INTERACTION OF HUMAN NOROVIRUS AND ITS SURROGATES WITH FRESH PRODUCE

by

Qing Wang

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal and Food Sciences

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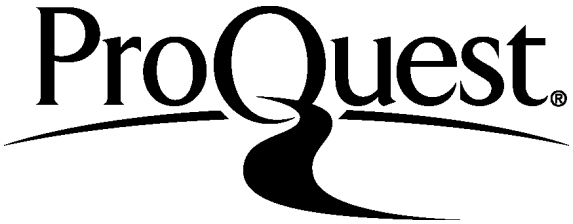

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Produce has been identified as the most common source of foodborne outbreaks in the United States. Among those outbreaks, human norovirus (huNoV) is the leading cause. HuNoV can contaminate produce at any point from farm to table. In order to reduce contamination events, the Food Safety Modernization Act (FSMA) has mandated the implementation of good agricultural practices. However, due to the variety of growing conditions, commodity and cultivar types, as well as pre- and post-harvest practices, it is still a great challenge to provide best practices to ensure produce safety.

Sprouted seeds have been involved in numerous foodborne outbreaks in the United States and across the world. Additionally, microgreens are gaining in popularity, but there is a lack of information pertaining to the microbiological safety of microgreens, particularly of those grown hydroponically. The potential risks associated with virus contamination of crops within a hydroponic system have not been studied to date. In order to better prevent foodborne outbreaks and protect public health, it is urgent to investigate the interaction between foodborne pathogens and fresh produce. Many studies have focused on bacterial pathogens, but little knowledge exits on the interaction between huNoV and fresh produce.

Furthermore, measuring norovirus infectivity is still a challenge due to a lack of appropriate cell lines and limitations associated with volunteer studies. Using surrogates to predict the behavior of huNoV is considered as a promising method to characterize its survivability in different environmental conditions. The objectives of

this project were to 1) investigate the survival and transfer of enteric viruses during seed storage and germination; 2) address how those viruses can be inactivated by intervention strategies before germination, including traditional chlorine washes as well as the novel non-thermal processing technology, application of aqueous ozone; 3) evaluate viruses and bacteria for their ability to become internalized from root to edible tissues of microgreens and secondly evaluate virus survival in re-circulated water without adequate disinfection; 4) increase the titers of Tulane virus (TV), and characterize the interaction between TV and Caco-2 cells, to better understand the mechanism of huNoV infection.

For seeds and sprouted seeds, viruses including murine norovirus (MNV), TV, and hepatitis A virus (HAV) were persistent and remained infectious for a prolonged period of time during seed storage with titers of 1.61 ± 0.19 log PFU/g, 0.85 ± 0.21 log PFU/g, and 3.43 ± 0.21 log $TCID_{50}$ /g after 50 days, respectively. Additionally, contaminated alfalfa seeds were allowed to germinate, virus was transferred from seeds to sprouts and was located in all tissues with low titers (~1-3 log PFU/g for MNV and TV, or ~2.5- 3.5 log $TCID_{50}$/g for HAV) as well as spent water (~1-3 log PFU/ml for MNV and TV, or ~2-3.5 log $TCID_{50}$/ml for HAV) during germination. These findings highlight the importance of sanitation and prevention procedures before germination. Further, traditional calcium hypochlorite treatment as well as a non-thermal technology of aqueous ozone were applied on inoculated seeds to determine their inactivation effectiveness. Data showed that both calcium hypochlorite and aqueous ozone resulted in significant reductions of viruses (and bacteria) inoculated on seeds. Calcium hypochlorite at 20,000 ppm was more effective than 2,000 ppm for all the organisms tested. The reductions of 20,000 ppm calcium

hypochlorite were 3.75 ± 0.42 log PFU/g and 2.29 ± 0.16 log PFU/g for MNV and TV in alfalfa seeds, respectively; whereas huNoV GII was reduced in seeds by 1.65 ± 0.40 log genomic copies/g. The effectiveness on viral inactivation decreased as the organic load increased. For calcium hypochlorite treatment, it is likely that both MNV and TV behave similarly at lower levels of hypochlorite; however, MNV is more sensitive to chlorine than is TV at relatively high levels (20,000 ppm) of calcium hypochlorite with ~ 1 log PFU/ml more reduction than that of TV. For aqueous ozone treatment, TV (reductions range from 1.66 to 3.83 log PFU/g) in alfalfa seeds was significantly more resistant compared to MNV (reductions range from 4.04 to 5.60 log PFU/g) in terms of infectivity. Interestingly, viral genomes were relatively resistant in seeds; reduction of TV genomic copies present in seeds was similar to that of huNoV with D-values (genomic copies) of 27.04 s and 27.73 s, respectively; whereas MNV had significantly greater reductions in genomic copies with D-value of 24.37 s. TV was determined to be more environmental robust than MNV with less reduction in infectivity observed both on seeds treated by calcium hypochlorite and aqueous ozone. Therefore, with greater retention of infectivity and more robust to disinfectant inactivation than MNV, TV makes it as a promising worst-case model for estimating huNoV.

For microgreens, both viruses and bacteria were detected in kale and mustard microgreen roots and were translocated to edible tissues via contaminated irrigation water. The levels of viral and bacterial uptake in edible portions and roots were relatively persistent during harvest (~1-2 log PFU/sample for viruses, and ~ 2-3 log CFU/sample for bacteria, respectively). Cross-contamination occurred easily. Even after an initial contamination event is removed, viruses can still be present and re-

circulated in water, taken up through the roots of microgreens, and transferred to edible tissues. These findings reinforce the need for adequate and diligent sanitation. The information on the transfer and internalization of viruses and bacteria in microgreens via contaminated water, as well as previously determined pre-harvest inactivation rates of pathogens present in fresh produce will be useful to conduct quantitative microbial risk assessment in the future, and the effectiveness of appropriate sanitation can be determined. Results showed that good agriculture practice as well as diligent sanitation are necessary to prevent foodborne outbreaks, which is the goal of produce guidelines and regulations.

INDEX WORDS: Human Norovirus, Murine Norovirus, Tulane Virus, Produce, Decontamination, Cross-contamination, Pre-harvest, Transfer, Survival

Chapter 1

INTRODUCTION

Many efforts have been made to increase consumer intake of fresh produce to meet federal dietary recommendations (1, 2). The U.S. Department of Agriculture (USDA) estimated that the per capita availability of fresh fruits and vegetables increased by 35.05% and 19.76% between 1970 and 2013, respectively (3). In turn, trends in fresh produce consumption in the U.S. have been increasing (1, 4). However, fresh produce usually consumed raw or uncooked can serve as vehicles for the transmission of foodborne pathogens and may pose a risk to public health. Foodborne outbreaks associated with fresh produce contaminated with microbial pathogens have been documented with an increasing trend (5). The Centers for Disease Control and Prevention (CDC) estimated that the incidence of foodborne illness attributed to the consumption of fruits and vegetables to be 4.9 million cases in the U.S. annually, accounting for 51% of the estimated annual illnesses (6). Importantly, human novovirus (huNoV) is recognized as one of the major etiologies causing produce-associated outbreaks of foodborne illnesses, accounting for 40% and 58% of all produce outbreaks between 1990 to 2005 and 2001 to 2010, respectively (7, 8).

Produce contamination can occur at any point from farm to table. Common pathways are thought to be through inadequately treated or contaminated irrigation water, animal feces used for fertilization or from wild animals, contaminated washing water during processing, and infected food handlers (9-11). There are still many knowledge gaps concerning binding or attachment of pathogens to plant tissues and

how this association contributes to foodborne outbreaks. Therefore, it is necessary to explain the complex means of pathogen contamination and mechanism of pathogen survival on plant tissues. Research on the pre-harvest interaction between pathogens and fresh produce mainly focused on bacterial pathogens. However, little knowledge exists on enteric viruses, especially huNoV. This dissertation aims to investigate the survival, transfer and interaction of foodborne pathogens, especially huNoV, with sprouted seeds and microgreens pre-harvest. The effectiveness of intervention strategies before seed germination was also determined. The role of diligent sanitation and good agricultural practices to mitigate pre-harvest risks is emphasized. As huNoV cannot be cultivated in cell culture, it is interesting to investigate several different surrogates in environmental conditions and compare their characteristics.

Results presented here provide information on how norovirus surrogates and hepatitis A (HAV) survive, attach, and internalize in seeds during storage. The survival and transfer of viruses during sprouting was also assessed after seeds were artificially contaminated (Chapter 3). The inactivation of pathogens (viruses and bacteria) present on external surface of seeds by calcium hypochlorite and aqueous ozone was measured (Chapters 4 and 5). Internalization of both viruses and bacteria through root uptake was observed in hydroponically grown microgreens during harvesting, and potential cross-contamination events were studied without complete disinfection (Chapters 6 and 7). The survival, transfer and inactivation rates of viruses in irrigation water can be used for a quantitative microbial risk assessment (QMRA) in the future to determine the role of appropriate sanitation and good agricultural practices during produce pre-harvest. Importantly, Tulane virus (TV) was shown to be environmentally robust based on results of virus survival and inactivation, indicating it

is a promising surrogate for huNoV. Efforts were made to increase the titers of TV (Chapter 8), since low titers of TV have been experienced in many laboratories.

This review highlights the research needs for enteric viruses in produce-associated outbreaks as well as viral inactivation by nonthermal processes. The quantitative microbial risk assessment is also reviewed to provide information on preventative strategies.

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Chapter 2

LITERATURE REVIEW

(A modified version will be submitted to Food Protection Trends)

2.1 Foodborne Illnesses Associated with Fresh Produce

Fresh produce has been identified as a vehicle for transmission of foodborne illness. Outbreaks associated with fresh produce have been documented with increased frequency in the United States. Over the last four decades, there has been a rising number as well as proportion of reported foodborne disease outbreaks associated with produce, increasing from 0.7% (13 of 1,875 outbreaks) in the 1970s, 6% (114 of 1,788 outbreaks) in the 1990s, 15% (684 of 4,638 outbreaks) from 1998 through 2007, to 17% (696 of 4,299 outbreaks) from 2001 to 2010 *(64, 66, 209)*. Recent studies estimated that the number of illnesses attributed to produce was highest among the different food commodities (categories), accounting for 46% of all the illnesses from outbreaks in U.S. *(189)*. Leafy vegetables (including herbs) are considered a commodity of highest priority in terms of fresh produce safety *(85)*.

The important role of viruses in foodborne outbreaks has been historically masked due to limitations in virus detection and clinical diagnosis. With the emergence of foodborne disease outbreak surveillance systems as well as improvements in new technologies and availability of molecular methods, viral pathogens have been recognized as a major cause of foodborne illnesses. A review of outbreaks associated with fresh produce summarized that viruses only caused 20% of

all produce-associated outbreaks in the U.S. from 1973 to 1997, and that HAV was the major etiology (causing 57% of viral outbreaks) followed by norovirus (causing 43% of viral outbreaks) *(209)*. Once more sensitive and widespread detection methods such as molecular assays for foodborne viruses came into use, the important contribution of viruses in foodborne illnesses became more obvious *(271)*. Norovirus was soon recognized as the most common cause of produce outbreaks from 1990 to 2005, accounting for 40% of all outbreaks associated with produce, whereas HAV accounted for 4% *(63)*. Recently, The Centers for Disease Control and Prevention (CDC) found that noroviruses caused 60.3% of produce-related outbreaks (150 of 262) between 1998 to 2008 *(189)*. During 2009-2012, vegetable row crops (e.g., lettuce and other leafy vegetables) and fruits accounted for 30% and 21% of foodborne norovirus outbreaks that were attributed to a single food category *(110)*. Similarly, norovirus was identified in 57% of all produce-associated outbreaks reported from 2002 to 2010 *(65)*. In addition, the average number of illnesses per outbreak involving produce was estimated at 35 illnesses, higher than the number of illnesses per outbreak associated with poultry (28), beef (21), and seafood (8), between 2002 to 2011 *(65)*. These findings emphasize the importance of studying norovirus to prevent produce-associated foodborne outbreaks.

2.2 Risk Associated with Sprouted Seeds and Microgreens

2.2.1 Sprouts

In the U.S., consumption of raw or lightly cooked sprouts has been associated with at least 49 foodborne outbreaks resulting in a total of 1,737 illnesses and three deaths between 1998 and 2014, and various serotypes of *Salmonella* and Shiga-toxin

producing *E. coli* were identified as the major bacterial etiologies *(44, 77)*. The seed germination process is unique for sprout production by providing an environment with warm temperatures, high moisture and nutrients. These ideal conditions can boost bacterial proliferation. The addition of a kill step prior to consumption can reduce the risks associated with sprout production. It has been well recognized that high levels of bacteria reside on sprouts during the germination process, which may exceed 10^8 CFU/g *(89, 216)*. Therefore, the total bacterial counts in sprouts is not a good way to evaluate the potential risk associated with microbial contamination; and thus the National Advisory Committee on Microbial Criteria for Foods (NACMCF) stated that the total bacteria counts in sprouts were not necessarily a concern *(77, 185)*. More attention and efforts are required to focus on the proliferation of pathogenic bacteria during germination.

Importantly, contaminated seeds with low-levels of pathogenic strains were a common source in most sprout-associated outbreaks, and germination is a key step for sprout safety and challenges the sprout industry *(185)*. Seed disinfection is considered a major effective approach to reduce the risks posed to the public health. However, when seeds were inoculated with high titers of bacteria or viruses, complete elimination was not obtained in finished sprouts by treatments with 20,000ppm calcium hypochlorite *(216, 262)*. These results emphasized the importance of end product testing for sprout production, even when processing follows recommended guidelines.

2.2.2 Microgreens

Hydroponic systems are gaining in popularity across the U.S. *(35)*. Specifically, growing microgreens hydroponically is a current trend in the food

industry *(35)*. Similar to sprouts, usually microgreens are grown within indoor facilities with controlled environmental conditions to minimize potential contamination by foodborne pathogens. Microgreens are different from sprouts; as they have been defined as salad crop shoots harvested for consumption within 10-20 days of seedling emergence with several harvests coming from one set of seeds *(151)*. Generally, microgreens are harvested by cutting the plants above the soil line when two fully developed cotyledon leaves and the first pair of true leaves have emerged or are partially expanded. In contrast, sprouts are usually collected when the cotyledon have just opened. Microgreens are considered as a gourmet food to add taste, color, and texture to dishes; they mainly appear in fine and upscale restaurants, and have been gaining popularity during the past few years due to their fresh appearance and health benefits *(47, 154, 186, 276)*. Previous studies revealed that microgreens could provide higher amounts of nutrients and antioxidant capacity than older or mature leaves *(154, 186)*.

With the increasing consumption of microgreens, concerns have been raised that food-borne illness outbreaks similar to those that encountered with sprouts may occur. Sprouts have been involved in at least 55 foodborne outbreaks across the world in which a few to as many as to thousands people became ill *(21, 45, 76, 127)*. Similar to sprout, contamination of microgreens with foodborne pathogens may occur at all stages from farm to table, such as by contact with contaminated soil, irrigation water, packaging materials, and handling at harvest and thereafter *(185)*. While no outbreak has been documented associated with microgreens so far, as outlined later in this dissertation, they are at risk for potential contamination. The U.S. Food and Drug

Administration (FDA) has yet to define commodity specific guidelines regarding microgreens.

2.3 Route of Contamination

Produce is a well-recognized source of foodborne outbreaks and a major vehicle for huNoV transmission *(37, 63, 64, 72)*. Produce that is consumed raw or with little or no processing may become contaminated with huNoV during postharvest handling (e.g., irrigation water, and amendments) and processing (e.g., washing, and packing), and also through contact with infected individuals who may handle the produce or seeds *(41, 63, 107, 172)*. As viruses attach to the produce, they can remain infectious and infect consumers at the end of the food chain.

2.3.1 Seeds

Contaminated seeds have been identified as major players in sprout-associated outbreaks *(75, 185)*. If the seeds are pathogen-tainted before germination, bacteria may grow and proliferate fast in the warm and humid germination environment *(43, 128)*. However, outbreaks associated with virus-contaminated seeds have not been reported, perhaps because of a lack of rapid and low-cost viral testing *(109)*. Nevertheless, it is still important to understand how viruses act in seeds and how they are distributed pre-harvest. It is known that hepatitis A virus (HAV) and the surrogates of huNoV (murine norovirus (MNV) and Tulane virus (TV)) are able to persist in alfalfa seeds for up to 50 days stored at room temperature *(261)*. When these seeds were allowed to germinate over a seven-day period, viruses were detected in all parts of the sprouts as well as in spent water. Therefore, in order to prevent cross-

contamination during sprouting, it is important to have pre-treatment before seed germination.

2.3.2 Soil

Enteric viruses contaminate soil by means of human or animal excreta *(34, 203)*. Infected individuals can shed huNoV in feces at a level of 10^{11} virus particles per gram of stool *(13)*. It is likely that soil that is contaminated by inappropriately treated sewage sludge (biosoilds) serves as a vector and source of human foodborne pathogens. Although huNoV has been detected in some animals (20), humans are considered the sole host for HuNoV. RNA from huNoV was found in swine and bovine fecal samples *(48, 176)*. Therefore, application of animal manure or biosolids as soil fertilizer can introduce huNoV to produce; subsequently, viruses can find their way to irrigation water and eventually crops.

It was observed that porcine parvovirus survived in soil with spiked anaerobic digestion residue and had no loss of its infectivity after 50 days at 15-18 °C *(134)*. Wei et al. observed that MNV and HAV could maintain infectivity in different animal manures and biosolids after incubation for 60 days at both 20 °C and 4 °C *(269)*. Importantly, the attachment of virus in biosolids and animal manure to lettuce followed by internalization by lettuce revealed the potential risk of fresh produce contamination *(268)*. Factors that can affect virus survival and transport in soil have been identified, including the soil type, soil texture, hydraulic condition, pH, humic substances, virus type, and cations *(34, 132)*.

To prevent the spread of viruses to the environment, it is necessary to have good practice of recycling fecal materials and apply it properly to soil as fertilizer. A variety of treatments such as composting, aerobic and anaerobic digestion, alkaline

stabilization, conditioning, dewatering, and heat drying have been considered. Temperature is a critical factor for virus survival in soil, and higher virus log reductions were gained at high temperatures *(61, 267)*. It was shown that virus and manure or biosoild type played an important role in virus survival, and that high pH can efficiently inactivate MNV and HAV *(269)*. Metals and metal oxides present in manure or biosoilds, such as aluminum salts and iron oxides, can be used as coagulants to embed viral particles in larger aggregates *(55, 173)*.

The U.S. Environmental Protection Agency (EPA) has provided regulations on the use of biosoilds (40 CFR Part 503). There are two classes of biosoilds with different requirements: especially for enteric viruses, pathogen reduction processes allow no more than 1 plaque forming unit (PFU) per 4 gram total solids (dry weight) for Class A. Besides, application of manure is regulated by USDA's National Organic Program (NOP) standards (7 CFR 205.203 (c)). To meet the compost standards, an initial carbon-nitrogen ratio should be between 25:1 and 40:1 with temperature maintained between 131 °F and 170 °F for 3 days using an in-vessel/static aerated pile system or for 15 days using a windrow composting system with a minimum of five turns. Furthermore, the FDA Food Safety Modernization Act (FSMA) final rules have established the microbial standards on bacterial testing to validate composting methods.

2.3.3 Irrigation water

Epidemiologic studies have found that 6 to 48% of ground water (e.g., wells) or surface water samples collected in the U.S., South Korea, or Japan contained at least one type of enteric viruses (e.g., enteroviruses, rotavirus, HAV, adenovirus, and norovirus) *(1, 33, 52, 93, 111, 149)*, and huNoV has been detected with varying

frequency *(1, 121)*. If ground or surface water is used as irrigation water, raw produce on the farm may be at risk of exposure to enteric viruses *(52)*.

Fresh produce can be contaminated by irrigation water through direct contact (e.g., spray or splash) or internalization into the plant tissue from roots. Several produce-associated outbreaks suggested that contamination may have occurred by irrigation or during harvest *(62, 81, 230)*, but no conclusive evidence was provided. These studies highlight the difficulties in identifying and tracing outbreaks back the point where the contamination occurred when retail or food service workers are not involved. Recently, four norovirus isolates associated with outbreaks in South Korea between 2008 and 2012 were linked to contaminated water *(53)*; emphasizing that irrigation water may serve as a source of pathogenic viruses.

To better understand risk from the use of virus-contaminated irrigation water, information on virus transfer during water irrigation is needed. Studies conducted in the field indicated that virus can directly contact and attach to vegetables and fruits from inoculated irrigation water, and the transfer rate depends on the type of produce and virus, and method of irrigation (surface or furrow irrigation) *(7, 54, 217)*. Importantly, internalization of huNoV through root uptake of produce via polluted irrigation water is another of the potential routes for plant contamination *(1, 172)*. Compared to bacteria, the behavior of viruses tends to be different, as viruses are much smaller in size and have different surface characteristics *(46, 116, 245)*. The great number of produce-associated illnesses caused by viruses *(64, 189)*, has resulted in the study of internalization of viruses in plants grown in artificially contaminated hydroponic systems *(40, 46, 67, 68, 79, 116, 118, 187, 245, 265, 266, 270)*. The driving force of water absorption facilitates internalization, and humidity in the plant-

growing environment significantly affects transpiration resulting in different levels of pathogen uptake *(270)*. Factors such as root integrity *(265)*, virus type *(245)*, and inoculation level *(270)* can affect virus internalization. Once enteric viruses are internalized into plants, there is no effective way to disinfect internalized pathogens due to lack of access and limited contact. Therefore, preventative strategies focusing on good agricultural and manufacturing practices during pre-harvest are essential to reduce risks of contamination.

2.3.4 Food handlers

Food handlers with or without infection are another important source of contamination *(102, 237)*. They have direct contact with food and can introduce viruses at any point in the food chain due to failure in good hygiene practice *(102)*. Improper hygiene practices may include contacting food with bare-hands, improper or/and inadequate hand washing, inadequate cleaning of processing or preparation equipment or utensils, and cross-contamination of ready-to-eat (RTE) food due to contaminated raw materials *(103, 237)*. According to data obtained from 1927 to 2006, viruses caused 60.2% (491/816) of outbreaks where food workers were involved, and norovirus was responsible for 33.6% (274/816) of these outbreaks *(102)*. In addition, 16.1% (44/274) of the foods associated with norovirus outbreaks were linked to produce *(102)*. More recently, it was found that improper handling during post production is the most common stage for produce to become contaminated by human pathogens, accounting for approximately 81% of all produce outbreaks from 1990 to 2007 *(74)*.

Norovirus outbreaks associated with food handlers are commonly reported *(91, 162, 205, 236, 257)*. Virus can be transferred to food handlers' hands, which can

further serve as a vehicle for virus spread and infections without proper food preparation practices *(6, 9, 139)*. A study investigated huNoV contamination during harvesting of bell peppers in Mexico and found that huNoV was absent on pickers' hands at the beginning of labor activity but detected 3 hours later *(153)*. This study indicated that the produce may be contaminated with huNoV and highlighted the role of hands in pathogen transfer during harvesting without frequent and proper hand washing practice. Viral contamination of produce by ill individuals or by asymptomatic individuals requires more attention. As mentioned previously, infected individuals can shed high levels of huNoV in feces (up to 10^{11} viral particles/gram) *(13)*, whereas only a small amount of viral particles (10-100) can result in illness *(191, 228)*. Through the fecal-oral route, virus transfer by infected food handlers can involve both symptomatic and asymptomatic individuals and importantly, asymptomatic and symptomatic individuals have similar levels of norovirus in their feces *(188)*.

Previous research has demonstrated the ease of virus transfer, and the transfer rates from hands/fingerpads to foods/food contact surfaces vary from 0.4% to 46%, and from 2.8% to 14% vice versa *(10, 17, 28, 30, 58, 78, 177)*. Variables that influence virus transfer rates were identified. Pressure and friction applied to the surface can facilitate virus transfer. When pressure was raised from 0.2 kg/cm^2 to 1.0 kg/cm^2, a 3-fold increase of HAV transfer was observed *(178)*. Transfer of MNV from food contact surfaces to lettuce significantly increased from 0 to 4% (100 g/cm^2) to 8 to 20% (1000 g/cm^2) *(78)*. It is likely that moisture plays a role in virus spread *(58)*. D' Souza et al. observed that virus was more easily transferred from stainless steel coupons to wet lettuce than to dry lettuce *(58)*. Characteristics of the food/food contact surface topography, material, stickiness, and the absorbent nature of the surface can

affect the transfer rate. Compared with other surfaces, smooth, hard and nonabsorbent surfaces, such as stainless steel, are poor virus receptors and donors *(30)*.

2.4 Mechanism of Viral Attachment to Produce

Viruses cannot replicate or multiply without a host, as they are obligate intracellular organisms. However, huNoV have properties that allow them to survive and remain infectious under harsh environmental conditions before consumption.

HuNoV can bind to a broad range of surfaces and easily spread via cross-contamination. Numerous outbreaks associated with huNoV in fresh produce have caused researchers to investigate the interaction of viruses with leafy greens and make attempts to identify binding sites. Understanding the mechanisms of virus attachment to produce is considered a key to developing effective detection methods and intervention strategies.

HuNoV virus-like particles (VLPs) were able to bind to Romaine lettuce leaf surfaces, especially to cut edges and occasionally to veins *(80, 92)*. Similarly, SYBR gold-labeled MNV was observed on lettuce piece surfaces, inside open cuts, and occasionally within stomata by confocal microscopy *(268)*. It is likely that viruses are heterogeneously dispersed on produce surfaces and some sites may attract virus binding better than others *(92, 268)*. The results also suggest the possibility that virus can internalize through cut edges or guard cells. Interestingly, MNV in biosolids was more likely to bind and internalize into lettuce than pure virus emphasizing the role of biosoilds (organic loads) in virus binding. In addition, extracts of produce *(92, 156)* or cell wall materials *(80)* were used to further investigate the molecules that are involved in binding, and it was suggested that binding can occur to Histo-blood group antigens (HBGAs) or HBGA analogs and other molecules.

Many factors that affect the strength of virus attachment to foods have been identified. The isoelectric point (pI) of viruses may affect their attachment to produce surfaces *(251, 264)*. It is known that the pI of huNoV is 5.9 to 6.0 *(97, 182)*, and the pH of surrounding solutions below or above the pI can affect the ultimate charge on virus surface, consequently affecting the attachment affinity. Virus recovery efficiencies from contaminated produce were determined by using electrolyzed water with different pHs (acidic, neutral, and basic), and the results showed that acidic electrolyzed water significantly decreased virus removal *(234)*. This study confirmed that the pH might interfere in the interaction between huNoV and produce surfaces. It was found that porcine sapovirus (SaV), another surrogate of huNoV, has the capability to attach to lettuce leaves significantly at its capsid isoelectric point (pH 5.0) *(264)*. Vega et al. also investigated the adsorption patterns of feline calicivirus (FCV), echovirus 11, bacteriophage ϕX174, and MS2 to lettuce in varying pH solutions *(251)*. The results showed variations in attachments among those four viruses, indicating that pI might not adequately explain attachment of viruses to lettuce. Vega et al. further compared the contributions of electrostatic and hydrophobic forces to attachment under three different conditions (1% Tween 80, 1M NaCl, and 1% Tween 80 with 1M NaCl) *(250)*. The desorbing effects of 1 M NaCl suggested that electrostatic forces play a major role in virus attachment.

Negatively charged carbohydrates like sialic acid and heparan sulfate are utilized as cellular receptors by many viruses and microorganisms to bind host cells *(123)*. HBGAs, which are a group of structurally related carbohydrates, have been identified as receptors for huNoV *(119, 120, 124, 125, 161, 168)*. It is assumed that the presence of carbohydrate moieties that resemble HBGAs can serve as binding site for

huNoV. Vegetables and fruits have abundant carbohydrate molecules and some of them might be similar to those in HBGAs and therefore could play a role in virus binding. Esseili et al. examined the role of carbohydrates in the attachment of huNoV VLPs to Romaine lettuce leaves *(80)*. The results showed that VLPs were distributed across the whole surface, but were especially attached to cut edges, stomata, and occasionally along minor veins; and the interaction between lettuce and viruses is associated with carbohydrate moieties present in plant cell walls. Cell wall materials (CWM) obtained from older leaves had significantly higher binding affinity (1.5- to 2-fold) to VLPs than those from younger leaves, indicating the differences in carbohydrate molecule abundances or composition between young and old leaves. Experiments involving the oxidation of carbohydrates and boiling of proteins in CWM suggested that virus binding is mediated mainly by carbohydrates of older leaves; whereas binding was mainly mediated by proteins in younger leaves. It is noted that several bacterial species can express HBGA-like sugars on the lipopolysaccharide O-chains *(197, 277)*, it is thus likely that huNoV can bind to bacteria already attached to leafy greens.

However, Gandhi et al. determined that some molecules in Romaine lettuce, other than HBGAs, might be responsible for binding recombinant Norwalk virus-like particles (rNVLPs) *(92)*. The rNVLPs localized in clusters along leaf veins rather than with an even distribution across the leaf. Therefore, specific ligand(s) present on leaf veins may be involved in binding, since veins have a thinner waxy coat than other leaf surfaces. Extracts of crushed Romaine lettuce leaves tended to bind rNVLPs in a dose-dependent manner over a 1000-fold range even though the extract did not appear to have any HBGA-like carbohydrates. Interestingly, the binding of Romaine extract to

rNVLPs was slightly enhanced by oxidation but decreased significantly by being boiled, which suggests proteins may be utilized for binding whereas sugars may mildly inhibit binding.

The effects of extracts from leafy greens on the binding ability of huNoV were also examined *(92, 156)*. Extracts of Romaine lettuce, cilantro, iceberg lettuce, celery, spinach, green onions, clover sprouts, and raspberries all bound rNVLPs, but HBGA-like sugars were not involved in binding *(92)*. Similarly, Li et al. showed that extracts of Romaine lettuce, cherry tomato, and spinach had little inhibitory effect on huNoV P particle binding to HBGAs *(156)*. However, extracts of vegetables may not be reflective of surface binding sites, authentic huNoV, P particles or VLPs may still behave differently from intact huNoV particles.

Those studies provided insights on virus binding and attachment, the electrostatic forces, pH, HBGA-like sugars, and other carbohydrates and proteins may all affect the binding affinity of huNoV to produce; however, further studies are still needed, as the mechanism of attachment of viruses in plants is not well understood.

2.5 Human Norovirus

2.5.1 Epidemiology

HuNoV is the leading cause of acute viral gastroenteritis. Person-to-person transmission remains the most common route and accounts for 62-84% of all reported outbreaks *(82, 248)*. The total number of illnesses caused by norovirus in the U.S. each year is estimated to be 19-21 million and an average of 5 episodes of norovirus gastroenteritis is experienced by each individual over a lifetime *(108)*.

Besides person-to-person transmission, foods play a role via the fecal-oral route. The CDC estimated that huNoV causes the most foodborne illnesses each year in the U.S. (5.5 million) annually, accounting for 58% of total foodborne illnesses *(204)*. Foodborne norovirus infections resulted in 15,000 hospitalizations and 150 deaths, and the economic burden was estimated to be ~$2 billion due to health care expenses and lost productivity *(22, 204)*. Similarly, huNoV causes the greatest number of foodborne illnesses in Canada, accounting for 65% of the total caused by known pathogens *(231)*. Norovirus can infect persons of all ages, and is transmitted via person-to-person spread or the fecal-oral route *(146, 246)*. Projectile vomiting can aerosolize the virus and pose a risk to exposed people *(191)*. The peak of huNoV disease outbreaks usually occurs in cold weather *(246)*. Generally, the incubation period of norovirus is 12-48 hours *(191, 233)*. Most common symptoms include acute onset of vomiting, watery diarrhea or both; other symptoms may include nausea, abdominal cramping and pain, malaise, anorexia, fever, headache and myalgia *(11)*. These symptoms will resolve in 1-3 days; however, virus shedding can last for up to three weeks with symptomatic or asymptomatic infection *(39, 191, 199)*.

HuNoV has distinguished characteristics that facilitate its persistence in the human population with high incidence of disease *(174, 252)*. HuNoV can be shed in vomit and feces at high levels (up to 3×10^7 viral particles/episode of vomiting; up to 10^{11} genomic copies/g of stool sample) for long durations (up to 22 d) *(2, 39, 114, 145, 169, 199)*, while its infectious dose is low (~10-1000 viral particles) *(144, 191, 228)*. Also, huNoV has diverse antigenic and genetic variations limiting the cross-protection among different genotypes, and repeated infections often occur due to short-term immune response *(146, 191)*. HuNoV is resistant to harsh conditions (i.e.,

low pH, moderate heating, and desiccation) and can persist in different environments (i.e., water and surfaces) for weeks to years *(5, 59, 78, 133, 141, 163, 175)*. In addition, many commonly used hand sanitizers and surface disinfectants (i.e., ethanol, quaternary, and anionic compounds) may have limited efficacy against huNoV as judged by their effects on surrogate viruses *(71, 105)*.

2.5.2 Classification and molecular biology

Noroviruses are members of the family *Caliciviridae*, which also include the genera, *Sapovirus*, *Lagovirus*, *Nebovirus*, and *Vesivirus (87, 100, 126)*. Another two proposed genera, *Recovirus* and *Becovirus*, are not yet accepted by the International Committee on Taxonomy of Viruses (ICTV). On the basis of similarity in amino acid sequences of their major capsid proteins, noroviruses are classified into six genogroups I-VI (GI-GVI), and each genogroup is further divided into genotypes or genetic clusters *(99)*. Genogroup GVI and tentatively proposed genogroup GVII contain canine noroviruses *(170, 239)*. The strains relevant to human disease belong to genetic clusters within GI, GII, and GIV *(278)*. GII and GI are the most common genogroups, causing 73 and 26% of all reported norovirus outbreaks from 1997 to 2000 in U.S., respectively *(83)*. Recent surveillance confirmed this trend and found that GII and GI were responsible for 89 and 11% of all reported outbreaks during 2009-2013 *(248)*. Among those outbreaks, GII.4 was the major cause and accounted for 72% of all outbreaks, and 94% of these isolates typed as either GII.4 New Orleans or GII.4 Sydney *(248)*. Several genotypes (GI.3, GI.6, GI.7, GII.3, GII.6, and GII.12) were also highlighted, as they are significantly more associated with foodborne transmission than person-to-person *(248)*.

