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HEAT INACTIVATION KINETICS OF TULANE VIRUS AND AICHI VIRUS

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HEAT INACTIVATION KINETICS OF TULANE VIRUS AND AICHI VIRUS

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Sukriti Ailavadi

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DEDICATION

To the loving memory of my grandmothers, Vidyavanti Ailavadi and Nirmal Paul for their trust in me.

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ABSTRACT

Human noroviruses (HNoVs) and Aichi virus (AiV) cause significant numbers of gastrointestinal diseases worldwide. Tulane virus (TV), a cultivable HNoV surrogate, is used to determine control measures against HNoV. The objectives of this study were to determine the heat inactivation kinetics of TV and AiV in cell-culture media and TV in spinach using the first-order and Weibull models. TV and AiV in cell-culture media at ~7 log PFU/ml in 2-ml glass vials were heated at 50-58°C [degree Celsius] up to 10 min in a circulating water-bath. Surviving infectious viruses were enumerated by standard plaque assays using confluent host cells in 6-well plates. First-order model D-values for TV at 52, 54, 56 and 60°C were 4.59±0.02, 2.91±0.01, 1.74±0.41 and 0.58±0.36 min, respectively, with a z-value of 9.09±0.01°C. The Weibull model showed $T_{d=1}$ [thermal decimal reduction time] values of 2.53±0.08, 1.99±0.10, 0.57±0.64 and 0.22±0.25 min, respectively at the same temperatures with a z-value of 6.99°C for cell culture media in 2-ml glass vials. D-values for TV in spinach in vacuum bags were 7.94±0.09, 4.09±0.04 and 1.43±0.07min and a z-value of 10.74±0.01°C by the first-order model and 4.89±0.02, 3.21±0.45 and 0.25±0.38 min for the Weibull model at 50, 54 and 58°C, respectively. TV may not be as suitable a surrogate as MNV-1 for HNoV heat inactivation studies in cell culture media in 2-ml glass vials or spinach in vacuum bags owing to its lower D and z-values (with $D_{50°C}$ of 36.28 min in cell-culture media and 14.57 min in spinach in 2-ml vials). D-values for AiV in 2-ml glass vials at 50, 54, and 58°C from the first-order model were 47.62±1.2, 7.14±1.13 and 2.12±0.04 min, respectively, with a z-value of 5.92°C, while the Weibull model showed $T_{d=1}$ values of 34.53±0.03, 2.59±0.05 and 0.91±0.06 min for the same temperatures, respectively. Thus, both TV and AiV have lower heat resistance than hepatitis A virus ($D_{56°C}$of 8.4 min in buffer in 2-ml glass vials and 8.43 min in spinach in vacuum bags). This study helped in understanding the heat-inactivation kinetics of AiV and TV that provide data for use in thermal processing to ensure food safety.

Keywords: Tulane virus, Aichi virus, Human norovirus, heat inactivation, D-value, z-value

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INTRODUCTION

Human noroviruses (HNoVs) and Aichi virus (AiV), known causative agents of gastroenteritis, have been increasingly associated with foodborne illnesses outbreaks worldwide. Currently, there are no known available vaccines or prescribed antiviral agents for HNoV or AiV infections. Therefore, effective inactivation strategies need to be researched to prevent the spread these viruses. Due to the difficulty in assessing infectivity and the current unavailability of animal models or reproducible cell culture infectivity assays for HNoVs, animal cultivable surrogates such as murine norovirus (MNV-1), feline calicivirus (FCV-F9), porcine enteric sapovirus (PEC), and Tulane virus (TV) are used *(19, 33, 49, 59, 68)*.

Thermal processes have been traditionally and most-widely used to inactivate pathogens in food, thus preserving food and extending the shelf-life. Heat is known to disrupt hydrogen bonding and destroy the structural integrity of proteins, thereby leading to protein denaturation of viral capsid proteins into non-infectious viral subunits and single individual proteins *(22, 107)*. Above 60°C, after the potential destruction of host-cell recognition and binding receptors occur, an alteration of the tertiary protein structure occurs, which facilitates the access of thermal energy to the nucleic acid material *(10)*. For industrial purposes and to understand heat inactivation kinetics of microorganisms, D- and z-values are determined. The D-value is defined as the time at a given temperature necessary to reduce a microbial population present in a defined medium by 90% and is indicative of the thermal resistance of a microorganism at a constant temperature *(13)*. The z-value is the change in temperature required to increase or decrease the D-value by 90% and is indicative of the temperature dependence of microbial inactivation *(13, 19)*. Inversion of the slope of log D versus temperature thus provides the z-value. Earlier research reports are available on cultivable human norovirus surrogates, feline calicivirus (FCV-F9), murine norovirus (MNV-1) as well as hepatitis A virus (HAV) in buffer and in food matrices such as spinach, deli meats and shellfish *(17, 21, 22, 129)*.

