Attachment, Internalization, and Dissemination of Human Norovirus and Animal Caliciviruses in Fresh Produce

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Erin Leigh DiCaprio, B.S.

Graduate Program in Food Science and Technology

The Ohio State University

2012

Master's Examination Committee:

Dr. Jianrong Li, Advisor

Dr. Yael Vodovotz

Dr. Ken Lee

Abstract

Fresh produce is a high risk food for human norovirus (NoV) contamination, because it can easily become contaminated at both the pre- and post-harvest stages of cultivation. Disease surveillance has shown that human NoV is attributed to 40% of all fresh produce related outbreaks reported each year in the U.S. However, the ecology, persistence, and interaction of human NoV and fresh produce are all poorly understood. Increasing outbreaks of viruses in fresh and fresh-cut vegetables and fruits give high urgency to understanding the interaction of human NoV with fresh produce in order to develop effective preventive measures. In this research, the attachment, uptake, internalization, and dissemination of human NoV and its surrogates (murine norovirus, MNV-1; and Tulane virus, TV) were evaluated.

First, the attachment of human NoV surrogates to fresh produce was visualized using confocal microscopy. Purified human NoV virus-like particles (VLPs), TV, and MNV-1 were conjugated with biotin, and subsequently applied to either Romaine lettuce or green onion. The biotinylated virus particles were visualized by incubation with streptavidin coated Quantum Dots (Q-Dots 655), which emit fluorescence that can be viewed using a confocal microscope. It was found that all three surrogates attached to the surface of Romaine lettuce leaves and were found aggregating in and around the stomata.

Similarly, human NoV VLPs, TV, and MNV-1 were found to attach to the surface of Romaine lettuce roots. In the case of green onions, human NoV VLPs were found between the cells of the epidermis 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. The results indicate that different viruses vary in their attachment patterns to different varieties of fresh produce.

A quantitative assessment of the level of attachment of a human NoV GII.4 strain, TV, and MNV-1 was executed using Romaine lettuce as a model system. Romaine lettuce roots and shoots were inoculated with varying levels of TV and MNV-1 and then washed with PBS to remove unattached viruses. It was found that simple washing removed less than 1 log of viruses from the shoots and 1-4 log of viruses from the roots, demonstrating that TV and MNV-1 bound more efficiently to Romaine lettuce leaves than to the roots. A human NoV GII.4 strain was inoculated at a level of 1×10^7 RNA copies/g to Romaine lettuce leaves and roots, and then washed with either PBS or 200ppm of chlorine. The human NoV GII.4 strain was found to attach similarly the both the Romaine lettuce leaves and roots, and that washing with 200ppm of chlorine removed less than 1 log of viral RNA copies from the tissues. The results demonstrate that different viruses attach differently to Romaine lettuce, and that washing is ineffective in removing viral contamination from fresh produce.

Next, it was determined whether human NoV and its surrogates could be internalized via roots and disseminated to edible portions of the plant. The roots of Romaine lettuce growing in hydroponic feed water were inoculated with 1×10^6RNA

copies/ml of human NoV GII.4 strain or 1-2×10^6PFU/mL of human NoV surrogates(TVand MNV-1), and plants were allowed to grow for 2 weeks. Leaves, shoots, and roots were harvested at days 0, 1, 3, 7, and 14 after virus inoculation. The plant tissues were homogenized and viral titers and/or RNA were determined by plaque assay and/or real-time RT-PCR. For human NoV, high levels of viral genome RNA (10^5-10^6 RNA copies/g) were detected in leaves, shoots, and roots at day 1 post-inoculation and remained stable over the 14 day study period. For MNV-1 and TV, relatively low levels of infectious virus particles (10^1-10^3 PFU/ml) were detected in leaves and shoots at days 1 and 2 post-inoculation, but reached a peak titer (10^5-10^6 PFU/g) at days 3 or 7 post-inoculation. In addition, human NoV had a rate of internalization comparable with TV as determined by real-time RT-PCR, whereas, TV was more efficiently internalized than MNV-1 as determined by plaque assay. To further confirm the viral internalization via lettuce roots, an identical experiment was performed with the exception that the harvested plant tissues were submerged in 50 ml of 1000 ppm chlorine for 5 min to eliminate any possible viral contaminations. The results showed that there were no significant differences observed in viral internalization in chlorine treated shoots and leaves on any of the study days compared to the untreated samples ($P>0.05$) during the experimental period. Taken together, these results demonstrated that human NoV and animal caliciviruses attached tightly to roots, became internalized via roots, and efficiently disseminated to the shoots and leaves of the lettuce.

In summary, this research elucidates a major gap in our understanding of the ecology of human NoV in fresh produce, specifically, our understanding of the fate of

human NoV after attaching to roots of growing lettuce. Elucidation of the mechanism of virus-plant interaction will facilitate the development of novel interventions to prevent viral attachment and internalization in plants.

Acknowledgments

I would like to thank my advisor Dr. Jianrong Li for his support and guidance in the execution of my research. I appreciate him giving me the opportunity to advance my education and for fostering my ability to think critically. I also extend my gratitude to the members of Dr. Li's research group who have been gracious in sharing their knowledge and expertise, as well as their kindness and humor, during my time here. The members of Dr. Li's laboratory include: Elbashir Araud, Dr. Hui Cai, Yue Duan, Dr. Junan Li, Fangfei Lou, Yuanmei Ma, Ana Puriganto, Dr. Yongwei Wei, and Yu Zhang. I would also like thank the other graduate students working in the food virology laboratory for their help and friendship throughout my studies. I would like to thank Dr. Vodovotz and Dr. Lee for acting as my committee members, their time and commitment is greatly appreciated.

I would like to thank my husband James for his unwavering confidence and support in my abilities. I would also like to thank my in-laws, Dr. Ralph DiCaprio and Dr. Laura DiCaprio, for their critical reading of my manuscript and thesis, and of course their love and support. Finally, I would like to thank my parents, Gavin and Pam Divers, and my brother Mitchell for their lifelong love and for always believing in me.

Vita

2001……Jefferson Forest High School

2005……B.S. Biology, Virginia Tech

2005……Corporate Reaction Team, Charles River Laboratories, Germantown, MD

2006-2007……...Lab Assistant, Novozymes Biologicals, Salem, VA

2007-2008……...Research Associate, Center for Gene Therapy, Research Institute at Nationwide Children's Hospital, Columbus, OH

2008-2009……...Microbiology Technician, Battelle Memorial Institute, Columbus, OH

2009-2010……...Medical Laboratory Technician, The Ohio State Medical Center, Columbus, OH

2010-present……....Graduate Research Associate, Department of Food Science and Technology, The Ohio State University, Columbus, OH

Publications

Erin DiCaprio, Anastasia Purgianto, John Hughes, and Jianrong Li. Attachment, internalization, and dissemination of human norovirus and animal caliciviruses in hydroponically grown Romaine lettuce. Applied and Environmental Microbiology. Submitted on April 04, 2012, under review.

Jianrong Li, Ashley Predmore, Fangfei Lou, and Erin Divers. Novel interventions against human norovirus: progress, opportunities, and challenges. Annual Review of Food Science and Technology 2012. 3:331-52.

Kurtis Feng, Erin Divers, Yuanmei Ma, and Jianrong Li. Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation. Applied and Environmental Microbiology 2011. 77(10):3507-17.

Killedar S., Julianne Rosario, Erin Divers, P.G. Popovich, Douglas M. McCarty, and Haiyan Fu. Mucopolysaccharidosis IIIB, a lysosomal storage disease, triggers a pathogenic CNS autoimmune response. Journal of Neuroinflammation 2010. 16:7.39.

Julianne Rosario, Erin Divers, C. Want, Jonathan Etter, Alyson Charrier, P. Jukkola, H. Auer, V. Best, D.L. Newsom, Douglas M. McCarty, and Haiyan Fu. Innate and adaptive immune activation in the brain of MPS IIIB mouse model. Journal of Neuroscience Research 2009. 87(4):978-90.

Fields of Study

Major Field: Food Science and Technology

Table of Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

ABBREVIATIONS

ANOVA – Analysis of variance
CaCV – Canine calicivirus
CDC – Centers for Disease Control and Prevention
CPE – Cytopathic effect
DMEM – Dulbecco's modified eagle medium
DNA – deoxyribonucleic acid
ELISA – Enzyme-linked immunosorbant assay
EM – Electron Microscopy
FBS – Fetal Bovine Serum
FCV – Feline calicivirus
FDA – Food and Drug Administration
HBGA – Histo-blood group antigen
Human NoV – human norovirus
LLC-PK – porcine renal epithelial cell line
MEM – Modified eagle medium
MK2-LLC – African green monkey kidney cell line
MNV-1 – Murine norovirus
MOI – Multiplicity of infection
NIAID – National Institute of Allergy and Infectious Disease
ORF – Open reading frame
PCR – Polymerase chain reaction
PBS – Phosphate buffered saline
PFU – Plaque forming unit
Ppm – Parts per million
qDot – Quantum dot
qPCR – Quantative polymerase chain reaction
RAW 264.7 – Mouse macrophage cell line
RdRp – RNA dependent RNA polymerase
RNA – Ribonucleic acid
RT-PCR – Reverse transcriptase polymerase chain reaction
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf9 – insect cell line
STAT-1 – Signal transducer and activator of transcription 1

TEM – Transmission electron microscopy
TV – Tulane virus
VLP – Virus-like particle
WHO – World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Traditionally the study of food safety has focused solely on the role of bacterial pathogens in causing food-borne disease. In recent years the importance of viruses as a cause of food-borne disease has been increasingly appreciated. Of the viruses commonly associated with food-borne disease, human norovirus (NoV) is decidedly the most important, accounting for greater than 50% of the food-borne illness reported every year (Fig. 1) (CDC, 2010,Atmar et al., 2008). The most recent data from the Centers of Disease Control (CDC) indicates that human NoV is responsible for over 23 million cases of illness annually, causing 95% of all non-bacterial gastroenteritis (CDC, 2010).

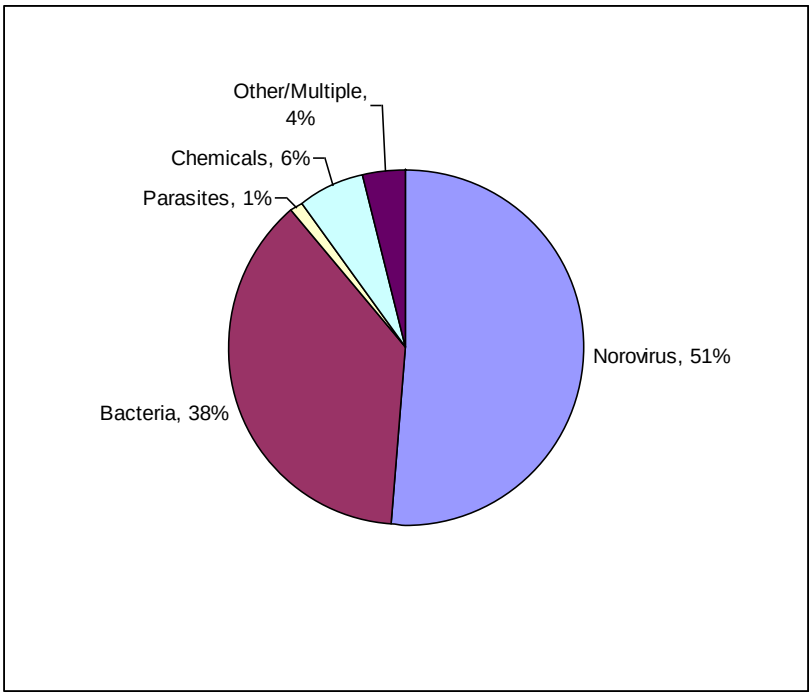


Figure 1. Known causes of foodborne illness outbreaks, U.S., 2006-2008 (Adapted from CDC, 2010)

Human NoV food-borne disease is commonly associated with foods that undergo little or no processing, or ready to eat foods in which the food handler may unknowingly transfer the virus to the foods they are preparing. Seafood such as shellfish, most notably oysters, mussels, and clams, and fresh produce, such as leafy greens and berries, account for much of the norovirus disease documented each year. Norovirus accounts for 40% of food-borne illness associated with fresh produce (Fig. 2) (DeWaal and Bhuiya, 2007). There is an urgent need to understand the virus-plant interactions in order to minimize the risk to public health.

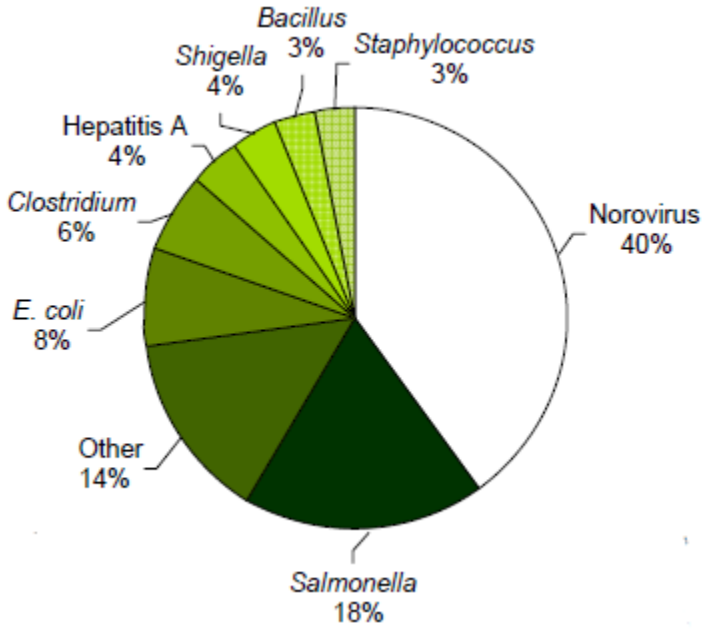


Figure 2. Respective percentages of pathogens linked to disease outbreaks due to fresh produce consumption between 1990-2005 (Adapted from DeWaal and Bhuiya, 2007)

Produce may become contaminated with human NoV in many steps during production and processing. Contaminated irrigation water may distribute norovirus on plant tissues, but there is little research on whether viral contaminants in the water supply can be internalized and disseminated to other plant tissues. Internalization may occur through direct contact with the root, by entry through natural openings in the aerial tissues of the plants, such as stomata, or through damaged plant tissues. Internalized

human NoV would be protected from all disinfectant procedures and therefore contaminated produce would never be rendered free of the pathogen. There is also little information about the interaction betweem human NoV and the surface of fresh produce. It is possible that the physical features of the plant protect the virus from removal or that the virus attaches to plant surface moieties making it difficult to remove by simple washing processes.

The purpose of this research is to determine (i) the attachment profile of human NoV and its surrogates to fresh produce, and (ii) to determine whether human NoV and its surrogates can be internalized via the roots and disseminated to the edible portion of growing plant tissues. This research will increase the understanding of enteric virus-plant interactions leading to targeted control measures during food production.

1.2. Overview of foodborne viruses

Viruses transmitted by food are defined as food-borne viruses. They include viruses from many families, however these viruses share many common characteristics. The main unifying trait of food-borne viruses is that the are non-enveloped viruses, lacking a lipid envelope. Non-enveloped viruses, in general, are more resistant to heat, pH, drying, and organic solvents than enveloped viruses. This environmental stability allows the non-enveloped viruses to be maintained in the food for long periods of time and to survive the acidic conditions found in the digestive tract. The stability of non-enveloped viruses also makes them more resistant to common sanitation methods and food processing technologies. A summary of food-borne viruses can be found in Table 1.

Table 1. Summary of the major foodborne viruses.

Virus	Genome	Envelope	Disease
Norovirus	+ssRNA	NO	Gastroenteritis
Adenovirus	dsDNA	NO	Gastroenteritis
Rotavirus	dsRNA	NO	Gastroenteritis
Sapovirus	+ssRNA	NO	Gastroenteritis
Astrovirus	+ssRNA	NO	Gastroenteritis
Aichivirus	+ssRNA	NO	Gastroenteritis
Hepatitis A	+ssRNA	NO	Jaundice, Hepatitis, Gastroenteritis
Hepatitis E	+ssRNA	NO	Juandice, Hepatitis, Gastroenteritis
Polio	+ssRNA	NO	Poliomyelitis

Note: +ssRNA: single-stranded positive-sense RNA virus; dsDNA: double-stranded RNA virus; dsRNA: double-stranded RNA viruses

1.2.1. Caliciviruses

Human NoV and human sapovirus are members of the family *Caliciviridae*. They have a single-stranded positive sense RNA genome, no envelope, and their capsid exhibits icoshedral symmetry. The transmission mode of members of this family is typically the fecal-oral route, but foods, hands, and fomites may carry the virus.

Norovirus has also been found to be transmitted by aerosolized vomitus or stool. The disease caused by these viruses is acute gastroenteritis, characterized by extreme nausea, vomiting, and diarrhea. There are no vaccines or anti-viral agents available to combat these viruses.

1.2.2. Adenovirus

Adenovirus is a member of the family *Adenoviridae*. It has a double-stranded DNA genome, which makes it the only food-borne virus with DNA as its genetic material. It has no envelope and exhibits icosahedral symmetry of its capsid. The transmission mode of adenovirus is typically fecal-oral, but it can also be transmitted through the respiratory tract or eyes. It is a latent virus, which means during latency it survives in the body without producing symptoms. The virus begins to replicate again during periods when the immune system is compromised, and disease symptoms return. Adenovirus causes gastroenteritis in children, but in adults it can cause disease in the respiratory tract and eyes. Currently there is a vaccine available for serotypes 4 and 7, but it is only approved for military use.

1.2.3. Rotavirus

Rotavirus is a member of the family *Reoviridae*. It has a double-stranded RNA genome which is composed of 11 segments. Rotavirus has no envelope and its capsid has icosahedral symmetry. The common transmission route is fecal-oral, but fomites are often found to harbor and then disseminate the virus. Rotavirus causes gastroenteritis

primarily in children aged 6 months to 2 years old; most people above this age have already developed immunity. Dehydration caused by diarrhea often leads to death, so a vaccine has been developed for this virus and is recommended for children at 2 months of age.

1.2.4. Hepatitis E virus

Hepatitis E virus is a member of the family *Hepeviridae*. It has a single-stranded positive sense RNA genome, no envelope, and its capsid has icosahedral symmetry. The transmission mode of hepatitis E virus is through the fecal-oral route. The virus causes gastroenteritis, liver damage, and jaundice. Hepatitis E virus infection has a high mortally rate among pregnant women, typically ranging from 10-20%. Hepatitis E virus causes sporadic disease in developed countries, but may be endemic and epidemic in some undeveloped countries where water and food sanitation practices are poor.

1.2.5. Picornaviruses

Polio virus, Aichivirus, and hepatitis A virus are members of the family *Picornaviridae*. They have a single-stranded RNA genome, no envelope, and a capsid with icosahedral symmetry. The transmission mode for all three viruses is the fecal-oral route. Polio virus transmission is commonly associated with drinking water that has been contaminated with fecal material. Aichivirus and hepatitis A virus are typically transmitted by food, although water is also a major transmission vehicle. Polio virus infection can lead to the disease poliomyelitis, which is a neuro-degenerative disease

causing muscle necrosis in severe cases. While polio outbreaks are rare in the U.S., it is still a significant problem in some developing countries. Aichivirus causes gastroenteritis. Hepatitis A virus causes gastroenteritis, live damage, and jaundice. There are vaccines available for both polio virus and hepatitis A virus.

1.2.6. Avian influenza virus

As Table 1 illustrates, none of the food-borne viruses have an envelope. There is controversy over whether avian influenza, an enveloped virus, should be characterized as a food-borne virus. While avian influenza is decidedly an important public health concern, influenza viruses are commonly spread by aerosols, not foods. Influenza viruses cause respiratory disease and are delivered to their host tissues predominantly through the nasal passages. Most enveloped viruses are not capable of surviving the acidic environment found in the stomach, which means the viruses become uninfectious if ingested. However, there are some isolated occurrences of people exhibiting an influenza infection after ingestion of under processed duck blood and animal meat (Loth et al., 2010). More research needs to be executed to determine if avian influenza should be characterized as a food borne virus.

1.3. Human NoV

Human NoV causes severe gastroenteritis characterized by vomiting, diarrhea, and stomach cramps. Vomiting is seen more commonly in children, while adults usually present with diarrhea. The diarrhea associated with the disease is free of blood, mucus,

and leukocytes (Glass et al., 2000). This differentiates norovirus associated diarrhea from diarrhea caused by bacterial pathogens such as *E. coli* O157:H7 where blood appears in the stools. The incubation period for the disease is usually 10-51 hours and the duration of the disease is 28-60 hours (Glass et al., 2000). Norovirus affects people of all ages and usually does not require hospitalization. However, severe disease may be observed in children, the elderly, or immunocompromised individuals, all of whom may require supportive care. Norovirus outbreaks seem to have no clear seasonality, but more cases are reported in the winter months.

The modes of transmission of human NoV are the fecal-oral route, through contaminated food, fomites, water, or aerosolized vomitus. The infectious dose of human NoV is very low, usually reported as <10 viral particles. A recent publication based on human volunteer studies and mathematical modeling, estimates that the probability of infection of a single human NoV particle is 0.5 viral (Teunis et al., 2008). Human NoV is shed in the stool of infected individuals and viral shedding peaks 1-3 days after infection. However, viral shedding has been reported up to 56 days post infection (Atmar et al., 2008). Approximately one-third of human NoV infected individuals are asymptomatic but actively shed the virus (CDC, 2011).

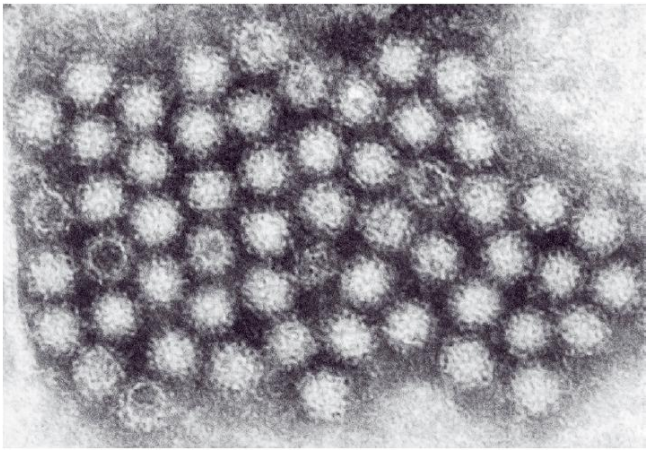


Figure 3. Transmission electron microscope image of human norovirus (Adapted from CDC, 2011).

The first documented human NoV outbreak occurred in 1968 in the town of Norwalk, Ohio. In 1972 the virus was officially identified using electron microscopy. The virus was termed Norwalk-like virus or small- round structured virus (SRSV). Human NoV is also commonly called the stomach flu or the cruise-ship disease because of its symptoms and its most commonly associated outbreak location, respectively. Human NoV is a member of genus *Norovirus* within the family *Caliciviridae*. The *Norovirus* genus is further subdivided into five genogroups (Fig. 4), GI-GV, with GI, GII, and GIV causing human disease. GIII are bovine noroviruses and GV includes murine norovirus. The genogroups are further divided into genotypes based on genome or viral capsid gene sequence. There are a total of 19 genotypes assigned to GII human NoV. Currently, the most prevalent human NoV belongs to genogroup II, genotype 4 (GII.4).

In the past ten years, more than three global pandemics have occurred, all of which were due to strains of GII.4 (Green et al., 2007).

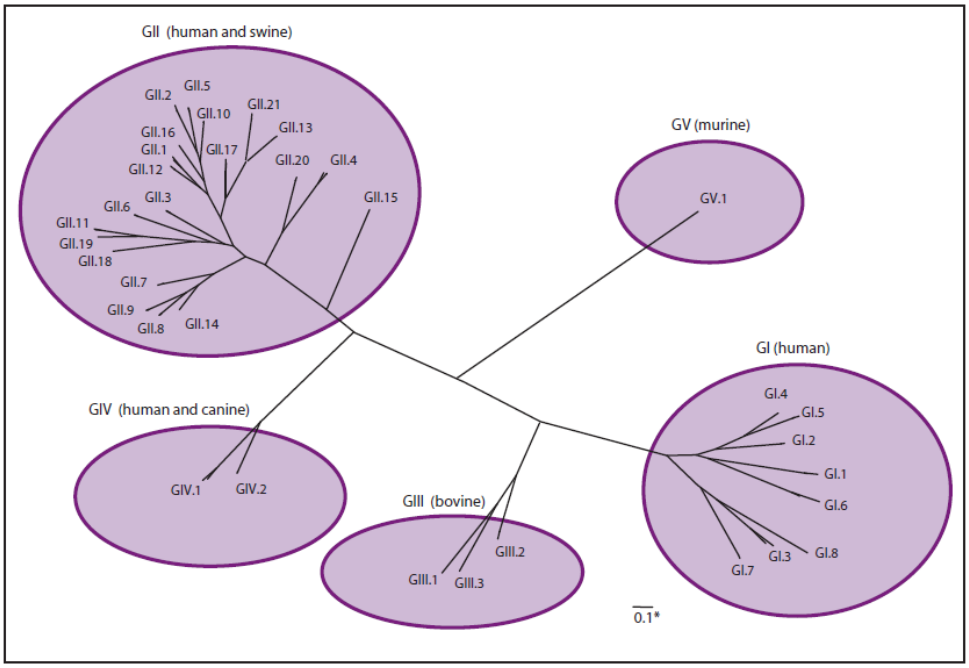


Figure 4. Norovirus genogroup and genotype characterization based on sequence homology of the VP1 (major capsid protein) gene (Adapted from CDC, 2011).

