4 samples of green onion and 2 samples of strawberry, in a range of 1.46×10^2–1.84×10^5 genomic copies of HAV per 25 g of sample. These findings represented a contamination of 33.3% of the analysed fresh produce. We could not establish any reasonable distribution pattern of positive samples per season.

Discussion

During the last decade, many organizations have stated the importance of the surveillance and diagnosis of foodborne viruses and they recommend including them in food safety regulations. Nevertheless, most of the countries do not consider pathogenic viruses monitoring in their food safety plans to date. The presence of enteric viruses in fresh produce is a subject of special relevance since fruits and vegetables belong to the group of ready-to-eat foods, which are not cooked before consumption. Molecular techniques, such as qPCR or RT-qPCR, are the most commonly used methods for the quantification of enteric viruses in food, in which the level of virus contamination is usually low (Bosch et al. 2011). Currently, increasing number of studies are focusing on the modification of these molecular methods to be able to discriminate between infectious and non-infectious viral particles. One of these adaptations, namely viability PCR (v-PCR), consists in coupling qPCR with nucleic acid intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA). These dyes may permeate damaged capsids and bind to DNA/

Table 4 Genome quantification of HAdV-2 inoculated on lettuce, green onion and strawberry sample eluates

	HAdV (Genome copies/ml)			
	Without DNase	DNase treatment (1U)		
	Undamaged HAdV	Undamaged HAdV	Inactivated HAdV	Log reduction
Lettuce	$2.4\ (\pm3.2) \times 10^9$	$6.6\ (\pm0.9) \times 10^7$	$3.2\ (\pm3.2) \times 10^6$	2.53 ± 0.57
Green onion	$8.7\ (\pm0.1) \times 10^7$	$1.3\ (\pm1.6) \times 10^7$	$7.9\ (\pm11.1) \times 10^7$	0.84 ± 1.55
Strawberry	$5.1\ (\pm2.0) \times 10^8$	$4.8\ (\pm3.5) \times 10^8$	$9.6\ (\pm13.3) \times 10^7$	1.44 ± 1.63

Table 5 Quantification of nucleic acids from undamaged viral particles of HAdV, HNoV GI, HNoV GII and HAV naturally occurring in lettuce, strawberry and green onion samples

	MNV-1 recovery[a]	HAdV[b]	HAV[b]	HNoV GI[b]	HNoV GII[b]
Spring					
Lettuce	30.75 ± 22.54	0/3	1/3	0/3	0/3
Green onion	15.32 ± 10.09	0/3	2/3	0/3	0/3
Strawberry	13.84 ± 12.10	0/3	0/3	0/3	0/3
Summer					
Lettuce	12.34 ± 13.81	0/3	2/3	0/3	0/3
Green onion	5.56 ± 4.19	0/3	1/3	0/3	0/3
Strawberry	4.22 ± 2.49	0/3	1/3	0/3	0/3
Autumn					
Lettuce	17.11 ± 8.38	0/3	0/3	0/3	0/3
Green onion	7.14 ± 5.50	0/3	0/3	0/3	0/3
Strawberry	3.65 ± 1.87	1/3	0/3	0/3	0/3
Winter					
Lettuce	58.58 ± 31.41	0/3	3/3	0/3	0/3
Green onion	8.56 ± 10.21	0/3	1/3	0/3	0/3
Strawberry	14.19 ± 6.71	0/3	1/3	0/3	0/3

[a] Mean ± SD (%). MNV-1 was used as a process control to monitor the efficiency of virus recovery

[b] Positive/total samples

RNA after exposure to intense visible light, preventing their amplification (Elizaquível et al. 2013). However, some researchers have also demonstrated that the effects of viability dyes vary depending on the type of virus and the type of dyes. For instance, previous works showed that EMA treatment before RT-qPCR showed a slight reduction of MNV infectivity (Kim et al. 2011) and PMA-RT-qPCR was not suitable to discriminate between infectious and non-infectious MNV particles (Kim and Ko 2012), which is considered the best surrogate for studying environmental survival of HNoV (Cannon et al. 2006). Moreover, another study determined that PMA differentiates infectious and non-infectious viruses only in particular conditions (Parshionikar et al. 2010). In this context, other studies employed the enzymatic treatment with RNase to detect intact MNV-1 (Diez-Valcarce et al. 2011), HNoV (Lamhoujeb et al. 2008; Topping et al. 2009) or HAV (Sánchez et al. 2012) in viral suspensions. Likewise, some improvements using PMAxx with a Triton pre-treatment have been done for the detection of NoV GI and GII in leafy vegetables (Randazzo et al. 2016). However, there is limited information about how these nucleases act in complex samples such as fresh produce and it has been demonstrated that the sample matrix plays an important role in the resistance of HNoV or the surrogates (Escudero-Abarca et al. 2014). In order to gain knowledge in this area, we evaluated whether qPCR and RT-qPCR preceded by enzymatic treatment with either DNase or RNase were appropriate to detect intact particles of HAdV, HAV, HNoV GI and HNoV GII in fresh produce samples.