Noroviruses are non-enveloped, single stranded, positive-sense RNA viruses *(11, 96)*. Each virus particle has an icosahedral capsid of approximately 27-35 nm in diameter with a genome of approximately 7.5 kb in length. The genome consists of three open reading frames (ORFs). ORF 1 encodes a nonstructural polyprotein, which self-cleaves posttranslationally into seven proteins (NS1 to NS7) used for viral replication. The mature proteins include N-terminal protein, NTPase, a picornavirus 3A-like protein, VPg, proteinase, and RNA-dependent RNA polymerase *(31, 101, 213)*. ORF 2 and ORF 3 encode major (VP1) and minor (VP2) structural proteins for viral capsids, respectively *(195)*. The VP1 protein has two defined major domains: the shell (S) and the protruding (P) domains. The S domain is used to form the inner parts of the capsid with a relatively well-conserved amino acid sequence, whereas the P domain forms arch-like protrusions with a more variable sequence. The P domain is then divided into the P1 and P2 subdomains. The P2 subdomain has the highest sequence variability among noroviruses, and its role in receptor binding and antigenic specificity has been proposed *(50, 51)*. It was shown that 90 dimers of VP1 can spontaneously self-assemble and form the major structure of the virion, *(131, 195)*, and only a few copies of VP2 are present in each virion *(94, 95)*.

2.5.3 HuNoV receptors

Based on binding assays and volunteer studies, it is well established that binding of huNoVs to its receptor(s) is complicated and may involve ABO, Lewis, and secretor-type HBGAs *(119, 120, 124, 125, 161, 168)*. The HBGAs presented on the cell surface are involved in virus entry. These carbohydrates are generated by several specific glycotransferases, especially fucosyltransferases, including FUT3 and FUT2, and are located at the distal end on the glycolipids and glycoproteins presented on the

surface of erythrocytes and mucosal epithelial cells as well as secreted glycoproteins of milk, saliva, and other secretions of the gastrointestinal tract. Other studies showed that the expression of ABH and Lewis carbohydrate antigens increases as enterocytes differentiate and travel up the villus *(98, 168, 241)*. As the HBGA receptors are expressed depending on the host's genetic make-up and the differentiation state of the host cells, norovirus binding and the host's susceptibility to infection is variable. These differences explain observations from previous human challenge studies that only a subset of individuals was susceptible to norovirus infection while the other subset was resistant to it *(161, 190)*. The transfection of glycosyltransferase complementary DNA can enhance norovirus binding to non-permissive cells *(168)*.

Besides HBGAs, noroviruses can also recognize heparan sulfate present on cellular membranes *(224)*. Heparan sulfate is a linear polysaccharide present on a wide variety of tissues as a component of certain proteoglycans rooted in the lipid bilayer of the plasma membrane *(69, 115, 142, 179)*. This specific binding was confirmed by blocking the VLP binding site with inhibitors, such as sulfated glycosaminoglycan and suramin. A marked reduction was observed in VLP bindings by the reagents that bind to cell surface heparan sulfate and the enzymes that specifically digest heparan sulfate. Importantly, by treatment of the cells with chlorate, sulfation of heparan sulfate was found mainly involved in this interaction between norovirus and heparan sulfate. This binding was variable among norovirus groups, VLPs derived from GII were more likely to bind heparan sulfate rather than those from GI. However, it is still unclear if heparan sulfate acts as the norovirus receptor, further studies are needed as heparan sulfate is not always involved in viral infection *(222)*.

2.5.4 HuNoV lifecycle

Norovirus infection starts with host cell attachment via carbohydrate receptors (HBGAs) and probably with other receptors (sialic acids). Norovirus then enters into cells and releases the VPg-linked RNA into cytoplasm. The VPg-linked RNA acts as an mRNA and initiates the translation through interactions with VPg and the cellular translation machinery. The ORF1 polyprotein is auto-catalytically cleaved and produces non-structural proteins for virus replication; whereas ORF2 and 3 encode the VP1 capsid protein and VP2 minor structural protein. After translation, a negative-strand intermediate is synthesized which is transcribed to make more mRNA and new ssRNA (+) genomes. As new virus particles are self-assembled, they can be released and infect neighboring cells after cell lysis *(232, 246)*. However, due to the unavailability of cell culture, the knowledge of the huNoV replicative cycle is still limited.

2.5.5 Detection methods

Currently, it is still not possible to cultivate huNoV in cell culture, which makes detection of infectious huNoV impossible. However, simple, rapid, and sensitive methods are required for detection of huNoV in food and water, or clinical samples to monitor the safety of foods as well as identify the source of contamination to control and reduce the spread of viruses *(254)*.

Historically, virus has been detected by using an electron microscope (EM) to reveal virus morphology. This procedure lacks sensitivity and requires expensive equipment and training. Therefore, this method is mainly for used for reference, but not widely used for laboratory diagnosis. Immunochromatographic (ICG) lateral flow assays can rapidly detect a large panel of huNoV genogroups with high specificity,

and specialized and costly equipment is not required; however, the overall sensitivity is quite low (35% to 52%) *(8)*. Enzyme immunoassays (EIAs) have high specificity and high throughput with sensitivity ranging from 57-76% *(57)*. Application of EIAs has been a challenge for clinical diagnostic purposes due to the number of antigenically distinct genotypes and the antigenic drift of certain strains *(145, 254)*. Currently, reverse transcriptase-polymerase chain reaction (RT-PCR) followed by DNA sequencing is the primary and most widely used approach to virus detection. With this method, huNoV can be detected in clinical specimens (feces or vomitus) as well as in foods, water, and environmental samples even when only small numbers of viruses are present *(246, 255)*. The relatively conserved regions of the RNA-dependent RNA polymerase (POL) in ORF1 *(24, 130, 258)* and the ORF1-ORF2 junction region *(137, 249)* have been selected most often for primer design to detect the majority of the circulating strains. As mentioned above, huNoVs are classified based on amino acid diversity of the VP1 protein. It has been proposed to use a dual-nomenclature system by investigating both POL region and VP1 sequences *(147)*. Therefore, RT-PCR can also be used to distinguish between strains or genotypes by targeting those two regions *(147)*.

2.6 Methods used to study of huNoV

2.6.1 HuNoV surrogates

The pathogenesis of huNoV is still not well understood due to the lack of appropriate cell culture models as well as the limitations of volunteer studies. In order to study and predict the behavior of huNoV, caliciviruses with close genetic and antigenic relatedness to huNoV have been widely used, such as FCV, MNV, TV, and

VLPs *(29, 87, 131, 272)*. Other viruses may also be considered, such as poliovirus and male-specific coliphage (MS2) *(16)*. Those surrogates make it possible to estimate huNoV survival under various environmental conditions.

FCV, a respiratory virus, belongs to the genus vesivirus *(16)*. It has historically been used as a surrogate for huNoV. The environmental persistence, transfer capacity, and resistance to disinfection of FCV have been extensively investigated *(29, 30, 71, 105, 210, 215, 238)*. Compared with MNV, FCV is more susceptible to low pH and elevated temperature *(38)*. MNV is genetically related to huNoV, and clustered in the norovirus genogroup. It has been widely used as a surrogate due to its similarity in size (28 to 35 nm in diameter), shape (icosahedral), buoyant density (1.36 ± 0.04 g/cm3), and genomic relatedness *(273)*. It was the first norovirus to be propagated in cell culture *(272)*. However, MNV causes systemic infection in mice but not gastroenteritis, and the susceptible cells are dendritic and macrophage cells rather than digestive epithelial cells via sialic acid moieties *(138, 227, 272)*. TV is a newly discovered calicivirus in the genus *Recovirus (87)*. It was isolated from the stool of captive rhesus macaques and can be cultivated in LLC-MK2 monkey kidney cells. It is also related to huNoVs with a similar size of ~36 nm, and has the property to bind type A and B HBGAs *(86)*. Several studies have showed the persistence of TV in the environment, indicating that it is a promising surrogate of huNoV *(261-263)*. The capsid protein can be expressed and self-assembled into VLPs in insect cell infected with recombinant baculoviruses. The VLPs share similarities in the morphology and antigenicity *(131)*. The VLPs are composed of the major capsid proteins (VP1) without nucleic acids and the minor capsid proteins (VP2). VP2 may help VP1 expression and be involved in particle maturation and stability *(26, 95)*. Therefore, the

VLPs might not be fully representative of all the properties of native virions. A lot of studies have been done to compare these surrogates *(38, 88, 117, 235)*, but it is still unclear which one(s) can truly represent survival, stability and sensitivity of huNoV.

2.6.2 Cell models

The most important limitation of a huNoV study is the lack of a routine *in vitro* model. Numerous efforts have been made to test different mammalian tissue cultures for huNoV viral propagation. Duizer et al. tried to grow 33 different huNoV strains in 27 cell lines including human gastrointestinal tract epithelia, monkey kidney epithelia, and other human and animal tissues, but cultivation of huNoV was not successful *(73)*. Guix et al. demonstrated that huNoV RNA isolated from fecal samples was infectious in human hepatoma Huh-7 cells with production of RNA and viral particles through one cycle, but the generated viruses were unable to infect neighboring cells *(104)*. This study showed that the RNA of huNoV was infectious and that an RNA-protein complex (VPg with viral genome) is important during infection. Overexpression of the human *FUT2* gene responsible for producing HBGAs, facilitates the binding between cells and virus, but is not sufficient for viral infection. This study suggested that huNoV replication *in vitro* might be blocked at the stages of attachment and uncoating *(104)*. Previously, it was found that MNV can infect and replicate in dendritic cells and macrophages *(272)*. Attempts to use those cell lines for huNoV replication failed, indicating that huNoV tropism is distinct from that of MNV *(148)*. Based on the success of MNV replication in those cell lines, another study hypothesized that huNoV and MNV share similar tropism to cells of the haematopoietic lineage *(155)*. By using adult duodenal tissues in *ex vivo* culture, infection and productive replication of huNoV was observed, and the results indicate that huNoV had a marked tropism for

glandular epithelial cells of the human duodenum. Further, a glandular epithelial cell line (HIEC-6) was selected for huNoV infection, but only up to a 2-log increase in viral genomic RNA was detected, and no observable cytopathic effect (CPE) was noted *(155)*.

It is considered that specific factors or features of cells during true differentiation may play a role in huNoV cultivation *(247)*. Three-dimensional (3-D) cell culture models have been tested for huNoV replication. Straub et al. used the 3-D organoid model of the human small intestinal epithelium (INT-407) for huNoV infection *(218)*. Cells were differentiated by growing on porous collagen-I coated microcarrier beads in a rotating-wall vessel, and the infection with GI and GII was confirmed with over five cell passages. CPE was displayed, associated with vacuolization and shortening of mirovilli. However, the crucial factors for huNoV infection and replication still need to be identified. It is likely that some molecules (e.g. receptors or co-receptors) may be present in the 3-D culture, which allows huNoV infection and spread among cells *(247)*. Further, another 3-D cell culture, Caco-2 cells derived from the large intestine, was developed for *in vitro* assays for huNoV in terms of cellular response (shortening of microvilli) and viral RNA amplification *(218)*. Therefore, huNoV infection and replication *in vitro* are more likely to occur in cell lines that (1) have a human gastrointestinal origin, (2) express apical microvilli, and (3) are positive secretor cell lines *(219)*. The 3-D cell culture models are promising, but the method is complicated and requires specific equipment as well as intensive labor.

Very recently, replication of huNoV GII.4 *in vitro* was successfully developed by using human B cells *(136)*. Interestingly, the results suggest that free HBGA or HBGA-expressing enteric bacteria can substantially enhance viral infection. The

reason that previous attempts to grow huNoV in different cell lines failed may be due to the absence of the stimulatory carbohydrate molecules. It is likely that the binding between huNoV and bacterially expressed HBGAs facilitates viral attachment to and infection in B cell, but the mechanism of bacterial enhancement during viral infection is still unclear. Further validation is still required since it has not been widely adapted in other laboratories, as reproducibility remains problematic.

2.6.3 Animal models and human challenge studies

Animal models (e.g. mice, chimpanzee, rhesus macaques, gnotobiotic pigs and calves) have been used to facilitate the progress in norovirus biology. Recently, Taube et al. challenged BALB/c Rag-γc-deficient mice engrafted with or without human $CD34^+$ hematopoietic stem cells (humanized or non-humanized) with huNoV stool isolates by combined peroral and intraperitoneal routes *(226)*. Replication of GII.4 was supported in both humanized and non-humanized mice by increased viral loads and viral protein expression, but no disease developed. This study demonstrated that genetically manipulated mice can be used for huNoV replication study; however, the infection was asymptomatic. Similarly, after seronegative chimpanzees were inoculated with huNoV intravenously, virus shedding in stool and serum antibody responses were similar to that observed in human, but no clinical signs of gastroenteritis were observed *(32)*. Susceptibility of other non-human primates including common marmosets, cotton top tamarins, cynomolgus, and rhesus macaques to huNoV infection was studied by oral inoculation *(200)*. Only rhesus macaques developed immune responses and had long-term viral shedding. Interestingly, gnotobiotic pigs *(49)* and calves *(214)* inoculated with huNoV via the peroral route developed mild diarrhea, which provides a unique perspective on huNoV pathogenesis

and immunity. Despite substantial efforts on animal models for huNoV infection studies, the mechanism of huNoV infection is still not fully understood.

Another important source of information and data is derived from human volunteer studies. In 1970s, volunteer studies were conducted to identify the clinical symptoms of huNoV *(70)*. Early studies showed that challenging volunteers with different strains resulted in immunity, which can be either of a long or short duration *(135, 190, 275)*. However, volunteers who developed symptoms could be re-infected with the same strain 27 to 42 months later *(190)*. Interestingly, some volunteers were resistant to huNoV and never became infected or developed symptoms *(190, 275)*. Later it was determined that huNoV infection requires the HBGA receptors present in the guts of volunteers. Susceptible volunteers were those that encoded a functional *FUT2* gene, which produces HBGAs for huNoV binding *(161)*. Importantly, the infectious dose of huNoV was also determined based on human challenge studies *(14, 228)*. Serum samples from volunteers that were challenged with huNoV can be used for vaccine development *(4, 25, 160, 223)*. Volunteer studies can also be used to validate the effectiveness of virus inactivation by new technologies (e.g. high pressure processing) in foods *(152)* as well as to investigate the persistence of huNoV on hands *(163)*. Human challenge studies provide valuable information on huNoV pathogenesis and immune responses as well as vaccine development; however, these studies are costly and require rigorous regulatory approvals. Therefore, those studies should be well designed.

2.7 Control and Prevention Strategies

2.7.1 Management

Produce-associated outbreaks reduce the confidence of consumers and recalls often cause a large amount of produce loss. It is important to ensure the safety of fresh produce for human health as well as the economy. Microbial monitoring can be an important means of assessing the safety of foods. However, routine virus tests are not often performed to monitor viral contamination in produce *(198)*, and it is likely that the levels of viruses present on produce can be lower than the detection limit. Fresh fruits and vegetables are consumed raw or with minimal processing, which means there is no killing step included before consumption. No one step will be sufficient to maintain the safety of produce, since pathogens can enter at any point from farm to table. In order to prevent virus-contaminated produce from entering the food chain, producers, manufacturers, retailers as well as consumers are responsible for safe production, harvesting, handling, storage, transport, marketing, and preparation.

Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs) as well as Good Hygiene Practices (GHP) should be maintained during the pre- and post-harvest environments. They serve as prerequisite programs to eliminate or significantly reduce viral pathogens from fresh produce. Based on that, programs like Hazard Analysis Critical Control Point (HACCP) can better control produce safety *(198, 212)*. HACCP program establishes a plan focusing on the hazard identification and prevention. Based on epidemiological data, the critical control points (CCPs) where pathogens are most likely to contribute to produce contamination will be highlighted and monitored to ensure proper operation of the system.

The Food and Agriculture Organization of the United Nations (FAO)/the Food Quality and Standards Service (AGNS) posted a practical approach to develop and implement quality assurance and food safety programs for fresh fruits and vegetables by providing access to reference information and training tools *(84)*. This approach enhances the application of Codex Alimentarius guidelines and recommendations *(194)*. Recently, the FDA released FSMA final rules on produce safety, which further provides science- and risk-based preventative steps to ensure produce safety and to prevent safety problems before they occur.

From a pre-harvest perspective, it is essential to use high quality water for irrigation and pesticide application, as viruses can be easily transferred and internalized into produce, and the strong attachments between viruses and produce surfaces make it difficult to remove them.

During post-harvest, washing is a common treatment for removing pathogens present on produce surfaces. Many factors affect the binding affinity as described above, leading to variation in levels of pathogen reduction by washing. In general, washing can significantly reduce viruses from produce surfaces by ~1 log PFU, but the reduction levels depend on washing time, water temperature, produce type, virus type, and the degree of virus contamination *(18, 19, 30, 42, 105)*. Many studies showed that washing is not sufficient to eliminate huNoV. Baert et al. found that only 1.01 log PFU of MNV was removed from spinach by one washing step of 2 min, and only obtained 1.26 PFU of MNV after three washing events with a initial titer of ~ 5 log PFU *(17)*. In addition, soaking produce in water may spread viruses from contaminated produce items to the whole batch *(198)*. It was determined that > 3 log PFU of MNV was easily transmitted to non-inoculated onion bulbs and spinach leaves

when they were washed in contaminated water which contained 5 log PFU/ml of MNV *(19)*.

Industrial sanitizers are commonly applied to disinfect produce and food-contact surfaces. Chlorine-based sanitizers are widely used in wash water for fresh produce *(27)*. Bacteriophages (MS2, ΦX174 and PRD1) and poliovirus type 1 from strawberry surfaces were significantly reduced (70.4 to 99.5%) by immersion in water containing chlorine (ranging from 0.3 to 300 ppm) for 2 min, but complete inactivation was not achieved *(166)*. Similarly, 90-99% of HAV and MS2 inoculated on strawberries, tomatoes, and lettuces were inactivated in 20 ppm chlorine solution *(42)*. The effectiveness of other commonly used sanitizers were also measured, but still viruses were still detectable *(17, 105, 166)*. A study investigated the efficiency of three commonly used disinfectants (5.25% sodium hypochlorite, 15% peroxyacetic acid-11% hydrogen peroxide, and 10% quaternary ammonium compounds) against FCV during rinsing of artificially contaminated strawberries and lettuce *(105)*. The results showed those sanitizers were unable to reduce PFU counts by at least 3 log at the manufacturer's recommended concentration for 10 min at room temperature. Compared with tap water, peroxyacetic acids (PAA) of > 250 mg/L is needed to obtain an additional 1 log PFU of both MNV and B40-8 *(18)*. After immersion in 1% trisodium phosphate (TSP), 0.5% hydrogen peroxide or 0.1% cetyplyridinium chloride, viruses (MS2, PRD1, and ΦX174, and poliovirus type 1) were still found on strawberries *(166)*. Su et al. observed that the effects of 2% TSP and sodium hypochlorite (200 mg/L) on the reduction of FCV and MNV on lettuce and jalapeno peppers (initially inoculated with ~4 log of viruses) were generally similar *(220)*. Those studies indicated that commonly used sanitizers can significantly inactivate, but

not completely eliminate viruses from fresh produce; since the viruses were artificially inoculated with relatively high titers for sanitizer studies, it is likely that the actual levels of viruses from produce are lower.

It is worth mentioning that it will be impossible to remove/inactivate viruses only by washing if they are already internalized into plant tissues, and those viruses may show little reduction post-harvest due to low temperature storage *(208)*.

During the preparation of fresh produce, food handlers can serve as virus carriers or vehicles, and they must follow good sanitary and hygiene practices to prevent contamination and cross-contamination. Contaminated foods and kitchen utensils should be appropriately disinfected to limit virus spread in restaurant/food establishments and home kitchens *(260, 261)*. Food safety education and training in the kitchen environment is effective to maintain proper personal hygiene and avoid cross-contamination *(180)*. Hand washing polices before and after handling foods are important, as contacts between hands and foods are common. It is recommended to maintain short nails, since they are less likely to harbor pathogens than long nails *(159)*. Wearing gloves is preferable to bare hand contact, and frequent glove changing or sanitation is crucial; importantly, wearing gloves does not lessen the need for hand washing as well as other hygiene practices *(15, 36, 184, 242)*. In addition, sanitation of kitchen utensils (e.g. knife, peeler, cutting board) as well as food preparation surfaces is necessary *(184)*. It is also recommended that food handlers report their health status or activities relating to diseases to the supervisor before working *(146, 242)*.

Extension and outreach activities focusing on disseminating of virological knowledge and preventative practices to stakeholders have been encouraged to bridge the knowledge and practice gaps in virus control from farm to table *(157)*. Besides,

interdisciplinary collaboration and interaction between virologist and food safety experts is desired *(157)*.

Vaccination is another important strategy to prevent huNoV infections in consumers. However, development of huNoV vaccine is still a challenge. Vaccine cross-protection may not be obtained due to variations in the antigencity among genotypes and strains *(246)*, and new variants appear frequently *(248)*. Moreover, without appropriate cell culture for huNoV, it becomes difficult to produce a live attenuated virus vaccine. In this situation, recombinant VLPs are used as immunogens; however, they cannot be produced on a large scale. Furthermore, vectored vaccines have been investigated, which are using viruses to transport pieces of norovirus *(106, 112, 167)*. Clinical trials (phase I and II) demonstrated those vaccines are promising, but more clinical trials (phase III) are needed for vaccine evaluation *(12, 259)*.

2.7.2 Non-thermal food processing technology

Generally, the effectiveness of newly food processing technologies on huNoV inactivation is difficult to assess, as huNoV cannot be cultivated in cell culture. In order to measure the effectiveness of those technologies, huNoV surrogates have been used. Scientists are trying to find surrogates that are more resistant to the processing treatments than huNoV.

Many studies have been conducted to evaluate the effectiveness of non-thermal processing techniques for virus reduction and elimination. Non-thermal technologies include gamma and electron beam radiation, ultraviolet (UV) light/pulsed light, ultrasound, cold plasma, gaseous antimicrobials, and high pressure processing (HPP).

Irradiation (electron beam and gamma irradiation) is generally not effective in virus inactivation *(88, 202, 279)*. A radiation dose of ~3 kGy electron beam is required

to cause approximately 1 log reduction/g of FCV on lettuces *(279)*. Less or up to a 1-log reduction of MNV was observed on cabbages and strawberries at doses 4 kGy of electron beam irradiation *(202)*. Similarly, 4 kGy delivered by electron beam provided < 2 log PFU reduction in both MNV and TV in phosphate-buffered saline (PBS) *(196)*. In addition, MNV is resistant to gamma irradiation, and only a 1.7-2.4 log MNV reduction was achieved in fresh produce at the dose of 5.6 kGy *(88)*. Virus inactivation by irradiation involves morphological change and capsid disruption as well as degradation of genomic materials *(88)*.

UV irradiation can inactivate a wide spectrum of foodborne microorganisms. One log reductions of MS2, PRD-1, and poliovirus type 1 were achieved by UV doses of 14, 8.7, and 4.1 mW s/cm^2 *(181)*. More than a 4-log $TCID_{50}$ (50% tissue culture infectious dose)/ml reduction of FCV on green onions and lettuce was achieved after exposure to UV light with doses of 120 and 240 mW s/cm^2 *(90)*. Additionally, a 1.6-log reduction of MS2 occurred with 30 mW s/cm^2 , whereas a 3.3-log inactivation occurred with 25 mW s/cm^2 *(150)*.

Pulsed-light has short and high-intensity pulses of light from the UV to near infrared region with an energy density of 0.01 to 50 J/cm^2 at the surface. After pulsed light treatment, less than a 1-log inactivation of MS2 was observed in an inoculated food matrix including powdered black pepper, garlic with 20 pulses at a fluence of 0.94 J/cm^2 per pulse *(23)*. With higher intensity pulsed light, reductions in MNV exceeded 3 log in less than 3 s with 5 pulses at a fluence of 3.45 J/cm^2 per pulse *(253)*. However, the penetration of UV is limited, and UV is not able to inactivate internalized viruses in fresh produce; it is therefore more likely to be used in surface decontamination *(90, 129)*.

Ultrasound with a frequency ranging from 20 kHz - 2 MHz can also be used for foodborne pathogen inactivation. In order to completely inactivate ~4 log PFU/ml of FCV, MS2, and MNV inoculated in PBS, 5-, 10-, and 30-min high-intensity ultrasound (HIUS) treatments are required, respectively *(221)*. However, virus inactivation by HIUS was significantly reduced when those viruses were inoculated in orange juice, implying that the food matrix can interfere with the treatment. Schultz et al. showed that only a 1-log reduction (~89%) of MN2 was obtained on fresh raspberries after 1 s of pressurized steam and high-power ultrasound (steam-ultrasound) treatment, and at this point texture damage of the raspberries was evident. This study indicated that steam-ultrasound may not be an appropriate method to decontaminate fragile berries *(207)*.

Cold plasma is a combination of photo-inactivating light, gaseous antimicrobials, and reactive species. It has a high degree of effectiveness and low cost, and has been used for produce decontamination *(143, 206)*. Exposure to 2.5-W argon (Ar) cold plasma resulted in a 5.5-log unit reduction in the FCV within 120 s *(3)*. Cold plasma treatment of an MS2 virus suspension for >30 s resulted in a reduction of >0.69 log *(274)*.

Gaseous antimicrobials were also tested, as the efficiency is not likely to be influenced by the location of viral contamination or pH. Exposure to hydrogen peroxide vapor (127 ppm) for 1 hour resulted in complete inactivation of all viruses tested, and > 4-log reductions for poliovirus, rotavirus, adenovirus, and MNV were observed *(240)*. Gaseous ozone with levels of 20-25 ppm resulted in a reduction of FCV by > 3 log within a hotel room (47.6 m^3) and a cruise liner cabin (36.4 m^3) after

20 min exposure *(122)*. Saturated steam vapor caused a 3-log reduction of MS2 on clay coupons after 2 s of exposure *(225)*.

High pressure processing (HPP) is considered a promising technology to inactivate viruses with minimal adverse effects on food quality (taste, flavor, texture, appearance, and nutritional value) *(157, 158, 164, 165)*. Factors that affect HPP effectiveness include pressure magnitude, temperature, holding time, virus type, and food matrix *(140, 165)*. It is sufficient to inactivate 6.85 log PFU of MNV by a pressure of 450 MPa for 5 min at 20 °C *(140)*. Pressure treatment of 600 MPa for 2 min barely caused any reduction in TV and MNV inoculated on un-wetted (dry) blueberries at both 4°C and 21°C; however, inactivation dramatically increased (> 3 log reduction) with lower pressure (<400 MPa) when blueberries were immersed in PBS during treatment *(158)*. More than a 5-log reduction was obtained in MNV-contaminated fresh-cut strawberries and lettuce with HPP treatment at 400 MPa at 4 °C for 2 min *(164)*. Further, it was demonstrated that HPP disrupted the viral capsid structure but genomic RNA remained intact *(164)*.

More research is still needed to determine which of these new technologies is most effective to reduce or eliminate viral pathogens in fresh produce.

2.7.3 Quantitative microbial risk assessment (QMRA)

It is known that zero risk cannot be achieved even with FSMA and other food safety programs (e.g. HACCP) *(60)*. With a comprehensive and integrative approach, quantitative microbial risk assessment (QMRA) can be conducted to determine the likelihood of a risk associated with foodborne pathogens to public health and measure the effectiveness of control strategies.

QMRA is a framework and approach to address the adverse health effects through environmental exposure to microbial agents with various mathematical models. The ultimate goals of QMRA are to support management decisions, develop and implement most favorable preventative solutions, and control the food safety risks. The degree of credibility in QMRA is mainly based on the quality and quantity of the data. The data can come from many sources, but they need to be quantitative to provide the numerical expression to estimate the risk with a point estimate and uncertainties. However, the actual epidemiological data needed for QMRA targeting a certain scenario are not always available *(113)*. In order to conduct a risk assessment properly, it is necessary to have appropriate assumptions and to maximally mimic the real situation. At the same time, surveillance and epidemiologic studies are needed to fill the gaps.

In the 1960s, the National Academy of Sciences (NAS) developed a risk framework "Red Book" to assess and control environmental pollution risks. Microbial risk assessment was then derived from this framework. Since 1999, the Codex Alimentarius has provided and revised the general principles and guidelines for the conduct of microbial risk assessment *(56)*. In 2012, the EPA provided microbial risk assessment guidelines with focus on food and water *(244)* followed by tools and methods for risk assessment on water media *(243)*.The risk assessment associated with microorganisms is different from that for chemicals. Unlike chemicals, microorganisms may multiply and can be inactivated throughout the food chain. The dose-response relation between microorganisms and illness/death also depends on the pathogen type as well as the susceptibility of different sub-populations. Those differences make it challenging to conduct an accurate QMRA *(256)*.

The formal framework of QMRA includes hazard identification, dose response, exposure assessment, risk characterization, and risk management. Hazard identification is to describe human health effects of specific pathogens, including severity, sensitive populations, and immunological response *(201)*. Dose-response is to characterize the relationship between various doses administrated and subsequent probability of infection or health effects. Exposure assessment is to determine the size and nature of the population exposed and the route, amount, and duration of exposure. Risk characterization is to integrate the information from exposure, dose response, and health steps to estimate magnitude of health risks.

QMRA has been widely used in drinking water safety *(171, 211, 229)*, and can also be applied to address contaminated irrigation water during pre-harvest of fresh produce *(183, 192, 193)*. The potential risk from human enteroviruses associated with the consumption of lettuce crops spray-irrigated with secondary-treated wastewater was estimated *(193)*. Infection rates were more likely to be affected by virus die-off rates on lettuce crops than the occurrence of high levels of viruses in irrigation water. Similarly, another study investigated the infection rates associated with the consumption of crops that were irrigated with waste stabilization pond effluents *(192)*. The sensitivity analysis showed that variations in effluent quality with assumed ratios of rotavirus to *E. coli* and reduction rates of pathogens greatly impacted the rotavirus infection rates. Interestingly, a QMRA model was developed to estimate norovirus risks from consumption of vegetables irrigated with human wastewater *(183)*. The results indicated that waste stabilization pond treatment was not sufficient, and further disinfection treatments were required to obtain acceptable levels of risk for consumption of cucumbers and broccoli.

In addition, risk perception and risk communication are also important parts of QMRA *(201)*. Experts and the public perceive risks differently. Experts are mainly driven by the expected numbers and data from risk assessment, whereas the public's perception of risk is influenced by many factors. After recognizing the different perceptions, risk communication efforts should focus on what people do not know.

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Chapter 3

SURVIVAL OF MURINE NOROVIRUS, TULANE VIRUS, AND HEPATITIS A VIRUS ON ALFALFA SEEDS AND SPROUTS DURING STORAGE AND GERMINATION

(A manuscript published in Applied and Environmental Microbiology)

3.1 Abstract

Human norovirus (huNoV) and hepatitis A virus (HAV) have been involved in several produce-associated outbreaks and identified as major foodborne viral etiologies. In this study, the survival of huNoV surrogates (murine norovirus (MNV) and tulane virus (TV)) and HAV was investigated on alfalfa seeds during storage and post-germination. Alfalfa seeds were inoculated with MNV, TV, or HAV with titers of 6.46 ± 0.06 log PFU/g, 3.87 ± 0.38 log PFU/g, 7.01 ± 0.07 log 50% tissue culture infectious dose ($TCID_{50}$) /g, respectively. Inoculated seeds were stored for up to 50 days at 22°C, and sampled during that storage period on days 0, 2, 5, 10, and 15. Following storage, virus presence was monitored over a one-week germination period. Viruses remained infectious after 50 days with titers of 1.61 ± 0.19 log PFU/g, 0.85 ± 0.21 log PFU/g, and 3.43 ± 0.21 log $TCID_{50}$ /g for MNV, TV, and HAV, respectively. HAV demonstrated greater persistence compared to MNV and TV without a statistically significant reduction over 20 days (<1 log $TCID_{50}$/g); however, relatively high levels of genomic copies of all viruses persisted over the testing time period. Low titers of viruses were found on sprouts and located in all tissues, as well as sprout-spent water sampled on days 1, 3, and 6 following seed planting. Results revealed the

persistence of viruses in seeds for a prolonged period of time and perhaps of greater importance this data suggests the ease of which virus may transfer from seeds to sprouts and spent water during germination. These findings highlight the importance of sanitation and prevention procedures before and during germination.

3.2 Introduction

With the increasing consumption of sprouted seeds due to health benefits (1), sprouts have been found associated with at least 55 foodborne outbreaks occurring worldwide resulting in a total of 15,233 illnesses (2). In 2011, the large outbreak in Europe associated with fenugreek seeds contaminated by *Escherichia coli* O104:H4 (3) renewed awareness for sprout and seed safety. Alfalfa sprouts historically have been a major player in foodborne outbreaks. According to the U.S. Food and Drug Administration (FDA), since 1990 there have been more than 30 reported outbreaks linked to the consumption of raw or lightly cooked alfalfa sprouts in North America, where *E. coli* O157:H7 and various serotypes of *Salmonella* were identified as the major bacterial etiologies (4). It is known that sprouts have potential for bacterial pathogen growth during germination which provides a warm, humid, and nutrient-abundant environment for sprouting. Recently, the FDA Food Safety Modernization Act (FSMA) Proposed Produce Safety Rule addressed the importance of sprout safety by requiring treatment immediately before sprouting to reduce microorganisms, and specific bacterial monitoring, including testing of sprouts and spent irrigation waters.

Many research studies have been conducted in attempts to better understand the interaction of bacterial pathogens with seeds and sprouts (5-12). If the seeds were contaminated prior to germination, bacterial pathogens such as *E. coli* O157:H7, *Vibrio cholerae* O1, and *S.* Typhi may grow and are more likely to be transferred to

outer surfaces and inner tissues (5, 6). Many factors that affect bacterial attachment were identified such as characteristics of surfaces, types of bacterial pathogens, and methods of disinfection. It was found that wrinkled/rough or damaged alfalfa seeds were likely to harbor more bacteria and this bacterial contamination was also more resistant to sanitizers compared to smooth and healthy seeds (8, 13). Barak et al. (7, 10) found that different serovars of *S. enterica* and plant-associated bacteria attached to alfalfa sprouts significantly better than *E. coli* O157:H7 during rinsing steps probably due to the presence of curli. Other factors affecting bacterial growth and survival on seeds were also identified such as homogenization methods, rinsing methods, soaking times, temperature, use of surfactants, irrigation systems, and sprouting devices (11, 12). However, little knowledge is known about the risk and survival associated with the viruses on seeds and sprouts. It is likely that viruses may be present in these moist environments that have previously been found to harbor contamination with pathogenic bacteria; however, the lack of epidemiological evidence is likely due to the lack of testing of foods and fecal samples for norovirus or other foodborne viruses.

Viruses are a great concern for produce safety, as viruses may be introduced from the pre-harvest environment at the farm, sprouting facility, and during preparation via infected food-handlers or cross-contamination in restaurant/food establishments (14-17). It was estimated that viruses cause over 5 million foodborne illnesses each year in the U.S., and human norovirus (huNoV) and hepatitis A virus (HAV) are identified as the most common viral etiologies of foodborne illnesses (18, 19). The low infectious dose of both huNoV and HAV with estimated averages as 10-

100 virus particles, means that even a small amount of contamination has the potential to cause illness (20-22).