It has long been debated whether long-term immunity is acquired after human NoV infection. Data is limited to a few volunteer studies involving just a few human NoV strains. It is thought that the diversity between strains of human NoV plays an integral part in its evasion of the immune system. Even closely related strains of human NoV

show major antigenic and receptor binding differences. Host susceptibility also plays an important role in human NoV infections. Early volunteer studies with human NoV strain GI.1 found that some individuals did not show symptoms of disease after exposure to the virus (Donaldson et al., 2008). Recent studies have shown the individuals with blood type O are more susceptible to GI.1 strain infections than people with other blood types. It has been established that human NoV attaches to histo-blood group antigens (HBGAs), which include A, B, H, and Lewis antigens (De Rougemont et al., 2011). HGBAs are found on erythrocytes and on epithelial cells, and as well as in some body secretions such as saliva. Hence, an individual's blood type and secretor/non-secretor status plays a role in susceptibility to infection with particular human NoV strains.

1.3.1. Human NoV genome

Human NoV has a single-stranded positive sense RNA genome. The genome is approximately 7.7 kb and has three open reading frames (ORF) (Fig. 5). The genome has a protein linked to the 5' end and is polyadenylated at the 3' end. ORF1 encodes the non-structural proteins, ORF2 encodes the major capsid protein VP1, and ORF3 encodes the minor capsid protein VP2. The order of genes in ORF1 is p48, NTPase, p22, VPg, $3CL^{pro}$, and RNA dependent RNA polymerase (RdRp) (Hardy, 2005). The function of many of these proteins has been deciphered by homologies found with viral and cellular proteins found in public databases.

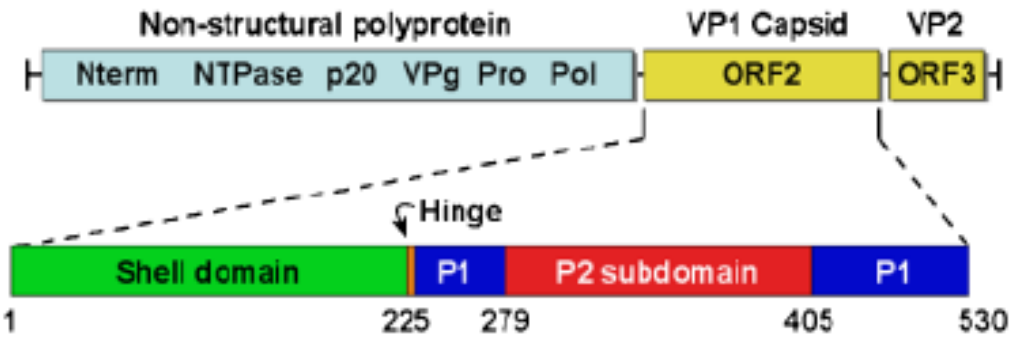


Figure 5. Human NoV genomic structure and capsid domains (Donaldson et al., 2008).

1.3.2. Human NoV capsid proteins, VP1 and VP2

The capsid of norovirus is made up of 90 dimers of the major capsid protein VP1 and one or two copies of the minor capsid protein VP2. Both VP1 and VP2 are encoded by a protein-linked subgenomic RNA that contains both ORF2 and ORF3. VP1 is composed of ~530-555 amino acids with a molecular weight that ranges from 58-60 kDa. There is a central variable region that is likely involved in strain specificity which is flanked on either side by two conserved domains. X-ray crystallography has revealed that the capsid contains 180 copies of VP1 arranged to form a T=3 icosahedral virion. VP1 folds to form two domains, shell (S) and protrusion (P), linked by a flexible hinge region (Fig. 6) (Hardy 2005). S domain is comprised of the N-terminal 225 amino acids of VP1. The S domain is important in the formation of the icosahedral structure. The P domain is made up of the remaining amino acids of VP1 and is further divided into P1

and P2 (Prasad et al., 1999). P2 is highly variable and plays an important role in the cell receptor binding ability of human NoV (Tan and Jiang, 2003).

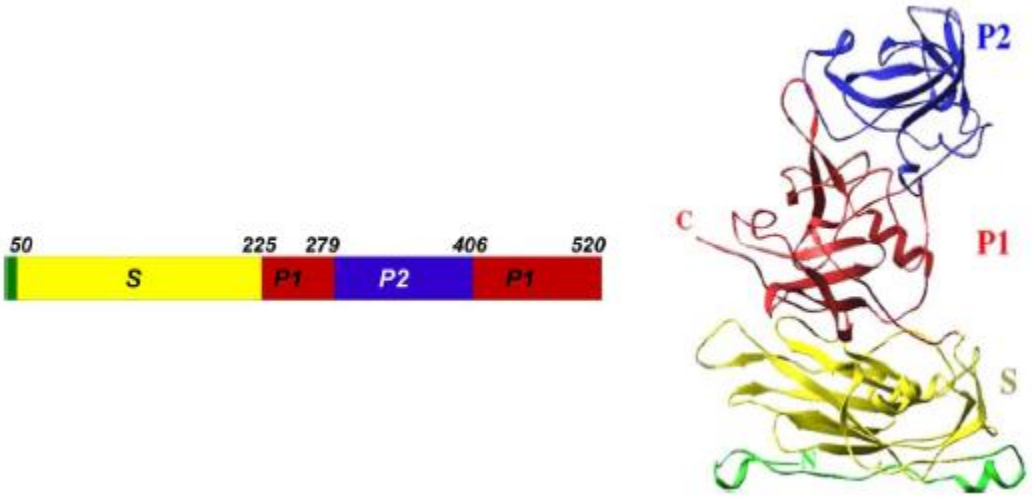


Figure 6. VP1 domains and ribbon diagram of VP1 monomer (Hardy, 2005)

VP2 is composed of ~208-268 amino acids with a molecular weight of ~22-29 kDa (Seah et al., 1999). The sequence of VP2 is highly variable between human NoV strains. VP2 is found in one or two copies per virion. While the function of VP2 has not been well described, it is thought that it is involved in stabilizing the capsid structure. Another possible role of VP2 is RNA binding, allowing the genome to be packaged within the capsid during viral replication (Glass et al., 2000).

1.3.3. Human NoV non-structural proteins

The nonstructural protein p48 has an amino acid sequence that does not share homology with other viral or cellular proteins in the public databases. Thus, the function of p48 is not understood. The nonstructural protein p41 has been identified as having NTPase activity. P41 is classified in the superfamily 3 of RNA helicases. Purified p41 was found to bind ATP in *vivo* and was able to hydrolyze ATP (Pfister and Wimmer, 2001). However, p41 was not able to unwind RNA:DNA heteroduplex, which indicates that it does not have helicase activity. The nonstructural protein p22 plays a role in the p22-VPg-3CL^{pro} precursor in the proteolytic processing pathway, but its additional activity is not well understood (Prasad et al., 1999).

The nonstructural protein VPg is covalently linked to genomic and subgenomic RNA (Burroughs and Brown, 1978). The experimental evidence for this protein-RNA linkage comes from the study of the animal caliciviruses. The function of VPg among virus families varies widely. It has long been known that calicivirus genomic RNA without VPg is not infectious (Burroughs and Brown. 1978). Experimental evidence suggests that VPg interacts specifically with the translation initiation factor eIF3 and with the 40S ribosomal subunit (Daughenbaugh et al., 2003). In this way, VPg could be involved in initiating viral protein synthesis. The nonstructural protein 3CL^{pro} is the single protease encoded by noroviruses (Hardy, 2005). Viral proteases in positive sense RNA viruses are important because they cleave the individual viral proteins from the polyprotein produced from mRNA translation. Experimental evidence suggests that

$3CL^{pro}$ contains a catalytic dyad composed of His30 and Cys139 (Someya et al., 2002). The last nonstructural protein encoded in human NoV genome is the RNA dependent RNA polymerase (RdRp) and has been found to have catalytic and structural elements similar to other RdRps of RNA viruses. The human NoV RdRp has the finger, palm, and thumb domains which are present in all RNA virus RdRps.

Although human NoV cannot be grown in the laboratory environment, much of what is known about its molecular biology has been elucidated using closely related surrogate viruses and sequence homology. Future studies using human NoV itself will provide the most precise data about the complex nature of this seemingly simple virus.

1.4. Epidemiology of human NoV

Transmission of human NoV norovirus usually occurs by one of three methods: fecal-oral route, person to person contact, and food/water borne (CDC, 2010). Transmission may also occur from ingestion of aerosolized vomitus or through contact with fomites that have become contaminated with virus (CDC, 2010). Recently, the focus on food borne transmission of the disease has increased. While viruses cannot grow in food, foods can become contaminated with viruses at many points during production. In the case of human NoV, it has been reported that fewer than 10 virus particles are sufficient to cause gastroenteritis.

Outbreaks of human NoV are most notably associated with cruise ships, but they can occur in any area where people are in close contact. Human NoV outbreaks have been reported in restaurants, retirement communities, schools, hospitals, nursing homes,

hotels, stadiums, and military installations (Seymor and Appleton, 2001). Of the reported cases of human NoV associated gastroenteritis monitored by the CDC from 1994-2006, 234 cases (35.4%) were associated with long-term care facilities, 205 cases (31.1%) were associated with restaurants or social events, 135 cases (20.5%) were associated with vacations including cruises, and 86 cases (13.0%) were associated with schools or the community (CDC, 2010). While contaminated food may be the primary culprit in transmitting the disease, human to human contact plays an important role in the secondary transmission of the virus (Seymor and Appleton, 2001).

Human NoV is highly stable in the environment, which makes it difficult to eradicate after primary infections have occurred. It has been estimated that the stool of an individual with an active norovirus infection may shed up to 100 billion virus particles per gram of feces (CDC, 2010). This fact, paired with the low infectious dose of human NoV, accounts for the rapid spread of the virus in a close community due to poor hygiene. It has also been demonstrated that approximately 30% of human NoV infections are asymptomatic, but individuals may actively be shedding the virus while appearing healthy (CDC, 2010). Consequently, asymptomatic carriers can pass human NoV to other people or to foods that they handle.

1.4.1. Foods commonly associated with human NoV outbreaks

Norovirus foodborne disease is commonly associated with foods that undergo little or no processing before consumption. High risk foods for human NoV contamination include fresh produce, seafood, and ready-to-eat food. The processing of

fresh produce, which usually entails a wash in 200ppm of chlorine, has little to no effect on the removal of norovirus or other viruses from the produce . Human NoV outbreaks have been associated with many types of food including the following: fresh cut fruit, lettuce, tomatos, melons, salads, green onions, strawberries, blueberries, raspberries, salsa, oysters, shellfish, clams, as well as many others (Herwaldt et al., 1994; Anon et al., 2005; Hjertqvist et al., 2006). While some of these foods may have become contaminated through the poor hygiene of food handlers, it has been shown that viral contamination can occur upstream in the food production process. An outbreak of human NoV associated with raspberries was linked to the use of sewage in irrigation water (Falkenhorst et al. 2005, Gaulin et al. 1999, Guyader et al., 2003). Outbreaks of human NoV have also been associated with oysters that were grown in water contaminated with human waste (Dowell et al., 1995, Morse et al., 1986).

1.4.2. Detection of human NoV in foods

Detection of human NoV in implicated foods is often difficult because of the complexity of the food matrix, the low level of virus in the food, and the genetic diversity of the virus (Guyader et al., 2003). Due to the fact that human NoV cannot be grown in cell culture, the detection methods are limited to reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Electron Microscopy (EM). In general, determination of food-borne outbreaks associated with human NoV relies on epidemiological investigations. The virus must be isolated from people who have become ill after consumption of the same food items. Sometimes an outbreak may be

traced to a food handler who also harbors human NoV. The recent trend in food microbiology to focus on viruses will certainly lead to improved molecular detection methods for human NoV in foods.

1.4.3. Recent outbreaks of human NoV

As described above, the epidemiological investigation of human NoV outbreaks is very complicated. Outbreaks associated with foods, water, and person-to-person contact will all be presented to describe the many ways in which this virus can be transmitted.

In January and February 2010 there were 70 cases of gastroenteritis in Ireland and England associated with the consumption of oysters (Dore et al., 2010). The oysters were all harvested from a particular area in Ireland. In Europe, the water where oysters are cultivated is classified as either A, B, or C based on the extent of fecal contamination. The extent of fecal contamination is based on the levels of *Escherichia coli* present in the water (Dore et al., 2010). The oysters implicated in the human NoV outbreak were harvested from a Class A shellfish harvesting area where *E. coli* counts were very low. Oysters that were associated with two gastroenteritis cases in Ireland were available for analysis and contained 2,040 and 2,350 human NoV genome copies per gram, respectively (Dore et al., 2010). Two companies producing oysters from the associated area voluntarily stopped production and implemented a recall on product that had already been distributed.

A military base in Germany was affected by an outbreak of human NoV in January 2009 (Wadl et al., 2010). 36 people presented with symptoms of acute

gastroenteritis and a subsequent investigation of food and food handlers was conducted. Affected individuals were questioned about their food consumption and it was found that prepared salad was common to most affected persons. Stool samples from affected individuals, food handlers, and environmental samples from the canteen were analyzed for bacterial and vial pathogens. Stool samples from affected individuals and from the environmental samples both tested positive for human NoV genotype GII.4.

In July 2005 there was an outbreak of acute gastroenteritis that occurred in a summer camp in Spain. All affected persons, 44 people in total, had eaten a common meal at lunch. One of the three food handlers responsible for preparing the meal (paella, round of beef, and fruit) had exhibited symptoms of human NoV infection two days before preparing the meal, but was asymptomatic at the time of the food preparation. Stool samples were collected from 10 affected individuals and from the three food handlers. The stool samples were tested for the presence of both bacterial and viral pathogens. Human NoV was detected in the stools of 7 of the 10 infected individuals, as well as two of the food handlers including the asymptomatic handler. The same strain of human NoV (GII.2) was detected in the affected individuals and the asymptomatic food handler (Barrabeig et al., 2010).

In the aftermath of Hurricane Katrina thousands of displaced New Orleans residents were relocated to the Reliant Park Complex in Houston, Texas. The evacuees arrived on August 31, 2005. An outpatient clinic was established at the facility and on September 2nd and physicians and staff noted a large increase in individuals presenting with symptoms of acute gastroenteritis. Beginning September 4th, stool and vomitus

samples were collected from patients with gastroenteritis. Samples were tested for bacteria, parasites, and enteric viruses. Human NoV was detected in stool samples using RT-PCR. Multiple strains of human NoV were isolated suggesting that a common source of contamination, such as food, was not the primary source of the outbreak. A total of 6985 visits were registered at the clinic and 1173 (17%) were because of gastroenteritis (Yee et al., 2007). This outbreak exemplifies how easily and quickly human NoV can be spread in a crowded community where poor hygiene allows for transmission.

An outbreak of gastroenteritis due to human NoV was shown to be caused by contaminated groundwater in a waterpark in Korea (Koh et al., 2011). In January 2008, a group of 180 students and 36 teachers visited a waterpark Gyeonggi-do in the Republic of Korea. Two days after visiting the park, 67 people in the group (31%) developed acute gastroenteritis. A formal epidemiological study that involved interviewing students, teachers, and food handlers, studying the food handling environment, and investigating the water supply and sewage system was executed to determine the etiologic agent responsible for the outbreak. Stool samples were collected from all affected individuals and tested for the presence of the bacterial pathogens (*Salmonella*, *Shigella*, *Camplyobacter*, and *Vibrio vulnificus)* and viral pathogens (adenovirus, rotavirus, astrovirus, and human NoV). No food samples were available for testing, but source water was tested for the presence of human NoV. Stool samples and groundwater samples tested positive for the GI.4 strain of human NoV using sequence analysis (Koh et al., 2011).

The outbreak data presented here demonstrates the many modes by which human NoV disease can be disseminated within a population. Determining human NoV as the causative agent of outbreaks is often hampered by the limited modes of detection of the virus and the genetic diversity found within the genus *Norovirus*. Because of the high number of human NoV strains circulating in the human population there is no cross-reactive antibody which can detect all the strains using ELISA assays. RT-qPCR detection is also hampered by low sequence homology because of the strain diversity. Also, EM analysis is very expensive and a highly trained observer is needed to distinguish human NoV from other enteric viruses. Finally, since human NoV is not cultivatable there is no cell based assay to detect human NoV in patient or environmental samples. As described above, the determination of human NoV as the causative agent of an outbreak is often determined by a combination of symptomology and the exclusion of other enteric pathogens as the culprit.

1.5. Human NoV surrogates

Due to the fact that human NoV cannot be grown in cell culture, most laboratory efforts to study the virus employ the use of surrogates. These surrogates include viruses that are closely related to human NoV in terms of genetic makeup, size, receptor binding, pathogenicity, and environmental stability. Other surrogates used for the study of human NoV include virus-like particles (VLPs) and P domain-particles (P-particles). These particles resemble portions of the human NoV protein capsid, which are important for receptor binding of the virus to the host cell and antigenic recognition of the virus by the

immune system. The particles are non-infectious due to the fact that they are composed only of protein and lack the viral genome component of the native virus. While the use of surrogates has aided in the understanding of human NoV, there are several limitations in comparing data generated from the use of surrogates to human NoV.

1.5.1. Human NoV virus-like particles (VLPs) and P domain particles (P-particles)

The norovirus virion is not enveloped and the protein capsid is composed of 180 monomeric protein units of VP1. These 180 proteins are further organized into 90 dimers. The VP1 protein can be divided into three domains; N, S, and P. While N and S function to stabilize the viral particle and are found internally, the P domain is positioned externally on the viral particle and can be further subdivided into P1 and P2 (de Rougemont et al., 2011). The P2 domain of VP1 is responsible for the receptor binding and the attachment to cells. While human NoV cannot be grown in cell culture, the use of recombinant baculovirus expression systems has allowed for the production of the VP1 protein in an insect cell line. The VP1 protein can then self-assemble to form human NoV virus-like particles (VLPs), which retain the same capsid structure and antigenicity as the native virus (Fig.7). The human NoV VLPs lack the genome component of the native virus and therefore are noninfectious. Interestingly, the expression of VP1 results in the formation of two sizes of VLPs. The diameters of the large and small particles were between 30-38 nm and 20-23 nm, respectively (Capucci et al.,1991, Taniguchi et al., 1981). The VLPs have been found to bind readily to the human NoV cellular receptor, the histo-blood group antigens (HBGAs) (Gandhi et al., 2010).

There are a number of advantages of using VLPs as a surrogate to study human NoV. First, human NoV VLPs can be produced in large quantities by expressing VP1 in a number of systems such as the recombinant baculovirus grown in insect cells, as described above. Secondly, damage to VLPs can be evaluated using biophysical and biochemistry methods such as electron microscopy (EM), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting assays. Finally, VLPs possess authentic receptor binding activity which is essential for viral infection. Hence, VLPs can be assayed for receptor binding activity as an indicator of virus survival.

Expression of the P domain of VP1 protein using a recombinant virus expression system resulted in the formation of ring- or pentagon-shaped structures with a diameter of 5 nm. This small particle was named the P particle. The P particles exhibited enhanced binding ability to HBGAs, which intuitively makes sense since the P domain of VP1 is responsible for the binding of the virus to its cellular receptor (HBGAs).

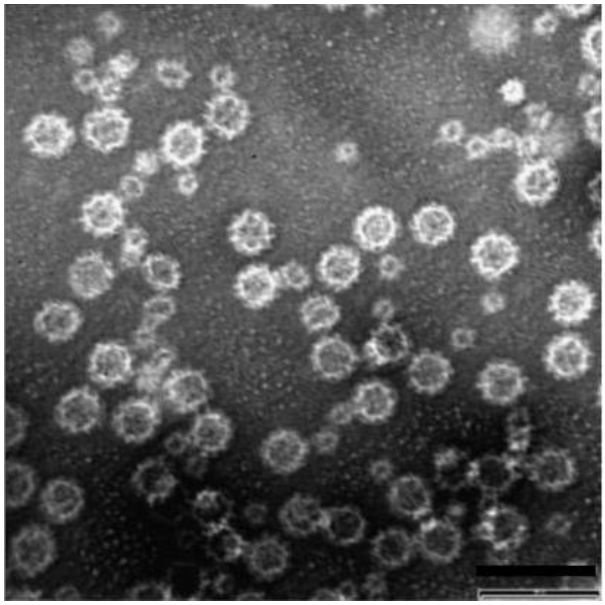


Figure 7. Transmission electron microscopy image of human NoV virus-like particles (VLPs) (Adapted from Feng el al., 2011)

1.5.2. Viral surrogates used for the study of human NoV

While VLPs serve as an effective tool for the study of receptor binding and capsid integrity of human NoV, they do not elucidate the complexities of pathogenesis, molecular biology, replication, or environmental stability of the virus. Therefore, surrogate viruses are necessary tools to study human NoV. These surrogate viruses include murine norovirus (MNV-1), feline calicivirus (FCV), porcine sapovirus, and Tulane virus (TV). A summary of these viruses can be found in Table 2.

Table 2: Comparison of human NoV to commonly used viral surrogates.

Virus	Genome	Viral Particle	Cellular Receptor	Cell Tropism	Host	Disease
Human norovirus	7.7 kb	28-35 nm	HBGAs	Intestinal epithelial	Humans	Gastroenteritis
Murine norovirus	7.3 kb	28-35 nm	Sialic Acid	Most cell types	Mice	Systemic Disease
Feline calicivirus	7.6 kb	28-35 nm	Sialic Acid	Most cell types	Felines	Respiratory Disease
Porcine sapovirus	7.3 kb	27-40nm	Unknown	Intestinal epithelial	Swine	Gastroenteritis
Tulane virus	6.7 kb	27-40 nm	HBGAs	Intestinal epithelial	Primates	Gastroenteritis

1.5.3. Murine norovirus (MNV-1)

Murine norovirus (MNV-1) has been used extensively as a surrogate for the study of human NoV. MNV-1 was isolated in 2003 from signal transducer and activator of transcription (STAT-1) defective mice and it was found that in these immunocompromised mice it caused a lethal systemic infection (Karst et al., 2003). MNV-1 is also a member of the genus *Norovirus*, and to date it is the only norovirus that has been adapted to cell culture. MNV-1 is able to replicate in murine macrophage cells (RAW 264.7) which makes it a good model to study human NoV replication. MNV-1 also resembles human NoV in terms of viral capsid structure, viral particle size, and genetic makeup. MNV-1 is also very stable at acidic pHs, when compared to other surrogates such as FCV (Cannon et al., 2006). Stability at low pHs is important for food-borne viruses as they must be able to survive the acidic conditions found in the digestive

tract to be able to cause infection. However, MNV-1 and human NoV differ with regard to their attachment receptors on cells. MNV-1 binds to the sialic acid residues found on the cell surface, while human NoV utilizes HBGAs as its functional receptor (Taube et al., 2009). In addition, MNV-1 does not cause the clinical manifestation of gastroenteritis which also differs from human NoV. The differences in receptors and pathogenesis of MNV-1 and human NoV may limit comparisons made between the viruses.

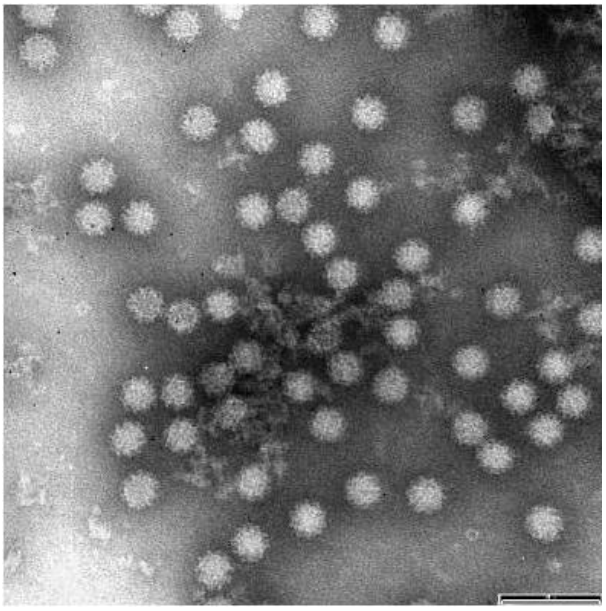


Figure 8. Transmission electron microscopy image of murine norovirus (MNV-1) (Adapted from Lou et al., 2011)

1.5.4. Feline calicivirus (FCV)

Feline calicivirus (FCV) is also commonly used as a surrogate for human NoV. FCV is a member of the genus *Vesivirus* and the family *Caliciviridae*, so it is closely related to human NoV. FCV was isolated in the 1950's and is one of the major causes of upper respiratory tract infections in cats. FCV does resemble human NoV in terms of genetic makeup, viral particle size, and viral capsid structure. FCV has been adapted to cell culture and can grow in feline kidney cells. The cellular receptor of FCV is sialic acid which also differs from the receptor used by human NoV. The use of FCV as a surrogate has declined due to the fact that it causes respiratory disease and is inactivated at low pHs.

1.5.5. Porcine sapovirus

Porcine sapovirus is another surrogate used for the study of human NoV. It is a member of the genus *Sapovirus* within the family *Caliciviridae*. Porcine sapovirus was isolated from the stool of swine in 1980 (Saif et al., 1980). It was later adapted to cell culture and grows in porcine kidney cells (LLC-PK) supplemented with intestinal content fluid filtrate. Unlike MNV and FCV, porcine sapovirus causes gastroenteritis in pigs and is classified as an enteric virus. Porcine sapovirus also resembles human NoV in genome size, genome organization, viral capsid structure, and viral particle size. The cellular receptor for porcine sapovirus has not been elucidated. Based on the fact that porcine sapovirus is an enteric virus, it serves as a good model to study the pathogenesis of human NoV.