Regarding the enzymatic treatment using RNase, different enzyme concentrations were evaluated and we observed differences in terms of the nuclease efficiency. We concluded that 10 μg/ml of RNase were able to degrade more than 99.99% of free RNA from inactivated MNV-1 without affecting the RNA from infectious virus extracted from lettuce, strawberry and green onion samples. Another study also demonstrated that RNase was effective in fresh produce samples and was not inhibited by this kind of food matrix and thus, this pre-treatment could help to detect RNA only from intact virus particles and avoid false-positive results in the RT-qPCR (Mormann et al. 2010). Besides being a good surrogate for studying environmental survival of HNoV, MNV-1 has also been indicated as a good candidate to simulate adhesion of enteric viruses to food surfaces (Deboosere et al. 2012). Contrarily, other studies have demonstrated that FCV is a poor model system for determining heat inactivation of HNoVs (Nowak et al. 2011; Topping et al. 2009). Therefore, as RNase treatment coupled with RT-qPCR worked well to detect intact MNV-1 in food samples, it could be useful to detect intact HNoV in fresh produce matrices. Although capsid integrity does not guarantee virus infectivity, it was already demonstrated that intact capsid is essential for infectivity (Levy et al. 2010; Nuanualsuwan and Cliver 2003). This fact, together with the fact that in vitro cultivation of HNoV to detect infectious particles is still incipient (Ettayebi et al. 2016), turns the RNase treatment into an attractive alternative to predict virus infectivity.

DNase degradation of free DNA from inactivated HAdV-2 did not work as expected and the enzyme only degraded the maximum of 2.5 $\log_{10}$ of free DNA (99.6%) in sample eluates, presenting high variation both among replicates and kind of fresh produce tested. It was also observed that, in some cases, DNase was not totally inactivated before extracting DNA from intact particles. Overall, it can be inferred that fresh produce contains some substances that may have interfered both in the action and inhibition of DNase, which did not occur in environmental samples such as drinking water (Fongaro et al. 2013) and water from swine manure treatment systems (Viancelli et al. 2012). Although the detection of HAdV is very important in food samples because it is considered a good indicator of faecal pollution (Albinana-Gimenez et al. 2009), results of infectious HAdV in fresh produce after DNase treatment are not fully reliable and it should be interpreted with caution. However, there are several cell culture-based methods which can replace molecular methods to detect infectious HAdV (Marti and Barardi 2016).

Concerning the naturally contaminated fresh produce, intact particles of HAV were detected in one-third of analysed samples, while they were all negative for the presence of HNoV GI and GII, and only one sample was positive for HAdV. On the other hand, the high prevalence of HAV in lettuce, strawberries and green onions, agrees with previous studies which identified fresh produce as an important vehicle for transmission of human pathogens (Lynch et al. 2009). Usually, enteric viruses reach the crops by irrigation with contaminated water or by fertilizing with sludge from wastewater treatment plants (Li et al. 2015). In Brazil, where this study was performed, HAV was previously detected in environmental water samples (de Paula et al. 2007; Rigotto et al. 2010), probably due to the deficient sewage treatment systems and/or leakage in septic tanks. Once in the crops, HAV can survive for many days, as it is one of the most persistent enteric viruses in environmental matrices such as soil and food (Rzeżutka and Cook 2004). Although HNoV and HAV are important foodborne viruses (Elizaquível et al. 2013), we only detected HAV but not HNoV, as food contamination by HNoV usually occurs at the last stage of post-harvest processing, normally in the kitchen (Lynch et al. 2009).

In conclusion, since the determination of viral infectivity of HNoV and most strains of HAV by plaque assay is not broadly available in many laboratories, enzymatic treatment of fresh produce eluates could allow the detection of undamaged RNA virus particles. This assay could be applied for routine microbiologic analyses of fresh produce, since it is not laborious and provides information about virus infectivity, contrarily to (RT)-qPCR. Moreover, this work provides data about the prevalence of enteric viruses in organic fresh produce from Brazil.

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