Currently there is no cell culture available for huNoVs in the laboratory; therefore, surrogates like murine norovirus (MNV) are used to predict norovirus behavior in environmental persistence studies (23). MNV was the first norovirus to be propagated in cell culture and shares similar genetic and structural features with human norovirus (23). Tulane virus (TV), a newly discovered calicivirus, belongs to the genus *Recovirus*, and is another potential surrogate (24, 25). TV has significant genetic diversity compared with MNV, but is capable of binding histo-blood group antigens (HBGA), which indicates that it shares structure similarity with huNoVs (26). Therefore, it is interesting to compare the survival of these two huNoV surrogates in environmental settings.

In this study, the behaviors of MNV, TV and HAV were investigated on intentionally contaminated alfalfa seeds during storage and on sprouts after a seven-day germination period. The degree of virus transfer to spent irrigation water was also investigated. Lastly, the distribution of viruses on contaminated sprouts was investigated. This study is important for determining the persistence of viruses on the seed surface, and for evaluating the potential risk associated with sprouting and irrigation water after seed contamination.

3.3 Materials and Methods

3.3.1 Virus cultivation and infectivity

Murine norovirus (MNV-1) (a gift from Dr. Herbert Virgin, Washington University School of Medicine, St. Louis, MO) was cultured in RAW 264.7 cells

(ATCC# TIB-71) in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), 100 U/ml penicillin G-streptomycin-0.25µg/ml Amphotericin B (Hyclone, Logan, UT), 2 mM L-alanine-L-glutamine (Gibco, Carlsbad, CA), and 1 mM sodium bicarbonate (Cellgro, Manassas, VA). Tulane virus (a gift from Dr. Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH) was propagated in LLC-MK2 cells (ATCC# CCL-7) in medium 199 (Hychlone, Logan, UT) supplemented with 10% FBS, and 100 U/ml penicillin G-streptomycin-0.25µg/ml Amphotericin B. After typically 48 h infection of 80-90% confluent monolayers for both MNV and TV, complete cytopathic effect (CPE) was observed. Hepatitis A virus (HAV) strain HM175 (ATCC# VR-1402) was propagated in fetal rhesus monkey kidney cells (FRhK-4) (ATCC# CRL-1688) in DMEM supplemented with 10% FBS, 100 U/ml penicillin G-streptomycin-0.25µg/ml Amphotericin B, and 1mM sodium bicarbonate. HAV was then infected in 80-90% confluent monolayer of FRhK-4 cells for typically 7 days to observe CPE. Viruses were obtained following three cycles of freeze-thawing infected cells, and centrifugation at 2000 × g for 15 min. The supernatant was filtered through by 0.2 µm membrane filter (Thermo, Rochester, NY) before storing viruses at -80 °C until use.

MNV and TV plaque assays were performed similarly to previous studies with slight modifications (23, 24). In brief, RAW 264.7 and LLC-MK2 cells were grown to 80-90% confluency in 6-well plates (Castar, Corning, NY), one hundred microliter of ten-fold serial dilutions of each virus sample prepared in Hank's balanced salt solution (HBSS) (Cellgro, Manassas, VA) was dispensed over monolayers in duplicate. The plates were incubated at 37 °C with 5% CO_2 for 1 h with gentle agitation every 15 min

followed by the addition of a 2 ml overlays. MNV-1 overlays consisted of 1.5% agarose (Lonza SeaPlaque, Rockland, ME) with complete Eagle's medium (MEM) (Hyclone, Logan, UT) supplemented with 2% FBS, 100 U/ml penicillin G-streptomycin-0.25µg/ml Amphotericin B, 2mM L-alanine-L-glutamine, and 1mM sodium pyruvate. TV overlays consisted of 1.5% agarose with complete medium 199 supplemented with 2% FBS, and 100 U/ml penicillin G-streptomycin and 0.25 µg/ml. After the incubation period (typically 48 h for MNV and TV), 1 ml of 0.2 g/L neutral red (Fisher, Fair Lawn, NJ) was added into each well followed by a 2-5 h incubation. Titers of virus were determined and expressed by plaque forming units (PFU).

The titer of HAV was determined by using 50% tissue culture infectious dose ($TCID_{50}$) in fetal rhesus monkey kidney cells (FRhK-4) (27). Cell monolayers were allowed to grow in 96-well plates containing complete DMEM supplemented with 2% FBS, 100 U/ml penicillin G-streptomycin-0.25µg/ml Amphotericin B, and 1mM sodium bicarbonate. Virus samples (100 µl) in ten-fold serial dilutions (eight replicates for each dilution) were inoculated onto confluent cells at 37 °C with 5% CO_2 for typically 15 days, and CPE was observed microscopically. Virus titers were determined and expressed by $TCID_{50}$ using the Reed-Muench method (27).

3.3.2 Virus genome quantification by real-time reverse transcription (RT)-PCR

The presence of MNV, TV and HAV genomic copies was detected on seeds, sprouts, and water samples. To generate a standard curve for each virus type, 1 ml of virus stock with known genomic copies (10^7 genomic copies/ml for both HAV and MNV, and 10^6 genomic copies/ml for TV) was 10-fold serially diluted with HBSS. RNA was extracted and reverse transcribed into cDNA by using QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) and Omniscript RT kit (QIAGEN) as reference

protocols, respectively. Three sets of primers were used for each type of virus as follows: forward primer (5'-CAGCACATCAGAAAGGTGAG-3') and reverse primer (5'-CTCCAGAATCATCTCCAAC-3') for HAV (28); forward primer (5'-CCAGCTTGATGTAGGCGATT-3') and reverse primer (5'-CTCAGCCATTGCACTCAAAG-3') for TV (26); forward primer (5'-TCTTCGCAAGACACGCCAATTTCAG-3') and reverse primer (5'-GCATCACAATGTCAGGGTCAACTC -3') for MNV (29). Real-time PCR reactions were performed in a total reaction volume of 20 μL containing 10 μL SYBR-Green PCR Master Mix (QIAGEN), 2 μL cDNA, and same set of primers with the protocol from QuantiTect SYBR Green PCR Kit (QIAGEN) on 384-well plates. Reactions were run on the Applied Biosystems 7900 HT Sequence Detection system (Applied Biosystems, Foster City, CA) with the following thermal conditions: 95 °C for 10 min followed by 40 cycles of 94 °C for 15 s, annealing temperature of each virus for 30 s (59 °C for MNV, 56 °C for HAV, 59 °C for TV), followed by a dissociation step at 60 °C for 15 s and 90 °C for 15 s. SYBR green signals were read in every cycle, and the logarithm of the increment in fluorescence was plotted versus the cycle number with fixed threshold level for all runs. Virus quantity was then determined by comparison to a standard curve and expressed as genomic copies. The standard curve was generated in duplicate for each qPCR run. The detection limits for all virus types were determined to be ~100 genomic copies/ml. MNV, TV and HAV in HBSS served as positive controls, and negative controls consisted of the environmental sample (seed, sprout or water) without virus.

3.3.3 Alfalfa seed preparation and storage

Alfalfa seeds (Johnny Seeds, Winslow, ME) were sterilized by submerging seeds in 70% ethanol for 5 min followed by soaking in a 10 % bleach solution for 20 min. Seeds were rinsed with deionized water and then dried under the laminar flow hood at room temperature overnight before dividing into 1 g samples in 1.5 ml microcentrifuge tubes prior to inoculation. After treatment, little effect was observed visually on sprouting percentage compared with untreated sprout seeds. Every 1 g seed sample was individually inoculated with 200 µl of MNV, TV, or HAV, and was stored for up to 50 days at 22°C in individual closed tubes. Seed samples were collected on sampling days (0, 2, 5, 10, 15, 20, 30, and 50), and every 1 g of seeds were carefully placed into 1 ml of HBSS and vortexed for 1 min, and the solution was retained for infectivity assays or/and real-time RT-PCR.

3.3.4 Alfalfa sprout germination and irrigation water collection

On sampling days (0, 2, 5, 10, and 15), another set of inoculated seed samples were germinated in the growth chambers (Victorio, Orem, UT). The growth chamber had three trays: the top tray was empty used for watering; the middle tray had rings to distribute seeds evenly and was used for germination; and the bottom tray was a holding container to collect spent irrigation water. During the 7-day germination period, 500 ml municipal tap water was added daily on the top tray. Water was then siphoned over seeds/sprout, and finally drained and collected in the bottom tray. The humidity and temperature inside of growth chambers containing uninoculated seeds/sprouts were measured and recorded daily by Traceable Therm./Clock/Humidity Monitor (Fisher, Pittsburgh, PA). Spent irrigation water samples (1 ml, duplicates) were collected on days 1, 3, and 6 following initial seed sprouting for each sample and

were processed for quantification of virus collected in the spent irrigation water. Sprouts (approximately 12 g sprouts from 1 g seeds after 7-day germination) were collected in 50 ml centrifuge tubes containing 10 ml HBSS, and vortexed for 1 min to elute the virus from the sprout for virus detection. In addition, 10 alfalfa sprouts geminated from inoculated day 0 seeds were randomly collected. The portions of sprouts including primary root, hypocotyl, true leaves, and seed coat, were cut separately by using scissors, and collected with forceps. The scissors and forceps were soaked in 10% bleach (Clorox, Oakland, CA) and neutralized in 5% sodium thiosulfate (Fisher, Fair Lawn, NJ) every time after being used to prevent cross-contamination. The presence of virus genomic copies from each portion of sprouts was determined.

3.3.5 Statistical Analysis

Experiments were conducted in triplicate. Results are reported as mean and standard deviation. Data were analyzed by ANOVA on JMP software (Version 9.0, SAS Institute Inc., Cary, N.C.), and significance was indicated if $p<0.05$.

3.4 Results

3.4.1 Virus recovery on the surface of alfalfa seeds after inoculation

Initial titers of viruses inoculated on seeds were determined to be 6.46 ± 0.06 log PFU/g (7.15 ± 0.50 log genomic copies/g) for MNV, 3.87 ± 0.38 log PFU/g (5.92 ± 0.45 log genomic copies/g) for TV, 7.01 ± 0.07 log $TCID_{50}$ /g (7.90 ± 0.37 log genomic copies/g) for HAV, respectively. After seeds were visibly dried after inoculation (approximately an hour) on day 0, MNV, TV and HAV were recovered from seeds with titers of 6.55 ± 0.24 log PFU/g (7.44 ± 0.06 log genomic copies/g),

3.43 ± 0.07 log PFU/g (5.73 ± 1.19 log genomic copies/g), and 5.60 ± 0.19 log $TCID_{50}$ /g (6.55 ± 0.15 log genomic copies/g), respectively. Log reductions were listed on day 0 (Table 2.1). The results showed significant reductions of HAV and TV on the surface of seeds after drying with values of 1.41 ± 0.19 $TCID_{50}$/g and 0.44 ± 0.07 log PFU/g ($p<0.05$), respectively. MNV was an exception where little reduction was observed.

Table 3.1 Infectivity reduction of HAV, MNV, and TV on alfalfa seeds and in HBSS stored at 22 °C for up to 50 days.

Virus	Matrix	Infectivity reduction of virus (log PFU/g or log $TCID_{50}$/g) [a]							
		Day 0	Day 2	Day 5	Day 10	Day 15	Day 20	Day 30	Day 50
HAV	Seeds	a1.41 ± 0.19^{A}	a1.44 ± 0.32^{A}	a2.02 ± 0.93^{A}	a1.61 ± 0.09^{A}	a1.78 ± 0.14^{A}	a2.15 ± 0.39^{A}	a3.68 ± 0.00^{B}	a3.58 ± 0.21^{B}
	HBSS	b0.00 ± 0.00^{A}	b0.05 ± 0.00^{A}	a0.80 ± 0.35^{B}	a1.80 ± 0.35^{C}	a1.80 ± 0.35^{C}	a2.38 ± 0.00^{CD}	a2.80 ± 0.35^{D}	a4.22 ± 0.24^{E}
MNV	Seeds	a-0.09 ± 0.24^{A}	a0.76 ± 0.63^{B}	a2.18 ± 0.03^{C}	a2.46 ± 0.04^{CD}	a2.86 ± 0.14^{DE}	a3.14 ± 0.21^{EF}	a3.90 ± 0.22^{F}	a4.85 ± 0.19^{G}
	HBSS	a0.00 ± 0.00^{A}	a-0.32 ± 0.43^{A}	a2.24 ± 0.11^{B}	b2.97 ± 0.03^{C}	a3.19 ± 0.10^{C}	a3.82 ± 0.14^{CD}	a4.22 ± 0.13^{D}	a4.94 ± 0.31^{E}
TV	Seeds	a0.44 ± 0.07^{A}	a0.57 ± 0.12^{B}	a0.81 ± 0.04^{B}	a1.53 ± 0.08^{C}	a2.01 ± 0.17^{D}	a2.15 ± 0.16^{D}	a2.19 ± 0.09^{D}	a2.58 ± 0.21^{E}
	HBSS	b0.00 ± 0.00^{A}	a0.03 ± 0.34^{A}	a0.77 ± 0.28^{B}	b0.91 ± 0.03^{BC}	b1.00 ± 0.06^{BC}	b1.24 ± 0.03^{C}	b1.69 ± 0.01^{D}	b1.84 ± 0.08^{D}

[a] Values are means ± SD of three replicates; values in rows with the same preceding letter indicate no significant difference (p>0.05) when comparing between seeds and HBSS for each virus; values in rows with the same following letter indicate no significant difference (p>0.05) when comparing virus survival between different sampling days.

3.4.2 Survival of viruses on alfalfa seeds and in HBSS during a 50-day storage at 22 °C

The survival rates of infectious virus particles for MNV, TV, and HAV on seeds after inoculation as well as in HBSS were determined at 21 °C (ranging from 17.9 to 23.4 °C) for up to 50 days. The infectivity reductions for each virus with log PFU or log $TCID_{50}$ were determined (Table 2.1). All viruses remained infectious on seeds for up to 50 days, with varying trends in reduction. Generally, the reductions observed in infectivity increased with extended storage time both on alfalfa seeds and in HBSS.

After initial decrease of 1.5 log $TCID_{50}$/g after drying, HAV persisted with no significant reduction on the surface of alfalfa seeds (< 1 log $TCID_{50}$/g) over 20 days ($p<0.05$), and decreased approximately 2 log $TCID_{50}$/g within 50 days; however both MNV and TV were reduced significantly within the first 2 days ($p<0.05$) on seeds. A greater reduction in MNV (almost 5 log PFU/g) was observed on the seed surface compared to TV (approximately 2 log PFU/g) after 50 days.

There was no significant reduction in HBSS within the first 2 days; however, a significant decrease was observed after 5 days ($p<0.05$), regardless of virus type. TV was relatively stable in HBSS with less than a 2 log PFU/g reduction after 50-days whereas a 4~5 log PFU/g or $TCID_{50}$/g reduction was found in both MNV and HAV.

Differences in virus survival were observed based on matrices (either seeds or HBSS). The reduction in virus infectivity from seeds and in HBSS were similar over this storage period for MNV, and no significant difference ($p>0.05$) was observed between seeds and HBSS over the storage period on day 10. TV decreased more quickly starting on day 0 in seeds, and significantly greater reductions ($p<0.05$) were

found in seeds beginning at day 10. In addition, after an approximately 1.5 log $TCID_{50}$/g reduction on day 0, HAV persisted on seeds and in HBSS.

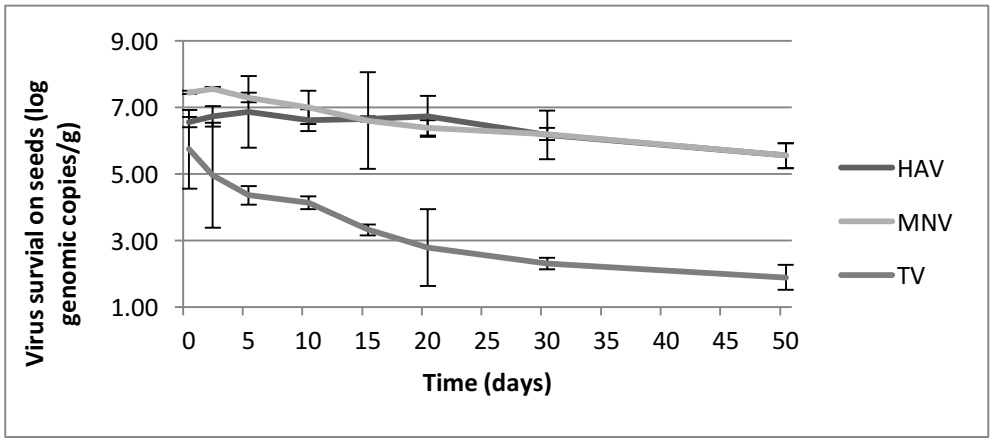

Figure 3.1 Genomic copies of HAV, MNV, and TV present on alfalfa seeds stored at 22 °C for up to 50 days.

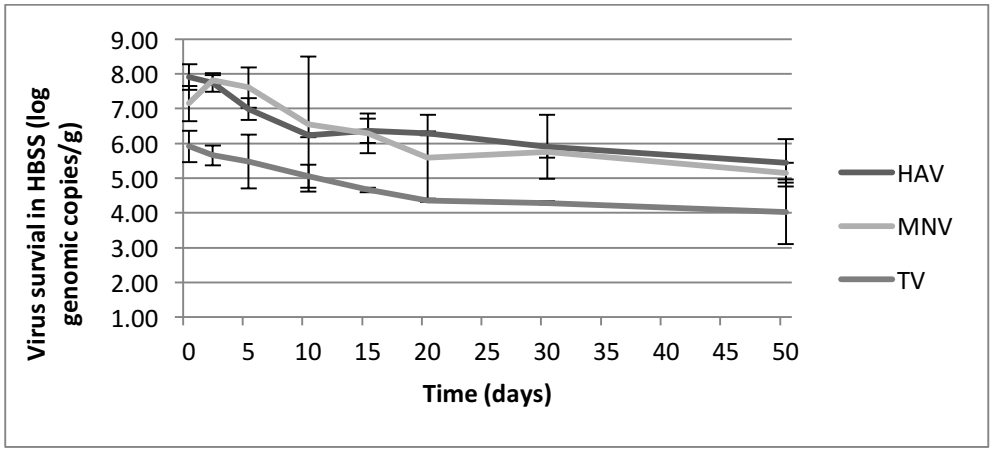

Figure 3.2 Genomic copies of HAV, MNV, and TV present in HBSS stored at 22 °C for up to 50 days.

The genomic copies of all the viruses were also determined over the time period and the data were displayed in Figures 2.1 and 2.2. The numbers of genomic copies for HAV and MNV were relatively constant in both matrices resulting in ~ 2 log reduction over 50 days. No significant difference in genomic copies of HAV was detected on seeds within the first 30 days, and MNV within the first 15 days. However, this trend was not observed for TV. The genomic copies of TV had similar trends to the plaque assay results, and significantly decreased after 10 days in both matrices. The reduction of TV genomic copies in HBSS was lower than that on seeds, which matched the plaque assay data as well.

3.4.3 Survival of viruses on alfalfa sprout after 7-day germination

The seeds were allowed to germinate for 0, 2, 5, 10, and 15 days post-inoculation with daily watering. After a 7-day germination period, the approximate weight of sprouts germinated from 1 g of seeds was ~ 12 g, and viruses were detected on sprout from seeds that were artificially contaminated. The humidity of sprouts was measured mainly above 55% in growth chamber, ranging from 36% to >90%. The levels of viruses detected on sprouts largely depended on the amounts of viruses that survived on seeds initially (Table 2.2). As there was no significant reduction of HAV on seeds within the first 15 days ($p>0.05$), similar levels of HAV ranging from 2.43 to 3.46 log $TCID_{50}$ were detected on the sprouts after germination for all the samples selected within this period, approximately 2.5 log $TCID_{50}$ lower than the initial titers on the inoculated seeds. In addition, the levels of MNV and TV found on sprouts decreased corresponding to the decreasing titers over the time. Interestingly the titers of TV associated with sprouts were <1 log PFU lower than that on seeds before germination, whereas more reductions were observed with HAV and MNV. Again the

numbers of genomic copies were similar with small fluctuations, which were 1~2 log higher than that determined by infectivity assays for all the sprout samples. As observed previously, the genomic copies of HAV and MNV declined but were persistent on sprouts. However, the TV genomic copies were found to be relatively stable over the course of the experiment.

Table 3.2 infectivity and genomic copies of HAV, MNV, and TV on alfalfa sprouts germinated (1 g seeds) on days 0, 2, 5, 10, and 15 after inoculation.

Day	Virus Survival on Sprout from Inoculated Seeds after Storage time (day 0, 2, 5, 10, and 15)[a]					
	HAV		MNV		TV	
	Infectivity (log $TCID_{50}$)	Genomic copies	Infectivity (log PFU)	Genomic copies	Infectivity (log PFU)	Genomic copies
0	3.46 ± 0.71^{A}	4.25 ± 0.47^{AB}	3.10 ± 0.08^{A}	4.73 ± 0.51^{A}	2.19 ± 0.06^{A}	3.36 ± 0.22^{A}
2	3.04 ± 0.59^{A}	4.57 ± 0.07^{A}	2.73 ± 0.36^{AB}	3.64 ± 0.69^{AB}	2.13 ± 0.09^{A}	3.58 ± 0.65^{A}
5	2.71 ± 0.35^{A}	4.19 ± 0.20^{AB}	2.31 ± 0.36^{B}	3.43 ± 0.50^{B}	2.08 ± 0.03^{A}	3.94 ± 0.36^{A}
10	2.54 ± 0.12^{A}	4.34 ± 0.65^{AB}	2.16 ± 0.05^{B}	3.29 ± 0.19^{B}	1.26 ± 0.12^{B}	3.49 ± 0.05^{A}
15	2.43 ± 0.04^{A}	3.58 ± 0.64^{B}	1.14 ± 0.05^{C}	2.78 ± 0.91^{B}	1.09 ± 0.12^{B}	3.47 ± 0.15^{A}

[a] Values are means ± SD of three replicates, values in columns with the same letter indicate no significant difference (p>0.05) when comparing virus survival by infectivity assay or genomic copies on sprouts from inoculated seeds with storage periods of 0, 2, 5, 10, and 15 days.

Table 3.3 Localization of HAV, MNV< and TV in the alfalfa sprouts (each sample represents pool of 10 sprouts). Sprouts were germinated from inoculated day 0 seeds.

Viruses	# positive/ total			
	Primary Root	Hypocotyl	First True Leaves	Seed Coat
HAV	3/3	3/3	3/3	3/3
MNV	2/3	3/3	3/3	3/3
TV	2/3	3/3	3/3	3/3

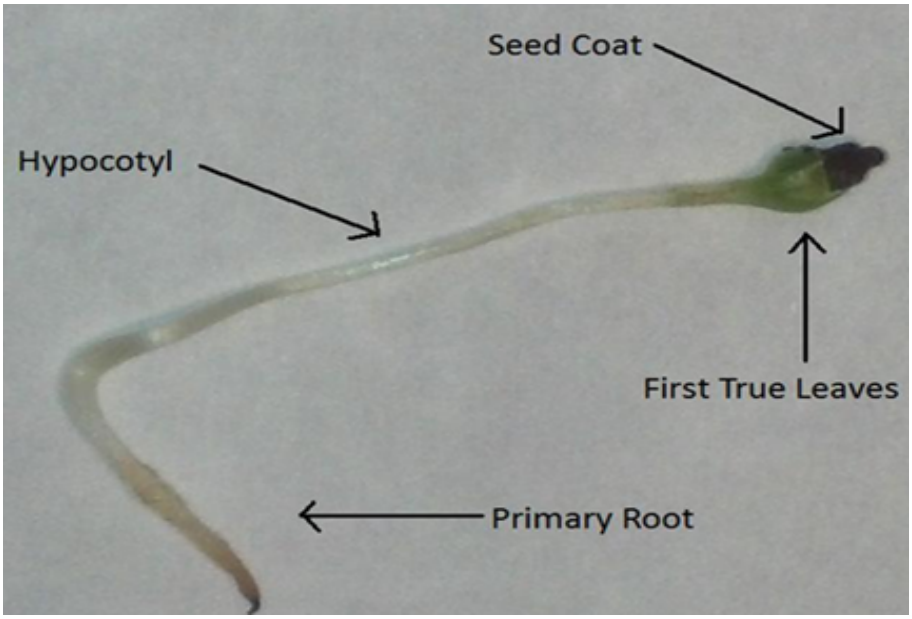

Figure 3.3 Anatomy of alfalfa sprout. The presence of viruses on each portion of sprouts was determined after 7-day period germination.

Virus was distributed within the alfalfa sprout. The anatomy of a sprout (Figure 2.3) including four parts: primary root, hypocotyl, true leaves, and seed coat; shows all portions of sprouts which were identified at least twice to be contaminated by each virus RNA genome (Table 2.3).

3.4.4 Presence of viruses in irrigation water during sprout germination.

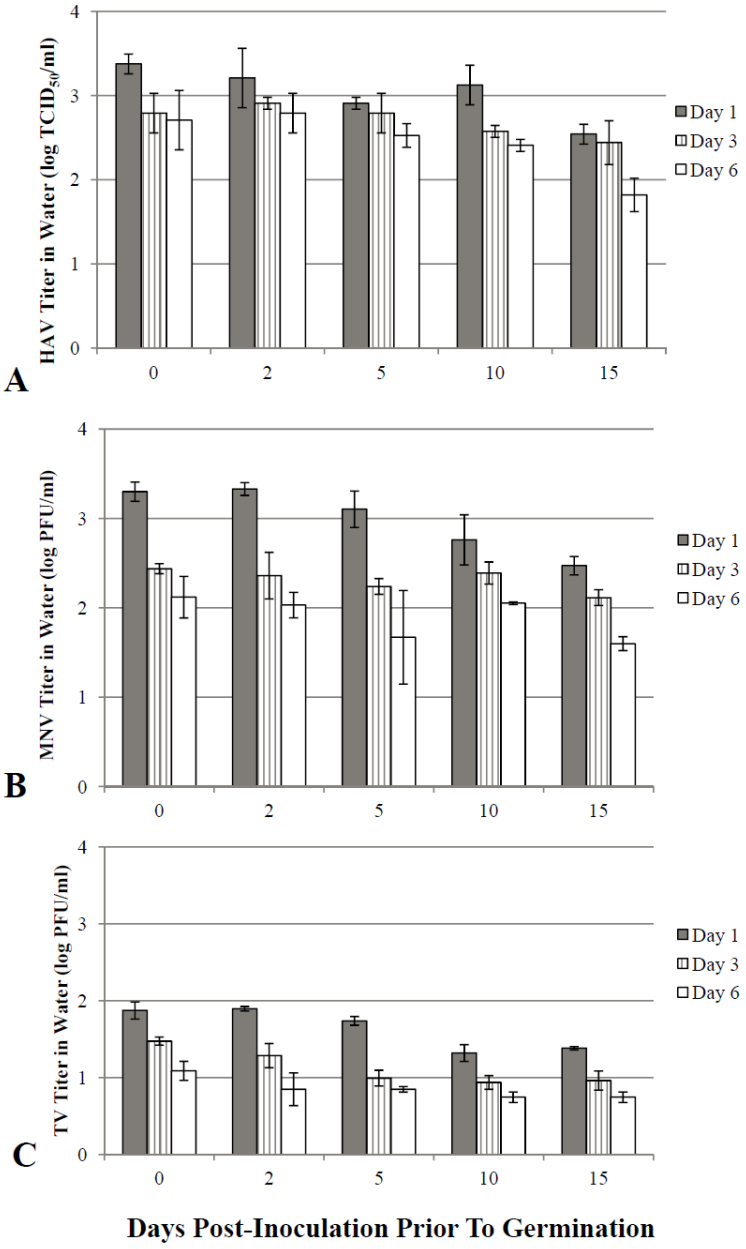

Figure 3.4 Presence of HAV (A), MNV (B), and TV (C) in spent irrigation water collected on day 1, 3, and 6 during alfalfa seed germination from seeds inoculated on day 0 and stored for up to 15 days.

The alfalfa seeds were watered daily and irrigation water was collected on days 1, 3 and 6 during a 7-day germination period to determine the presence of each virus. The levels of HAV, MNV, and TV transferred from seeds/sprouts to irrigation water are shown in Figure 2.4 (A, B, and C). Viruses were detected in all the irrigation water samples over the germination period. Due to the initial inoculum levels, the levels of MNV and HAV were higher than TV with approximately 2 log PFU and 2 log $TCID_{50}$ respectively in irrigation water on germination day 1 within this 15-day storage period, whereas more than 1 log PFU of TV was found on germination day 1 for all samples. A general trend of decreasing number of viruses in irrigation water from day 1 to day 6 was observed during sprouting, and in most cases significantly higher amount of viruses were detected on day 1 rather than on day 3 and 6 ($p<0.05$). As well, the titers of each virus in water on the same germination day decreased with extending time. Little reduction of HAV and MNV was observed in water on each germination day, likely the levels of TV were similar on day 6. The genomic copies of each virus were found relatively persistent with 1~2 log higher than that determined by infectivity assays.

3.5 Discussion

Alfalfa sprouts may become contaminated from a number of sources, including contaminated seeds, water, or mishandling/cross-contamination during food preparation (30-32). Contaminated seeds were previously identified as the major cause for sprout-associated outbreaks (17). In this study, we demonstrated that MNV, TV, and HAV can persist on the surface of alfalfa seeds for a prolonged period, and these viruses could contaminate sprouts after germination and be transferred to spent-irrigation water. This result is not surprising, and is supported by previous viral

infectivity studies at room temperature in tap water/ seawater/ groundwater which demonstrated long-term infectivity of MNV (> 30 days), TV (> 30 days), huNoV (> 61 days), and HAV (> 60 days) (25, 33, 34).

Virus survival varied depending on virus types and matrices. Different survival patterns were observed on seeds and in HBSS for all the viruses. HAV was relatively persistent over the first 20 days, followed by small reductions within 50 days on seeds, which confirmed the conclusion that HAV persisted better under dry conditions, as stated in other studies (35). On day 0 viruses were recovered from seeds after drying, and for both HAV and TV recoveries from seeds after inoculation decreased significantly . Previous studies showed that HAV did not lose infectivity after drying in plasma or culture medium (36). Observed reductions may be explained by differences in recovery which reinforces the strong attachment between alfalfa seeds and viruses. The influential factors including electrostatic and hydrophobic forces, as well as isoelectric point (pI) of the capsid proteins, environmental conditions (e.g., pH, ionic strength, humidity, darkness, and temperature) were identified to be involved in virus binding to similar matrices (34, 37-41). Little additional reduction was observed after MNV was recovered from seeds on day 0, which revealed relatively weak attachment.

It appears that alfalfa seeds can provide niches for virus survival and protect viruses from harsh conditions. With their oval shape, the surfaces of alfalfa seeds are relatively uniform. The seed surface contains small hills and narrow valleys which are not likely to allow entrapment of bacteria (8); however, these valleys might offer spots for virus attachment as viral particles are much smaller than bacterial pathogens. It is possible that viruses harbored within seed coat crevices may escape the environmental

effects of light, temperature/ pH fluctuations. Additionally, surface crevices could also prevent removal or inactivation of foodborne pathogens by washing and reduce contact with disinfectants, resulting in ineffective virus removal and inactivation.

The survival rates of MNV and TV were slightly different on seeds as well as in HBSS. However, recent studies showed more similar patterns of both MNV and TV survival in tap water over 25 days at 20 °C (25).. In most cases, viruses tend to survive better at lower temperatures (25, 35, 42, 43), and the temperature fluctuations could result in virus inactivation. In addition, alfalfa sprouts provided a neutral pH (44), and pH ranging from 6 to 8 has been shown to be preferable for virus survival with decreased rates of inactivation (25, 42, 43, 45). HBSS contains salts and provides a stable pH at ~7.25 which is within that range. The difference observed here may be in part explained by the levels of initial inoculums. The initial titer of TV was much lower than MNV, and it is possible that the virus could persist for a long period of time at low levels. Moreover, it was shown that HAV survival was inversely proportional to the level of relative humid (35), whereas MNV acted in the opposite manner (42).

Attempts to correlate virus infectivity with the number of genomic copies as determined by real time RT-PCR provided useful information on the relative stability of the virus itself. The genomic copies of HAV and MNV on seeds were relatively persistent regardless of their infectivity, whereas for TV, the genomic copies decreased in a similar manner to the number of infective virus as determined by plaque assay. This may indicate loss of HAV and MNV infectivity as a result of capsid changes rather than from denaturation that could impact genome integrity. In addition, the inactivation of TV may lead to degradation of RNA more easily.

However, the genomic materials detected in this study were small segments for each virus, and it should be noted that these do not represent the whole genome. On the contrary, the levels of TV genomic copies detected on sprouts and spent water were stable without significant difference ($p<0.05$) probably due to the high level of humidity.

As huNoV surrogates, TV may be more environmental robust than MNV with less reduction in infectivity observed both on seeds and in HBSS, and the genomic copies were capable to persist in HBSS regardless of infectivity. This indicates TV could be another possible surrogate for huNoV in environmental studies, especially in the conditions of high humidity. Sinclair et al. (2012) provided the criteria for surrogate selection to conduct risk assessment in the environment emphasizing both surrogate attributes (e.g., practical, biological, and environmental attributes) and experimental context (46). In order to determine if TV is the ideal surrogate, it is necessary that the characteristics of TV are similar or very close to that of huNoV in natural or engineered systems. TV is cultivable in cell culture and still genetically related to huNoVs, although not as similar as MNV. The most interesting property of TV is its functional morphology to bind HBGA (26). In this study, TV displayed similar environmental attributes to MNV, which was relatively tolerant at room temperature and neutral pH regardless of humidity, and generally exhibited greater resistances than MNV in infectivity. Considering both attributes, TV can be selected as a tentative surrogate of huNoVs in environmental survival studies. However, one surrogate might not be able to present the full properties of huNoVs under different environment conditions nor treatments, the genomic copies of TV in drying conditions was very persistent and decreasing in the similar pattern to its infectivity. Therefore, it

remains necessary to employ several surrogates for study to better understand the potential behavior of huNoVs, as surrogates exhibit slight differences in each attribute.

