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Attachment and localization of human norovirus and animal caliciviruses in fresh produce

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ABSTRACT

Fresh produce is a high risk food for human norovirus (NoV) contamination. To help control this pathogen in fresh produce, a better understanding of the interaction of human NoV and fresh produce needs to be established. In this study the attachment of human NoV and animal caliciviruses (murine norovirus, MNV-1; Tulane virus, TV) to fresh produce was evaluated, using both visualization and viral enumeration techniques. It was found that a human NoV GII.4 strain attached efficiently to the Romaine lettuce leaves and roots and green onion shoots, and that washing with PBS or 200 ppm of chlorine removed less than 0.4 log of viral RNA copies from the tissues. In contrast, TV and MNV-1 bound more efficiently to Romaine lettuce leaves than to the roots, and simple washing removed less than 1 log of viruses from the lettuce leaves and 1–4 log PFU of viruses from roots. Subsequently, the location of virus particles in fresh produce was visualized using a fluorescence-based Quantum Dots (Q-Dots) assay and confocal microscopy. It was found that human NoV virus-like particles (VLPs), TV, and MNV-1 associated with the surface of Romaine lettuce and were found aggregating in and around the stomata. In green onions, human NoV VLPs were found between the cells of the epidermis and cell walls of both the shoots and roots. However, TV and MNV-1 were found to be covering the surface of the epidermal cells in both the shoots and roots of green onions. Collectively, these results demonstrate that (i) washing with 200 ppm chlorine is ineffective in removing human NoV from fresh produce; and (ii) different viruses vary in their localization patterns to different varieties of fresh produce

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1. Introduction

Human norovirus (NoV) is the leading causative agent of food-borne outbreaks associated with fresh produce, accounting for over 40% of all fresh produce related illness reported in the US each year (Heaton and Jones, 2008; Seymour and Appleton, 2001). This pathogen has been linked to outbreaks in lettuce, fresh cut salads, green onions, and various types of berries (Abbaszadegan et al., 1999; Doyle and Erickson, 2008; Falkenhorst et al., 2005; Heaton and Jones, 2008; Le Guyader et al., 2004; Lynch et al., 2009). Despite the high prevalence of human NoV associated outbreaks in fresh produce, little is known about the interaction of the virus with these high risk food commodities. Unlike bacterial pathogens, viruses associated with food-borne outbreaks are unable to multiply in the foods due to the fact that viruses are obligate intracellular organisms. However, the most commonly associated food-borne viruses are known to be highly stable in the environment and are also shed at a very high titer from their hosts. The high prevalence of these viruses within the human population coupled with the ability of the viruses to remain infectious under extreme pH, their resistance to desiccation in the environment, and stability at low (even freezing) temperatures, makes most foods that undergo limited processing, such as fresh produce, susceptible to contamination.

To date, most of our understanding of the stability and persistence of human NoV in foods comes from the study of surrogate viruses. Three cultivable animal caliciviruses, feline calicivirus (FCV), canine calicivirus (CaCV), and murine norovirus (MNV-1), have been extensively used as human NoV surrogates. Although these animal caliciviruses share variable degrees of genetic relatedness with human NoV, they differ from human NoV in clinical manifestations, host receptors, susceptible cell types, pathogenesis, and immunity (Cannon et al., 2006; Jaykus and

Abbreviations: NoV, norovirus; MNV-1, murine norovirus; TV, Tulane virus; VLPs, virus-like particles; Q-Dots, quantum dots; FCV, feline calicivirus; CaCV, canine calicivirus; CPE, cytopathic effect; HBGAs, histo-blood group antigens; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; MOI, multiplicity of infection; Opti-MEM, low serum Eagle's minimum essential medium; HBSS, Hank's balanced salt solution; RT-PCR, reverse transcriptase polymerase chain reaction; HABA, 4′-hydroxyazobenzene-2-carboxylic acid; RT-qPCR, real time reverse transcriptase polymerase chain reaction.