1.5.6. Tulane virus (TV)

Tulane virus (TV) is a newly recognized surrogate for human NoV. TV was isolated from the stool of rhesus macaques showing symptoms of gastroenteritis in 2008 (Farkas et al. 2008). TV is a member of a newly created genus, *Recovirus*, within the family *Caliciviridae*. TV has been adapted to cell culture and grows in monkey kidney cells (LLC-MK2). TV also closely resembles human NoV in regards to genome size, genome organization, viral capsid structure, and viral particle size (Fig. 9). One exciting characteristic of TV is that it shares the same cellular receptors as human NoV, (HBGAs). This makes TV an important surrogate for the study of human NoV pathogenesis. Since TV is cultivatable it may be used to test experimental human NoV vaccines efficacy in vivo. However, the environmental stability of TV has not been elucidated and further study of this virus is needed to determine its potential as a surrogate.

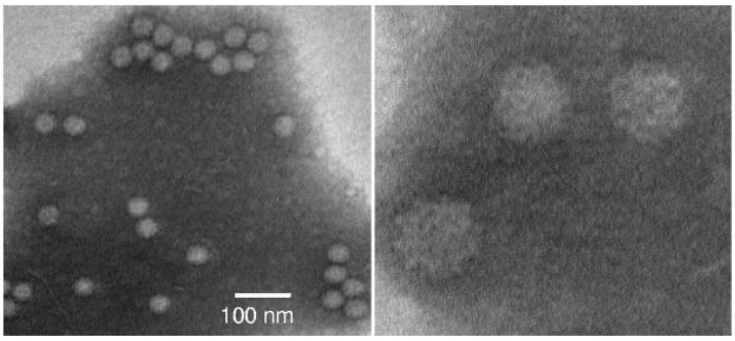


Figure 9. Tranmission electron microscopy image of Tulane virus (TV) (Adapted from Farkas et al., 2008)

1.6. Factors contributing to viral persistence in fresh produce

Human NoV has been shown to be transmitted easily in foods and recent work has focused on understanding the interaction between norovirus and food products. Bioaccumulation of human NoV from the irrigation or feed water or specific receptor binding of human NoV to food may play a role in its persistence in the food. The small size of human NoV (38nm) increases its potential to enter the tissues of plants. Bacterial pathogens have been shown to gain entry into the internal structures of plants and, on average, viruses are approximately 1000 times smaller than bacteria. It is feasible to assume that a smaller pathogen would enter the plant tissues more easily compared to larger pathogens. It has been shown that simple washing of fresh produce with 200ppm chlorine removes less than 1 log of virus, indicating that viruses attach tightly to the produce surface (Predmore and Li, 2010). In this section irrigation water as a potential vehicle for human NoV contamination of fresh produce, virus interaction with soil, and

the contributing factors of plant physiology that make fresh produce susceptible to human NoV contamination, will be discussed.

1.6.1. Environmental stability of foodborne viruses

The persistence of viruses in the environment plays a critical role in their ability to contaminate produce. Several chemical, physical, and biological factors affect viral stability. These factors include heat, light, desiccation, pressure, pH, salinity, enzyme activity, and microbial activity (Bosch, 1983). As previously described, human NoV is highly stable in the environment, as are many non-enveloped enteric viruses. They are highly resistant to environment stresses such as desiccation, temperature, and pH changes. This environmental stability increases the potential for irrigation water or soil to become contaminated with viruses and then deliver the virus to crops. This stability also plays a role in the post-harvest contamination of foods with viruses. Human NoV and murine NoV have been found to be stable on stainless steel coupons for several weeks (Takahashi et al., 2011). Also, washing and hand sanitizers have been shown to be ineffective in removing virus from hands (Liu et al., 2010). Hence, virally contaminated utensils, cutting surfaces, and food handlers can easily transfer virus to foods.

1.6.2. Irrigation water as a source of virus for the contamination of fresh produce

Agriculture is responsible for the largest usage of freshwater worldwide and about 70% of this usage is for irrigation. Nearly 17% of all cropland is irrigated, which correlates to one third of the world wide food supply being exposed to irrigation water

(Shanan et al., 1998). The use of feces for fertilization or fecally contaminated irrigation water has been known to play a role in spreading enteric microorganisms. For this reason, the use of night soil or irrigation with untreated human waste water is illegal in the U.S. and is not recommended by the World Health Organization (WHO). However, nearly 70% of all the irrigated crop land is found in developing countries where irrigation water regulations may not exist (Choi et al., 2004).

Enteric viruses may also contaminate sources of surface or ground water. This contamination can come from sewage discharge, septic tanks, recreational bathers, etc. Groundwater is generally regarded as being free of microbial contamination and is considered a safe source of irrigation water. However, recent studies in the U.S. indicate that 8-31% of ground water is contaminated with viruses (Abbaszadegan et al., 2003, Borchardt et al., 2003). These sources of irrigation water are not monitored for microbial contamination and their usage potentially poses another risk for disseminating enteric viral disease.

1.6.3. The effect of the soil matrix on viral contamination of fresh produce

The soil matrix also plays an important role in controlling viral transport to crops. These factors may include soil type, water saturation, pH, conductivity of the water, and organic matter. The soil type influences viral movement significantly. Fine-textured soils tend to absorb viruses more readily than coarse-textured soils (Bosch, 1983). A highly water saturated soil also allows for more viral movement, as all the pores in the soil are open and the virus has less interaction with the soil particles (Santamaria and

Toranzos, 2003). Under acidic conditions viruses normally possess a negative charge. Therefore, the virus may adhere to positively charged materials found in the soil and be tightly bound in the soil matrix. In neutral or alkaline soils, the virus will not bind to materials in the soil and can easily be disseminated throughout the soil matrix (Sobsey et al., 1980). The presence of cations in the soil also favors viral absorption. The presence of cations limits the amount of repulsive forces between the virus and the soil. However, soluble organic solids, humic acid, and fulvic acid will compete with viruses for soil absorption sites and reduce the level of viral attachment to the soil (Sobsey and Hickey, 1985).

1.6.4. Plant physiology

The physiology of plants makes them susceptible to contamination by enteric viruses. Possible ports of entry for viruses found in plants include the roots and stomata, as well as breaks in the cuticle caused by damage. Once a virus has entered the plant it may be transported along with water and nutrients through the vascular tissues, disseminating the virus throughout the plant. It is theorized that this viral movement would be isolated to the xylem and phloem, as the plasmodesmata between most other plant cells are too small for viruses to penetrate and gain entry to the individual cells. Also, the surfaces of plant leaves are complex and have many surface structures that may help to bind the virus. Lipids, carbohydrates, and proteins have all been described on the leaf surface and they may serve to anchor the virus. This binding may be specific

(receptor mediated) or non-specific, though current research suggests that it may be specific.

The vascular tissues of plants are responsible for disseminating water, nutrients, and the products of photosynthesis throughout the plant tissues. There are two types of vascular tissues present in plants, xylem and phloem. The xylem is primarily responsible for the movement of water and dissolved minerals throughout the plant. The cells that make up the xylem include tracheids and vessel members (Hopkins and Huner, 2004). Tracheids are long, tapering cells while vessel members are shorter, cylindrical and joined together to form long tubes (Hopkins and Huner, 2004). Both cell types have a thickened secondary cell wall and when mature lack protoplasm (Hopkins and Huner, 2004). Phloem tissue is responsible for the movement of organic molecules within the plant tissues. These molecules are transported from source to sink, meaning from photosyntheticly active or storage tissues to actively growing portions of the plant. Phloem tissue is primarily composed of sieve cells. The sieve cells are long, joined end to end, and devoid of nuclei at maturity (Hopkins and Huner, 2004).

In terms of viral movement within the plant, the xylem would be responsible for the primary transportation from the roots to the aerial portions of the plants. Once viral contamination has reached the leaves, it is possible that it may be present in leaves which are photosyntheticly active. Then further dissemination of the virus may occur via the phloem as nutrients are shuttled to growing plant tissues. In contrast, if virus was first present in the leaves, it would likely be the phloem that would be responsible for

transporting the virus to the roots, as roots serve as a storage location for the products of photosynthesis.

The epidermis is the outer layer of cells that covers the leaves, roots, stems, and fruits. The outer surface of plants is usually covered by a waxy cuticle that limits water loss. There may also be specialized hairs called trichomes present on the epidermis. These hairs function to reduce solar energy absorption and water loss by leaves, salt secretion, and provide protection from insects (Hopkins and Huner, 2004). Leaves have specialized pores called stomata that interrupt the epidermis. The stomata allow for gas exchange (CO_2 and O_2) between the leaf and the environment. The stomata are surrounded by specialized guard cells, which can swell and constrict in response to osmotic pressure (Hopkins and Huner, 2004). While these stomata often close in response to common plant pathogens, the plant defense system would have no recognition of human enteric viruses. It is possible that viruses could contaminate the leaves and possibly enter the stomata. In cases where CO_2 is actively entering the leaf, the virus could be pulled inside of the leaf itself.

In the roots, the epidermis has been replaced by the periderm. The periderm is composed of phelloderm and a layer of protective, non-living cork cells (Hopkins and Huner, 2004). The root that develops from the seed is called the primary root and branching of this root results in what are referred to as lateral roots. The center of the root is called the stele. The center of the stele contains xylem and phloem surrounded by the pericycle and endodermis. Surrounding the stele is the cortex of the root and encapsulating the cortex is the epidermis or periderm. The primary functions of the root

are anchorage, storage, and absorption of water and nutrients (Hopkins and Huner, 2004). Root hairs are formed by the elongation of a single cell along the periderm (Steudle and Peterson, 1998). These root hairs increase the surface area of the root allowing for increased water and mineral exchange. There are pores on the root hair and epidermis called plasmodesmata that allow for the absorption of water and nutrients into the vascular tissues. The average diameter of the plasmodesmata has been reported between 40-60nm (Turner et al., 1994). However, the size exclusion principle indicates that the size of a molecule that can enter through these pores passively is 700kDa (Turner et al., 1994). There are active transport mechanisms used by the plant to increase the size of the plasmodesmata to allow for larger molecules to enter the plant (Tuner et al., 1994). It is possible that enteric viruses could be actively transported into the vascular system of the plant via root pores, but it is more likely that breaks in the roots due to transplantation or root growth would allow direct penetration of the virus into the vascular system of the plant.

1.7. Pathogen attachment, internalization, and dissemination in fresh produce

Pathogenic bacteria, protozoa, and viruses causing human enteric disease have been associated with fresh produce because these food commodities undergo little or no processing before consumption. However, the complex mechanisms by which these pathogens interact with produce has only recently been the subject of investigation. The objectives of many research efforts have focused on the ability of pathogens to attach to the surface of produce, as well as the internalization of pathogens into the internal

structures of the produce and dissemination of the pathogens throughout the plant tissues. Most studies have focused bacterial pathogens, such as *E. coli* and *Salmonella* species, which are highly associated with outbreaks of enteric disease caused by leafy greens. However, the interaction of foodborne viruses with fresh produce is poorly understood. Due to the fact that human NoV accounts for nearly 40% of fresh produce associated outbreaks, researchers have begun to investigate the persistence, attachment, internalization, and dissemination of enteric viruses in fresh produce. Using both microscopic evaluation and pathogen enumeration techniques, several important food-borne pathogens have been shown to attach and be internalized in many types of fresh produce.

1.7.1. Bacterial attachment to fresh produce

The attachment of bacterial pathogens to fresh produce has been evaluated using both scanning electron microscopy and confocal microscopy. Several studies have demonstrated the attachment of *E. coli* O157:H7 to lettuce using fluorescently labeled antibodies and confocal microscopy (Seo and Frank, 1999, Takeuchi and Frank, 2000). Lettuce samples were submerged in liquid containing the bacteria and then the surface was washed and disinfected with 20-200ppm of chlorine. *E. coli* O157:H7 was found inside the stomata, attached to cut edges, and 10-100μm below the surface, even after chlorine treatment indicating the protection of the bacteria from inactivation by the plant physical structure. Similar studies utilizing lettuce as a model system have found that *Salmonella enterica* strains exhibit similar attachment profiles as *E. coli* O157:H7, with

Salmonella enterica aggregating within the stomata, along cut edges, and within the upper interior layers of the lettuce tissue (Brandl, 2008, Kroupitski et al., 2009, Kroupitski et al., 2011).

1.7.2. Viral attachment to fresh produce

The attachment profile of human NoV surrogates to fresh produce has been demonstrated in several independent studies. The determination of where the virus is aggregating or attaching on the fresh produce can provide insight to the mechanisms of persistence of the viruses in the foods and may lead to targeted methods of removal. Rawsthorne et al., (2009) first established a protocol for visualizing MNV-1 in green onions utilizing Quantum Dots (Q-Dots). MNV-1 was subjected to purification followed by the conjugation of biotin to the purified virus capsid protein. It was found that the biotinylation of MNV-1 had no effect on the ability of the virus to replicate in cell culture. The MNV-1 complexed with biotin was applied to green onions and then subsequently stained with streptavidin coated Q-Dots655 which emit fluorescence at 655nm. The virus then was able to be visualized in green onions using confocal microscopy. It was found that MNV-1 bound to the surface of the epidermal cells of the green onion, and while washing decreased the MNV-1 signal slightly, a significant amount of virus still remained attached to the green onion.

Another study to observe viral attachment to produce used purified MNV-1 stained with SYBR gold to visualize the virus on the surface of Romaine lettuce leaves using a confocal microscope (Wei et al., 2010). It was observed that the viral particles

attached to the surface of the lettuce and were seen inside of stomata. This may be evidence that virus may enter plants through stomatal openings and perhaps become internalized into the plant tissue. Unfortunately, the use of SYBR gold to stain virus particles has been criticized because the SYBR gold dye is much larger than the virus particles and may lead to false positives (Rawsthorne et al., 2009).

Gandhi et al. (2010), used human NoV VLPs to investigate the attachment of virus to romaine lettuce. Using confocal microscopy and fluorescently labeled antibodies, it was found that VLPs localized along the veins of lettuce leaves, indicating that there may be specific ligand binding involved. Romaine lettuce extract (RE) was prepared and used to coat ELISA plates. The RE was tested against anti-HBGA antibodies to determine if HBGA-like polysaccharides were present in lettuce. Based on lack of antibody binding to RE, it was concluded that the RE lacked HBGA like polysaccharides. The epidermal cells of the leaf surface are covered by a cuticle composed of cutin, polysaccharides, and waxes with long chain fatty acids which may serve as alternative binding sites for norovirus.

Esseili et al. (2012), also evaluated the attachment of human NoV VLPs to Romaine lettuce. Using antibodies specific to human NoV VLPs and confocal microscopy, it was found that human NoV VLPs attached to the surface of Romaine lettuce, and were aggregating in stomata and along cut edges of the lettuce. Furthermore, it was demonstrated using an ELISA assay that human NoV GII.4 VLPs bound to the cell wall material of young and old leaves, the green leaf lamina, and also the principle vein of Romaine lettuce (Esseili et al., 2012). This binding was found to be strongest in the

cell wall material of old leaves and the green leaf lamina, compared to other plant tissues tested. This was believed to be due to the fact that the cell walls of older leaves are more complex and contain a higher carbohydrate concentration. To further demonstrate that the human NoV VLPs were binding to carbohydrates, sodium periodate treatment was used to oxidize carbohydrates in the cell wall extract and this treatment significantly reduced the binding efficiency of the human NoV VLPs. Further research is needed to determine the specific types of carbohydrates involved in the norovirus-fresh produce interaction. An understanding of the specific mechanism of norovirus-fresh produce binding will facilitate the development of targeted control measures.

1.7.3. Bacterial internalization and dissemination in fresh produce

Varying rates of bacterial internalization in fresh produce have been observed in laboratory experiments. Differences have been seen in the internalization and dissemination rates of bacteria due to the bacterial species, bacterial strains within the same species, the plant model system used, the growth medium of the plant, the environmental conditions of plant growth, the mode of inoculation of the plant, and the number of bacteria inoculated. In most agricultural practices irrigation occurs either through spray irrigation, where the leaves and soil may contact the irrigation water, or through drip irrigation, where the roots and soil are the main points of contact for the irrigation water. Therefore, most studies have focused on the inoculation of bacterial pathogens to the leaves, to mimic spray irrigation, or to the soil, to model drip irrigation. In addition, most crops are seeded and germinated under greenhouse conditions and then

transferred to the field. Transplantation may cause damage to the roots of the plants that may increase pathogen internalization, and this factor has also been evaluated. Finally, some seedling cultivation and also mature crop cultivation occurs under green house conditions with the plants being grown hydroponically. The internalization of bacterial pathogens in the feed water for hydroponic growth has also been investigated. Due to the large amount of publications dealing with bacterial internalization in fresh produce, the focus of this review will be on *E. coli* sp. and *Salmonella* sp. internalizing in leafy greens.

The rate of bacterial internalization due to the presence of bacteria on the aerial portions of fresh produce has been evaluated in several studies. It was found that spray irrigation water of field growing spinach which was inoculated with varying levels (10^2-10^6 CFU/ml) of *E. coli* O157:H7 resulted in detection of viable *E. coli* O157:H7 in leaves after surface disinfection only in the spinach receiving the highest dosage level of 10^6 CFU/ml (Erickson et al., 2010a). Similarly, it was found that increasing the inoculation level to 10^8 CFU/ml of *E. coli* O157:H7 in spinach and lettuce, lead to increased detection of the bacteria at 48 h post inoculation (Erickson et al. 2010a). A study evaluating the internalization of a *Salmonella enterica* sp., found that the presence or absence of light played a significant role in the internalization of the bacteria into the tissues of iceberg lettuce (Kroupitski et al., 2009). Even with a high inoculum of *Salmonella enterica* sp. (10^8 CFU/ml), it was found that in dark conditions no internalization of the bacteria occurred. However, after exposure to light, the bacteria was found in the stomata and the upper internal plant tissues (Kroupitski et al., 2009).

However, other published work has demonstrated that bacterial internalization does not occur due to contamination of aerial portions of the plants (Zhang et al., 2009).

Although less frequently associated with fresh produce related outbreaks, the internalization of parasitic pathogens in fresh produce has also been investigated. The oocytes are the reproductive structures of many *Cryptosporidium sp*. It was found that spinach leaves sprayed with irrigation water containing 10^4 *Cryptosporidium parvum* oocytes per mL, had oocytes present in their stomatal openings and at the mesophyll level (Macarisin et al., 2010).

Internalization of bacterial pathogens via the root has also been studied extensively. *E. coli* O157:H7 at a level of 7 log CFU was applied to the soil of mature lettuce plants and it was found that the 40-80% of the aerial portions of the plant tested positive for the pathogen (Solomon et al., 2002). However, when *E. coli* O157:H7 was injected into the root zone of spinach at the same level (7 log CFU) only 10 of 60 (16%) plants tested positive for the bacteria (Mitra et al., 2009). In the Solomon et al., (2009) study, no surface decontamination step was used. In contrast, in Mitra et al., (2009) study, the surface of plants was decontaminated by 70% ethanol, followed by 10% bleach surface disinfection, which allowed measurement of only internalized pathogens. This discrepancy in internalized bacterial detection supports the idea that experimental design, as well as plant cultivar, plays a significant role in the varying detection of internalization and dissemination of pathogens in fresh produce.

Several other studies have investigated the internalization of *E. coli* sp. when inoculated to soil of growing spinach. These studies all employed to use of surface

decontamination with 2,000-4,000ppm of sodium hypochlorite and it was found that the bacteria was only detected within the roots, but not the leaves of the lettuce (Warriner et al., 2003, Hora et al., 2005). Franz et al. (2007), compared the internalization rates of *E. coli* O157:H7 and *S. Typhimurium* applied to the soil of lettuce seedlings and found that the internalization rate was higher for *S. Typhimurium*. The internalization rates of different *Salmonella* serovars *Dublin, Enteritidis, Montevidea,* and *Typhimurium* in lettuce were found to be 59%, 85%, 93%, and 89%, respectively, indicating that even bacteria within the same species behave differently in regards to their internalization rates in the same plant cultivar under the same growth and environmental conditions (Klerks et al., 2007). It has also been demonstrated that the plant growth media has a significant impact on bacterial pathogen internalization. A study comparing the internalization rates of *E. coli* O157:H7 and *Salmonella* sp. was greater in hydroponically grown leafy greens compared to soil cultivated leafy greens (Franz et al., 2007, Sharma et al., 2009).

1.7.4. Viral internalization and dissemination in fresh produce

As compared to bacterial pathogens, viral internalization and dissemination in fresh produce is poorly understood. The potential internalization and dissemination of human NoV in fresh produce caused by the use of contaminated irrigation water poses a significant risk to consumers because the internalized virus is protected from all surface decontamination measures. A study by Wei et al., (2010) investigated the internalization of murine norovirus MNV-1 via the leaves of romaine lettuce. It was found that MNV-1 in cell culture medium and MNV-1 suspended in animal manure both attached to the

surface of lettuce. MNV-1 inoculated lettuce was subsequently wiped with 1% Vikron to eliminate surface viruses and MNV-1 was detected in samples, post decontamination. The detection of MNV-1 after disinfection indicates that virus may have been internalized through openings in the lettuce leaves. However, the efficiency of the disinfectant to remove all surface viruses from the lettuce leaf has been brought into question.

In a recent study by Wei et al. (2011), the rate of MNV-1 internalization via roots of Romaine lettuce using contaminated irrigation solution was tested. Both soil and hydroponic growing conditions were used to test the rate of MNV-1 internalization and dissemination. The lettuce grown in soil was kept under normal greenhouse conditions, while the lettuce grown hydroponically was kept in a growth chamber at either 99% or 70% relative humidity (RH). The differences in RH were used to determine the effect of the transpiration rate of the lettuce on the amount of viral uptake. The transpiration rate of lettuce grown in 70% RH was found to be about 10 fold higher than that of the plants grown in 99% RH. Only 1 out of 8 plants grown in 99% RH was found to harbor MNV-1 RNA, while the plants grown in 70% RH had detectable MNV-1 RNA in 7 out of 8 plants, indicating the importance of the plant transpiration rate on the level of uptake of virus. For lettuce grown in soil, MNV-1 RNA was detected in leaves at days 1, 3, and 5 post inoculation. However, in cell culture infectivity assays, only 3 samples from the entire study tested positive, and this was 5 days after inoculation. Although the rate of infectious MNV-1 detected in lettuce leaves was lower than the detection of MNV-1

RNA, these results indicate that a risk of contracting viral disease due to internalized virus exists.

There have been inconsistent results among studies investigating the internalization of enteric viruses in fresh produce. Studies using canine calicivirus (CaCV) at a concentration of 10^6-10^9 RNA copies/ml to inoculate Romaine lettuce growing in soil and hydroponically, showed that virus could occasionally be detected in leaves (Urbanucci et al., 2009). However, similar studies using a human NoV genogroup II (GII) isolate found no viral RNA in the plant aerial tissues (Urbanucci et al., 2009). It was reported that less than 2 log PFU/g was detected in hydroponically grown beans when the plants were inoculated with approximately 10^{10} PFU/ml of bacteriophage f2 (Ward and Mahler, 1982). Green onions grown both hydroponically and in soil showed 100% positive for hepatitis A virus RNA (Chancellor et al., 2006). Differences among results could be due to differences in plant cultivars, viral differences, and differences in experimental conditions. Since only a few viral particles are sufficient to cause disease in humans, viral internalization and dissemination poses a high risk for food safety even if the internalization rates are low. It is apparent that many factors play a role in the rate of pathogen internalization in fresh produce. However, in the case of viruses there is much more research needed to understand the complex mechanisms by which these pathogens persist in foods.

The current understanding of pathogen attachment, internalization, and dissemination in fresh produce can be summarized as the following. First, it is firmly established that bacterial pathogens can be internalized and disseminated in fresh produce

via both leaves and roots of plants. Second, a low level of human NoV surrogates (murine NoV and canine calicivirus) internalization and dissemination occurs in lettuce via roots. This is based on only two studies (Urbanucci et al., 2009; Wei et al., 2011) showing that a high level of RNA can be detected in plants by real-time RT-PCR whereas no to low number of infectious virus particles (less than 2 log) can be detected by plaque assay. Third, no viral internalization and dissemination was detected by real-time RT-PCR when a human NoV strain was used. This is based on one study performed by Urbanucci et al., (2009). Clearly, more research is needed to determine whether human NoV can be internalized and disseminated in fresh produce and to determine the factors that influence the rate of attachment and internalization of viruses in fresh produce and this is the main goal of our research.

CHAPTER 2

Qualitative and quantitative assessment of the attachment of human novovirus and animal caliciviruses to fresh produce

2.1. Abstract

Fresh produce is a high risk food for human norovirus (NoV) contamination. Currently, the mechanism of viral attachment to fresh produce is poorly understood. 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 human NoV virus-like particles (VLPs), TV, and MNV-1 attached to the surface of Romaine lettuce and were found aggregating in and around the stomata using a fluorescence-based Quantum Dots (Q-Dots) assay and observed under a confocal microscope. In the case of green onions, human NoV VLPs were found between the cells of the epidermis 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. In addition, the effectiveness of washing on the removal of human NoV and its surrogates from Romaine lettuce were determined. It was found that a human NoV GII.4 strain had similar attachment efficiency to the Romaine lettuce leaves and roots, and that

washing with PBS or 200ppm of chlorine removed less than 1 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 of viruses from roots. Collectively, these results demonstrate that (i) different viruses vary in their attachment patterns to different varieties of fresh produce; and (ii) washing is ineffective in removing human NoV from fresh produce.