Virus transmission in water is an important concern for the sprout industry, based on this study. The seeds were watered daily for germination, and the rates of virus survival on seeds were significantly reduced after the first watering, but the viruses spread through water to contaminate the entire batch of sprouts including all the portions of sprouts. Three types of viruses all survived and were still infectious in the irrigation water during the process of germination, and the virus titers depended on the initial levels on the seeds. TV survival in the germination water was found to be less than that of HAV and MNV due to the original lower inoculum. A similar conclusion was drawn from previous reports (47-49). It was previously observed that viruses in contaminated water could be easily absorbed by vegetables after being immersed in water and viruses persisted during storage (50). Washing without any application of disinfectant or sanitizers can result in reduction but does not guarantee a complete decontamination (50). In view of the fact that alfalfa sprouts are most likely consumed raw or may be just slightly cooked as an ingredient for different recipes, adequate hygienic measures both in production and during preparation are necessary to reduce foodborne illness.

Studies showed the presence of viruses in used irrigation water at room temperature for a short storage period, and that viruses could be transmitted to produce by washing with contaminated water or internalized via root (50-52). Wastewater or irrigation water can be another source of contamination if reused (53). The risks can be increased by virus persistence as well as by the heterogeneous distribution of viruses (54). It is advisable to test the irrigation water to obtain an indication of the

amount of contamination of sprouts grown from seeds and avoid cross-contamination (55). Other techniques to reduce contamination on seeds can be utilized, such as high pressure processing (56), irradiation (57), heat, and calcium hypochlorite (58).

In this study, alfalfa seeds were selected as a model to understand the behavior of foodborne viruses during a prolonged storage, as well as the interaction of viruses with sprouted seeds and their transfer to irrigation water during germination. These findings suggest that viruses may survive for a relatively long period of time on seeds and reveal the ease with which viruses may transfer and spread during the germination process. Thus, it is imperative to apply appropriate disinfectants to remove pathogens from seeds, and implement good agricultural and manufacturing practices, including worker hygiene and sanitation, during sprouting to limit contamination as well as cross contamination. Attention should be paid to the re-use of irrigation water, which could be a potential source of pathogens.

3.6 Acknowledgements

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Chapter 4

EFFECTIVENESS OF CALCIUM HYPOCHLORITE ON VIRAL AND BACTERIAL CONTAMINATION OF ALFALFA SEEDS

(A manuscript published in Foodborne Pathogens and Disease)

4.1 Abstract

Alfalfa sprouts have been involved in numerous foodborne outbreaks which has increased the awareness for seed and sprout safety. This study compared the effectiveness of $Ca(OCl)_2$ on the inactivation of bacteria and viruses on alfalfa seeds and in the presence of a simulated organic load. Alfalfa seeds were inoculated with human norovirus (huNoV) genogroup II (GII), murine norovirus (MNV), Tulane virus (TV), *Escherichia coli* O104:H4, and *Salmonella enterica* serovar Agona. Seeds were treated with $Ca(OCl)_2$ (2,000 ppm or 20,000 ppm with the average of free chlorine 1,388 ± 117 mg/L and 11,472 ± 1500 mg/L, respectively, pH adjusted to 7.00). The reduction of huNoV genomic copies indicated that huNoV was relatively resistant to $Ca(OCl)_2$ regardless of concentrations. Significant reductions were observed in the order of TV < *Salmonella* Agona < MNV < *E. coli* O104:H4 at 20,000 ppm $Ca(OCl)_2$. A similar trend was found at 2,000 ppm $Ca(OCl)_2$ in the order of TV, *Salmonella* Agona, MNV < *E. coli* O104:H4. $Ca(OCl)_2$ at 20,000 ppm was more effective than 2,000 ppm for all the organisms tested. This trend was also observed in samples containing an artificial organic material load. $Ca(OCl)_2$ activity on virus inactivation decreased as the organic load increased. Reduction was greater in FBS-containing samples compared to alfalfa seeds, indicating a close relationship between the

organisms and alfalfa seeds. $Ca(OCl)_2$ could not completely inactivate bacteria or viruses inoculated on seeds, and high levels of *E. coli* O104:H4 and *S.* Agona were present on sprouts from sanitized seed samples following a 7-day germination period.

4.2 Introduction

Sprouted seeds have been involved in numerous foodborne outbreaks across the world, and since 1990 more than 30 reported outbreaks were linked to the consumption of raw or lightly cooked alfalfa sprouts in the U.S. (Bari *et al.*, 2011; CDC, 2012; Erdozain *et al.*, 2011; IFSN, 2009). The number of foodborne illnesses associated with these outbreaks ranged from as few as a single number to as large as thousands including dozens of deaths, as observed with the large outbreak in Germany in 2011 (Buchholz *et al.*, 2011; Erdozain *et al.*, 2011; IFSN, 2009). Many sources are identified as routes for sprout contamination, including pathogen-tainted seeds, contact with soil, fertilizer, irrigation water, harvesting, storing, processing, distribution, and contamination during food preparation (Erdozain *et al.*, 2013; NACMCF, 1999; Yang *et al.*, 2013). Importantly, contaminated seeds were identified as the major source in most sprout-associated outbreaks, and germination is a key step for sprout safety and challenges the sprout industry (NACMCF, 1999). During sprouting, seeds are first soaked in water and then placed in a warm and humid environment which is ideal for bacterial growth (NACMCF, 1999). If bacteria, such as pathogenic *Escherichia* and *Salmonella* spp., both identified as major etiologies in outbreaks associated with sprouted seeds (Erdozain *et al.*, 2011; IFSN, 2009), are present on seeds before germination, they can easily proliferate. Seed disinfection is a preventive approach to reduce the risk associated with contaminated seeds. Soaking seeds in 20,000 ppm calcium hypochlorite ($Ca(OCl)_2$) solution before sprouting is considered as an

appropriate treatment (NACMCF, 1999); while the Canadian Food Inspection Agency (CFIA) also describes a treatment with 2,000 ppm of calcium hypochlorite or sodium hypochlorite for 15-20 min for use as an antimicrobial treatment for seeds (CFIA, 2008). It is necessary to evaluate seed disinfection treatments, and many studies have aimed to investigate the efficacy of $Ca(OCl)_2$ on foodborne bacteria on seeds (Brooks *et al.*, 2001; Buchholz and Matthews, 2010; Gandhi and Matthews, 2003; Holliday *et al.*, 2001; Kim *et al.*, 2003; Lang *et al.*, 2000; Liao, 2009; Suslow *et al.*, 2002; Zhao *et al.*, 2010); however, no substantial research has been conducted assessing viruses. Within this study the effectiveness of treatment on virus is put in perspective by comparative evaluation of bacteria.

The U.S. Centers for Disease Control and Prevention (CDC) estimate that human norovirus (huNoV) causes the greatest number of illnesses associated with a known pathogen each year in the U.S. (5.5 million), accounting for up to 58% of foodborne illnesses (Scallan *et al.*, 2011). In addition, recent studies revealed that the total number of illnesses caused by norovirus in U.S. each year was 19-21 million, and an average of 5 episodes of norovirus gastroenteritis would be experienced by each individual in a lifetime (Hall *et al.*, 2013). Noroviruses are classified into at least 5 genogroups I-V (GI-GV), and a novel genogroup VI containing canine norovirus was recently proposed (Mesquita *et al.*, 2010). The strains relevant to human disease belong to genetic clusters within GI, GII, and GIV (Zheng *et al.*, 2006). GII is the most common genogroup, causing 73% of all reported norovirus outbreaks from 1997 to 2000 in U.S. (Fankhauser *et al.*, 2002). The fact that norovirus has low infectious dose of 10-100 particles with a median of 18 particles reinforces how foodborne illnesses can occur easily (Patel *et al.*, 2009; Teunis *et al.*, 2008). Due to the lack of

cell culture or animal models for routine study of huNoV, surrogates are relied on for the study of huNoV. Two surrogates were selected for use in this study, including murine norovirus (MNV) and Tulane virus (TV) (Cannon *et al.*, 2006; Hirneisen and Kniel, 2013a). Previous studies showed that viruses, especially MNV and TV, persist and survive for up to 50 days in alfalfa seeds (Wang *et al.*, 2013).

During seed disinfection, the presence of organic material may alter the effectiveness of $Ca(OCl)_2$ on the seeds. Previous studies showed that relatively low levels of organics, like 0.5% bovine serum albumin (BSA), did not substantially interfere with the antimicrobial activity of sodium hypochorite (NaOCl) (Sassone *et al.*, 2003); however, it was demonstrated that high concentrations of BSA significantly reduced the antimicrobial activity of NaOCl, calcium hydroxide, and iodine potassium iodide in bacterial inactivation (Pappen *et al.*, 2010; Portenier *et al.*, 2001). It is hypothesized that the high organic loads could substantially decrease the effectiveness of $Ca(OCl)_2$.

In this study, alfalfa seeds were selected as a model to better understand the efficacy of seed treatment with $Ca(OCl)_2$. HuNoV GII and its surrogates MNV, and TV, and two bacterial sprout isolates *Escherichia coli* O104:H4 and *Salmonella* Agona, were used to assess microbial inactivation on alfalfa seeds by $Ca(OCl)_2$, and the effect of organic loads was also investigated. Comparative disinfection parameters were observed for the microorganisms tested. Additionally, post-disinfection, bacterial growth was assessed following a 7-day germination period.

4.3 Materials and Methods

4.3.1 Virus cultivation and infectivity

Murine norovirus (MNV-1) (a gift from Dr. Herbert Virgin, Washington University School of Medicine, St. Louis, MO) was cultured in RAW 264.7 cells (ATCC# TIB-71) in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), 100 U/mL penicillin G-streptomycin-0.25µg/mL Amphotericin B (Hyclone, Logan, UT), 2 mM L-alanine-L-glutamine (Gibco, Carlsbad, CA), and 1 mM sodium bicarbonate (Cellgro, Manassas, VA). Tulane virus (TV) (a gift from Dr. Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH) was propagated in LLC-MK2 cells (ATCC# CCL-7) in medium 199 (Hychlone, Logan, UT) supplemented with 10% FBS, and 100 U/mL penicillin G-streptomycin-0.25µg/mL Amphotericin B. After typically 48 h infection of 80-90% confluent monolayers for both MNV and TV, complete cytopathic effect (CPE) was observed. Viruses were obtained following three cycles of freeze-thawing infected cells, and centrifugation at 2,000 × g for 15 min. The supernatant was filtered through by 0.2 µm membrane filter (Thermo, Rochester, NY) before storing viruses at -80 °C until use.

MNV and TV plaque assays were performed similarly to previous studies with slight modifications (Farkas *et al.*, 2008; Wobus *et al.*, 2004). In brief, RAW 264.7 and LLC-MK2 cells were grown to 80-90% confluency in 6-well plates (Castar, Corning, NY), one hundred microliter of ten-fold serial dilutions of each virus sample prepared in Hank's balanced salt solution (HBSS; Cellgro, Manassas, VA) was dispensed over monolayers in duplicate. The plates were incubated at 37 °C with 5% CO_2 for 1-3 h with gentle agitation every 15 min followed by the addition of a 2 mL

overlays. MNV-1 overlays consisted of 0.5% agarose (Lonza SeaKem LE, Rockland, ME) with complete Eagle's medium (MEM; Hyclone, Logan, UT) supplemented with 2% FBS, 100 U/mL penicillin G-streptomycin-0.25μg/mL Amphotericin B, 2mM L-alanine-L-glutamine, and 1mM sodium pyruvate. TV overlays consisted of 0.5% agarose with complete medium 199 supplemented with 2% FBS, and 100 U/mL penicillin G-streptomycin- 0.25 μg/mL Amphotericin B. After the incubation period (typically 48 h for MNV), 1 mL of 0.2 g/L neutral red (Fisher Scientific, Fair Lawn, NJ) was added into each well followed by a 2-5 h incubation. After typically 48 -72 h of the incubation period for TV, 2 mL of 3.7% formaldehyde (Fisher Scientific, Fair Lawn, NJ) in phosphate-buffered saline (PBS; pH 7.2) was added into each well, followed by at least 2 h incubation, stained with 0.05% (wt/vol in 10% ethanol) crystal violet (Fisher Scientific, Kalamazoo, MI). Titers of virus were determined and expressed by plaque forming units (PFU).

4.3.2 Human norovirus preparation

Human norovirus genogroup II (huNoV GII) was supplied by Megan Davis, South Carolina Department of Health and Environmental Control. HuNoV purification from stool samples was performed similarly to the protocols in previous studies with slight modifications (Hirneisen and Kniel, 2013b; Lewis and Metcalf, 1988). Stool samples were added into 0.01 M phosphate buffered saline (PBS) to make 10% (*v/v*) slurry. After being vortexed vigorously, the suspension was centrifuged at 2,000 ×g for 20 min to remove the solids. The supernatant was retained and polyethylene glycol (PEG) (Fisher Scientific, Waltham, MA) was then added to make the final concentration of 8% (wt/vol). The suspension was stirred for 4 h at 4°C and then centrifuged at 10,000 ×g for 30 min. PEG supernatant was discard and pellet was

suspended in 0.15 M Na_2HPO_4 (pH 9.0) and shaken for 20 min at 250 rpm. After another centrifugation at 10,000 ×g for 30 min, the supernatant was processed through 0.2 μm membrane filter (Thermo, Rochester, NY) to remove bacteria and debris. Filtrate was diluted in PBS and stored in aliquots before freezing at -80 °C.

4.3.3 HuNoV genome quantification by real-time reverse transcription (RT)-PCR

The level of huNoV was quantified by real-time RT-PCR. To generate a standard curve for each virus type, 1 mL of virus stock with known genomic copies (10^7 genomic copies/mL for huNoV) was 10-fold serially diluted with HBSS. RNA of virus samples was extracted and reverse transcribed into cDNA by using QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) and Omniscript RT kit (QIAGEN) as reference protocols, respectively. The primers used for huNoV GII were shown as follows: forward primer VP1-FP3 (5'-TGGGTGCTCCCAAGTTATTC-3') and reverse primer VP1-RP3 (5'-CTGGAGCTGCCTCTTGGTAG-3') (Hirneisen and Kniel, 2013b). Real-time PCR was performed in a total reaction volume of 20 μL containing 10 μL SYBR-Green PCR Master Mix (QIAGEN), 2 μL cDNA, and same set of primers with the protocol from QuantiTect SYBR Green PCR Kit (QIAGEN) on 384-well plates. Reactions were run on the Applied Biosystems 7900 HT Sequence Detection system (Applied Biosystems, Foster City, CA) with the following thermal conditions: 95 °C for 10 min followed by 40 cycles of 94 °C for 15 s, annealing step at 56 °C for 30 s, and extension step at 72°C for 30s, followed by a dissociation step at 60 °C for 15 s and 90 °C for 15 s. SYBR green signals were read in every cycle, and the logarithm of the increment in fluorescence was plotted versus the cycle number with fixed threshold level for all runs. Virus quantity was then determined by

comparison to a standard curve and expressed as genomic copies. The standard curve was generated in duplicates in each run of qPCR. The detection limits for all virus types were determined to be ~ 100 genomic copies/mL. HuNoV in HBSS served as positive controls, and negative controls consisted of the seed and HBSS sample without virus as well as PCR blanks.

4.3.4 Bacterial growth and quantification

Escherichia coli O104:H4 (ATCC# BAA-2326) and *Salmonella enterica* serovar Agona (ATCC# 51957), which were both previously implicated in foodborne outbreaks associated with sprouts, were used in this study. They were cultured at 37 °C in 10 mL of LB broth (Fisher Scientific, Fair Lawn, NJ) overnight. Cells of each strain were collected by centrifuge at 2,000 × g for 15 min at room temperature (22 ± 1 °C). Supernatant was then discarded, and cells were resuspended in equal volume of HBSS to remove all the organic load in growth medium. Bacteria were prepared fresh before use. After treatments, samples including controls were 10-fold diluted in HBSS, and plated 100 µL in duplicate on XLT-4 Agar (BD, Sparks, MD) and Sorbitol MacConkey agar (BD, Sparks, MD) for *S.* Agona and *E. coli* O104:H4, respectively. Plates were incubated at 37 °C for 24 h. Colonies were enumerated and determined by colony forming units (CFU).

4.3.5 Calcium hypochlorite preparation

Calcium hypochlorite (Fisher Scientific, Fair Lawn, NJ) was prepared fresh by dissolving 0.400 g or 4.00 g in 200 mL deionized water to make the final concentrations of 2,000 ppm and 20,000 ppm, respectively. pH was adjusted to 7.00 by adding 0.1 M HCl. The free chlorine concentrations were measured by High Range

Chlorine Test Kit (HACH, Loveland, CO) with the average of 1,388 ± 117 mg/L and 11,472 ± 1500 mg/L, respectively. The $Ca(OCl)_2$ solutions were used immediately after preparation.

4.3.6 Alfalfa seed preparation

Alfalfa seeds (*Medicago sativa*) (Johnny's, Winslow, ME) were sterilized by submerging seeds in 70% ethanol for 5 min followed by soaking in a 10 % bleach solution for 20 min. Seeds were rinsed with deionized water and then dried under the laminar flow hood at room temperature overnight before dividing into 1 g samples in 15 mL centrifuge tubes prior to inoculation. After treatment, little effect was observed visually on sprouting percentage compared with untreated sprout seeds. Every 1 g seed sample was individually inoculated with 500 µL of huNoV GII, TV, MNV, *E. coli* O104:H4, and *S.* Agona, respectively. In order to determine the effectiveness of $Ca(OCl)_2$, the initial titers of pathogens and surrogates inoculated on alfalfa seeds were 7.70 ± 0.01 logs genomic copies of huNoV GII, 7.04 ± 0.18 log PFU of MNV, 6.16 ± 0.23 log PFU of TV, 9.72 ± 0.12 log CFU of *E. coli* O104:H4, and 9.19 ± 0.65 log CFU of *S.* Agona, respectively. Inoculum was then allowed to dry visually for an hour at 20°C. In order to measure the survival rates of the microorganisms inoculated on seeds after drying, seeds were vortexed for 1 min to eluted virus/bacterium with 5 ml HBSS after an hour drying. For each type of virus/bacterium and concentration level of $Ca(OCl)_2$, three treatment seed samples and one recovery control were prepared for each of three trials of experiment (a total of 9 treatment samples and 3 recovery controls). One seed sample was also included in each trial by adding 500 µL HBSS without virus inoculation, and it served as controls for $Ca(OCl)_2$ neutralization and cytotoxicity testing.

4.3.7 Effects of calcium hypochlorite on bacteria or viruses inoculated on alfalfa seeds and on bacteria or viruses in the presence of organic materials

After the virus inoculum or HBSS was visibly dry on 1 g alfalfa seeds, 1 mL $Ca(OCl)_2$ was added into inoculated seed samples and un-inoculated controls. All the samples were carefully placed on the shaking platform at 150 rpm to mix thoroughly for 20 min. After 20 min seed treatments, the free chlorine was measured again with the values of ~250 mg/L and ~5000 mg/L, respectively To neutralize any available disinfectant, 4 mL FBS was added immediately to each treated sample after 20 min. To study the effects of $Ca(OCl)_2$ in the presence of organic load, similar experiment was conducted. Viruses were prepared in HBSS containing FBS to make final concentration 10%, 30%, and 50%, respectively. Five hundred µL of $Ca(OCl)_2$ was added to 500 µL FBS-containing virus sample for 20 min, and all the samples were shaken at 150 rpm. HBSS containing 10%, 30%, and 50% FBS without viruses was included in each trial, which served as controls for neutralization and cytotoxicity testing. To neutralize any available disinfectant, FBS was added immediately after 20 min to 100 µL of each 2,000 ppm and 20,000 ppm $Ca(OCl)_2$ treated sample (90-99% final concentration). Neutralization buffers for treated seeds without viruses were tested for cell cytotoxicity. Viruses or bacteria mixed with the series 10-fold dilutions of neutralization buffers served as neutralization controls. Samples were then tested for quantification. The log reduction was obtained by subtracting the amount of bacteria/viruses recovered after $Ca(OCl)_2$ treatments from the amount of bacteria/viruses recovered from samples that did not undergo treatments. If the sample volume was large due to neutralization, Amicon ultra centrifuge filters of 100KDa (Millipore, Billerica, MA) were used to concentrate samples in a small volume followed the protocol provided. Recovery control was included, and no significant

difference was observed between virus samples with and without concentration step ($p>0.05$).

4.3.8 Germination of bacteria-inoculated seeds after $Ca(OCl)_2$

Another set of bacteria-inoculated seeds with or without $Ca(OCl)_2$ treatments were allowed to germinate in the sprout growth chambers (Victorio, Orem, UT) by watering daily. The growth chamber had three trays: the top tray was empty used for watering; the middle tray had rings to distribute seeds evenly and was used for germination; and the bottom tray was a container to collect spent irrigation water. During the 7-day germination period, 500 mL municipal tap water (free chlorine was under the detection limit < 10 mg/L) was added daily on the top tray, and siphoned over seeds/sprouts, and finally drained and collected in the bottom tray. The humidity and temperature inside of the growth chambers containing uninoculated seeds/sprouts were measured and recorded daily using a Traceable Therm./Clock/Humidity Monitor (Fisher, Pittsburgh, PA). The humidity in the growth chamber averaged >70%, with a range from 36% to >90%, and the temperature was 20.0 ± 1.27 °C. After a 7-day germination period, sprouts (approximately 12 g) germinated from 1 g seeds sample were collected in a 50 mL centrifuge tube containing 10 mL HBSS. Samples were vortexed for 1 min to elute bacteria from the sprouts to investigate bacterial growth during germination.

4.3.9 Statistical Analysis

All experiments were conducted three times, and the reported results are means and standard deviations. Data were analyzed by ANOVA on JMP software (Version

10.0, SAS Institute Inc., Cary, N.C.). Significant differences in least-squares means were indicated if $p < 0.05$.

4.4 Results

4.4.1 Virus and bacterial recovery from alfalfa seeds after inoculation

In this study, the survival rates of microorganisms (both viruses and bacteria) on alfalfa seeds were determined after drying seeds for 1 h at room temperature. The log reductions of all the pathogens and surrogates tested are listed in Table 3.1. After 1 h drying, the levels of huNoV GII, TV, and *S.* Agona recovered from alfalfa seeds decreased significantly regardless of differences in initial titers ($p<0.05$). The reductions ranged from approximately 1 to 2 logs as shown in Table 3.1. Little difference was observed in titers of MNV and *E. coli* O104:H4 before and after drying in alfalfa seeds ($p>0.05$).

Table 4.1 Log reduction of huNoV GII, MNV, TV, *E. coli* O104:H4, and *S.* Agona post-drying (1 h) at room temperature (22 ± 1 °C).

Viruses and Bacteria	Initial titer	Log reduction due to drying [a]
HuNoV GII (log genomic copies/g seeds)*	7.70 ± 0.01	1.43 ± 0.21
MNV (log PFU/g seeds)	7.04 ± 0.18	0.27 ± 0.20
TV (log PFU/g seeds)*	6.16 ± 0.23	0.92 ± 0.07
E. coli (log CFU/g seeds)	9.72 ± 0.12	0.16 ± 0.06
S. Agona (log CFU/g seeds)*	9.19 ± 0.65	1.09 ± 0.52

[a] Values are means ± SD of three replicates; virus or bacterium noted with an asterisk indicates significant difference ($p<0.05$) when comparing the levels of viruses or bacteria before and after drying.

4.4.2 Inactivation of viruses and bacteria from contaminated alfalfa seeds by $Ca(OCl)_2$ treatments

Significant reductions were observed for viruses and bacteria after either 2,000 ppm or 20,000 ppm $Ca(OCl)_2$ treatments (Tables 3.2). Following treatment with 2,000 ppm $Ca(OCl)_2$, huNoV GII had ~ 1 log reduction in genomic copies; whereas the infectivity of its surrogates TV and MNV had significantly higher reductions with ~1.7 log PFU/g seeds ($p<0.05$). Similar trends were observed at 20,000 ppm $Ca(OCl)_2$ treatment, however, the reduction of MNV was significantly greater than that of TV by approximately 1.5 log PFU/g seeds ($p<0.05$). *E. coli* O104:H4 achieved significantly greater reductions than *S.* Agona at both concentration levels of $Ca(OCl)_2$ ($p<0.05$, Table 3.2). When comparing the inactivation of both viruses and bacteria treated by either 2,000 ppm or 20,000ppm $Ca(OCl)_2$, *E. coli* O104:H4 had significantly greater reductions than any other pathogens ($p<0.05$); however, the genomic copies of huNoV were most stable ($p<0.05$). To be more exact, significant log reductions were observed in the order of TV< *S.* Agona < MNV < *E. coli* O104:H4 at 20,000 ppm $Ca(OCl)_2$, and the order was TV, *S.* Agona, MNV < *E. coli* O104:H4 at 2,000 ppm $Ca(OCl)_2$. In addition, $Ca(OCl)_2$ at 20,000 ppm was more effective than 2,000 ppm for all viruses and bacteria, but complete inactivation was not obtained for the pathogens and surrogates studied, which may be in part due to the high titers inoculated in alfalfa seeds in this study to best assess inactivation.

Table 4.2 Reduction of huNoV GII (log genomic copies/g seeds), MNV and TV (log PFU/g seeds), and *E. coli* O104:H4 and *S.* Agona (log CFU/g seeds) on inoculated alfalfa seeds after $Ca(OCl)_2$ treatments.

Viruses/Bacteria	Initial titer	$Ca(OCl)_2$ Treatments (log genomic copies/g seeds, log PFU/g seeds, or log CFU/g seeds) [a]	
		2,000 ppm (free chlorine 1,388 ± 117 mg/L)	20,000 ppm (free chlorine 11,472 ± 1500 mg/L)
HuNoV GII	6.27 ± 0.23	1.08 ± 0.59 [A]	1.65 ± 0.40 [B]
MNV	6.77 ± 0.32	a 1.74 ± 0.35 [A]	c 3.75 ± 0.42 [B]
TV	5.24 ± 0.26	a 1.78 ± 0.32 [A]	a 2.29 ± 0.16 [B]
E. coli O104:H4	9.63 ± 0.02	b 3.85 ± 0.25 [A]	d 5.97 ± 0.17 [B]
S. Agona	8.11 ± 0.01	a 1.84 ± 0.23 [A]	b 3.10 ± 0.36 [B]

[a] Values are means ± SD of three replicates; values in columns with the same preceding letter indicate no significant difference ($p>0.05$) when comparing MNV, TV, *E. coli* O104:H4, and *S.* Agona inactivation after treatments; values in rows with the same following letter indicate no significant difference ($p>0.05$) when comparing between treatments for each virus/bacterium.

4.4.3 Effects of organic load in $Ca(OCl)_2$ inactivation of viruses and bacteria

The activity of $Ca(OCl)_2$ in the presence of artificial organic loads was investigated (Table 3.3). Bacteria and viruses inoculated in FBS-containing HBSS were slightly diluted, with initial titers of 7.10 ± 0.01 log genomic copies of huNoV GII, 5.01 ± 0. 30 log PFU of TV, 6.46 ± 0.26 log PFU of MNV, 8.42 ± 0.07 log CFU of *E. coli* O104:H4, and 8.06 ± 0.01 log CFU of *S.* Agona, respectively. The reductions of pathogens and surrogates in the presence of FBS were >2 log greater than that inoculated in seeds. Microbial inactivation as a result of $Ca(OCl)_2$ treatment substantially decreased as the concentration of FBS increased, especially in the presence of FBS at >30%.

All viruses had great reduction in genomic copies or infectivity regardless of the concentration of FBS present, but complete inactivation was not obtained at 2,000 or 20,000 ppm $Ca(OCl)_2$. Viral genetic material from huNoV GII was detected in all samples treated with both 2,000 ppm and 20,000 ppm $Ca(OCl)_2$. MNV compared with TV was inactivated more readily as shown by the number of positive samples/total number of samples tested (Table 3.3). Greater reductions were obtained for MNV, with more than 40% of samples under the detection limit at 2,000 ppm $Ca(OCl)_2$, and with limited increase in inactivation at 20,000 ppm. Viruses were much more resistant compared to bacteria.

Table 4.3 Reduction of huNoV GII (log genomic copies/mL), MNV and TV (log PFU/mL), and *E. coli* O104:H4 and *S.* Agona (log CFU/mL) by $Ca(OCl)_2$ treatments in the presence of an artificial organic load.

Viruses/ Bacteria	Organic load	2,000 mg/L $Ca(OCl)_2$ (free chlorine 1,388 ± 117 mg/L) Reduction (log genomic copies/mL, log PFU/mL, or log CFU/mL) [a]	Ratio [b]	20,000 ppm $Ca(OCl)_2$ (free chlorine 11,472 ± 1500 mg/L) Reduction (log genomic copies/mL, log PFU/mL, or log CFU/mL) [a]	Ratio [b]
HuNoV GII	10% FBS	a 4.51 ± 0.23 [A]	9/9	a 4.51 ± 0.05 [A]	9/9
	30% FBS	b 3.12 ± 0.14 [A]	9/9	a 4.36 ± 0.28 [B]	9/9
	50% FBS	b 2.80 ± 0.06 [A]	9/9	a 4.02 ± 0.03 [B]	9/9
MNV	10% FBS	a 5.71 ± 1.10 [A]	2/9	a 6.27 ± 0.22 [A]	0/9
	30% FBS	a 5.35 ± 0.99 [A]	5/9	a 6.18 ± 0.46 [B]	1/9
	50% FBS	a 5.17 ± 1.21 [A]	5/9	a 5.96 ± 0.85 [A]	2/9
TV	10% FBS	a 3.45 ± 0.69 [A]	9/9	a 4.99 ± 0.62 [B]	0/9
	30% FBS	ab 3.09 ± 0.52 [A]	9/9	ab 4.45 ± 0.46 [B]	3/9
	50% FBS	b 2.79 ± 0.52 [A]	9/9	b 4.16 ± 0.68 [B]	4/9
E. coli O104:H4	10% FBS	a 8.07 ± 1.20 [A]	2/9	a 8.62 ± 0.54 [A]	0/9
	30% FBS	ab 7.87 ± 1.44 [A]	3/9	a 8.44 ± 0.92 [A]	1/9

	50% FBS	b 6.91 ± 1.72 [A]	6/9	a 7.79 ± 1.43 [A]	4/9
	10% FBS	a 8.26 ± 0.50 [A]	0/9	a 8.27 ± 0.50 [A]	0/9
S. Agona	30% FBS	a 8.09 ± 0.81 [A]	1/9	a 8.27 ± 0.50 [A]	0/9
	50% FBS	a 8.06 ± 1.15 [A]	1/8	a 8.27 ± 0.50 [A]	0/9

[a] Values are means ± SD of three replicates; values in columns with the same preceding letter indicate no significant difference (p>0.05) when comparing the effect of organic loads for each virus/bacterium after treatments; values in rows with the same following letter indicate no significant difference (p>0.05) when comparing between treatments for each virus/bacterium.

Overall, the majority of bacterial samples were decreased to below the detection limit as shown by the number of positive samples/total number of samples tested (Table 3.3). The addition of FBS and enhanced organic loads had no effect on bacterial reduction, as the $Ca(OCl)_2$ was very effective to inactivate bacteria inoculated in HBSS with different levels of FBS; and the ratio of the number of positive samples/total number of samples tested indicated most samples were under the detection limit.

4.4.4 Bacterial growth on alfalfa seeds/sprout during germination after $Ca(OCl)_2$ treatments

Following $Ca(OCl)_2$ treatments, alfalfa seeds were germinated in the growth chamber over 7 days with daily watering. At the conclusion of the 7-day period, bacterial levels present on sprouts were determined, and compared with those from germinated untreated sprouts (Table 3.4). Bacteria were not completely eliminated by disinfection treatments in this study. After the treatments, titers of *E. coli* O104:H4 and *S.* Agona were found to be 5.78 ± 0.24 and 6.27 ± 0.22 CFU/g seeds following treatment at 2,000 ppm $Ca(OCl)_2$ and 3.67 ± 0.16, and 5.01 ± 0.36 CFU/g seeds from seeds treated with 20,000 ppm. No significant differences in levels of *E. coli* O104:H4 were found between untreated and 2,000 ppm treated samples, nor between 2,000 ppm and 20,000 ppm treated samples ($p>0.05$). However, the level of *E. coli* O104:H4 was significantly greater in sprouts germinated from untreated seeds than that from seeds treated with 20,000 ppm treated ones ($p<0.05$). For *S.* Agona, significant differences were observed between untreated, 2,000 ppm and 20,000 ppm $Ca(OCl)_2$ treatments. The alfalfa sprouts germinated from 20,000 ppm $Ca(OCl)_2$ treated seeds had significantly lower level of *S.* Agona than that from 2,000 ppm $Ca(OCl)_2$ treated seeds

($p<0.05$), the difference (~0.6 log CFU/g seeds) was slight though. Similar differences observed with *S.* Agona also existed between sprouts germinated from untreated seeds and that from 2,000 ppm $Ca(OCl)_2$ treated seeds.

Table 4.4 *E. coli* O104:H4 and *S.* Agona growth on sprouts from 1 g seed samples at 7-day post inoculation following $Ca(OCl)_2$ treatments.

Bacteria	Treatments		
	Untreated	$Ca(OCl)_2$ 2,000 ppm (free chlorine 1,388 ± 117 mg/L)[a]	$Ca(OCl)_2$ 20,000 ppm (free chlorine 11,472 ± 1500 mg/L)[a]
E. coli O104:H4	9.33 ± 0.04 ^{A}	9.00 ± 0.29 ^{AB}	8.68 ± 0.40 ^{B}
S. Agona	9.50 ± 0.26 ^{A}	8.87 ± 0.17 ^{B}	8.45 ± 0.10 ^{C}

[a] Values are means ± SD of three replicates; values in rows with the same following letter indicate no significant difference ($p>0.05$) when comparing between treatments for each bacterium

4.5 Discussion

As the National Advisory Committee on the Microbiological Criteria for Foods mentioned in its guidelines, soaking seeds in 20,000 ppm $Ca(OCl)_2$ before sprouting is an appropriate procedure used in seed decontamination (NACMCF, 1999). Recently, the Food Safety Modernization Act (FSMA) also proposed rules regarding enhanced safety for sprout production requiring treatment of seeds immediately before sprouting to reduce pathogenic microorganisms. However, these guidelines do not contain details on pH, temperature, time of treatment, testing for free chlorine levels, concentration of $Ca(OCl)_2$, nor any recommendation for physical force required. With these gaps in knowledge, the inactivation rates of bacterial pathogens have been investigated (Brooks *et al.*, 2001; Buchholz and Matthews, 2010; Gandhi and

Matthews, 2003; Holliday *et al.*, 2001; Kim *et al.*, 2003; Lang *et al.*, 2000; Liao, 2009; Suslow *et al.*, 2002; Zhao *et al.*, 2010), but little to no knowledge exists about the effectiveness of $Ca(OCl)_2$ decontamination on viruses.