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Escudero-Abarca, 2010; Koopmans and Duizer, 2004; Li et al., 2012; Wobus et al., 2004). Therefore, whether these surrogates truly represent human NoV remains unknown. Recently, a new primate calicivirus, Tulane virus (TV), was discovered in the stool of rhesus macaques housed in the Tulane National Primate Research Center (Farkas et al., 2008). TV replicates *in vitro* in rhesus monkey kidney cells and causes typical cytopathic effects (CPE). Importantly, TV also recognizes histo-blood group antigens (HBGAs) as its functional cellular receptors, similar to human NoV (Farkas et al., 2008). TV is also genetically closely related to human NoV (Cromeans et al., 2014). Thus, TV could serve as a useful surrogate for human NoV.

Commonly in the food industry, fresh produce receives limited treatment to eliminate pathogens. Produce harvested from the field is often transported to processing facilities where bulk product is submerged in washing tanks to remove physical hazards such as soil, stones, and woody material. Often, this submersion tank may also include chlorine; however the industry is limited to the use of less than 200 ppm chlorine in the wash tanks (Li et al., 2012; Predmore and Li, 2011). This chlorine level must be constantly monitored, as the presence of pathogens, as well as organic matter will react with and decrease the active chlorine levels in the wash tanks. While 200 ppm chlorine has been shown to be effective in eliminating many bacterial pathogens such as *Escherichia coli* and *Salmonella* species, it has little to no effect on the removal of viral pathogens (Le Guyader et al., 2009; Li et al., 2012; Lynch et al., 2009; Oie et al., 2008; Predmore and Li, 2011; Seymour and Appleton, 2001). Specifically, research utilizing human NoV surrogates (such as MNV-1 and FCV) has shown that less than 1 log reduction in viral titer was achieved by using 200 ppm chlorine to remove viruses from fresh produce (Predmore and Li, 2011). This data demonstrates that enhanced sanitation mechanisms specifically targeting viruses may be necessary in the food industry.

In order to gain a better understanding of the interaction of human NoV and fresh produce, the objectives of this research were to: (i) determine the effect of simple washing with 200 ppm chlorine on the removal of human NoV and its surrogates from Romaine lettuce and green onions and (ii) visualize and compare the localization of human NoV surrogates on fresh produce.

2. Materials and methods

2.1. Viruses and cell culture

Murine norovirus (MNV-1) was generously provided by Dr. Herbert W. Virgin IV, Washington University School of Medicine. Tulane virus (TV) was a generous gift from Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center. MNV-1 and TV were propagated in confluent monolayers of the murine macrophage cell line RAW 264.7 and the monkey kidney cell line MK2-LLC (ATCC, Manassas, VA), respectively. RAW 264.7 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), at 37 °C under a 5% CO_2 atmosphere. For growing MNV-1 stock, confluent RAW 264.7 cells in T-150 flasks were infected with MNV-1 at a multiplicity of infection (MOI) of 0.1. After 1 h incubation at 37 °C, 15 ml of DMEM with 2% FBS was added. The virus was harvested 2 days post inoculation by three freeze-thaw cycles and low speed centrifugation at 1000 ×*g* for 30 min. MK2-LLC cells were cultured in low serum Eagle's minimum essential medium (Opti-MEM, Invitrogen), supplemented with 2% FBS, at 37 °C under a 5% CO_2 atmosphere. For growing TV stock, MK2-LLC cells in T-150 flasks were washed with Hank's balanced salt solution (HBSS) and subsequently infected with TV at an MOI of 0.1. After 1 h incubation at 37 °C, 15 ml of Opti-MEM with 2% FBS was added. The virus was harvested 2 days post inoculation and subjected to three freeze-thaw cycles, followed by centrifugation at 1000 ×*g* for 30 min.