TV has similarities to HNoV such as being similar in size, shape and structure (30-35 nm, icosahedral shape, with a positive-sense single-stranded RNA), recognition of histo blood antigen receptors A and B, and disease symptoms *(46)*. Previous studies have shown high resistance of TV as compared to other HNoV

surrogates (MNV-1 or FCV-F9) to different environmental conditions such as pH, high hydrostatic pressure, aq. chlorine *(33)*, making it a suitable surrogate for studying HNoV inactivation approaches.

Owing to the variety of food products associated with foodborne virus contamination, their fecal-oral route of transmission and low infectious dose, there is a vital need for the development of cost-effective inactivation techniques.

The objectives of this study were to:

Objective 1: Determine the heat inactivation kinetics of TV as HNoV surrogate in cell culture media in 2-ml glass vials and in spinach within vacuum bags, with comparison of Linear and Weibull models.

Objective 2: Determine the heat inactivation kinetics of AiV in cell culture media in 2-ml glass vials with comparison of Linear and Weibull models.

Based on these results, the heat-inactivation kinetics would be determined that could be potentially used in industrial settings for appropriate processing of foods to prevent outbreaks.

CHAPTER I

LITERATURE REVIEW

1 FOODBORNE VIRUSES

1.1 Human Noroviruses

Human noroviruses (HNoV) are the leading causative agents of acute gastroenteritis worldwide and are responsible for illness of 1 in every 15 people, with about 56,000-71,000 hospitalizations and 570-800 deaths each year *(56)*. Fever, diarrhea, vomiting, chills and severe dehydration are some of the common symptoms of HNoV infection which develop within 12-48 hours after infection and can last for 24-72 hours *(95, 109, 126)*. As analyzed by the Centers for Disease Control and Prevention (CDC), there were 1,008 HNoV outbreaks in the 43 states of USA from 2009-2012, out of which 92% resulted from the consumption of contaminated vegetables, fruits and mollusks *(56)*, minimally processed food and fresh produce, shellfish, ready-to-eat meals, sandwiches, baked products, water and ice *(96, 132)*. These viruses pose a potential risk to public health as even a small amount of contamination can cause/result in disease outbreaks and spread through water, food, person to person contact and aerosolized vomitus *(59)*. This varied means of transmission along with their environmental stability, low infectious dose, association with a wide range of produce and food commodities and resistance to disinfectants and mild processing conditions contribute towards the challenge of controlling HNoVs.

Human noroviruses belong to the *Caliciviridae* family and are non-enveloped, 30-35 nm virions with an icosahedral symmetry that contain a single-stranded positive-sense RNA of 7.5 to 8.5 kb in length enclosed in a capsid *(15, 46)*. Out of the five genera of *Caliciviridae* family which are Norovirus, Sapovirus, Vesivirus, Norovirus and Lagovirus, HNoV belongs to the genus Norovirus *(15)*. Their RNA genome is known to have three open reading frames (ORFs) with ORF1 encoding nonstructural proteins including the RNA dependent RNA polymerase (RdRp); ORF2 encoding major viral proteins that form the capsid (VP1) and has protruding (P) domain and shell (S) domain; and ORF3 encoding minor viral protein (VP2) *(39)*. The ORF1 encodes a polyprotein that is post-translationally cleaved into seven non-structural mature proteins (NS1 to NS7) that are involved in viral replication *(126)*.

The P domain was shown to have better binding ability than VP1 to the viral histo-blood group antigens receptors *(84)*. Human histo-blood group antigens (HBGAs) are complex carbohydrates linked to glycoproteins or glycolipids that are

present on red blood cells and mucosal epithelial cells or as free antigens in biological fluids, such as blood or saliva *(117)*. Six different HBGA binding patterns shown by HNoVs are - recognition of HBGAs of type A and O secretors; recognition of A, B, and O secretors; A and B secretors; A secretors and two Lewis-epitope binders *(117)*.

Based on the capsid sequences, currently there are seven known genogroups of HNoV out of which GI, GII and GIV affect humans, GIII and GV affect bovines and GVI and GVII affect canines *(46, 126)*. Based on the complete VP1 and the RNA dependent RNA polymerase (RdRp) amino acid sequence, currently there are 9 genotypes of GI, 22 of GII, 2 of each GIII, GIV, GV and GVI, and 1 in the tentative new GVII *(126)*. Among the GII strains, GII.4 has been the most prevalent for example, HNoV GII.4 Minerva, along with the new emerging GII.17 strain *(29, 38)*.

