MODELING OF NOROVIRUS TRANSMISSION DURING SLICING AND QUANTITATIVE RISK ASSESSMENT OF HUMAN NOROVIRUS TRANSMISSION IN A FOODSERVICE SYSTEM

by

Di Li

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and approved by

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ABSTRACT OF THE DISSERTATION

Quantitative Risk Assessment of Human Norovirus Transmission in a Foodservice System

By Di Li

Dissertation Director:
Dr. Donald W. Schaffner

Human norovirus is the leading cause of outbreaks of acute non-bacterial gastroenteritis worldwide. Recent epidemiological evidence indicated that preparation of fresh produce for use as ingredients in ready-to-eat food in commercial settings has been a significant source of the norovirus infections in the United States.

In this dissertation, to help understand the cross-contamination of norovirus during preparation and service of fresh produce product in foodservice systems, we analyzed spread pattern of norovirus from a single tomato to many others via the use of a commercial slicer. Murine norovirus (MNV) was used as a surrogate. A non-linear regression equation was generated: $y = -0.903^* \ln(x) + 7.945$, ($R^2 = 0.91$), where y = log MNV per slicing and x = tomato number. The MNV levels transferred generally decreased as the number of tomatoes sliced increased, with some exceptions. Infrequent but erratic transfers, where the MNV level of a subsequent tomato was higher than that of a preceding tomato, occurred in later transfer of some trials. This study illustrates the complex nature of risk prediction associated with norovirus cross-contamination during food preparation in commercial establishments.

We also developed a simulation model to quantify the overall effect of norovirus cross-contamination in a food service establishment. For each possible source of initial contamination, using a manual tap versus a hands-free faucet were studied at 0, 30, 50, 70, and 100% handwashing compliance levels to check the number of salads and employees that may end up carrying more than 10 norovirus. When a lettuce/tomato was the initial source of contamination, change of knife and cutting board/slicer every 100 lettuce/tomato units was simulated. Change of tongs after preparation of every 100 salads was also simulated when a manual tap was used. Our model provides valuable information that can be considered for the control of NoV outbreaks. The results from our study suggested that multiple factors should be considered to control the spread of NoV, and that no one or even multiple combinations of factors will completely control NoV transmission risk.

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DEDICATION

This dissertation is lovingly dedicated to my parents, for their constant love and encouragement. Without their continuous support and counsel I could not have completed this process.

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1.1 History of Human Norovirus

Human norovirus (NoV), also known as Norwalk-like virus (NLV) and small round structured virus (SRSV), belongs to the genus *Norovirus* in the family of Caliciviridae (*63*). The earliest description of human NoV infection can be traced back to 1929 when acute non-bacterial gastroenteritis was first described by Zahorsky and known as "winter vomiting disease", because the outbreaks typically peaked during the winter months (*192*). At that time, the causative agent had not yet been identified (*58*, *75*). In 1968, an outbreak of acute non-bacterial gastroenteritis occurred at an elementary school in Norwalk, Ohio, US and it was reported that the primary infection was among teachers and students with an attack rate of 50%. The secondary infection was among family members with an attack rate of 32%. Although numerous initial attempts were made, no specific pathogen was identified until 1972, when Kapikian et al., applied a relatively novel technique, immune electron microscopy, to stool filtrates collected from a volunteer who was experimentally inoculated with a fecal sample from the original outbreak (*90*). In this study, a 27-nm viral particle was observed and following serological evidence from experimental and natural infections, the prototype NoV species, Norwalk virus, was determined to be the causative agent of the outbreak of acute gastroenteritis. This was also the first time a virus had been identified as the aetiological agent of gastroenteritis in humans. In 2002, the name "Norovirus" was approved by the International Committee on Taxonomy of Viruses (ICTV) (*84*).

1.2 Norovirus Classification

1.2.1 Taxonomy

NoV belongs to the family Caliciviridae (*137*). The word "calici" is derived from the Greek calyx, meaning cup, referring to the cuplike depressions seen on the surface of small virions of 27-40 nm (*64*).

According to ICTV(*84*), there are five genera in the Caliciviridae family including *Norovirus*, *Vesivirus*, *Lagovirus*, *Sapovirus*, and *Nebovirus* (*Becovirus*). Both human NoV and murine norovirus (MNV) are found in the genus *Norovirus* and feline calicivirus (FCV) is found in the genus *Vesivirus*. Both norovirus and sapovirus can cause acute viral gastroenteritis in human adults. Additional genera have been proposed including *Recovirus*, which can infect rhesus monkeys (*51*), and *Valovirus*, which can infect swine (*110*). All genera in Caliciviridae family can infect animals.

NoV is a non-enveloped, positive-sense single-stranded RNA (where positive-sense means the viral RNA genome can be directly translated by the host cell to make viral proteins) virus possessing a genome of 7.4-7.7 kb which is packaged into a small naked icosahedral virion of 27 to 32 nm in diameter (*6*, *61*, *90*, *191*). An aggregate virus particle may contain hundres of genome copies (*172*).

Because an in-vitro cell culture system of human NoV is not yet available, classification of NoV is difficult to perform using methods such as serotyping and no uniform classification system for NoV currently exists (*193*). Currently, classification of NoV is based on phylogenic grouping of complete open-reading frames (ORFs) sequences (*64*, *99*).

The NoV genome contains three ORFs: ORF1, ORF2 and ORF3. ORF1 encompasses around 2/3 of the NoV genome, and encodes a 200 kDa polyprotein, which undergoes proteolytic cleavage mediated by the virus-encoded "3C-like" proteinase, located in the

upstream of the RNA-dependent RNA polymerase. The proteolytic process is rapid, co-translational and results in the production of six non-structural proteins (*116*, *169*). ORF2 is about 1.8 kb in length and encodes the major capsid protein (viral protein 1, VP1) of 60 kDa, which is responsible for capsid related functions, including capsid assembling and formation, host interactions and immunogenic reactivity of the virus (*35*, *87*). VP1 capsid can be divided into two domains: the S domain, which includes the N-terminal within the capsid and the intermediate shell, and the protruding P domain (*147*). The P domain can be further sub-divided into the P1 and P2 sub-domains. P2 sub-domain is highly variable and most of the cellular receptor interactions and immune recognition epitopes are thought to be located in this sub-domain (*33*, *141*). ORF3 is about 0.6 kb in length and encodes a 25 kDa small basic structural protein (viral protein 2, VP2) involved in up regulation of expression and stability of VP1 (*14*, *64*, *162*) and prevents disassembly of VP1. It has been reported that VP2 protein is involved in the formation of infectious viral particles (*5*, *94*). A 42 to 78 nucleotide non-translated region is located in the downstream of ORF3 and attached to a polyadenylated tail (*64*). Based upon VP1 amino acid sequences, NoV can be divided into five separate genogroups: GI, GII, GIII, GIV, and GV (*193*).

Norovirus genogroups, GI, GII and GIV, are associated with human gastroenteritis (*64*, *193*). NoV GII also contains porcine strains. The most commonly identified strain in both outbreaks and sporadic settings is genotype 4 in genogroup II (GII.4). GV contains murine strains (*193*). More recently, NoV has been detected in a wide range of animals including lion, dog, sheep, pig, and cow (*117*, *130*, *131*, *180*, *187*). The norovirus strains that infect

sheep and cows belong to genogroup III, and the strains that infect lion and dog belong to genogroup IV.

The NoV genogroups can be further delineated into more than 36 genotypes, or clusters (*101*, *193*). The capsid nucleotide sequences of the NoV strains vary from 31.3 to 52.0% with an average of 45.8% between the five genogroups and divergence is typically 15% within the same genogroup (*193*).

Due to the lack of the mammalian cell culture system for NoV, two animal caliciviruses: FCV and MNV have been commonly used as surrogates in laboratory studies for NoV because they can be grown in cells outside the host (*2*, *25*, *37*, *44*, *78*, *134*). Before the discovery of MNV, FCV was the most commonly used surrogate in human NoV study. Different from human NoV, FCV is not an enteric virus but a respiratory virus. FCV has similar resistance to dehydration as human NoV, however, the suitability of FCV has been questioned due to its poor stability and susceptibility to low pH, organic solvents, elevated temperature, and inability to persist on environmental surfaces for extended periods of time (*25*). MNV is a natural enteric pathogen of mice and is more resistant to organic solvents and more tolerant than FCV to both high and low pH values (*25*) (Figure 1.1). Generally, enteric viruses are resistant to low pH and it has been reported that NoV can cause infection in humans after a three hour incubation at pH 2.7 (*41*). Therefore, the capability to survive at a low pH environment makes MNV a more suitable surrogate for human NoVs compared to FCV.

1.2.2 Norovirus GII: the Predominant Genogroup Lead to Foodborne Outbreaks

Although all three NoV genogroups, GI, GII and GIV have been indicated in acute gastroenteritis outbreaks, NoV GII is the predominant cause of NoV outbreaks (*106*). It

was reported that of 217 NoV fecal samples received by CDC between 1997 and 2000, GII was responsible for 73% of all reported NoV outbreaks (*50*). GII.4 is the most commonly reported cluster associated with NoV outbreaks since 1995. During the past decade, new GII.4 strains have emerged every 2 to 3 years, replacing previously predominant GII.4 strains (*32*). In the United States, the NoV strain US95/96 was first recognized in the mid 90s and then identified as the predominant strain type during the next few years. From 2000 to 2004, the Farmington Hills strain took the place of the US95/96, and became the dominant stain. Since 2006, the GII.4-2006a ("Laurens-like" strain) and GII.4-2006b ("Minerva-like" strain) variants became dominant (*166*, *194*). In 2009, the New Orleans strain emerged and replaced the previously dominant strain, but did not spark an increase in NoV outbreaks. In March 2012, GII.4 Sydney, a new strain of NoV which was first detected in Australia, appeared and has caused acute gastroenteritis outbreaks in multiple countries since then (*178*). GII.4 Sydney has been spreading nationwide in the US and is currently the leading cause of NoV outbreaks. More than 140 outbreaks in the United States have been reported caused by the Sydney strain since September 2012, and a statistically significant increase in the proportion of outbreaks caused by GII.4 Sydney was noted (*32*): from 19% in September to 46% in October and 58% in November 2012. Among the 141outbresks, 72 outbreaks were caused by direct person-to-person transmission, and 29 outbreaks were foodborne.

1.2.3 Murine Norovirus

In 2003, murine norovirus, in NoV genogroup five, which can cause severe disease in immuno-compromised mice was identified by Herbert W. Virgin's group at Washington University (*93*). MNV shares many molecular and biological properties with human NoV.

MNV can be cultured in a mouse macrophage and dendritic cell line. The development of the in vitro cell culture system for the cultivation of MNV-1 provided the first small animal model to understand the biology and pathogenesis of human NoV (*186*). Similar to human NoV, MNV genomes are continually evolving. To date, numerous strains of MNV have been isolated and sequenced (*12*, *80*, *168*, *173*). Compared to the relatively low (46%) nucleotide identities in human NoV, MNV strains are related to each other with full-length nucleotide identities ranging from 87.0 to 94.1% (*95*).

1.3 Survival, Stability, and Inactivation of Norovirus

1.3.1 Survival and Stability

Norovirus is extremely stable. NoV can survive over a wide range of temperatures (-20 °C to 60 °C), and it has been reported that it is minimally affected when stored at refrigeration temperature (4 °C) (*25*, *44*, *46*). NoV is acid tolerant and shows resist low pH (gastric pH levels). NoV can also survive in relatively high concentration of chlorine, up to 10 ppm (*144*). Additionally, it has been reported that NoV may survive up to 12 days on a contaminated carpet (*34*, *49*).

Several studies have been conducted to investigate the survivability of NoV under different temperatures and pH conditions. Clay and co-workers (*37*) investigated the survival of NoV on various surfaces such as telephone buttons, telephone receivers, wires, computer mouse, keyboard keys, and brass using FCV as the surrogate. The results showed that at room temperature, FCV can survive on telephone buttons and receivers for two to three days. D'Souza and co-worker (*45*) found that FCV could survive up to seven days at room temperature on surfaces of stainless steel, formica, and ceramics. Figure 1.2 shows

the time to 1-log reduction of FCV on environment surfaces using data collected from Clay and D'Souza's studies.

1.3.2 Norovirus Inactivation

The environmentally stable NoV is resistant to most of the commonly used cleaning agents and multiple chemical agents, and there are not many effective conventional cleaners against it (*44*, *46*, *82*, *88*, *174*, *177*).

The quaternary ammonium compounds (QAC), soluble in both water and oil-based media, are widely used as disinfectants. However, studies have shown that quaternary ammonium compound disinfectant cleaners were not highly effective against FCV (*44*) even at higher concentrations, up to twice of the manufacturer's recommendation.

Study on the effect of chlorinated cleaning products against NoV showed that 3,000 ppm (or higher) concentration of sodium hypochlorite was needed to completely inactivate (> 5 log10) FCV in suspension at room temperature for 30 minutes (*46*), and no infectious virus was detected after exposure to a concentration of 5,000 ppm hypochlorite (*44*). Both pH and temperature can influence cleaning capacity (*174*).

Heat is another common way to inactivate pathogens. When feline calicivirus was exposed to a temperature of 63°C or greater, a rapid reductions of infectious virus was detected within the first few minutes (*25*, *44*, *46*) and no virus was recovered after exposure to 70 °C for 5 minutes (*44*). While FCV can be inactivated after boiling for one minute, steaming shellfish may not completely inactivate the virus and prevent NoV infection (*98*). Data collected from published studies showed a linear rrelationship between log time for a decimal reduction (seconds) and temperature (°C) (*9*, *25*). The higher the temperature, the less the time was required to reach a 1 log MNV-1 reduction (Figure 1.3).

Ethanol is commonly used in laboratory settings for disinfection. However, ethanol alone has so far proven to be an inadequate disinfectant against NoV (*44*, *46*). Most hand treatments are more effective against NoV when applied for a longer time (104) (Figure 1.4). Effects of different antimicrobial compounds vary with their compositions as well as the items they were applied to (68) (Figure 1.5).

1.3.3 Detection of Norovirus

Immune Electron Microscopy

NoV was first visualized using immune electron microscopy. Immune electron microscopy enables the identification of NoV by their characteristic morphology, however, the technique is insensitive (requires at least 10^6 viral particles per ml of stool for visualization) and time consuming for routine test of enteric viruses in stool specimens collected for investigations during an outbreak (*40*).

Enzyme Linked Immunosorbent Assay

An enzyme linked immunosorbent assay (ELISA) has been developed for the detection of NoV antigen in stool samples. Compared with the immune electron microscopy method, ELISA is more efficient and can be used for screening a large number of samples. However, the low sensitivity (the ability to detect a pathogen when a pathogen is present) between 44 and 59% of ELISA assay has limited its use for detection of NoV and diagnosing outbreaks due to the antigenic diversity among different NoV strains (*60*).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Because human NoV cannot be cultured in routine cell lines, the development of sensitive diagnostic assays has been hampered. The appearance of RT-PCR amplification has greatly improved and facilitated sequencing and genome characterization of NoV

strains (*95*, *105*, *119*, *191*). This molecular detection method uses a two-step variation of PCR. It first converts RNA to complementary DNA (cDNA) from messenger RNA (mRNA) using deoxynucleotide triphosphates (dNTPs) and enzyme reverse transcriptase (RT). This is followed by PCR which allows the amplification of the cDNA. RT-PCR can be used to test for the presence of NoV in both environmental samples such as surface swabs, foods, and water as well as clinical samples such as stool and vomit samples. With RT-PCR, identification of NoV is best made from stool specimens taken two to three days after onset of symptoms, although good results can still be obtained on samples taken as long as five days after illness. RT-PCR is able to detect NoV from samples of up to two weeks after patient recovery and even longer (*121*).

Due to the great amount of genetic diversity exists among NoV clusters (*95*), the diagnosis of NoV infection in humans still remains a challenging task since no single virus-like particle can be used to detect all strains of NoV circulating in humans. RT-PCR offers the most information; however, it is expensive and labor-intensive.

Immune electron microscopy requires a sample with high virus concentration. ELISA assays are relatively inexpensive and quick but much less sensitive. Therefore, RT-PCR has remained the most reliable means for detection of NoV and diagnosis of NoV infection as it offers the possibility of detecting a low quantity of virus and is the most sensitive routine method used (*19*, *22*, *148*, *153*). There are hardly any fast and efficient methods that can be applied outside research laboratory settings, and the progress in detecting and managing outbreaks of disease caused by human NoV is often delayed. Currently, clinical and epidemiologic criteria are widely used for NoV outbreaks (*7*).

1.3.4 Clinical Manifestation, Susceptibility, and Immunity of Human Norovirus Infection

Epidemiology

NoV is the leading cause of outbreaks of acute non-bacterial gastroenteritis worldwide (*48*). It is estimated that human NoV causes more that 20 million acute gastroenteritis, 70,000 hospitalizations and 800 deaths annually in the United States (*27*). Outbreaks of NoV commonly take place in areas of close human contact which facilitate the spread of the virus, such as restaurants, nursing homes, hospitals, schools, day care centers, vacation settings, and cruise ships (*47*, *50*, *52*, *62*, *85*, *92*, *128*). Since the late 1990's, global epidemics of NoV outbreaks have been caused by a dominant genotype, GII.4 (*21*, *123*, *181*, *182*). Almost 40% of all NoV outbreaks occurred in restaurant settings, most often due to poor hygiene practice and cross contamination (*143*, *144*). NoV infections occur throughout the year, with a predominant seasonal occurrence of outbreaks during the winter months (*108*, *139*). Cases of NoV infections peaking during the spring have also been reported (*62*, *76*, *139*).

Clinical Picture of Human Norovirus Infection

Clinical Manifestation and Treatment

NoV associated gastroenteritis is characterized mainly by clinical symptoms of projectile vomiting and non-bloody diarrhea, and often accompanied with variable systemic symptoms including nausea, fever, headache, chills or muscle pain (*1*, *42*). Typically, symptoms of NoV infection last for 12 to 72 hours, with an incubation period of 24 to 48 hours after exposure (*91*), although presentation of symptoms may be prolonged in some cases and infection may progress to chronic norovirus infection, particularly in the

elderly, young children or immune compromised patients (*97*, *108*, *122*, *140*, *141*, *154*). In rare occasions, infections may be lethal due to serious dehydration (*160*). Asymptomatic infections after experimental inoculations have also been described (*59*).

Kaplan and Feldman proposed clinical and epidemiologic criteria for the identification of outbreaks caused by NoV infection in absence of laboratory detection, which includes the following four clinical features: 1. Vomiting in more than half of symptomatic cases; 2. Mean (or median) incubation period of 24 to 48 hours; 3. Mean (or median) duration of illness of 12 to 60 hours and, and 4; No bacterial pathogen isolated in stool culture (*91*). Currently, the Kaplan criteria are still the most useful and discriminating diagnostic tool for the identification of NoV associated foodborne outbreaks (*176*).

Gastroenteritis caused by NoV infection in immune-competent patients is generally mild, self-limiting, and no specific therapy is required other than rehydration (*121*). In severe cases of NoV infection, administration of an oral fluid and electrolyte treatment is often necessary to replace the loss of fluids (*170*). To date, there is still no vaccine to prevent this illness.

Human Susceptibility to Norovirus Infection

NoV is extremely infectious. Teunis et al. reported a low ID_{50} (the number of pathogens at which 50% of a population will be infected) of less than 20 virus (*172*). NoV can infect patients of all ages. It has been indicated that usceptible populations, such as elderly people (> 65 years), children (< 5 years) are more vulnerable to NoV infection and may suffer from more severe disease than healthy individuals (*135*).

Immunity of Human Norovirus Infection

The nature of the host immunity associated with NoV infection is not yet clear. Volunteer challenge studies were conducted to investigate the host immune responses to NoV infection. Volunteers were challenged by oral immunization of bacteria-free fecal filtrates containing infectious virus (*133*, *145*). Not all volunteers were symptomatic. According to challenge studies, some degree of short term immunity appeared to be present, and was strain specific (*133*, *145*, *190*). Infections were not induced in volunteers who became ill after initial challenge, when rechallenged with homologous virus 6 to 14 weeks later. However, long term immunity was suspected since individuals who were symptomatic after initial challenge became ill again when rechallenged 27 and 42 months later (*133*). More interestingly, individuals who did not develop symptoms of NoV gastroenteritis remained asymptomatic, which indicated an inherent natural resistance to NoV infection (*11*, *65*, *145*). It is still not clear yet what role antibodies play in regard to the prevention and resistance of NoV infection. Most symptomatic individuals experienced increased serum antibody titers after each challenge and were more susceptible to infection than individuals who had a non-detectable or had low levels of serum antibodies after challenge with the same strain (*11*, *65*, *145*). This indicates that factors other than serum antibodies appeared important for NoV immunity since increased antibody titers did not offer protection.