2.2. Characterization of a human norovirus GII.4 strain

Human NoV clinical isolate 5 M was originally isolated from an outbreak of acute gastroenteritis in Ohio. The stool samples were diluted 1:10 in PBS, shaken vigorously for 10 min at 4 °C and centrifuged for 10 min at 5000 ×*g*. The sample was first filtered through a 0.45 μm filter, then a 0.22 μm filter, aliquoted and stored at −80 °C until use. The entire genomic cDNA of the human NoV strain 5 M was amplified by RT-PCR using five to six overlapping fragments. The PCR products were then purified and cloned into a pGEM-T-easy vector (Promega), and sequenced at the Plant Microbe Genetics Facility at The Ohio State University. The full-length genome of the viral isolate was 7558 nt in length and has been deposited into GeneBank at accession number JQ798158. Sequence comparison found that the strain belongs to the norovirus genotype GII.4. The genomic RNA level was then quantified by real-time RT-PCR by targeting VP1 gene and the GII.4 isolate 5 M was found to have 6.7×10^6 genomic RNA copies/ml.

2.3. Production and purification of human NoV virus-like particles (VLPs) in a baculovirus expression system

The capsid VP1 gene of human NoV GII.4 strain was amplified by high fidelity PCR and cloned into a pFastBac-Dual expression vector (Invitrogen) at *Sma* I and *Xho* I sites under the control of the p10 promoter, which resulted in construction of the expression vector, pFastBac-Dual-VP1. The correct insertion of the VP1 gene was confirmed by DNA sequencing. Subsequently, pFastBac-Dual-VP1 was transformed into DH10Bac and the baculovirus expressing VP1 protein was generated by transfection of bacmids into *Spodoptera frugiperda* (Sf9) cells (ATCC no. CRL-1711™, Manassas, VA) using a Cell-fectin Transfection kit (Invitrogen), according to the manufacturer's instructions. Human NoV VLPs were purified from insect cells as previously described with minor modifications (Ma and Li, 2011). Briefly, Sf9 cells in T-150 flasks were infected with baculovirus at a MOI of 10, and the infected Sf9 cells and cell culture supernatants were harvested at 6 days post-inoculation. The VLPs were purified from cell culture supernatants and cell lysates by ultracentrifugation through a 40% (w/v) sucrose cushion, followed by CsCl isoponic gradient (0.39 g/cm^3) ultracentrifugation. Purified VLPs were analyzed by SDS-PAGE, Western blot, and electron microscopy. The protein concentration of the VLPs was determined using Bradford reagent (Sigma Chemical Co., St. Louis, MO).

2.4. Purification of murine norovirus (MNV-1) and Tulane virus (TV)

The purification of MNV-1 and TV was performed using previously described methods with minor modifications (Lou et al., 2011). The virus suspension was centrifuged in a Sorvall SS-34 rotor (Kendro Laboratory Products, Germany) at 8000 ×*g* for 15 min to remove cellular debris. The supernatant was then incubated with DNase I (10 μg/ml) and $MgCl_2$ (5 mM) at room temperature. After 1 h of incubation, 10 mM EDTA and 1% lauryl sarcosine were added to stop nuclease activity. Virus was concentrated by centrifugation at 82,000 ×*g* for 6 h at 4 °C in a Ty 50.2 rotor (Beckman Coulter, Fullerton, CA). The pellet was resuspended in phosphate-buffered saline (PBS) and further purified by centrifugation at 175,000 ×*g* for 6 h at 4 °C through a sucrose gradient (7.5 to 45%) in an SW55 Ti rotor (Beckman). The final virus-containing pellets were resuspended in 100 μl PBS. The virus titer was determined by plaque assay. Viral protein concentration was measured by Bradford reagent (Sigma Chemical Co.) using a Bovine Serum Albumin (BSA) standard curve.

2.5. Biotinylation of MNV-1, TV, and human NoV VLPs

Purified MNV-1, TV, and human NoV VLPs were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions. Briefly,

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