The many recent outbreaks related to HNoV worldwide are summarized in Table **1**. In mid-March 2015, two patients in a geriatrics ward suffered from diarrhea in a healthcare facility in Belgium, and though the outbreak was controlled in 22 days, 27 patients had experienced diarrhea, 12 of whom tested positive for HNoV genogroup II *(100)*. From 2004–2008, in the US approximately 66% of all HNoV nosocomial infections occurred in elderly adults, with mortality rates roughly 200% higher among those ≥65 years compared to <5 year olds *(78)*. In older adults, HNoVs cause approximately 10–20% of gastrointestinal hospitalizations, 10–15% of gastroenteritis deaths *(78)*. Among older adults HNoV GII.4 was the most predominant genotype related to inpatient, long term care facility and community settings, potentially due to a combination of novel GII.4 mutations *(78)*.

When 16 healthy secretor positive adults orally received different dosages of HNoV inoculum (10-fold dilutions ranging from 4.8 to 4,800 RT-PCR units), gastroenteritis developed in 11 persons *(8)*. The researchers observed that virus shedding (measured by immunomagnetic capture RT-PCR) was first detected at a median of 36 hours after inoculation and lasted a median of 28 days after inoculation *(8)*. Seronegative chimpanzees inoculated with HNoV GI.1, were not shown to have any sign of gastroenteritis, but there were similarities in the onset and duration of virus shedding in stool and serum antibody responses to that observed in humans *(15)*. When two infected chimpanzees were rechallenged 4, 10, or 24 months later with NV, the chimpanzees were reported to be resistant to reinfection, possibly due to the presence of HNoV-specific serum antibodies (13). Moreover, intramuscular vaccination of these chimpanzees with GI virus like particles (VLPs) caused protection

from HNoV infection when challenged 2 and 18 months later, whereas GII virus like particles (VLPs) vaccine or a placebo did not cause protection *(15)*. Hence, further research on vaccination schemes are needed.

With regards to HNoV propagation in the lab, some researchers have shown that HNoV infection is possible with cells in 3D systems. One-day human intestinal epithelium cells in 3 dimensions (3-D) on 24-well plates containing collagen-I–coated porous microcarrier beads in rotating-wall vessel (RWV) bioreactors were infected four times until passage 5 with HNoV GI and GII, that were extracted from stool samples obtained from cruise-ship and nursing home outbreaks *(109)*, and showed cytopathic effects (CPE) after 24-48 h post infection. Successful norovirus replication was observed through all 5 passages in the 3-D small intestinal model, which was confirmed by CPE, RT-PCR, and fluorescence in situ hybridization (FISH) with genogroup-specific molecular beacons *(109)*.

On the other hand, some researchers show that 3D cell aggregates do not allow infection or replication of HNoV *(88)*, without any infection or CPE withGII.4 on differentiated 3D cell culture systems of human embryonic intestinal epithelial cells (Int-407) and human epithelial colorectal adenocarcinoma cells (Caco-2) grown on collagen-I porous micro carrier beads in a rotating bioreactor as analysed by RT-PCR *(88)*. After 48 h of infection, no increase in the viral RNA titer compared with 1h post inoculation was observed*(88)*.GII.4-Sydney HNoV-positive stool sample with 6 log genome copy numbers inoculated onto the BJAB human B cell line, showed a significant 10-fold and 25-fold increase in viral genome copy number at 3 and 5 dpi, respectively, compared with input levels*(63)*.Filtration of the HNoV-positive stool sample over a 0.2µm membrane, caused decrease in genome replication indicating the presence of a filterable cofactor. The researchers showed that HNoV genomes increased fourfold and 20-fold at 3 and 5 dpi, respectively, after inoculation with passage 0 (P0) virus, which indicates that primary BJAB infection results in the production of new infectious virus particles *(63)*.

HNoV GII.4-Sydney-positive stool when applied into the apical supernatant fluid of polarized human colon adenocarcinoma cell line HT-29 intestinal epithelial cells (IECs), caused a 600-fold increase in viral genome copy number in the B cell fraction of infected cultures at 3 dpi *(63)*, without any increase in the number of viral genome copy in the absence of B cells. Moreover, filtration of the stool sample ablated B cell–associated viral genome replication, which was consistent with the results observed

for direct B cell infections *(63)*.These researchers observed that there was no increase in infectivity of HNoV with either *Escherichia coli* (which did not express H antigen) or lipopolysaccharide (LPS, a component of the outer membrane of Gram-negative bacteria), whereas synthetic H antigen restored infectivity of filtered stool comparably with *E. cloacae (63)*.

