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

Keywords: human norovirus, fresh produce, attachment, localization, Tulane virus, murine norovirus

47 **Abbreviations:**

48 NoV – norovirus

49 MNV-1 – murine norovirus

50 TV – Tulane virus

51 VLPs – virus-like particles

52 Q-Dots – quantum dots

53 FCV – feline calicivirus

54 CaCV – canine calicivirus

55 CPE – cytopathic effect

56 HBGAs – histo-blood group antigens

57 DMEM – Dulbecco's modified Eagle medium

58 FBS – fetal bovine serum

59 MOI – multiplicity of infection

60 Opti-MEM – low serum Eagle's minimum essential medium

61 HBSS – Hank's balanced salt solution

62 RT-PCR – reverse transcriptase polymerase chain reaction

63 HABA – 4'-hydroxyazobenzene-2-carboxylic acid

64 RT-qPCR – real time reverse transcriptase polymerase chain reaction

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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 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 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% CO₂ 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 $1000 \times g$ 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% CO₂ 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 $1000 \times g$ for 30 min.

2.2. Characterization of a human norovirus GII.4 strain

Human NoV clinical isolate 5M 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 $5,000 \times g$. 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 5M 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 5M 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 isopycnic gradient (0.39 g/cm³) 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 $8,000 \times g$ for 15 min to remove cellular debris. The supernatant was then incubated with DNase I (10 $\mu\text{g/ml}$) 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 $82,000 \times g$ 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 $175,000 \times g$ 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, 1-10mg/ml of protein was incubated with 20-fold molar excess of biotin reagent on ice for 60 min. Following incubation, unbound biotin was removed using a Zebra Desalt Spin Column (Pierce Biotechnology). To confirm biotin binding to the MNV-1, TV, and human NoV VLP capsid protein, the biotinylated sample was subjected to the 4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay, a chromogenic binder of biotin used for quantification, according to the manufacturer's protocol. Briefly, 180 μl of HABA/avidin solution was added to the wells of a 96-well plate and absorbance readings were taken at 500 nm in an ELISA plate reader (Molecular

Devices, Sunnyvale, CA). Next, 20 µl of biotinylated sample was added to the wells containing the HABA/avidin solution and mixed well. After 1 min of incubation at RT, the plate was read at 500 nm and the absorbance value was recorded. The absorbance values for the HABA/avidin solution and the HABA/avidin/biotin solution were used to calculate the level of biotin incorporation correlating to the protein concentration of each sample with calculations based on the Beer Lambert Law. Previously, the infectivity of MNV-1 was not found to be altered following biotinylation (Rawsthorne et al., 2009). Similarly, we found that MNV-1 and TV were infective in cell culture following biotinylation using standard plaque assay. Based on the fact that the biotinylated viruses retained the ability to replicate in cell culture it can be assumed that they also retained the ability to attach to their cellular receptor.

2.6. Detection of biotinylated viruses by Q-Dot and confocal microscope

The imaging of viral attachment on fresh produce was executed using previously described methods (Rawsthorne et al., 2009). Pieces of Romaine lettuce leaves and roots were rinsed with tap water, dried, and then cut into approximately 2 cm squares using a scalpel and placed in the well of a 96-well plate. Next, 100 µl of 10 µg protein/mL of either biotinylated MNV-1, TV, or human NoV VLPs was applied to each lettuce sample and incubated at RT for 2 h. After incubation, samples were washed three times with 1 ml PBS. Samples were then treated with 100 µl of streptavidin coated Q-Dots 655 and incubated at RT for 30 min to allow for binding. Samples were washed three times with 1 ml of PBS to remove unbound Q-Dots. Samples were then transferred to a well-slide

and mounted with a coverslip. Samples were then viewed on an Olympus Spectral Confocal Microscope at The Ohio State Campus Microscope and Imaging Facility.

2.7. Virus enumeration by plaque assay

MNV-1 and TV were quantified by plaque assay in RAW 264.7 and LLC-MK2 cells, respectively. Briefly, cells were seeded into six-well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2×10^6 cells per well. After 24 h incubation, RAW 264.7 and MK2-LLC cell monolayers were infected with 400 μ l of a 10-fold dilution series of MNV-1 or TV, respectively, and the plates were incubated for 1 h at 37°C with gentle agitation every 10 min. The cells were overlaid with 3 ml of Eagle minimum essential medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine. After incubation at 37 °C and 5% CO₂ for 2 days, the plates were fixed in 10% formaldehyde. The plaques were visualized by staining with 0.05% (w/v) crystal violet. Viral titer was expressed as mean log₁₀ plaque forming unit (PFU)/ml $\pm$ standard deviation.