In this study, we showed $Ca(OCl)_2$ could significantly reduce the levels of huNoV GII, MNV, TV, *S.* Agona, and *E. coli* O104:H4 inoculated on alfalfa seeds, but complete inactivation was not achieved. Incomplete reduction indicated limited efficacy of $Ca(OCl)_2$, in part likely due to high levels of inoculums. Alfalfa seeds treated with higher concentrations of $Ca(OCl)_2$ resulted in greater reductions in both viruses and bacteria. Bacteria that survived the treatments grew to >8 log CFU in alfalfa sprouts after 7-day germination period, the levels were close to those without treatment.

Microorganisms recovery from alfalfa seeds after drying varies depending on bacteria or virus type. Significant reduction of huNoV genomic copies and TV infectivity was observed, but nearly full infectivity remained in the MNV recovered from alfalfa seeds. Similar results in previous studies showed that TV recovery from alfalfa seeds after inoculation significantly decreased, whereas little reduction of MNV was observed (Wang *et al.*, 2013). For bacteria, little reduction was found in *E. coli* O104:H4 after recovery from alfalfa seeds, but ~1 log reduction of *S.* Agona was observed. Similarly, a higher reductions of *Salmonella* on inoculated alfalfa seeds compared to *E. coli* were previously observed (Zhao *et al.*, 2010). The variations in recovery rates could be explained by loss of genomic copies or inactivation of infectivity during the drying step, and the surface properties of each type of pathogens and surrogates attached to alfalfa seeds. The factors that affect attachment between microorganisms and alfalfa seeds includes electrostatic and hydrophobic forces, as

well as environmental conditions (Vega *et al.*, 2008; Wang *et al.*, 2011). After $Ca(OCl)_2$ inactivation and neutralization, the log titers recovered from alfalfa seeds were determined and subtracted from recovery values to calculate log reductions for each microorganisms.

$Ca(OCl)_2$ has previously resulted in an average reduction of 2.5 log CFU/g seeds at 2,000 mg/L (2,000 ppm), and 3.0~3.5 log CFU/g seeds at 20,000 mg/L (20,000 ppm), in enteric bacterial pathogens at room temperature (Ding *et al.*, 2013; Montville and Schaffner, 2004). Here, we showed similar reduction levels and obtained similar conclusions that antimicrobial activity of hypochlorite increased with increasing concentrations (Erkmen, 2003; 2010). We found the reductions of *S.* Agona were 1.84 and 3.10 log CFU/g seeds at different levels of $Ca(OCl)_2$, respectively. Inactivation rates were similar to results obtained by Nei et al. where ~3 log CFU/g of *Salmonella* in alfalfa seeds were inactivated by 20,000 ppm $Ca(OCl)_2$ after 20 min treatment (Nei *et al.*, 2011). Other studies indicated a range of reductions; Zhao et al. found > 6.0 log CFU/g reduction of *S.* Typhimurium on alfalfa seeds after 20min treatment of 20,000 ppm $Ca(OCl)_2$ at 21°C (Zhao *et al.*, 2010), whereas Buchholz et al. showed only ~1.5 log CFU/g reduction of *S.* Stanley in alfalfa seeds at 20,000 ppm for 15 min with rotary shaking (100 rev/min) at a temperature range from 21-23 °C (Buchholz and Matthews, 2010). The variation in *Salmonella* inactivation could be attributed to the experiment protocols, such as use of specific strains, inoculation level and procedure, source of alfalfa seeds, treatment time, temperature, and physical force.

Interestingly, *E. coli* O104:H4 showed greater inactivation compared to *Salmonella* with 3.10 and 5.97 log CFU/g seeds at 2,000 and 20,000 ppm treatment, respectively. Previous studies focused on the inactivation of *E. coli* O157:H7 by

$Ca(OCl)_2$, while little is known about *E. coli* O104:H4. Taormina et al. found that 2,000 ppm $Ca(OCl)_2$ resulted in only ~2.0 log CFU/g reduction of *E. coli* O157:H7 (Taormina and Beuchat, 1999), and Beuchat et al. and Holliday et al. both showed the ~2.0 log CFU/g reduction of *E. coli* O157:H7 at 20,000 ppm $Ca(OCl)_2$ in alfalfa seeds (Beuchat *et al.*, 2001; Holliday *et al.*, 2001). The reduction levels of *E. coli* O104:H4 by $Ca(OCl)_2$ shown here were generally higher than those of *E. coli* O157:H7 in previous studies. These discrepancy may be explained by differences in experiment design and by the characteristics of *E. coli* strain types. As an enteroaggregative *E. coli* (EAEC) with shiga toxin and some enterohemorrhagic *E. coli* (EHEC) characteristics (Mellmann *et al.*, 2011; Pierard *et al.*, 2012), the *E. coli* O104:H4 strain is perhaps different in its response to $Ca(OCl)_2$.

Viruses were generally more resistant to $Ca(OCl)_2$ treatments than bacteria. The oval shape with uniform surfaces composed of hills and narrow valleys (Fransisca and Feng, 2012) were not likely to protect bacteria from disinfectants, but could provide numerous hiding sites for viruses. The accessibility of chlorine to react with pathogens hidden in crevices or between cotyledon and seed coat of alfalfa seeds could be the major reason (Ding *et al.*, 2013; Yang *et al.*, 2013). The number of genomic copies are determined; however, as human norovirus cannot be cultivated in cell culture, infectivity may not be inferred from this data. The numbers of genomic copies of huNoV GII were relatively stable and more resistant to the treatments with limited reductions observed (< 2 log genomic copies/g seeds). Shin et al. assessed the norovirus resistance to chlorine and found only a 2 log reduction in water containing a 1 mg/L (1 ppm) dose of free chlorine (Shin and Sobsey, 2008). Another study showed a 3.75 mL/L (3.75 ppm) dose of chlorine was not effective to inactivate norovirus

which remained infectious to volunteers (Keswick *et al.*, 1985). It is worth mentioning that the presence of genomic copies certainly does not equal infectivity, nor does it represents infectivity. $Ca(OCl)_2$ is a strong oxidizer and likely causes damage to the viral capsid (Nuanualsuwan and Cliver, 2003); however, the integrity of the norovirus capsid is unknown, which might be measured by RNase treatment or cell binding assay in the future (Li *et al.*, 2012; Topping *et al.*, 2009), and the genomic copies detected in this study were small segments, which are not representative of the whole genome. Approximate reductions of 1.7 log PFU/g of both MNV and TV in alfalfa seeds were observed following 2,000 ppm $Ca(OCl)_2$, but a significantly higher reduction of MNV (~ 4 log PFU/g) was observed following treatment with 20,000 ppm. Previous studies obtained much higher reduction by hypochlorite, it was found that both TV and MNV had > 5 log PFU reductions after treatment of 2,000 ppm $Ca(OCl)_2$ in water (Hirneisen and Kniel, 2013a). Belliot *et al.* showed that 36.4 mM NaOCl resulted in at least a 4 log drop in MNV infectivity after only 0.5 min of exposure time (Belliot *et al.*, 2008). The greater inactivation could be explained by the different matrix and levels of organic materials as well as the crevices on seed coats which provide sites protecting viruses from disinfectants. It is likely that both MNV and TV behave similarly at lower level of hypochlorite; however, MNV is more sensitive to chlorine than TV at relatively high levels of $Ca(OCl)_2$. Thus, MNV may not be the worst-case model for estimating huNoV inactivation. TV was found to be more robust than MNV to disinfectant inactivation indicating it could be another possible surrogate for huNoV.

The effect of organic materials on antimicrobial activity of $Ca(OCl)_2$ was also evaluated. The inactivation of each microorganism inoculated on alfalfa seeds was

significantly lower than that in FBS-containing HBSS, indicating alfalfa seeds contain more organic materials and more protection reducing direct interaction with $Ca(OCl)_2$ and that it is not the organic load alone. Generally, the antimicrobial activity of $Ca(OCl)_2$ in FBS-containing HBSS decreased as the organic load increased when the FBS >30% for viruses. A similar conclusion was obtained showing organic materials could inhibit the chlorine inactivation of bacteria including *Listeria monocytogenes*, *Salmonella, Staphylococcus,* and *E. coli* (Buncic and Sofos, 2012; Pappen *et al.*, 2010; Valderrama *et al.*, 2009). In another study, the reaction between hypochlorite and egg albumin resulted in degradation of protein and reduction of hypochlorite with 2-9 molecules of hypochlorite interacting with each amino acid residue attached (Baker, 1947). Given this information, bacteria were inactivated beyond the detection limit in this study regardless of FBS concentration. Bacterial inhibition is affected by both chlorine and organic concentrations.

$Ca(OCl)_2$ treatments (2,000 ppm and 20,000 ppm) were not able to completely inactivate either *S.* Agona nor *E. coli* O104:H4 inoculated on alfalfa seeds. The bacteria survived after the treatments could grew to > 8 log CFU in sprouts after 7-day germination period, close to those without treatments. Fransisca et al. found that *E. coli* that survived the 20,000 ppm chlorine treatment in radish seeds with 3.21 log grew to as high as 6.2 log within 3 days of sprouting (Fransisca *et al.*, 2011). Gandhi et al. reported that *S.* Stanley reached > 7.0 log on sprouts grown from *S.* Stanley inoculated alfalfa seeds that were treated with 20,000 ppm $Ca(OCl)_2$ (Gandhi and Matthews, 2003). The results revealed high level of chlorine treatment might kill most of the natural microflora on seeds, which allows both *S.* Agona and *E. coli* O104:H4 to replicate quickly without competition. Following treatment, while it seemed that large

amounts of bacteria were inactivated, upon germination these treated bacteria recovered and increased numbers were observed on sprouts. It has been shown previously that *S. enterica* and *E. coli* could grow to higher levels without competition from other bacteria (Cooley *et al.*, 2003; Liao, 2008).

These results suggest that more effective strategies are in urgent need to control the sprout safety. Alternative treatments and multi-hurdle approaches should be considered to decontaminate seeds prior to germination as well as throughout the process of sprouting, such as inclusion of organic acids (Lang *et al.*, 2000; Zhao *et al.*, 2010), H_2O_2 (Holliday *et al.*, 2001), combinations of heat and chemicals (Bang *et al.*, 2011; Bari *et al.*, 2009), electrolyzed water (Bari *et al.*, 2003; Jadeja *et al.*, 2013), ozone or ozonated water (Sharma *et al.*, 2002; 2003), irradiation (Bari *et al.*, 2003; Waje and Kwon, 2007), high pressure (Neetoo and Chen, 2010; Neetoo *et al.*, 2009), or competitive inhibition (Cooley *et al.*, 2003; Liao, 2008). Processing methods, like dipping, soaking, spraying, or fumigation, may also be considered to help target the disinfectant at the microorganisms tightly bound to the seeds.

4.5.1 Acknowledgments

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Chapter 5

INACTIVATION OF HUMAN NOROVIRUS AND ITS SURROGATES ON ALFALFA SEEDS BY AQUEOUS OZONE

(A manuscript published in the Journal of Food Protection)

5.1 Abstract

Alfalfa sprouts have been associated with numerous foodborne outbreaks. Previous studies investigated the effectiveness of aqueous ozone on bacterial-contaminated seeds, yet little is known about the response of human norovirus. This study assessed aqueous ozone for the disinfection of alfalfa seeds contaminated with huNoV and its surrogates. The inactivation of viruses without a food matrix was also investigated. Alfalfa seeds were inoculated with huNoV GII, Tulane virus (TV) and murine norovirus (MNV); viruses alone or inoculated on seeds were treated in deionized water containing 6.25 ppm aqueous ozone with agitation at 22 °C for 0.5, 1, 5, 15, or 30 min. Data showed that aqueous ozone resulted in reductions of MNV and TV infectivity from 1.66 ± 1.11 to 5.60 ± 1.11 log PFU/g seeds; for all treatment times significantly higher reductions were observed for MNV ($p<0.05$). Viral genomes were relatively resistant with a reduction of 1.50 ± 0.14 to 3.00 ± 0.14 log genomic copies/g seeds; reduction of TV inoculated in seeds was similar to that of huNoV, whereas MNV had significantly greater reductions in genomic copies ($p<0.05$). Similar trends were observed in ozone-treated viruses alone, with significantly higher levels of inactivation ($p<0.05$), especially with reduced levels of infectivity for MNV and TV. Significant inactivation by aqueous ozone indicates that ozone may be a plausible

substitute for chlorine as an alternative treatment for seeds. The behavior of TV was similar to huNoV, and makes it a promising surrogate for these types of scenarios.

5.2 Introduction

The safety of sprouted seeds is recognized as a significant concern after numerous foodborne illnesses have been associated with the consumption of sprouts *(4)*. During the last two decades, there have been more than 30 reported outbreaks associated with the consumption of raw or lightly cooked alfalfa sprouts in the U.S. *(1, 3, 4, 14)*. Contamination of foodborne pathogens can occur anywhere from farm to table, and in many cases seed contamination has been identified as a major point of concern following an investigation *(20)*. During germination, seeds are soaked in water and sprouted in warm and humid conditions. This environment facilitates the growth of bacteria if seeds are already contaminated, and even a small amount of bacteria can proliferate to a large number. However, there is no complete inactivation or successful kill step for treating sprouted seeds. Therefore, immediate disinfection of seeds before germination is a preventive approach to reduce microorganisms of public health significance. The FDA Food Safety Modernization Act (FSMA) proposed rules for produce safety emphasize that seed treatments for sprouted seeds must be scientifically validated methods. Chlorine-based disinfection, such as 20,000 ppm calcium hypochlorite treatment, has been suggested and widely used as a practical method within the sprout industry, where typical treatments (10 to 15 min) have little effect on seed germination rates and have varying degrees of effectiveness on microbial inactivation *(2, 7, 8, 12, 18, 20)*. Nevertheless, the potential unfavorable impacts on the environment and production of chlorine by-products force the sprout industry and scientists alike to continue to seek potential alternatives.

Ozone is a strong oxidizer, like chlorine, and should be considered for enhancing seed and sprout safety. Ozone is environmentally friendly, and can inactivate a wide range of foodborne pathogens, including viruses, bacteria, protozoa, and some other higher forms such as worms and mites. It can act in either the gaseous state or while dispersed in water, and it works without formation of residues or by-products *(24)*. A few studies have been conducted to evaluate the effectiveness of ozone on bacterial-contaminated seeds *(24, 25, 28)*, yet little is known about the efficacy of ozone on viruses contaminated on seeds.

Human norovirus (huNoV) is a leading cause of produce-associated outbreaks. It is estimated that huNoV causes over 5 million illnesses each year in the U.S. *(23)*, and it is likely that an individual may experience an average of 5 episodes of norovirus gastroenteritis within a lifetime *(9)*. Due to its low infectious dose of 10-100 virus particles, even a small amount of contamination has the potential to cause illness *(22, 27)*. However, the pathogenesis of huNoV is still not well understood due to the lack of a cell culture model and the limitations of human volunteer studies. Various members of the *Calicivirus* family with close genetic and antigenic relatedness to huNoV have been widely used to predict norovirus behavior in studies, including murine norovirus viruses (MNV) and Tulane virus (TV). In this study, alfalfa seeds were selected as a model to better understand the efficacy of seed treatment by aqueous ozone. A GII huNoV strain and its surrogates MNV and TV were used to assess virus inactivation on alfalfa seeds. Comparison of huNoV to these two surrogates within this unique environment advances our knowledge of huNoV behavior *(17)*.

5.3 Materials and Methods

5.3.1 Virus cultivation

Murine norovirus (MNV-1) (a gift from Dr. Herbert Virgin, Washington University School of Medicine, St. Louis, MO) was cultured in RAW 264.7 cells (ATCC# TIB-71) in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), 100 U/ml penicillin G-streptomycin-0.25µg/ml Amphotericin B (Hyclone, Logan, UT), 2 mM L-alanine-L-glutamine (Gibco, Carlsbad, CA), and 1 mM sodium bicarbonate (Cellgro, Manassas, VA). Tulane virus (TV) (a gift from Dr. Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH) was propagated in LLC-MK2 cells (ATCC# CCL-7) in 199 medium (Hyclone, Logan, UT) supplemented with 10% FBS, and 100 U/ml penicillin G-streptomycin-0.25µg/ml Amphotericin B. After typically 48 h infection of 80-90% confluent monolayers for both MNV and TV, complete cytopathic effect (CPE) was observed. Viruses were obtained following three cycles of freeze-thawing infected cells, and centrifugation at 2,000 × g for 15 min. The supernatant was filtered through a 0.2 µm membrane filter (Thermo, Rochester, NY) before storing viruses at -80 °C until use.

5.3.2 Human norovirus preparation

Human norovirus genogroup II (huNoV GII) was supplied by Megan Davis, South Carolina Department of Health and Environmental Control. Norovirus purification from stool samples was performed using a modified protocol *(30)*. Stool samples were added into 0.01 M phosphate buffered saline (PBS) to make a 10% (*v/v*) slurry. After vigorous vortexing, the suspension was centrifuged at 2,000 ×g for 20 min to remove the solids. The supernatant was retained and polyethylene glycol (PEG)

(Fisher Scientific, Waltham, MA) was added to a final concentration of 8% (wt/vol). The suspension was stirred for 4 h at 4°C and then centrifuged at 10,000 ×g for 30 min. PEG supernatant was discarded and the pellet suspended in 0.15 M Na_2HPO_4 (pH 9.0) and placed on a shaker for 20 min at 250 rpm at room temperature. After another centrifugation at 10,000 ×g for 30 min, the supernatant was processed through a 0.2 μm membrane filter (Thermo, Rochester, NY) to remove residual bacteria and debris. Filtrate was diluted in PBS and stored in aliquots before freezing at -80 °C.

5.3.3 Alfalfa seed preparation and virus inoculation

Alfalfa seeds (Johnny's, Winslow, ME) were sterilized by submerging seeds in 70% ethanol followed by soaking seeds in a 10 % fresh commercial bleach solution (~8000 ppm sodium hypochlorite). Seeds were rinsed with deionized water and then dried completely. This treatment did not result in loss of germination compared to untreated seeds (data not shown). Seed samples (1 g) and sterile deionized water (49.5 ml) were individually inoculated with 500 μl MNV, TV or huNoV GII with initial titers of 6.66 ± 0.14 log PFU, 4.27 ± 0.88 log PFU, or 7.73 ± 0.05 log genomic copies, respectively. Seeds were allowed to dry for 60 min at 22°C within a biosafety cabinet.

5.3.4 Effects of aqueous ozone on virus inoculated on alfalfa seeds/in water

Samples of viruses alone or viruses inoculated on alfalfa seeds were treated in 50 ml deionized water containing aqueous ozone delivered at 0.9 g/h at a flow rate of 2.4 L/min (6.25 ppm) (Golden Buffalo, Orange, CA) with agitation at 22 °C for 0.5, 1, 5, 15, or 30 min, as described previously *(11, 31)*. Residual ozone concentrations were measured with a HACH ozone test kit (HACH Company, Loveland, CO) and were found to be 0.0, 0.15, 0.20, 0.40, and 0.60 ppm for the times listed above. Ten ml

Sodium thiosulfate (5%) was added post-treatment to quench residual ozone; 1 g seed samples were collected, and viruses were eluted from 1 g seeds with 2 ml Hanks Balanced Salt Solution (HBSS). Inoculated seeds soaked in 50 ml agitated water without ozone were included as controls. Neutralizer controls and cytotoxic controls were also included to confirm the cell culture viability was not affected by sodium thiosulfate or residual ozone. The neutralization controls confirmed that ozone residues were quenched, and there was no cytotoxicity apparent on cell cultures from observational controls.

5.3.5 Virus quantification by plaque assay and real-time reverse transcription (RT)-PCR

Virus was quantified by plaque assay and RT-PCR as previously described *(29, 30)*. To determine the infectivity of MNV and TV, RAW 264.7 and LLC-MK2 cells were grown to 80-90% confluency in 6-well plates (Costar, Corning, NY), 100 µl of ten-fold serial dilutions of each virus sample was dispensed over monolayers in duplicate. The plates were incubated at 37 °C with 5% CO_2 for 1 h with gentle agitation every 15 min followed by the addition of a 2 ml overlay. For MNV-1 the overlay consisted of 1.5% agarose (Lonza SeaPlaque, Rockland, ME) with complete Eagle's medium (MEM); and for TV the overlay consisted of 1.5% agarose with complete 199 medium. After the incubation period (typically 48 h for MNV and TV), 1 ml of 0.2 g/L neutral red (Fisher, Fair Lawn, NJ) was added into each well followed by a 2-5 h incubation. Titers of virus were determined and expressed as plaque forming units (PFU). Viral loss due to recovery was repeatedly determined to be < 1 log. The presence of the genomic copies of huNoV GII, MNV or TV was detected using a two-step RT-PCR. RNA of virus samples was extracted and reverse

transcribed into cDNA with a QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) and Omniscript RT kit (QIAGEN), respectively. Three sets of primers were used for each type of virus as follows: forward primer VP1-FP3 (5'-TGGGTGCTCCCAAGTTATTC-3') and reverse primer VP1-RP3 (5'-CTGGAGCTGCCTCTTGGTAG-3') for huNoV GII *(10)*; forward primer (5'-TCTTCGCAAGACACGCCAATTTCAG-3') and reverse primer (5'-GCATCACAATGTCAGGGTCAACTC -3') for MNV *(13)*; forward primer (5'-CCAGCTTGATGTAGGCGATT-3') and reverse primer (5'-CTCAGCCATTGCACTCAAAG-3') for TV *(5)*. Real-time PCR reactions were performed in a total reaction volume of 20 µl containing 10 µl SYBR-Green PCR Master Mix (QIAGEN), 2 µl cDNA, and same set of primers with the protocol from QuantiTect SYBR Green PCR Kit (QIAGEN). Reactions were run on a Rotor-Gene Q (QIAGEN) thermocycler with the following conditions: 95 °C for 10 min followed by 40 cycles of 94 °C for 15 s, annealing temperature of each virus for 30 s (60 °C for huNoV GII, 59 °C for MNV, and, 59 °C for TV), followed by a dissociation step at 60 °C for 15 s, and 90 °C for 15 s. SYBR green signals were read in every cycle, and the logarithm of the increment in fluorescence was plotted versus the cycle number with fixed threshold level for all runs. Virus quantity was determined by comparison to a standard curve and expressed as genomic copies. HuNoV GII, MNV, and TV stocks served as positive controls, and negative controls consisted of the samples (seed or water) without virus. Log reductions of viruses were calculated by comparing treated samples with untreated controls.

5.3.6 Statistical analysis

All virus inactivation experiments were completed in triplicate (9 samples per treatment time), and reported results are means with standard deviations. The D-values (decimal reduction time (seconds): time required to obtain 1 log reduction of the viruses at 22°C) of each virus by 6.25 ppm aqueous ozone treatment were also determined. Data were analyzed by ANOVA on JMP software (Version 10.0, SAS Institute Inc., Cary, N.C.). Significant differences in least-squares means were indicated if $p < 0.05$.

5.4 Results and Discussion

In order to investigate the response of huNoV to the aqueous ozone treatment, huNoV GII was used as it has been well documented in a large number of outbreaks. However, huNoV cannot be cultivated in cell culture, the number of genomic copies detected could not completely represent its characteristics. To better understand its behavior to ozone treatments, surrogates such as MNV and TV were also used. In this study, MNV, TV, and huNoV inoculated on alfalfa seeds or suspended in sterile water alone were treated with ozone (6.25 ppm) at various time intervals (0.5 to 30 min) in sterile water, and subsequent reductions in virus infectivity and/or genomic materials were measured (Tables 4.1 and 4.2).

Table 5.1 Log reduction of viruses (log PFU/g seeds, log genomic copies/g seeds) on inoculated alfalfa seeds after aqueous ozone treatment or after water agitation without aqueous ozone treatment.

Detection Method	Viruses Inoculated on Seeds	Viral Reduction [a]					
		Time (min)					
		0.5	1	5	15	30	30 (water only)*
Plaque assay (log PFU/g seeds)	MNV	a 4.04 ± 0.38 A	a 4.27 ± 0.28 A	a 4.56 ± 0.44 AB	a 4.90 ± 0.66 B	a 5.60 ± 1.11 C	0.60 ± 0.45
	TV	b 1.66 ± 1.11 A	b 2.03 ± 1.22 AB	b 3.00 ± 1.72 BC	b 3.45 ± 1.42 C	b 3.83 ± 1.01 C	1.40 ± 0.30
RT-PCR (log genomic copies/g seeds)	huNoV GII	a 1.50 ± 0.14 A	ab 1.67 ± 0.59 AB	ab 2.05 ± 0.54 BC	ab 2.38 ± 0.61 C	ab 2.50 ± 0.39 C	0.82 ± 0.36
	MNV	a 1.68 ± 0.63 A	a 2.16 ± 0.85 B	a 2.47 ± 0.75 BC	a 2.57 ± 0.22 C	a 3.00 ± 0.14 D	0.88 ± 0.40
	TV	a 1.59 ± 0.80 A	b 1.65 ± 0.82 A	b 1.88 ± 0.97 A	b 1.92 ± 1.01 A	b 1.98 ± 0.93 A	0.21 ± 0.15

[a]Values are means ± standard deviation of three replicates; values within columns with the same preceding letter indicate no significant difference ($p > 0.05$) when comparing reductions in infectivity or genomic copies for each virus after the same treatments; values within rows with the same following letter indicate no significant difference ($p > 0.05$) when comparing between treatments for each virus.

* Log reductions resulting from 30 min water agitation,

huNoV GII, human norovirus genogroup II; MNV, murine norovirus; PFU, plaque-forming units; TV, Tulane virus.

Table 5.2 Log reduction of infectivity/genomic copies of viruses (log PFU/ml water, log genomic copies/ml water) on inoculated in water after aqueous ozone treatment.

Detection Method	Viruses Inoculated in Water	Viral Reduction [a]				
		Time (min)				
		0.5	1	5	15	30
Plaque assay (log PFU/ml water)	MNV	a 4.13 ± 0.57 A	a 4.32 ± 0.73 A	a 4.98 ± 0.25 AB	a 5.43 ± 0.48 B	a 6.66 ± 0.17 C
	TV	a 2.85 ± 2.20 A	a 3.34 ± 1.71 A	a 4.27 ± 1.06 A	a 4.27 ± 1.06 A	b 4.27 ± 1.06 A
RT-PCR (log genomic copies/ml water)	huNoV GII	a 2.57 ± 0.94 A	a 2.47 ± 0.55 A	a 2.73 ± 0.52 A	a 1.71 ± 0.11 A	b 2.95 ± 0.46 A
	MNV	b 1.45 ± 0.33 A	a 1.74 ± 0.14 A	b 4.32 ± 0.90 B	b 5.16 ± 0.82 BC	a 4.89 ± 0.80 C
	TV	b 1.43 ± 0.85 A	a 1.51 ± 1.00 A	a 1.73 ± 1.03 A	a 1.85 ± 1.11 A	c 1.70 ± 1.03 A

[a]Values are means ± standard deviation of three replicates; values within columns with the same preceding letter indicate no significant difference ($p > 0.05$) when comparing reductions in infectivity or genomic copies for each virus after the same treatments; values within rows with the same following letter indicate no significant difference ($p > 0.05$) when comparing between treatments for each virus.

huNoV GII, human norovirus genogroup II; MNV, murine norovirus; PFU, plaque-forming units; TV, Tulane virus.

Ozone can inactivate viruses rapidly by reacting with capsid proteins and nucleic materials *(16)*. In this study, aqueous ozone significantly reduced the infectivity of MNV and TV as well as the genomic integrity of huNoV GII present on contaminated alfalfa seeds and on virus alone in sterile water ($p<0.05$). After 0.5 min ozone treatment, an immediate loss of > 4 log PFU MNV was obtained on inoculated alfalfa seeds; and the reductions slightly increased to 5.6 log PFU after 30 min of ozone exposure (Table 4.1). Much lower reduction of TV was observed on inoculated seeds with a loss of 1.66 log PFU after 0.5 min ozone treatment, and a reduction of only 3.83 log PFU after 30 min (Table 4.1). TV was significantly more resistant compared to MNV in terms of infectivity ($p<0.05$). The number of genomic copies was relatively resistant to reduction with approx. 1~2 log genomic copies/g of seeds destroyed for all viruses (Table 4.1). Interestingly, the reduction observed in the number of huNoV GII genomic copies was similar to that of both MNV and TV (Table 4.1).

Inoculated seeds that were soaked in agitated water without ozone treatment served as a control to quantify how much virus was removed by the agitation in water alone. Compared with the initial inoculum, small but significant log reductions (generally < 1 log PFU or genomic copies/g seeds) were observed on inoculated seeds after being soaked in agitated water without ozone treatment over a 30 min period ($p<0.05$) (Table 4.1). The limited reductions as a result of water agitation were much lower than those following ozone treatments.

Similar reductions were observed for viruses suspended in water treated with ozone (Table 4.2). Great reductions were achieved within only 0.5 min of ozone treatment, and then reductions increased slightly over the increasing treatment time.

Higher amounts of infectious MNV and TV were inactivated in water alone compared to that on seeds (Table 4.2). After 30 min of treatment with 6.25 ppm aqueous ozone, > 6 log PFU of MNV and >4 log PFU of TV inoculated in water alone were inactivated (Table 4.2). No infectious MNV or TV was observed in contaminated water after 30 min of ozone treatment with a limit of detection of 100 virus particles per sample; whereas, complete viral inactivation was not achieved in the presence of inoculated alfalfa seeds. The presence of organic material from alfalfa seeds may hinder the effectiveness of ozone and as shown previously, and it is also likely that viruses may be present in the crevices on seed surfaces *(30)*. A significantly higher reduction of MNV genomic copies was observed as the treatment time increased compared to the other viruses ($p<0.05$) (Table 4.2). The number of genomic copies of TV and huNoV remained relatively stable and was more resistant to the treatment with limited reduction observed (<2 log in water samples) (Table 4.2).

Inactivation rates rose more steeply during the first 0.5-1 min in terms of reductions in both infectivity and genomic copies, and flattened out as the treatment time increased (Tables 4.1 and 4.2). Dramatic reductions were observed during the first 0.5-1 min; and to better compare the inactivation rates, D-values (time required to obtained 1 log reduction by 6.25 ppm aqueous ozone treatment at 22°C) were calculated based on the reductions obtained after 0.5 and 1 min treatments (Table 4.3). The question of which is the better surrogate remains and the D values obtained here can be useful in comparing norovirus surrogates. While MNV and TV are both widely used as surrogates for huNoV, they differ in capsid structure and composition. MNV is the most genetically related to huNoV, and clustered in norovirus genogroup V (GV). It was the first norovirus to be propagated in cell culture and shares similar genetic and

structural features with huNoV *(32)*. Whereas, TV which was isolated from the stool of a rhesus monkey is a Calicivirus in the genus *Recovirus,* and is cultivable *in vitro (6)*. TV has more capsid similarity to huNoV, as it binds type A and B histo-blood group antigens (HBGA) like huNoV *(5)*. Based on reductions of infectivity, the D-value indicated that TV was more resistant to aqueous ozone treatment than MNV, as TV requires significant more time to obtain 1 log PFU reduction than MNV ($p<0.05$) (Table 4.3). Moreover, no significant difference was observed between the D-values of the three viruses comparing genomic copies detected in the presence of contaminated seeds ($p>0.05$); when the three viruses were exposed to ozone in the water alone, the behavior of TV genomic material was similar to that of huNoV, and the genomic material of MNV was more susceptible (Table 4.2). Therefore, TV may be the better model for estimating the characteristics of huNoV concerning inactivation or treatment with ozone. In order to achieve a 3-log reduction in infectivity of MNV and TV on alfalfa seeds, 5 min of ozone treatment at 6.25 ppm was needed; whereas 1 min was required to obtain a similar reduction for both viruses suspended in water alone.

Table 5.3 D-values (seconds) of NoV GII, MNV and TV (infectivity or genomic copies) by 6.25 ppm aqueous ozone treatment at 22°C inoculated on seeds or in water based on log reductions observed after 0.5 and 1 min.

	Virus	D-value (infectivity) (s)	D-value (genomic copies) (s)
Seeds	huNoV	-	27.73 ± 11.17 A
	MNV	10.80 ± 3.51 A	24.37 ± 8.28 A
	TV	18.53 ± 6.91 B	27.04 ± 12.49 A
Water	huNoV	-	19.17 ± 8.41 A
	MNV	10.76 ± 4.15 A	28.20 ± 7.90 AB
	TV	22.81 ± 12.18 B	31.69 ± 17.99 B

[a] Values within columns with the same letter indicate no significant difference ($p > 0.05$) when comparing different viruses inoculated in seeds/water treated with ozone.
huNoV GII, human norovirus genogroup II; MNV, murine norovirus; PFU, plaque-forming units; TV, Tulane virus.

Here, we found that low concentrations (6.25 ppm) of ozone and short contact time (0.5 min) were sufficient to inactivate viruses by > 1 log PFU or 1 log genomic copies in both inoculated seeds and suspended water samples, and virus inactivation increased with the treatment time. Lim *et al* found a rapid decrease in virus survival within the first 30 s at ca. 1 ppm ozone concentration *(19)*. Hirneisen *et al* showed that less than 1 log PFU of MNV was inactivated on fresh produce (lettuce and green onions), and ~2 log PFU was inactivated in water by 30 s at 6.25 ppm ozone; and this inactivation increased to 3 and 5 log PFU after 10 min for MNV on lettuce and green onions *(11)*. Interestingly, we found higher reduction levels here, ~4 log PFU of MNV was inactivated after 30 s of treatment on alfalfa seeds and in water. The success of the treatment may be explained by the agitation during treatment, which facilitated the reaction between ozone and viruses. Alternative treatments, especially chlorine, have been studied to decontaminate viruses *(15, 21, 26, 30)*. When viruses were treated in water alone, Keswick et al. showed a 3.75 ml/L (3.75 ppm) dose of chlorine (sodium hypochlorite) was not effective to inactivate Norwalk virus which was still infectious to volunteers *(15)*. Shin et al. found only a 2 log reduction of Norwark virus in water containing a 1 mg/L (1 ppm) dose of free chlorine (sodium hypochlorite) at 3 min contact time *(26)*. Park et al. determined that the reduction of MNV suspended in a 10% fecal sample was less than 1 log PFU, if the applied sodium hypochlorite concentration was ≤ 2500 ppm, suggesting the organic load significantly influences the effectiveness of chlorine *(21)*. When viruses were inoculated on seeds, only ~1.7 log and ~2-3 log PFU reductions of MNV and TV were achieved by very high levels of calcium hypochlorite at 2,000 ppm and 20,000 ppm for 20 min, respectively *(30)*. Those studies indicated that chlorine might not be as effective as aqueous ozone for

the inactivation of viruses; where to achieve similar viral reductions higher concentration of chlorine and longer treatment time were needed.