When monolayers of human intestinal enteroids (HIE) were inoculated with HNoV GII.4, 1.5-2.5 log increase in genome equivalents (using RT-qPCR) at 96 hours post-infection was reported (45). HNoV GII.3 and GII.4 viral replication (measured by RT-qPCR and immunofluorescent assays) was promoted when the researchers used human, sow and commercially available bovine and porcine bile which was due to bile effect on HIEs (45). This marks the beginning of the cultivation and propagation of HNoVs in the laboratory, though still expensive and is still at the infancy stage at the time of this writing. Few studies on inactivation of HNoV are reported where HNoV GII.4 was reduced by only 1.69 log genomic equivalents/ml on exposure to cold atmospheric plasma (at 30 mW/cm^2, 23.5°C and 40% relative humidity for 15 min *(3)*. About 3 and 2.8 log receptor captured viral genomic signal (RCVGS) reduction on exposure to UV at 1 J/cm^2 and 100°C for 2min, respectively was reported for HNoV GII.4 *(127)*. A 2.9 log reduction in RNA level of HNoV GII.4 has been reported on exposure to high hydrostatic pressure processing (HHP) at 250 MPa at 1°C *(76)*.

Since, there were no reproducible cell-culture based systems or infectivity assays for HNoV propagation and detection at the time of this study, cultivable surrogates were used to determine inactivation, transmission and persistence in the environment. An ideal surrogate for HNoV should be similar in structure and size, have greater resistance, should be non-pathogenic and easy to use in lab. Currently, Feline Calicivirus (FCV-F9), Murine Norovirus (MNV-1), Tulane Virus (TV), Porcine Sapovirus (SaV), bacteriophages and virus like particles (VLPs) are therefore used as cultivable HNoV surrogates *(33, 35, 130)*.

1.2 HNoV surrogates

1.2.1 Murine Norovirus

Murine Norovirus (MNV-1) belongs to genogroup V of the genus Norovirus in the family *Caliciviridae*. MNV-1 is 28 to 35 nm in diameter, icosahedral in shape, non-

enveloped, and has a buoyant density of 1.36 g/cm^3 which is characteristic of HNoVs. It has a positive-sense single-stranded 7.5 kb RNA genome. It is replicable in the laboratory using murine macrophage RAW 264.7 cells *(131)*. It is known to infect mice deficient in components of innate immunity and cause diarrhea and lethality *(120)*. ORF1 of MNV-1 encodes a predicted 187.5kDa polyprotein containing the 2C helicase, 3C protease, and 3D polymerase motifs found in other caliciviruses and picornaviruses. ORF2 of MNV-1 encodes a 58.9 kDa capsid protein that can self-assemble into virus-like particles while ORF3 of MNV-1 encodes a 22.1 kDa basic protein *(120, 131)*.

MNV-1 has been reported to show a 3.4 and 0.9 log PFU/ml reduction on high hydrostatic pressure (HHP) treatment at 400 MPa at 21°C for 2min in cell culture media at neutral pH *(76)*. Thermal inactivation at 56°C of MNV-1 in cell culture media showed a D-value of 3.65 and 3.74 min in glass capillary tubes and 2 ml glass vials, respectively *(18, 22, 33)*, while it was reported to show a 5 log $TCID_{50}$/ml reduction in polypropylene microcentrifuge tubes at 56°C *(33)*. It was highly sensitive to UV showing complete reduction at 10 mW/cm^2 for 2min, 4.1 log PFU/ml reduction in viral titer on using either 6% wt/wt ozone for 10 min or pulsed light on polyethylene disks at 8.98 J/cm^2 *(79, 95, 125)*. It is highly pH resistant as it has been reported to show 0.2 and 2 log PFU/ml reduction in infectivity using 100mM citric acid (pH 2) and 100mM carbonate buffer (pH 10) for 30 min at 37°C while there was 1.4 log PFU/ml reduction with commercial bleach at 1,000 ppm chlorine for 5 min at room temperature on stainless steel discs *(33)*.

1.2.2 Feline Calicivirus

Feline Calicivirus is a non-enveloped icosahedral virus which belongs to the genus Vesivirus is known to cause respiratory diseases in both domestic and exotic cats with morbidity up to 30%. It is 27-40 nm in size with a positive sense single stranded 7.7 kb RNA. It is cultivable in lab using the host Crandell-Reese feline kidney cell line *(28)*.