2.8. Enumeration of viral genomic RNA by real time reverse transcriptase PCR (RT-qPCR)

Since human NoV cannot be grown in cell culture, real-time RT-PCR was used to quantify viral genomic RNA copies. Briefly, total RNA was extracted from samples using an RNeasy Kit (Qiagen), followed by reverse transcription and real-time PCR. First strand cDNA was synthesized by SuperScriptase III (Invitrogen) using the primer

VP1-P1 (5'- TTATAATACACGTCTGCGCCC-3'), which targets the VP1 gene of human NoV. The VP1 gene was then quantified by real-time PCR using custom Taqman primers and probes (Forward primer: 5'-CACCGCCGGGAAAATCA-3') (Reverse primer: 5'-GCCTTCAGTTGGGAAATTTGG-3') (Reporter: 5'-FAM-ATTTGCAGCAGTCCC-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). PCR reaction 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 seconds prior to cycling, followed by 50 cycles of 95°C for 15 second for annealing and 60°C for 60 seconds for extension. Standard curves and StepOne Software v2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as mean $\log_{10}$ genomic RNA copies/ml $\pm$ standard deviation.

To compare the attachment-rate between human NoV and TV, 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 TV. The VP1 gene was then quantified by real-time PCR using custom Taqman primers and probes (Forward primer: 5'-TTGCAGGAGGGTTTCAAGATG-3') (Reverse primer: 5'-CACGGTTTCATTGTCCCCATA-3') (Probe: 5'-FAM-TGATGCACACATGTGGGA-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems). PCR reaction, cycling parameters, and quantification method were identical to those used for human NoV.

2.9. Quantification of viral attachment to Romaine lettuce and green onion roots and shoots

Romaine lettuce (*Latuca sativa*) was grown under greenhouse conditions in 4" pots for 30 days post germination. Shoot and roots were then harvested and roots were washed with tap water to remove soil. Following harvest, 1g samples of both roots and shoots were placed in sterile stomacher bags. Green onions (*Allium fistulosum*) were purchased from a local grocery store in Columbus, OH. Roots and shoots were separated, and 1g samples of both roots and shoots were placed in sterile stomacher bags. Lettuce samples were then surface inoculated with either MNV-1 or TV at a titer of 10^7 PFU/g. After the addition of virus, stomacher bags were heat sealed and allowed to incubate at RT for 1 h with gentle rocking to allow for viral attachment. Samples were in constant contact with viral inoculum and remained wet after 1 h incubation. After 1 h incubation, control samples were directly transferred to a new sterile stomacher bag. Samples subjected to washing, had the viral inoculum liquid removed from stomacher bags and then lettuce samples were rinsed 5 times with 2 ml of sterile PBS to wash unattached virus from lettuce tissue. Washed lettuce tissue was then transferred to a new sterile stomacher bag, 5 ml of sterile PBS was added to each sample, and samples were subsequently stomached for 3 min at room temperature. Sample lysate was then extracted from the stomacher bag and transferred to a sterile collection tube. Viral titer in the sample lysate was determined by plaque assay.

To determine the effectiveness of washing on the removal of human NoV from Romaine lettuce and green onion, the same experimental procedure as above was used, but all samples were treated with 1×10^7 RNA copy/g of human NoV strain 5M or 1×10^7

RNA copy/g of TV. Again the control samples received no wash and were transferred directly to new sterile stomacher bag to serve as the baseline for the level of viral attachment. The treated samples were rinsed 5 times with either 2 ml of sterile PBS or 200 ppm chlorine. Following washing, samples were processed as above and quantification of viral genomic RNA was carried out using real time RT-PCR.