In conclusion, aqueous ozone is a promising disinfection technology for sprout production with relatively high effectiveness for decontaminating sprouts of huNoV and its viral surrogates, MNV and TV. These results also suggest that TV is a better surrogate for huNoV in disinfection studies using ozone.

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Chapter 6

SURVIVAL AND TRANSFER OF MURINE NOROVIRUS WITHIN A HYDROPONIC SYSTEM DURING KALE AND MUSTARD MICROGREEN HARVESTING

(A manuscript published in Applied and Environmental Microbiology)

6.1 Abstract

Hydroponically-grown microgreens are gaining in popularity, but there is a lack of information pertaining to the microbiological safety of microgreens, particularly of those grown hydroponically. The potential risks associated with virus contamination of crops within a hydroponic system have not been studied to date. Here the human norovirus (huNoV) surrogate (murine norovirus (MNV)) was evaluated for its ability to become internalized from roots to edible tissues of microgreens. Subsequently, virus survival in re-circulated water without adequate disinfection was assessed. Kale and mustard seeds were grown on hydroponic pads (7-days, harvest at days 8-12), edible tissues (10-g) were cut 1-cm above the pads, and corresponding pieces (4×4 cm^2) of pads containing only roots were collected and treated as one sample. Samples were collected from a newly contaminated system (re-circulated water inoculated with ~ 3 log PFU/ml MNV on day 8), and from a previously contaminated system. Viral titers and RNA copies were quantified by plaque assay and real-time reverse transcription (RT)-PCR. The behavior of MNV was similar in kale and mustard microgreens ($p>0.05$). MNV was detected in edible tissues and roots after 2 hours post-inoculation and the levels were generally stable

during the first 12 hours. Relatively low levels (~2.5 to ~1.5 log PFU/sample of both edible tissues and roots) of infectious viruses were found with a decreasing trend over time from harvest days 8-12. However, the levels of viral RNA present were higher and consistently stable (~4.0 to ~5.5 log copies/sample). Re-circulated water maintained relatively high levels of infectious MNV over the period of harvest from 3.54 to 2.73 log PFU/ml. Importantly, cross-contamination occurred easily; MNV remained infectious in previously contaminated hydroponic systems for up to 12 days (2.26 to 1.00 PFU/ml), and MNV was detected in both edible tissues and roots. Here we see that viruses can be re-circulated in water, even after an initial contamination event is removed, taken up through the roots of microgreens, and transferred to edible tissues. Ease of product contamination shown here reinforces the need for proper sanitation.

6.2 Introduction

Hydroponics are gaining in popularity with consistent growth across the United States (1). Specifically growing microgreens hydroponically is a new trend in the food industry (1). Microgreens have been defined as salad crop shoots harvested for consumption within 10-20 days of seedling emergence (2). Microgreens are considered as a gourmet food to add taste, color, and texture to dishes; they mainly appear in fine and upscale restaurants, and have been gaining attention and popularity during the past few years due to the fresh appearance and health benefits (3, 4). Both microgreens and sprouts are usually grown within indoor facilities with controlled environmental conditions to minimize potential contamination of foodborne pathogens. However, microgreens are different from sprouts. Generally, microgreens have two fully developed cotyledon leaves with the first pair of true leaves emerged or

partially expanded, and during harvest they are cut above the soil line; whereas sprouts are mainly soaked in the water and younger with cotyledon just opened or not.

With the increasing consumption of microgreens, concern for a situation similar to the sprout boom is occurring. As previously reported, sprouts have been involved in at least 55 foodborne outbreaks across the world with illnesses ranging from as few as one to as large as thousands (5, 6). While no outbreak has been documented associated with microgreens so far, as outlined in this study they are at risk for potential contamination. The U.S. Food and Drug Administration (FDA) has yet to define commodity specific guidelines regarding microgreens.

Human Norovirus (huNoV) causes over 5 million illnesses each year in the United States, and is the most common viral etiology of foodborne illnesses (7). It is likely that an individual may experience an average of 5 episodes of norovirus gastroenteritis within a lifetime (8). Produce safety is of great concern as fresh produce serves as the major vehicle for huNoV transmission (9, 10). Produce that is consumed raw or with little or no processing may become contaminated with huNoV during postharvest handling (e.g., irrigation water, and amendments) and processing (e.g., washing, and packing), and also through contact with infected individuals who may handle the produce or seeds (11-13). Previously, huNoV has been detected in surface water and ground water with varying frequency (14, 15). It is likely that viruses can be spread by water, and internalization of huNoV through root uptake of produce via polluted irrigation water is one of the potential routes for plant contamination (13, 15). However, without an appropriate cell culture model, the behavior of huNoV is still not well understood.

In order to predict the characteristics of huNoV, murine norovirus (MNV) with close genetic and antigenic relatedness have been widely used (16, 17). MNV was the first norovirus to be propagated in cell culture and is being clustered in norovirus genogroup V (GV) (17). For these reasons along with the fact that MNV is non-pathogenic, MNV was chosen as the surrogate for huNoV in this study.

Epidemiology suggests that a great number of produce-associated illnesses are caused by viruses (10, 18), resulting in the study of internalization of viruses in plants. It has been observed that plants that were grown in artificially contaminated hydroponic systems can take up viral pathogens (19-25). The driving force of water absorption facilitates internalization, and humidity in the plant growing environment significantly affects the transpiration (21). In addition, factors such as root integrity (19), virus type (25), and inoculation level (21) can affect the levels of virus internalization. However, it is still poorly understood whether virus internalization occurs in produce grown in contaminated hydroponic systems and if virus particles can accumulate in edible plant tissues (24).

In this study, kale and mustard microgreens were selected as a model to better understand the virus uptake, persistence, distribution, and transmission in microgreens grown in an artificially contaminated hydroponic system. MNV, a huNoV surrogate, was used to investigate the behavior of human norovirus.

6.3 Materials and Methods

6.3.1 Hydroponic system

Microgreens were grown at the Fisher Greenhouse at the College of Agriculture and Natural Resources, Newark, DE. The nutrient film technique (NFT)

hydroponic system was prepared by tilting three platforms at a 30 degree angle in order to allow water to flow through the system (Shown in Figure 1). Each set had 4 trays and its own water vessel containing 4000 ml of tap water supplemented with 30 ml of a nutrient solution A that contained $Ca(NO_3)_2$ (120.0 g/L, YaraLiva, Tampa, FL), and 30 ml of a solution B that contained 5-11-26 Hydro-Sol (120.0 g/L, Peters Professional, Dublin, OH), $MgSO_4$ (1.17 g/L, Giles Chemical, Waynesville, NC), and Sprint 330 (0.58 g/L, Becker Underwood, Ames, IA). A pump was placed in the water vessel and pumped water to the top of the system through a tube at a constant rate (~10 ml/s). The pump was set to run continuously over time cycling on for 5 min and then off for 10 min. The water flowed down the platforms due to gravity, then back into the water vessel and re-circulated through the NFT system.

Figure 6.1 Hydroponic systems including control, kale, and mustard.

6.3.2 Disinfection of hydroponic system

After completing each trial of experiments, hydroponic system was disinfected as followed. Re-circulated water and microgreen plants including hydroponic pads were removed. Firstly, whole system was then sprayed with 5% bleach in water (v/v) (Clorox, Oakland, CA). Then, water vessel was filled with 8,000 ml of tap water

containing 400 ml bleach (Clorox) with same concentration (5% bleach). The circulation system was on and kept water running the whole system for 24 hours. After complete disinfection was conducted, system was rinsed thorough with tap water first, and then 10,000 ml of tap water was re-circulated for another 24 hours to remove the chlorine residues. Samples (including microgreens and water) were tested negative before inoculation.

6.3.3 Plant cultivation

Seeds of microgreens including kale (*Brassica napus*) and mustard (*Brassica juncea*) (Johnny's, Winslow, ME) were planted and grown on micro-mats hydroponic grow pads (Handy Pantry, West Springville, UT), which were soaked in circulating water. Three individual sets of 12 micro-mats hydroponic grow pads (33.00 cm × 6.35 cm) were placed in each hydroponic system (3 pads/tray) including kale, mustard, and a positive control with no plants and circulating virus alone. On day 0, seeds were distributed evenly on pads; and each pad had 6.75 g kale and 3.75 g mustard seeds, respectively. Water was supplemented with a nutrient solution and re-circulated by pumping, as described above in the section of hydroponic system. The microgreens were germinated and ready to harvest beginning on day 8 to day 12. The temperature of the greenhouse was 22.3 °C with an average humidity of 51%. Seeds were germinated in 12 hours of daylight of averaged radiation 1057.2 J/cm^2 and 12 hours of dark of 0.4 J/cm^2 daily (greenhouse parameters were provided by Priva greenhouse monitoring system).

6.3.4 Inoculation of circulating water

Each of three water vessels held a total volume of 4000 ml fresh feed water that was inoculated with 200 ml of MNV on day 8 with the starting titer of ~3.5 Log PFU/ml. The microgreens were maintained in virus-inoculated feed water from days 8 to 12. An inoculated positive control was included in circulating water without seeds on the pads. A negative control was also included in a smaller setting due to space limitation. Due to evaporation, fresh water was added to maintain the initial water level daily, but no additional virus inoculum was added after the initial virus inoculation.

6.3.5 Sample collection

The virus titer in water was monitored throughout the experiment. Water samples (10 ml) were collected directly from each water vessel at each sampling time including controls before virus inoculation without further concentration step. Starting from day 9, microgreens (edible portion) and root pads were sampled, respectively. The microgreens (edible portion) were cut 1 cm above the pad with pruners (Fiskars, Sauk City, WI), 10 g microgreens samples (kale or mustard), and correspondingly two pieces of 4×4 cm^2 pads containing roots without edible portion were collected separately in two homogenizer bags (Fisher Scientific, Pittsburgh, PA), and treated as one microgreen edible tissue and one root sample. Microgreen edible tissue and root samples were mixed with 10 ml and 5 ml phosphate buffed saline (PBS, pH 7.2), respectively. Samples were then smashed by a 16-ounce rubber mallet hammer (Craftsman, Hoffman Estates, IL) and followed by stomaching for 2 min. The homogenates were collected and transferred to new collection tubes. Samples were then frozen at -20 °C for less than 2 weeks before being processed by plaque assay and

real-time reverse transcription (RT)-PCR. Chloroform extraction was conducted prior to analysis with a ratio of 1:1 (*v:v*) to avoid the interference of bacteria and tissue residue. After phase separation by centrifuge (6,000 × g, 10 min, at 4 °C), the aqueous phase was retained for analysis without further concentration step. Inhibitor controls were included by adding MNV stock directly into environmental samples (smashed microgreen edible and root tissues, as well as water samples) followed with chloroform extraction. It was determined that plant and water matrix has little effects on virus titers in both assays.

6.3.6 Virus survival and uptake in the newly contaminated hydroponic system

The newly contaminated hydroponic system was obtained by MNV inoculation in re-circulated water on day 8. The survival and transfer of virus was monitored in two separate studies, including short (12 hours) and long (harvesting time from day 8 to day 12) time periods. Virus uptake in the first 12 hours was investigated directly following inoculation. Water and microgreen (edible tissue and roots) samples were collected at 2, 4, 8, and 12 hours to determine the rate of detectable virus taken up by the microgreens. In addition, virus survival and transfer during harvesting from days 8 to 12 were measured. The amount of virus detected from external surface of microgreen edible tissues by rinsing the surface instead of smashing the tissues was consistently under the detection limit, indicating no external contamination occurred

6.3.7 Cross-contamination in a previously contaminated hydroponic system

Three sets of hydroponic systems including kale, mustard, and an unplanted control were first used for growing microgreens, and MNV was inoculated on day 8 as described previously, to obtain virus contaminated hydroponic systems. After

harvesting on day 12, microgreens, pads, and water were removed without washing or disinfection, and the hydroponic system was considered as previously contaminated. Immediately, a new set of pads and microgreens seeds were applied for germination without inoculation, and the extent of virus transfer to these microgreens was determined from days 8 to 12 via the previously contaminated system. The titer of virus present in the fresh water was also monitored from day 0 to 12.

6.3.8 Detection of background flora in the hydroponic system

Sampling for bacterial growth was performed in triplicate from a newly contaminated system (on days 8, 9 and 12), and from a previously contaminated system (on days 1, 8, and 12) simultaneously. Samples were collected from each system including water, microgreen edible tissues and roots. The samples were serially-diluted in sterile PBS (pH 7.2), and enumeration performed on Tryptic Soy Agar (TSA, Remel, Lenexa, KS) to monitor the background flora. In addition, the water samples were analyzed using Colilert according to manufacturer's instructions with Quanti-Tray/2000 (IDEXX, Westbrook, Maine) to detect coliforms and *Escherichia coli*.

6.3.9 Virus prorogation and infectivity quantification

Murine norovirus (MNV-1) (a gift from Dr. Herbert Virgin, Washington University School of Medicine, St. Louis, MO) was cultured in RAW 264.7 cells (ATCC# TIB-71) in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), 100 U/ml penicillin G-streptomycin-0.25µg/ml Amphotericin B (HyClone, Logan, UT), 2 mM L-alanine-L-glutamine (Gibco, Carlsbad, CA), and 1

mM sodium bicarbonate (Cellgro, Manassas, VA). Cells were infected with MNV at a multiplicity of infection (MOI) of 1. After 48 h infection of 80-90% confluent monolayers, complete cytopathic effect (CPE) was observed. Viruses were obtained by three cycles of freeze-thawing infected cells, followed by centrifugation at 2000 × g for 15 min. The supernatant was filtered through a 0.2 μm membrane filter (Thermo, Rochester, NY) before storing at -80 °C.

6.3.10 Quantification of infectious virus

MNV plaque assay was performed similarly to previous studies (17, 26). Briefly, after RAW 264.7 cells reached 80-90% confluency in 6-well plates (Castar, Corning, NY), 100 μl of ten-fold serial dilutions of MNV sample prepared in Hank's balanced salt solution (HBSS) (Cellgro, Manassas, VA) were dispensed over monolayers in duplicate. The plates were incubated at 37 °C with 5% CO_2 for 1 hour with gentle agitation every 15 min followed by addition of 2 ml overlays. MNV-1 overlays consisted of 1.5% agarose (Lonza SeaPlaque, Rockland, ME) with complete Eagle's medium (MEM) (HyClone, Logan, UT) supplemented with 2% FBS, 100 U/ml penicillin G-streptomycin-0.25μg/ml amphotericin B, 2mM L-alanine-L-glutamine, and 1mM sodium pyruvate. After the incubation period (typically 48 hours for MNV), 1 ml of 0.2g/L neutral red (Fisher Scientific, Fair Lawn, NJ) was added to each well followed by a 2-5 hour incubation. Titers of virus were determined and expressed as plaque-forming units (PFU) with a limit of detection of 10 virus particles/sample.

6.3.11 Virus genome quantification by real-time reverse transcription (RT)-PCR

MNV RNA was extracted and reverse transcribed into cDNA using QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) and Omniscript RT kit (QIAGEN) as reference protocols, respectively. Primers used for MNV were: forward primer (5'-TCTTCGCAAGACACGCCAATTTCAG-3') and reverse primer (5'-GCATCACAATGTCAGGGTCAACTC -3') (27). Real-time PCR reactions were performed in a total reaction volume of 20 µl containing 10 µl SYBR-Green PCR Master Mix (QIAGEN), 2 µl cDNA, and primers (described above) with the protocol from QuantiTect SYBR Green PCR Kit (QIAGEN). Reactions were run on a Rotor-Gene Q thermocycler (QIAGEN) with the following conditions: 95 °C for 10 min followed by 40 cycles of 94 °C for 15 s, annealing temperature 59 °C for 30 s, followed by dissociation step which is 60 °C for 15 s, and lastly 90 °C for 15 s. SYBR green signals were read in every cycle, and the logarithm of the increment in fluorescence was plotted versus the cycle number with fixed threshold level for all runs. The detection limits for MNV were determined to be ~100 genomic copies/ml of sample solutions. Virus quantity was determined by comparison to a standard curve and expressed as genomic copies. Positive controls tested were MNV stocks. Negative controls were also collected during harvesting period, which consisted of the environmental samples (microgreen edible tissues, roots, or water) without virus inoculation.

6.3.12 Statistical analysis

Experiments were conducted in triplicate. In each trial, samples were collected in triplicate expect water samples (one replicate), and then each of those samples were analyzed in duplicate. Results are reported as mean and standard deviation. The

kinetics of MNV survival in the re-circulated water were characterized by fitting the plaque assay data from the both newly and previously contaminated systems to linear, exponential, and Weibull models, respectively. The statistical criterion applied to distinguish among the survival models was p-value. Data were analyzed by ANOVA on JMP software (Version 11.2, SAS Institute Inc., Cary, N.C.), and significant differences were indicated if $p<0.05$.

6.4 Results

6.4.1 MNV was efficiently taken up via roots and transferred into edible tissues during the first 12 hours from virus inoculated in water (short-term study)

At full maturation on day 8, MNV was inoculated in the circulating water with a starting titer of 2.63 ± 0.66 log PFU/ml. The amount (log PFU/sample) and ratio (number of positive samples over number of samples tested) of MNV disseminated in kale and mustard microgreens 2, 4, 8, and 12 hour post-inoculation is shown in Table 1. The occurrence of MNV was similar in kale and mustard microgreens ($p>0.05$), and MNV was present in all edible tissues and roots tested. High levels of MNV were detected in both kale and mustard edible tissues as soon as 2 hour post-inoculation with average of 3.47 log PFU/sample, and the levels were stable during the first 12 hours without significant change ($p>0.05$) (Table 1). A similar trend of MNV genomic materials (~4 log copies/sample) was observed in edible tissues (Figure 5.2 A). As expected, MNV was found in roots since hydroponic pads that contained roots were soaked in the virus-contaminated water. The levels of MNV detected in the roots 2 hours post-inoculation were 1.98 and 2.59 log PFU/sample for kale and mustard, respectively (Table 1). The viral levels in kale roots significantly increased to 2.63 log

PFU/sample 4 hours post-inoculation ($p<0.05$), and maintained up to 12 hours with slight increase; whereas, the titers detected in mustard roots were stable at ~ 2.7 log PFU/sample over the time (Table 1). The number of MNV genomic copies in kale and mustard roots was ~5-6 log copies/sample and this amount was stable within the first 12 hours (Figure 5.2 B). Importantly, the titer of infectious MNV in roots was significantly lower than that in edible tissues ($p<0.05$). These results suggest that MNV was efficiently taken up via roots, internalized, and transferred into microgreen edible tissues.

Furthermore, the viral titer in re-circulated water was also monitored during the 12-hour period. Compared with the original inoculation levels, MNV in water was maintained at ~2 log PFU/ml without significant reduction in all three systems (control, kale, and mustard) over the time (Table 2). This trend was also confirmed by detection of MNV genomic materials present in water (Figure 5.3).

Table 6.1 Transfer of MNV in kale and mustard microgreens grown hydroponically within the first 12 hours.

Portion	Microgreens	Virus titers (log PFU/sample)* and ratio (positive counts/samples tested)							
		2 h		4 h		8 h		12 h	
Edible tissues	Kale	a3.47 ± 0.24^{A}	(7/7)	a3.62 ± 0.12^{A}	(9/9)	a3.59 ± 0.14^{A}	(9/9)	a3.50 ± 0.15^{A}	(8/8)
	Mustard	a3.47 ± 0.27^{A}	(9/9)	a3.69 ± 0.20^{A}	(9/9)	a3.53 ± 0.27^{A}	(9/9)	a3.58 ± 0.32^{A}	(9/9)
Roots	Kale	a1.98 ± 1.14^{A}	(9/9)	a2.63 ± 0.24^{B}	(9/9)	a2.66 ± 0.28^{B}	(9/9)	a2.68 ± 0.34^{B}	(9/9)
	Mustard	a2.59 ± 0.51^{A}	(9/9)	a2.75 ± 0.44^{A}	(9/9)	a2.79 ± 0.50^{A}	(9/9)	a2.77 ± 0.33^{A}	(9/9)

*Values are means ± SD of three trials with three samples each; values in columns with the same proceeding letter indicate no significant difference of virus titers when comparing the edible tissue/root between kale and mustard on each sampling time; values in rows with the same following letter indicate no significant difference of virus titers within the portions of kale or mustard over time.

Table 6.2 The survival of MNV in re-circulated water during harvest of kale and mustard microgreens with the first 12 hours.

		Virus titers (log PFU/ml)*				
		0 h	2 h	4 h	8 h	12 h
Water	Control	a2.26 ± 0.15^{A}	a2.87 ± 1.11^{A}	a2.82 ± 1.04^{A}	a2.69 ± 1.05^{A}	a2.68 ± 0.99^{A}
	Kale	a2.27 ± 0.24^{A}	a2.89 ± 0.91^{A}	a2.82 ± 0.84^{A}	a2.75 ± 1.49^{A}	a3.14 ± 1.01^{A}
	Mustard	a2.35 ± 0.39^{A}	a2.28 ± 0.58^{A}	a1.91 ± 0.19^{A}	a2.15 ± 0.15^{A}	a1.91 ± 0.13^{A}

*Values are means ± SD of three trials with three samples each; values in columns with the same proceeding letter indicate no significant difference of virus titers when comparing the water samples of control, kale and mustard on each sampling day; values in rows with the same following letter indicate no significant difference of virus titers in water samples from control, kale, or mustard system over time.

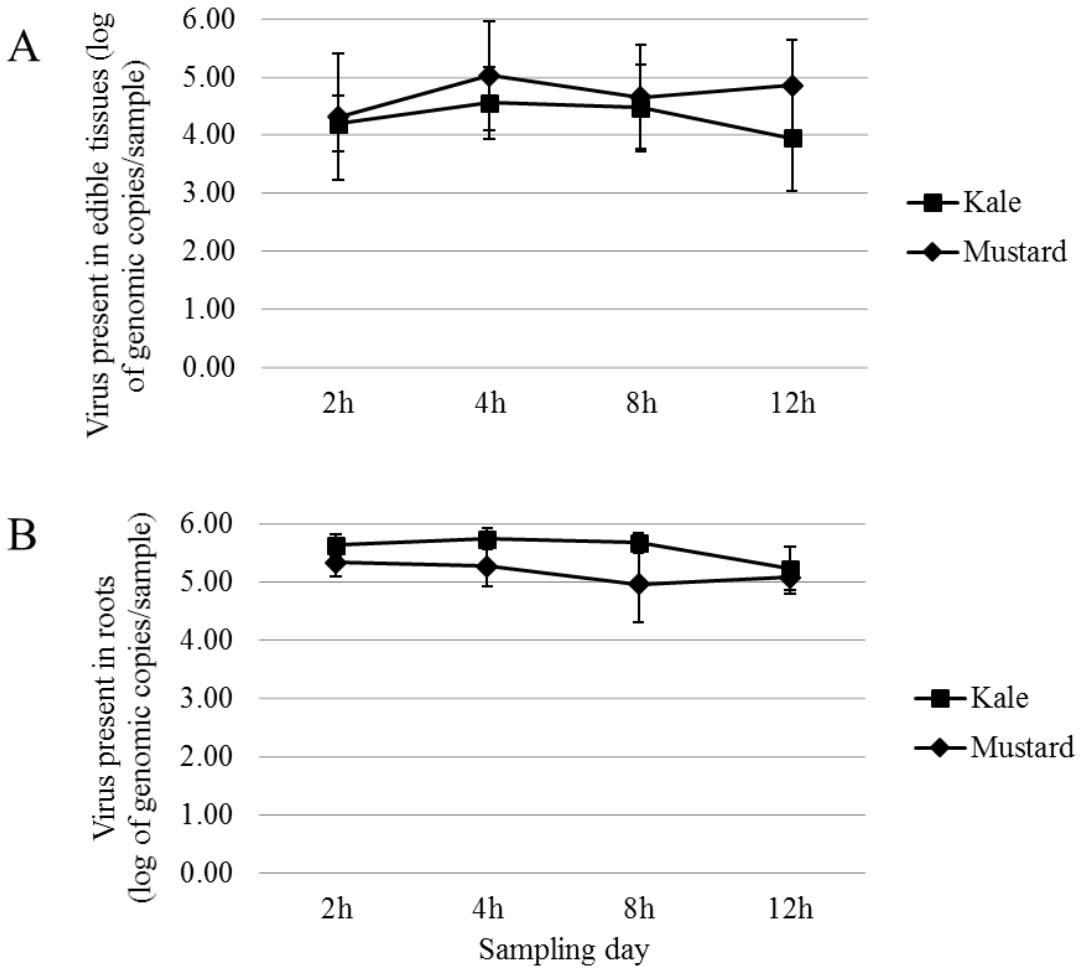

Figure 6.2 Presence of MNV genomic copies in kale and mustard edible tissues (A) and roots (B) within the first 12 hours during harvest from a newly contaminated hydroponic system (virus inoculated in water on day 8).

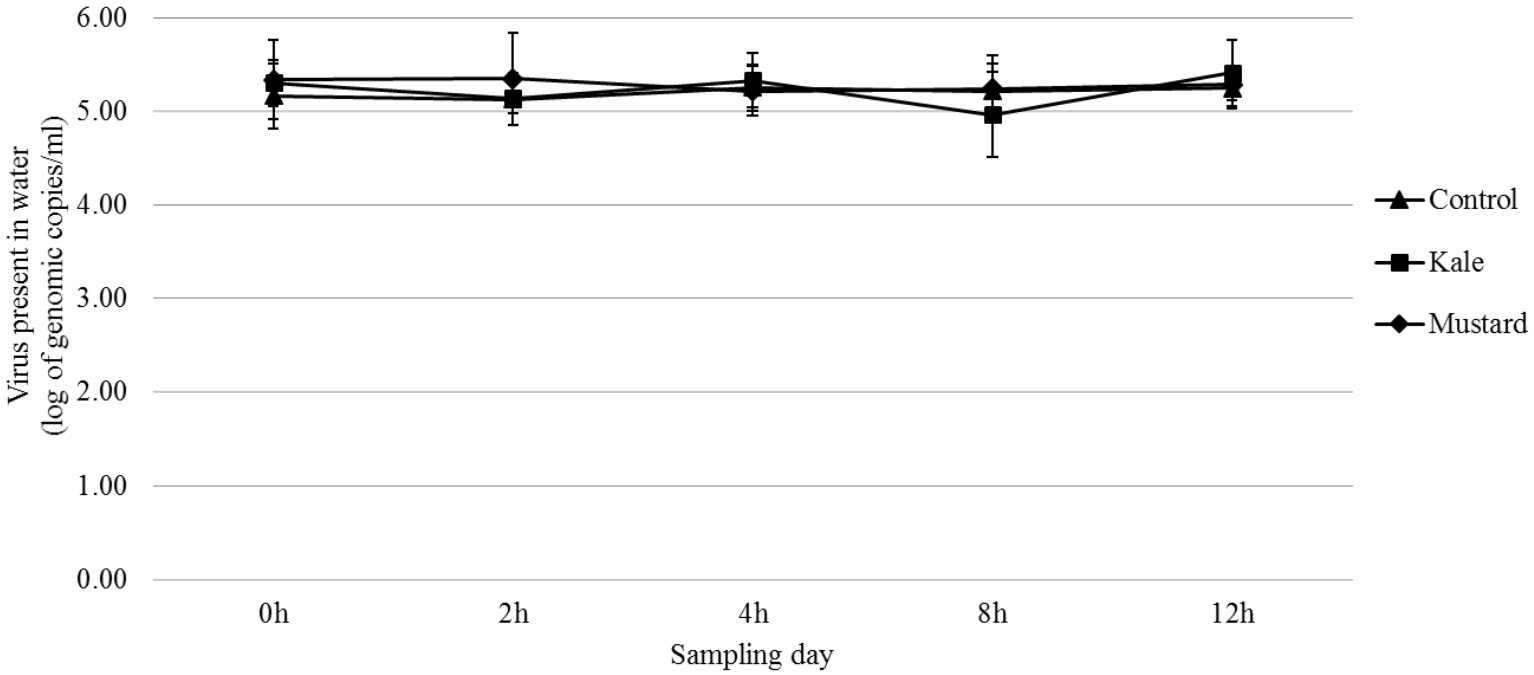

Figure 6.3 Presence of MNV genomic copies in re-circulated water within the first 12 hours during harvest from a newly contaminated hydroponic system (virus inoculated in water on day 8).

6.4.2 MNV remained infectious and gradually decreased in the roots and edible tissues of kale and mustard microgreens during the harvesting period (long-term study)

In order to observe virus behavior for a longer period of time, the survival and transfer of MNV from days 9-12 were also determined. The starting titer of MNV in re-circulated water (day 8) was 3.42 ± 0.49 log PFU/ml, and Table 3 shows MNV uptake and transfer at days 8-12. The occurrence of MNV remained similar in kale and mustard, with contamination observed in more than half of the samples (Table 3). The number of positive samples decreased over time. MNV was detected in both kale and mustard edible tissues one day post-inoculation with an average titer of 2.30 ± 1.02 and 2.49 ± 0.39 log PFU/sample, respectively (Table 3). The levels of infectious MNV in both kale and mustard edible tissues gradually decreased through day 12. At day 12, the titer dropped to 1.55 ± 1.17 and 1.61 ± 0.93 log PFU/sample, respectively (Table 3). MNV detected in mustard edible tissues was significantly higher at days 9 and 10 than that at day 12 ($p<0.05$), but no significant difference was observed in kale (Table 3). MNV genomic materials were persistent in edible tissues at ~4 log copies/sample (Figures S1 A and S3 A). Similarly, infectious MNV was also detected in the root samples of kale and mustard on all days tested with a decreasing trend (Table 3). The viral titers in the kale and mustard roots on day 9 were 2.53 ± 0.28 and 2.23 ± 0.37 log PFU/sample, respectively, and dramatically decreased to <1.50 log PFU/sample on day 12 ($p<0.05$) (Table 3). It is interesting that the viral titers in roots were close to that found in edible tissues during days 9-12 (Table 3). An increased amount of MNV genomic copies (~ 4-5 log copies/sample) was observed in roots, but remained stable over time (Figure 5.4 B). However, the genomic copies in roots were slightly lower than that within the first 12 hours (Figures S1 B and S3 B).

Table 6.3 Transfer of MNV in kale and mustard microgreens grown hydroponically at days 9-12.

Portion	Microgreens	Virus titers (log PFU/sample)* and ratio (positive counts/samples tested)							
		Day 9		Day 10		Day 11		Day 12	
Edible tissues	Kale	a2.30 ± 1.02^{A}	(8/9)	a2.23 ± 0.92^{A}	(8/9)	a1.96 ± 1.16^{A}	(7/9)	a1.55 ± 1.17^{A}	(6/9)
	Mustard	a2.49 ± 0.39^{A}	(9/9)	a2.37 ± 0.37^{A}	(9/9)	a2.12 ± 0.36^{AB}	(9/9)	a1.61 ± 0.93^{B}	(7/9)
Roots	Kale	a2.53 ± 0.28^{A}	(9/9)	a2.14 ± 0.34^{A}	(9/9)	a2.01 ± 0.79^{AB}	(8/9)	a1.47 ± 0.85^{B}	(7/9)
	Mustard	a2.23 ± 0.37^{A}	(9/9)	a1.71 ± 0.98^{AB}	(7/9)	a1.51 ± 0.88^{AB}	(7/9)	a1.42 ± 0.82^{B}	(7/9)

*Values are means ± SD of three trials with three samples each; values in columns with the same proceeding letter indicate no significant difference of virus titers when comparing the edible tissue/root between kale and mustard on each sampling day; values in rows with the same following letter indicate no significant difference of virus titers within the portions of kale or mustard over time.

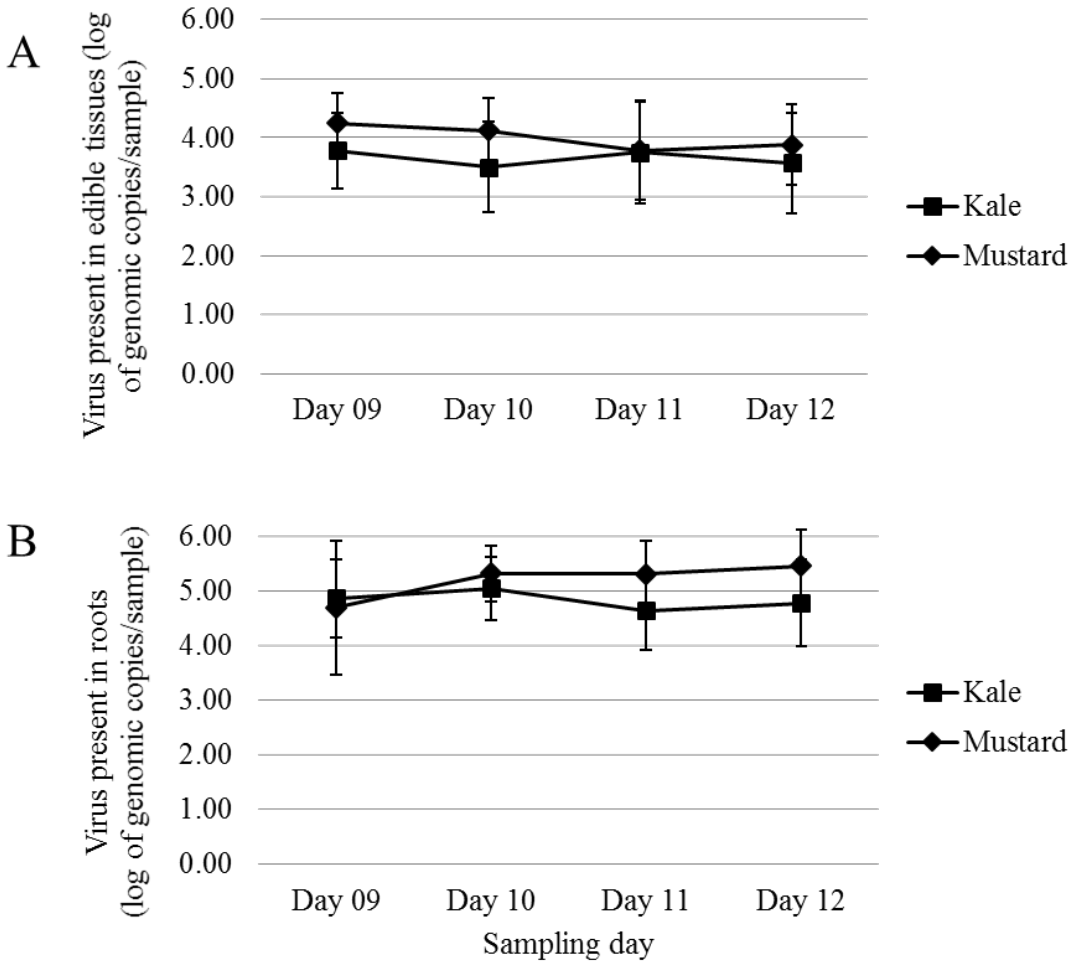

Figure 6.4 Presence of MNV genomic copies in kale and mustard edible tissues (A) and roots (B) at days 9-12 during harvest from a newly contaminated hydroponic system (MNV inoculated in water on day 8).