2.2. 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 (DeWaal and Bhuiya, 2007). This pathogen has been linked to outbreaks in lettuce, fresh cut salads, green onions, and various types of berries (Abbazadegan et al., 2003, Doyle and Erickson, 2008, Heaton and Jones, 2008, 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 and the ability of the viruses to remain infectious under extreme pH and their resistance desiccation in the environment, makes most foods that undergo limited processing susceptible to

contamination by contaminated water and soil sources or by transmission via processors and food handlers.

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), 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. Therefore, whether these surrogates truly represent human NoV remains unknown. Recently, a new primate calicivirus, TV, was discovered in the stool of rhesus macaques housed in the Tulane National Primate Research Center. TV replicates in vitro in rhesus monkey kidney cells and causes typical cytopathic effect (CPE). Importantly, TV also recognizes the histo-blood group antigens (HBGAs) in a similar manner to human NoV. The complete genome of TV has been sequenced and the TV is genetically closely related with human NoV compared with other caliciviruses. 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 in 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 200ppm chlorine in the wash tanks. 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 200ppm chlorine has been shown to be effective in eliminating many bacterial pathogens such as *Esherichia coli* and *Salmonella* species, it has little to no effect on the removal of viral pathogens. Specifically, research utilizing human NoV surrogates (such as murine norovirus and feline calicivirus) has shown less than 1 log reduction in viral in titer was achieved by using 200ppm 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 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 difficultly 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. 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. 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. 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.

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

2.3. Materials and Methods

2.3.1. 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^{o}C under a 5% CO_2 atmosphere. For growing MNV-1 stock, confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 0.1. After 1 h incubation at 37^{o}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^{o}C under a 5% CO_2 atmosphere. For growing TV stock, MK2-LLC cells 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^{o} 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.

2.3.2. 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 4ºC and centrifuged for 10 min at 5,000×g. The sample was filtered through a 0.45 μm filter, aliquoted and stored at −80ºC 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 was then quantified by real-time RT-PCR and the GII.4 isolate 5M was found to have 6.7×10^{6} genomic RNA copies/ml.

2.3.3. 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 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/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).

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

The purification of MNV-1 and TV was performed using the method described by Lou et al. (2011) with minor modifications. The virus suspension was centrifuged in a Sorvall SS-34 rotor (Kendro Laboratory Products, Germany) at 8,000 × *g* for 15 min to

remove cellular debris. The supernatant was then digested 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.

2.3.5. 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.

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

The imaging of viral attachment on fresh produce was executed using a protocol adapted from Rawsthorne et al., (2009). Pieces of Romaine lettuce leaves and roots were cut into approximately 2 cm squares using a scalpel and placed in the well of a 48-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.

2.3.7. 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 µl of a 10-fold dilution series of MNV-1 or TV, respectively, and the plates were incubated for 1 h at 37^{o}C with gentile 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% CO_2 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 ± standard deviation.

2.3.8. 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 1 second for annealing and 60°C for 20 seconds for extension. Standard curves and StepOne Software v2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as mean log10 genomic RNA copies/ml ± standard deviation.

2.3.9. Quantification of viral attachment to Romaine lettuce roots and shoots

Romaine lettuce 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. Samples were then inoculated with either MNV-1 or TV at a titer of 10^7, 10^6, and 10^5 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. After 1 h incubation, control samples were directly transferred to a new sterile stomacher bag. Samples subjected to washing, viral inoculation media was removed from stomacher bags and then lettuce samples were washed 5 times with 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. 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, the same experimental procedure used above was followed with a few exceptions. First, only one inoculation level was tested and all samples were treated with 1×10^7 RNA copy/g of human NoV strain 5M. Again the control samples received no wash, and treated samples were washed 5 times with either PBS or 200ppm chlorine. Following washing, samples were processed as above and quantification of viral genomic RNA was carried out using real time RT-PCR.

2.3.10. 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.

2.4. Results

2.4.1. Attachment of MNV-1, TV, and human NoV VLPs to the leaves and roots of Romaine lettuce

To visualize the attachment of virus to the surface of Romaine lettuce leaves and roots, a fluorescent streptavidin-labeled Quantum dots (Q-Dots) assay was developed.

The capsid proteins of the purified MNV-1, TV and human NoV VLPs were labeled with biotin using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce Biotechnology). Biotinylated viruses and VLPs were then applied to the surface of Romaine lettuce leaves and shoots, followed by the addition streptavidin coated Q-Dots 655, which emit fluorescence at a wavelength of 655nm. The flouescence of the biotinylated virus/streptavidin Q-Dot complex was then observed using a confocal microscope, allowing for visualization of the areas on the produce were viruses were bound.

The attachment of MNV-1, TV, and human NoV VLPs was observed on Romaine lettuce leaves at 100 × magnification, however there was no obvious differences in binding pattern (data not shown). When the magnification was increased to 400 ×, the viruses and VLPs are found to be aggregating in and around the stomata of the lettuce leaves (Fig. 10). The localization of signal in and around the stomata suggests that the viruses and VLPs are either binding more specifically to these structures or that the natural contours of the leaf leads to an increase in the localization of viruses and VLPs in the areas on the leaves where stomata are found. 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. 11).

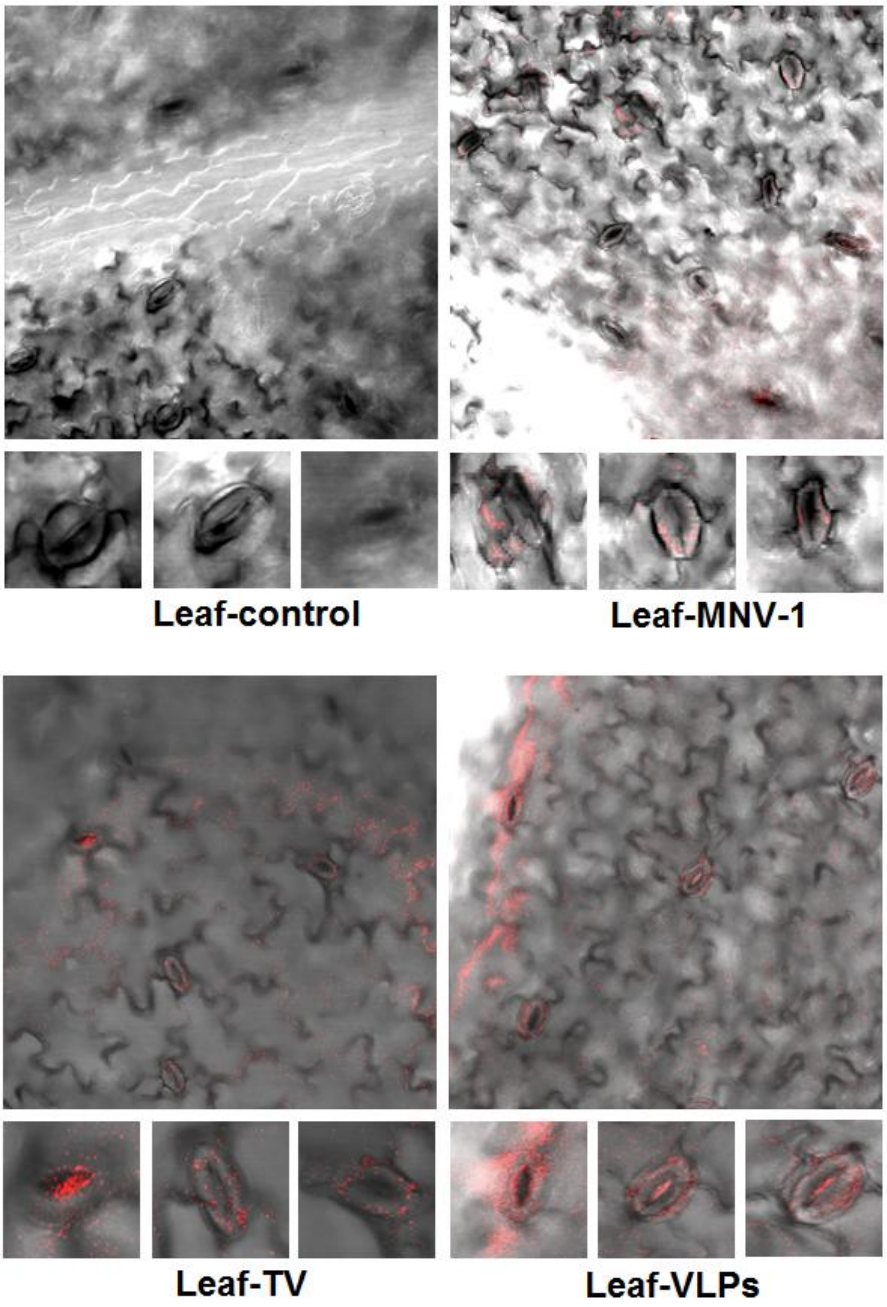


Figure 10. Confocal microscopic evaluation of the binding patterns of human NoV surrogates to the leaves of Romaine lettuce under 400 × magnification. Red signal indicates virus particles.

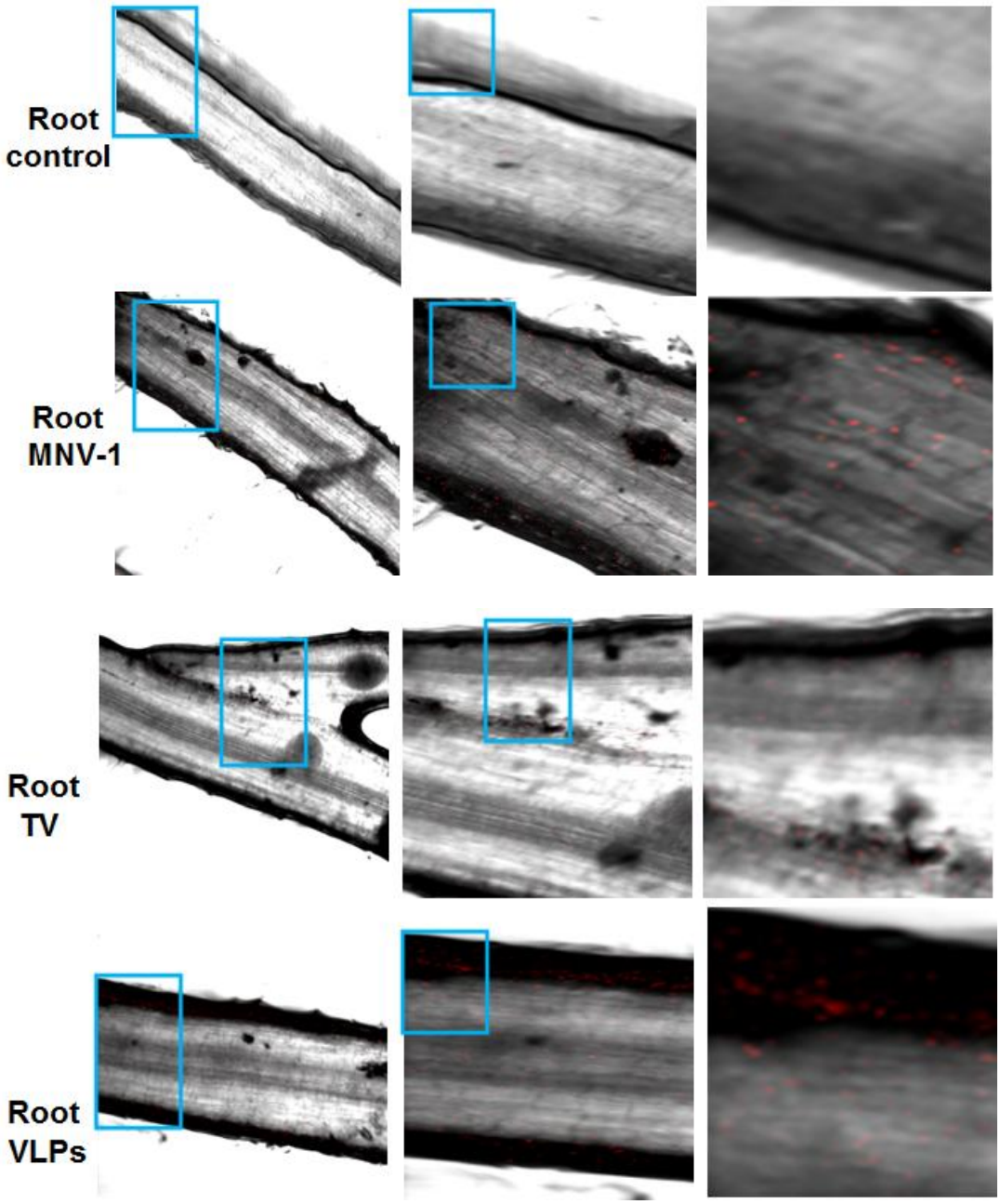


Figure 11. Confocal microscope evaluation of the binding patterns of human NoV surrogates to Romaine lettuce roots under 100 × magnification. Red signal indicates viral particles.

The visualization of attached MNV-1, TV, and human NoV VLPs to Romaine lettuce even after washing 5 times indicates that they are very tightly bound to both the roots and the leaves of the lettuce. The localization of virus and VLP signal in and around the stomata provides evidence of the feasibility for these plant structures to serve as a port of entry for the internalization of viruses. Also, this is the first evidence showing the attachment of viruses to the roots of Romaine lettuce. The roots of the lettuce serve as another potential site for the internalization of virus, and the ability of virus to bind tightly to roots may facilitate their internalization through the root pores.

In addition, the same method was used to visualize the attachment of viruses and VLPs to the surface of green onion shoots and roots. Fig. 12 shows the attachment of human norovirus surrogates to the 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 (Fig. 12). However, for MNV-1 and TV this pattern was diminished with the viruses being found covering the surface of the green onion cells (Fig. 13). This pattern was also observed by Rawsthorne et al., (2009) showing MNV-1 covering the entire green onion cell. The binding pattern of VLPs to green onion roots was also similar to that seen in the shoots (Fig. 13). The VLP signal was found in between the cells of the root epidermis (Fig. 13). A similar, but less pronounced pattern was also observed for MNV-1 (Fig. 13). However, TV was again found to be covering the entire surface of the root cell (Fig. 13).

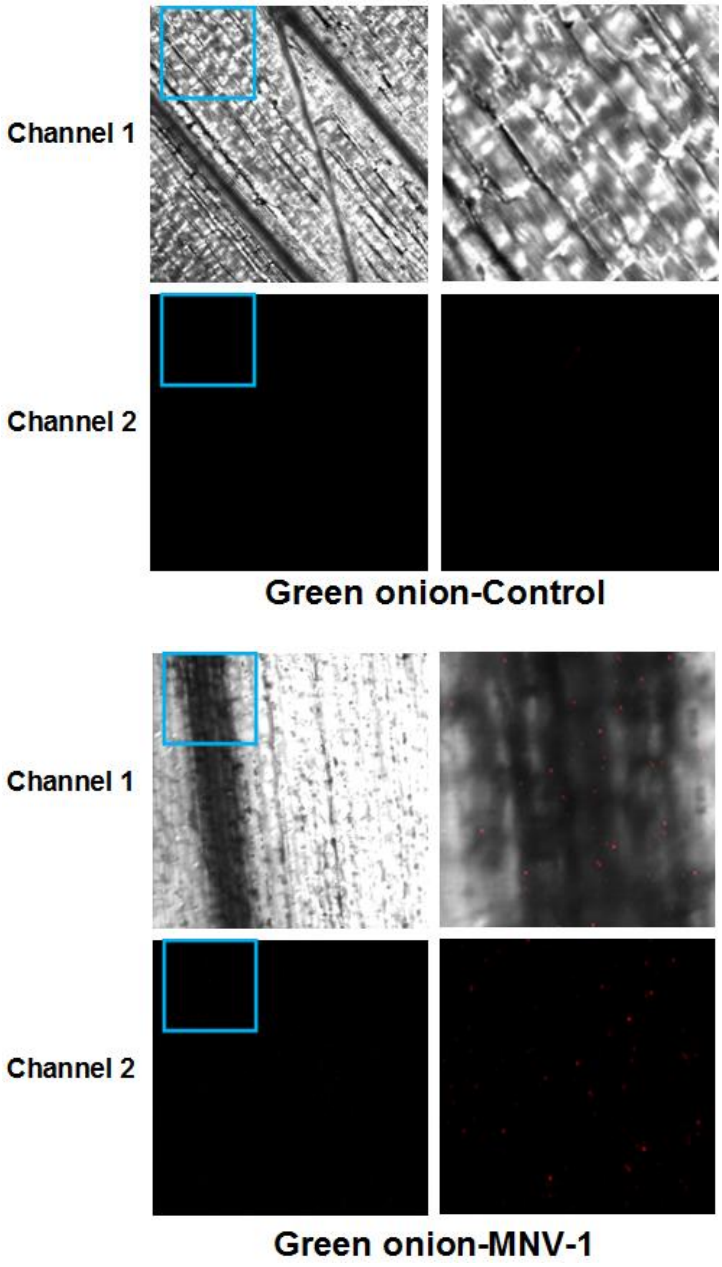
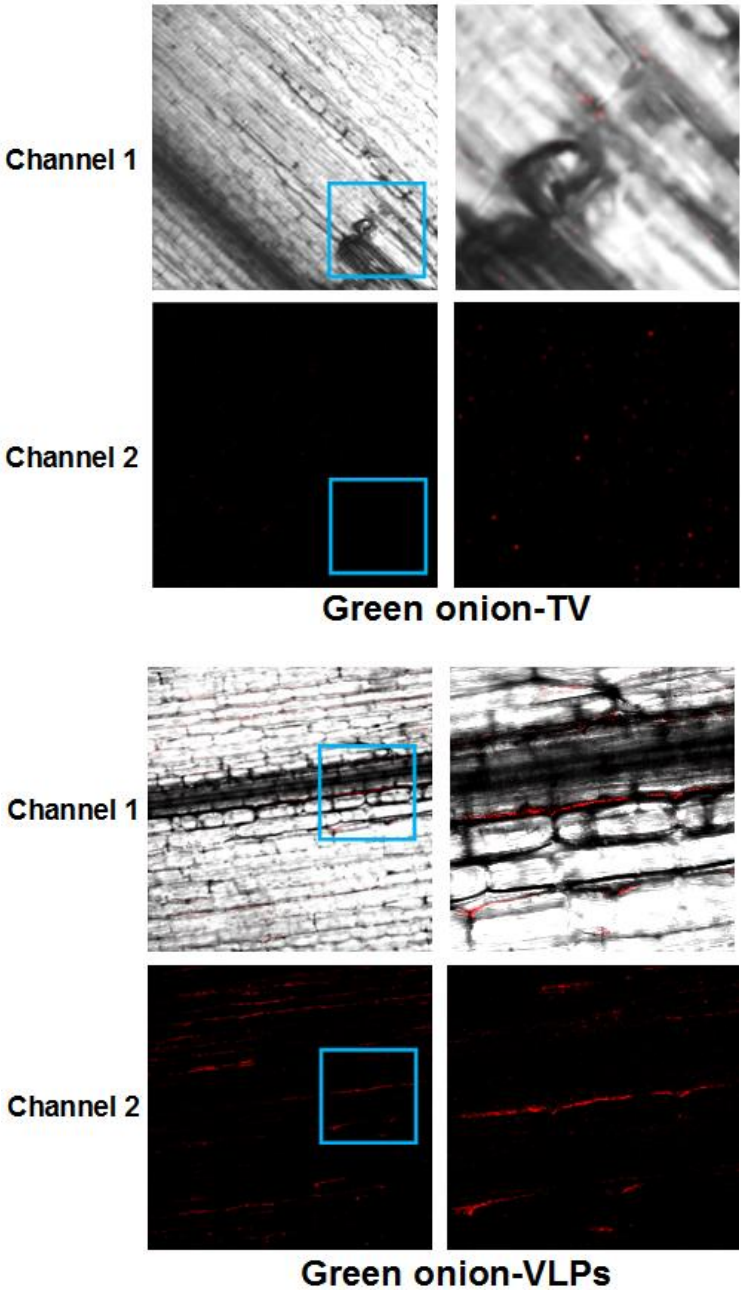


Figure 12. Confocal microscope evaluation of the binding patterns of human NoV surrogates to green onion shoots under 100 × magnification. Red signal indicates virus particles.

continued

Figure 12., continued



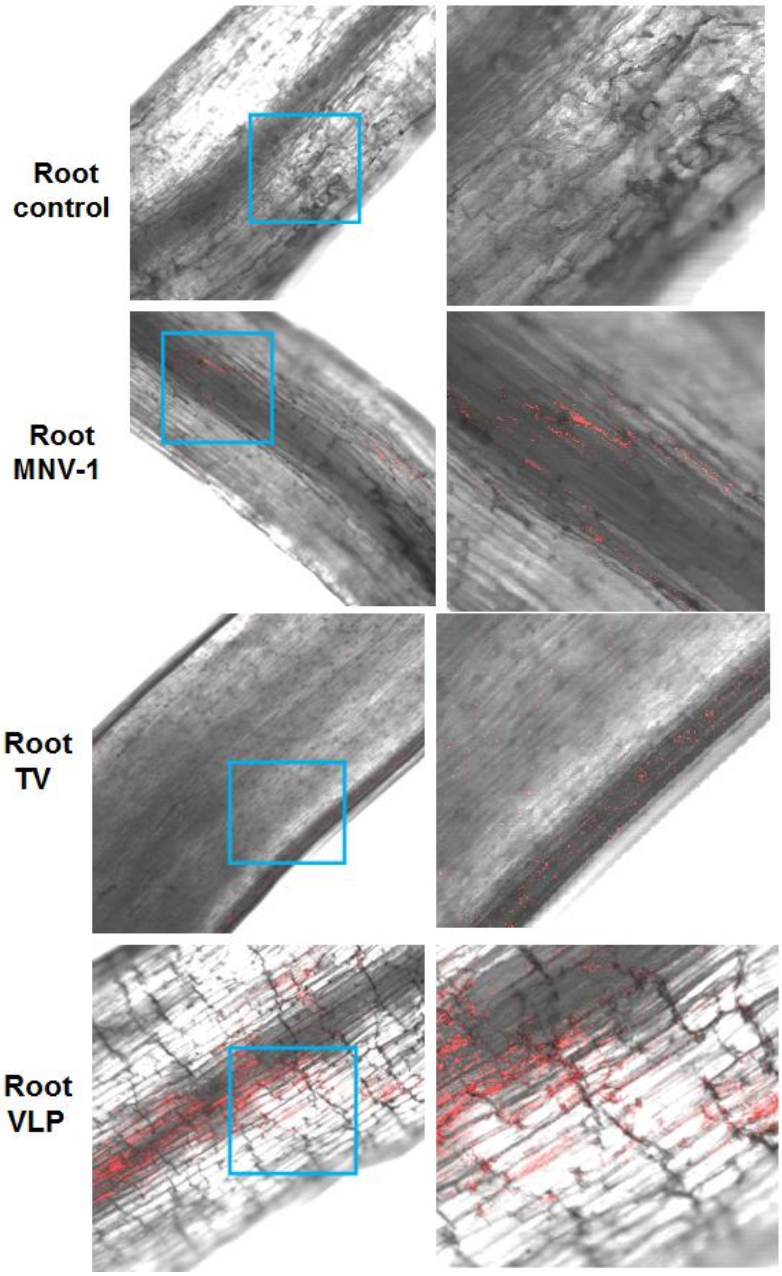


Figure 13. Confocal microscope evaluation of the binding patterns of human NoV surrogates to green onion roots under 100 × magnification. Red signal indicates virus particles.

Differences in the localization of each human NoV surrogate were observed in green onion shoots and roots. This result indicates that viruses differ in the mechanisms of persistence in green onions. For VLPs, the signal was localized in between cells which may physically protect them from removal by washing, however a binding interaction cannot be ruled out. For both MNV-1 and TV, the signal was found on the surface of the green onion cells of the shoots and roots, indicating that viral binding to the green onion surface was occurring.

2.4.2. Quantitative assessment of the level of attachment of MNV-1 and TV to Romaine lettuce roots and shoots

Romaine lettuce shoots and roots were inoculated with either MNV-1 or TV at a titer of 10^7, 10^6, and 10^5 PFU/g. Control samples received no washing and treated samples were rinsed 5 times with sterile PBS. All samples were then subjected to stomaching for 3 min and sample lysate was subjected to viral enumeration by plaque assay.

In Romaine lettuce shoots inoculated with MNV-1, the control inoculated with 10^7 PFU/g had a viral titer of 4.8×10^6 PFU/g after stomaching and the washing treatment had a viral titer of 3.2×10^6 PFU/g (Fig. 14A). There was no statistical difference between these groups ($P>0.05$). Similarly, lettuce shoots inoculated with 10^6 PFU/g with no wash had 5.5×10^5 PFU/g of MNV-1 present and with the wash had 8.7×10^5 PFU/g (Fig. 14A). Lettuce shoots inoculated with 10^5 PFU/g with no washing had viral titers of 4.5×10^4 PFU/g and with wash had 6.2×10^3 PFU/g (Fig. 14A). The additional washing treatment

did not have a significant effect on the viral titer detected. These results indicate that washing Romaine lettuce leaves with PBS does not have a significant effect (<0.5 log reduction) on removing MNV-1.

Fig. 14A.