2.10. Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed by one-way multiple comparisons using Minitab 16 statistical analysis software (Minitab Inc., State College, PA). A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Quantitative assessment of the level of attachment of human NoV GII.4, TV, and MNV-1 to Romaine lettuce roots and shoots

To determine the effectiveness of washing on the removal of viruses from fresh produce, the human NoV GII.4 strain 5M was inoculated to Romaine lettuce leaves and roots at a level of 1×10^7 RNA copies/g. Control samples received no washing step and treated samples were washed 5 times with either PBS or 200 ppm of chlorine. Following treatment, samples were stomached for 3 min and sample lysate was then used to quantify viral genomic RNA by RT-qPCR.

In Romaine lettuce leaves inoculated with human NoV, the control group had 9.9×10^5 RNA copies/g detected by RT-qPCR (Fig. 1). Washing treatment did not significantly remove human NoV from leaves, with 8.1×10^5 and 7.1×10^5 RNA copies /g

detected for PBS and 200 ppm of chlorine wash, respectively (Fig. 1). The control group for Romaine lettuce roots inoculated with human NoV had a titer of 2.7×10^6 RNA copies/g (Fig. 1). Washing roots with 200 ppm of chlorine did not have significant reduction in the viral genomic RNA detected (2.0×10^6 RNA copies/g) (Fig. 1). In addition, washing roots with PBS only lead to 0.4 log reduction in human NoV RNA, with a titer of 3.5×10^5 RNA copy/g (Fig. 1).

Romaine lettuce shoots and roots were also inoculated with either MNV-1 or TV at a titer of 10^7 PFU/g followed by washing. In Romaine lettuce shoots inoculated with MNV-1, the control had a viral titer of 4.8×10^6 PFU/g after stomaching and the PBS washing treatment had a viral titer of 3.2×10^6 PFU/g (Fig. 2A). There was no statistical difference between these groups ($P > 0.05$). The additional washing treatment with chlorine did not have a significant effect on the viral titer detected. These results demonstrate that washing Romaine lettuce leaves with PBS does not have a significant effect (< 0.5 log reduction) on removing MNV-1. The effectiveness of washing on removal of MNV-1 in roots of lettuce was also determined. Lettuce roots inoculated with 10^7 PFU/g of MNV-1 had a titer of 2.3×10^7 PFU/g, however after washing the titer was 8.7×10^5 PFU/g, which represents 1.8 log virus reduction (Fig. 2A). In contrast to the results of washing lettuce shoots, it appears that simple washing can remove 1-2 logs of MNV-1 from lettuce roots.

Similarly, the effectiveness of washing on removal of TV was determined. Lettuce shoots inoculated with 10^7 PFU/g of TV had titer of 6.2×10^6 PFU/g and after washing with PBS the titer of the shoot was 8.7×10^5 PFU/g (Fig. 2B). In contrast to lettuce shoots inoculated with MNV-1, simple washing with PBS removed approximately

1 log of TV from lettuce shoots (Fig. 2B). In addition to plaque assay, RT-qPCR was used to quantify the amount of TV RNA remaining on lettuce shoots after washing. In the unwashed control lettuce shoots 4.24×10^6 RNA copy/g was detected, washing with PBS and 200ppm chlorine reduced this amount to 8.16×10^5 and 5.47×10^5 RNA copy/g, respectively (Fig. 2C). The reduction in the TV RNA level due to washing was less than 1 log and was similar to human NoV. Lettuce roots inoculated with 10^7 PFU/g of TV had an initial titer of 1.8×10^7 PFU/g and after washing with PBS the titer had decreased to 1.2×10^5 PFU/g (Fig. 2B). Washing reduced to detectable titer of TV in roots by approximately 2 logs in all samples tested. Using RT-qPCR as the detection method, the control lettuce roots had an average RNA level of 4.0×10^6 RNA copy/g and washing with PBS and 200ppm chlorine reduced the RNA level to 2.59×10^6 and 4.03×10^5 RNA copy/g, respectively (Fig. 2C). Taken together, this data demonstrate that (i) commonly used washing strategies are ineffective in removing human NoV and animal caliciviruses from lettuce; and (ii) both MNV-1 and TV were removed from roots more easily than from shoots.

3.2. Quantitative assessment of the level of attachment of human NoV GII.4 and TV to green onion roots and shoots

Human NoV GII.4 strain 5M was inoculated to the green onion leaves and roots at a level of 1×10^7 RNA copies/g. Control samples received no washing step and treated samples were washed 5 times with either PBS or 200 ppm of chlorine. Following treatment, samples were stomached for 3 min and sample lysate was then used to quantify viral genomic RNA by RT-qPCR. Additionally, green onion shoots and roots

were inoculated with either TV at a titer of 10^7 RNA copy/g and processed as described for human NoV.