In addition, the viral titers in re-circulated water were measured daily from day of inoculation (day 8) until the microgreens were completely harvested (day 12). The starting titer of the re-circulated water used for this long-term study (days 9-12) was approx. 1 log PFU/ml higher than that used for short-term study (0-12 hours). As virus uptake via root to microgreen edible tissues over the period of harvest, viruses present in water gradually decreased (Table 4). The titer in water after inoculation (day 8) was 3.26 ± 0.40 log PFU/ml for kale, which was significantly higher than that on days 11 and 12 with values of 2.68 ± 0.25 and 2.75 ± 0.16, respectively ($p<0.05$). Virus titers also decreased in control and mustard water; however, no significant difference was observed over the period of time. The number of MNV genomic copies remained at > 3 log copies/ml in all water samples tested (control, kale, and mustard) with no significant reduction from day 8 to 12 ($p>0.05$) (Figure 5.5). These results suggested that MNV was stable and persistent in re-circulated water.

Table 6.4 The survival of MNV in re-circulated water during the harvest of kale and mustard microgreens at days 8-12.

		Virus titers (log PFU/ml)*				
		Day 08	Day 09	Day 10	Day 11	Day 12
Water	Control	a3.54 ± 0.49^{A}	a3.36 ± 0.66^{A}	a3.21 ± 0.67^{A}	a3.09 ± 0.63^{A}	a2.73 ± 0.65^{A}
	Kale	a3.26 ± 0.40^{A}	a3.11 ± 0.20^{AB}	a2.85 ± 0.29^{AB}	a2.68 ± 0.25^{B}	a2.75 ± 0.16^{B}
	Mustard	a3.46 ± 0.72^{A}	a3.21 ± 0.85^{A}	a3.20 ± 0.37^{A}	a3.10 ± 0.39^{A}	a2.77 ± 0.52^{A}

*Values are means ± SD of three trials with three samples each; values in columns with the same proceeding letter indicate no significant difference of virus titers when comparing the water samples of control, kale and mustard on each sampling day; values in rows with the same following letter indicate no significant difference of virus titers in water samples from control, kale, or mustard system over time.

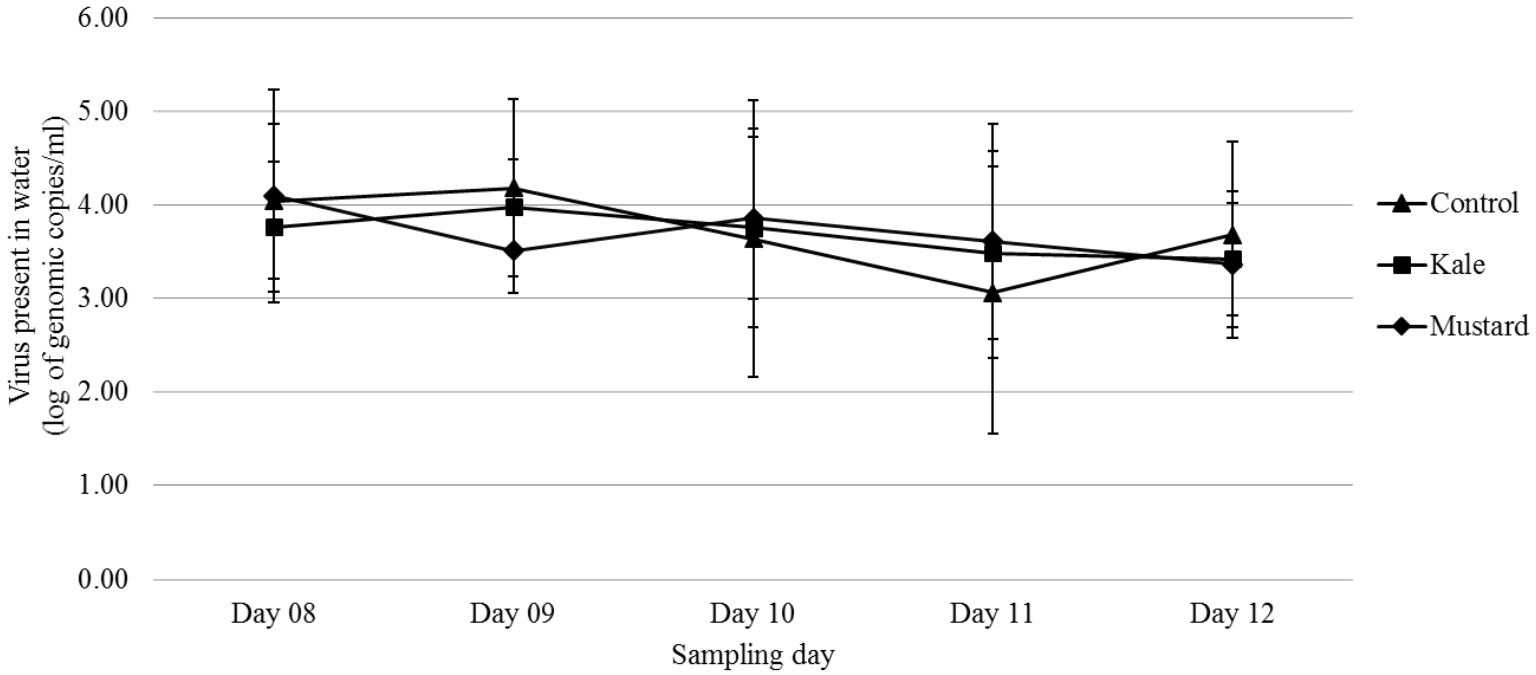

Figure 6.5 Presence of MNV genomic copies in re-circulated water at days 8-12 during harvest from a newly contaminated hydroponic system (virus inoculated in water on day 8).

6.4.3 Cross-contamination of MNV in a previously contaminated hydroponic system easily occurred

Immediately following the completion of the original study, an identical experimental design and procedures were performed without disinfection, and the potential risks associated with the previous contaminated system was investigated. Contamination occurred in almost all the samples examined (Table 5). Generally, lower titers of infectious MNV (~ 1.5-2.5 log PFU/sample) were found in both edible tissues and roots compared with the newly contaminated system (Table 5). Virus titers in the edible tissues of kale and mustard at day 12 were 2.61 log PFU/sample, significantly higher than titers on day 8 which was 1.99 ± 0.82 and 2.26 ± 0.28, for kale and mustard respectively ($p<0.05$) (Table 5). The level of MNV genomic copies present in edible tissues persisted at ~4 log copies/sample (Figure 5.6 A). This indicates that viruses can accumulate in microgreen edible tissues over longer exposure times (days 0-12). MNV was detected in roots as well. Viral titers in kale roots were consistent at ~2 log PFU/sample, whereas viral titers increased in mustard roots from ~1.5 to 2.2 log PFU/sample (Table 5). The genomic copies present in roots dropped to ~ 3 log copies/sample (Figure 5.6 B), significantly lower than that found in newly contaminated system (Figure 5.4 B). This provides evidence that the integrity of MNV genomic materials decreases gradually in roots.

Table 6.5 Transfer of MNV in kale and mustard microgreens grown in previously contaminated hydroponic system.

		Virus titers (log PFU/sample)* and ratio (positive counts/samples tested)					
		Day 08		Day 09		Day 12	
Microgreens	Kale	a1.99 ± 0.82^{A}	(8/9)	a2.43 ± 0.15^{AB}	(9/9)	a2.61 ± 0.14^{B}	(9/9)
	Mustard	a2.26 ± 0.28^{A}	(9/9)	a2.49 ± 0.12^{B}	(9/9)	a2.61 ± 0.14^{B}	(9/9)
Roots	Kale	a2.03 ± 0.20^{A}	(9/9)	a2.09 ± 0.17^{A}	(9/9)	a2.17 ± 0.18^{A}	(9/9)
	Mustard	a1.69 ± 0.66^{AB}	(8/9)	a1.52 ± 0.89^{A}	(7/9)	a2.21 ± 0.11^{B}	(9/9)

*Values are means ± SD of three trials with three samples each; values in columns with the same proceeding letter indicate no significant difference of virus titers when comparing the edible tissue/root between kale and mustard on each sampling day; values in rows with the same following letter indicate no significant difference of virus titers within the portions of kale or mustard over time.

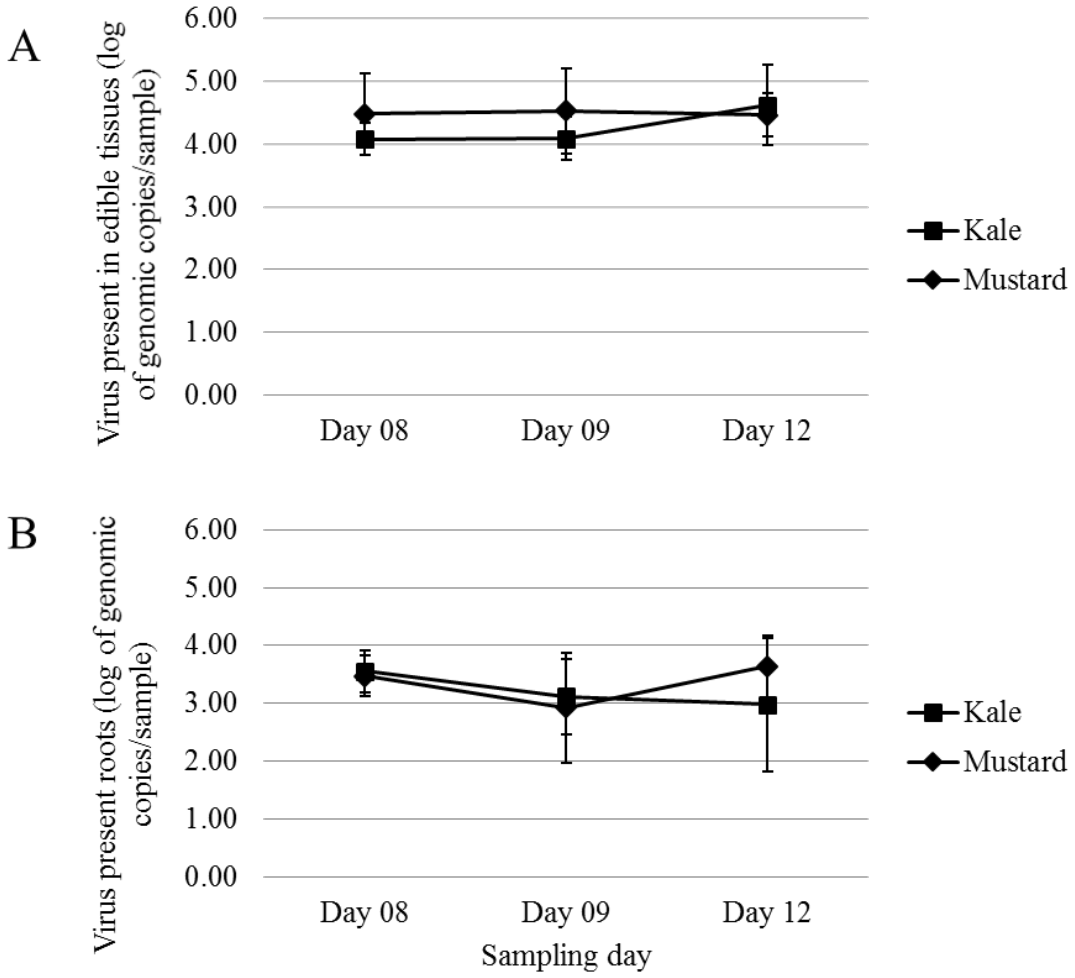

Figure 6.6 Presence of MNV genomic copies in kale and mustard edible tissues (A) and roots (B) at days 9-12 during harvest from a previously contaminated hydroponic system.

Greater than 2 log PFU/ml infectious MNV was detected in fresh water at day 0 after the inoculated water was discarded; however, these MNV levels were significantly lower compared with the original newly contaminated hydroponic system (Table 6). Viral titers decreased over time from ~2 log PFU/ml at day 0 to ~1 log PFU/ml at day 12 in all systems tested ($p<0.05$) (Table 6). MNV genomic materials of also gradually decreased through day 12 without significant difference ($p>0.05$) (Figure 5.7). These results demonstrate that MNV can persist in the environment and is able to remain infectious over a long period of time. Without proper cleaning and sanitation, viruses were transferred and contaminated the whole hydroponic system via re-circulated water. Even with fairly low titers, MNV was efficiently internalized and disseminated in microgreens grown hydroponically.

Table 6.6 The survival of MNV in re-circulated water in previously contaminated hydroponic system.

		Plaque assay (log PFU/ml)								
		Day 00	Day 01	Day 02	Day 03	Day 05	Day 08	Day 09	Day 10	Day 12
Water	Control	a2.26 ± 0.02^{A}	a2.00 ± 0.03^{B}	a1.94 ± 0.10^{B}	a1.81 ± 0.07^{BC}	a1.65 ± 0.14^{CD}	a1.68 ± 0.07^{CD}	a1.47 ± 0.12^{E}	a1.61 ± 0.13^{DE}	a1.44 ± 0.13^{E}
	Kale	a2.24 ± 0.23^{A}	a2.15 ± 0.12^{AB}	a2.01 ± 0.12^{AB}	ab1.99 ± 0.21^{AB}	b1.89 ± 0.13^{BC}	a1.67 ± 0.19^{CD}	a1.59 ± 0.14^{DE}	a1.50 ± 0.08^{DE}	ab1.32 ± 0.15^{E}
	Mustard	a2.11 ± 0.12^{A}	a2.08 ± 0.14^{A}	a1.87 ± 0.04^{A}	ab1.93 ± 0.07^{B}	a1.52 ± 0.07^{B}	a1.51 ± 0.22^{B}	a1.32 ± 0.15^{B}	a1.45 ± 0.05^{B}	b1.00 ± 0.30^{C}

*Values are means ± SD of three trials with three samples each; values in columns with the same proceeding letter indicate no significant difference of virus titers when comparing the water samples of control, kale and mustard on each sampling day; values in rows with the same following letter indicate no significant difference of virus titers in water samples from control, kale, or mustard system over time.

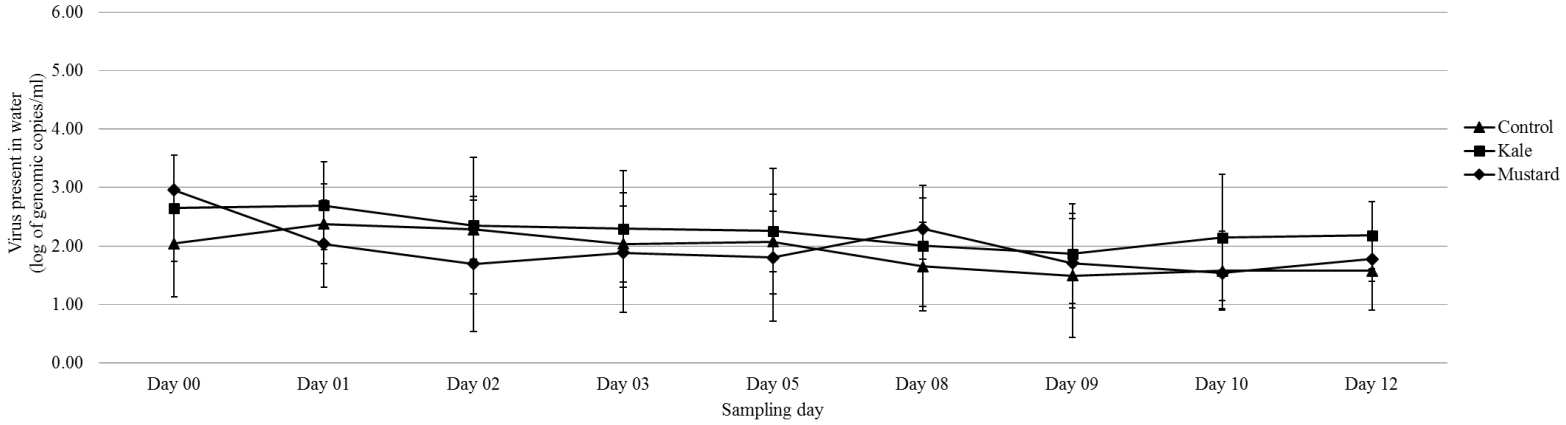

Figure 6.7 Presence of MNV genomic copies in re-circulated water during germination and harvesting from previously contaminated hydroponic system.

6.4.4 Bacterial background in the hydroponic system

The bacterial background in the hydroponic system was examined in this study. In the newly contaminated hydroponic system, the bacteria flora present in water increased from day 8 to 9 with average of 2.22 ± 0.25 log CFU/ml to > 5.35 log CFU/ml (Table S1). The level of bacteria at day 9 was similar to that at day 12, indicating bacterial levels were maintained over the harvest period. The highest bacteria levels of ~ 8 log PFU, were detected in kale and mustard samples with no significant change over the time of harvesting ($p>0.05$) (Table S1). Interesting, the starting levels of bacteria detected in water from the previously contaminated hydroponic system were much higher with average of > 4 log CFU/ml, and remained stable over the 12 days (Table S2). Similar levels of bacteria were also present in kale/mustard edible tissues and roots from the previously contaminated hydroponic system. In addition, the Colilert method showed *E. coli* was absent in the re-circulated water (data not shown). The number of coliforms was much lower, but the growth trend of coliforms was similar to that determined by TSA plate count (Tables S3 and S4).

6.4.5 Kinetic of MNV survival in re-circulated water

Three different models were compared to evaluate the kinetics of virus survival in re-circulated water. Compared with exponential and Weibull models, linear model was best fit for water samples obtained from a newly contaminated hydroponic system (Table S5). Whereas, both linear and Weibull models are appropriate to estimate the trend of MNV in the re-circulated water from a previously contaminated hydroponic system (Table S5).

6.5 Discussion

Use of hydroponic systems for growing produce crops along with aquaculture or aquaponics-raised fish is increasing, but there is limited information pertaining to the microbiological safety of microgreens. Epidemiological data revealed that huNoV is a leading cause of produce-associated outbreaks (10). Recent surveillance of produce-associated outbreaks in the United States and the European Union from 2004-2012 indicated that >50% of outbreaks were caused by huNoV, and recommended the produce industry following the Good Agricultural Practices Guides and avoiding contamination by food handlers who are infected by huNoV (9). Microgreens can be contaminated by huNoV at any point from farm to table. Even though microgreens share some similarities with sprouts, there are currently no standards or practices for microgreen production. HuNoV can contaminate irrigation water via cross-contamination. It is possible that food handlers in greenhouse may introduce viruses to water when they are infected asymptomatically. In this study, a nutrient film technique hydroponic system was chosen as a model to mimic the large-scale production of microgreens, and investigated for the potential risks of virus transfer and survival after a contamination event. Our results show that MNV can be efficiently taken up and internalized into microgreen edible tissues via roots through contaminated re-circulated water as soon as 2 hours post water inoculation. Importantly, we found that MNV remained in the system once water and plants were removed and could survive for a long period of time in microgreens as well as in the hydroponic system. Without appropriate cleaning and disinfection procedures, virus cross-contamination could easily occur where viruses were infectious in the new set of plants grown in the previously contaminated hydroponic system. The levels of MNV used here were similar to the levels of huNoV detected in environmental samples (e.g., water samples)

ranging from ~10 to ~5×10^4 genomic copies/ml (28-30). However, those levels were much lower than that huNoV shed in vomit and feces (up to 3×10^7 viral particles/episode of vomiting; up to 10^{11} genomic copies/g of stool samples) (31).

Infectious MNV (~2-3 log PFU/sample) was detected in both kale and mustard microgreen edible tissues as soon as 2 hours following inoculation. During the first 12 hours following inoculation and recirculation of contaminated water, the titers were relatively stable. It is possible that virus uptake occurred immediately after inoculation, and quickly saturated in plant edible tissues. The saturation can also be confirmed by the stable levels of genomic materials detected in microgreen edible tissues and roots (Figure 5.2). Similarly, Hirneisen et al., determined that the levels of MNV internalized into green onions within a floating hydroponic system were also consistent with an average of ~4 log PFU/sample from day 1 to day 5 after inoculation, suggesting that saturation was reached within 24 hours (32). Ward and Mahler reported that the uptake and transfer of bacteriophage f2 occured rapidly in bean plants within 16 hours after exposure, and virus reached the maximal levels in stems and upwards in leaves via cut roots (19). Whereas, DiCaprio et al., used Romaine lettuce in a hydroponic growth system with one-time inoculated water with aeration; and they found that MNV internalized in Romaine lettuce increased and reached the peak titer on day 3 post water inoculation (22). Chancellor et al., used fluorescent microspheres to investigate hepatitis A virus uptake in green onions, and determined that florescence accumulated and nearly doubled between day 1 and 2 and reached a plateau at day 7(33). In this study, higher titers of MNV reached peaks within shorter time in both microgreen edible tissues and roots. The variations in the length of time required to reach peak virus concentration may be attributed to the

experiment protocol, such as the virus types and inoculation levels, procedures, types of plants, growth stage, integrity of roots, and hydroponic system (19-22, 25, 32, 33). The growth stage of plants in this study was much younger (days 8-12), whereas the plants used in previous studies mentioned above were at least 3 weeks old following germination (19, 21). It is possible that root growth can increase contact surface with re-circulated water; allowing the microgreens to concentrate more viruses in their tissues. Low humidity may facilitate virus uptake. Wei et al observed a 10-fold higher internalization of MNV at 70% humidity compared to humidity at 99%, as humidity significantly affects the transpiration (21). The environmental humidity of microgreen growth conditions was much lower (~51%), which may increase the rate of transpiration resulting in higher levels of viruses being internalized. In addition, the water was continuously re-circulated in the system, which may facilitate virus uptake. The roots being bound to hydroponic pads can increase the direct contact and exposure time to surrounding recirculating water. Moreover, the stage of plant development may present different composition of carbohydrates (e.g., monosaccharide and raffinose) (34), which can potentially affect the virus binding affinity. Esseili et al found that norovirus virus-like particles were likely to bind older and younger leaves differently by cell wall materials (23).

From days 9-12, the concentration of infectious MNV present on microgreens remained persistent with only a slight decrease (~ 1 log PFU/sample) over the time. Whereas, Ward and Mahler observed 2 to 3 log reduction of bacteriophage f2 in bean plant tissues (roots, stem, and leaves) within a week (19). It is likely that the hydroponic pads absorbed viruses from the recirculating water. Interestingly, microgreen edible tissues contained slightly more infectious viruses compared with

the number of viruses present in roots regardless of the type, but there was no significant difference ($p>0.05$). This is similar to what was observed in previous studies. Chancellor et al., used fluorescent microspheres as a biomarker to determine how hepatitis A virus contaminate green onions. Chancellor's group observed that significantly more fluorescence was detected at the bottom than at the middle or top of green onion 1 day post-inoculation; however, the levels in those three sections (top, middle, and bottom) became similar as the time increased (33). Also, the levels of MNV, Tulane virus, and huNoV GII.4 RNA distributed in Romaine lettuce sections from a hydroponic system including roots, shoots, and leaves, were also similar (22); occasionally the levels of viruses (MNV, Tulane virus, or hepatitis A virus) detected in leaf, shoot/stem sections of plants were higher than that in roots (22, 32, 33). On the contrary, Ward and Mahler found distinct lower levels of virus in leaves than that in roots of 3-week old bean plants; and they considered that the interiors of the plants act as molecular sieves and permit only a portion of the bacteriophage f2 to be moved from one barrier to the next (19). These observed differences may be explained by the maturity of the plants and the differences between the sections such as components and structures, as well as the persistence of viruses within different conditions.

MNV concentrations present in the recirculating water for the long-term study gradually decreased during the experimental period, as virus was uptaken by microgreens. However, in most cases, no significant difference was observed between kale/mustard and control water for both short and long-term studies. It is possible that the decrease of virus titers in water was not detectable. The hydroponic study was conducted in a large scale, and 4000 ml of virus-inoculated water was circulated. The viruses transferred into the microgreens might be negligible. By comparing different

models, it was determined that linear model was the best fit for all the water samples ($p<0.05$), which may be useful to predict the behavior of MNV in a similar environment (Table S5).

We also found that MNV survived in the previously contaminated hydroponic system (up to 16 days in previously contaminated system), and be continuously circulated. Studies have shown that MNV can survive in water for a month with ~ 1 log PFU/ml reductions (35). Here in the previously contaminated, even after the inoculated circulated water was removed, without proper cleaning or disinfection, the hydroponic system on its own still reserved a large amount of viruses on the surfaces of water vessel and hydroponic platforms. Cross-contamination could easily occur within a hydroponic system, and MNV were easily transferred to microgreens seeds. It is very likely that all portions of microgreens would be contaminated by virus during germination (36), and the surface of microgreens may provide sites for virus accumulations. During the harvesting period (days 8-12), viruses were detected in the microgreen edible tissues and roots. The viruses detected in edible tissues may also include those present on the external surface. The information obtained here enforces the need for proper sanitation and provides useful information required for development of preventative strategies. MNV was measured by both plaque assay and real-time RT-PCR, providing different prospectives of virus behavior. The genomic copies in microgreen edible tissues were consistent over the time (Figures S1 A, S3 A, S5 A). With relatively large amounts of viruses available in the water used in this study, microgreen edible tissues may become saturated with MNV and the capsids of MNV were more likely to be damaged rather than genomic materials. Interestingly, the genomic materials degraded faster when the virus was located in microgreen roots

(Figures S1 B, S3 B, S5 B). The possible toxic effects of the plants on viruses (viral capsids or genomic materials), especially different sections of the plant, should be further explored (37, 38).

Generally, the behavior of MNV in both kale and mustard was similar and no significant difference was observed. It may be explained that they both belong to *Brassica* species. In the future, it will be interesting to investigate virus uptake among different genera of plants.

In conclusion, virus inoculated in water was taken up into the edible tissues of the microgreens via the roots. The internalization of viruses into produce poses a potential risk, as it will become more difficult to be removed or inactivated (33). Besides, if the system was not properly disinfected or cleaned, cross-contamination can occur. This study on survival and transfer of MNV in hydroponic system is important to identify the routes of virus contamination, and provides useful information to develop efficient preventative strategies, further better to conduct risk assessment regarding viral contamination in the hydroponic system.

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Chapter 7

BACTERIAL SURVIVAL AND TRANSFER IN HYDROPONICALLY GROWN MICROGREEN S DURING HARVESTING

(A manuscript to be submitted to Foodborne Pathogens and Disease)

7.1 Abstract

This study investigated uptake of *Escherichia coli* O157:H12 and *Salmonella* enterica serovars Typhimurium and Agona by kale and mustard microgreens grown hydroponically. Kale and mustard seeds were allowed to germinate and grow on hydroponic growth pads for 7 days. On day 8, hydroponic circulated water was inoculated with 3.5 - 4.0 log CFU/ml of the nonpathogenic strains of *Escherichia coli* O157:H12 and *Salmonella* Typhimurium LT2 and the fate of these bacteria was investigated in a greenhouse setting. *Salmonella* Agona was introduced the plants in a laboratory setting. During the harvesting period (Days 9, 10 and 12), the edible portions of the microgreens were harvested by cutting them 1 cm above the pad. The hydroponic pads containing the roots were collected as root samples. Both types of samples were analyzed by bacterial enumeration and enrichment. Results show that the bacteria were internalized in both kale and mustard edible tissues as soon as 1 day post-inoculation at average levels of 1.54 to 3.66 log CFU/sample. The two strains were also found in roots at 1.00 to 3.48 log CFU/sample. The levels of *E. coli* O157:H12 and *S.* Agona were similar in both kale and mustard microgreens ($p>0.05$), whereas *S. Typhimurium* LT2 present in kale and mustard microgreens was significantly different ($p<0.05$). These findings revealed that bacteria may be re-

circulated throughout hydroponic systems and efficiently translocated to the edible portion of microgreens, reinforcing the need for adequate and diligent sanitation.

7.2 Introduction

Produce safety is of great concern as fresh produce serves as a vehicle for foodborne pathogen transmission (DeWaal and Bhuiya 2007; DeWaal and Glassman 2013; Doyle and Erickson 2008). Usually, produce is consumed raw or with little or no processing, and can be easily contaminated with foodborne pathogens preharvest (e.g., irrigation water, and amendments), during processing (e.g., washing, and packing), and by contact with infected individuals who may handle the produce (Carter 2005; DeWaal and Bhuiya 2007; Hall and others 2012; Mathijs and others 2012). Microgreens have been gaining more attention and becoming popular across the U.S.; and as such they will likely share a significant portion of sprout markets (Brentlinger 2007). Microgreens are salad crop shoots of various species harvested within 10-20 days following seedling emergence, they are typically associated with the emergence of the true leaves (Lee and others 2004). Microgreens are usually grown under controlled environmental conditions within a greenhouse or indoor facility to prevent the introduction of microbial pathogens. However, little is known concerning the risk profile of microgreens, as no outbreak associated with microgreens has been reported in the U.S.. As the consumption of microgreens increases, there is a need to identify possible contamination routes and risks associated with pre-harvest production to provide useful information for guidelines regarding microgreen production.

It has been reported that pathogenic *Escherichia coli* and *Salmonella* spp have been isolated from irrigation water used for produce production, including use in

hydroponic systems (Benjamin and others 2013; Greene and others 2008; Micallef and others 2012). Extensive research has been conducted to provide evidence and insight on foodborne pathogen transfer, including internalization into plant tissues through contaminated water in hydroponic systems (Bernstein and others 2007; Cooley and others 2003; Deering and others 2012b; Dong and others 2003; Franz and others 2007; Guo and others 2002; Jablasone and others 2005; Kutter and others 2006; Sharma and others 2009; Warriner and others 2003). The presence of bacteria has been detected in a variety of different tissues in plants, such as vasculature tissues, lateral root junctions, xylem, phloem, and internal portions of leaf and stomata (Brandl 2008; Cooley and others 2003; Deering and others 2012b; Hirneisen and others 2012; Kutter and others 2006; Solomon and others 2002). Generally, two major routes of internalization were identified. Bacteria can either be pulled into plant tissues via water or enter through natural openings on the plant surface (Deering and others 2012a), and irrigation water is considered a critical source of potential contamination (Steele and Odumeru 2004). Many studies have confirmed the presence of bacteria from "above-the-ground" or edible portions of the plants after exposure to contaminated irrigation water (Bernstein and others 2007; Cooley and others 2003; Dong and others 2003; Guo and others 2002; Howard and Hutcheson 2003; Itoh and others 1998; Jablasone and others 2005; Kutter and others 2006; Sharma and others 2009; Warriner and others 2003). Those studies indicated that the driving force of water absorption facilitates bacterial internalization into the plant. The extent of foodborne pathogens taken up into plants is likely dependent on several factors, like root damage, type of pathogens, type and age of plants, exposure time, inoculum levels, and humidity (Bernstein and others 2007; Cooley and others 2003; Dong and

others 2003; Guo and others 2002; Jablasone and others 2005; Kutter and others 2006; Sharma and others 2009). Once produce is contaminated by internalized pathogens, the effectiveness of sanitizing agents would be limited due to the pathogens' inaccessibility and lack of contact with the agents (Solomon and others 2002). Furthermore, studies have shown that the levels of *E. coli* and *Salmonella* spp. increased within the plants (Cooley and others 2003; Jablasone and others 2005; Warriner and others 2003).

If microgreens are growing with contaminated irrigation water, it is very likely that pathogens would be taken up through the roots and internalized to edible or foliar portions, posing potential health risks. In this study, kale and mustard microgreens were selected to examine the risk of water contamination and internalization of *E. coli* O157:H12 and *Salmonella* spp. in hydroponically grown microgreens during harvesting. Nonpathogenic strains were used in a nutrient film technique system within a greenhouse setting, whereas the pathogenic strain was studied in the laboratory.

7.3 Materials and Methods

7.3.1 Bacterial strains, culture, and inoculum preparation

Non-pathogenic nalidixic acid resistant *Escherichi coli* O157:H12 (*E. coli* O157:H12-Nal) (a gift from Dr. Manan Sharma, USDA, ARS, Beltsville, MD) was originally isolated from a watershed sample. Another nonpathogenic strain, *Salmonella* enterica serovar *Typhimurium* LT2 (ATCC# 700720) was selected with spontaneous rifampicin resistance (*S. Typhimurium* LT2 -Rif) by transferring colonies on XLT-4 agar (BD, Sparks, MD) containing 80 μg/ml rifampicin (Fisher Scientific,

Fair Lawn, NJ). Both of the nonpathogenic strains were used in the Fischer greenhouse at the College of Agriculture and Natural Resources. A pathogenic *Salmonella* enterica serovar Agona (ATCC# 51957) with rifampicin resistance (*S.* Agona-Rif) was used in a laboratory setting. The strains were stored at -80 °C in 20% glycerol. For experiments, bacteria were grown from a glycerol stock and streaked onto Sorbitol MacConkey agar (BD) supplemented with 50 μg/ml nalidixic acid and XLT-4 supplemented with 80 μg/ml rifampicin for *E. coli* O157:H12 and *Salmonella* spp., respectively. Plates were incubated at 37 °C for 24 h. An isolated colony was inoculated into a tube containing 9 ml of Luria Bertani (LB) broth (BD) containing 50 μg/ml nalidixic acid and 80 μg/ml rifampicin for *E. coli* O157:H12 and *Salmonella* spp., respectively. After culturing for 24 h, 900 μl culture was transferred into a tube containing 9 ml of LB broth. When the OD_{600} reached ~ 0.5 (exponential growth phase), 1 ml was inoculated onto an LB plate, spread with beads and incubated overnight Five ml of 0.1% buffered peptone water (BPW) (1g/liter) was added to one of the plates and a sterile plastic inoculation loop or a scraper was used to scrape the lawn of bacteria from the plate. The suspension was pipetted off the plate surface and placed into a sterile tube. This process was repeated once for a total of 10 ml from each plate. The 10-ml suspension was pelleted in BPW by centrifugation at 2000 × g for 10 min at room temperature (22 ± 1°C) to achieve a high bacterial concentration (10^7 or 10^8 CFU/ml).