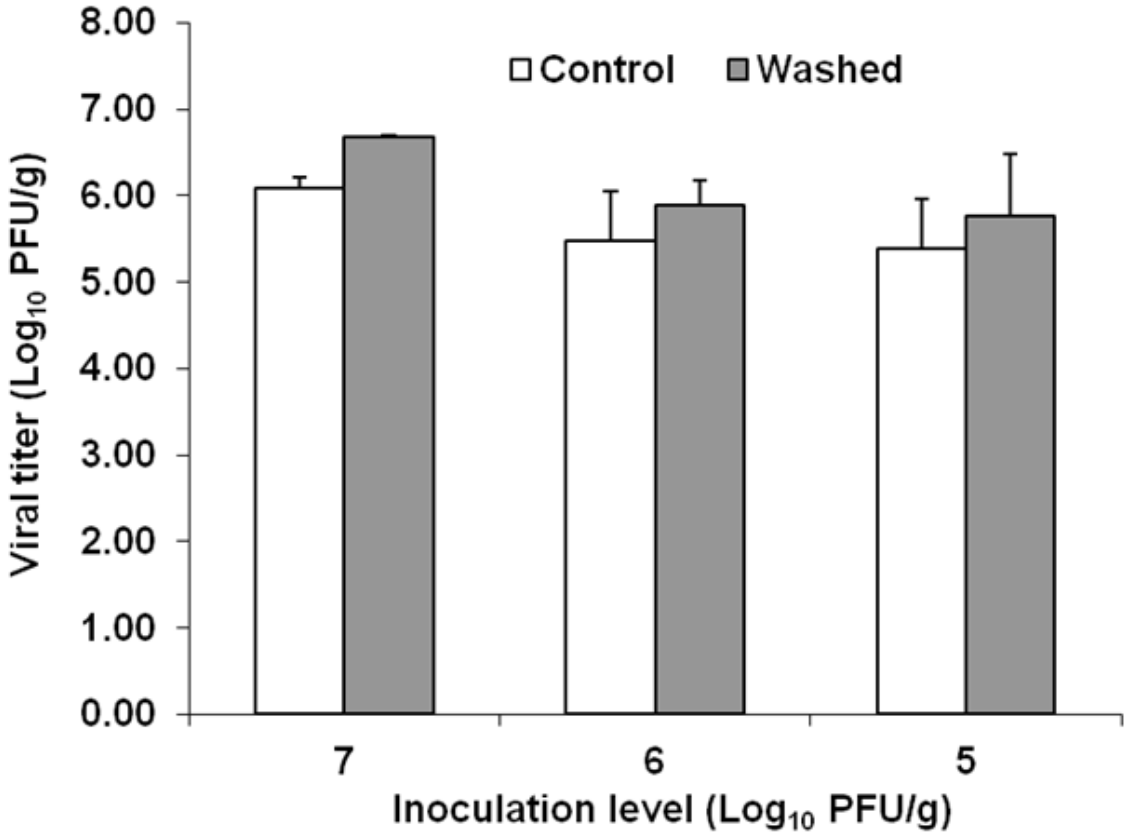
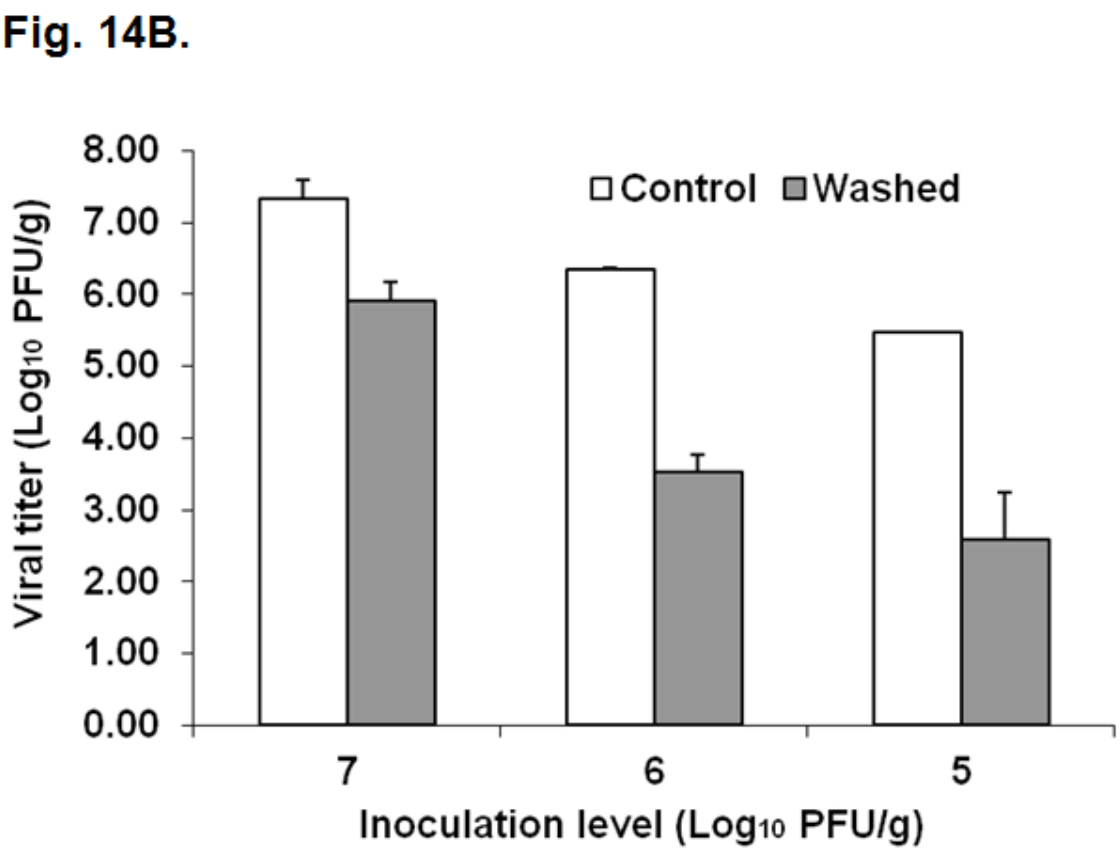


Figure 14. The effectiveness of PBS washing in removal of MNV-1 from Romaine lettuce shoots (A) and roots (B).

continued

Figure 14., continued



The effectiveness of washing on removal of MNV-1 in roots of lettuce as 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. 14B). In roots inoculated with 10^6 PFU/g of MNV-1 virus was detected at a level of 2.2×10^6 PFU/g and after washing the titer was reduced to 3.6×10^3 PFU/g which represents an almost 3 log reduction (Fig. 14B). Likewise, in roots

inoculated with 10^5 PFU/g of MNV-1 also had >2 log reductions in viral titer after washing (Fig. 14). In contrast to the results of washing lettuce shoots, it appears that simple washing can remove 1-3 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 6.2×10^6PFU/g and after washing the titer of the shoot was 8.7×10^5 PFU/g (Fig. 15A). Shoots inoculated with 10^6 PFU/g of TV had an initial titer of 3.9×10^5 PFU/g and after washing the titer was 3.4×10^4 PFU/g (Fig. 15A). Shoots inoculated with 10^5 PFU/g had an unwashed titer of 5.9×10^4 PFU/g and after washing the titer had dropped to 2.0×10^3 PFU/g (Fig. 15). In contrast to lettuce shoots inoculated with MNV-1, washing removed approximately 1 log of TV from lettuce shoots (Fig. 15A).

Fig. 15A.

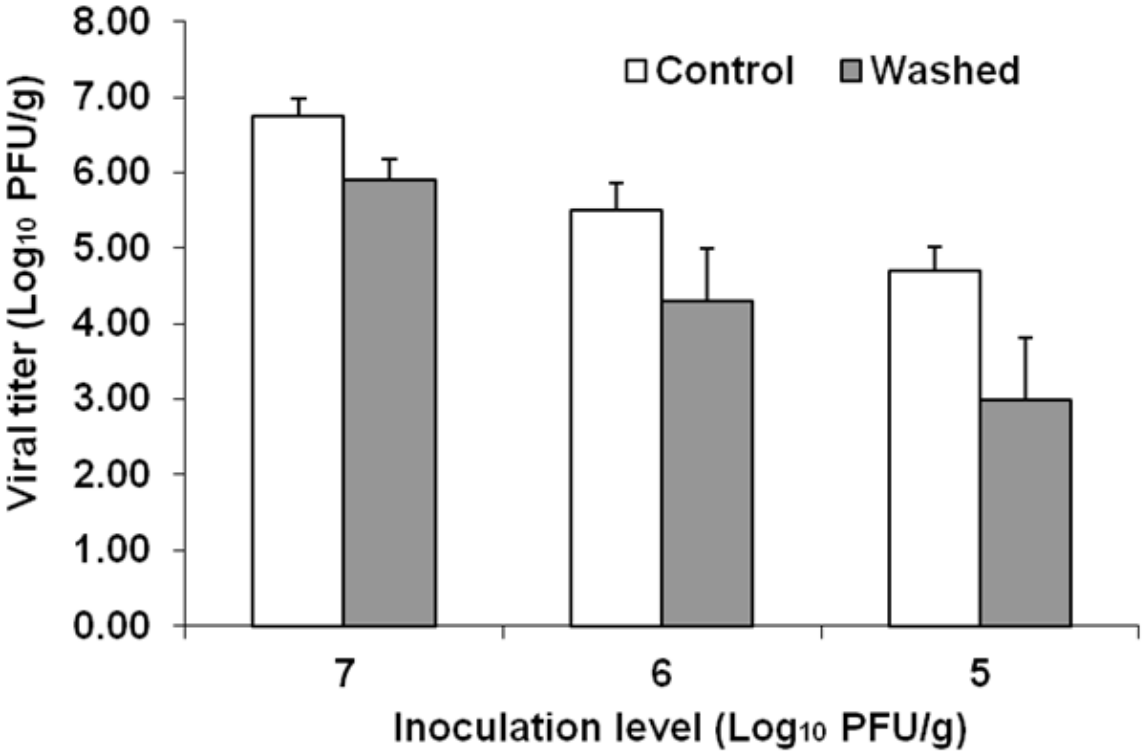
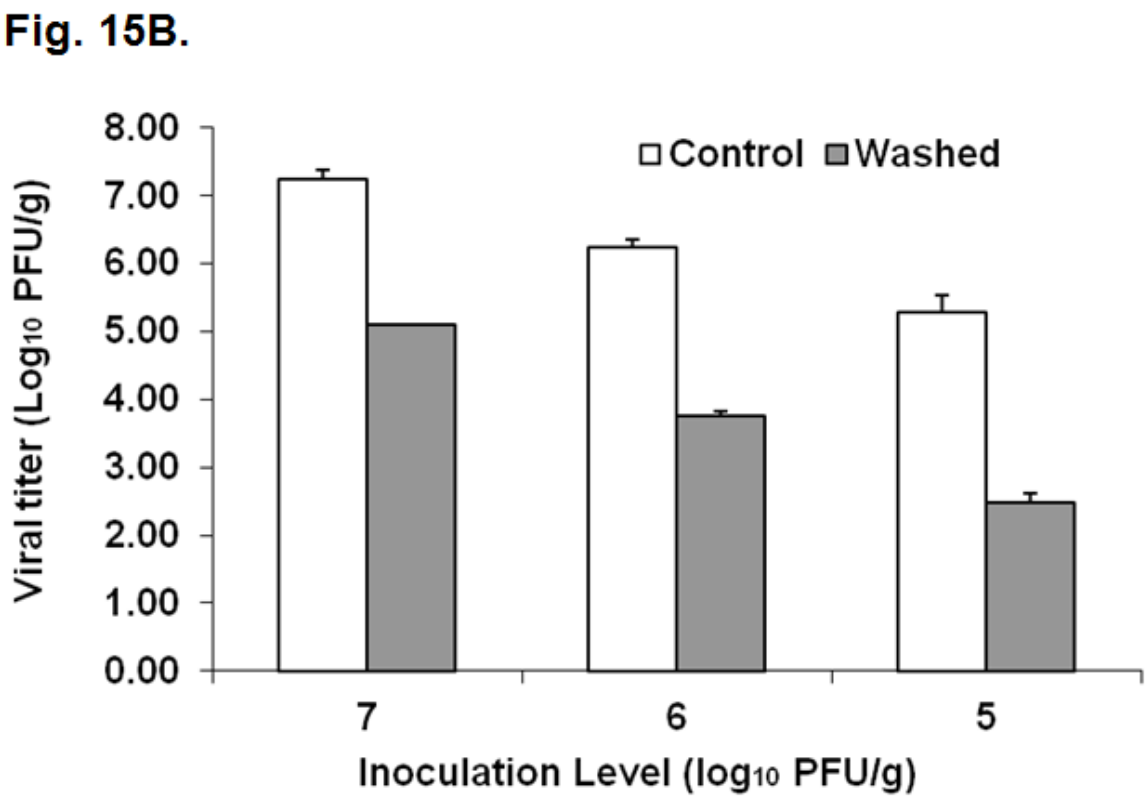


Figure 15. The effectiveness of PBS washing in the removal of TV from Romaine lettuce shoots (A) and roots (B).

continued

Figure 15., continued



Lettuce roots inoculated with 10^7 PFU/g of TV had an initial titer of 1.8×10^7 PFU/g and after washing the titer had decreased to 1.2×10^5 PFU/g (Fig. 15B). Roots incubated in the presence of 10^6 PFU/g of TV had a titer of 1.7×10^6 PFU/g and after washing a drop in titer to 5.6×10^3 PFU/g was observed (Fig. 15B). Similarly, when roots were inoculated with 10^5 PFU/g of TV the initial titer was 2×10^5 PFU/g and washing decreased the titer detected in the lysate to 3.1×10^2 PFU/g (Fig. 15B). Washing reduced to detectable titer of TV in roots by approximately 2-3 logs in all samples tested.

Taken together, this data indicates that (i) MNV-1 attaches more efficiently than TV to Romaine lettuce shoots; and (ii) both MNV-1 and TV were removed from roots more easily than from shoots.

2.4.3. Quantitative assessment of the level of attachment of a human NoV GII.4 strain and TV to Romaine lettuce shoots and roots

To determine the effectiveness of washing on the removal of human NoV 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. 16A). 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. 16A). The control group for Romaine lettuce roots inoculated with human NoV had a titer of 2.7×10^6 RNA copies /g (Fig. 16B). Washing roots with 200 ppm of chlorine did not have significant reduction in viral genomic RNA detected (2.0×10^6 RNA copies/g) (Fig. 16B). 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. 16B).

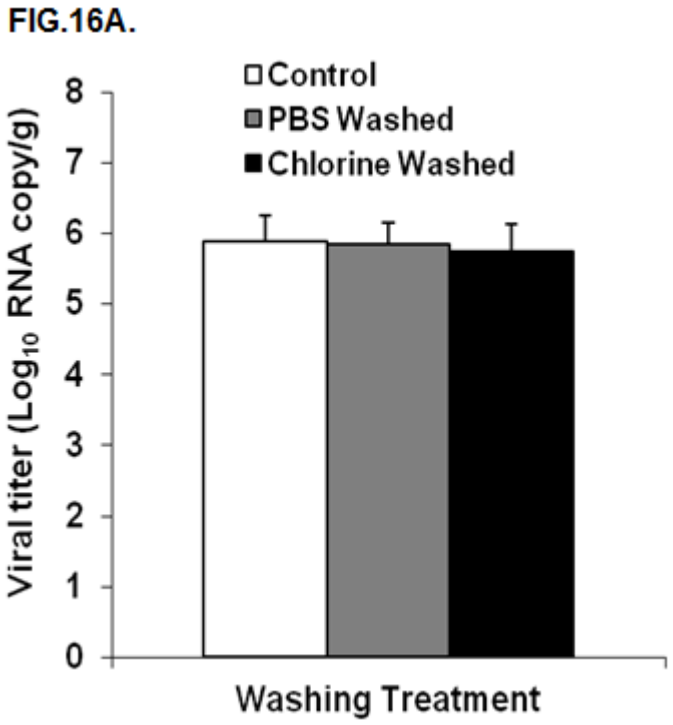
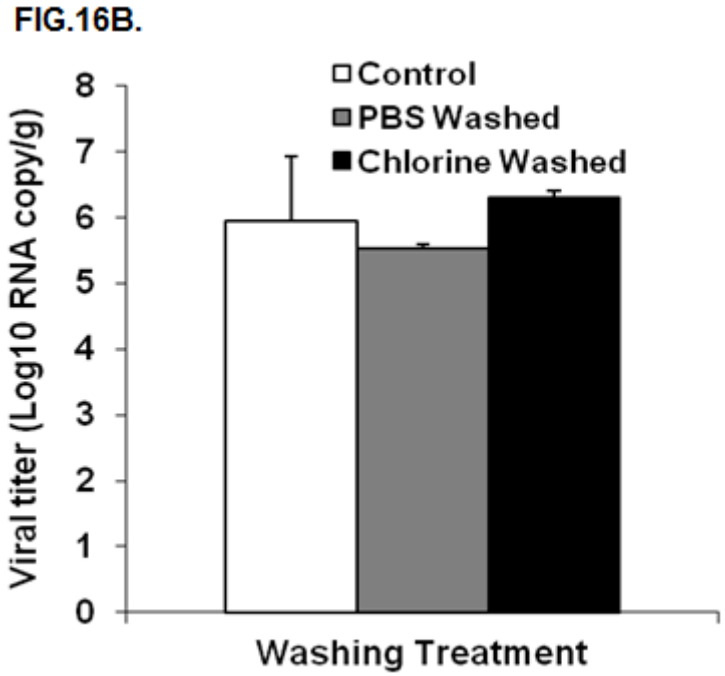


Figure 16. The effectiveness of PBS and 200ppm chlorine washing in the removal of a human NoV GII.4 strain from Romaine lettuce shoots (A) and roots (B).

continued

Figure 16., continued



The data indicates that (i) the human NoV GII.4 strain attaches tightly to Romaine lettuce shoots and roots, and (ii) 200ppm of chlorine and PBS wash are not effective in removing human NoV from Romaine lettuce shoots and roots.

2.5. Discussion

Despite the fact that human NoV accounts for more than 40% of fresh produce-associated outbreaks, the mechanism of human NoV persistence and the interaction of human NoV with fresh produce are poorly understood. In this study, a fluorescent based

Q-Dot assay was developed to visualize the attachment of viruses to Romaine lettuce and green onion. It was found that human NoV surrogates (MNV-1 and TV) and VLPs aggregated in and around the stomata on the lettuce leaves. In green onions, human NoV VLPs were observed aggregating between the epidermal cells of the green onion surface. However, both MNV-1 and TV were found to be attaching to the surface of green onion cells. These results indicate that each virus has an individually specific binding pattern which varies with different types of fresh produce.

It has been a challenge to visualize virus in fresh produce because of their small size and complexity of food matrix. To date, only three studies have successfully visualized the attachment of viruses to fresh produce. Rawsthorne et al., (2009) established a protocol for visualizing MNV-1 and hepatitis A virus in green onions utilizing purified virus conjugated with biotin and streptavidin-coated Q-Dots. It was found that both viruses attached to the surface of green onions. 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. 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. 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 were found to behave differently in green onions. Human NoV VLPs were observed aggregating between the epidermal cells of the green onion surface whereas MNV-1 and TV were found to be attaching to the surface of green onion cells. To our knowledge, this is the first evidence that different viruses have varying binding patterns depending on the variety of fresh produce.

Although the physical characteristics of the produce 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. It is known that human NoV utilizes the histo-blood group antigen (HBGA), a carbohydrate moiety, for receptor binding. Carbohydrate moieties are abundant in fresh produce, and carbohydrates constitute 90% (dry weight basis) of the plant cell wall (Esselli et al. 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, but VLP binding to Romaine lettuce was the highest compared to the other produce. Interestingly, denaturization of protein in extracts reduced the level of binding to human NoV VLPs to Romaine lettuce

extract, indicating that proteins were involved in the attachment of human NoV VLPs to the lettuce. Oxidation of the carbohydrates was found to increase the binding of the human NoV VLPs to the lettuce extract, further implicating the importance of proteins or other plant surface ligands in the attachment of NoV to lettuce.

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. Homogenates of cell wall extract and also isolates from the green leaf lamina and the principle vein were used to coat ELISA plates. The plates were subsequently treated with human NoV VLPs, followed by treatment with antibodies specific for the VLPs. It was found that the human NoV VLPs attached to the cell wall material from older (outer most) leaves and also the cell wall material from young (inner most) leaves of Romaine lettuce. Human NoV VLPs were also found to attach to the green leaf lamina and principle vein material isolated from Romaine lettuce. In this study 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.

In this study, the difficulty in removal of viruses from different portions of lettuce was compared. For MNV-1, it was found that simple washing removed less than 0.5 log of virus from lettuce shoots. For TV, simple washing achieved approximately 1 log virus

reduction in lettuce shoots. These results indicate that MNV-1 and TV have different affinities for attaching to Romaine lettuce. This could be due to the differences in the receptor binding of these two viruses. MNV-1 is known to bind to sialic acid residues, while TV binds to HBGAs, which is analogous to human NoV. It is possible that Romaine lettuce has more sialic acid analogs than HGBA-like molecules present on the surface. However, for both MNV-1 and TV, the removal of viruses from the roots was much more successful than from the shoots. Washing of roots incubated with MNV-1 and TV lead to 1-3 log and 2-3 log reduction respectively, depending on the virus inoculation level. It is likely that this may be related to the differences in the physical structures of shoots and roots. It is also possible that the lettuce shoot surface has more exposed carbohydrate moieties that resemble viral cellular receptors that allow for enhanced attachment.

Prior to this study, data was limited on the effectiveness of washing on the removal of human NoV from fresh produce. In this study, the effectiveness of a commonly used sanitizer (200ppm of chlorine) and PBS in removing a human NoV GII.4 strain from shoots and roots was determined. It was found that both chlorine solution and PBS were not effective in removing human NoV from lettuce shoots and roots. Less than 0.4 log RNA copies were removed from shoots and roots due to washing. In contrast, PBS solution was able to remove 1-3 logs and 2-3 logs of MNV-1 and TV from roots, respectively. This suggests that human NoV may be more difficult to remove from roots than MNV-1 and TV. These observations also indicate that human NoV and its

surrogates (MNV-1 and TV) may have different binding affinities to fresh produce although all of them use carbohydrate-like molecules as receptors.

In summary, we demonstrated that (i) a human NoV GII.4 strain attached efficiently to Romaine lettuce leaves and roots and that simple washing with PBS or 200ppm 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 may 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.

CHAPTER 3

Attachment, internalization, and dissemination of human norovirus and animal caliciviruses in hydroponically grown Romaine lettuce

3.1. Abstract

Fresh produce is a major vehicle for the transmission of human norovirus (NoV) because it is easily contaminated during both pre- and post-harvest stages however, the ecology of human NoV in fresh produce is poorly understood. In this study, we determined whether human NoV and its surrogates can be internalized via roots and disseminated to edible portions of the plant. The roots of Romaine lettuce growing in hydroponic feed water were inoculated with 1×10^6RNA copies/ml of human NoV GII.4 strain or $1\text{-}2\times10^6$PFU/mL of animal caliciviruses (Tulane virus, TV; and murine norovirus, MNV-1), and plants were allowed to grow for 2 weeks. Leaves, shoots, and roots were homogenized and viral titers and/or RNA were determined by plaque assay and/or real-time RT-PCR. For human NoV, high levels of viral genome RNA (10^5-10^6 RNA copies/g) were detected in leaves, shoots, and roots at day 1 post-inoculation and remained stable over the 14 day study period. For MNV-1 and TV, relatively low levels of infectious virus particles (10^1-10^3 PFU/ml) were detected in leaves and shoots at days 1 and 2 post-inoculation, but reached a peak titer (10^5-10^6 PFU/g) at days 3 or 7 post-inoculation. In addition, human NoV had a rate of internalization comparable with TV as

determined by real-time RT-PCR. Whereas, TV was more efficiently internalized than MNV-1 as determined by plaque assay. Taken together, these results demonstrated that human NoV and animal caliciviruses attached tightly to roots, became internalized via roots, and efficiently disseminated to the shoots and leaves of the lettuce.

3.2. Introduction

The Caliciviridae family includes a number of enteric viruses that cause gastroenteritis in humans and animals. Examples of these viruses include human norovirus (NoV), human sapovirus, and the newly discovered monkey calicivirus (Tulane virus, TV). Human NoV is the leading cause of nonbacterial gastroenteritis worldwide, contributing to over 95% of all non-bacterial acute gastroenteritis each year, and more than 60% of all foodborne illnesses reported annually (Atmar et al.,2008). The virus is highly infectious, resistant to common disinfectants, and has a low infectious dose (Wei et al., 2010, 2011). However, human NoV remains difficult to study because it cannot be grown in cell culture and it lacks a small animal model (Atmar et al., 2008). For these reasons, human NoV is classified as a Category B biodefense agent by the National Institute of Allergy and Infectious Diseases (NIAID).

In recent years, the consumption of fresh produce has increased as individuals strive to maintain a healthy diet. However, disease surveillance has shown that vegetables and fruits are major vehicles for the transmission of human NoV, since they normally undergo little or no processing and are easily contaminated pre- and post-harvest through irrigation, fertilizers, soil, wildlife, domestic animals, packaging, and

food handlers (Abbaszadegan et al., 2003, Doyle and Erickson, 2008, Heaton and Jones, 2008, Lynch et al. 2009, Rawsthrone et al. 2009). It has been reported that norovirus accounts for more than 40% of outbreaks caused by fresh produce in the US annually (Seymour, 2001). Fresh-produce related outbreaks caused by noroviruses have been reported in lettuce, salad, fruit salad, tomato, carrot, melon, strawberry, raspberry, orange juice, fresh cut fruit, coleslaw, spring onion, and other vegetables (Abbazadegan, 2003, Doyle and Erickson, 2008, Heaton and Jones, 2008, Lynch et al., 2009, Rawsthorne et al., 2009). In another survey it was found that, salads, lettuce, and fruits contributed 67%, 47%, and 67% respectively, to human norovirus gastroenteritis in the US from 1990-2005 (DeWaal and Bhuiya, 2007). Increasing outbreaks of viruses in fresh produce gives high urgency to understanding the ecology of enteric viruses in vegetables and fruits and the mechanism of viral contamination and persistence in fresh produce.