For human NoV GII.4 the control group, which received no washing, had a viral titer of 1.1×10^7 RNA copies/g remaining on the shoots and 1.7×10^7 RNA copies/g remaining on the roots (Fig. 3). Simple washing with PBS did not have a significant effect on the level of human NoV GII.4 attachment to either the green onion shoots or roots, with 4.2×10^6 RNA copies/g detected in the shoots and 5.8×10^6 RNA copies/g remaining in the roots (Fig. 3). Similarly, washing with 200 ppm chlorine did not enhance the removal of virus from green onion shoots, with 5.7×10^6 RNA copies/g residual virus remaining after washing (Fig. 3). However, washing the green onion roots with 200 ppm chlorine was successful in reducing the RNA copies detected to 4.1×10^5 RNA copies/g, an approximate 1.5 log reduction (Fig. 3).

Using RT-qPCR as the detection method, TV RNA was detected in the unwashed control green onion shoots at a level of 1.57×10^7 RNA copy/g and in the green onion roots at a level of 8.43×10^6 RNA copies/g (Fig. 4). Washing of the green onion shoots with PBS reduced the RNA level to 3.94×10^6 RNA copies/g and 200 ppm chlorine reduced the level to 1.58×10^6 RNA copies/g (Fig. 4). Green onion roots subjected to PBS washing had a TV RNA level of 4.54×10^6 RNA copies/g while 200 ppm chlorine washing reduced the RNA level to 1.66×10^6 RNA copies/g (Fig. 4).

3.3. Localization of MNV-1, TV, and human NoV VLPs to the leaves and roots of Romaine lettuce and green onions

Using a fluorescent streptavidin-labeled Quantum dots (Q-Dots) assay, the localization of MNV-1, TV, and human NoV VLPs was observed on Romaine lettuce leaves at 400 × magnification. Highly purified VLPs were used in these studies as the biotin may adhere to other proteins found in the human NoV clinical isolates and give false positive results. VLPs also retain the cellular receptor binding ability of the native virus, so they serve as a good model to determine human NoV binding to fresh produce. The viruses and VLPs were found to be aggregating in and around the stomata of the lettuce leaves (Fig. 5). MNV-1, TV, and human NoV VLPs can also be seen attaching to the roots of Romaine lettuce and the viruses and VLPs were found covering the entire root surface (Fig. 6).

In addition, the same method was used to visualize the localization of viruses and VLPs to the surface of green onion shoots and roots. Figure 7 shows the localization of human norovirus surrogates in green onion shoots. For VLPs, a very specific pattern can be observed with the signal localizing in between the cells of the green onion epidermis and cell walls (Fig. 7). However, for MNV-1 and TV this pattern was diminished with the viruses being found covering the surface of the green onion cells (Fig. 7). The binding pattern of VLPs to green onion roots was also similar to that seen in the shoots (Fig.8). The VLP signal was found in between the cells of the root epidermis and cell walls (Fig. 8). A similar, but less pronounced pattern was also observed for MNV-1. However, TV was again found to be covering the entire surface of the root cell (Fig.8).

4. Discussion

411 Despite the fact that human NoV accounts for more than 40% of fresh produce-
412 associated outbreaks, the mechanisms of persistence and the interaction with fresh
413 produce are poorly understood. Here, the ability of human NoV and its surrogates to be
414 removed from the surface of fresh produce by simple washing were evaluated. In
415 addition, a fluorescent based Q-Dot assay was developed to visualize the localization of
416 viruses on the surface of Romaine lettuce and green onion. Determining where human
417 NoV is located on the surface of fresh produce and the efficacy of washing on the
418 removal virus from produce can provide insight into high prevalence of outbreaks
419 associated with the virus.