Human histo-blood group antigens (HBGAs) are complex carbohydrates linked to glycoproteins or glycolipids on the cellular membranes of red blood cells and epithelial cells of the gut and secreted bodily fluids such as saliva (*107*, *151*). According to NoV outbreak investigations and human challenge studies, HBGAs have been indicated as the receptor for NoV infection and play an important role in human susceptibility to NoV

infection (*73*, *81*, *83*, *115*, *155*). Individuals who express HBGAs in saliva and mucosa (*127*) carry a gene encoding a functional alpha-1, 2-fucosyltransferase (FUT2) are termed secretor-positive. The FUT2 gene encodes a fucosyltranferase responsible for generating the H type 1 and H type 3 antigens from disaccharide precursors, which are expressed on mucosal surfaces and have been shown to bind NoV VLPs (*127*). Secretor-negative individuals who do not show expression of the FUT2 gene are rarely infected by any genotype of NoV. Saliva binding studies have demonstrated that different NoV strains exhibit different HBGAs binding patterns (*26*, *165*). NoV GII.4, the global dominant strains, has the broadest binding range and can bind to all secretor-positive individuals, regardless of their ABO blood group phenotypes, but not to secretor-negative individuals (*69*, *81*). NoV GII strains have also been shown to bind to the saliva of secretor-negative individuals (*114*, *165*), which may contribute to the higher prevalence of NoV strains in GII. One foodborne outbreak with a secretor-independent susceptibility pattern was described recently (*142*).

Overall, the lack of sustained immunity gained after NoV infection could be due to the great mutation rate and high genetic variability within human NoV strains and genogroups (*100*, *144*). It was reported that a single amino acid change in the antigenic region (P2 domain) of the MNV capsid protein was sufficient to avoid immune neutralization (*118*). In human NoVs, amino acid divergence at key antigenic motifs within the capsid VP1 can be seen between pandemic GII.4 variants (*112*, *113*, *167*). Infection with NoV of one genotype may not provide immunity against another genotype or even variants within the same genotype (*70*, *113*, *167*). The capsid protein evolves over time by antigenic drift,

which allows repeated infection of previously exposed individuals (*112*, *113*), and thus contribute to epidemic potential.

1.4 Spread of Norovirus

Contamination of food items can occur at any time point from original production in the farm to the point of being served on table for consumption. Foods can be contaminated with pathogenic microorganisms through cross contamination such as: washing with contaminated water, preparation using contaminated utensils, contact with contaminated environment surfaces, and poor personal hygiene.

1.4.1 Cross-Contamination

Cross-contamination occurs when bacteria and viruses from one surface are transferred to another, such as transfer between contaminated hands, produce, knife, cutting board etc. Cross-contamination plays a significant role in transferring harmful pathogens to food product. According to the World Health Organization (WHO) (*188*), 25% of foodborne outbreaks are associated with cross-contamination events. Foods that require minimal processing before consumption are more likely to spread pathogenic bacteria and viruses around once contaminated (*66*). Preparation of multi-ingredient foods (e.g. salads and sandwiches) may have more chance of cross-contamination due to increased number of manipulation steps. Recontamination of processed food after final preparation has also been highlighted as a source of pathogenic microorganisms (*152*). According to an investigation by Rangel and coworkers, about 50% of produce-associated *E. coli* O157 outbreaks were caused by kitchen level cross-contamination during 1982-2002, and lettuce alone accounted for 34% of produce outbreaks (*150*). Fresh tomatoes are commonly linked to salmonellosis outbreaks in the US (*53*) and approximately 42,000 laboratory-confirmed

cases of salmonellosis are reported each year in the United States (*158*). It has been observed that *Salmonella* could spread from a contaminated tomato to an uninoculated tomato during washing procedures (*149*), and can be transferred from surface to flesh during cutting (*111*). NoV can be transmitted through person to person contact, fecal-oral cross contamination, consumption of contaminated food or water, and airborne droplets of vomitus (*102*, *128*, *156*). A review of more than 800 foodborne outbreaks associated with infected foodhandlers showed that 33% were associated with NoV (*67*).

1.4.2 Norovirus Transmission

Person to Person Transmission

Person to person transmission is the most common spread mode of NoV in outbreaks (*4*, *16*, *29*). Generally, NoV is spread from person to person either by the fecal-oral route or through aerosolized vomitus from projectile vomiting (*121*).

Numerous NoV outbreaks have been associated with foodhandlers who were ill or freshly recovered (*126*, *185*). Barker et al., found that contaminated fingers could sequentially contaminate up to seven additional clean surfaces without reinoculation of the fingers (*10*). NoV infection features a high frequency of vomiting in more than 50% of the cases. When a projectile vomiting occurs, NoV can be transmitted over distances easily through aerosolized vomitus (*121*). Therefore, the airborne transmission route plays an important role in person to person spread. A study of a NoV outbreak in a hotel restaurant reported that people who were seated at the same table had the highest attack rate of NoV infections, and the attack rate of individuals who were seated at the adjacent table to the index person could be more than 70%. The attack rate was proportional to the distance between the previously healthy diner and the ill individual (*128*).

Foodborne and Waterborne Routes

Many NoV outbreaks have been reported due to the consumption of contaminated food, such as shellfish, vegetables, and frozen fruits (*36*, *77*, *109*, *120*, *132*, *138*, *159*), and water, including drinking water, ice, and water in swimming pools (*29*, *43*, *46*). NoV particles are highly resistant and can still be present in high levels in water after various treatment processes (*39*, *72*). Bivalve molluscs (oysters, mussels, and clams) are filter feeders. When their living environment is polluted with NoV, bivalve molluscs are susceptible to picking up, accumulating and retaining virus particles in their gut from water (*171*, *175*). Contaminated bivalves, if consumed without proper cooking, can lead to NoV infection. Using contaminated water for irrigating or washing has also been reported to cause contamination of fruits and vegetables (*125*, *136*).

Environmental Contamination

A NoV particle is highly persistent in the environment and can maintain its infectivity for months (*34*). During an outbreak, NoV particles can be transferred from one object to another in a chain of relocation events. Door handles and food preparation surfaces often appear to be the areas with high contact frequency (*37*). Projectile vomiting can spread virus far away and cause contamination of a large area. If a contaminated surface or object is not identified and cleaned, it can serve as a virus reservoir and may cause an outbreak (*49*, *103*, *179*).

NoV outbreaks are often the result of a mixture of more than one mode of transmission (*121*). Contaminated hands can facilitate the spread of virus to previously uncontaminated surfaces. A study on the spread of NoV has shown that NoV can be transferred from a contaminated source to clean hands and then to other surfaces, such as telephone receivers

and door or tap handles (*10*). Once present on an object or surface, the environmentally stable virus particle can be spread by multiple routes to the next individual. Asymptomatic shedding after the recovery (up to 2 weeks) of patients provides further opportunity for the spread of pathogenic virus (*8*, *154*).

1.4.3 Handwashing

Foodhandlers, who have direct contact with food items, can pass pathogens easily to food during preparation. Good hygiene practices are critical to reduce the spread of foodborne pathogens and hands are one of the major vehicles for cross-contamination to Ready-to-eat (RTE) foods. Effective handwashing can decrease the risk of pathogen transmission and may therefore help to prevent the spread of pathogens in a food preparation environment (*86*).

A food safety behavior study conducted among young adults showed that 60% of all participants washed their hands before preparing food but only 16% performed as recommended by FDA Food Code (*23*). In this same study, only 40% of participants washed their hands with soap and water after touching raw food and before handling RTE produce during food preparation (*23*). Foodhandlers are usually trained in the principles of food hygiene and food safety; however the training does not always translated into good hygiene practices (*79*). Adequate hand hygiene practice was observed in only 14% of participants from 29 catering operations, where “adequate” was defined based on related industry standards and guidelines (*38*). The type of hand wash facilities (automated handwashing vs. manual) may influence the efficacy of virus reduction, and an automated system could prevent cross-contamination between hands and spigots, which may happen during a manual handwashing procedure. Hand drying, the last stage of the handwashing

process, can help further reduce the likelihood of pathogen transmission. Different hand drying methods such as hot-air dryers, paper towels and cloth towels, as well as different designs of hand drying equipment (e.g. paper-towel dispenser types, hot-air dryer speed), work differently on pathogen reduction (*74*, *79*).

Experiments conducted by Bidawid et al., showed that touching food items with virus contaminated finger pads for 10 seconds resulted in a transfer rate of 46, 18, and 13% of the virus to ham, lettuce and stainless steel, respectively (*15*). The number of viruses transferred from finger tips were reduced to less than 5% by rinsing with water, washing with liquid soap and water, and with 62% or 75% alcohol-based hand rubs (*15*). This study revealed that a high level of viral transfer may occur between heavily contaminated fingers and surfaces, and that handwashing can reduce the level of cross-contamination from hands. However, no further information on the secondary transfer of viruses from the "now-contaminated" food items to other food items or on the extent of cross-contamination throughout the subsequent food preparation steps was reported.

1.5 Norovirus Outbreaks and Sporadic Cases

According to CDC, an outbreak of NoV is defined as an occurrence of two or more similar illnesses resulting from a common exposure (*28*). Human NoV is currently the leading cause of non-bacterial gastroenteritis (*25*) and NoV outbreaks and sporadic cases are estimated to cause about 21 million cases of acute gastroenteritis, which includes 800 deaths and 70,000 hospitalizations each year in the United States (*27*). In a study of more than two thousand acute gastroenteritis outbreaks due to cross contamination by person to person contact reported during 2009-2010, 89% were suspected or confirmed to be caused solely by NoV (*184*). NoV outbreaks typically occur in settings where a large number of

people are present in close proximity, such as restaurants, cruise ships, schools, and healthcare institutions (*18*, *24*, *189*). Statistical data from January 1996 through November 2000 showed that 39% of reported NoV outbreaks occurred in restaurants (*143*, *144*). Generally, most of NoV outbreaks can be explained by the following five factors: 1) relatively low median infection dose (ID_{50} =18 viruses) coupled with its high concentration in stool of infected humans; 2) environmental stability; 3) multiple transfer routes; 4) prolonged asymptomatic shedding period; and 5) lack of long term immunity (*13*).

According to a recent study, during 2000 to 2008, NoV alone has caused nearly 5.5 million cases of foodborne illness, which represented 58% of the cases attributed to known agents, (*158*). Meanwhile, a great proportion of the estimated 38 million gastroenteritis cases of unknown etiology may also be caused by NoV (*157*). A wide range of food types have been indicated as vehicles of infection, including shellfish, fruits, salads, meat, fruit, soups, and bakery products (*55*, *57*, *183*). CDC has suggested carefully washing fruits and vegetables, and cooking shellfish thoroughly to prevent NoV infection, however, washing is of limited effectiveness and foods handled after washing or cooking can still become contaminated. Therefore, great attention should be paid to food items such as salads that are typically eaten raw, and sandwiches, which have no further cooking after handling (*121*). Food handlers play an important role in virus transmission, and food handlers who were sick prior to or during preparation of the implicated food have been identified in many foodborne outbreaks (*121*).

An outbreak of NoV at a wedding reception in the U.K., where 55 of the 111 guests became ill with gastroenteritis, was linked to a kitchen assistant who suddenly became ill on the eve of the reception. The sick employee vomited into a sink that was used the next

morning for preparation of potato salad after cleaning and disinfection with a chlorine-based disinfectant. The sink was identified as the vehicle of infection. This event highlights the persistence of NoV and its resistance to disinfectants (*146*). In 2000, a multi-state NoV outbreak in the US caused at least 333 infection cases across 13 states after consuming catered salads prepared by infected food handlers (*3*). In a 2004 outbreak, two groups of people and some individuals who had dined at a restaurant on the same evening in Florida became infected with NoV due to the consumption of a salad dish. Lettuce, which was used as an ingredient for the salads, was handled by a pre-symptomatic food worker, who was implicated as the source of the NoV contamination by the subsequent survey (*96*). In 2005, a multisite NoV outbreak occurred in a franchise restaurant. More than 125 customers were sick after consumption of party-sized submarine sandwiches. Later investigation showed that an asymptomatic lettuce-chopper, who prepared lettuce for sandwiches, was ill with NoV symptoms on the previous day and came back to work right after recovery. The sink used for washing and preparing lettuce was also used for handwashing, and no sanitizing step was performed before or after washing lettuce, which may have provided the opportunity for cross-contamination (*30*). Most NoV foodborne illness outbreaks have been caused by direct contamination of food by a food handler and these outbreaks highlight the ease of human NoV transmission from infected food handlers to RTE foods.

GII.4 is the dominant genotype found in most of NoV outbreaks. It accounts for approximately 60-90% of reported cases of NoV gastroenteritis (*20*, *56*, *161*). This may be due to its high biological fitness (*20*), the large variety of receptor specificity (*165*), and its' high mutation rate (*167*). During the past decade, a new GII.4 strain has evolved every two

or three years, and replaced the previously predominant strain. The new dominant strain - GII.4 Sydney, which was first identified in Australia in March 2012, spread rapidly and has caused acute gastroenteritis outbreaks in multiple countries (*178*). Human NoV outbreaks caused by this new strain have been increased from 19% in September to 58% in December 2012 in the US (*32*).

NoV is difficult to detect in food matrices. Although, numerous research studies have been conducted attempting to seek efficient detection method for NoV, no single detection method is sensitive to all NoV strains, and there is still no ideal way for routine testing and monitoring for local regulatory laboratories to date (*71*, *164*).

Human NoV outbreaks not only put a health risk for infected patients, but also cause a great financial burden. For instance, the cost of a large outbreak of NoV infection in a tertiary care hospital in Maryland in 2004 was estimated over $650,000, including total lost revenue of more than $400,000, and sick leave, cleaning, and supplies replacement as a result of the outbreak for about $240,000 (*89*). It has been suggested that the best ways to reduce the risk of NoV transmission in foodservice settings is to: perform good hygiene practices including thorough and frequent hand washing, regular cleaning of food contact surfaces, disposal or disinfection of contaminated materials, exclusion of affected staff from work until 48 h after the cessation of symptoms, and the closure of affected sites to limit the spread of infection until thorough disinfection is properly performed (*31*). Enhanced caution, monitoring, education, and regulation could be beneficial and helpful for reducing the incidence of NoV outbreaks. More information is needed to understand the cross-contamination of human NoV through the kitchen environment.

1.6 Risk of Norovirus infection in Food Service Systems

In recent years, NoV has drawn lots of attention from policy makers and researchers due to the high prevalence of NoV-related outbreaks. NoV has been identified as a major cause of acute gastroenteritis outbreaks worldwide. CDC reported that NoV accounts for about 90% of acute gastroenteritis outbreaks caused by person-to-person transmission with reported etiology (*27*).

Many studies have been conducted to develop effective and reliable methods to recover and detect the virus from food and stool samples and to determine its survival and transmission. However, limited work has been done on the spread of NoV in an integrated foodservice system.

In a foodservice system, food handlers' hygiene practices play an important role in pathogen transmission and cross-contamination. Food items and food contact surfaces can be easily contaminated during preparation. Most frequently, foodborne NoV outbreaks are due to contamination caused by NoV-infected food handlers and their poor hygiene practices (*124*). Because of its environmental stability and multiple transmission schemes, human NoV, once in the system, can spread rapidly and make transmission difficult to control unless it is recognized early and effective interventions are taken. A single vomiting incident following NoV infection may produce 30 million viral particles (*129*). Food preparation areas can easily become contaminated by transfer of fecal matter via hands or vomitus if infected personnel were not kept away. The extended asymptomatic viral shedding duration after recovery may silently increase the risk for NoV to spread if personal hygiene practices, such as handwashing after using the bathroom or before handling food items, are not strictly performed at all times.

Ready-to-eat foods such as salads, sandwiches, and deli foods are at greater risk of NoV transmission than other cooked foods (*183*) because considerable human contact is required during preparation, including handling, chopping/slicing and mixing, and the lack of an intervention step prior to consumption. Semi-liquid foods such as salad dressings may further accelerate the spread of NoV during mixing. Fresh produce, such as lettuce, tomatoes, baby spinach and onions, are frequently used ingredients for RTE foods and have often been implicated as the source of contamination in many NoV outbreaks. Contaminated cold cut meats, cut fruit and ice have also been reported (*17*, *163*). The issue of NoV contamination of fresh produce was noted in the 2007 International Lettuce and Leafy Greens Food Safety Research Conference report on food safety research priorities for lettuce and leafy greens. Academic scientists, state and federal regulators and industry representatives from all over the world were invited and asked to develop a prioritized list of research need to address human pathogens in and on lettuce and leafy greens. A number of research needs were identified and two points in particular are of relevance to this study: 1) evaluate the risk of NoV contamination in lettuce and leafy greens at retail and in foodservice; 2) determine pathogen-food contact surface transfer coefficients for postharvest and fresh cut produce handling (*54*).

To help understand the cross-contamination of NoV during preparation and service of fresh produce product in foodservice systems, it is important to understand the characteristics of NoV transmission between different foods and food contact surfaces as well as food handling behavior among food workers. The availability of such information is important for quantitative microbial risk model development. Experiments on the transfer of norovirus from a single tomato to many others via the use of an 11-horizontal

blade slicer were conducted to investigate the dissemination of NoV and a model was generated on the number of virus transferred to each sliced tomato (Chapter 2). A simulation model that mimics the complex interactions involved in NoV transmission that may take place in a foodservice system was developed to identify steps and interventions throughout the food preparation and serving process in food service operations that may affect the spread of NoV and provide the scientific basis needed to develop risk management strategies and educational materials and help reduce public health risk caused by NoV (Chapter 3).

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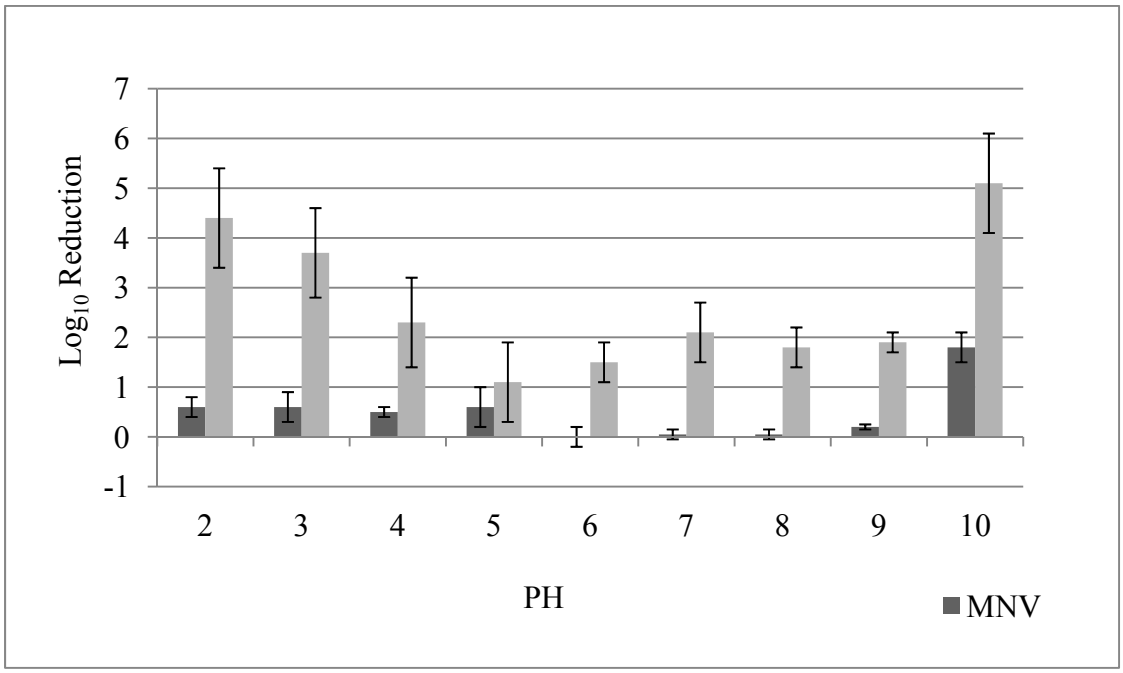
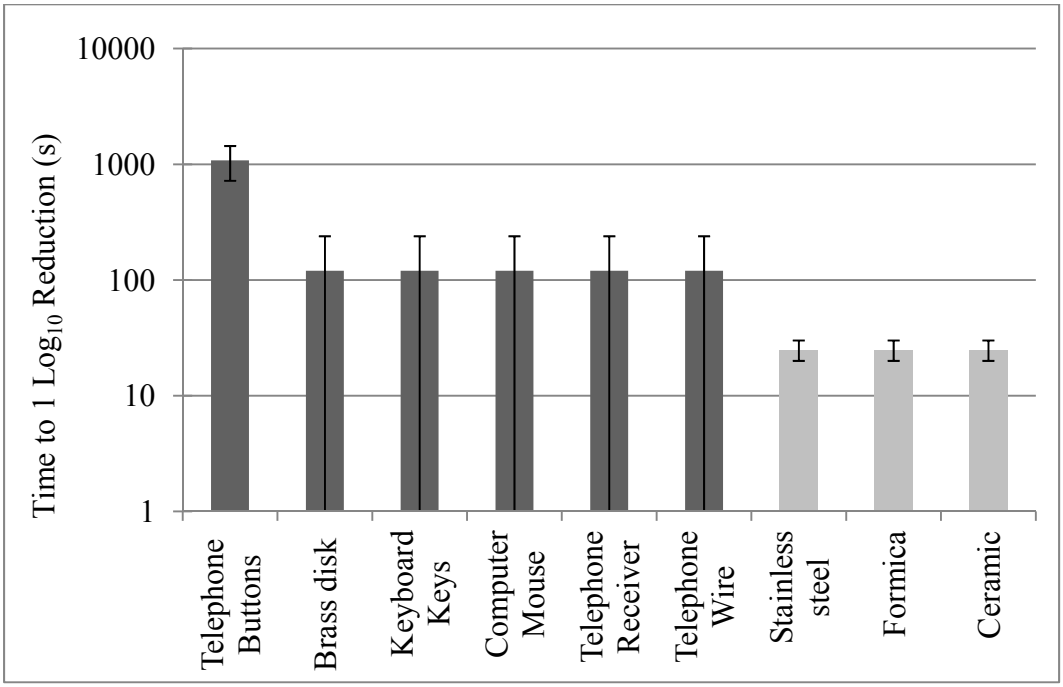
1.8 Figures

Figure 1.1 Histograms showing pH effect on survival of FCV and MNV-1 after 30 min at 37 °C.