Internalization of pathogens is considered one of the major routes for contamination of fresh produce. It has been well established that foodborne bacterial pathogens such as *E. coli.* O157:H7 and *Salmonella* sp., become internalized and disseminated in plant crops, including lettuce, spinach, tomato, and mung bean shoots via the plant root systems, through wounds in the cuticula, or through stomata (Bernstein et al., 2007, Jablasone et al., 2005, Aruscavage et al., 2008). The efficiency of the internalization of bacterial pathogens in plants can be affected by many factors such as the type of plant, plant stress, bacterial species and strains, bacterial dose, and environmental humidity and temperature (Bernstein et al., 2007, Doyle and Erickson, 2008, Jablasone et al., 2005, Seymore and Appleton, 2001, Aruscavage et al., 2008).

However, the penetration, uptake, internalization, dissemination, and persistence of foodborne viruses in plants is poorly understood. The feasibility of internalization of human enteric viruses by plants is supported by the ability of plants to internalize their own viral pathogens, which can be taken up from soil and water. As the size of a virus is approximately 1000 times smaller than bacteria, in theory, the efficiency of a smaller pathogen to enter and disseminate in plants would be elevated. Since human enteric viruses may be present in sewage-contaminated soil or water, they may also be taken into the plant through the roots and/or leaves. The dissemination of the viruses via the vascular system of the plant could also facilitate movement of the virus from the inedible portions of the plant (roots) to the edible portions of the plant (leaves).

To date, only two studies have examined whether human NoV and its surrogates can be internalized and disseminated in plants. Urbanucci et al., (2009) found that canine calicivirus (CaCV) RNA could be detected in the aerial tissues of lettuce grown both hydroponically and in soil, though not all samples in the treatment groups tested positive. In contrast, when a human NoV G.II strain was used under the same experimental conditions; no viral RNA in the lettuce was detected even when challenged with a high level of human NoV (Urbanucci et al.,2009). Most recently, Wei et al., (2011) found that less than 2 logs of infectious MNV-1 could be detected in leaf samples from days 1 to 5 when the roots were challenged with high level of MNV-1 (5×10^{8}PFU/ml). However, no infectious virus was detected when the roots were challenged with low level of MNV-1 (5×10^{5}PFU/ml). Furthermore, infectious MNV-1 was undetectable when lettuce was grown in soil even inoculated with high level of MNV-1 (5×10^{8}PFU/ml) (Wei et al.,

2011). These two studies demonstrated that low levels of virus internalization of human NoV surrogates, such as MNV-1 and CaCV, can occur in growing lettuce. However, based on Urbanucci's 2009 study, it seems that human NoV cannot be internalized via roots and disseminated to leaves of lettuce. The basis for the differences seen in the rate of internalization between human NoV and its surrogates has not been elucidated.

The objectives of this study were to determine the attachment of human NoV to the roots of lettuce and to evaluate the internalization and dissemination of human NoV in hydroponically growing lettuce using a GII.4 human NoV strain, which is currently the prevalent strain circulating in many countries. In addition, we compared the efficiency of viral internalization and dissemination of different caliciviruses (MNV-1, TV, and human NoV) in lettuce.

3.3. Materials and Methods

3.3.1. Viruses and cell culture

Murine norovirus strain MNV-1 was generously provided by Dr. Herbert W. Virgin IV, Washington University School of Medicine. Tulane virus 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)

(Invirogen), at 37^{o}C in a 5% CO_2 atmosphere. For growing MNV-1 stock, confluent RAW 264.7 cells 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), supplemented with 2% FBS, at 37^{o}C in a 5% CO_2 atmosphere. For growing TV stock, MK2-LLC cells 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 1006 × g for 30 min.

3.3.2. Plant cultivation for hydroponic growth

Seeds of romaine lettuce (Lactuca sativa) were planted in 2 inch plug trays and grown under greenhouse conditions. Twenty days after germination, plants were removed from the soil and inserted in the hydroponic growth system. The hydroponic feed water was supplemented with a nutrient solution containing nitrogen, phosphorus, and potassium. The feed water was also supplemented with 1% penicillin, kanamycin, and streptomycin to control microbial growth. After viral inoculation, the plants were grown in the lab under a fluorescent light cycle of 12 hours light and 12 hours darkness. The temperature and relative humidity was maintained at 20^{o}C and 40%, respectively.

3.3.3. Viral inoculation and sample collection

The hydroponic feed water was inoculated with either MNV-1 or TV. The total volume of the hydroponic feed water reservoir was 100 ml which was inoculated with 5 ml of viruses having a starting titer of 1×10^6 PFU/ml. Controls received no viral inoculation in feed water. At days 0 (before viral inoculation), 1, 2, 3, 7, and 14 the leaves, shoots, and roots were harvested and weighed. The samples were homogenized by freezing with liquid nitrogen and grinding with a mortar and pestle. Homogenized tissue was resuspended in 5 ml phosphate buffered saline (PBS, pH 7.0). Sample homogenates were centrifuged at 1000 × g to remove cellular debris and the virus containing supernatant was transferred to a new collection tube for viral enumeration by plaque assay. At days 0, 1, 2, 3, 7, and 14, 500 µl samples of feed water were collected for determination of viral titer by plaque assay. For chlorine treated samples, following harvest each tissue was submerged in a 50 ml conical tube containing 1000 ppm chlorine and incubated at room temperature for 5 min. After chlorine wash, samples were placed in a new 50mL tube containing tap water and submerged for 5 min with gentile agitation. Following tap water wash, samples were placed in a 50 ml tube containing 0.25 M sodium thiosulfate to neutralize residual chlorine. All solutions were changed between samples to maintain the oxidation potential of the chlorine solution. Samples were then homogenized and processed as described above. For human NoV, the feed water was inoculated to a starting concentration of 1×10^6 RNA copies/ml, while controls received no viral RNA. Sample collection methods were the same as above. Quantification of viral genomic RNA was executed using RT-qPCR.

3.3.4. 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 µl of a 10-fold dilution series of MNV-1 or TV, respectively, and the plates were incubated for 1 h at 37^{o}C with gentile agitation every 10 min. The cells were overlaid with 3 ml of Eagle's minimum essential medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg kanamycin/ml, 0.05 mg 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 ± standard deviation.

3.3.5. Quantification of viral RNA by real-time RT-PCR

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 1 second for annealing and 60°C for 20 seconds for extension. Standard curves and StepOne Software v2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as mean log10 genomic RNA copies/ml ± standard deviation.

To compare the internalization rate between human NoV and Tulane virus, Tulane virus 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 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 with human NoV.

3.3.6. RNase treatment of lettuce tissues

Following harvest, processed Romaine lettuce samples were stored at -80°C. Samples were then thawed and 100µl aliquots were incubated with (0.5µg/µl) of RNase (Invitrogen) at 37°C for 1 hr. Samples were subjected to RNA extraction using the RNeasy Kit (Qiagen), followed by real time RT-PCR using the procedures listed above.

3.3.7. 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

3.4. Results

3.4.1. TV was efficiently internalized and disseminated in Romaine lettuce grown hydroponically

The TV feed water in the reservoir for Romaine lettuce hydroponic growth had a starting titer of 1.25×10^6 PFU/ml. To prevent contamination, the leaves, shoots, and roots of lettuce were harvested separately at days 0, 1, 2, 3, 7, and 14 after viral inoculation (Fig.17). The kinetics of the internalization and dissemination of TV was monitored. TV was detected in leaves as soon as day 1 post-inoculation with an average titer of 6.1×10^1 PFU/g. The viral titer in the leaves gradually increased through day 14 (Fig. 18). At day 7 post-inoculation, the viral titer reached 9.8×10^5 PFU/g, which was significantly higher than days 1, 2, and 3 (Fig. 18). The TV titer in the leaves on day 14

was 6.3 $\times 10^5$, which was comparable to day 7 (Fig. 18). Similarly, infectious TV was also detected in the shoots on all days tested, with a viral titer in the shoots of 7.8 $\times 10^3$ PFU/g on day 1 (Fig. 18). The viral titer gradually increased and reached a peak titer of 2.4×10^6 PFU/g on day 7. The TV titer in the shoots on day 14 was 1.3×10^6, which was a slight decrease compared to day 7. During the experimental period, the viral titer in shoots was significantly higher than that in leaves ($P<0.05$). As expected, TV was detected lettuce roots since they were in direct contact with virus-contaminated feed water. On day 1, the titer in roots was 1.5×10^5 PFU/g, and increased in titer until day 14 (Fig. 18). The TV titer in the roots on day 7 and 14 was 1.2×10^6 PFU/g and 1.0×10^6 PFU/g, respectively., and the viral titer in shoots at day 7 was higher than that found in roots ($P<0.05$). These results suggest that TV efficiently attached to roots, internalized in roots, and disseminated into shoots and leaves of the lettuce. Concurrently, the titer of the feed water was also monitored each day until the plants were harvested. Consistent with the increasing viral titer in lettuce, the titer of the TV in the feed water gradually decreased during the experimental period. On days 1, 2, 3, 7, and 14, the titer of the feed water was 3.75 $\times 10^5$, 7.5×10^5, 3.5 $\times 10^4$, 7.5×10^4, and 5.0×10^3 PFU/ml, respectively. To further confirm that the decreasing titer in feed water was due to internalization via roots and not to the instability of TV in feed water, TV was diluted in feed water (without lettuce) and viral titer was monitored until day 14. TV was found to be highly stable in the feed water alone over the 14 day period with no significant reduction in viral titer (data not shown). Taken together, these results suggest that TV was internalized via roots and disseminated to shoot and leaf portion of the plants.

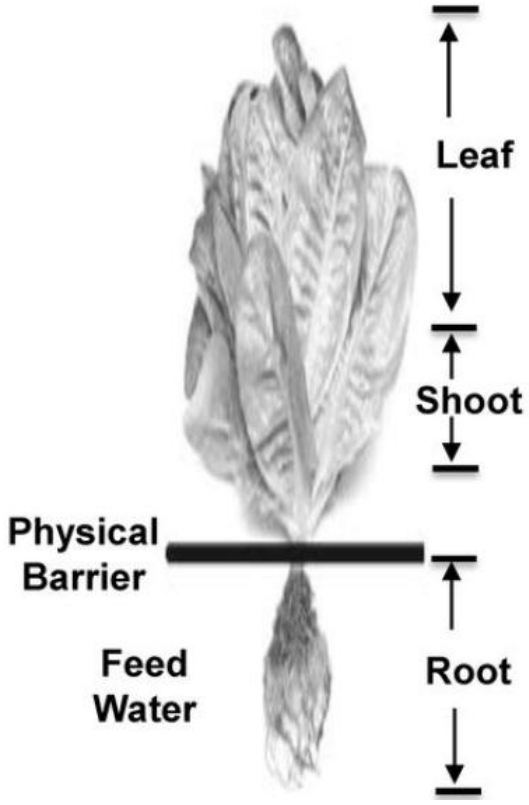


Figure 17. Schematic of harvesting procedure of Romaine lettuce. Leaf tissue represents the aerial tissues of the lettuce starting 2 inches above the root juncture and was harvested first. Shoot tissue represents the 2 inch portion of the aerial tissue connected to the root juncture and was not in contact with the feed water, which was harvested second. Root tissue consists of all lettuce roots and was in direct contact with the feed water and was harvested third.

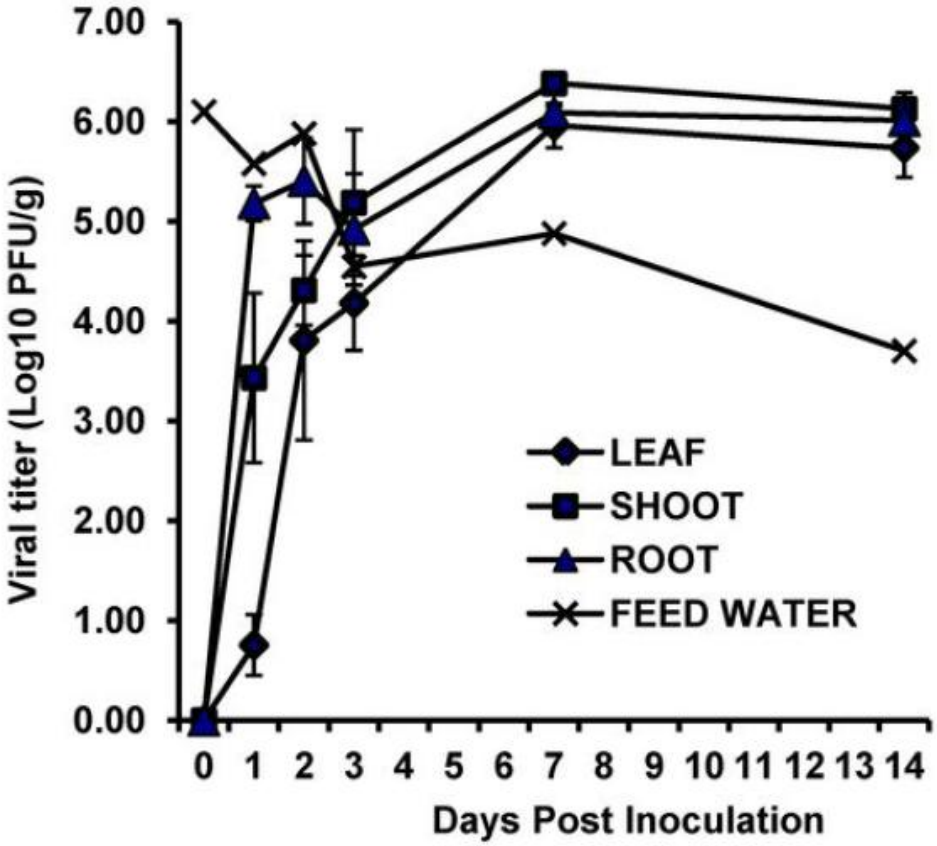


Figure 18. Internalization of TV in Romaine lettuce grown hydroponically. Viral titer is reported as PFU/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

As the plants were grown hydroponically, it is possible that the shoots and leaves of lettuce may have been contaminated by virus moving on the external surface of the plant through capillary action. To exclude this possibility, we an identical experiment where the harvested plant tissues were submerged in 50 ml of 1000 ppm chlorine for 5

min was performed. It was found that TV was completed inactivated when incubated with 1000 ppm of chlorine for 2 min (data not shown). Theoretically, treatment of lettuce with 1000 ppm of chlorine for 5 min should be sufficient to inactivate any virus that may present on the surface of the shoots and leaves. As shown in Fig. 19, there were no significant differences observed in TV internalization in chlorine treated shoots and leaves on any of the study days compared to the untreated samples ($P>0.05$) during the experimental period. However, there was a significant difference in the detection of TV between untreated roots and chlorine treated roots on day 1 ($P<0.05$). Presumably, this is due to the inactivation of the surface virus by chlorine because roots directly contacted the virus-contaminated feed water. However, there were no differences in TV internalization in the chlorine treated roots on days 2, 3, 7, or 14 compared to the roots receiving no treatment ($P>0.05$). This experiment confirmed that TV was indeed absorbed by roots and disseminated to shoots and leaves of lettuce.

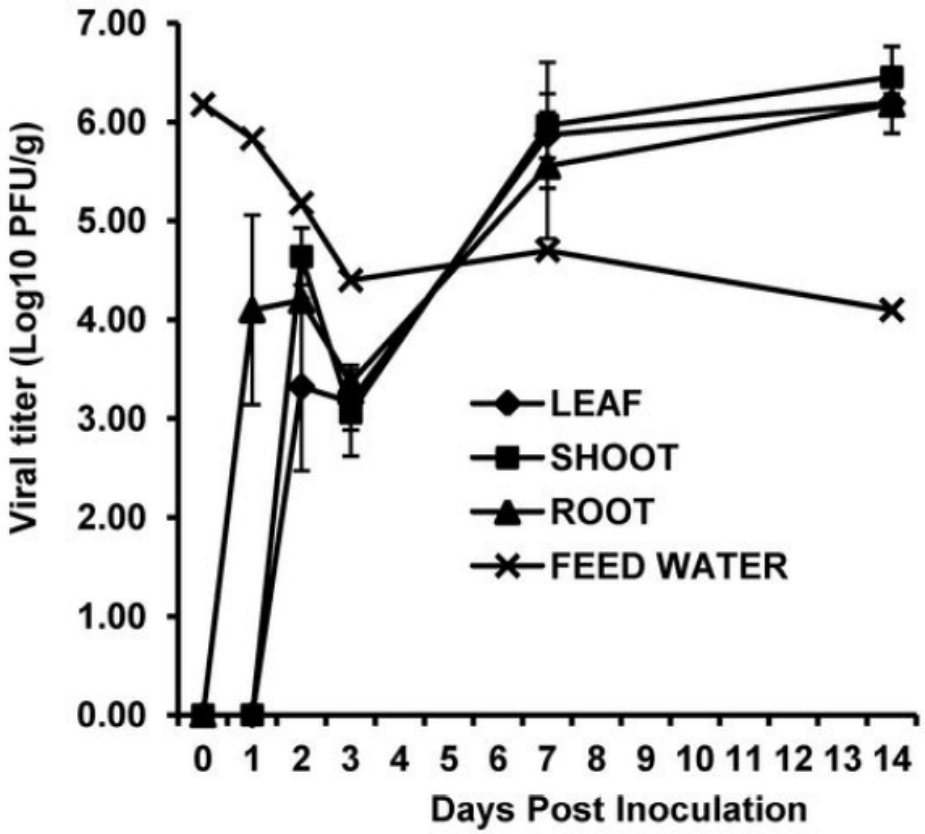


Figure 19. Chlorine treatment of lettuce tissue after TV internalization and dissemination. Viral titer is reported as PFU/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

3.4.2. Internalization and dissemination of MNV-1 in Romaine lettuce grown hydroponically.

The kinetics of MNV-1 internalization in lettuce was also determined. The starting titer (day 0) of MNV-1 feed water in the reservoir for hydroponically grown Romaine lettuce was 2.5×10^6 PFU/ml. The experimental design was identical to that

described above. Fig. 20 shows the dynamics of MNV-1 titer in leaves, shoots, roots, and feed water. In leaf tissues, MNV-1 was detected on days 1, 2, 3, 7, and 14 using plaque assays. On day 1, the viral titer detected in the leaves was 5.9×10^{1} PFU/g and increased to 3.3×10^{5} PFU/g on day 3, and remained at this level for the duration of the study (Fig.20). Similarly, all shoots harvested from days 1 to 14 were positive for infectious MNV-1. On day 1, 5.9×10^{1} PFU/g of MNV-1 was detected in the shoots and increased until day 3 to 3.3×10^{5} PFU/g, and again the level of virus detected in the shoots remained stable until day 14 (Fig. 20). All plaque assay results for roots were positive. MNV-1 was detected in the roots on day 1 at 6.5×10^{3} PFU/g and increased until day 3 to reach a titer of 2.5×10^{5} PFU/g, and the MNV-1 titer was maintained in the roots until day 14 (Fig. 20). MNV-1 titer in the feed water gradually decreased. The initial titer (day 0) in feed water was 2.5×10^{6} PFU/ml. On day 1, the titer decreased to 2.5×10^{5} PFU/ml and on day 2, the titer was further decreased to 2.5×10^{4} PFU/ml, and maintained similar titer until day 14 (Fig. 20). As a control, MNV-1 titer was not significantly decreased in feed water without lettuce (data not shown). This result indicates that the decreasing titer in feed water of growing lettuce was due to the internalization of MNV-1 via roots of lettuce, and not to the instability of MNV-1 in feed water. The starting titer of the feed water of both TV and MNV-1 was comparable. However, there was a significantly higher titer of TV detected in the roots on days 1, 7, and 14 compared to MNV-1 titer in roots. The TV titer detected in the shoots on days 7 and 14 was also significantly higher than the MNV-1 titer detected in shoots. However, the TV titer in the leaves was only

significantly higher than MNV-1 on day 7. These results indicate that TV was more efficient in attachment, internalization, and dissemination in lettuce than MNV-1.

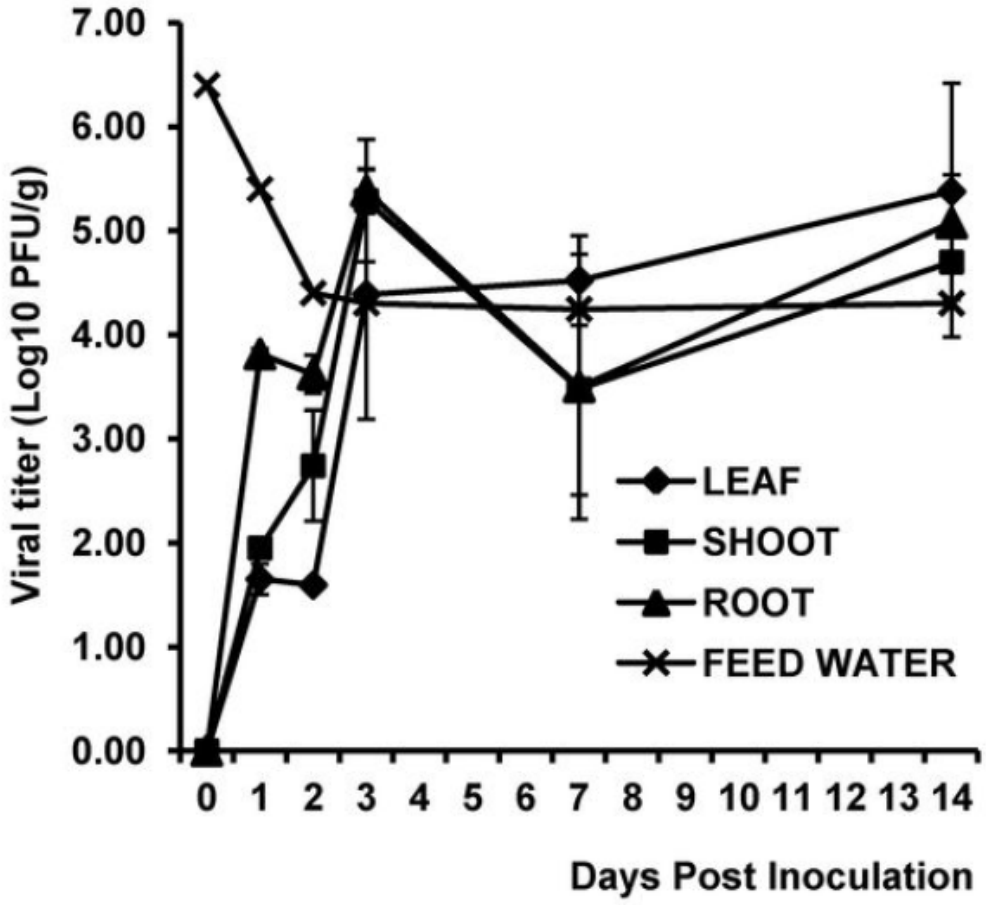


Figure 20. Internalization of MNV-1 in Romaine lettuce grown hydroponically. Internalization kinetics plot was determined by plaque assay and results are reported as PFU/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

3.4.3. Internalization and dissemination of human NoV in Romaine lettuce grown hydroponically

To determine the rate of human NoV internalization, Romaine lettuce was grown hydroponically and the feed water source was inoculated with human NoV GII.4 isolate 5M at a starting titer of 2.9×10^6 RNA. The experimental design and procedures were identical as described above. The kinetics of viral RNA in leaf, shoot, root, and feed water was quantified by real-time RT-PCR. A high level of human NoV RNA (6.9×10^5 RNA copies/g) was detected in the leaf tissue of the lettuce on day 1 post inoculation and the human NoV RNA detected in the leaves remained stable over the 14 day study period (Fig. 21). Human NoV RNA was also detected in the shoots of lettuce on day 1 post inoculation at a titer of 2.1×10^6 RNA copies/g (Fig. 21), which was significantly higher than that in leaves ($P<0.05$). Similarly, the RNA copies detected in the shoots remained stable over the study period to a final titer of 4.4×10^5 RNA copies/g on day 14 (Fig. 21). Root samples were also positive for human NoV RNA on day 1 post inoculation with a titer of 3.9×10^5 RNA copies/g (Fig. 21). The human NoV RNA detected in the roots reached a peak titer (3.15×10^6 RNA copies/g) at day 3 post-inoculation and decreased to 1.95×10^4 RNA copies/g on day 14. The human NoV RNA copies present in the feed water gradually decreased to a final titer of 1.8×10^5 RNA copies/mL on day 14 (Fig. 21). These results demonstrated that human NoV was efficiently internalized and disseminated in lettuce grown hydroponically.

