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Detection of human adenoviruses in organic fresh produce using molecular and cell culture-based methods

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ABSTRACT

The consumption of organic fresh produce has increased in recent years due to consumer demand for healthy foods without chemical additives. However, the number of foodborne outbreaks associated with fresh produce has also increased. Contamination of food with enteric viruses is a major concern because the viruses have a low infectious dose and high persistence in the environment. Human adenovirus (HAdV) has been proposed as a good marker of faecal contamination. Therefore, the aim of this study was to evaluate the efficiency of the plaque assay (PA), real time PCR (qPCR) and integrated cell culture-RT-qPCR (ICC-RT-qPCR) for the recovery of HAdV from artificially and naturally contaminated fresh produce. Organic lettuce, strawberries and green onions were selected because these fresh products are frequently associated with foodborne outbreaks. The virus extraction efficiencies from artificially contaminated samples varied from 2.8% to 32.8% depending on the food matrix and the quantification method used. Although the HAdV recoveries determined by qPCR were higher than those determined by PA and ICC-RT-qPCR, PA was defined as the most reproducible method. The qPCR assays were more sensitive than the PA and ICC-RT-qPCR assays; however, this technique alone did not provide information about the viability of the pathogen. ICC-RT-qPCR was more sensitive than PA for detecting infectious particles in fresh produce samples. HAdV genome copies were detected in 93.3% of the analysed naturally contaminated samples, attesting to the common faecal contamination of the fresh produce tested. However, only 33.3% of the total samples were positive for infectious HAdV particles based on ICC-RT-qPCR. In conclusion, this study reported that HAdV can be an efficient viral marker for fresh produce contamination. Good detection of infectious HAdV was obtained with the ICC-RT-qPCR and PA assays. Thus, we suggest that the ICC-RT-qPCR and PA assays should be considered when quantitative microbial risk assessment (QMRA) studies are required and to establish reliable food safety guidelines.

1. Introduction

Foodborne viruses are increasingly recognized worldwide as the most important cause of foodborne outbreaks (Baert et al., 2011; Stals et al., 2013). In the European Union, the European Food Safety Authority (EFSA) reported that 14% of the foodborne outbreaks that occurred during 2012 were caused by viral agents, which represented an increase in viral outbreaks of 44.3% compared with 2011 (EFSA/ECDC, 2014). Minimally processed foods such as fresh produce are usually implicated in foodborne outbreaks because most of these foods are consumed raw or without peeling (Rodríguez-Lázaro et al., 2012). Contamination of fresh produce can occur by two main routes: i) by infected food handlers during the harvesting, packaging and preparation of the food or ii) pre-harvesting contamination by irrigating with polluted water (Cheong et al., 2009) or fertilizing fields with contaminated manure (Stals et al., 2013). Because conventional wastewater treatment processes are not completely efficient for the removal or inactivation of enteric viruses (Maunula et al., 2013) and the demand for organic products is rising (Heaton and Jones, 2008), foodborne outbreaks associated with fresh produce have become a matter of public health concern.

Although many studies investigating the bacterial contamination of fresh produce have been performed, there is a lack of knowledge concerning viral contamination of food (Predmore and Li, 2011). Furthermore, several studies have demonstrated that the levels of indicator bacteria only slightly correlate with the virus levels, and the correlation is even lower when the faecal indicator concentrations are low (Contreras-Coll et al., 2002). Enteric viruses are a major concern because they have a low infectious dose (approximately 10–100 virus particles) (Teunis et al., 2008) and exhibit high persistence in the environment due to their non-enveloped nature (Barker et al., 2013; Rodríguez-Lázaro et al., 2012). Therefore, even though the viral loads on fresh produce may be low and enteric viruses do not multiply in foods, viruses can remain as infectious particles on food surfaces for long periods, thereby increasing the possibility of transmission to

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humans (Bosch et al., 2011). In this context, studies are needed to assess the prevalence of enteric viruses in fresh produce and their persistence in the production chain.

The viruses most frequently involved in foodborne infections are noroviruses (NoV) and hepatitis A virus (HAV), which are responsible for gastroenteritis and hepatitis, respectively (Bosch et al., 2011). Human adenoviruses (HAdVs) are frequently associated with other diseases (*i.e.*, respiratory, conjunctivitis or otitis) but some strains can also cause gastroenteritis, usually in children and immune-compromised people (Jiang, 2006). Although they are not common causative agents of foodborne outbreaks (EFSA/ECDC, 2014), HAdVs have become a subject of growing interest because they possess characteristics that make them an ideal indicator of faecal pollution (Albinana-Gimenez et al., 2009; Hundesa et al., 2006). First, HAdVs are non-enveloped, double-stranded DNA viruses and thus are up to 60-fold more resistant to UV irradiation than RNA viruses (Fong and Lipp, 2005). Indeed, HAdV is considered the most resistant pathogenic virus to UV radiation (Linden et al., 2007). Moreover, these human viruses can survive for long periods outside of their hosts (Horowitz and Mold, 2007) and, because they have a DNA genome, they present lower mutation rates compared to RNA viruses, which facilitates their detection by PCR (Wyn-Jones et al., 2011).