420 In this study, the difficulty in removal of viruses from different portions of lettuce
421 was compared. Overall, all three viruses bound efficiently to fresh produce, with viral
422 recovery from the control samples within 1 log PFU or RNA copy of the inoculum
423 applied. For MNV-1, it was found that simple washing removed less than 0.5 log of
424 virus from lettuce shoots. For TV, simple washing achieved approximately 1 log virus
425 reduction in lettuce shoots. These results indicate that MNV-1 and TV have different
426 affinities for attaching to Romaine lettuce. This could be due to the differences in the
427 receptor binding of these two viruses. MNV-1 is known to bind to sialic acid residues,
428 while TV binds to HBGAs, which is analogous to human NoV. It is possible that
429 Romaine lettuce has more sialic acid analogs than HGBA-like molecules present on the
430 surface. However, for both MNV-1 and TV, the removal of viruses from the roots was
431 much more successful than from the shoots. Washing lettuce roots incubated with MNV-
432 1 or TV lead to 1 and 2 log reductions in infectious virus detection, respectively. It is
433 likely that this may be related to the differences in the physical structures of shoots and

434 roots. It is also possible that the lettuce shoot surface has more exposed carbohydrate
435 moieties that resemble viral cellular receptors that allow for enhanced attachment.

436 Prior to this study, data was limited on the effectiveness of washing on the
437 removal of human NoV from fresh produce. However, it has been shown that 200 ppm
438 chlorine has a limited anti-viral effect on human NoV surrogates, which less than a 0.5
439 $\log_{10}$ reduction following a 5 min contact time (Cromeans et al., 2014). It was found that
440 both chlorine solution and PBS were not effective in removing human NoV RNA from
441 lettuce shoots and roots. Less than 0.4 log RNA copies were removed from shoots and
442 roots due to washing. In contrast, PBS solution was able to remove 1-3 logs and 2-3 logs
443 of infectious MNV-1 and TV from lettuce roots, respectively. This suggests that human
444 NoV may be more difficult to remove from lettuce roots than MNV-1 and TV. However,
445 it was also determined that high levels of TV RNA remained in lettuce after washing,
446 similar to the results obtained for human NoV. This could be due to the increased
447 sensitivity of RT-qPCR compared to cell culture based assays or due to the fact that RT-
448 qPCR cannot discriminate between RNA from infectious or non-infectious viral particle.
449 Therefore, RT-qPCR based methods for determining human NoV persistence in fresh
450 produce may overestimate the actual abundance of infectious virus.

451 It has been a challenge to visualize virus in fresh produce because of their small
452 size and complexity of food matrix. To date, only three studies have successfully
453 visualized the attachment of viruses to fresh produce. Rawsthorne et al., (2009)
454 established a protocol for visualizing MNV-1 and hepatitis A virus in green onions
455 utilizing purified virus conjugated with biotin and streptavidin-coated Q-Dots. It was
456 found that both viruses attached to the surface of green onions (Rawsthorne et al., 2009).

In another study, human NoV VLPs were found to be localized along the veins and cut edges of both the lettuce and cilantro (Gandhi et al., 2010). Esseili et al., (2012) evaluated the attachment human NoV VLPs in Romaine lettuce using human NoV VP1 antibody staining, followed by confocal microscopy. It was found that human NoV VLPs were attaching to the surface of Romaine lettuce, and human NoV VLPs were aggregating in stomata and along cut edges of the lettuce (Esseili, Wang and Saif, 2012). To date, direct comparison of viruses binding to different fresh produce has not been reported.

In this study, we compared the binding pattern of MNV-1, TV, and human NoV VLPs to two different types of fresh produce, lettuce and green onion. It was found that all three viruses exhibited similar binding patterns to the leaves and roots of lettuce after washing. Under higher magnification, it was found that all surrogates aggregated in and around the stomata on the lettuce leaves. This was similar to results observed by other groups investigated VLP localization on lettuce (Esseili, Wang and Saif, 2012). The localization of signal in and around the stomata indicates that the viruses and VLPs were either binding more specifically to these structures, that the natural contours of the leaf lead to an increase in the localization of viruses and VLPs in the areas on the leaves where stomata are found, or that ionic interactions between the virus capsid and other charged particles on the leaf surface contributes to the aggregation of viruses in that area. The presence of viruses and VLPs in stomata indicates that they may be protected from removal due to physical features of the plants. The presence of these surrogates in the stomata may also pose a risk for the potential internalization of viruses via these pores in the aerial portions of the plant. However, the three viruses reported in this study were

found to behave differently in green onions. Human NoV VLPs were observed aggregating between the epidermal cells and cell walls of the green onion surface whereas MNV-1 and TV were found to be attaching to the surface of green onion cells. Previously, others have also shown the MNV-1 and HAV attached to the surface of green onions (Rawsthorne et al., 2009). To our knowledge, this is the first evidence that different viruses have varying binding patterns depending on the variety of fresh produce. This may also contribute to the high levels of human NoV 5M RNA detected in green onion both before and after washing treatment. It is possible the human NoV is attaching or localizing in these regions, which allows for the accumulation of the virus at a higher level and also confer protection from washing.

