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Internalization and dissemination of human norovirus and Tulane virus in fresh produce is plant dependent

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ABSTRACT

Human norovirus (NoV) is a leading cause of fresh produce associated outbreaks. Previous research indicates that the roots of growing leafy greens and berries internalize human NoV. However the effect of plant type and inoculum level on internalization rates has not been directly compared. In this study we compared the internalization and dissemination rates of human NoV and its surrogate, Tulane virus (TV) in green onion, radishes, and Romaine lettuce. We also evaluated the effect inoculum level and plant growth matrix on the rate of viral internalization. In the hydroponic growth system, we detected internalization and dissemination of human NoV RNA in green onions. In hydroponically growing green onions inoculated with high titer TV, we found higher rates of internalization and dissemination compared to green onions inoculated with low titer TV. In soil growth systems, no infectious TV was detected in either green onion or radishes. However, in Romaine lettuce plants grown in soil approximately 4 $\log_{10}$ PFU/g was recovered from all tissues on day 14 p.i. Overall, we found that the type of plant, growth matrix, and the inoculum level influences the internalization and dissemination of human NoV and TV.

1. Introduction

Human norovirus (NoV) is one of the major causes of acute gastroenteritis worldwide (Allwood et al., 2004; Atmar and Estes, 2006; Dicaprio et al., 2013; Glass et al., 2009; Robilotti et al., 2015). The virus is a leading cause of foodborne illness in the United States, causing nearly 58% of foodborne disease (Dicaprio et al., 2013; Scallan et al., 2011). Fresh produce has been identified as a leading cause of foodborne illness and is a major high risk food associated with human NoV outbreaks in the US (Hall et al., 2013; Li et al., 2012; Lynch et al., 2009). Vegetable row crops (such as leafy greens) and fruits were responsible for 30% and 21%, respectively, of human NoV foodborne outbreaks in the US (2009–2012) (Hall et al., 2012).Vegetables and crops can be contaminated with human NoV at any point from farm to fork. Evidence has shown that the source of pre-harvest contamination mainly comes from soil, fertilizer, or irrigation water. Though human NoV is a major contributor to fresh produce associated outbreaks, the modes of contamination and persistence of the virus in vegetables remains poorly understood.

Human NoV is a non-enveloped single stranded positive sense RNA virus of the family *Caliciviridae* (Robilotti et al., 2015). The major challenge in human NoV research is that currently there is no robust cell culture system for the virus. Recently, two cell culture systems were developed for human NoV, but have not yet been optimized to produce high viral titers (Ettayebi et al., 2016; Jones et al., 2015). Therefore, much of the understanding of human NoV molecular biology, pathogenesis, and environmental stability has come from the study of surrogate viruses (Li et al., 2012; Richards, 2012). Many viruses within the family *Caliciviridae* have been utilized as human NoV surrogates, including murine norovirus (MNV), feline calicivirus (FCV), and Tulane virus (TV) (Cannon et al., 2006; Cromeans et al., 2014; Farkas, 2015; Hirneisen and Kniel, 2013b; Kniel, 2014; Li et al., 2012). Tulane virus (TV) is a newly recognized surrogate for human NoV and is member of the genus

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Abbreviations

NoV	norovirus
TV	Tulane virus
MNV	murine norovirus
FCV	feline 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
RT-qPCR	real time reverse transcriptase polymerase chain reaction

Recovirus within *Caliciviridae* (Farkas et al., 2008). TV was shown to have similar pH stability to human NoV and other surrogates at ranges from pH 3 to pH 8 (Hirneisen and Kniel, 2013b). TV causes enteric infection in primates and also recognizes histo-blood group antigens as a cellular attachment receptor, similar to human NoV (Farkas et al., 2010).

Internalization of pathogens in growing produce is considered one of the potential routes for contamination of fresh produce (Erickson, 2012; Heaton and Jones, 2008; Warriner et al., 2003; Warriner and Namvar, 2010). Bacterial pathogens, such as *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7, have been shown to be internalized in vegetables including lettuce, radishes, alfalfa and green onions (Erickson et al., 2010a,b; Erickson et al., 2010a,b; Franz et al., 2007; Ge et al., 2012, 2014; Golberg et al., 2011; Hora et al., 2005; Jablasone et al., 2005; Kroupitski et al., 2009; Mitra et al., 2009; Pu et al., 2009; Sharma et al., 2009; Warriner et al., 2003). Human NoV has also been shown to be internalized in growing produce such as lettuce, spinach, and green onion (DiCaprio et al., 2015a,b; Dicaprio et al., 2012; DiCaprio et al., 2015a,b; Esseili et al., 2012; Hirneisen and Kniel, 2013a; Wei et al., 2010, 2011). However, few studies exist where multiple types of produce have been evaluated for viral internalization in parallel. In addition, the influence of initial inoculum levels on viral internalization in different types of produce has not been evaluated extensively. In this study, green onion, radishes, and Romaine lettuce were selected to evaluate the effect of growth matrix, inoculum level, and vegetable type on the internalization of human NoV and TV in fresh produce.