Virus detection is primarily based on the following two principles: the determination of infectious viruses by propagation in cell culture or the quantification of viral genomes by molecular amplification techniques such as qPCR or RT-qPCR. At present, qPCR is the gold standard for virus detection due to its sensitivity, specificity, speed and ability to provide quantitative data (Martin-Latil et al., 2012). However, interpretation of the results is not always straightforward and several factors should be taken into consideration (Stals et al., 2013). One of the major limitations of qPCR is its inability to differentiate between infectious and non-infectious viruses. In contrast, cell culture allows the detection of infectious viruses because it is primarily based on the detection of the cytopathic effects produced in host cells during virus replication. HAdV has an advantage over other enteric viruses because its infectivity can be evaluated by plaque assay (PA) due to the virus's ability to propagate in cell culture (Cromeans et al., 2008). Recently, the integrated cell culture-RT-qPCR assay (ICC-RT-qPCR), which is based on the quantification of viral mRNA produced during virus replication in host cells, was developed to evaluate the capacity of HAdV to replicate *in vitro* (Fongaro et al., 2013). This technique overcomes the disadvantages of long incubation periods in cell culture and eliminates the possibility of non-infectious particle detection when qPCR alone is employed. ICC-RT-qPCR is also more robust than ICC-qPCR because this former technique can eventually detect non-infectious viruses that remain adsorbed to cell surfaces but are not able to enter cells and complete the replication cycle. Although ICC-RT-qPCR assay was previously employed for the detection of enteric viruses in environmental samples, to the best of our knowledge, this is the first report to propose the use of ICC-RT-qPCR to detect infectious HAdVs on fresh produce.

The main objective of this study was to evaluate the efficiency of the PA, qPCR and ICC-RT-qPCR assays in the recovery of HAdVs from artificially and naturally contaminated fresh produce. The following three different fresh products frequently associated with foodborne outbreaks were selected for this study: lettuce, strawberries and green onions. All of the products tested were organic. HAdV was chosen due to its double role as a human pathogen and indicator of faecal contamination.

2. Materials and methods

2.1. Cell lines and virus stocks

Human adenovirus type 2 (HAdV-2) stocks were produced by infecting A549 cells (permissive cells derived from human lung carcinoma cells), kindly provided by Dr. Rosina Gironès from the University of Barcelona, Spain. The A549 cells were cultured in a monolayer with Eagle's Minimum Essential Medium (MEM; Gibco, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum (FBS) and 1 mM sodium pyruvate and incubated at 37 °C with 5% CO_2. The cell cultures were infected with HAdV-2. After 48 h of incubation, the cells were subjected to three freezing and thawing cycles to lyse the cells. The cell lysates were harvested and centrifuged at 1600 × *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.

2.2. Food samples and virus inoculation

Fresh lettuce, strawberries and green onions with an organic certification (Ecocert, IBD and Ecovida) were purchased from a local store and stored at 4 °C prior to analysis. Based on the ISO technical specifications (norovirus and hepatitis A virus analyses from food and animal feed; ISO/TS 15216-1:2013), 25 g of each fresh produce sample was weighed and cut into smaller pieces when necessary. Naturally contaminated samples were analysed immediately. Artificially contaminated samples were seeded with 100 μl of a HAdV-2 suspension at a concentration of 10^8 Plaque Forming Units/ml (PFU/ml), corresponding to 10^9 genomic copies/ml (GC/ml), that was uniformly distributed as small drops onto the sample surface. Then, the samples were placed in a biosecurity cabinet for approximately 1 h until the drops dried.

2.3. Virus elution and concentration

HAdV was recovered from the naturally and artificially contaminated samples following the previously described method (Dubois et al., 2007). The experiments were conducted in five replicates for each type of fresh produce. Briefly, the samples were placed into a sterile bag (BagLight®, Interscience, Saint Nom, France) 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, and the pH was monitored and adjusted to 9.0 with 1 N NaOH if necessary. A prolongation of the agitation period by 10 min was performed after each adjustment. 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 volumes of a 50% (*w*/*v*) polyethylene glycol (PEG) 8000/1.5 M NaCl solution were 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 with 500 μl of PBS and stored on ice prior to HAdV quantification by PA, qPCR and ICC-RT-qPCR.

2.4. Cytotoxicity tests

Cytotoxicity tests were performed to determine the potential toxicity of the fresh produce matrices used in this study on the A549 cell line. A549 cell monolayers (1.5×10^5 cells/ml) were propagated in 24-well microplates and incubated at 37 °C with 5% CO_2 for 24 h. Then, two-fold serial dilutions (1 to 1:64) of uncontaminated eluted samples were prepared in MEM, and 100 μl of each dilution was inoculated in duplicate onto the A549 plates. After 1 h of incubation, the inoculum was removed and the cells were supplied with 1 ml of culture medium supplemented with 2% FBS. After 7 days, the medium was removed and the cellular integrity was confirmed by staining the cell monolayer with 250 μl of naphthalene black (Sigma-Aldrich) for 15 min at 37 °C. To evaluate the cytotoxic effects, the wells corresponding to each sample dilution were compared with the control containing only an untreated cell monolayer and medium.