In order to develop strategies to eliminate viruses from fresh produce, the interaction between the virus and the fresh produce must be established. Several possibilities could account for the difficulty in the removal of viruses from fresh produce. First, the virus could be binding specifically to the fresh produce, if the surface of the produce contained moieties that closely resemble the viral cellular receptor (Esseili, Wang and Saif, 2012; Gandhi et al., 2010; Wei et al., 2010). Another possibility is that the virus could be binding non-specifically to the fresh produce due to ionic interactions of the protein capsid and the surface of the fresh produce (Tian et al., 2011; Vega et al., 2008; Vega et al., 2005). Third, the persistence of viruses in produce could be due to the small size of the virus, which would allow them to enter small crevices and spaces on the produce surface and hence be protected from removal or inactivation (Wei et al., 2010). Finally, viruses may be internalized via roots and/or leaves, and disseminated to other portions of the plants which would render traditional sanitization

strategies ineffective against internalized viruses (DiCaprio et al., 2015; Dicaprio et al., 2012; Esseili, Wang, Zhang, et al., 2012; Hirneisen and Kniel, 2013; Wei et al., 2011).

Although the physical characteristics of the vegetable seems to be playing an important role in protecting the viruses from removal, specific and non-specific binding of the viruses to the produce is still likely to influence attachment. Carbohydrate moieties are abundant in fresh produce, and carbohydrates constitute 90% (dry weight basis) of the plant cell wall (Esseili, Wang and Saif, 2012). Recent evidence suggests that HBGA-like molecules may play a role in binding of human NoV to fresh produce. Gandhi et al., (2010) found that human NoV VLPs bound to extracts of Romaine lettuce, cilantro, iceberg lettuce, and spinach, and VLP binding to Romaine lettuce was the highest compared to the other produce. Similarly, Esseili et al., (2012) investigated the attachment of human NoV VLPs to specific parts of the lettuce leaf and also to cell wall material using an ELISA. It was observed that boiling the lettuce extracts prior to ELISA to denature proteins had little to no effect on the level of human NoV binding, indicating that proteins may not be involved in binding. However, pre-treatment with sodium periodate to oxidize carbohydrates did decrease specific human NoV VLP binding to the lettuce extracts. These results indicate that specific binding to carbohydrate moieties, similar to the HBGAs, may play a role in the binding of human NoV VLPs to Romaine lettuce (Esseili, Wang and Saif, 2012).

In summary, we demonstrated that (i) a human NoV GII.4 strain attached efficiently to both Romaine lettuce leaves and roots and green onion shoots and roots, and that simple washing with PBS or 200 ppm chlorine did not remove the virus from the shoots and roots; (ii) human NoV surrogates (MNV-1 and TV) were more difficult to

remove from lettuce leaves than roots; and (iii) different viruses appear to have independent binding patterns in fresh produce. Understanding the attachment mechanisms of human NoV to fresh produce will facilitate the development of specific control measures to eliminate viral hazards in fresh produce. Future work should focus on methods to enhance viral removal from fresh produce during washing, such as using surfactants. Also, determining whether human NoV can bind specifically to plant carbohydrates would help explain the difficulty in removing viruses from fresh produce.

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FIGURE LEGENDS:

FIG 1. Effect of washing on the removal of human NoV GII.4 from Romaine lettuce leaves and roots. 1 g samples of Romaine lettuce leaves and roots were inoculated with human NoV GII.4 stock to achieve a titer of 1.0×10^7 RNA copy/g of lettuce tissue. Samples were incubated for 1 h at room temperature with gentle agitation. Fresh produce samples were washed 5 times with either PBS or 200ppm chlorine, and the samples were stomached. The level of human NoV GII.4 RNA was determined by RT-qPCR and is expressed as log₁₀ RNA copy/g. Bars are the average of 3 replicates. Error bars represent +/- 1 standard deviation.