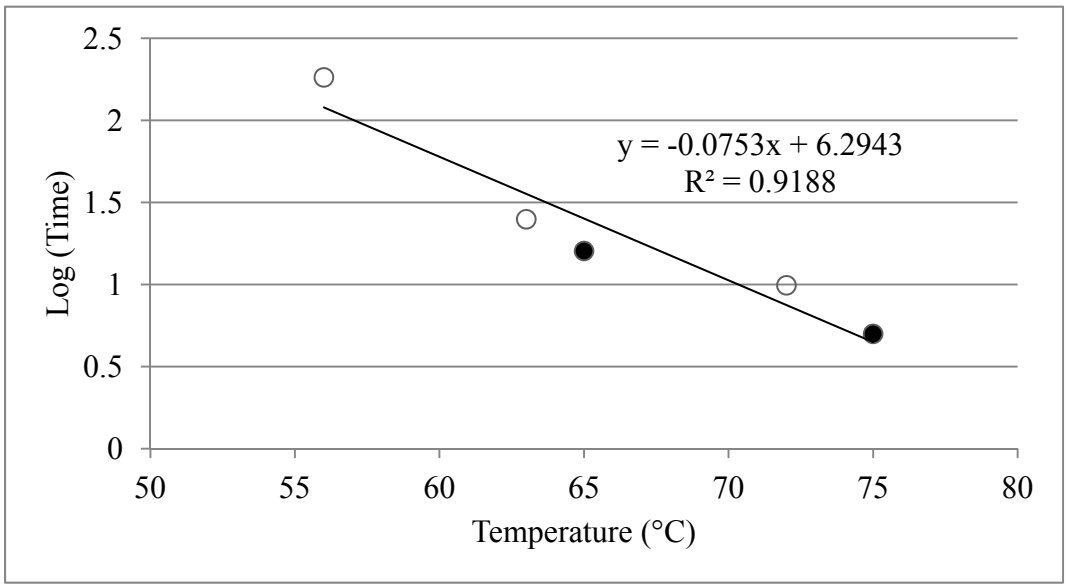
* Data were collected from Cannon et al., 2006 (25).

Figure 1.2 Histograms showing the time (seconds) required to reach a 1-log reduction of FCV (PFU) on different surfaces at room temperature.



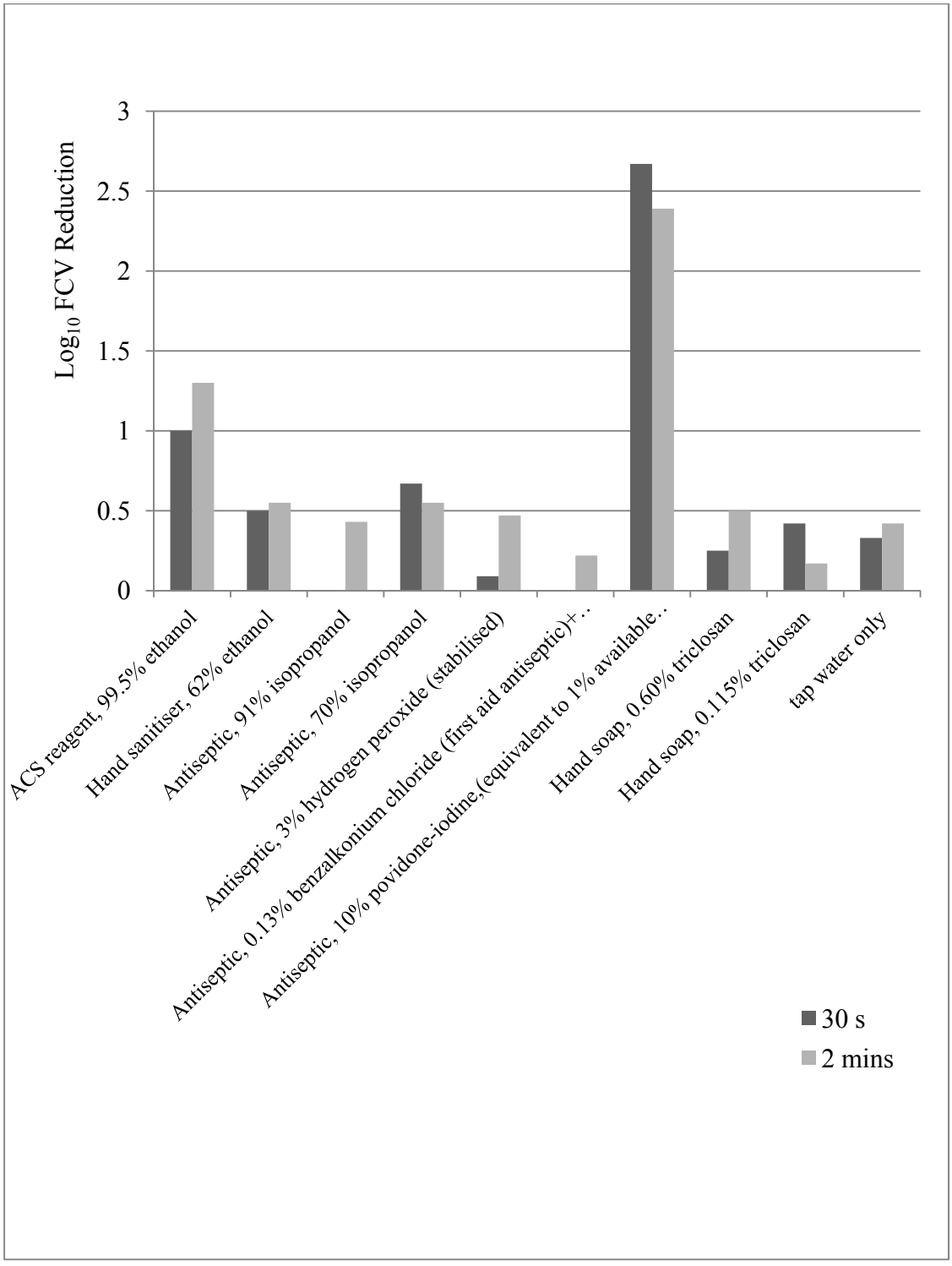
* Data were collected from 2 separate studies (dark gray: Clay et al., 2006 (37); light gray: D'Souza et al., 2006 (45)).

Figure 1.3 Effect of temperature on time (seconds) for 1-log reduction on MNV-1.



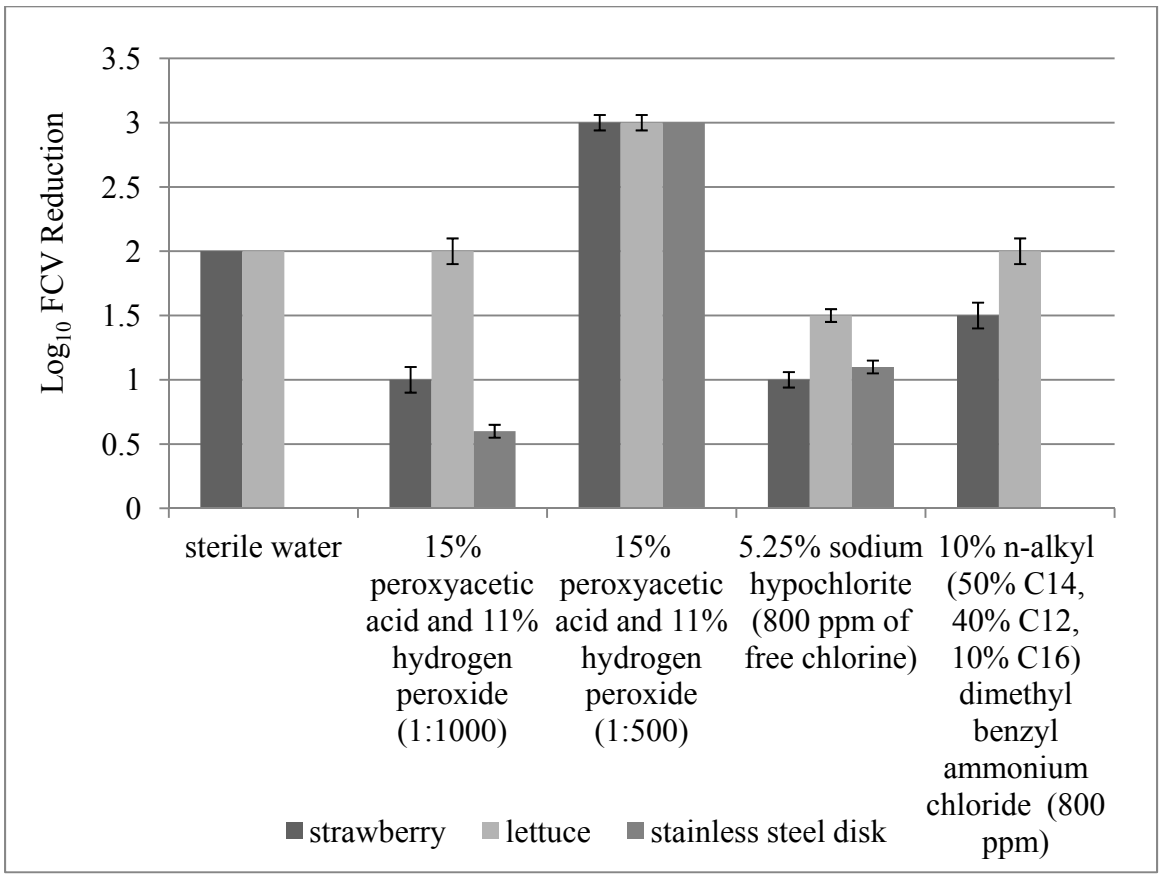
* Data were collected from 2 separate studies (open circle: Cannon et al., 2006 (25); closed circle: Baert et al., 2008 (8)).

Figure 1.4 Histograms showing log reduction of FCV on fingerpad using different hand treatments for 30-seconds and 2-minutes respectively.



* Data were collected from Lages et al., 2008 (104).

Figure 1.5 Histograms showing the effect of various antimicrobial compounds (20µl) on reducing FCV inoculated on strawberry, lettuce and stainless steel disk after 10 minutes.



* Data were collected from Gulati et al., 2001 (68).

Chapter 2: Modeling Norovirus Transmission during Mechanical Slicing of Globe Tomatoes[1]

2.1 Abstract

Recent epidemiological evidence indicates that preparation of fresh produce for use as ingredients in ready-to-eat food in commercial settings has been a significant source of the norovirus (NoV) infections in the U.S. This research investigated the dissemination of NoV from a single tomato to many others via the use of an 11-horizontal blade slicer commonly found in restaurants or sandwich shops. A total of eight trials were conducted. The source of contamination in each trial was a soak-inoculated, air-dried globe tomato containing ~8 log murine norovirus (MNV). Each trial began by slicing a single un-inoculated tomato in the slicer, followed by slicing an inoculated tomato. This was then followed by slicing from 9 to 27 uninoculated tomatoes. A similar and constant hand pressure on the slicer was used in every trial. Three slices from each tomato were collected for virus elution, concentration, and extraction before RT-PCR detection of MNV. The change in MNV per sliced tomato was averaged over all eight trials, and two mathematical models were fit to the average concentration using a second order logarithmic model or a second order power model. Regression analysis determined that the equation that best fit the data was $y = -0.903^* \ln(x) + 7.945$, where y = log MNV per slicing and x = tomato number. An acceptable fit ($R^2 = 0.91$) was indicated. The MNV levels transferred (y) generally decreased as the number of tomatoes sliced (x) increased, with some exceptions. Infrequent but erratic transfers, where the MNV level of a subsequent tomato was higher

[1] Di Li conducted the data analysis and modeling. Donald W. Schaffner provided oversight of data analysis and modeling and edits to the draft, Carol Shieh supervised laboratory data collection and wrote the first draft of the manuscript. Mary Lou Tortorello provided portions of the first draft and edits Gregory J. Fleischman provided comments on the draft and data analysis. This manuscript has been submitted to the International Journal of Food Microbiology.

than that of a preceding tomato, occurred in later transfer of some trials. In contrast, the first and second transfers of each trial were always shown to have sharply decreased levels of MNV from the inoculum. The MNV log reduction per slicing event changes throughout the process: with a predicted 0.63 log reduction from tomato 1 to tomato 2 (76% reduction); a 0.07 log reduction predicted from tomato 13 to tomato 14 (a 14% reduction); and 0.03 log reduction predicted from tomato 27 to 28 (a 7% reduction). Clearly virus transfer is variable even given the consistent slicing procedure used throughout each trial. This study illustrates the complex nature of risk prediction associated with NoV cross-contamination during food preparation in commercial establishments.

2.2 Introduction

Noroviruses (NoVs) far exceed the other known agents of gastroenteritis as a cause of illness in the U.S. (Scallan et al., 2011b). For the years 2000 - 2008, over 5 million cases of foodborne illness were caused each year by NoV alone, representing nearly 60% of the cases attributed to known agents, more than all other bacterial and parasitic pathogens combined (Scallan et al., 2011b). NoV also probably accounts for a large proportion of the estimated 38 million gastroenteritis cases of unknown etiology based on its epidemiological characteristics (Scallan et al., 2011a; CDC, 2012). Among the single food commodities identified as sources of NoV infection outbreaks, fresh produce accounted for the majority of outbreaks (Hall et al., 2012). When complex foods were implicated, fresh produce-containing ready-to-eat foods (e.g., salads or sandwiches) were often linked. When points of contamination of the fresh produce items could be identified, the majority of NoV outbreaks were linked to food preparation or food service procedures, rather than to production or processing (Hall et al., 2012). Commercial settings, especially restaurants

and delicatessens, accounted for 83% of the food preparation settings linked to the outbreaks, and food handler contact during preparation of uncooked or ready-to-eat foods contributed to foodborne NoV outbreaks most commonly (Hall et al., 2012). Fresh produce in the commercial food preparation setting is clearly a significant source of the NoV disease burden.

NoVs are highly contagious with only 10 to 18 virus particles needed to cause infection in some cases (Teunis et al., 2008; CDC, 2013b). They are easily transmitted, by person-to-person contact as well as through contact with contaminated surfaces (CDC, 2013a). The U.S. National Outbreak Reporting System (NORS) indicated that the number of NoV disease outbreaks attributed to contaminated foods during 2009-2010 actually declined compared to the previous 5-year period (CDC 2013a). NORS allowed reporting of transmission vehicles other than food or water for the first time; thus, NoV contamination scenarios, perhaps involving multiple transmission routes, may be more accurately documented in the future (CDC, 2013a). Environmental vehicles including utensils, equipment, and gloves have been contributing factors in NoV outbreaks, although they are not the predominant transmission route (Dreyfuss, 2009). Although studies of virus transfer from food handlers and environmental vehicles to food matrices have been published (Bidawid et al., 2000; Bidawid et al., 2004; D'Souza et al., 2006; Wei et al., 2010), there are many knowledge gaps and the mechanisms by which NoVs are transmitted are not well understood. It would be beneficial to develop a more thorough understanding of NoV transmission characteristics, so that specific control measures can be evaluated and implemented.

The transfer of microorganisms during slicing of foods has been a topic of research in the past with the focus on bacterial transfer in meats. Earlier research (Farrell et al., 1998; Flores and Tamplin, 2002) showed that *E. coli* O157:H7 in beef could be disseminated to specific areas of the meat grinder and to portions of ground beef, with more *E. coli* O157:H7 distributed in portions collected immediately after contamination was introduced. When deli meats were inoculated with *Listeria monocytogenes* and sliced with a commercial deli slicer, prolonged transfer of the pathogen to over at least 30 slices was observed (Vorst et al., 2006). In addition, *L. monocytogenes* could be transferred from an inoculated slicer onto meats (Lin et al., 2006). The transfer rate of strong biofilm-forming *L. monocytogenes* was reported to be slightly greater than that of weak biofilm-forming *Listeria* (Keskinen et al., 2008), and *Listeria* transferred more readily to lean turkey meat compared to more fatty salami.

More recently, mathematical models for bacterial transfer have been developed. Aarnisalo et al. (2007) showed that lower numbers of *L. monocytogenes* were transferred to salmon slices when the inoculum level was lower, when the temperature was colder or the attachment time was longer. These researchers also showed a progressive exponential reduction in *L. monocytogenes* transfer during slicing. Two empirical models for *L. monocytogenes* transfer from contaminated slicers to salami loaf were developed by Sheen (2008). These models were reasonably accurate with high starting concentrations (>5 log CFU) and less accurate with lower levels of *Listeria.* Møller et al. (2012) developed a five parameter semi-empirical model for *Salmonella* transfer from a contaminated grinder to pork meat. This model hypothesized that transfer occurred from two environmental matrices inside the grinder, and satisfactorily predicted the observed concentrations of

Salmonella during grinding of up to 110 pork slices. Hoelzer et al. (2012) summarized the probability distributions and mathematical models of *L. monocytogenes* transfer as an aid to quantitative microbial risk assessment.

This study used a mechanical, commercial-grade slicer, and investigated its capability for disseminating, transferring or cross-contaminating viruses from a single produce item to many others. Mechanical slicers are frequently used in foodservice, especially for high moisture content produce that is not precut prior to arrival in the restaurant (e.g. globe tomatoes or cucumbers). The tomato matrix was chosen in the current study, because it has been one of the most common ingredients of vegetable salads and sandwiches. Other types of fresh produce frequently sliced by a mechanical slicer also include cucumber, onions, and green peppers. The current research is the first to characterize virus transfer to fresh produce during mechanical slicing with the intent to develop data and models for use in future risk assessment.

2.3 Materials and Methods

2.3.1 MNV-soaked tomato sliced by a mechanical slicer

As a part of the virus transfer experimental design, each slicing trial began by slicing a single inoculated tomato. Soak-inoculation was chosen due to the impracticality of using spot-inoculation over the large surface area of a ~180 g globe tomato. The tomato was soaked in 15 ml of MNV stock solution [prepared by replicating MNV-1 in Raw cells 264.7 (Wobus et al., 2004)] in a Fisher sampling bag. The sampling bag-top was sealed by a vacuum sealer (ULINE, Chicago, IL), to maximize contact between MNV and tomato surface. The sealed bag containing the tomato and MNV solution was gently shaken in an ice bath (to maintain MNV viability) at 35 rpm for 20 min, with a change to the

bag-position after 10 min. The inoculated tomato was transferred to a sterile Petri dish and placed in a bio-safety hood level II for approximately 40 min until it appeared completely dry. A commonly used restaurant-type mechanical slicer with 11 horizontal stainless steel blades 1/4" apart (Easy Tomato Slicer II, Nemco Food Equipment, Hicksville, OH) was used to slice 10 to 28 globe tomatoes per trial, with a total of eight trials used in the study. This slicer produced 11 to 12 tomato slices with a single hand-push. An un-inoculated tomato was sliced prior to beginning the trial. An inoculated tomato was then sliced, followed by up to 27 individual un-inoculated tomatoes sliced one by one.

To avoid experimental error or laboratory cross-contamination during slicing, 3 individuals with gloves handled (1) clean tomatoes to be placed on the slicer, (2) the slicer handle and tomato slices' sampling, and (3) each sample bag with 120 ml-eluent to be sealed with a sealer. MNV transfer patterns were examined by collecting three slices of each tomato and quantifying MNV from the 3 slices. The selection of the 3 representative slices (no. 3, 5, and 7) was designed and determined by the contamination pattern method described as the most probable contamination areas for hand contamination of a tomato (see Results section). Briefly, the selection experiments were conducted as follows. Four volunteers were asked to pick up stem-down globe tomatoes. The areas where volunteers' fingertips contacted the surface of tomatoes were marked with a permanent marker and sliced through the 11 blade mechanical slicer. The slices within the marked areas were tallied for each tomato, with the stem end counted as slice #1 (data in Table 2.2).

2.3.2 MNV eluted from tomato slices

Three slices of each tomato were aseptically transferred to a sampling filter bag containing 120 ml of ice-cooled eluent 0.3% beef extract (BE powder, Becton Dickinson, Sparks, MD), pH adjusted to 8.5. The eluent was prepared by tissue culture grade-PBS 10-fold dilution of 3% BE (originally made in water). Sealed filter bags (BagPage F, 63 micron porosity, Interscience, France) from each trial were shaken together at 125 rpm for 10 min at 10°C. The eluates were collected for direct qRT-PCR examination or RNA extraction before qRT-PCR. Direct qRT-PCR was conducted by diluting the eluates 25-fold with water and heat-releasing RNAs at 100ºC for 10 min. In 6 of 8 trials, the first 5 tomato eluates were examined by direct qRT-PCR. From the 6^{th} to 28^{th} tomato, the eluates were RNA-extrated prior to qRT-PCR quantification. For the remaining 2 trials, tomato eluates were all RNA-extracted with some later samples concentrated first (with Amicon Ultra columns approximately 18-fold concentrated, 9 ml concentrated to 0.5 ml) before RNA extraction. When different detection or concentration procedures were used in a trial, at least two samples were randomly selected and run by different procedures in order to normalize all data in the same trial.