7.3.2 Qualification and Quantification of bacterial inoculum

Samples including controls were collected and immediately ten-fold serial dilutions were performed in PBS (pH 7.2) and 100 μl were plated in duplicate on Sorbitol MacConkey agar (BD) containing 50 μg/ml nalidixic acid and XLT-4 Agar

(BD, Sparks, MD) containing 80 μg/ml rifampicin and for *E. coli* O157:H12 and *Salmonella* spp., respectively. Plates were incubated at 37 °C for 24 h. Colony-forming units (CFU) were enumerated. All samples were stored at 4 °C for up to 48 h for further analysis if needed.

If no CFU were observed by direct plating, bacterial enrichment was conducted. For *E. coli* O157:H12 enrichment, an equal volume of modified 2 × EHEC medium (Biocontrol, Bellevue, WA) was added to the sample and incubated at 37 °C for 5 h. Nalidixic acid was added to a final concentration of 50 μg/ml and the cultures were incubated at 42 °C for 18-24 h. enrichment loopful of the culture was streaked onto Sorbitol MacConkey Agar with 50 μg/ml nalidixic acid and incubated for 24 h at 37 °C. If typical colonies were present, enrichment was recorded as *E. coli* O157:H12 positive. For *Salmonella* spp. enrichment, equal volumes of 2 × lactose broth (BD) was added to the sample and incubated at 37 °C for 24 h. One ml of enrichment culture was added to 9 ml tetrathionate broth (TT; Oxoid, Basingstoke, Hampshire, England), and 0.1 ml of this culture was added to 9.9 ml Rappaport-Vassiliadis R10 (RV; Oxoid). TT broth and RV broth tubes were incubated at 37 °C for 24 h and at 42 °C for 48 h, respectively. Following incubation, a 10-μl loopful broth from both tubes was streaked onto XLT-4 agar containing 80 μg/ml rifampicin. XLT-4 plates were incubated at 37 °C for 24 h. If typical colonies were present, enrichment was recorded as *Salmonella* spp. positive.

7.3.3 Hydroponic system

Microgreens were grown at the Fisher Greenhouse at the College of Agriculture and Natural Resources, Newark, DE. The nutrient film technique (NFT) hydroponic system was prepared by tilting three platforms at a 30 degree angle in

order to allow water to flow through the system (Shown in Figure 6.1). Each set had four trays and its own water vessel containing 4000 ml of water supplemented with 30 ml of a nutrient solution A that contained $Ca(NO_3)_2$ (120.0 g/L, YaraLiva, Tampa, FL), and 30 ml of a solution B that contained 5-11-26 Hydro-Sol (120.0 g/L, Peters Professional, Dublin, OH), $MgSO_4$ (1.17 g/L, Giles Chemical, Waynesville, NC), and Sprint 330 (0.58 g/L, Becker Underwood, Ames, IA). A pump was placed in the water vessel to pump water to the top of the system through a tube at a constant rate (~10 ml/s). The pump was set to run continuously but with cycling on for 5 min and then off for 10 min. The water flowed down the platforms due to gravity, then back into the water vessel and recirculated through the Nutrient film technique (NFT) system.

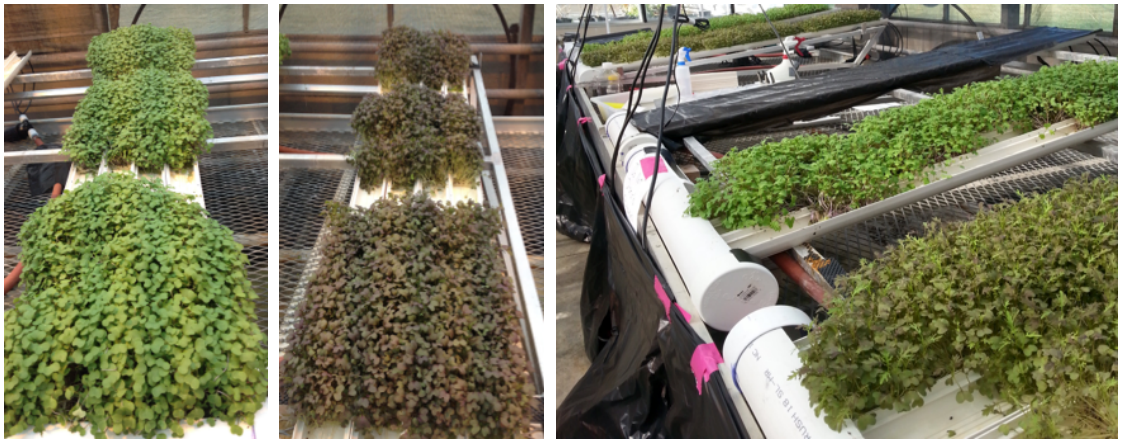

Figure 7.1 Nutrient film technique (NFT) hydroponic system including control, kale and mustard.

7.3.4 Plant cultivation

Kale (*Brassica napus*) and mustard (*Brassica juncea*) (Johnny's, Winslow, ME) were grown on micro-mats hydroponic grow pads (Handy Pantry, West

Springville, UT) soaked in circulating water. Three individual sets of 12 micro-mats hydroponic grow pads (33.00 cm × 6.35 cm) were placed in each hydroponic system (3 pads/tray). Positive controls were included with no plants and circulating bacteria alone, and negative controls consisted of the environmental sample (microgreen edible tissues, roots, or water) before inoculation. On day 0, 6.75 g kale and 3.75 g mustard seeds, were distributed evenly on the pads, respectively. Water was supplemented with a nutrient solution and circulated. Microgreens were mature one-week post germination, and were ready to harvest by days 8-12. The temperature of the greenhouse was 22.3 °C with an average humidity of 51%. Microgreens were grown in 12 h of daylight of averaged radiation of 1057.2 J/cm^2 and 12 h of darkness of 0.4 J/cm^2 daily.

7.3.5 Inoculation of circulating water

Fresh feed water (4000 ml) was inoculated on day 8 with a starting titer of 3.80 ± 0.24 log CFU/ml and 3.57 ± 0.35 log CFU/ml in water for *E. coli* O157:H12 and *S. Typhimurium* LT2, respectively. The microgreens were maintained in bacteria-inoculated feed water from day 8 to day 12. As positive control, pads without plants were placed in the system with inoculated water circulated alone. Due to evaporation, fresh water was added to maintain the initial water level daily, but no additional bacterial inoculum was added after the initial inoculation.

For the pathogenic strain, the experiment was conducted in the laboratory from days 8-12. On day 8, kale and mustard microgreens were removed from greenhouse and fully covered with the bottoms of three Nalgene polypropylene trays (324 mm L × 257 mm W × 105mm H) (Thermo Scientific, Vernon Hill, IL), respectively. Each tray contained 300 ml of *S.* Agona-inoculated water (starting titer 3.93 ± 0.54 log CFU/ml)

and the water barely covered the pads. As positive control, pads without plants were soaked in 300 ml contaminated water. The temperature was 22.0 °C with an average humidity of 61%. Light/dark periods lasted for 12 h each.

7.3.6 Bacterial survival and uptake in the contaminated hydroponic system

Survival and transfer of bacteria was monitored during the harvesting time on days 8-12. Bacteria uptake was investigated directly following inoculation. Water and microgreen (edible tissue and roots) samples were collected at days 9, 10 and 12 to determine the speed of detectable bacteria taken up by the microgreens.

7.3.7 Sample collection

The levels of bacteria in water were monitored throughout the experiment. Water samples were collected from each water vessel at each sampling time, and 10-fold series dilutions were prepared before plating. If the levels of targeted bacteria were low, samples (10 or 100 ml) were filtered through a 0.45-mm pore-size filter (Microcheck II Beverage Monitor, Pall Corporation, Port Washington, NY). The filter was then transferred to selective agar plate.

Starting from day 9, microgreens (edible portion) and root pads were both sampled with pruners (Fiskars, Sauk City, WI). The microgreens (edible portion) were cut 1 cm above the pad, 10 g microgreens samples (kale or mustard), and correspondingly two pieces of 4×4 cm^2 pads containing roots without edible portion were collected in homogenizer bags (Fisher Scientific, Pittsburgh, PA), and treated as one microgreen edible tissue/root sample. Microgreen edible tissue and root samples were mixed with 10 ml and 5 ml phosphate buffed saline (PBS, pH 7.2), respectively. Samples were then smashed by a hammer followed by stomaching for 2 min. The

homogenates were collected and transferred to new collection tubes. Ten-fold dilutions were analyzed by plating count/enrichment. Two trials with two replicates in each trial were completed.

7.3.8 Statistical Analysis

Experiments were conducted in two trials with two samples each. Results are reported as mean and standard deviation. Data were analyzed by ANOVA on JMP software (Version 11.2, SAS Institute Inc., Cary, N.C.), and significant differences were indicated if $p<0.05$.

7.4 Results and Discussion

7.4.1 Bacteria was efficiently taken up via roots and transferred into edible tissue during the harvesting period.

After full maturation, each strain was inoculated in the circulating water on day 8 with a start titer of 3.5 - 4.0 log CFU/ml. In order to prevent cross-contamination, microgreen edible tissues and root pads were cut separately. Here, we found that without direct contact between the microgreen edible tissues and contaminated irrigation water, nonpathogenic strains including *E. coli* O157:H12 and *S. Typhimurium* LT2, as well as pathogenic strain *S.* Agona transferred to the aerial part of the plants via the roots. The amount (log CFU/sample) and ratio (number of positive samples over number of samples tested) of bacteria internalized in microgreen edible tissues is shown in Table 6.1. Each strain was detected in both kale and mustard edible tissues as soon as 1 day post-inoculation ranging from 1.75 - 3.06 log CFU/sample (Table 6.1). Similarly, detection of bacteria (e.g., *E. coli*, *Salmonella* spp. and *Listeria* spp.) in aerial parts of plants hydroponically grown in contaminated water

has been reported in maize (Bernstein and others 2007), lettuce (Franz and others 2007), tomato (Guo and others 2002), and spinach (Warriner and others 2003). Via personal communication, it was observed that hydroponic system might potentially cause root damage and facilitate bacteria uptake. As expected, strains were also found in roots since hydroponic pads that contained roots were soaked in the bacteria-contaminated water with averages of 2.52 - 3.13 log CFU/sample for most of samples on day 9 (Table 6.1). Both *E. coli* O157:H12 and *S.* Agona were detected in all the microgreen edible tissue and root samples. However, *S. Typhimurium* LT2 was not recovered in all the samples tested.

Table 7.1 Transfer of bacteria in kale and mustard microgreens grown hydroponically at days 9-12.

		Microgreens	Bacterial levels in log CFU/sample[a] (ratio of positive counts/samples tested)		
			Day 9	Day 10	Day 12
E. coli O157:H12[b]	Edible Tissue	Kale	2.83 ± 0.15A (4/4)	3.66 ± 0.88A (4/4)	3.08 ± 0.42A (4/4)
		Mustard	2.26 ± 0.59A (4/4)	3.39 ± 0.62B (4/4)	2.68 ± 0.28AB (4/4)
	Roots	Kale	2.71 ± 0.91AB (4/4)	2.87 ± 0.38A (4/4)	1.37 ± 0.52B (4/4)
		Mustard	+ * (4/4)	3.46 ± 0.40A (4/4)	2.72 ± 0.31B (4/4)
S. Typhimurium LT2[b]	Edible Tissue	Kale	2.30 ± 0.17A (2/4)	1.77 ± 0.53A (2/4)	2.13 ± 0.00A (2/4)
		Mustard	1.99 ± 0.48A (2/4)	1.89 ± 0.34A (2/4)	1.83 ± 0.40A (2/4)
	Roots	Kale	2.52 ± 0.46A (2/4)	1.35 ± 0.07A (2/4)	1.00 (2/4)
		Mustard	3.13 ± 0.13A (2/4)	3.06 ± 0.44A (4/4)	3.05 ± 1.07A (2/4)
S. Agona[c]	Edible Tissue	Kale	3.06 ± 0.82A (4/4)	3.04 ± 1.51A (4/4)	1.62 ± 0.47A (4/4)
		Mustard	1.75 (4/4)	1.72 ± 0.32A (4/4)	1.54 ± 0.09A (4/4)
	Roots	Kale	3.10 ± 1.17A (4/4)	3.48 ± 1.37A (4/4)	2.40 ± 0.25A (4/4)
		Mustard	3.12 ±1.07A (4/4)	2.96 ± 1.47A (4/4)	1.58 ± 0.39A (4/4)

[a] values are mean ± SD of two trials with two samples each; values in rows with the same letter indicate no significant difference within the portions of kale or mustard over time.
[b] experiments on *E.coli* O157:H12 and *S.*LT2 uptake were conducted in a greenhouse setting.
[c] experiments on *S.*Agona uptake were conducted in a laboratory setting.
* + indicates where samples were detected by enrichment only.

Generally, the levels of each strain were maintained over the harvest period without significant change ($p>0.05$). Exceptions include *E. coli* O157:H12 detected in mustard edible tissues, as well as both kale and mustard root samples. The levels of *E. coli* O157:H12 in mustard edible tissues were significantly increased from day 9 to day 10 ($p<0.05$); whereas the levels in root samples was increasing from day 9 to day 10, and significantly dropped on day 12 ($p>0.05$).

The behavior of *E. coli* O157:H12 and *S.* Agona was similar in kale and mustard microgreens ($p>0.05$), whereas the levels of *S. Typhimurium* LT2 present in kale and mustard microgreens were significantly different ($p<0.05$) (Figures 6.2 and 6.3). This finding suggests that internalization may depend on bacterial type (Dong and others 2003; Guo and others 2002; Jablasone and others 2005; Kutter and others 2006).

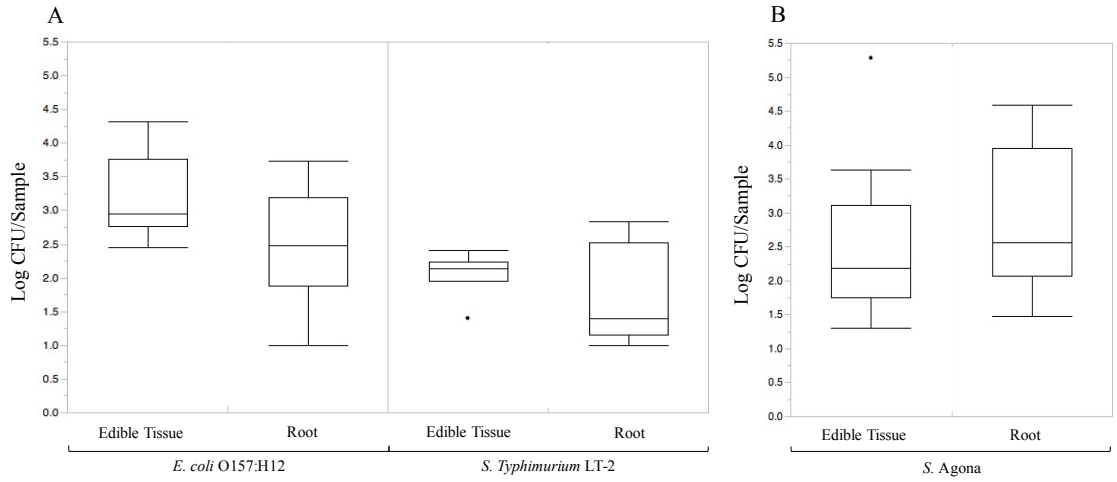

Figure 7.2 Transfer of nonpathogenic (A) and pathogenic (B) bacteria in kale microgreens grown hydroponically during harvesting period. The box-plots encompass the lower and upper quartiles, lines within the box are the median values, and the whiskers indicate the degree of dispersion of the data. Outliers are shown as dots. Enrichment data were not included.

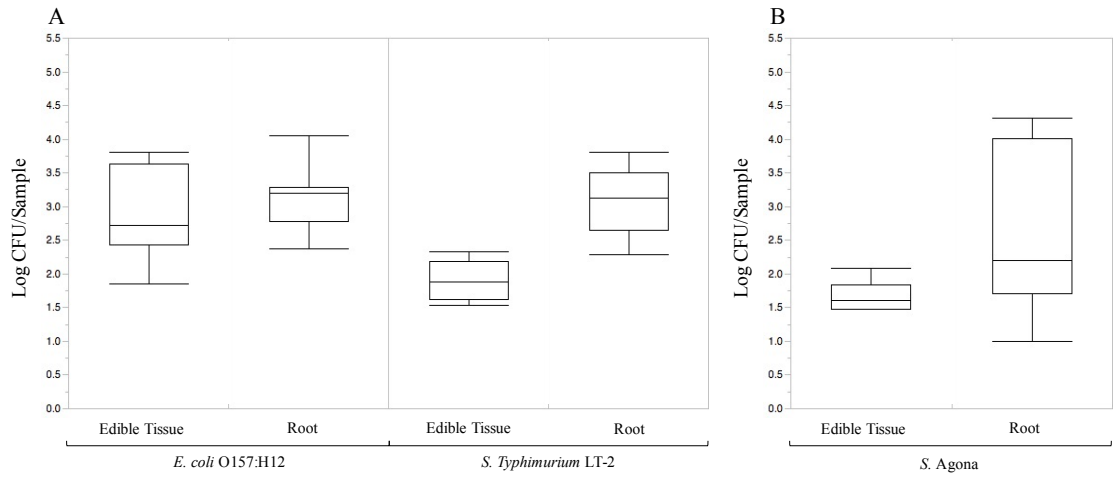

Figure 7.3 Transfer of nonpathogenic (A) and pathogenic (B) bacteria in mustard microgreens grown hydroponically during harvesting period. The box-plots encompass the lower and upper quartiles, lines within the box are the median values, and the whiskers indicate the degree of dispersion of the data. Outliers are shown as dots. Enrichment data were not included.

In addition, the levels of nonpathogenic strains present in kale microgreen edible tissues were lower than that in kale root samples (Table 6.1, Figure 6.2). The plants act as molecular sieves and only a portion of strains can be transferred to the aerial parts of plants. Previously, Guo et al. observed a similar trend and found that the population sizes of salmonellae were in the order of leaves < stem< hypocotyls and cotyledons (Guo and others 2002). On the contrary, the levels of each strain present in mustard roots were higher than those in mustard microgreen edible tissues (Table 6.1, Figure 6.3). It is possible that root exudates from mustard promoted bacterial replication (Bolton and others 1993). Plant type needs to be considered (Jablasone and others 2005).

7.4.2 Bacteria survived in re-circulated water during the harvesting period

In order to observe the survivability of bacteria in re-circulated water during the harvesting period, the levels of each strain were monitored (Shown in Table 6.2, Figure 6.4). The starting levels of the re-circulated water used for this study were 3.80 ± 0.24, 3.57 ± 0.35, and 3.93 ± 0.54 log CFU/ml in water for *E. coli* O157:H12, *S. Typhimurium* LT2, and *S.* Agona, respectively. For water samples, nonpathogenic strains *E. coli* O157:H12 and *S. Typhimurium* LT2 were mainly detected by enrichment on days 8 and 9 (Table 6.2). The nonpathogenic strains were then recovered on days 10 and 12. It is possible that those nonpathogenic strains were suffering environmental stress right after inoculation in circulation water, and gradually recovered as the time increased. Besides, it is also likely that microbial background may affect the levels of each strain present in the re-circulated water, which may either stimulate or inhibit foodborne pathogens (Mandrell 2009). Differently, *S.* Agona was much more robust and more easily recovered from water (Table 6.2). Compared with *S.* Agona, the survivability of *S. Typhimurium* LT2 shown here indicates it may not be a good surrogate for *S.* Agona in environmental studies.

Table 7.2 The survival of bacteria in re-circulated water during harvest of kale and mustard microgreens at days 8-12.

Water		Bacterial levels in log CFU/sample [a] (ratio of positive counts/samples tested)			
		Day 8	Day 9	Day 10	Day 12
E. coli O157:H12[b]	Control	3.85 ± 0.07A (4/4)	2.82 ± 1.53AB (4/4)	3.73 ± 0.33AB (2/4)	2.02 ± 0.07B (2/4)
	Kale	+ *(2/4)	+ *(2/4)	2.39 ± 0.35A (4/4)	2.61 ± 0.15A (4/4)
	Mustard	3.71 ± 0.44A (2/4)	+ *(2/4)	2.11 ± 0.77B (4/4)	3.22 ± 0.07A (4/4)
S. Typhimurium LT2[b]	Control	+ *(2/4)	+ *(2/4)	3.57 ± 0.35 (4/4)	1.48 (2/4)
	Kale	+ *(2/4)	+ *(3/4)	1.30 (4/4)	1.00 (2/4)
	Mustard	4.75 ± 0.00 (2/4)	+ *(2/4)	2.85 (2/4)	2.34 (2/4)
S. Agona[c]	Control	4.05 ± 0.33A (2/4)	5.16 ± 0.31A (2/4)	3.20 ± 1.91A (4/4)	3.25 ± 1.53A (4/4)
	Kale	3.71 ± 0.23A (4/4)	3.29 ± 0.47A (4/4)	2.60 ± 1.28A (4/4)	1.39 ± 0.36B (4/4)
	Mustard	4.10 ± 0.81A (4/4)	3.01 ± 0.98A (4/4)	1.00 (2/4)	1.18 (4/4)

[a] values are mean ± SD of two trials with two samples each; values in rows with the same letter indicate no significant difference within the portions of kale or mustard over time.

[b] experiments on survival of *E.coli* O157:H12 and *S. Typhimurium* LT2 were conducted in a greenhouse setting.

[c] experiments on survival of *S.* Agona were conducted in a laboratory setting.

* + indicates where samples were detected by enrichment only.

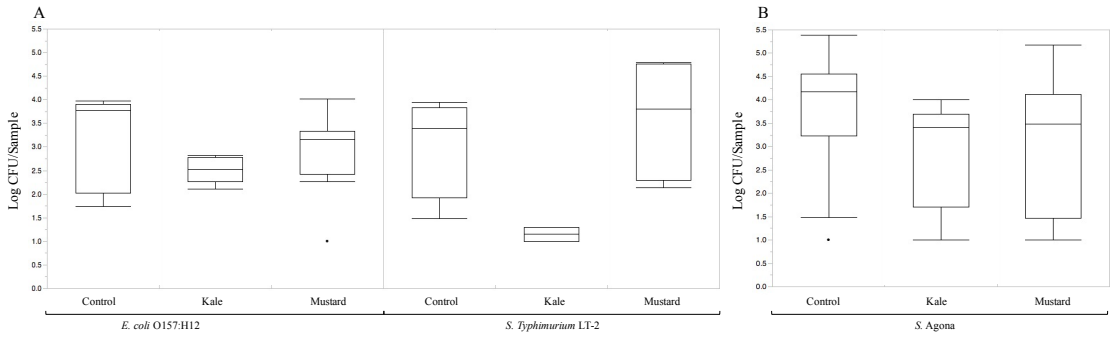

Figure 7.4 Survival of nonpathogenic (A) and pathogenic (B) bacteria in re-circulated water during harvest of kale and mustard microgreens at days 8-12. The box-plots encompass the lower and upper quartiles, lines within the box are the median values, and the whiskers indicate the degree of dispersion of the data. Outliers are shown as dots. Enrichment data were not included.

In conclusion, we have demonstrated that bacteria can internalize in hydroponically grown microgreens via the roots and translocate to microgreen edible tissues. The levels of bacteria present in edible tissues ranged widely likely due to a wide variety of factors. The significance of these findings to microgreen safety revealed the need for continued attention to safe irrigation water as well as the importance of adequate and diligent system sanitation. The data in this study can further be used in the development of microgreen production guidelines.

7.5 Acknowledgements

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Chapter 8

THE EFFECTS OF MULTIPLICITY OF INFECTIONS IN PROLIFORATION OF TULANE VIRUSES

(A manuscript submitted to Food and Environmental Virology)

8.1 Abstract

Tulane virus (TV) is a human norovirus (HuNoV) surrogate widely used to estimate the behavior of HuNoV. Low titers of TV have been experienced by many laboratories and this may limit the use of TV for a variety of research projects, including environmental virology applications. Previously, by manipulating the multiplicity of infection (MOI) (virus plaque forming unit and host cell ratio) virus production and foreign gene expression from the insect cell-baculovirus system were measured. It was determined that MOI plays an important role in both virus production and foreign gene expression. In this study, the LLC-MK2 cell line was cultured and infected with TV at different MOIs ranging from 1×10^{-6} to 1×10^{-2}, and the titers of TV were analyzed after complete cytopathic effects (CPE) were observed. This study showed that TV infection significantly increased as the MOI decreased. The highest MOI of 1×10^{-2} resulted in the lowest production of TV, while as MOIs $\leq$ 1×10^{-3} significantly increased the titers of TV ($p<0.05$) by 5-10 times. Our experiments indicated that the titers of TV could be increased significantly by decreasing the MOI, and this may be explained by the consequences of simultaneous virus infection and cell replication. This method can be used to improve and increase

the currently low titers of TV, and may be also useful for further understanding of the infection process of TV

8.2 Introduction

Human norovirus (huNoV) is a leading cause of foodborne illness in the U.S. accounting for 58% of all foodborne illness (Scallan et al. 2011). It is estimated that the total number of cases of huNoV in the U.S. each year ranges from 19 to 21 million, with an average of 5 episodes of huNoV gastroenteritis experienced by each individual within a lifetime (Hall et al. 2013; Payne et al. 2013). HuNoV has specific properties making it relatively easy to spread and highly contagious. HuNoV infection is likely due to several factors, including a low infectious dose of 10-100 virus particles, long duration of shedding with potential risk of secondary spread, strong stability in the environment, and diverse strains that stimulate a short immune response (Teunis et al. 2008; Patel et al. 2009). The pathogenesis of huNoV is still not well understood, as an appropriate repeatable cell culture model has not yet been discovered (Jones et al. 2014). Given these important details, various surrogates have been used *in vitro* to predict the behavior of huNoV, including viruses in the *Caliciviridae* family with close genetic and antigenic relatedness to huNoV. Tulane virus (TV) is a recently discovered calicivirus and belongs to the genus *Recovirus*, which has been used as a huNoV surrogate in laboratories. It was isolated from the stool of a rhesus monkey, and can be cultivable *in vitro* (Farkas et al. 2008). Importantly, TV is a promising surrogate to investigate the binding property as it has the ability to bind type A and B histo-blood group antigens (HBGA) (Farkas et al. 2010). Previous studies showed that TV is robust surrogate in the face of different inactivation methods (e.g., UV, alcohols, ozone, high hydrostatic pressure) and

environmental conditions (Cromeans et al. 2014; Drouaz et al. 2015; Wang and Kniel 2015; Tian et al. 2013). These findings indicated TV might be a good candidate for studying the behavior of huNoV and investigate its survivability for the benefit of pubic health.

Low titers of TV have been experienced in many laboratories limiting its use for a variety of research projects. Compared to another huNoV surrogate murine norovirus (MNV-1), the typical cultivated titer of TV is about 2-3 logs lower (Tian et al. 2013; Wang et al. 2013; Cromeans et al. 2014; Li et al. 2013). In order to facilitate the use of TV as an alternative surrogate for huNoV, the aim of this study was to increase the titers of TV by optimizing the multiplicity of infection (MOI).

8.3 Materials and Methods

8.3.1 Virus cultivation

Tulane virus (TV) (a gift from Dr. Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH) was propagated in LLC-MK2 cells (ATCC# CCL-7) in cell growth medium which was 199 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS), and 100 U/ml penicillin G-streptomycin-0.25μg/ml Amphotericin B in a humidified CO_2 incubator (37°C, 5% CO_2). After a typical 48 h infection of confluent monolayers, complete cytopathic effect (CPE) was observed. Viruses were obtained following three cycles of freeze-thawing infected cells, and centrifugation at 2,000 × g for 15 min. The supernatant was filtered through a 0.2 μm membrane filter (Thermo, Rochester, NY) before storing viruses at -80 °C until use.

8.3.2 Infection with different MOI's

The total number of cells at ~90% confluency in T-75 flasks was estimated to be 5.8×10^6 per T-75 flask by hemocytometer method. Briefly, after LLC-MK2 cells were ~90% confluent, cells were detached by Trypins-EDTA (Life Technologies, Carlsbad, CA) and suspended in cell growth medium as described above. The cell suspension was transferred into a test tube with an equal volume of 0.4% trypan blue solution (Cellgro, Manassas, VA) to achieve 10-50 cells/mm^2 for accurate counts. The mixture was then introduced into both V-shaped wells of a hemocytometer with approximately 20 μL each. The counts of clear cells (alive and intact cells) were recorded and determined under an inverted microscope. After the cells were ~90% confluency in T-75 flasks, cells were rinsed with Hank's Balanced Salt Solution (HBSS), and 12 ml of cell growth medium with 2% FBS was added into each flask. A viral stock solution was also added in amounts corresponding to MOI's (virus plaque forming unit and host cell ratio) of 1×10^{-6}, 2×10^{-6}, 5×10^{-6}, 1×10^{-5}, 2×10^{-5}, 5×10^{-5}, 1×10^{-4}, 2×10^{-4}, 5×10^{-4}, 1×10^{-3}, 2×10^{-3}, 5×10^{-3}, or 1×10^{-2}. The flasks were then placed in the incubator at 37°C, 5% CO_2 until CPE was observed. The incubation period ranged from 48 to 120 h, as it required more time for low MOI infection.

8.3.3 Tulane virus titer determination

After complete CPE was observed in T75 flasks with different MOI treatments. Virus was obtained by the way as described above. The titers of TV were quantified by plaque assay as previously described (Wang et al. 2013; Wang and Kniel 2014). To determine the infectivity of TV, LLC-MK2 cells were grown to 80-90% confluency in 6-well plates (Costar, Corning, NY), 100 μl of ten-fold serial dilutions of each virus sample was dispensed over monolayers in duplicate. The plates were

incubated at 37 °C with 5% CO_2 for 1 h with gentle agitation every 15 min followed by the addition of a 2 ml overlay. The overlay consisted of 1.5% agarose with complete 199 medium. After the incubation period (typically 48 h), 2 ml of 3.7% formaldehyde (w/v in phosphate-buffered saline) (Fisher Scientific, Pittsburg, PA) was added in each well for at least 2 h, and the plaques were visualized by staining with 0.05% crystal violet (w/v in 10% ethanol). Virus titers were determined and expressed as plaque forming units (PFU)/ml.

8.3.4 Statistical Analysis

All virus experiments were completed in triplicate with 2 samples each, and reported results are means with standard deviations. Data were analyzed by ANOVA on JMP software (Version 10.0, SAS Institute Inc., Cary, N.C.). Significant differences in least-squares means were indicated by $p < 0.05$.

8.4 Results and Discussion

This study optimized infection strategies for increasing virus titers. Initially, same amount of LLC-MK2 cells were infected with TV at a wide range of MOIs (1×10^{-6} to 1×10^{-2}), and virus was only collected when CPE was observed. During infection, there is a trend that the time course extended as the MOI decreased. A longer incubation time (~120 hr) was required for the lower MOIs, especially at 1×10^{-6}. The highest MOI of 1×10^{-2} resulted in the lowest production of TV of 5.21 log PFU/ml; while MOIs $\leq 1\times10^{-3}$ showed significantly increased titers of TV ($p<0.05$) by 5-10 times, with levels ranging form 5.92 to 6.21 log PFU/ml (Table 1). Interestingly, a wide range of MOI's from 1×10^{-3} to 5×10^{-1} was used to obtain TV stocks for experiments in different laboratories (Li and Chen 2015; Tan et al. 2015; Xu et al.

2015; Wang et al. 2014; Esseili et al. 2015; Cromeans et al. 2014). However, generally the titers of TV obtained were 2-3 log PFU/ml lower than MNV (Tian et al. 2013; Wang et al. 2013; Cromeans et al. 2014; Li et al. 2013). Thus, it is necessary and important to increase TV titers in the laboratory to better investigate huNoV and compare with other surrogates at similar levels.

Table 8.1 Titers of TV (log PFU/ml) obtained from virus proliferation by different MOIs.

	MOIs *												
	1×10^{-6}	2×10^{-6}	5×10^{-6}	1×10^{-5}	2×10^{-5}	5×10^{-5}	1×10^{-4}	2×10^{-4}	5×10^{-4}	1×10^{-3}	2×10^{-3}	5×10^{-3}	1×10^{-2}
Titers of TV (PFU/ml)	5.93 ± 0.54^{A}	5.92 ± 0.19^{A}	6.05 ± 0.38^{A}	6.06 ± 0.20^{A}	6.09 ± 0.39^{A}	6.21 ± 0.39^{A}	6.03 ± 0.55^{A}	5.93 ± 0.55^{A}	6.18 ± 0.35^{A}	5.94 ± 0.57^{A}	5.71 ± 0.31^{AB}	5.72 ± 0.29^{AB}	5.21 ± 0.90^{B}

* Values are means ± standard deviation of three replicates; values with the same following capital letter indicate no significant difference ($p > 0.05$) when comparing titers in infectivity of TV after different MOI treatments.

Previously, to obtain a high level of virus production and gene expression, various methods were considered for insect cell-baculovirus systems (Licari and Bailey 1992; Power et al. 1994; Reid et al. 1995; Wong et al. 1996; Radford et al. 1997; Liebman et al. 1999; Maranga et al. 2003; Zhang and Merchuk 2004; Carinhas et al. 2009). A low MOI (1×10^{-1}-5×10^{-1}) was recommended to produce high levels of virus whereas a high MOI (5-10) was used for foreign gene expression (Merrington et al. 1997; Kollewe and Vilcinskas 2013). Given a certain amount of cell, virus, medium, and cell density at the time of infection and the time of harvest (observation of CPE), the key optimization parameter to obtain high titers is the MOI (Licari and Bailey 1992). Based on prior knowledge, it has been recommended to use low MOIs to efficiently produce higher amounts of both recombinant virus and proteins (Wong et al. 1996; Yamada et al. 2009). It may be that the infection process is synchronous at higher MOIs (>1 plaque forming unit per cell) given that all cells are infected immediately. Cells can barely grow due to the immediate infection; however, at lower MOIs, only a portion of cells is initially infected (primary infection), which allows the remaining cells to continue to grow and proliferate. With multi-infection cycles, those cells only get infected at a later point (secondary, tertiary, etc. infections) when the initial infected cells release the progeny viruses. It has been shown that low MOIs permit cell growth (Reid et al. 1995; Liebman et al. 1999; Radford et al. 1997), which may result in higher viral titer when compared with high MOIs. Xu et al., found that released TV virions were only detected after 24 hr post infection at an MOI of 2 (Xu et al. 2015). It is likely that infected cells may stay intact for a longer time period (>24 hr) and accumulate more intracellular virions before lysis with even lower MOIs (<1). The problems associated with defective interfering particles from high MOI virus

stocks at high passage numbers may also be prevented with low MOIs (Reid et al. 1995). We recommend that in order to efficiently produce higher titers of TV scientists reduce the MOIs, which at the same time give a 10^2 to 10^5 reduction in the demand for virus stock.

8.5 Acknowledgements

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Appendix D

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