2. Materials and methods

2.1. Viruses and cell culture

Human NoV GII.4 strain 707 (GenBank accession number JQ798158) was originally isolated from an outbreak of acute gastroenteritis in Ohio. The virus genomic RNA was quantified by reverse transcriptase quantitative PCR (RT-qPCR) and then stored at −80 °C. TV was generously provided by Xi Jiang at Cincinnati Children's Hospital. TV was propagated in confluent monolayers of the monkey kidney cell line MK2-LLC (ATCC, Manassas, VA). MK2-LLC cells were cultured at 37 °C in a 5% CO_2 atmosphere in low-serum Eagle's minimum essential medium (Opti-MEM; Invitrogen, Carlsbad, CA) supplemented with 2% FBS. Before virus infection, MK2-LLC cells were washed with Hanks' balanced salt solution (HBSS) and subsequently infected with TV at an MOI of 1. After 1 h of incubation at 37 °C, 18 ml of Opti-MEM with 2% FBS was added. The virus was harvested 2 days post infection by three freeze thaw cycles followed by centrifugation at 3000 × g for 10 min to remove cell debris. After centrifugation, the supernatant was collected and virus stocks were stored at −80 °C. TV titer was determined by plaque assay.

2.2. Virus enumeration by plaque assay

TV was quantified by plaque assay in LLC-MK2 cells by seeding the cells into 6-well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of approximately 3×10^6 cells per well. After one day incubation, cell monolayers were infected with 400 μl of a 10-fold dilution series of samples, respectively, and the plates were incubated for 1 h at 37 °C with gentle agitation every 10 min. The cells were then overlaid with 2.5 ml of overlay medium (Eagle's minimum essential medium with 1% agarose, 2% FBS, 1% sodium bicarbonate, 15 mM HEPES, and 100 units/ml penicillin, kanamycin, and streptomycin) (Invitrogen). After incubation at 37 °C for 2 days, the cells were fixed with 10% formaldehyde (Fisher Scientific, Waltman, MA) overnight. The plaques were visualized by staining with 0.05% (wt/vol) crystal violet (Sigma-Aldrich, St. Louis, MO).

2.3. Quantification of human NoV by RT-qPCR

Human NoV RT-qPCR was carried out as previously described (22). Total RNA was extracted from 100 μl of sample using an RNeasy kit (Qiagen, Hilden, Germany), followed by RT-qPCR. First-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) using the primer VP1-P1 (5′- TTATAATA-CACGTCTGCGC CC-3′), which targets the VP1 gene of human NoV. The VP1 gene was then quantified by real-time PCR using custom primers and TaqMan probe (Forward primer, 5′-CACCGCCGG-GAAAATCA-3′, reverse primer, 5′-GCCTTCAGTTGGGAAATTTGG-3′, and reporter, 5′-FAM- ATTTGCAGCAGTCCC NFQ-3′) on a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). TV RNA was also quantified by RT-qPCR. First strand cDNA was synthesized by SuperScriptase III (Invitrogen) using the primer TVRT (5′-AATTCCACCTTCAACCCAAGTG -3′), which targets the VP1 gene of Tulane virus. The VP1 gene was then quantified by real-time PCR using custom primers and TaqMan probe (Forward primer: 5′-TTGCAGGAGGGTTTCAAGATG-3′) (Reverse primer: 5′-CACGGTTT-CATTGTCCCCATA-3′) (Probe: 5′-FAM-TGATGCACACATGTGGGA-NFQ-3′). PCR and cycling parameters followed the manufacturer's protocol (Invitrogen). Briefly, TaqMan Fast Universal Master Mix was used for all reactions. For cycling parameters, a holding stage at 95 °C was maintained for 20 s prior to cycling, followed by 50 cycles of 95 °C for 1 s for annealing and 60 °C for 20 s for extension. A standard plasmid for human NoV and TV was constructed by inserting the sequence of entire ORF2 (VP1 gene) into pGEM T-easy vector (Promega, Madison, WI). The purified plasmids for human NoV and TV with known concentration was then ten-fold serial diluted to generate a standard curve for RT-qPCR. Standard curves and StepOne software version 2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as the mean $\log_{10}$ genomic RNA copies/ml ± standard deviation.

2.4. Plant cultivation

Seeds of green onion (*Allium fistulosum* L.) and radish (*Raphanus sativus*) were purchased from Livingston Seed (Columbus, Ohio) and stored at 4 °C. Before sowing, the seeds were soaked for 1 day at room temperature (20 °C) in a 500 ml beaker of tap water. Subsequently, the water was drained and the seeds were covered with 2 layers of wet Kimwipes (Fisher Scientific, USA) for 2 days to