2.3.3 MNV in eluates detected by qRT-PCR, with and without RNA extraction

Tomato eluate samples or eluate concentrates, 300 µl per sample, were RNA-extracted using RNeasy mini kit (Qiagen, Hilden, Germany), with the eluate concentrates first prepared by Amicon Ultra (100K) centrifugal filter units (Millipore, Billerica, MA). The final 60 µl RNA per sample was immediately frozen at -80°C. Three types of templates were incorporated into qRT-PCR reactions of UltraSense qRT-PCR kit (Invitrogen, Carlsbad, CA): (1) RNAs of eluates, (2) RNAs of eluate concentrates, and (3) directly diluted eluate samples (for high MNV titer samples only). In each RT-PCR reaction, 6 µl

of each RNA sample or a sample diluted 25-fold in water were added to a microtube containing forward primer, reverse primer, 0.5 µl RNAse inhibitor (Promega, Madison, WI), and water. All reaction mixtures were placed in a closed microtube, heated 10 min at 100°C in PTC 200 Thermal cycler (Bio-RAD, Hercules, CA), and cooled down to 4°C in the cycler. Each of the heated and cooled mixtures was spun first, adding 4 µl of 5X reaction mix, fluorogenic probe (final 500 nM), RT-PCR enzymes, Mg solution (final 3.5 mM), and DNA-grade water to make up a total reaction volume of 20 µl. Thermocycling conditions used in DNA Engine Opticon (Bio-RAD, Hercules, CA) and StepOnePlus System (Applied Biosystems, Foster City, CA) were 50°C for 20 min (reverse transcription), 95°C for 2 min (Taq-activation), and cycling 50 times at 95°C for 15 sec (denaturing), 55°C for 15 sec (annealing), 72°C for 15 sec (extension). The MNV-1 primers and probe sequences were extracted from the publication of Muller et al. (2007) with a modification on the probe labeling. The nucleotide sequences of the primers were 5'-AGAGGAATCTATGCGCCTGG-3' and 5'-GAAGGCGGCCAGAGACCAC-3'. The probe was 5'-FAM-CGCCACTCCGCACAAACAGCCC-BHQ1-3' (manufactured by Integrated DNA Technologies, Carolville, IA).

2.3.4 Detection limits of analyzing MNV in tomato

The detection limits were refined through the course of the tomato slicing study. Direct RT-PCR detection was used initially, with a detection limit of 10^5 MNV PCR units per reaction, where each reaction contained 6 µl templates that were derived from the 25-fold water-diluted 120 ml eluate for each tomato. When RT-PCR detection of RNA-extracted eluates was used the detection limit was no higher than 10^4/reaction, where each reaction contained 6 µl of 5-fold RNA concentrates from 0.3 ml eluate of 120 ml used for each

tomato. Finally, the RT-PCR detection of RNA-extracted and Amicon-concentrated samples had the lowest detection limit of ~ 10^3 MNV PCR units/reaction, where each reaction contained 6 µl of 5-fold RNA concentrates derived from 18-fold Amicon-concentrated eluate of 120 ml for each tomato.

2.3.5 Standard curve for virus quantification

Five- or ten-fold diluted MNV stock samples were prepared and quantified by qRT-PCR. The highest dilution with detectable MNV in the sample was assumed to contain 1 RT-PCR unit (PCR-U). All Ct values along with their corresponding PCR-U were used to formulate a standard curve. MNV units in tomato eluates were quantified and calculated via StepOnePlus PCR system analysis and Microsoft Excel.

2.3.6 MNV transfer data analyses

The change in PCR-U per tomato as slicing occurred was averaged over all eight trials, and two mathematical models were fit to the average concentration using Excel (Microsoft, Redmond, WA) using a second order logarithmic model or a second order power model. Those two models were:

$$PCR\text{-}U = a * \ln(\text{slice number}) + b$$

$$PCR\text{-}U = a' * (\text{slice number})^{b'}$$

Where a, b, a' and b' are regression parameters, and where PCR units are predicted as a function of slice number.

2.4 Results

2.4.1 Optimizing MNV qRT-PCR with Mg Concentration, Template Dilution, and RNA Extraction of Eluates

To optimize qRT-PCR for MNV detection, Mg concentrations of 3, 3.5, 4, 5, and 6 mM in the reactions were tested. It was observed that in some trials 3 mM Mg was the best for amplifying MNV with 1 cycle lower Ct versus 4 mM Mg. In other trials, 4 mM Mg allowed better detection of MNV, e.g. a Ct of 23.8 for 4 mM vs. 24.7 for 3 mM. Therefore, the average concentration of 3.5 mM $MgCl_2$ was chosen for subsequent qRT-PCR analysis for MNV. Dilution of the tomato eluates and RNA extraction were also compared to determine which method might allow greater removal of qRT-PCR inhibitors. These purification steps also present drawbacks since dilution may reduce the level of MNV to below the detection limit, and RNA extraction step may not allow 100% recovery. For this comparison, randomly selected eluate samples from 4 of the 8 trials were RNA-extracted or simply diluted 10, 25 or 50 fold prior to quantification using qRT-PCR. Results in Table 2.1 illustrate that samples b and c with 25-fold dilutions had identical log MNV levels, as compared those determined by 10-fold dilutions. The first sample (sample a) showed slightly greater level of MNV (6.9 logs) as determined by 25-fold diluted sample which could be due to an experimental error, because the MNV level determined by 50-fold dilution was also 6.8 logs (data not shown). Only sample d presented slightly wider variability between two dilutions (diluted 25- and 10-fold). In fact, inhibitors should not be an issue in sample d, because greater inhibition did not exist in the less diluted (10-fold) sample, and resulted in a higher level of MNV. All 4 representative samples indicated that the 10- to 25-fold dilution had sufficient removal of natural inhibitors from the 120 ml-eluate to allow accurate quantification by qRT-PCR. On the other hand, eluate samples which were subjected to RNA extraction all presented lower MNV levels than diluted samples, possibly due to the recoveries of <100% compared to non-RNA extracted

samples. RNA-extracted samples were regularly tested by inserting another target in the study, and were shown to have no inhibitor. Overall, the detection efficiency in 25-fold diluted eluate samples was greater than that of the RNA extracts of eluates among all samples tested. The $\log_{10}$ MNV derived from the Ct values of the 25-fold diluted samples were 13 to 23% higher than those derived from their corresponding RNA extracts, shown in the last column in Table 2.1. On average, dilution of the eluates allowed 20% greater detection of MNV with an average factor of 1.2 (averaged from 6 trials in Table 2.1) between direct dilution and RNA extraction in determining MNV levels in samples. This factor was incorporated into the trials containing both direct-diluted and RNA extracted samples.

2.4.2 Hand-contamination Patterns of Tomato during Handling

The hand contamination study, performed for determining the most likely hand contamination area of a globe tomato during handling or picking, showed that slices 3 to 7 had the highest probabilities of contamination (Table 2.2), ranging from 64 to 73% (7 or 8 positives out of 11 trials). Three slices (no. 3, 5 and 7) of each tomato representing the most likely contamination areas were selected for virus analysis during slicing. This allowed for reduced volumes of eluent (as compared to the volumes needed to elute from all 10 slices) and better representation of the small quantity of sample to be analyzed by qRT-PCR.

2.4.3 MNV Transfer through a Mechanical Slicer during Tomato Slicing

As sampling and detection protocols were optimized, the transfer study was initiated with a single inoculated tomato in each trial. Ten to 28 globe tomatoes per trial were used during the 8 continuous tomato-slicing trials. The individual MNV transfer pattern differed between trials, with 3 representative trials with similar starting inocula (8.35 ± 0.26 logs of

MNV PCR-U per tomato) shown in Fig. 2.1. Each data point in Fig. 2.1 was the average MNV level per tomato calculated from duplicate reactions of RT-PCR in each of a minimum of two rounds of extraction, concentration and detection per slicing trial. As the number of tomatoes sliced increased, the MNV levels transferred showed the descending trend with some exceptions (Fig. 2.1). Infrequently, the titer of a later tomato was higher than that of the proceeding tomatoes. The largest MNV level increase was 2.19 logs in tomato #14 of the trial indicated by filled triangles (in Fig. 2.1 with 4.54 and 6.63 logs of MNV in tomato 12 and 14 respectively). This erratic transfer was observed more frequently in later tomatoes of a trial, possibly due to transfer of virus-laden tomato debris from an earlier tomato trapped in the slicer. In contrast, the first and second transfers of each trial were shown to have sharp decreases of MNV from the inoculum (Fig. 2.1).

2.4.4 Regression Analysis of MNV Transfer during Slicing

All MNV levels at each specific tomato order were averaged to derive a single virus transfer pattern since similar virus inocula were used in the 8 trials. The average MNV level for each slicing from all 8 trials is shown in Figure 2.2. It is clear from the pattern that MNV concentration generally declines with each slicing, but that the rate of decline is not linear. Regression analysis determined that the equation best fitting the data was $y = -0.903^* \ln(x) + 7.945$, where y = log MNV per slicing and x = tomato number. The R^2 value (0.91336) indicates a good fit. The log reduction per slicing event changed throughout the process: with a predicted 0.63 log reduction from tomato 1 to tomato 2 (76% reduction); a 0.07 log reduction predicted from tomato 13 to tomato 14 (14% reduction); and 0.03 log reduction predicted from tomato 27 to 28 (7% reduction). In addition, Fig. 2.2 (bottom curve) also shows the predicted average accumulated log

reduction of MNV over the course of the trials. Clearly the greatest MNV reductions occurred in the first few sliced tomatoes, such that by the third slicing, 90% of all MNV introduced by the contaminated tomato have left the system. The second log reduction, corresponding to 99% of all the MNV in the system, occurred by the 9th sliced tomato, while the third log reduction (99.9% of all MNV) did not occur until the 28th sliced tomato.

2.5 Discussions

An average of 1 foodborne NoV outbreak is reported every day in the U.S. (Hall et al., 2012). Produce-implicated NoV outbreaks have been epidemiologically linked to RTE food preparation or food handlers, but details on how viral cross-contamination occurs during food preparation in retail settings are very limited. Although bacterial cross-contamination during grinding and slicing has been modeled (Hoelzer et al., 2012), similar data for viruses have not been reported. Recently viruses were described to be transferred to 7 sequentially prepared fresh produce batches using hand knife-cutting (Wang et al., 2013), but MNV levels transferred were not quantified and can not be readily used for modeling.

The virus transfer patterns among the 8 trials presented were somewhat biphasic, i.e., a phase of rapidly reduced MNV levels in tomatoes followed by another phase with tailing effects. The fastest reductions of MNV occurred within the first few tomatoes sliced, specifically the first two tomatoes (#2 and 3) after the inoculated tomato (#1) passed through the slicer. An earlier analysis using simple linear regression modeling had only R^2 of 0.7 when describing the relationship between MNV levels on tomatoes and slicing order number (data not shown). A non-linear equation with high correlation ($R^2 = 0.91$) was developed where Y (log PCR-U per tomato) = -0.903*ln (X, slicing order) + 7.945, capable

of predicting MNV concentration from the first tomato with ~8 logs MNV through the last tomato measured was developed. This model is similar but not identical to other models developed for *Listeria* transfers during slicing deli meats (Sheen and Hwang, 2008), or for slicing salmon fillets (Aarnisalo et al., 2007). Generally, cross-contamination during slicing has a descending pattern of pathogen transfer, but with various rates of transfer. All four slicing models including ours use either a natural logarithm, or exponential function to describe the pattern. In contrast, *E. coli* or *Salmonella* transfers during grinding of meats appears to be different from those for slicing, as blending may have had an additional impact on pathogen transfer (Flores and Tamplin, 2002; Moller et al., 2011).

In transfer studies performed with other foods, various factors were observed to affect transfer. Vorst et al. (2006) observed prolonged transfer of *Listeria* in salami over many slices, and they hypothesized that this might be due to its high fat (36%) and low moisture content. In comparison with our results, Vorst et al. observed a 2-log reduction in *Listeria* after meat slice #20, whereas we observed a 2 log-reduction by tomato slicing #9. This difference supports the hypothesis that fat and moisture play a role, as tomatoes have essentially no fat (< 0.2%) and high moisture content. However, this difference could also be explained by the obvious differences between MNV and *Listeria* (e.g. pathogen size and surface chemistry, etc.). The latter explanation is supported by the observation of Aarnisalo et al., (2007) since salmon fillets showed greater prolonged *Listeria* transfer compared to salami, yet only contain 12% fat, along with 20% protein and 67% moisture.

Another possible factor affecting pathogen transfer is the physical action of mechanical slicing since slicing force and speed may affect transfer. The mechanical slicer used in the current study required hand pushing of the slicer-handle to force a tomato through the

11-horizontal blades. If the force is too little, the tomato is not sliced through, and if the force is too great, the tomato slices fall out of the slicer track. These factors may have served to minimize the variation in the force, which may have impacted viral transfer during mechanical slicing.

Using the derived model equation Y = - 0.903*ln (X) + 7.945 where Y is the log PCR-U and X is the slicing number of tomato, we estimate that continuous slicing of ~100 tomatoes would achieve a total accumulated reduction of 4 logs of MNV. Carrying this further, the model predicts that it is theoretically possible to cross-contaminate over one thousand tomatoes with a single tomato containing 8 log PCR-U of virus. However, considering the observation by Sheen (2008) who noted that slicing transfer models were more accurate when developed using high vs. low levels of *Listeria*, further validation may be needed before our model can be applied to low levels of viral contamination. Given that an infected individual frequently sheds millions to billions of NoV for days even before and after symptoms appearance (CDC, 2013b), the extensive cross-contamination observed in the current study and low dosages needed to cause infection (Teunis et al. ,2008; CDC, 2013b), it is not surprising that large numbers of illnesses are frequently associated with the foodborne NoV outbreaks.

2.6 Conclusion

This research investigated the dissemination of NoV from a single tomato to many clean tomatoes via an 11-horizontal blade, hand-operated slicer commonly found in restaurants or sandwich shops. The virus levels in cross-contaminated tomatoes showed a generally descending trend as the number of tomatoes sliced increased. Regression analysis determined that the best-fit model equation for virus transfer during tomato slicing

is Y (log PCR-U per tomato) = - 0.903*ln (X, tomato order number) + 7.945 (log PCR-U inoculum), $R^2 = 0.91$. The nonlinearity of this model shows that the virus transfer rates change throughout the slicing process. This study provides an important indication of how extensive NoV cross-contamination during food preparation in a retail setting may be. As with other studies using different foods, this study shows that the cross-contamination has a quantifiable trend, the delineation of which is valuable for risk assessment and prediction.

2.7Acknowledgements

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2.9 Tables

Table 2.1 MNV Levels in Tomato Eluates Determined by qRT-PCR, with and without RNA Extraction

Eluates	Log of MNV PCR units per tomato [a] detected using:			Ratio [d] of 25 fold direct diluted to RNA-extracted
	RNA extraction [b]	Direct dilution and heating [c]		
		10-fold	25-fold	
1	6.0 (26.4±0.2)[e]	6.8 (30.7±0.9)	6.9 (32.1)	1.15
2	4.9 (31.7±0.9)	6.0 (34.6±1.4)	6.0 (36.5)	1.23
3	-[f]	7.9 (25.0±0.8)	7.9 (27.0±0.3)	-
4	-	6.5 (32.2±0.3)	6.2 (35.6)	-
5	5.2 (29.5±0.1)	-	5.9 (37.3±0.9)	1.13
6	5.4 (28.4±0.1)	-	6.2 (35.7±0.7)	1.15
7	5.5 (28.5±0.1)	-	6.4 (34.5±0.1)	1.16
8	5.3 (29.5±0.4)	-	6.1 (36.4±2.3)	1.15

[a] Each MNV level was calculated by applying its Ct value (expressed as mean±sd) to the standard curve formula:
Y= -0.199X + 7.596, where Y represents the MNV level in log and X represents Ct value.
[b] Tomato eluate (300 µl) was RNA-extracted into 60 µl, 5-fold concentrated. Six µl of the RNA extract was used
for RT-PCR assay for quantification.
[c] Tomato eluate was not RNA-extracted, but directly diluted with H_2O in 10-, and 25-fold to reduce the inhibitory interference.
Six µl of the RNA extract was also used for RT-PCR assay for quantification.
[d] Ratio was calculated via dividing log MNV of 25-fold direct dilution by log MNV of RNA extraction protocol.
[e] Mean Ct value and standard deviation per reaction. [f] Experiment not performed.

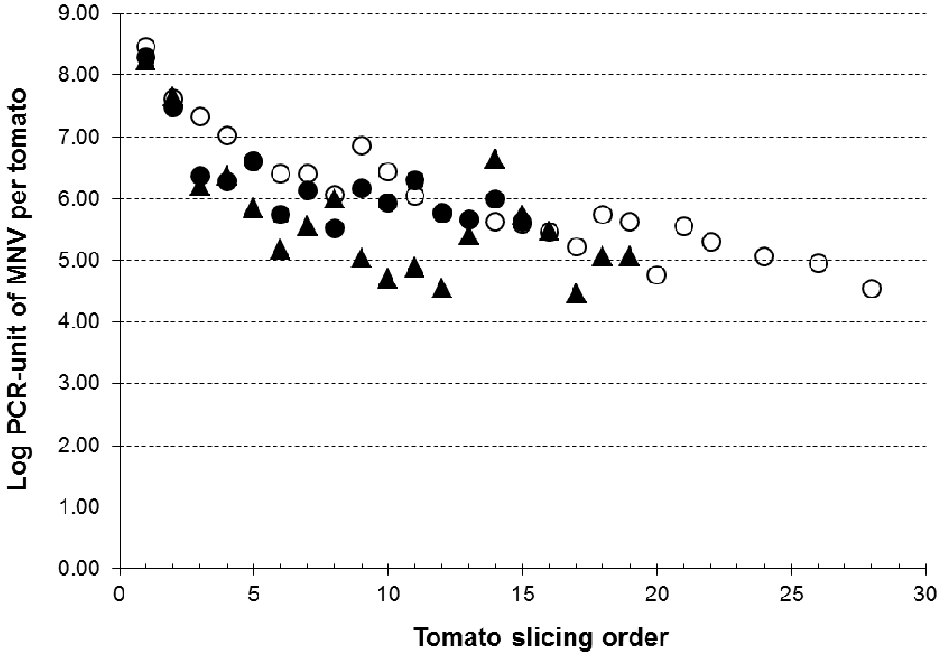
Table 2.2 Fingertip-touched Pattern of a Tomato by Hand Picking

Tomato	Weight (g)	Individual [a]	Tomato slice number [b]										Number of slices contaminated
			1	2	3	4	5	6	7	8	9	10	
A	191	W	-[c]	+[d]	+	+	-	-	-	-	-	-	3
B	186	W	-	+	+	+	+	+	+	-	-	-	6
C	209	W	-	-	+	+	+	+	-	-	-	-	4
D	194	X	-	+	+	+	+	+	+	+	-	-	7
E	142	X	-	+	+	+	+	+	+	-	-	-	6
F	183	X	-	-	-	-	-	+	+	+	-	-	4
G	170	Y	-	-	-	-	+	+	+	+	+	+	6
H	190	Y	-	-	-	-	+	+	+	+	-	-	4
I	219	Y	-	-	+	+	+	+	+	-	-	-	5
J	150	Y	+	+	+	-	-	-	-	-	+	+	5
K	147	Z	-	+	+	+	+	-	-	-	-	-	4
Average weight	180±25												
Frequency of hand contact[e]			1	6	8	7	8	8	7	4	2	2	

[a] Four volunteers identified by letter
[b] Contact points between the volunteer's fingertips and each tomato were marked with a permanent marker. The tomato was sliced through the 10-blade commercial slicer. Tomato slices were numbered 1 to 10, with the first slice being the stem-end slice.
[c] Not touched by hand while picking up the tomato. [d] Finger touch potential contamination area. [e] The number designates the total number out of 11 trials that individuals' hands had touched the specific slice.

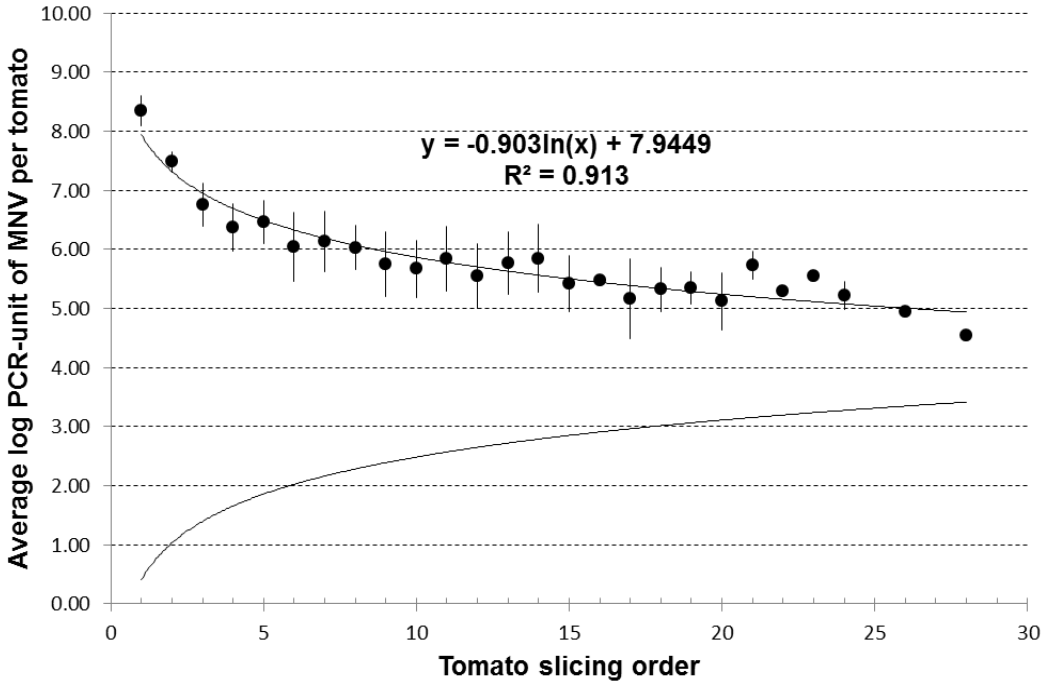
2.10 Figures

Figure 2.1 MNV Level (in log PCR units) per Tomato Sliced and Detected during Slicing Trials #1, 2, and 3.