FIG 2. Effect of washing on the removal of MNV-1 (A) and TV (B, C) from Romaine lettuce leaves and roots. (A) Quantification of MNV-1 by plaque assay. 1 g samples of Romaine lettuce leaves and roots were inoculated with MNV-1 to achieve a titer of 1.0×10^7 PFU/g of lettuce tissue. Samples were incubated for 1 h at room temperature with gentle agitation. Fresh produce samples were washed 5 times with either PBS or 200 ppm chlorine, samples were stomached, and the remaining virus was quantified by plaque assay. Data shown was expressed as log₁₀ PFU/g. Bars are the average of 3 replicates. Error bars represent +/- 1 standard deviation. **(B) Quantification of TV by plaque assay.** The method was identical to panel A except the virus was changed to TV. **(C) Quantification of TV by RT-qPCR.** Data shown was expressed as log₁₀ RNA copy/g. Bars are the average of 3 replicates. Error bars represent +/- 1 standard deviation.

FIG 3. Effect of washing on the removal of human NoV GII.4 from green onion shoots and roots. 1 g samples of green onion shoots and roots were inoculated with human NoV GII.4 stock to achieve a titer of 1.0×10^7 RNA copy/g of lettuce tissue. Samples were incubated for 1 h at room temperature with gentle agitation. Samples were washed 5 times with either PBS or 200 ppm chlorine, and were stomached. Human NoV GII.4 RNA was quantified by RT-qPCR and is expressed as log₁₀ RNA copy/g. Bars are the average of 3 replicates. Error bars represent +/- 1 standard deviation.

FIG 4. Effect of washing on the removal of TV from green onion shoots and roots.

1 g samples of green onion shoots and roots were inoculated with TV to achieve a titer of 1.0×10^7 RNA copy/g of lettuce tissue. Samples were incubated for 1 h at room temperature with gentle agitation. Samples were washed 5 times with either PBS or 200 ppm chlorine, and were stomached. TV RNA was quantified by RT-qPCR and is expressed as log₁₀ RNA copy/g. Bars are the average of 3 replicates. Error bars represent +/- 1 standard deviation.

FIG 5. Localization of human NoV VLPs, TV, and MNV-1 on Romaine lettuce

leaves. Purified MNV-1, TV, and human NoV VLPs were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit. Pieces of Romaine lettuce leaves were cut into approximately 2 cm squares, inoculated with 100 µl of 10 µg protein/mL of either biotinylated MNV-1, TV or human NoV VLPs, and were incubated at RT for 2 h. After incubation, samples were washed three times with 1 ml PBS. Samples were then treated with 100 µl of streptavidin coated Q-Dots 655 and incubated at RT for 30 min to allow for binding. Samples were washed three times with 1 ml of PBS to remove unbound Q-Dots. Samples were then transferred to a well-slide and mounted with a coverslip. Samples were then viewed on an Olympus Spectral Confocal Microscope at The Ohio State Campus Microscope and Imaging Facility. Red signals indicate virus particles.

FIG 6. Localization of human NoV VLPs, TV, and MNV-1 on Romaine lettuce

roots. Romaine lettuce roots were contaminated with 100 µl of 10 µg protein/mL of

biotinylated MNV-1, TV or human NoV VLPs, washed, and virus particles were visualized by Q-Dots assay and confocal microscope.

FIG 7. Localization of human NoV VLPs, TV, and MNV-1 on green onion shoots.

Green onion shoots were contaminated with 100 µl of 10 µg protein/mL of biotinylated MNV-1, TV or human NoV VLPs, washed, and virus particles were visualized by Q-Dots assay and confocal microscope.

FIG 8. Localization of human NoV VLPs, TV, and MNV-1 on green onion roots.

Green onion roots were contaminated with 100 µl of 10 µg protein/mL of biotinylated MNV-1, TV or human NoV VLPs, washed, and virus particles were visualized by Q-Dots assay and confocal microscope.

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 691
 692

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Fig.5

Fig. 6

**Root
control**

**Root
MNV-1**

**Root
TV**

**Root
VLPs**

Figure 7

Fig.7

Fig.8