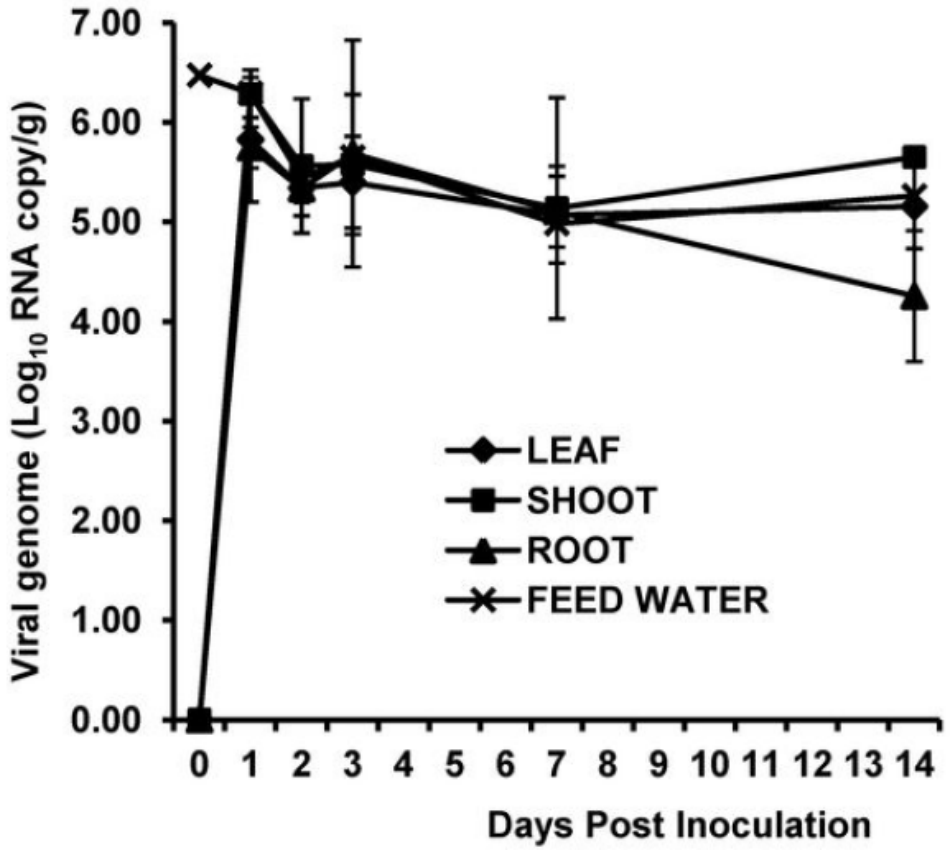


Figure 21. Detection of internalized human NoV GII.4 RNA in Romaine lettuce grown hydroponically. Internalization kinetics plot was determined by RT-qPCR and results are reported as RNA copies/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

Subsequently, the internalization rate between human NoV and Tulane virus was compared. Tulane virus was quantified by both plaque assay and real time RT-PCR. As shown in Fig. 22, Tulane virus RNA was detected at a high titer in the leaves (1.9×10^6 RNA copies/g) on day 1 post inoculation and remained stable over the 14 day study

period. Similarly, the RNA detected in the shoots was also detected at day 1 post inoculation at a titer of 1.2×10^6 RNA copies/g (Fig. 22). The TV RNA detected in the shoots also remained stable over the 14 day study period, with no significant change in the RNA detected throughout the study ($P>0.05$). TV RNA was also detected in the roots of lettuce on day 1 post inoculation at a titer of 3.2×10^6 RNA copies/g (Fig. 22). The RNA titer found in the roots remained stable over the 14 day study period, and was similar to the results obtained for RNA copy in the leaf and shoot tissue. Tulane virus RNA copies present in the feed water gradually decreased to a final titer of 1.8×10^5 RNA copies/ml on day 14 (Fig. 22).

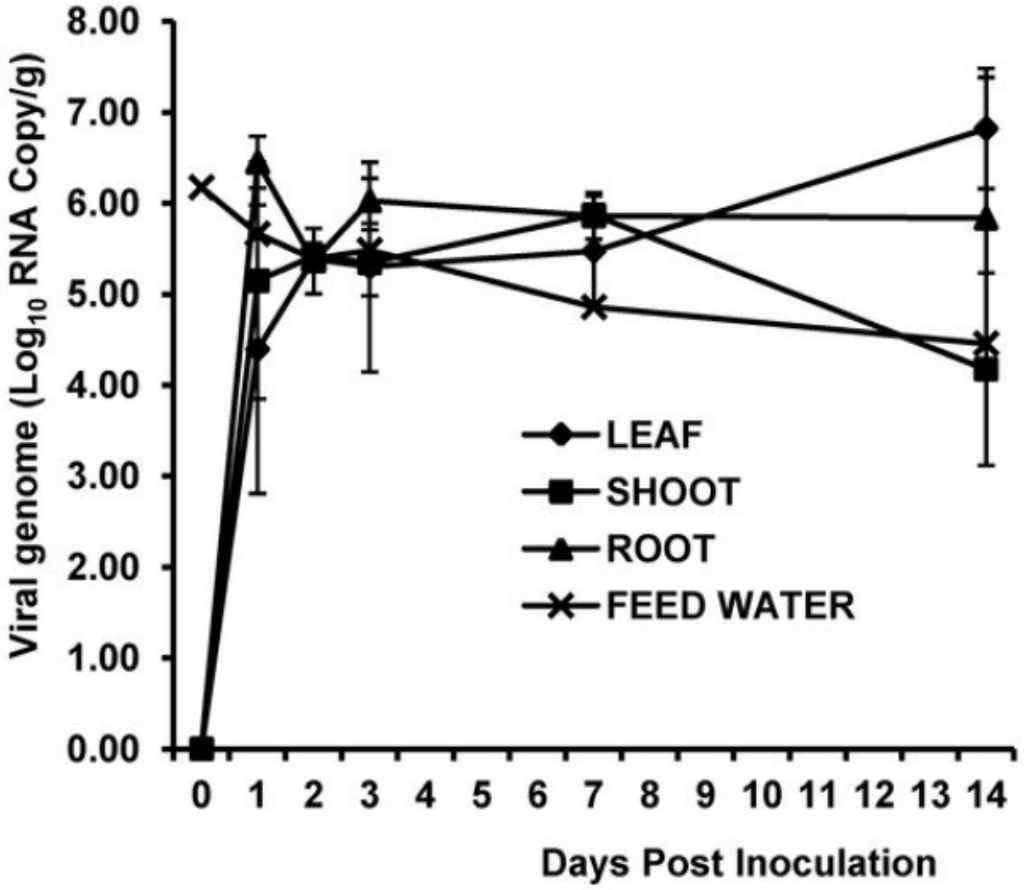


Figure 22. Detection of internalized TV RNA in Romaine lettuce grown hydroponically. Internalization kinetics plot was determined by RT-qPCR and results are reported as RNA copies/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

Upon comparison it was realized there was a difference in kinetics of Tulane virus internalization determined by the two detection methods, real time RT-PCR and plaque assay. A higher level of Tulane virus RNA (2.5×10^4 to 1.4×10^4 RNA copies/g) was

detected in leaves and shoots at days 1 and 2 post inoculation using real-time RT-PCR, compared to a relatively low level of infectious viral particles (1-3 log PFU/g) in leaves and shoots at days 1 and 2 using plaque assay. It was hypothesized that there may be noninfectious viral particles or naked RNA present in leaves and shoots at days 1 and 2. To address this possibility, all of the samples were treated with 5 µg of RNase A to degrade any exogenous RNA before RNA extraction, and viral RNA was then quantified by real-time RT-PCR. In all day 1 samples tested there was an approximately 2.5 log reduction in the amount of Tulane virus RNA detected in the roots after RNase treatment, compared to samples that were not treated with RNase (Fig. 22). Also, day 1 shoots treated with RNase had approximately 1.3 log reduction in viral compared to untreated samples (Fig. 23). On all other study days tested, there was less than a 1 log reduction in Tulane virus RNA detected due to RNase treatment (Fig. 23). This indicates that some naked viral RNA was present in the plant tissues which was degraded by RNase treatment. It is likely that the naked viral RNA originated from the virus particles which were damaged within the plant tissues.

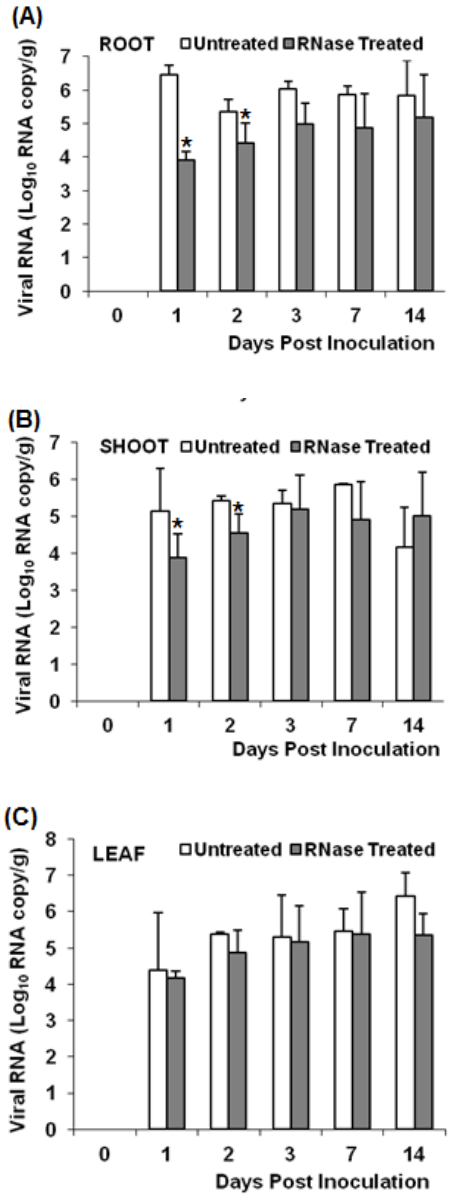


Figure 23: Detection of internalized TV RNA in Romaine lettuce treated by RNase. Viral RNA was quantified by RT-qPCR and results are reported as RNA copies/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation and * denotes statistical difference.

To determine whether the amount of human NoV RNA detectable in lettuce samples was affected by pre-treatment with RNase, the same RNase treatment used on Tulane virus lettuce samples was applied to human NoV samples. In contrast to TV, pre-treatment with RNase did not have a significant effect on the amount of human NoV RNA that was detected in the day 1 root samples (Fig. 24). There was approximate 1 log reduction in the viral RNA detected in the shoots after treatment with RNase (Fig. 24). In both TV and human NoV samples, there was not a significant reduction of the amount of RNA detected in the leaf tissue on day 1 post inoculation (Fig. 22, Fig. 24). The RNase treatment reduced the amount of viral RNA detected in the plant tissues by approximately 0.5-1.5 log on all other study days (Fig. 24). Finally, we increased the RNase treatment level to 25 μg, with the same sample concentration and incubation period as above with treatment the of 5 μg of RNase (data not shown).

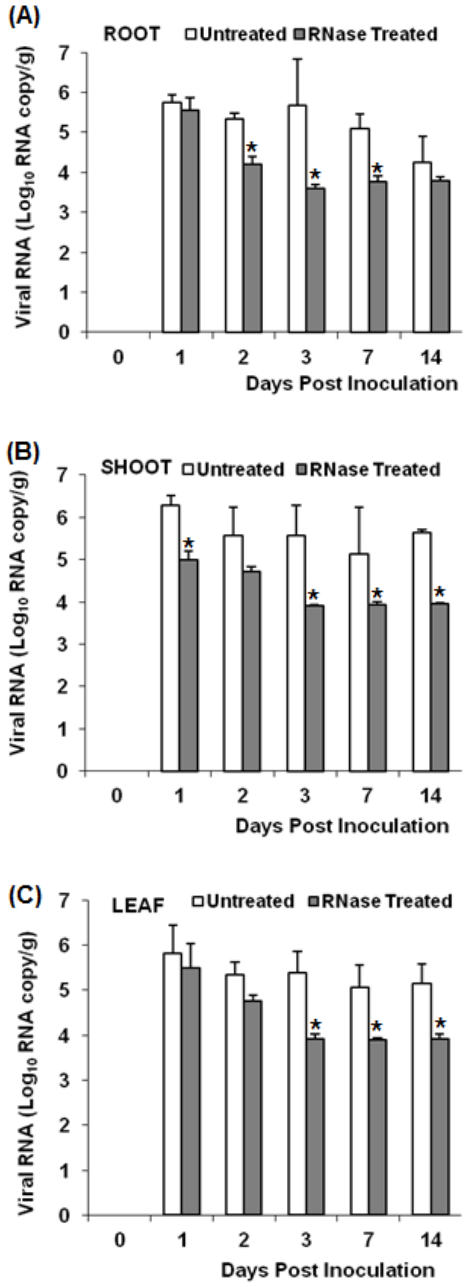


Figure 24. Dectection of internalized human NoV RNA in Romaine lettuce treated by RNase. Viral RNA was quantified by RT-qPCR and results are reported as RNA copies/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation and * denotes statistical difference.

These results indicate (i) the levels of human NoV RNA detected are from a mixture of intact viral particles and naked viral RNA in the plant tissues; (ii) RNase treatment degraded the naked viral RNA; and (iii) intact virus particles persisted in plant tissues for at least 14 days.

3.5. Discussion

Human NoV is the leading causative agent of fresh produce-associated outbreaks. However, the interaction of human NoV with fresh produce is poorly understood. In this study, we experimentally demonstrated that human NoV and its surrogates attached to roots, became internalized, and efficiently disseminated to the shoots and leaves of the plants using hydroponically grown Romaine lettuce as a model. Although it has been documented that a low level of internalization and dissemination of MNV-1 and CaCV occurrs in lettuce (Urbanucci et al., 2009, Wei et al., 2010), this is the first report of the successful detection of internalization and dissemination of human NoV in plants.

Fresh produce is one of the major high risk foods for human NoV contamination because it can become contaminated at any point during processing, including both pre-harvest and post-harvest stages. These results indicate that viral internalization through the roots may be an important route for human NoV contamination and persistence in fresh produce. Previously, it was shown that hepatitis A virus RNA could be detected inside green onions which were grown hydroponically in feed water inoculated with this virus (Chancellor et al., 2006). Poliovirus was found in leaves of tomato plants after

growth in soil irrigated with poliovirus contaminated water at level of 10^3-10^4PFU/ml. (Oron et al., 1995). Bacteriophage f2 was also detected in beans grown hydroponically when challenged with 10^{10}PFU/ml of the virus (Ward and Mahler, 1982). These results indicate that viral internalization during hydroponic growth of crops does occur, although the level of virus detected varies among experiments. Since human NoV may be present in sewage-contaminated soil or water, it may also be taken into the plant through the roots. Once viruses are internalized, it would be significantly more challenging to eliminate them, since traditional sanitation measures usually target the pathogens on the surface of fresh produce. Of further concern is that these internalized viruses can potentially survive for long periods (weeks to months) in fresh produce since human NoV is highly stable in the environment.

During either the pre-harvest, or field growing stage of produce production, the use of irrigation water contaminated with norovirus poses the most significant risk in disseminating disease. Agriculture is responsible for the largest usage of freshwater worldwide and about 70% of this usage is for irrigation. Nearly 17% of all cropland is irrigated, which equates to one third of the world wide food supply being exposed to irrigation water (Bosch, 1983). The use of feces or fecally contaminated irrigation water has been shown to play a role in spreading enteric microorganisms. For this reason, the use of night soil or irrigation with untreated human waste water is illegal in the U.S. and is not recommended by the World Health Organization. However, nearly 70% of all the irrigated crop land is found in developing countries where irrigation water regulations may not exist (Choi et al., 2004). Groundwater is generally regarded as being free of

microbial contamination and is considered a safe source of irrigation water. However, recent studies in the U.S. indicate that 8-31% of ground water is contaminated with viruses (Abbaszadegan et al., 2007, Borchardt et al., 2003). While irrigation water is commonly screened for fecal coliforms, it is rarely tested for the presence of viruses. All these factors contribute to irrigation water posing a significant risk for distributing viral pathogens to fresh produce.

Previously, Urbanucci et al., (2009) investigated the internalization of human NoV in lettuce. However, no viral RNA was detected in leaves when lettuce was grown hydroponically or in soil after challenge with a high level (10^{6-7} RNA copies/ml) of human NoV. In contrast, in this study it was found that high level of human NoV RNA was detected at day 1 and was persistent in roots, shoots, and leaves at least for 14 days when the roots were challenged with a 10^6 RNA copies/ml of human NoV. Several factors may be responsible for this apparent discrepancy. One possibility is that variations in the experimental conditions between studies, such as, environmental growth conditions, the type of lettuce tested, viral strain, and the amount of viral inoculum. In this study, Romaine lettuce was used whereas Rapid lettuce was used in Urbanucci's study (2009). It is known that environmental factors (such as temperature and relative humidity conditions) have an affect on the transpiration rate of the lettuce, which may have a significant effect on viral internalization and dissemination. In our experiments, the plants were grown at 20^{o}C at relative humidity of 40% but, the growth conditions were not reported in Urbanucci's study (2009). Thus, we cannot directly compare if these environmental factors contributed to the difference in results. Plant transpiration rate

increases as the relative humidity of the air decreases, and this increase in transpiration seems to correlate to an increase in viral internalization and dissemination. For example, Wei et al., (2011) showed a significant increase in MNV-1 internalization in lettuce when the relative humidity was 80% compared to 95%. In our study, we decreased the relative humidity to 40%, and the dissemination of MNV-1 to leaves was increased to 4-5 log PFU/g (Fig. 20) compared to the results reported by Wei et al, (2009) at 80% relative humidity.

Similar to bacterial internalization, it is also possible that different viral strains may have differing rates of internalization and dissemination. In our study, we used a genogroup II genotype 4 (GII.4) strain of human NoV. Although Urbanucci et al. (2009) also used a GII virus, the specific genotype was not reported in their study (Urbanucci et al., 2009). Within genogroup II, at least 33 human NoV genotypes have been identified (Zheng et al., 2006). It is well known that different human NoV genotypes have different binding affinity to its functional receptor, the histo-blood group antigens (HBGAs) (Huang et al., 2005, Hutson et al., 2002, Tan and Jiang, 2001). HBGAs are carbohydrate complexes that are present on the surface of erythrocytes as well as the intestinal, genitourinary, and respiratory epithelia. There are three major families of HBGAs, Lewis, ABO, and secretor, and each is specifically recognized by different human NoV strains. Recent studies have shown that human NoV binds to HBGA-like molecules which exist in fresh produce (such as lettuce, blueberries, and strawberries) (Gandhi et al., 2010, Tian et al., 2007). In fact, with some carbohydrate moieties, the analogues of human NoV receptors, such as glucose and glycan, are highly abundant in vegetables and

fruits. It is possible that these HBGA-like molecules may play a role in viral attachment, internalization, and dissemination.

A recent study by Esseili et al. (2012), demonstrated that human NoV GII.4 virus-like particles (VLPs) bound to the cell wall material of young and old leaves, the green leaf lamina, and also the principle vein of Romaine lettuce. This binding was found to be strongest in the cell wall material of old leaves and the green leaf lamina, compared to other plant tissues tested. This was believed to be due to the fact that the cell wall of older leaves are more complex and contain a higher carbohydrate concentration compared to younger leaves. To further demonstrate that the human NoV VLPs were binding to carbohydrates, sodium periodate treatment was used to oxidize carbohydrates in the cell wall extract and this treatment significantly reduced the binding efficiency of the human NoV VLPs (Esseili et al., 2012). The fact that human NoV GII.4 VLPs have been shown to attach specifically to carbohydrates found in Romaine lettuce may explain the high amount of bioaccumulation of human NoV GII.4 RNA observed in this study. This possibility is further supported by the fact that that HBGA-like receptors exist in gastrointestinal epithelial cells of oysters, mussels, and clams which are also a high risk food for human NoV contamination (Maalouf et al., 2011, Tian et al., 2007). These HBGA-like receptors were shown to play an essential role in bioaccumulation of human NoV in oysters, mussels, and clams (Le Guyader et al., 2006, Maalouf et al., 2011,Tian et al., 2007). Furthermore, different human NoV strains are known to have different binding affinities to shellfish because of their differences in receptor usage. In this study, we also demonstrated that human NoV and TV have similar efficiency in internalization

and dissemination in lettuce (Fig. 21, Fig. 22) under the same experimental conditions, whereas TV appears to have a much higher internalization rate than MNV-1 (Fig. 18., Fig. 20). The difference in internalization kinetics may also be related to the properties of each virus such as surface structure, receptor binding affinity, and charge. A recent study has shown that TV also binds to HBGAs, the functional receptor of human NoV (Farkas et al., 2010), but further studies are required to identify whether receptor binding contributes to the bioaccumulation of human NoV in fresh produce.

Since human NoV is not cultivable in cell culture, real-time RT-PCR is frequently used for the detection of human NoV. The major disadvantage of real-time RT-PCR is that it cannot discriminate infectious and noninfectious particles. Thus, one may argue that the high level of RNA copies detected in lettuce may due to the presence of naked human NoV RNA, rather than infectious viral particles. To rule out the possibility, we treated all samples with RNase to degraded naked viral RNA, followed by RNA extraction and real-time RT-PCR. RNase treatment decreased 0.5-1.5 log of human NoV RNA copies in lettuce tissues from days 2 to 14, suggesting that naked human NoV RNA is present in these samples which may come from damaged human NoV particles (Fig. 24). High levels of Tulane virus RNA were detected in leaves and shoots at days 1 and 2 post inoculation (Fig. 23), whereas low levels of infectious viral particles were isolated from leaves and shoots at days 1 and 2 using plaque assay (Fig.18). After RNase treatment, there was an approximate 2.5 log reduction in the TV RNA detected in the shoots (Fig. 23). In leaves harvested on day 1, there was $2.5x10^4$RNA copies/g detected by real time RT-PCR (Fig. 23), whereas less than 1 log of infectious virus was detected

by plaque assay (Fig. 18). However, RNase treatment did not significantly reduce the amount of TV RNA detected in leaves ($P>0.05$) indicating that some noninfectious viral particles, and not naked RNA, were present in the leaves. A similar phenomenon was observed by Wei at al. (2011), where MNV-1 RNA, but not infectious MNV-1, was detected in the leaves of Romaine lettuce inoculated with MNV-1 at the root juncture. It is likely that these viral particles were damaged and hence rendered noninfectious, whereas the viral RNA persisted in the plant tissues. Although plants lack an immune system analogous to the human immune system, plant have developed an array of structural, chemical, enzymatic, and protein-based defenses aimed at detecting and eliminating invading organisms (DeWit 2007, Postel and Kemmerling, 2009). It is possible that different stability against these varying plant defenses. Therefore, the most compelling data presented in this study may be on the rate of human NoV internalization and dissemination in Romaine lettuce, compared to the data from human NoV surrogates.

In summary, our study elucidates a major gap in our understanding of ecology of human NoV in fresh produce, specifically, our understanding of the fate of human NoV after attaching to roots of growing lettuce. Dissection of the mechanism of virus-plant interactions will facilitate the development of novel interventions to prevent viral attachment and internalization in plants.

CHAPTER 4

CONCLUSION

Firstly, this research demonstrates that different viruses vary in their attachment profiles to fresh produce. Human NoV VLPs, TV, and MNV-1 were observed attaching similarly to the surface of Romaine lettuce leaves, and were found to aggregate in and around the stomata. Additionally, the viruses and VLPs were found to attach similarly to the roots of Romaine lettuce. However, differences were seen between the attachment of human NoV VLPs and viral surrogates to green onions. Human NoV VLPs were found to be localized between the epidermal cells of green onion shoots and roots, while TV and MNV-1 were found to attach to the surface of the epidermal cells.

Secondly, this research evaluated the level of viral attachment to Romaine lettuce leaves and roots. A human NoV GII.4 strain was applied to Romaine lettuce shoots and roots and was found to attach to both tissues similarly. Washing with either PBS or 200ppm of chlorine reduced the attached virus detected by less than 1 log of human NoV RNA copies, indicating that human NoV attaches tightly to both lettuce shoots and roots. It was also found that both MNV-1 and TV bound to Romaine lettuce leaves very efficiently, and that simple washing resulted in less than 1 log reduction in viral titer. However, it was found that MNV-1 and TV bound to lettuce roots less efficiently and

simple washing removed 2-3 logs of virus. These results indicate that human NoV may be adhering to the lettuce leaves and roots more efficiently compared to TV and MNV-1. Also, simple washing is not sufficient to remove viral pathogens from lettuce.

Thirdly, it was shown that a human NoV GII.4 strain, TV, and MNV-1 were all internalized and disseminated in Romaine lettuce grown hydroponically when applied to the feed water. This is the first evidence of a human NoV strain being internalized and disseminated in fresh produce when applied to the roots. High levels of viral RNA (5-6 log RNA copies) were detected in shoots and leaves at day 1 post virus inoculation, and persisted in the plant tissues for at least 14 days. Although infectious human NoV cannot be detected using RT-qPCR, RNase treatment was employed to degrade exogenous RNA and this treatment resulted in a reduction of 0.5-1.5 log RNA copies detected in the lettuce tissues. The results indicate that human NoV may be susceptible to degradation within the plant tissues, but a sufficient amount of intact viral particles survive to cause human disease. Also, two cultivable animal caliciviruses were efficiently internalized and disseminated in Romaine lettuce grown hydroponically, demonstrating that the internalized viruses remains infectious in plant tissues.

In summary, human NoV and its surrogates attach tightly to the surface of Romaine lettuce and that the viruses can become internalized through the roots and disseminated to the aerial portions of the plants tissues. This is the first evidence of a human NoV strain found in the internal structures of fresh produce after inoculation to the roots. This research suggests that viral internalization and dissemination may be an important mechanism of viral contamination and persistence in fresh produce.

Internalized human NoV poses a significant risk to public health, because commonly used sanitizers in the fresh produce industry would have no effect on the internalized virus.