Trial 1 is shown in filled circle ●, from tomato #1 (8.3 logs of MNV) till tomato #15, 5.62 logs of MNV. Trial 2 is shown in filled triangle ▲, from tomato #1 (8.25 logs of MNV) till tomato #19, 5.07 logs of MNV. Trial 3 is shown in empty circle ○, from tomato #1 (8.46 logs of MNV) till tomato #28, 4.54 logs of MNV.

Figure 2.2 The Best-fit Curve for MNV Level per Tomato throughout Tomato-slicing.



Upper curve: MNV level (in log PCR unit) per tomato averaged from all 8 trials, with the best-fit regression curve of Y (log MNV per tomato) = - 0.903 * ln (X, tomato order number) + 7.945 logs, $R^2 = 0.91$. The standard deviation among 8 trials at each slicing order is indicated by the vertical bar. Lower curve: the predicted curve for accumulated reduction of MNV (in log PCR unit)

Chapter 3. Quantitative Risk Assessment of Human Norovirus Transmission in a Foodservice System

3.1 Abstract

Human norovirus is the leading cause of non-bacterial acute gastroenteritis in humans worldwide and has caused great concern globally. Norovirus causes an estimated 58% of all foodborne illnesses cases in the United States annually. A great portion of reported norovirus outbreaks is due poor hygienic practices of food handlers. The objective of this study was to provide a quantitative assessment of the risk of passing norovirus infection to co-workers and producing contaminated food products through multiple cross-contamination events through modeling perparation of lettuce and tomato salads to go in a quick service restaurant. The effects of handwashing compliance, faucet type, original source of contamination, and periodic replacement of utensils were investigated, using a simulation model developed using the software program Arena. Our results showed that risk increases at low handwashing compliance levels when a manual tap was used for handwashing. This is due to the fact that the manual tap served as a cross-contamination hub for norovirus to spread to employees' hands and then to food products and contact surfaces. This risk is not offset when handwashing compliance is low. A hands-free faucet can reduce such risk by breaking norovirus transmission between hand and tap. Regular change of utensils such as slicer and tongs may also reduce the risk by clearing attached viral particles out the system. The risk model also shows that changing one single factor may not be sufficient and control of multiple factors works best for norovirus risk management.

3.2 Introduction

Human norovirus (NoV) causes half of all gastroenteritis outbreaks worldwide and is considered the most common cause of foodborne disease in the United States (*7*). The Centers for Disease Control and Prevention (CDC) estimates that 21 million cases of acute gastroenteritis and approximately 800 deaths attributed to human NoV occur each year in the US (*7*). NoV is a highly infectious, environmentally stable, and constantly evolving organism (*7*, *14*, *15*). Human NoV can spread easily through various routes such as contact with and/or ingestion of contaminated food or water, direct human contact, contact with soiled surfaces, as well as splashing of feces or aerosols from vomiting (*23*, *26*). Once a person is infected, billions of virus particles are shed in that person's stool and vomit and the infected individuals can continue to shed the virus two or more weeks after symptoms resolve (*7*). Moreover, asymptomatic NoV infection has also been reported (*2*, *11*, *20*, *21*). Since as few as 10 virus particles may be sufficient to infect an individual (*26*), once a single infection occurs, virus can spread rapidly through multiple routes.

NoV outbreaks commonly occur in restaurants, cruise ships, schools, colleges, camps and nursing homes. Isolation of infected individuals, assurance of proper hand hygiene practice, and effective disinfection of environmental surfaces are all suggested for managing, controlling and preventing NoV outbreaks (*17*). No human NoV vaccines are currently available due to the fact that the organism is constantly evolving and cannot be grown in cell culture (*1*).

Foods can become contaminated with NoV at any point from farm to fork during growth, harvest, shipping, and processing. Many foodborne NoV outbreaks have been linked to the consumption of food contaminated by an infected food handler in the foodservice environment where considerable human contact can take place during food

preparation, including washing, chopping/slicing and mixing. Foods that are eaten raw, such as leafy vegetables and fruits are at high risk for NoV contamination (*3*, *24*), since there is often no effective pathogen reduction step (e.g. cooking) for such food prior to consumption (*6*).

Hands are a common vehicle for the transfer of harmful microbes and can be easily become contaminated through handling contaminated foods, contact with soiled surfaces or poor hygienic practices when using the toilet. Poor personal hygiene is one of the major risk factors that can lead to the occurrence of foodborne illness in retail and foodservice establishments, and effective handwashing compliance continues to be a primary concern for regulatory agencies (*10*). Proper handwashing can reduce fecal-oral transmission of infectious agents and is one of the most important measures to minimize the contamination of food by employees (*10*).

In recent years, human NoV has drawn attention from policy makers and researchers due to the high prevalence of NoV related outbreaks (*13*). Limited work has been done, however, to estimate NoV transmission within an integrated foodservice system where a vast potential for complicated cross-contamination exists (*18*). Many studies have investigated the survival and transmission of NoV and surrogates on and between different surfaces under a variety of conditions including those associated with outbreaks on cruise ships and aircrafts, as well as hospitals (*12*, *25*, *27*, *28*). Quantitative microbial risk assessment (QMRA), which contains four elements: hazard identification, exposure assessment, hazard characterization, and risk characterization (*8*) can help identify risk factors and interventions leading to specific outcomes through hazard identification, and qualitative and quantitative evaluation. QMRA is a valuable tool for decision making and

food safety hazard control through systematical analyzation and interpretation of existing information.

In this study, we build a QMRA simulation model that mimics some of the complex interactions involved in NoV transmission that may take place in a foodservice setting, which prepares and serves lettuce and tomato salads to go . The QMRA can be used to determine the risk factors that either accelerate and lead to further spread of viruses or slow and prevent the spread of viruses during food cutting, mixing, packaging and delivery to customers in a simulated quick service restaurant (QSR). The risk assessment model can be a useful tool for policy makers and foodservice risk managers to reduce morbidity and economic loss currently associated with NoV outbreaks.

3.3 Material and Methods

3.3.1 Data and Assumptions

Laboratory Data Analysis

Cross-contamination during food preparation is a major food safety concern and has been considered as an important factor leading to foodborne outbreaks. Our collaborators from the Illinois Institute of Technology (IIT) and the U.S. Food and Drug Administration (FDA) conducted experiments on cross-contamination of NoV between different objects using murine norovirus as the surrogate of human NoV (see Appendix 1). Transfer coefficients between: a) lettuce and hands; b) lettuce and knife; c) lettuce and cutting board; d) slicer and tomatoes were generated using data collected from their laboratory research and incorporated into our simulation model. The transfer coefficients were calculated using the following formula: number of viruses on recipient surface / number of viruses on donor surface ×100, as described in Chen *et al.* (9). The reduction of norovirus

through handwashing was performed by IIT and was calculated as: (number of viruses on hands before washing - number of viruses on hands after washing) / number of viruses on hands before washing ×100. Each pair of final and initial counts was used to determine one coefficient. Arena input analyzer (Rockwell Automation, Warrendale, PA) was used to fit probability distributions to each set of data.

Model Overview

Our simulated restaurant serves purely lettuce and tomato to go salads. In our model, some parameters were generated from experimental data provided by our collaborators, some were collected from published studies and the rest were either based on observation or assumption when data were otherwise unavailable. We assumed that the transfer rate between tomato and hand/tong were the same as between lettuce and hand/tong and transfer rate between tong and lettuce/tomato was the same as the transfer rate between lettuce and knife. The transfer rate from hands to contact surfaces such as the top of the tongs and salad container boxes was assumed to be 2/3 of the transfer rate from hand to lettuce and the reverse transfer rate was assumed to be 1/2 of the transfer rate from lettuce to hand based on the ratio of transfer rate between hands and different subjects reported in the Bidawid (5) study. It was assumed that employees would only wash their hands after using the restroom and handwashing compliance levels were the same for all employees. All transfer parameters are listed in Table 3.1.

To assess the risk of human NoV transmission in a typical quick service (fast food) restaurant, the model simulated the spread of NoV, as affected by the source of contaminant (food or infected food handler) and handwashing compliance. We simulated the spread of NoV starting with produce (lettuce or tomato) arrival at the appropriate work

station, followed by the transfer of each food item through two preparation stations: cutting/slicing, mixing and packaging, followed by delivery of prepared salad packages to customers. During cutting or slicing, NoV cross-contamination points included in the model were between: (a) tomato or lettuce and hand; (b) tomato and slicer or lettuce and knife; and (c) lettuce and cutting board. During the filling of salad containers, transfer of NoV between tongs and tomato or lettuce, as well as transfer between hand and the top of the tongs was considered. Any virus carried by lettuce and tomatoes were summed to represent total contamination in the salad. Transfer of NoV from hand to the outside of salad container was included in packaging step. As previously mentioned, simulated food employees use the restroom on occasion. If hands were washed after using restroom, NoV transfer between hands and tap would occur during handling the tap if a manual tap was used. During handwashing, the reduction of NoV number on hands was calculated as indicated in Table 3.1. At the final step in the process (sale/purchase), we assumed possible transmission between counter staff hands and the outside of the salad container. The number of viruses on each related objects/surfaces was updated every time a contact between two objects occurred or handwashing was performed. We assumed the infectivity of NoV in the system did not change over time and total number of virus can only be reduced through handwashing and utensil change. We also assumed all transfer events were independent except norovirus transfer from slicer to tomatos. For example, after cutting one unit of lettuce, contamination level of lettuce (LContL) is updated and the calculation is: LContL= (PreLContL*(1 - TrLh - TrLk - TrLb)) + (PreLHContL * TrHl) + (PreKLContL * TrKl) + (PreBLContL * TrBl), where PreLContL is contamination level of lettuce before cutting, TrLh, TrLk, and TrLb are the transfer rates of NoV particles from

lettuce to hands, knife and cutting board respectively, PreLHContL is contamination level of hand of lettuce handler before cutting, TrHl is the transfer rate of NoV particles from hand to lettuce, PreKLContL is contamination level of knife before cutting, TrKl is the transfer rate of NoV particles from knife to lettuce, PreBLContL is contamination level of cutting board before cutting, and TrBl is the transfer rate of NoV particles from cutting board to lettuce. We used a simple measure of risk, as an alternative to using a dose-response function. It was assumed that all salads sold to customers that contained more than 10 NoV could put consumers at risk of NoV infection and all employees who carried more than 10 NoV may be at risk of NoV infection.

Our simulation consisted of four employees per shift, with two eight-hour shifts per day and assumed that lettuce and tomato handlers started working 30 minutes earlier than other employees. In each shift, an employee was assigned one of four different jobs: cutting lettuce, slicing tomato, salad mixing and packaging, or taking customer orders. Simulated employees were allowed four restroom visits per shift as long as the entire batch (20 units) of produce or the package of the salad being prepared was finished. We assumed that this restaurant would serve exactly 800 salads per day, with each salad made from of one unit of lettuce and one unit of tomato. In any given scenario, contamination would arise from only a single source, either from the first food product (one lettuce or tomato unit) prepared that day, or from a first shift employee infected with NoV. A constant number of 4×10^9 virus particles were assumed to represent the initial contamination level, whether the source was hands or produce.

We assumed the QSR contained four stations: lettuce cutting, tomato slicing, salad mixing and packaging, and the point of sale counter. The four employees who work on the

same shift had a pre-assigned workstation and they did not switch stations. We assumed that lettuce and tomatoes were prepared in batches and each batch contains 20 units. The simulation starts with a batch of 20 lettuce or tomato units arriving at the cutting station. Once all 20 units in the batch were cut, they were sent as a group to the mixing and packaging station. The mixing and packaging employee would start filling, mixing, and packing salads as long as at least one unit of lettuce and tomato was available. The salad boxes were considered ready to sell once packing was finished. Once a customer arrived and an order was placed, the counter staff would pick a salad container and pass it to the customer. We assumed that 400 salad bowls were prepared during a working shift (i.e. 400 unit tomatoes and lettuce were prepared, mixed and packed). We also assumed that each customer could only make one purchase and each purchase would contain one salad box. We assumed that all orders were "to go" and no customer restroom was provided.

3.3.2 Model Development

The simulation model was developed using Arena® software (Rockwell Automation, Warrendale, PA). Arena® is a commercial, off-the-shelf, high-end, discrete-event simulation package which can be used to conduct the simulation of time-dependent phenomena. The discrete event simulation enables the model to track the spread route and distribution of NoV throughout a foodservice operation, (e.g., whether viruses arrive in a contaminated batch of product or via the hands of an infected food handler, and at which stages the number of NoV is decreased and how much reduction is achieved). The model was built to mimic a simplified version of the complex interactions involved in a QSR setting, including working status of restaurant employees, the transfer of foods between stations, and the possibility of virus spread throughout the entire process from preparing to

delivery. The simulation model was run 1000 times for each scenario. For each possible source of initial contamination: contaminated lettuce, contaminated tomato, NoV infected lettuce handler, tomato handler, mixing employee, and counter staff, handwashing using a manual tap versus a hands-free faucet were investigated at 0, 30, 50, 70, and 100% handwashing compliance. Handwashing compliance was defined as the fraction of the time the employee wash hands after using the restroom. Replacement of tongs after preparation of every 100 salads was also simulated when a manual tap was used. When lettuce or tomato was the initial source of contamination, replacement of knife and cutting board/slicer after every 100 lettuce or tomato units was checked as well as replacement of knife and cutting board/slicer together with tong replacement.

3.4 Results

3.4.1 Spread of Norovirus when a Manual Tap was used for Handwashing

Norovirus from a Contaminated Food Product

Norovirus Spread from a Contaminated Lettuce

In the case when NoV was initially brought into a QSR setting by contaminated lettuce, simulation results showed that the mean of the number of lettuce units contaminated with more than 10 NoV particles after cutting decreased from 398, when no handwashing was performed to 237, when handwashing was performed all the time (Table 3.2). The manual tap used for handwashing can serve as a hub for NoV transmission and subsequently spread NoV to other employees who then spread contamination further to food products or contact surfaces through cross-contamination. When no handwashing was performed, no NoV was transferred to other employees and therefore no subsequent transfer to food or contact surfaces occurs either. With increased handwashing compliance, in the

contaminated lettuce scenario, the number of tomato unit contained >10 NoV particles after slicing increased. At the end of the day, about half of salad boxes sold was contaminated with more than 10 NoV particles when no handwashing was performed, and this number decreased from ~400 to ~300 at high handwashing compliance levels (Table 3.2).

Table 3.3 shows contamination patterns for employees. When no handwashing was performed, only the first shift lettuce handler carried >10 NoV particles at the end of the shift and none of the rest of the employees was at risk when no handwashing was performed since there was no hand-tap-hand NoV transmission. When handwashing was performed 30% of the time, the risk for every employee to carry more than 10 NoV particles was greater than the risk when no handwashing was performed. Although handwashing can cause a 98% reduction in NoV contamination on the hands (Table 3.1), handwashing compliance at 30% alone was not enough to reduce the risk brought by NoV transmission through direct contact between hand and tap right before and after handwashing. When the handwashing compliance level was increased to 50%, the risk of lettuce handlers carrying more than 10 NoV particles at the end of shift reduced while the risk for all the other employees increased. When handwashing compliance level increased to 70% or further to100%, the benefit of NoV reduction through handwashing overcame the risk brought by NoV spread through hand-tap contact and the risk of carrying more than 10 NoV particles reduced for all employees except the mixing employee (Table 3.3). The continued increasing in risk to the mixing employee with the increased handwashing compliance level was due to NoV transfer from hands to the top of tongs. In this case the top of the tongs serves as a reservoir for NoV. NoV accumulates through repeated contact

between hand and tong. This does not occur for the lettuce handler, tomato handler, or counter staff, where there was only one type of direct contact between hands and food or surfaces: hand-lettuce, hand-tomato, and hand-packaging box respectively. During salad mixing, there were multiple contact routes between hand and different surfaces such as hand and tong for picking up lettuce, hand and tongs for picking up tomato, and hand and salad packaging boxes. Therefore, NoV on mixing employees' hands decreased faster than other employees through multiple routes and the increased handwashing compliance provided the opportunity for hands to pick NoV from the handwashing tap and therefore increased the chance of having > 10 NoV particles on the hands at the end of the shift.

Norovirus Spread from a Contaminated Tomato

When a tomato was the original source of NoV contamination, every unit of sliced tomato used for each salad was contaminated with more than 10 NoV particles (Table 3.2). Additionally, all 800 salad boxes contained more than 10 NoV particles even with perfect handwashing compliance. In this case, the source of NoV contamination for all tomato units except the first one was the slicer, which was contaminated after cutting the originally contaminated tomato. The slicing model used: $Y = - 0.903*\ln(X) + 7.945$ (where Y is the logarithm of the number of NoV particles transferred to tomato and X is the slice number), essentially dictates that all the subsequent tomato slices would contain more than 10 NoV particles. As handwashing compliance increases, the number of lettuce unit contained >10 NoV particles after cutting increased (Table 3.2). When no handwashing was performed, the first shift tomato handler was the only employee who ends up carrying more than 10 NoV particles (Table 3.3). When handwashing was performed 30% of the time, the risk for every employee to carry more than 10 NoV particles was greater than the risk when no

handwashing was performed. When handwashing compliance was increased to 50%, the risk of tomato handlers carrying more than 10 NoV particles at the end of shift reduced while the risk for lettuce handler increased. When handwashing compliance increased to 70% or 100%, the risk reduced for both tomato and lettuce handlers (Table 3.3); similar to the pattern seen when the lettuce was the source of contamination. The risks for mixing employee and counter staff to carry more than 10 NoV particles at the end of the shift also showed similar trend as when lettuce was the initial source of contamination.

Norovirus from an Infected Employee

Norovirus Spread from Infected Lettuce Handler

When NoV contamination was originated from the first shift lettuce handler, simulation results showed that when no handwashing was performed, all 400 lettuce cut on first shift were contaminated with more than 10 NoV (Table 3.2). The number increased to 441 at 30% handwashing compliance level (due to carry over of virus particles from first shift to second shift via the handwash tap) and decreased from 441 as handwashing compliance went up to 50, 70 and 100%. As handwashing compliance increased, the number of sliced tomato contaminated with more than 10 NoV was also increased. Compared with zero handwashing compliance, the number of salad mix (mix of chopped lettuce and tomato slices) with more than 10 NoV particles increased when handwashing was performed 30% of the time and then decreased when handwashing was performed more often. The number of final salad packages with > 10 NoV, however, was increased when handwashing compliance level increased from 0 to 50% and leveled off at 70 and 100% (Table 3.2). When no handwashing was performed, no employee (other than the first shift lettuce handler) would carry more than 10 NoV because in this simulation there is no

other route for the NoV to spread except from the first shift lettuce handler's hand to lettuce. When handwashing was performed, the chance of lettuce handler, tomato handler, or counter staff carrying more than 10 NoV at the end of shift peaked at either 30 or 50% handwashing compliance level and decreased at higher levels (Table 3.3). The mixing employee had an increasing risk of carrying more than 10 NoV particles, which was again due to multiple contacts between hand and different contact surfaces.

Norovirus Spread from Infected Tomato Handler

When first shift tomato handler brought NoV into the system at the beginning of the day, simulation results showed all tomatoes sliced on the first shift were contaminated with more than 10 NoV when no handwashing was performed (Table 3.2). The number increased when handwashing compliance increased from 0 to 30% and decreased at higher compliance level. As handwashing compliance went up, the number of lettuce samples with > 10 NoV increased. The number of contaminated salad mixes and final salad packages followed the same trend as when the lettuce handler was the initial carrier of NoV (Table 3.2). When no handwashing was performed, no other employees ended up with > 10 NoV on their hands (Table 3.3). When handwashing was performed, the chance of mixing employee and counter staff carrying more than 10 NoV at the end of the shift followed the same trend as when the lettuce handler was the initial carrier of NoV (Table 3.3). The similar trend was due to the fact that when a lettuce/tomato handler was initial carrier of NoV, NoV only spread between lettuce/tomato handlers hand and lettuce/tomato. Cutting/slicing utensils which could make different impact were not involved in the transmission of NoV.

Norovirus Spread from Infected Mixing Employee

In the scenario when the first shift mixing employee, who was responsible for mixing lettuce and tomato, and packing salad boxes, was infected and brought NoV into the system, all salad sold were contaminated with more than 10 NoV even when handwashing was performed 100% of the time (Table 3.2). When no handwashing was performed, NoV spread only to surface of salad boxes and no produce was contaminated. The number of cut lettuce, sliced tomato and salad mix unit contaminated with more than 10 NoV particles increased with the increased handwashing compliance (Table 3.2). When no handwashing was performed, no lettuce or tomato handlers would carry more than 10 NoV at the end of shift, however, NoV did spread to counter staffs' hands through external surface of the salad packaging box (Table 3.3). The number of lettuce and tomato handlers with more than 10 NoV at the end of the shift increased as more handwashing was performed and all mixing employee and counter staff ended up carrying more than 10 NoV. Increased handwashing compliance did not help in reducing the risk (Table 3.3). The fact that a greater fraction of all employees end up with more than 10 NoV compared with lettuce and tomato handler scenarios was due to the reservoir effect from the top of tongs. The two tongs used for picking lettuce and tomato reduced the number of NoV on mixing employee' hand by accumulating of NoV on top of tongs. Therefore the number transferred from hand to tap during handwashing was less than other employees when infected, which made the spread of NoV less intensive and long lasting.

Norovirus Spread from Infected Counter Staff

The infected counter staff on first shift spreads NoV to 400 salad packaging boxes through hand-box contact (Table 3.2) when no handwashing was performed, no other items were contaminated. When handwashing was performed, NoV was spread to

cut/sliced produce items by cross-contamination via the handwashing tap. The number of processed produce item as well as salad unit contained more than 10 NoV increased with improved handwashing compliance and leveled off at high handwashing compliance level (Table 3.2).

When no handwashing was performed, contamination generally did not spread to employees other than the originally ill employee (Table 3.3). The one exception is in the case of the mixing employee, who spreads contamination to the second shift mixing employee via hand-tong contact and the counter employee via the outside of the salad box. More employees ending up with more than 10 NoV when handwashing compliance increased from 30% to 50% and then decreased as handwashing compliance level increased from 50% to 100%, except when the mixing employee was the source of the contamination. In this case, as handwashing compliance increased up to 100%, the number of lettuce and tomato handlers ending up with more than 10 NoV increased steadily.

3.4.2 Spread of Norovirus when a Hands-free Faucet was used for Handwashing

Norovirus from a Contaminated Food Product

Norovirus Spread from a Contaminated Lettuce

When a hands-free faucet was used, the spread of NoV can be highly reduced due to the elimination of hand-tap transfer. When NoV was originally from one contaminated lettuce head, no NoV spread to any other restaurant employees (Table 3.4) and therefore no spread to any sliced tomato, tongs or packaging boxes. Only salads made from lettuce units contaminated with more than 10 NoV particles were at risk (Table 3.5). With increased handwashing compliance, the number of lettuce unit and salad contaminated with more than 10 NoV decreased (Table 3.5). The number of lettuce handlers who end up carrying

more than 10 NoV particles also decreased and the more reduction was observed (Table 3.4) compared to using of a manual tap during handwashing (Table 3.3).

Norovirus Spread from a Contaminated Tomato

When a tomato was the initial source of NoV contamination and a hands-free faucet was used for handwashing, no lettuce was contaminated, and all sliced tomato units therefore all salads were contaminated with more than 10 NoV particles due to the spreading property between slicer and tomato (Table 3.5). Only the first shift tomato handler may end up with more than 10 NoV on his hands and the risk can be decreased by improving handwashing compliance. Greater reduction was observed when a hands-free faucet was used for handwashing (Table 3.4) compared to using a manual tap for handwashing (Table 3.3).

Norovirus from an Infected Employee

Use of a hands-free faucet in the simulation stops the NoV spread from an infected employee's hand to tap, from where NoV can be subsequently transferred to other employees' hands and then to food and contact surfaces (Table 3.4). When the first shift lettuce handler, tomato handler or counter staff was infected and brought NoV into the system, this employee could only pass NoV from hands to a single item through direct contact. The transmission was one-way and there was only one time contact between hand and every single item. When a hands-free faucet was used for handwashing instead of a manual tap, the number of salad with more than 10 NoV was reduced at all handwashing compliance levels and the number was decreased with increased handwashing compliance (Table 3.5). When handwashing was performed 100% of the time, using a hands-free faucet can lead to more than 50% reduction on the number of salad contaminated with

more than 10 NoV (Table 3.5) compared to using a manual tap (Table 3.2). No other employees working on the same day end up with any NoV except the original carrier employee. When the first shift mixing employee was a carrier, NoV was passed to counter staff because each employee's hands contact the outside of the salad box. The simulation result showed that when the first shift mixing employee was initial carrier of NoV, all salad boxes would end up with more than 10 NoV (Table 3.5) and all mixing employees and counter staffs working on the same day would end up with > 10 NoV on their hands by the end of the shift (Table 3.4). Note that NoV accumulated on the top of tongs during the first shift was transferred to the second shift mixing employee's hand through hand-tong contact, and the contamination then spread to second shift counter staff as well.

3.4.3 Effect of Replacement of Utensil on Norovirus Transmission

Effect of Cutlery Replacement

NoV can spread between cutting utensils and produce through direct contact. In the simulation model, NoV could only be passed to knife and cutting board from lettuce, and passed to the slicer from the initially contaminated tomato, and then to other tomatoes from the slicer. When the source of original contamination was lettuce, the simulation results showed that replacing the knife and cutting board after cutting every 100 lettuce units did not have much effect on reducing the number of salad contaminated with more than 10 NoV particles (Table 3.2, 3.5, 3.6) or transfer of contamination to employees hands (Table 3.3, 3.4, 3.7). This was due to relatively low transfer rate from lettuce to knife (~2.2%) and cutting board (~2.5%) and relatively high transfer rate from knife (~76%) and cutting board (~41%) to lettuce (Table 1). A large number of NoV from the first contaminated lettuce unit were transferred to the first shift lettuce handler's hand. Because hands have a

low transfer rate (~1.8%), once contaminated with NoV, they repeatedly transfer small amount of NoV to lettuce. Since the amount transferred from hands to lettuce exceeded the 10 NoV limit in most cases, many lettuce units became contaminated.

The simulation results showed that when the source of contamination was tomato, changing the slicer after every 100 tomato units cut, reduced the number of tomato units contaminated with more than 10 NoV by ~50% (Table 3.6) compared to when the same slicer was used throughout the day without cleaning (Table 3.2, 3.5). Note that even changing the slicer after 100 units are sliced, still results in a high number of contaminated slices (~400, Table 3.6), because the tomato workers hands become contaminated after handling the first slice. No effect was observed on the reduction of risks for employees carrying more than 10 NoV at the end of their shifts (Table 3.3, 3.4, 3.7).

Effect of Tong Replacement

As described above, tongs played an important role on NoV accumulation and re-distribution. Simulation results showed that when tongs were changed regularly (every 100 salad boxes), the number of salad sold to customer contained more than 10 NoV particles were highly reduced. Compared to using same tongs without cleaning throughout the day (Table 3.2), ~ 50% reduction can be reached when handwashing was performed 100% of the time (Table 3.8). When lettuce was the initial source of NoV contamination, changing tongs, knife and cutting board regularly reduced the number of salad with more than 10 NoV (Table 3.2, 3.8), which was mainly due to the reduced number of NoV transferred from hand to packaging boxes. Changing tongs, knife and cutting board regularly along with using a manual tap did not provide as much reduction on the number of salad with more than 10 NoV (Table 3.8) as using a hands-free faucet for handwashing

(Table 3.5). When tomato was the source of initial contamination, changing both slicer and tongs regularly provided more reduction on the number of contaminated salad with more than 10 NoV (Table 3.8) compared to changing slicer alone when handwashing was performed using a manual tap (Table 3.6 a), but less reduction compared to changing slicer alone and using a hands-free faucet (Table 3.6 b). The number of employees with > 10 NoV was also greatly reduced especially for mixing employee and counter staff when utensils were changed regularly (Table 3.9) compared to using same tongs during the day when a manual tap was used for handwashing (Table 3.3).

3.5 Discussion

The simulation results presented in this study showed that NoV can be transferred in multiple ways and no single factor can uniformly control the spread of NoV with perfect efficacy. When handwashing is regularly performed after visiting the restroom, the portion of simulated food that may cause human NoV infection, as well as the chance that an employee hands may become contaminated can be reduced in some, but not all cases, especially when a manual tap was used. Previously reports in the literature indicate that increased handwashing compliance can lead to a reduction of food contamination (*4*, *18*). Hands play an important role in transmitting NoV to foods and surfaces as well as to other people through cross contamination. Although handwashing can provide a ~2-log reduction of NoV on hand, use of the manual tap provides opportunities for pathogens to spread to the tap, then to other employees' hands and further to food products, package surfaces, and utensils through direct contact as previously reported (*9*). The hands-free faucet prevents hand-and-tap contact and therefore stops the spread of NoV during handwashing in the simulation. When a hands-free faucet is used, no NoV spreads to other

employees or further to food and contact surfaces via tap. Using a hands-free faucet can reduce the final number of products with more than 10 NoV particles and stop some employees hands from becoming contaminated. Our results also showed that changing slicers regularly can also help reduce the number of contaminated food products by removing NoV present on those items. The lettuce and tomato tong handles (used by the salad mixing employee), which have close and high frequency contact with the employees hands can serve as a reservoir and accumulate NoV and then subsequently spread NoV particles back to hands at a later time. The simulation results showed that handwashing alone may not necessarily reduce the number of salad boxes and employees with more than 10 NoV particles even when a hands-free faucet was used especially at low handwashing compliance levels. The number of contaminated salads with more than 10 NoV may not reduce even when tongs are replaced regularly and handwashing is performed 100% of the time. If tongs were not changed often, all prepared salads, and all mixing employees and counter staff would end carrying more than 10 NoV particles when the salad mixing employee was the original source of contamination. Such results are consistent with the observation that NoV outbreaks are often the result of a mixture of more than one mode of transmission (*16*). The simulation results show that cross-contamination through hands, cutlery, and food and contact surfaces are all of importance in NoV spread. Even when knife, slicer and tongs are regularly changed and handwashing was performed 100% after of all restroom visits, the simulation results showed that some salads would be contaminated with more than 10 NoV particles and some foodhandlers would end up with > 10 NoV particles on their hands. Therefore, the only truly effective NoV control measure is to prevent the organism from entering the restaurant.

Limited studies have developed risk models for cross contamination, especially RTE food products. Very often, models were developed to study cross-contamination through a specific route or single process (*19*). Pouillot and coworkers (*22*) studied the spread of *Campylobacter* and *Salmonella* during chicken preparation which including an cooking process and pointed out replacement of utensils, cutting boards and containers could reduce the risk of cross-contamination. Mokhtari and Jaykus (*18*) studied influence of handwashing efficiency and handwashing compliance on the spread of NoV in an 8-hour retail shift using a quantitative model. In the Mokhtari and Jaykus simulation model employees were not assigned specific jobs, and generic (rather than specific) transfer parameters between food and contact surfaces, and hand and contact surfaces were used throughout the model. The Mokhtari and Jaykus model was also designed such that the source of initial NoV contamination could only be from infected employees. In our model, different jobs were assigned to different employees working on the same shift, and NoV transfer routes were simulated differently based on the jobs and processes occurring.

Our model provides valuable information that can be considered for the control of NoV outbreaks. The results from our study suggested that multiple factors should be considered to control the spread of NoV, and that no one or even multiple combinations of factors will completely control NoV transmission risk.

3.6 References

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3.7 Tables

Table 3.1. Transfer parameters and corresponding probability distributions.

Donor	Receiver	Distribution	Source
Produce	Hand	BETA(0.915, 2.1039)	IIT Data
Hand	Produce	BETA(0.924, 2.23646)	IIT Data
Lettuce	Knife	BETA(0.732, 2.26599)	IIT Data
Knife	Lettuce	TRIA(0.34, 0.935, 1)	IIT Data
Lettuce	Board	BETA(1.45, 2.56243)	IIT Data
Board	Lettuce	BETA(0.765, 1.10613)	IIT Data
Hand	Tap	BETA(0.867, 2.36018)	IIT Data
Tap	Hand	BETA(1.15, 2.16814)	IIT Data
Produce	Tong	BETA(0.732, 2.26599)	IIT Data
Tong	Produce	TRIA(0.34, 0.935, 1)	IIT Data
Hand	Tong top / Container	(2/3)*BETA(0.924, 2.23646)	Assumed
Tong top / Container	Hand	(1/2)*BETA(0.915, 2.1039)	Assumed
Handwashing Reduction		98%	IIT Data

Table 3.2. Number of produce or salad item contaminated with more than 10 norovirus particles when a manual tap was used for handwashing.

Source of contamination	Item at risk (contains >10 norovirus particles)	Number of servings contains >10 noroviruses per 800 units [a]				
		Handwashing compliance - Manual				
		0%	30%	50%	70%	100%
Lettuce	Lettuce	398±1	362±4	317±5	285±5	237±5
	Tomato	0	26±4	38±4	45±4	53±4
	Lettuce + Tomato	399±1	373±4	332±5	299±5	253±5
	Final Salad	399±1	401±6	366±7	344±7	304±7
Tomato	Lettuce	0	29±4	37±4	44±4	49±4
	Tomato	800	800	800	800	800
	Lettuce + Tomato	800	800	800	800	800
	Final Salad	800	800	800	800	800
Lettuce Handler	Lettuce	400	441±5	425±5	391±4	342±4
	Tomato	0	96±8	132±8	145±7	156±6
	Lettuce + Tomato	403	477±6	459±6	418±5	368±4
	Final Salad	403	579±10	599±10	613±10	613±11
Tomato Handler	Lettuce	0	97±8	131±8	140±7	153±6
	Tomato	400	444±5	424±5	394±4	345±4
	Lettuce + Tomato	403	483±7	460±6	420±5	371±4
	Final Salad	403	589±10	602±10	608±10	617±11
Mixing Employee	Lettuce	0	158±9	263±9	327±9	404±8
	Tomato	0	155±9	255±10	329±9	397±8
	Lettuce + Tomato	0	263±10	396±9	477±8	547±6
	Final Salad	800	800	800	800	800
Counter Staff	Lettuce	0	102±8	149±8	154±7	155±6
	Tomato	0	105±8	144±8	152±7	151±6
	Lettuce + Tomato	0	174±11	231±9	231±7	219±6
	Final Salad	400	609±10	651±10	660±9	648±10

[a] Expressed as mean (95% confidence interval)

Table 3.3. Number of times a restaurant employee would carry more than 10 norovirus particles at the end of shift when a manual tap was used for handwashing. Simulation results vary between 0-2 employees per job type, for each of 1000 simulations. Results were multiplied by 1000 for ease of comparison.

Source of contamination	Employee at risk (carries >10 norovirus particles)	Number of employees who carried >10 noroviruses per 2000 simulated employees [a]				
		Handwashing compliance - Manual				
		0%	30%	50%	70%	100%
Lettuce	Lettuce Handler	1000	1042±20	842±30	651±30	346±30
	Tomato Handler	0	346±30	410±30	354±30	218±30
	Mixing Employee	0	621±50	852±60	1107±60	1214±60
	Counter Staff	0	450±40	497±40	428±30	271±30
Tomato	Lettuce Handler	0	380±30	441±30	383±30	239±30
	Tomato Handler	1000	1042±20	842±30	678±30	344±30
	Mixing Employee	0	685±50	885±60	1076±60	1246±50
	Counter Staff	0	491±40	508±40	468±30	284±30
Lettuce Handler	Lettuce Handler	1000	1313±30	1259±30	1107±20	1058±10
	Tomato Handler	0	676±50	840±40	820±30	801±30
	Mixing Employee	0	901±50	1366±50	1646±40	1834±30
	Counter Staff	0	1019±50	1229±50	1328±40	1293±40
Tomato Handler	Lettuce Handler	0	690±50	883±40	849±30	798±30
	Tomato Handler	1000	1327±30	1242±30	1104±20	1064±20
	Mixing Employee	0	947±50	1346±50	1645±40	1830±30
	Counter Staff	0	1062±50	1248±50	1304±40	1307±40
Mixing Employee	Lettuce Handler	0	1036±50	1527±40	1734±30	1873±20
	Tomato Handler	0	1033±50	1482±40	1725±30	1892±20
	Mixing Employee	2000	2000	2000	2000	2000
	Counter Staff	2000	2000	2000	2000	2000
Counter Staff	Lettuce Handler	0	686±50	953±40	915±30	874±30
	Tomato Handler	0	672±50	929±40	910±40	887±30
	Mixing Employee	0	887±50	1365±50	1654±40	1808±30
	Counter Staff	1000	1583±30	1630±30	1572±30	1501±30

[a] Expressed as mean (95% confidence interval)

Table 3.4. Number of times a restaurant employee would carry more than 10 norovirus particles at the end of shift when a hands-free faucet was used for handwashing. Simulation results vary between 0-2 employees per job type, for each of 1000 simulations. Results were multiplied by 1000 for ease of comparison.

Source of contamination	Employee at risk (carries >10 norovirus particles)	Number of employees who carried >10 noroviruse per 2000 simulated employees [a]				
		Handwashing compliance - Hands-free				
		0%	30%	50%	70%	100%
Lettuce	Lettuce Handler	1000	819±20	598±30	405±30	185±20
	Tomato Handler	0	0	0	0	0
	Mixing Employee	0	0	0	0	0
	Counter Staff	0	0	0	0	0
Tomato	Lettuce Handler	0	0	0	0	0
	Tomato Handler	1000	820±20	619±30	413±30	208±30
	Mixing Employee	0	0	0	0	0
	Counter Staff	0	0	0	0	0
Lettuce Handler	Lettuce Handler	1000	1000	1000	1000	1000
	Tomato Handler	0	0	0	0	0
	Mixing Employee	0	0	0	0	0
	Counter Staff	0	0	0	0	0
Tomato Handler	Lettuce Handler	0	0	0	0	0
	Tomato Handler	1000	1000	1000	1000	1000
	Mixing Employee	0	0	0	0	0
	Counter Staff	0	0	0	0	0
Mixing Employee	Lettuce Handler	0	0	0	0	0
	Tomato Handler	0	0	0	0	0
	Mixing Employee	2000	2000	2000	2000	2000
	Counter Staff	2000	2000	2000	2000	2000
Counter Staff	Lettuce Handler	0	0	0	0	0
	Tomato Handler	0	0	0	0	0
	Mixing Employee	0	0	0	0	0
	Counter Staff	1000	1000	1000	1000	1000

[a] Expressed as mean (95% confidence interval)

Table 3.5. Number of produce or salad item contaminated with more than 10 norovirus particles when a hands-free faucet was used for handwashing.

Source of contamination	Item at risk (contains >10 norovirus particles)	Number of servings contained >10 noroviruses per 800 units [a]				
		Handwashing compliance - Hands-free				
		0%	30%	50%	70%	100%
Lettuce	Lettuce	398	338±5	294	257	214±5
	Tomato	0	0	0	0	0
	Lettuce + Tomato	399	339±5	295	258	216±5
	Final Salad	399	339±5	295	258	216±5
Tomato	Lettuce	0	0	0	0	0
	Tomato	800	800	800	800	800
	Lettuce + Tomato	800	800	800	800	800
	Final Salad	800	800	800	800	800
Lettuce Handler	Lettuce	400	386±2	360±4	329±5	282±5
	Tomato	0	0	0	0	0
	Lettuce + Tomato	403	388±2	362±4	330±5	283±5
	Final Salad	403	388±2	362±4	330±5	283±5
Tomato Handler	Lettuce	0	0	0	0	0
	Tomato	400	385±2	361±4	332±5	287±5
	Lettuce + Tomato	403	387±2	363±4	333±5	288±5
	Final Salad	403	387±2	363±4	333±5	288±5
Mixing Employee	Lettuce	0	0	0	0	0
	Tomato	0	0	0	0	0
	Lettuce + Tomato	0	0	0	0	0
	Final Salad	800	800	800	800	800
Counter Staff	Lettuce	0	0	0	0	0
	Tomato	0	0	0	0	0
	Lettuce + Tomato	0	0	0	0	0
	Final Salad	400	391±2	373±3	346±4	303±5

[a] Expressed as mean (95% confidence interval)

Table 3.6. Number of produce or salad item contaminated with more than 10 norovirus particles when lettuce/tomato was the original source of contamination and knife and cutting board/slicer was replaced after cutting every 100 lettuce/tomato units.

a. A manual tap was used for handwashing

Source of contamination	Item at risk (contains >10 norovirus particles)	Number of servings contained >10 noroviruses per 800 units [a]				
		0%	30%	50%	70%	100%
Lettuce	Lettuce	398±1	362±4	317±5	285±5	237±5
	Tomato	0	26±4	38±4	45±4	53±4
	Lettuce + Tomato	399±1	373±4	332±5	299±5	253±5
	Final Salad	399±1	401±6	366±7	344±7	304±7
Tomato	Lettuce	0	29±4	37±4	44±4	49±4
	Tomato	398±1	360±4	322±5	291±5	243±5
	Lettuce + Tomato	399±1	373±5	336±5	307±5	260±5
	Final Salad	399±1	405±7	371±7	349±7	312±8

[a] Expressed as mean (95% confidence interval)

b. A hands-free faucet was used for handwashing

Source of contamination	Item at risk (contains >10 norovirus particles)	Number of servings contained >10 noroviruses per 800 units [a]				
		0%	30%	50%	70%	100%
Lettuce	Lettuce	398±1	338±5	294±6	257±6	214±5
	Tomato	0	0	0	0	0
	Lettuce + Tomato	399±1	339±5	295±6	258±6	216±5
	Final Salad	399±1	339±5	295±6	258±6	216±5
Tomato	Lettuce	0	0	0	0	0
	Tomato	398±1	337±5	296±6	259±6	216±5
	Lettuce + Tomato	399±1	339±5	298±6	261±6	217±5
	Final Salad	399±1	339±5	298±6	261±6	217±5

[a] Expressed as mean (95% confidence interval)

Table 3.7. Number of times a restaurant employee would carry more than 10 norovirus particles at the end of shift when lettuce/tomato was the original source of contamination and knife and cutting board/slicer was replaced after cutting every 100 lettuce/tomato units. Simulation results vary between 0-2 employees per job type, for each of 1000 simulations. Results were multiplied by 1000 for ease of comparison.

a. A manual tap was used for handwashing

Source of contamination	Employee at risk (carries >10 norovirus particles)	Number of employees who carried >10 noroviruses per 2000 simulated employees [a]				
		0%	30%	50%	70%	100%
Lettuce	Lettuce Handler	1000	1042±20	842±30	651±30	346±30
	Tomato Handler	0	346±30	410±30	354±30	218±30
	Mixing Employee	0	621±50	852±60	1107±60	1214±60
	Counter Staff	0	450±40	497±40	428±30	271±30
Tomato	Lettuce Handler	0	380±30	441±30	383±30	239±30
	Tomato Handler	1000	1042±20	842±30	678±30	344±30
	Mixing Employee	0	685±50	885±60	1076±60	1246±50
	Counter Staff	0	491±40	508±40	468±30	284±30

[a] Expressed as mean (95% confidence interval)

b. A hands-free faucet was used for handwashing

Source of contamination	Employee at risk (carries >10 norovirus particles)	Number of employees who carried >10 noroviruses per 2000 simulated employees [a]				
		0%	30%	50%	70%	100%
Lettuce	Lettuce Handler	1000	819±20	598±30	405±30	185±20
	Tomato Handler	0	0	0	0	0
	Mixing Employee	0	0	0	0	0
	Counter Staff	0	0	0	0	0
Tomato	Lettuce Handler	0	0	0	0	0
	Tomato Handler	1000	820±20	619±30	413±30	208±30
	Mixing Employee	0	0	0	0	0
	Counter Staff	0	0	0	0	0

[a] Expressed as mean (95% confidence interval)

Table 3.8. Number of produce or salad item contaminated with more than 10 norovirus particles when knife and cutting board/slicer was changed after handling 100 lettuce/tomato units and tongs were changed after every 100 salad boxes.