LIST OF REFERENCES

Abbaszadegan M., M. LeChevallier, and C.P. Gerba. 2003. Occurrence of viruses in US groundwater. J Am Water Works Assoc. 95:107-120.

Agrios G.N. (ed.). (2005) Plant Pathology, Fifth Edition. Elsevier Inc., Oxford, UK.

Aruscavage D., S.A. Miller, M.L. Lewis-Ivey, K. Lee, and J.T. LeJeune. 2008. Survival and dissemination of *Escherichia coli* O157:H7 on physically and biologically damaged lettuce plants. J Food Prot. 71(12): 2384-2388.

Atmar R.L., A.R. Opekun, M.A. Gilger, M.K. Estes, S.E. Crawford, F.H. Neill, and D.Y. Graham. 2008. Norwalk Virus Shedding after Experimental Human Infection. Emer. Infect Dis. 14: 1553-1557.

Bae J., and K.J. Schwab. 2008. Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface and ground water. Appl Environ Microbiol. 74: 477-484.

Baert L., J. Debevere, and M. Uyttendaele. 2009. The efficacy of preservation methods to inactivate foodborne viruses. Int J Food Microbiol. 131: 83-94.

Barrabeig I., A. Rovira, J. Buesa, R. Bartolome, R. Pinto, H. Prellezo, and A. Dominguez. 2010. Foodborne norovirus outbreak: the role of an asymptomatic food handler. BMC Inf Dis. 10:269.

Belliot G., S.V.Sosnovtsev, T. Mitra, C. Hammer, M. Garfield, et al. 2003. In vitro proteolytic processing of the MD145 norovirus ORF1 nonstructual polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells. J Virol. 77:10957-10974.

Bernstein N., S. Sela, R. Pinto, and M. Ioffe. 2007. Evidence for internalization of Esherichia coli into the aerial parts of maize via the root system. J Food Prot. 70:471-475.

Borchardt M.A., P.D. Bertz, S.K. Spencer, and D.A. Battiagelli. 2003. Incidence of enteric viruses in groundwater from household wells in Wisconsin. Appl Environ Microbiol. 69:1172-1180.

Bosch A., G. Sanchez, F. Le Guyader, H. Vanaclocha, L. Haugarreau, and R. Pinto. 2001. Human enteric viruses in Coquina clams associated with a large hepatitis A outbreak. Water Sci Technol. 43:61-65.

Bosch J.C. 1983. Viruses in environmental waters, in: Viral Pollution of the Environment (G. Berg, ed.). CRC Press, Inc., Boca Raton, FL. pp:177-148.

Brandl M.T. 2008. Plant lesions promote the rapid multiplication of Escherichia coli O157:H7 on postharvest lettuce. Appl Environ Microbiol. 74:5285-89.

Burroughs J.N. and Brown F. 1978. Presence of covalently linked protein on calicivirus RNA. J Gen Virol. 76: 443-446.

Cannon J.L., E. Papafragkou, G.W. Park, J. Osborne, L.A. Jaykus, and J. Vinjé. 2006. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. J Food Prot. 69:2761-2765.

Centers for Disease Control and Prevention. Morbidity and Mortality Weekly Report (MMWR) September 9, 2011. 60(35): 1197-1202.

Centers for Disease Control and Prevention. 2011. Updated norovirus outbreak management and disease prevention guidelines. MMWR 2011. 60(3): 1-15.

Centers for Disease Control and Prevention. 2010. CDC reports 1 in 6 get sick from foodborne illnesses each year. Atlanta, GA: CDC. http:www.cdc.govmediapressrel2010r101215.html

Chancellor D.D., S. Tyagi, M.C. Bazaco, S. Bacyinskas, M.B. Chancellor, V.M Dato, and F. de Miguel. 2006. Green onions: potential mechanism for hepatitis contamination. J Food Prot. 69: 1468-1472.

Choi C, I. Song, S. Stine, J. Pimentel, and C.P. Gerba. 2004. Role of irrigation and wastewater: comparison of subsurface irrigation and furrow irrigation. Water Sci Technol. 50:61-68.

Croci L, D. De Medici, C. Scalfaro, A. Fiore, and L. Toti. 2002. The survival of hepatitis A virus in fresh produce. Int J Food Microbiol. 73: 29-34.

Currier M. 1996. Foodborne outbreak. Diary, Food, and Environ. Sanitation. 16:32-33.

De Rougemont A., N. Ruvoen-Clouet, B. Simon, M. Estienney, C. Elie-Caille, S. Aho, P. Pothier, J. Le Pendu, W. Bioreau, and G. Belliot. 2011. Qualitative and quantitative

analysis of the binding of GII.4 norovirus variants onto human blood group antigens. J Virol. doi:10.1128/JVI.02077-10.

De Wit P.J. 2007. How plants recognize pathogens and defend themselves. Cell Mol Life Sci. 64:2726-2732.

DeWaal C.S. and Bhuiya F. Outbreaks by the numbers: fruits and vegetables 1990-2005. Presented at Annu. Meet. Int. Assoc. Food. Prot., Orlando, Florida, http://www.cspinet.org/foodsafety/IAFPPoster.pdf.

Daughenbaugh K., C.S. Frasier, and M.E. Hardy. 2003. The genome linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. EMBO J. 22: 2852-2859.

Donaldson E.F., L.C. Lindesmith, A.D. LoBue, and R.S. Baric. 2010. Viral shape-shifting: norovirus evasion of the human immune system. Nature Reviews. 8:231-241.

Donaldson E.F., L.C. Lindessmith, A.D. LoBue, and R.S. Baric. 2008. Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. Immunological Reviews. 225: 190-211.

Dore B., S. Keaveney, J. Flannery, and P. Rajko-Nenow. 2010. Management of health risks associated with oysters harvested from a norovirus contaminated area, Ireland, February-March 2010. Euro Surveill. 15(19): 45-53.

Dowell S.F., C. Groves, K.B. Kirkland, H.G. Cicirello, T. Ando, J.R. Gentsch, S.S. Monroe, C.D. Humphrey, C. Slemp, et al. 1995. A multistate outbreak of oyster-associated gastroenteritis: implications for interstate tracing of contaminated shellfish. J Infect Dis. 171: 1497-503.

Doyle M.P. and M.C. Erickson. 2008. Summer meeting 2007-the problems with fresh produce: an overview. J Appl Microbiol. 105:317-330.

Duizer E., K.J. Schwab, F.H. Neill, R.L. Atmar, M.P. Koopmans, and M.K. Estes. 2004. Laboratory efforts to cultivate noroviruses. J Gen Virol. 85:79-87.

Erickson M.C., C.C. Webb, J.C. Diaz-Perez, S.C. Phatak, J.J. Silvoy, et al. 2010a. Infrequent internalization of *Escherichia coli* O157:H7 into field-grown leafy greens. J Food Prot. 73:1023-29.

Erickson M.C., C.C. Webb, J.C. Diaz-Perez, S.C. Phatak, J.J. Silvoy, et al. 2010b. Preharvest internalization of *Escherichia coli* O157:H7 into lettuce leaves, as affected by insect and physical damage. J Food Prot. 73:1809-16.

Esseili M.A., Q. Wang, and L.J. Saif. 2012. Human GII.4 norovirus-like partcles (VLPs) bind to carbohydrates of Romaine lettuce leaf cell wall materials. Appl Environ Microbiol. 78:786-794.

Estes M.K., B.V. Prasad, and R.L. Atmar. 2006. Noroviruses everywhere: has something changed? Curr Opin Infect Dis. 19: 467–474.

Falkenhost G., L. Krusell, M. Lisby, S.B. Madsen, B. Bottiger, and K. Molbak. 2005. Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005. Euro Surveill. 10:21-27.

Farkas T., K. Sestak K., C. Wei, and X. Jiang. 2008. Characterization of a rhesus monkey calicivirus representing a new genus of *Caliciviridae*. J Virol. 82:5408-5416.

Feng K., E. Divers, Y. Ma, and J. Li. 2011. Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation. Appl Environ Microbiol. 77:3507-3517.

Franz E., A.A. Visser, A.D. Van Diepeningen, M.M. Klerks, A.J. Termorshuizen, and A.H.C. can Bruggen. 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Food Microbiol. 24:106-12.

Gandhi K.M., R.E. Mandrell, and P. Tian. Binding of Norwalk virus viral-like particles to veins of romaine lettuce. 2010. Appl Environ Microbiol. doi:10.1128/AEM.01566-10

Gaulin C.D., D. Ramsay, P. Cardinal, and M.A. D'Halevyn. 1999. Epidemic of gastroenteritis of viral origin associated with eating imported raspberries. Can J Public Health. 90:37-40.

Glass P.J., L.J. White, J.M. Ball, I. Leparc-Goffart, M.E. Hardy, and M.K. Estes. 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. J Virol. 74: 6581-6591.

Griffin M.R., J.J. Surowiec, D.I. McCloskey, B. Capuano, B. Pierzynski, M. Quinn, R. Wojnarski, W.E. Parkin, H. Greenberg, and G.W. Gary. 1982. Foodborne Norwalk virus. Am J Epidemiol. 115:178-184.

Hardy, M.E. 2005. Norovirus protein structure and function. FEMS Microbiology Letters. 253: 1-8.

Heaton J.C. and K. Jones. 2008. Microbial contamination of fruit and vegetables and the behavior of enteropathogens in the phyllosphere: a review. J App Microbiol. 104: 613-626.

Herwaldt B.L., J.F. Lew, C.L. Moe, D.C. Lewis, C.D. Humphrey, S.S. Monroe, E.W. Pon, and R.I. Glass. 1994. Characterization of a variant strain of Norwalk virus from a foodborne outbreak of gastroenteritis on a cruise ship in Hawaii. J Clin. Microbiol. 32:861-866.

Hopkins W.G. and Huner N.P.A (ed.). 2004. Introduction to Plant Physiology, Third Edition. John Wiley and Sons, Inc., Hoboken, NJ.

Hora R., K. Warriner, B.J. Shelp, and M.W. Griffiths. 2005. Internalization of Esherichia coli O157:H7 following biological and mechanical disruption of growing spinach plants. J Food Prot. 68:2506-9.

Huang P., T. Farkas, W. Zhong, M. Tan, S. Thornton, A.L. Morrow, and X. Jiang. 2005. Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns. J Virol. 79:6714-6722.

Hutson A.M., R.L. Atmar, D.Y. Graham, and M.K. Estes. 2002. Norwalk virus infection and disease is associated with ABO histo-blood group type. J Infect. Dis. 185:1335–1337.

Jablasone J., K. Warriner, and M. Griffiths. 2005. Interactions of Escherichia coli O157:H7, Salmonella typhimurium and Listeria monocytogenes with plants cultivated in a gnotobiotic system. Int J Food Microbiol. 99:7-18.

Karst S.M., C.E. Wobus, M. Lay, J. Davidson, and H.W. Virgin 4th. 2003. STAT 1-dependent innate immunity to a Norwalk-like virus. Science 299:1575-1578.

Klerks M.M., E. Franz, M. van Gent-Pelzer, C. Zijlstra, and A.H.C. van Bruggen. 2007. Differential interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. ISME J. 1:620-31.

Koh S.J., H.G Cho, B.H. Kim, and B.Y. Choi. 2011. An outbreak of gastroenteritis caused by norovirus-contaminated groundwater at a waterpark in Korea. J Korean Med Sci. 26: 28-32.

Kroupitski Y., D. Golberg, E. Belausov, R. Pinto, D. Swartzberg, et al. 2009. Internalization of *Salmonella enterica* in leaves in induced by light and involves chemotaxis and penetration through open stomata. Appl Envirol Microbiol. 75:6076-86.

Kroupitski Y., R. Pinto, E. Belausov, and S. Sela. 2011. Distribution of *Salmonella typhimurium* in romaine lettuce leaves. Food Microbiol. 28:990-97.

Kuo H.W., D. Schmid, S. Jelovcan, A.M. Pichler, E. Magnet, S. Reichart, and F. Allerberger. 2009. A foodborne outbreak due to norovirus in Austria, 2007. J Food Prot. 72(1): 193-196.

Le Guyader F.S., F. Loisy, R.L. Atmar, A.M. Hutson, M.K. Estes, N. Ruvoen-Clouet, M. Pommepuy, and Le Pendu Jacques. 2006. Norwalk virus-specific binding to oyster digestive tissues. Emerg Infect Dis. 12(6): 931-936.

Le Guyader F.S., C. Mittelholzer, L. Haugarreau, K.O. Hedlund, R. Alsterlund, M. Pommepuy, and L. Svensson. 2004. Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. Int J Food Microbiol. 97: 179-86.

Leib S. 1985. Norwalk virus gastroenteritis outbreak associated with a cafeteria at a college. Am J of Epidemiol. 121: 259-268.

Li J., A. Predmore, E. Divers, and F. Lou. 2012. New interventions against human norovirus: progress, opportunities, and challenges. Annu Rev Food Sci Technol. 3:331-352.

Liu P., Y. Yuen, H.M. Hsiao, L.A. Jaykus, and C. Moe. 2010. Effectiveness of liquid soap and hand sanitizer against Norwalk virus on contaminated hands. Appl Environ Microbiol. 76(2):394-9.

Lo S.V., A.M. Connolly, S.R. Palmer, D. Wright, P.D. Thomas, and D. Joynson. 1994. The role of pre-symptomatic food handler in a common source outbreak of food-borne SRSV gastroenteritis in a group of hospitals. Epidemiol and Infect. 113:513-521.

Lou F., H. Neetoo, H. Chen, and J. Li. 2011. Inactivation of a human norovirus surrogate by high-pressure processing: effectiveness, mechanism, and potential application in the fresh produce industry. Appl Environ Microbiol. 77:1862-1871.

Lou F., H. Neetoo, H. Chen, and J. Li. 2011. Lack of correlation between virus barosensitivity and the presence of a viral envelope during inactivation of human rotavirus, vesicular stomatitis virus, and avian metapneumovirus by high-pressure processing. Appl Environ Microbiol. 77:8538-8547.

Lynch M.F., R.V. Tauxe, and C.W. Hedberg. 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiol Infect. 137:307-315.

Ma Y. and J. Li. 2011. Vesicular stomatitis virus as a vector to deliver human norovirus virus-like particles: a new vaccine candidate against an important non-cultivable virus. J. Virol. 85:2942-2952.

Maalouf H., J. Schaeffer, S. Parnaudeau, J. Le Pendu, R.L. Atmar, S.E. Crawford, and F.S. Le Guyader. 2011. Strain-dependent norovirus bioaccumulation in oysters. Appl Environ Microbiol. 77(10): 3189-96.

Maalouf H., M. Zakhour, J. Le Pendu, J.C. Le Saux, R.L. Atmar, and F.S. Le Guyader. 2010. Distribution in tissue and seasonal variation of norovirus genotype I and II ligands in oysters. App Env Microbiol. 76(16); 5621-5630.

Mandrell R., L. Gorski, and M. Brandl. 2006. Attachment of microorganisms to fresh produce, p. 34–61. In G. M. Sapers, J. R. Gorny, and A. E. Yousef (ed.), Microbiology of fruits and vegetables. CRC, Taylor and Francis, Boca Raton, FL.

Macarisin D., G. Bauchan, and R. Fayer. 2010. *Spinacea oleracea* L. leaf stomata harboring *Cryptosporidium parvum* oocysts: a potential threat to food safety. Appl Environ Microbiol. 76:555-59.

Mead P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe.1999. Food-related illness and death in the United States. Emerg. Infect Dis. 5:607-625.

Marks P.J., I.B. Vipond, F.M. Regan, K. Wedgwood, R.E. Fey, and E.O. Caul. 2004. A school outbreak of Norwalk-like virus: evidence of airborne transmission. Epidemiol Infect. 131: 727-736.

Mitra R., E. Cuesta-Alonso, A. Wayadande, J. Talley, S. Gilliland, and J. Fletcher. 2009. Effect of route of introduction and host cultivar on the colonization, internalization, and movement of the human pathogen Escherichia coli O157:H7 in spinach. J Food Prot. 72:1521-30.

Morse D.L., J.J. Guzewich, J.P. Hanrahan, R. Stricof, M. Shavengani, R. Deibel, J.C. Grabau, N.A. Nowak, J.E. Merrmann, G. Cukor et al. 1986. Widespread outbreaks of clam- and oyster-associated gastroenteritis. Role of Norwalk virus. N Engl J Med. 314: 678-81.

Oron G., M. Goemans, Y. Manor, and J. Feyen. 1995. Poliovirus distribution in the soil-plant system under reuse of secondary wastewater. Wat Res. 29(4): 1069-1078.

Patterson W., P. Haswell, P.T. Fryers, and J. Green. 1997. Outbreak of small round structured virus gastroenteritis arose after kitchen assistant vomited. Communicable Disease Report 7:R101-3.

Pfister T. and E. Wimmer. 2001. Polypeptide p41 of a Norwalk-like virus is a nucleic acid-independent nucleoside triphosphatase. J Virol. 75: 1611-1619.

Ponka A., L. Maunula, C.H. Von Bonsdorff, and O. Lyytikainen. 1999. An outbreak of calicivirus associated with the consumption of frozen raspberries. Epidemiol and Infect. 123:469-474.

Postel S. and B. Kemmerling B. 2009. Plant systems for recognition of pathogen-associated molecular patterns. Semin. Cell Dev Biol. 20:1025-1031.

Prasad B.V., M.E. Hardy, T. Dokland, J. Bella, M.G. Rossman, and M.K. Estes. 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science. 286: 287-290.

Predmore A. and J. Li. 2011. Enhanced Removal of a Human Norovirus Surrogate from Fresh Vegetables and Fruits by a Combination of Surfactants and Sanitizers. Appl Environ Microbiol. 77:4829-4838.

Rawsthorne H., T.G. Phister, and L.A. Jaykus. 2009. Development of a fluorescent in situ method for visualization of enteric viruses. App Environ Microbiol. 75(24); 7822-7827.

Richards G.P. 2012. Critical review of norovirus surrogates in food safety research: rationale for considering volunteer studies. Food Environ Virol. 4:6-13.

Saif L.J., E.H. Bohl, K.W. Theil, R.F. Cross, and J.A. House. 1980. Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. J Clin Microbiol. 12(1):105-11

Santamaría J. and G.A. Toranzos. 2003. Enteric pathogens and soil: a short review. Int Microbiol. 6(1):5-9.

Scipioni A., A. Mauroy, J. Vinje, and E. Thiry. 2008. Animal noroviruses. Vet. J. 178: 32–45.

Seah E.I., I.C. Gunesekee, J.A. Marshall, and P.J. Wright. 1999. Variation in ORF3 of genogroup 2 Norwalk-like viruses. Arch Virol. 144:1007-1014.

Seo K.H. and J.F. Frank. 1999. Attachments of *Escherichia coli* to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using Confocal scanning laser microscopy. J Food Prot. 62:2-9.

Seymour I.J. and H. Appleton. 2001. Foodborne viruses and fresh produce. J App Microbiol. 91: 759-773.

Sharma M., D.T. Ingram, J.R. Patel, P.D. Millner, X. Wang, et al. 2009. A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in soil and hydroponic medium. J Food Prot. 72:1513-20.

Sobsey M.D. and A.R. Hickey. 1985. Effects of humic and fulvic acids on poliovirus concentration from water by microporous filtration. Appl Environ Microbiol. 49(2):259-64.

Sobsey M.D., C.H. Dean, M.E. Knuckles, and R.A. Wagner. 1980. Interactions and survival of enteric viruses in soil materials. Appl Environ Microbiol. 40(1):92-101.

Solomon E.B., S. Yaron, and K.R. Matthews. 2002. Tranmission of Esherichia coli O157.H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl Environ Microbiol. 68: 397-400.

Someya Y., N. Yakeda, and T. Miyamura. 2002. Identification of the active-site amino acid residues in the Chiba virus 3C-like protease. J Virol. 76:5949-5958.

Steudle E. and C.A. Peterson. 1998. How does water get through roots? J Exp Botany. 49(322):775-88.

Takahashi H., A. Ohuchi, S. Miya, Y. Izawa, and B. Kimura. 2011. Effect of food residues on norovirus survival on stainless steel surfaces. PLoS One. 6(8):e21951. Epub 2011 Aug 22.

Takeuchi K. and J.F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. J Food Prot. 63:434-40.

Tan M., P. Huang, J. Meller, W. Zhong, T. Farkas, and X. Jiang. 2003. Mutations within the P2 domain of norovirus capsid affect binding to human histo-blood group antigens: evidence for a binding pocket. J Virol. 77: 12562-12571.

Tan M. and X. Jiang. 2005. Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle. Trends Microbiol. 13: 285-293.

Taube S., J. Perry, K. Yetming, S.P. Patel, H. Auble, L. Shu, H.F. Nawar, C.H. Lee, T.D. Connell, J.A. Shayman, and C.E. Wobus. 2009. Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine norovirus. J Virol. 83(9): 4092-4101.

Teunis P.F.M., C.L. Moe, P. Liu, S.E. Miller, L. Lindesmith, R.S. Baric, J. Le Pendu, and R.L. Claderon. 2008. Norwalk virus: how infectious is it? J Med Virol. 80: 1468-1476.

Tian P., A.L. Engelbrektson, X. Jiang, W. Zhong, and R.E. Mandrell. 2007. Norovirus recognizes histo-blood group antigens on gastrointestinal cells of clams, mussels, and oysters: a possible mechanism of bioaccumulation. J Food Prot. 70:2140-2147.

Tian P., D. Yang, and R. Mandrell. 2011. Differences in the binding of human norovirus to and from romaine lettuce and raspberries by electrolyzed waters. J Food Prot. 74:1364-1369.

Turner A., B. Wells, and K. Roberts. 1994. Plasmodesmata of maize root tips: structure and composition. J Cell Sci. 107:3351-61.

Urbanucci A., M. Myrmel, I. Berg, C.H. von Bonsdorff, and L. Maunula L. 2009. Potential internalization of caliciviruses in lettuce. Int J Food Microbiol. 135: 175-178.

Visser H., L. Verhoef, W. Schop, and H.M. Gotz. 2010. Outbreak investigation in two groups of coach passengers with gastroenteritis returning from Germany to the Netherlands in February 2009. Euro Surveill. 15(28):pii=19615.

Wadl M., K. Scherer, S. Nielsen, S. Diedrich, L. Ellerbroek, C. Frank, R. Gatzer, M. Hoehne, R. Johne, G. Klein, J. Koch, J. Schulenburg, U. Thielbein, K. Stark, and H. Bernard. 2010. Food-borne norovirus-outbreak at a military base, Germany, 2009. BMC Inf Dis. 10:30.

Wang D., Q. Wu, X. Kou, L. Yao, and J. Zhang. 2008. Distribution of norovirus in oyster tissues. J Appl Microbiol. 105: 1966-1972.

Ward R.L. and R.J. Mahler. 1982. Uptake of bacteriophage f2 through plant roots. Appl Environ Microbiol. 43: 1098-1103.

Warriner K., F. Ibrahim, M. Dickenson, C. Wright, and W.M. Waites. 2003. Interaction of Esherica coli with growing spinach plants. J Food Prot. 66:1790-97

Webby R.J., K.S. Carville, M.D. Kirk, G. Greening, R.M. Ratcliff, S.K. Crerar, K. Dempsey, M. Sarna, R. Stafford, M. Patel, and G. Hall. 2007. Internationally distributed

frozen oyster meat causing multiple outbreaks of norovirus infection in Austrailia. Clin Inf Dis. 44:1026-31.

Wei J., Y. Jin, T. Sims, and K.E. Kniel. 2010. Manure- and biosolids-resident murine norovirus 1 attachment to and internalization by romaine lettuce. Appl Environ Microbiol. 76(2): 578-583.

Wei J., Y. Jin, T. Sims, and K. Kniel. 2011. Murine norovirus-1 internaliztion into Latuca sativa during irrigation. Appl Environ Microbiol. doi:10.1128/AEM.02701-10.

Westrell T., V. Dusch, S. Ethelberg, J. Harris, M. Hiertgvist, N. Jourdan-da Silva, A. Koller, A. Lenglet, M. Lisby, and L. Void. 2010. Norovirus outbreaks linked to oyster consumption in United Kingdom, Norway, France, Sweden, and Denmark, 2010. Euro Surveill. 15(12): pii=19524.

Wobus C.E., L.B. Thackray, and H.W. Virgin. 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. J Virol. 80(11): 5104-5112.

Yee E.L., H. Palacio, R. Atmar, U. Shah, C. Kilborn, M. Faul, T.E. Gavagan, R.D. Feigin, J. Versalovic, F.H. Neill, A.L. Panlilio, M. Miller, J. Spahr, and R.I. Glass. 2007. Widespread outbreak of norovirus gastroenteritis among evacuees of hurricane Katrina residing in a large "megashelter" in Houston, Texas: lessons learned for prevention. Clin Inf Dis. 44: 1032-9.

Zelner J.L., A.A. King, C.L. Moe, and J.N.S. Eisenberg. 2010. How infections propagate after point-source outbreaks: an analysis of secondary norovirus transmission. Epidemiology. 21(5): 711-718.

Zhang G., L. Ma, L.R. Beuchat, M.C. Erickson, V.H. Phelan, and M.P. Doyle. 2009. lack of internalization of *Escherichia coli* O157:H7 in lettuce (Lactuca sativa L.) after leaf surface and soil inoculation. J Food Prot. 72:2028-37

Zheng D.P., T. Ando, R.L. Fankhauser, R.S. Beard, R.I. Glass, and S.S. Monroe. 2006. Norovirus Clasification and Proposed Strain Nomenclature. J. Virol. 346:312–323