Source of contamination	Item at risk (contains >10 norovirus particles)	Number of servings contained >10 noroviruses per 800 units [a]				
		Handwashing compliance - Manual				
		0%	30%	50%	70%	100%
Lettuce	Lettuce	398±1	362±4	317±5	285±5	237±5
	Tomato	0	26±4	38±4	45±4	53±4
	Lettuce + Tomato	399±1	373±4	332±5	299±5	253±5
	Final Salad	399±1	385±5	343±5	311±5	264±5
Tomato	Lettuce	0	29±4	37±4	44±4	49±4
	Tomato	398±1	360±4	322±5	291±5	243±5
	Lettuce + Tomato	399±1	373±5	336±5	306±5	243±5
	Final Salad	399±1	386±5	348±5	318±5	271±5
Lettuce Handler	Lettuce	400	441±5	425±5	390±4	341±4
	Tomato	0	96±8	132±8	144±7	155±6
	Lettuce + Tomato	403	475±6	457±6	416±4	366±4
	Final Salad	403	521±8	489±7	437±5	382±4
Tomato Handler	Lettuce	0	97±8	131±8	140±7	152±6
	Tomato	400	444±5	424±5	394±4	344±4
	Lettuce + Tomato	400	481±7	459±6	418±5	369±4
	Final Salad	400	527±8	490±7	440±5	386±4
Mixing Employee	Lettuce	0	99±7	141±7	159±7	181±6
	Tomato	0	97±8	136±8	157±7	175±6
	Lettuce + Tomato	0	170±10	224±9	243±7	254±5
	Final Salad	400	527±7	502±7	465±5	423±3
Counter Staff	Lettuce	0	102±8	148±8	153±7	154±6
	Tomato	0	104±8	144±8	151±7	150±6
	Lettuce + Tomato	0	173±11	230±9	229±7	217±6
	Final Salad	400	555±9	536±8	482±7	421±5

[a] Expressed as mean (95% confidence interval)

Table 3.9. Number of times a restaurant employee would carry more than 10 norovirus particles at the end of shift when knife and cutting board/slicer was changed after handling 100 lettuce/tomato units and tongs were changed after every 100 salad boxes. Simulation results vary between 0-2 employees per job type, for each of 1000 simulations. Results were multiplied by 1000 for ease of comparison.

Source of contamination	Employee at risk (carries >10 norovirus particles)	Number of employees who carried >10 noroviruses per 2000 simulated employees [a]				
		Handwashing compliance - Manual				
		0%	30%	50%	70%	100%
Lettuce	Lettuce Handler	1000	1042±20	842±30	650±30	345±30
	Tomato Handler	0	346±30	410±30	354±30	214±30
	Mixing Employee	0	313±30	354±30	344±30	211±30
	Counter Staff	0	425±40	460±30	389±30	213±30
Tomato	Lettuce Handler	0	377±30	440±30	383±30	239±30
	Tomato Handler	1000	1041±20	841±30	677±30	343±30
	Mixing Employee	0	347±40	346±30	342±30	235±30
	Counter Staff	0	458±40	457±30	411±30	229±30
Lettuce Handler	Lettuce Handler	1000	1311±30	1240±30	1068±20	1008±10
	Tomato Handler	0	673±50	820±40	775±30	706±30
	Mixing Employee	0	648±40	795±40	818±30	806±30
	Counter Staff	0	907±50	996±40	922±30	709±30
Tomato Handler	Lettuce Handler	0	683±40	866±40	798±30	720±30
	Tomato Handler	1000	1324±30	1220±30	1070±20	1008±10
	Mixing Employee	0	681±40	787±40	839±30	834±20
	Counter Staff	0	944±50	1009±40	931±30	746±30
Mixing Employee	Lettuce Handler	0	799±40	976±40	933±30	898±20
	Tomato Handler	0	800±40	959±40	946±30	922±20
	Mixing Employee	1000	1338±30	1188±20	1075±20	1012±10
	Counter Staff	1000	1484±30	1302±30	1123±20	987±10
Counter Staff	Lettuce Handler	0	681±50	931±40	851±30	797±30
	Tomato Handler	0	667±50	904±40	865±30	796±30
	Mixing Employee	0	691±50	868±40	895±30	878±20
	Counter Staff	1000	1483±30	1381±30	1162±20	1019±10

[a] Expressed as mean (95% confidence interval)

OVERALL CONCLUSION

Norovirus can be transferred through multiple routes through direct contact and cross-contamination during food preparation. Our study on the dissemination of norovirus from a single tomato to a series of clean tomatoes through a hand-operated slicer showed a descending trend on the number of virus particles in cross-contaminated tomatoes. As the number of tomatoes being sliced increased, a nonlinear regression model could estimate the number of norovirus particles transferred to each clean tomato: Y (log PCR-U per tomato) = - 0.903*ln (X, tomato order number) + 7.945 (log PCR-U inoculums), $R^2 = 0.91$. The tomato slicing study illustrated how extensive norovirus cross-contamination can be during food preparation in a commercial establishment. The nonlinear model developed is valuable for risk assessment and prediction.

Our study on simulating norovirus transmission allowed for the estimation of the overall effect of cross-contamination in a food service establishment. The results showed that norovirus transmission can be affected by multiples factors, including the initial source of contamination, handwashing efficiency, the type of tap provided for handwashing, as well as the frequency of utensil replacement. Our study showed that any single factor alone many not necessarily reduce risk of norovirus transfer. Multiple controls should be considered for reducing the spread of norovirus but even when multiple controls are used, risk of contamination is not eliminated. This study provided the preliminary scientific basis needed to develop risk management strategies, which may help reducing public health risk caused by norovirus infection.

Appendices

Appendix 1: Transfer rate distributions generated using raw data provided by our collaborators from the Illinois Institute of Technology (IIT)

Statistical distributions were generated using Arena output analyzer for the purpose of incorporating transfer parameters for the simulation model development.

Transfer rate from lettuce to hand

Volunteer	Transfer %
201107042	0.88
201107043	1.42
201107002	2.25
201107003	4.33
201107005	0.56
201107006	0.53
201107010	1.38
201107011	1.83
201107013	1.52
201107014	2.09
201107015	3.73
201107017	0.82
201107018	0.22
201107019	0.30
201107023	0.51
201107024	2.01
201107025	1.38

Distribution: BETA(0.915, 2.1039)

Transfer rate from hand to lettuce

Volunteer	Transfer %
201107010	2.34
201107011	0.64
201107013	0.14
201107014	0.23
201107015	1.21
201107016	1.23
201107017	1.44
201107018	2.30
201107019	1.52
201107023	1.63
201107024	5.11
201107025	4.78

201107026	2.79
201107027	1.09
201107028	0.45
201107029	0.30
201107030	0.10
201107031	2.21
201107032	1.48
201107033	3.66
201107034	0.99

Distribution: BETA(0.924, 2.23646)

Transfer rate from lettuce to knife

Volunteer	Transfer %
201107010	0.19
201107011	0.30
201107013	1.08
201107014	0.65
201107015	0.29
201107017	1.31
201107018	2.06
201107019	2.31
201107023	0.16
201107024	1.19
201107025	0.41
201107027	0.31
201107028	0.63
201107030	0.61
201107032	7.95
201107033	4.33
201107034	5.85
201107035	1.61
201107036	2.39
201107037	0.88
201107040	6.49
201107041	2.32
201107042	3.22

Distribution: BETA(0.732, 2.26599)

Transfer rate from knife to lettuce

Volunteer	Transfer %
201113061	279
201113064	132

201113133	103
201113195	176
201108171	117
201108172	137
201108173	40
201108174	80
201108261	41
201108262	132
201108263	104
201108301	74

Distribution: TRIA(0.34, 0.935, 1)

Transfer rate from lettuce to cutting board

Volunteer	Transfer %
201110281	1.39
201110282	2.17
201110283	0.98
201110284	0.11
201111011	0.92
201111012	0.12
201111013	0.08
201111014	0.23
201111181	0.09
201111182	0.28
201111183	0.31
201111184	2.33
201111221	0.71
201111222	2.53
201111223	0.32
201111224	0.70
201111225	1.19

Distribution: BETA(1.45, 2.56243)

Transfer rate from cutting board to lettuce

Volunteer	Transfer %
201113061	55.28
201113064	39.42
201113133	8.40
201108261	87.49
201108262	30.35
201108301	14.30
201109074	1.61

201109201	91.13
201109202	33.85

Distribution: BETA(0.765, 1.10613)

Transfer rate from tap to hand

Volunteer	Transfer %
201006006	19.59
201006006	54.14
201007002	27.64
201007002	10.00
201007005	79.60
201007005	28.60
201007009	22.80
201007009	27.37
201007017	57.21
201007017	90.43
201007026	6.67
201007028	45.26
201007028	27.02
201007035	13.01
201007035	12.62
201107020	39.55
201107021	6.67
201107022	11.38

Distribution: BETA(1.15, 2.16814)

Transfer rate from hand to tap

Volunteer	Transfer %
201006005	0.34
201007001	0.06
201007006	0.79
201007014	0.46
201007018	0.69
201007025	1.36
201007027	0.48
201007034	0.70
201007041	3.57
201007000	2.48
201007001	1.11
201007004	0.63
201007009	0.65

201007012	0.20

Distribution: BETA(0.867, 2.36018)

Appendix 2: Norovirus Cross-contamination during Preparation of Fresh Produce

Stephen F. Grove[1]*, Annamalai Suriyanarayanan[1], Balasubramanyam Puli[1], Heng Zhao[1], Mingming Li[1], Di Li[2], Don Schaffner[2], and Alvin Lee[1],

[1]Institute for Food Safety and Health, Illinois Institute of Technology, 6502 S. Archer Road, Bedford Park, IL 60501-1957, USA

[2]Rutgers University, New Brunswick, NJ 08901-8520, USA

* Corresponding author: Institute for Food Safety and Health, 6502 S. Archer Road, Bedford Park, IL 60501-1957, USA; E-mail: sgrove@iit.edu; Tel: (708) 563-8176; Fax: (708) 563-8176

Abstract

Infection with norovirus (NoV) is considered the most common cause of foodborne illness in the U.S. Foodborne NoV outbreaks may result from consumption of food contaminated by an infected food handler in the foodservice environment, in which bare-hand contact, inadequate cleaning, and gloved hand contact are common contributing factors.

The goal of this study was to examine cross-contamination of a NoV surrogate, murine norovirus (MNV-1), during common procedures used in preparation of fresh produce in a food service setting.

Introduction

Infection with human norovirus (HuNoV) is considered the most common cause of foodborne illness in the U.S. (CDC, 2013; Scallan et al., 2011). Many outbreaks of foodborne HuNoV are associated with the consumption of salads, sandwiches, and fresh produce (Widdowson et al., 2005). HuNoV outbreaks often involve the preparation of food by an infected food handler in the foodservice environment, in which bare-hand contact, inadequate cleaning, and gloved hand contact have been identified as common contributing factors (CDC, 2006).

Fresh fruits and vegetables have been implicated as vehicles for HuNoV (Ponka *et al.*, 1999; Schmid *et al.*, 2007); in fact the pathogen-commodity pair of HuNoV and leafy vegetables was attributed to the highest number of outbreak-related illnesses (4,011) reported to CDC between 1998 and 2008 (CDC, 2013). Preparation of fresh fruits and vegetables usually involves considerable human contact, including handling, chopping/slicing and mixing, and since these foods are often consumed raw, there is often no effective pathogen reduction steps prior to consumption.

In foodservice environments such as restaurants, large amounts of food may be prepared in a relatively confined area, involving the interaction of multiple employees. People ill with HuNoV may excrete large numbers of virions via feces and vomit during each episode, and therefore the potential exists for the contamination to spread throughout the food preparation area and large numbers of people fall ill.

The transfer of norovirus by hands and/or during food preparation has been investigated on few occasions, using either cultivatable surrogate viruses for HuNoV or bacteriophages when hand contact is involved (Bidawid et al., 2004; Julian et al., 2010), and HuNoV itself for transfer between inanimate objects (D'Souza et al., 2006; Escudero et al., 2012; Stals et al., 2013). These studies examined transfer resulting from contact between objects at a standardized level of force to ensure consistency between replicates. To date, there is a lack of studies investigating the variability in virus transfer between a variety of volunteer subjects performing standardized food preparation tasks. The purpose of this study was to examine cross-contamination of NoV during common procedures used in preparation of fresh produce in a food service setting. A volunteer study was conducted to capture the inherent variability of virus transfer observed between participants, without the concern of keeping pressure of contact, tightness of grip, etc. constant. This variability is important for consideration when developing a quantitative risk assessment.

Materials and methods

Virus and cell culture propagation. Murine norovirus (MNV-1), provided by Dr. Herbert W. Virgin, Washington University, St. Louis MO, was used in this study as a surrogate for the unculturable HuNoV, due to the close genetic traits they share (Wobus *et al.,* 2006). MNV-1 was propagated by infecting a monolayer of RAW 264.7 cells (American Type

Culture Collection (ATCC), Manassas VA; ATCC number TIB-71™) at approximately 90% confluency, grown in Dulbecco's Modified Essential Medium (DMEM; Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS; Life Technologies). Infected cells were incubated at 37°C, 5% CO_2 until complete cytopathic effect was observed (approx. 72 h). The virus-containing media underwent three freeze-thaw cycles between -80°C and 4°C to release virions from infected cells, and centrifuged at 3,000 x*g* for 20 min to pellet debris. Virus-containing supernatant was transferred into filter units (Amicon® Ultra; EMD Millipore Corporation, Billerica, MA) and centrifuged at 2,600 x*g* for 15 min to concentrate the virus stock between 10 and 15 x (final titer of approximately 8.0-log plaque-forming units (PFU)/mL). Concentrated virus stock was filtered through a 0.2 um membrane syringe filter and frozen at -80°C prior to use.

Infectious MNV-1 was quantified in all samples by plaque assay in 6-well plates containing overnight confluent monolayers of RAW 246.7 cells. Each well was inoculated with 0.5 ml sample and incubated for one hour to promote virus attachment. Inoculum was then removed and 2 ml overlay (1:1 mixture of 2 x Minimal Essential Media (MEM; Life Technologies) containing 10% new-born calf serum (Life Technologies) and 3% low melting point agarose (Lonza, Allendale, NJ) was added. After 48 h incubation at 37°C and 5% CO_2, a second overlay was added, containing 0.003% neutral red stain (Sigma-Aldrich, St. Louis, MO), in order to visualize plaques, which were then counted at 72 h post-infection.

Study participants. Approximately 80 volunteers participated in this study to perform the various transfer scenarios (Figure 1). The volunteers, who were staff and students located at the Institute for Food Safety and Health, were asked a series of questions regarding their

current overall health, and checked for any obvious cuts, welts or abrasions on their hands prior to participating in the study. Each participant signed a consent form (approved by the institutional review board at Illinois Institute of Technology and the Research Involving Human Subjects Committee at the Food and Drug Administration) prior to participating in the study. In total, 150 individual transfer events were performed by the 80 participants, in order to achieve at least 10 independent replicates per transfer event.

Transfer of MNV-1 between contaminated spigots and bare hands. The surface of a stainless steel spigot was inoculated with MNV-1 by spotting 0.1 ml of the inoculum on the handle surface with a pipette. After air drying for 30 min in a biological safety cabinet, a volunteer was instructed to 'turn on' the tap once, and 'turn off' the handle once with one bare hand. The volunteer's now-contaminated hand was tested for MNV-1 by the glove juice method, modified from Casanova *et al*. (2008). A clean vinyl glove was filled with 25 ml DMEM + 10% FBS (5 ml per finger), and a participant's hand was inserted into the glove, taking care not to contaminate the glove's outer surface. Recovery was performed by massaging the hand for 2 min (1 min massage of fingers, 1 min massage of palm) to detach virions from the surface and into the eluent.

To measure the transfer in the opposite direction, the palm and fingers of a volunteer's two hands were contaminated with MNV-1. After air drying for 10 min in a biological safety cabinet, one hand was used to 'turn on' and then 'turn off' the spigot. The handle was swabbed to measure MNV-1 transfer, and the second inoculated hand was tested for initial MNV-1 levels by the glove juice method.

Effectiveness of handwashing as an intervention step. Two commonly-available soaps were purchased from a local retail store. One was a liquid soap and the other was a foaming

soap. The palm and fingers of a volunteer's left hand was inoculated with 0.1 ml (approximately 6-log PFU/hand) MNV-1 and air-dried in a biological safety cabinet for 10 min. The participants then washed their hands by either rinsing hands for 5 s under running tap water; washing hands for at least 20 s with the liquid soap; or washing hands for at least 20 s with the foaming soap. After reaching the minimum time requirement, participants were instructed to stop washing once their hands felt clean.

The three handwashing scenarios tested were designed to compare the differences between a brief rinse of hands with running water and handwashing that complied with the requirements in the FDA 2009 Food Code (FDA, 2009).

Soap was rinsed from participants' hands using tap water, and then hands were dried thoroughly with paper towels. Virions remaining on each hand after washing were recovered using the glove juice method.

Transfer of MNV-1 between Romaine lettuce and gloved and ungloved hands. The palm and fingers of bare or gloved (polyvinyl, vinyl or nitrile gloves; Daydots, Fort Worth, TX) hands were inoculated with 0.1 ml (approximately 6-log PFU/hand) MNV-1 and air-dried in a biological safety cabinet for 10 min.

Romaine lettuce hearts were purchased from a local market, and leaves were cut into squares of approximately 5 x 5 cm. Approximately 5 g lettuce leaf pieces (containing 4-5 pieces) were chopped with a sterile stainless steel knife on a sterile cutting board (standardized using 4 horizontal cuts and 4 vertical cuts), and then all diced pieces were removed from the board by the volunteer with one inoculated hand/glove and transferred to a stomacher bag for analysis. The diced lettuce was diluted in 25 ml DMEM + 10% FBS and homogenized by stomaching at 230 RPM for 1 min.

To measure transfer from lettuce to hands/gloves, lettuce squares of approximately 5 x 5 cm were inoculated with 0.025 ml each, and dried in a biosafety cabinet for 10 min. These inoculated squares (5 g; 4-5 pieces) were chopped with a sterile stainless steel knife on a sterile cutting board as described, and then all diced pieces were removed from the board by clean gloved or ungloved hands. Hands/gloves were then sampled for transferred virus by the glove juice method.

Transfer of MNV-1 to knife and cutting board during chopping of Romaine lettuce. After inoculated Romaine lettuce was chopped as described above, the amount of MNV-1 that transferred to the knife blade and cutting board was determined by swabbing those surfaces with a sterile sponge soaked in 5ml DMEM. Virions were extracted from the sponge by stomaching in 45 ml DMEM for 1.5 min, and quantified by plaque assay.

In some trials, either the knife or board, contaminated after chopping the inoculated lettuce, was used to chop 5 g piles of Romaine lettuce squares as described. The diced lettuce was aseptically transferred to a stomacher bag, diluted in 25 g DMEM and homogenized by stomaching for 1 min as described.

Results

MNV-1 transfer data from all scenarios examined were log-transformed to obtain normally distributed data suitable for statistical analysis.

Transfer of MNV-1 between contaminated spigot and bare hands. MNV-1 transfers more readily from a contaminated spigot to a clean hand (35 ± 25%) compared to transfer from a contaminated hand to a clean spigot (0.6 ± 0.4%; Figure 2). In addition, the distribution of log transfer % appears to be wider, and thus more variable, for transfer to the spigot than from the spigot (Figure 3). The concentration of MNV-1 recovered from the hands of

participants after handling the contaminated spigots ranged from 4.81-log to 6.45-log PFU/hand, while between 3.72-log and 5.92-log PFU/spigot was transferred to the sterile spigot after handling by a contaminated hand.

Effectiveness of handwashing as an intervention step. Reductions of 1.8- to 4.1-log were achieved by rubbing contaminated hands under water for at least 5 s, while washing hands for at least 20 s achieved reductions of 2.3- to 3.7-log with liquid soap, and 2.5- to 3.9-log with foaming soap (Figure 4). No significant difference ($p>0.05$) between these three methods was observed.

Transfer of MNV-1 between Romaine lettuce and gloved and ungloved hands. No significant difference ($p>0.05$) between bare and gloved hands was observed in log transfer % of MNV-1 from Romaine lettuce (Figure 5a). In contrast, transfer of MNV-1 to Romaine lettuce was affected by glove type, with a greater average transfer of MNV-1 ($p<0.05$) recorded from vinyl gloves than bare hands and the other glove types (Figure 5a). Distribution plots indicate the frequency with which transfer coefficients were recorded (Figure 6), with variability in transfer distributions appearing to be larger for transfers involving nitrile gloves and bare hands compared to the other glove types.

Transfer of MNV-1 during chopping of Romaine lettuce. During the chopping of Romaine lettuce, MNV-1 transfer coefficient was lower in the direction from contaminated lettuce to polyethylene chopping board ($p<0.05$) than from a contaminated chopping board to lettuce (Figure 7). This same trend was observed between the stainless steel knife blade and lettuce, with significantly more ($p<0.05$) MNV-1 being transferred to lettuce from the contaminated blade than in the reverse direction. Greater range of distributions was noted

in transfers between Romine lettuce and knife blade during chopping, than compared to transfers between lettuce and cutting board (Figure 8).

Discussion

MNV-1 transferred more readily from a contaminated spigot to a clean hand compared to transfer from hand to clean spigot. This finding appears to indicate a higher affinity of MNV-1 for human hands than for the smooth stainless steel surface of the spigot. This finding was not observed for transfers between lettuce and bare hands, where no difference was observed in the log transfer % between each direction.

During the chopping of Romaine lettuce, MNV-1 transferred more readily from the knife blade or cutting board to lettuce than from lettuce, indicating that once present on surfaces used during chopping, noroviruses may spread widely to additional fresh produce under preparation. The material of the gloves worn by food service staff also affect norovirus transfer, with greater transfer coefficients for MNV-1 observed from vinyl gloves to Romaine lettuce than from nitrile gloves and bare hands. Vinyl gloves are commonly used in foodservice establishments, but their relatively smooth surface may not promote strong viral attachment, thus promoting the transfer to other surfaces.

The cutting board is a more constant object during chopping than the knife, which introduces random contact events (each slice) with the Romaine lettuce. This would be expected to affect transfer, and contribute to the variation that was observed.

The variability in transfer observed is a reflection of the use of different volunteers, performing the various tasks in a manner that feels ‘normal’ to them (e.g. tight or loose grip). Despite the variability, the results indicate the high potential for norovirus spread through a food service setting.

Bidawid *et al.* (2004) studied enteric virus transfer during food preparation using feline calicivirus (FCV) as a surrogate virus for HuNoV. Transfer between hands and ham, lettuce and stainless steel was investigated, by pressing inoculated volunteer fingertips onto each surface for 10 s at a measured pressure. Up to 46, 18, and 13% of the FCV inoculum was transferred to ham, lettuce and stainless steel, respectively; greater transfer coefficients than those observed in the current study. This may be attributable to the variable nature of volunteer tasks performed in the current study, as opposed to maintaining consistency by measuring pressures of contact.

The use of FCV as a surrogate to NoV has come into question due to its sensitivities to pH extremes and inability to persist on environmental surfaces for extended periods of time (Cannon *et al*., 2006). A similar caution must be used when interpreting results for MNV-1 transfer, but advantages of using MNV-1 as a NoV surrogate include its similarity in genetic relatedness, persistence in the environment, and stability against sanitizers. Handwashing is effective in removing >2.5-log MNV-1, but does not remove viral contamination completely. Millions of virions can be released during vomiting and diarrhea events, yet less than 100 may be all that is required to initiate infection. MNV-1 was always detected on contaminated hands after washing, indicating that a risk of transferring virions even after washing hands exists, and additional controls to limit norovirus spread during food preparation are needed.

Washing hands with either of the soaps in this study did not reduce levels of MNV-1 on the hands significantly more than rinsing inoculated hands under running tap water for at least 5 sec. The Food Code lists requirements for hand washing, and a number of studies have investigated factors related to improvements in hand hygiene compliance. Allwood *et al*.

(2004) and Green *et al*. (2007) reported the benefits of training to increase the rate of hand washing by food handlers. Green *et al*. (2006) reported that food handlers have on average 8.6 work activities per hour that should involve handwashing opportunities, while Paez *et al.* (2007) suggested that food preparers and food servers should wash their hands 6 and 11 times per hour, respectively. Glove use reportedly reduced hand washing frequency (Green *et al*., 2006, 2007). Our work highlights the reduction that may be achieved in levels of norovirus on hands as a result of washing, but suggests that additional intervention strategies are needed to adequately prevent the transfer of viruses from hands.

It is well established that handling of food by an ill food worker is the primary contributing factor to viral foodborne illness, especially in foodservice settings (Guzewich and Ross, 1999; CDC (a), 2006). Guzewich and Ross (1999) evaluated risks related to microbiological contamination of ready-to-eat (RTE) food prepared by food preparation workers, as well as interventions to minimize those risks. Exclusion of ill individuals from the work place was a primary intervention identified, as was hand hygiene and prevention of bare-hand contact with RTE foods. It is anticipated that the data collected in this study can be useful in the continued development of risk analyses that may estimate the relative risk or benefits of these and other common food handling practices.

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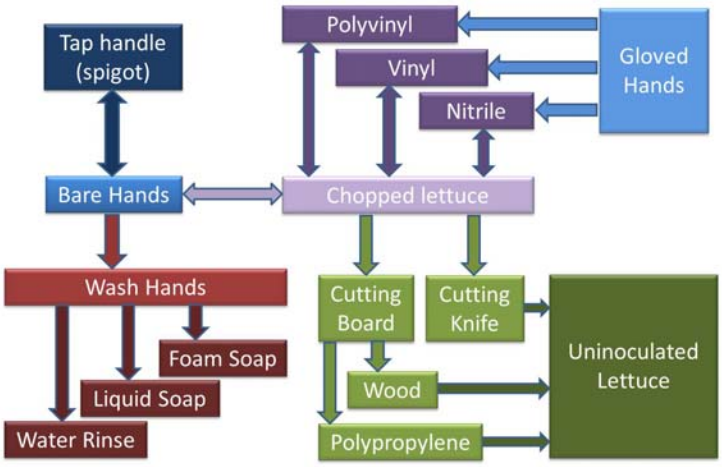


Figure 1. Transfer scenarios investigated during volunteer trials. Arrows point in the direction of the virus transfer tested.

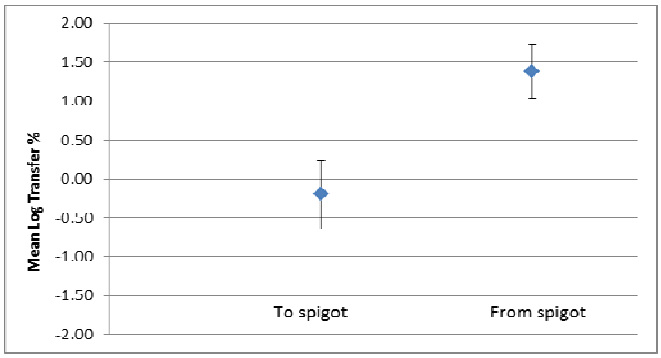
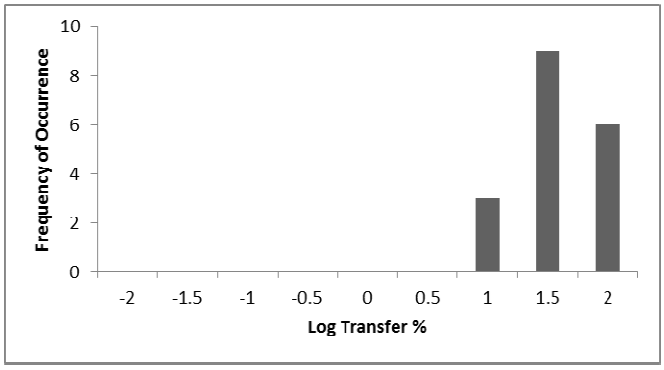


Figure 2. Transfer of MNV-1 between bare hands and tap handle (spigot)

(a)



(b)

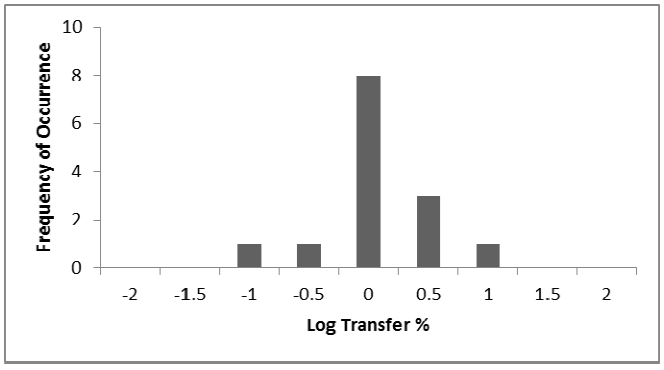


Figure 3. Distribution of MNV-1 log transfer % from (a) spigot to hand, and (b) hand to spigot.

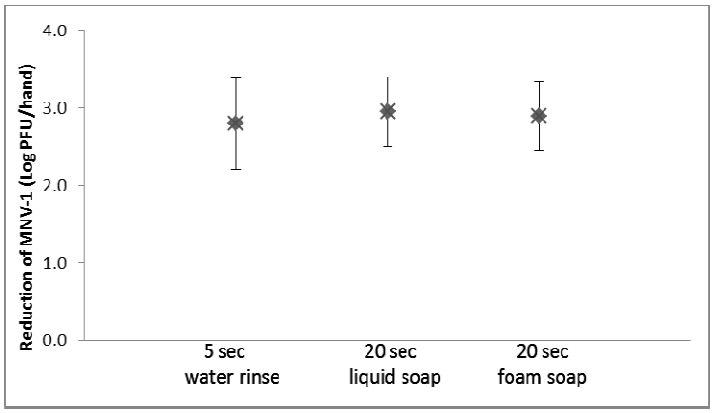
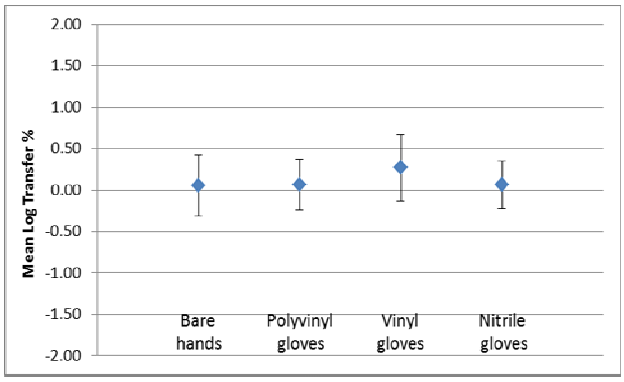


Figure 4. MNV-1 reduction from hands (log PFU/hand) after a water rinse (for at least 5 s), or after washing (for at least 20 s) with either liquid soap or foaming soap.

(a)



(b)

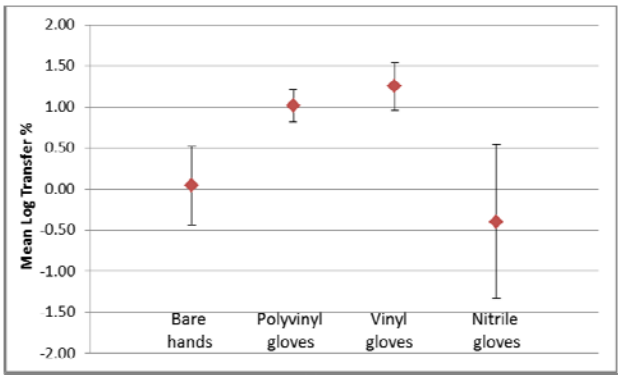
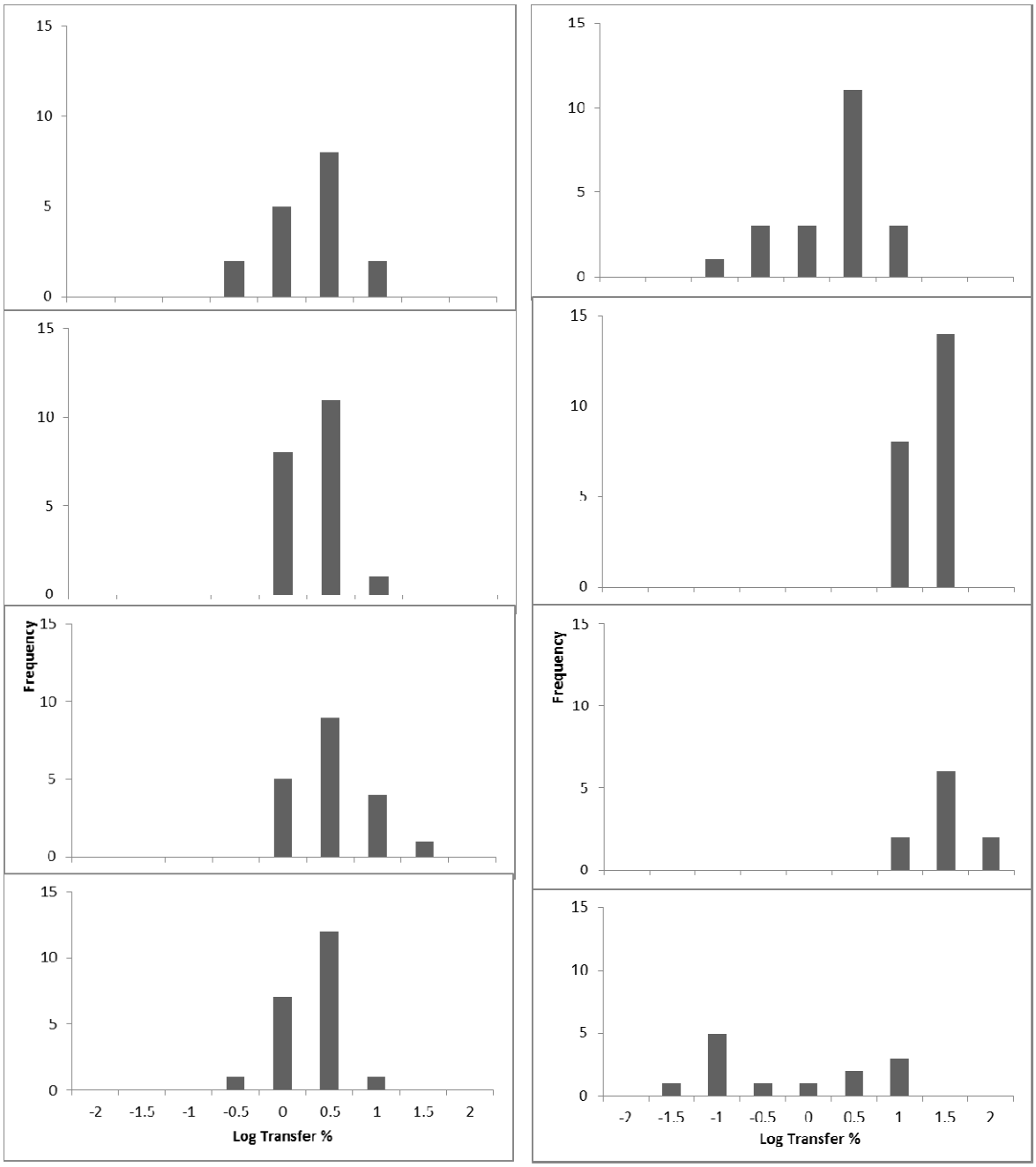


Figure 5. MNV-1 log transfer % from (a) lettuce to bare and gloved hands, and from (b) bare and gloved hands to lettuce.



(a) (b)

Figure 6. Distribution of MNV-1 log transfer % from (a) lettuce to gloved and ungloved hands, and (b) from gloved and ungloved hands to lettuce. From top graph to bottom: bare hands; polyvinyl gloves; vinyl gloves; and nitrile gloves.

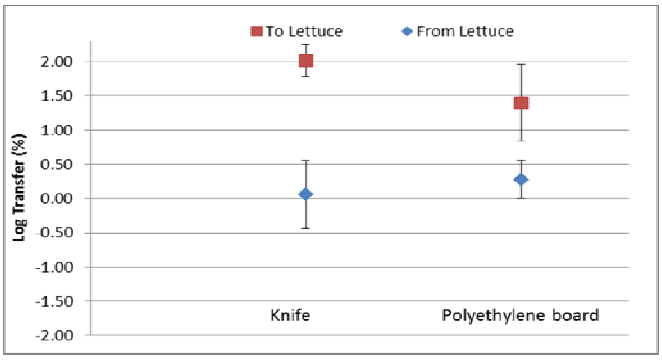


Figure 7. MNV-1 log transfer % from Romaine lettuce to a stainless steel knife and polyethylene cutting board or from a contaminated knife or cutting board to uncontaminated lettuce during chopping.

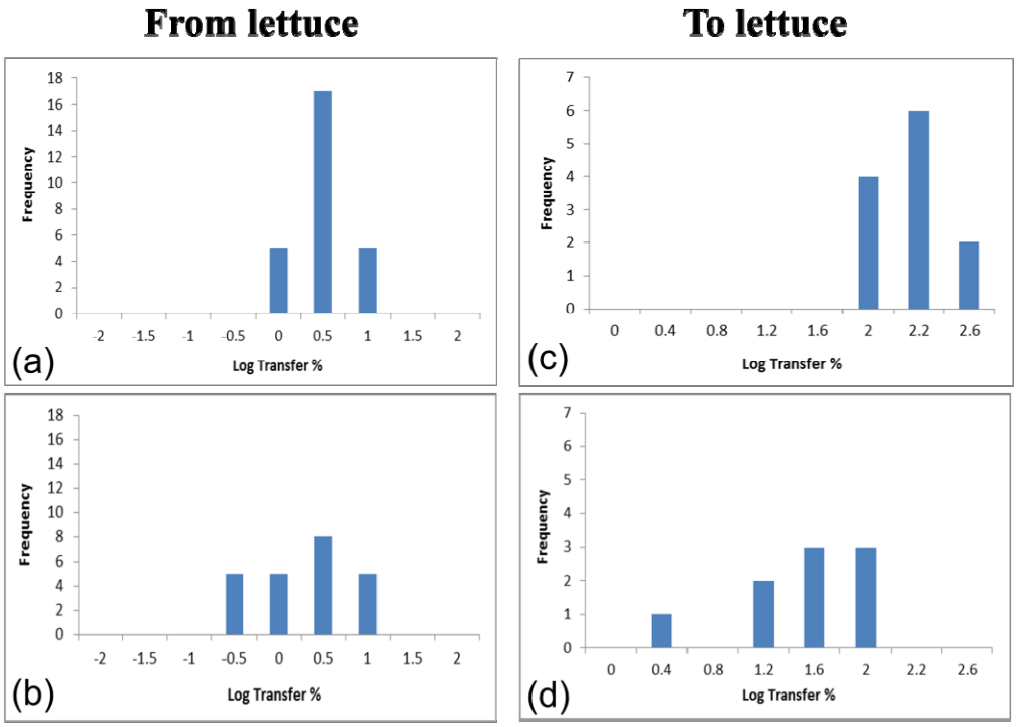


Figure 8. Frequency of MNV-1 transfer from contaminated Romaine lettuce to (a) polyethylene cutting board and (b) stainless steel knife blade, and of MNV-1 transfer from contaminated (c) polyethylene cutting board and (d) stainless steel knife to Romaine lettuce.

CURRICULUM VITA

Di Li

1999 - 2003	B.S. in Biological Sciences, Yunnan University, Kunming, China
2005 – 2007	M.S. in Statistics, Rutgers University, the State University of New Jersey, New Brunswick, NJ
2010 – 2012	M.S. in Food Science, Rutgers University, the State University of New Jersey, New Brunswick, NJ
2004 -2006	Research Assistant in Department of Ecology and Evolution, Rutgers University New Brunswick, NJ
2006 – 2008	Teaching Assistant in Division of Life Sciences, Rutgers University, New Brunswick, NJ
2008 - 2011	Research Assistant in Department of Food Science, Rutgers University, New Brunswick, NJ

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