Reports indicate a 4 log PFU/ml reduction in FCV-F9 viral titer on exposure to 29 mJ/cm^2 UV in cell culture media *(90)* and a 4 log $TCID_{50}$/ml reduction upon exposure to Ar atmospheric plasma for 180s at 1.5W *(1)*. Thermal inactivation studies of FCV-F9 in cell culture media shows D-values at 56°C as 6.3 min using both capillary tubes and 2-ml glass vials *(18, 22)* and reduction to non-detectable levels in

microcentrifuge tubes after 20min at 56°C *(33)*. It has shown to be reduced to non-detectable levels upon treatment with 100mM citric acid (pH 2) and 100mM carbonate buffer (pH 10) for 30 min at 37°C and 5.3 log PFU/ml reduction using commercial bleach at 1,000 ppm chlorine for 5 min at room temperature on stainless steel discs *(33)*.Due to its respiratory transmission route, vulnerability and low sensitivity to pH, environmental stress, elevated temperatures and chemical disinfectants, it may not accurately predict HNoV stability or inactivation and may not be the most suitable HNoV surrogate *(18, 27, 44)*.

1.2.3 TV

Tulane virus was recently isolated from stool samples of rhesus monkeys and belongs to the genus Recovirus in the *Caliciviridae* family*(129)*. It is small, non-enveloped, and icosahedral with positive sense, single stranded RNA genome which is 6714 nt in length and 40 nM in diameter *(46)*. Both, HNoV and TV have three open reading frames (ORF) wherein ORF1 encodes non-structural protein, ORF 2 encodes capsid protein (VP1) and ORF 3 encodes minor structural proteins (VP2) *(46)*. The 90 dimers of capsid protein are divided into two domains, the Shell domain (S) which is an eight folded jellyroll structure and forms the icosahedral shell and the protruding domain (P) with subdomains P1 and P2, that are responsible for the viral entry into the host cell *(139)*. The VP1 (capsid protein) is 534 amino acid (aa) with a molecular mass of 57.9 kilo Daltons, whereas VP2 (minor structural protein) is 218aa and has a molecular mass of 22.8 kDa. It is cultivated in the lab using LLC-monkey kidney cells*(46)*.Similar to HNoV, it binds to histo-blood group antigens and is closely related to HNoV GII *(59)*. Hence, this cultivable virus is used to determine appropriate inactivation methods against HNoV.

TV shows reduction in infectivity of 1.2 log PFU/ml when treated with commercial bleach at 1,000 ppm chlorine for 5 min at room temperature on stainless steel discs *(33)*. TV is also known to be highly resistant to high pressure processing showing 6 log PFU/ml reduction at 600 MPa for 1 min at 4°C and shows 0.2 and 2 log PFU/ml reduction in infectivity using 100mM citric acid (pH 2) and 100mM carbonate buffer (pH 10) for 30 min at 37°C *(33)*. These previous findings suggest that in comparison to other HNoV surrogates, TV is most resistant to change in pH, pressure or chlorine concentration under the above given conditions, and is therefore a suitable

surrogate for HNoV for the above mentioned processing and inactivation approaches.

1.2.4 Porcine Sapovirus

The prototype strain of human Sapovirus SaV, the Sapporo virus, was identified originally in faecal specimens by electron microscopy (EM) from an outbreak in an orphanage in Sapporo, Japan, in 1977 *(128)*. SaVs are known to cause enteric diseases in humans of all ages but more so in younger children. Based on complete capsid sequence, out of the five genogroups, GI, GII, GIV, and GV are found to infect humans and GIII infects pigs. The genomes of the SaVs are organized into two ORFs *(71)*. A number of Sapovirus strains are also predicted to contain an additional small ORF overlapping with the 5' end of the capsid gene *(70)*. It can be cultivated in the lab using LLC-porcine kidney cells. It has been reported to show 2.2, 1.2 and 2.38 log $TCID_{50}$/ml reduction with 70% ethanol for 30s,10 mg/l sodium hypochlorite for 1 min and heat treatment at 56°C for 30 min, respectively *(130)*.

1.2.5 Bacteriophage MS2

Bacteriophage MS2 is an icosahedral single stranded RNA virus (26nm) that belongs to RNA coliphages group I in the *Leviviridae* family. It has *Escherichia coli* as its natural host and is therefore commonly found in sewage waste and animal faeces *(35)*. Like HNoVs, it is adapted to intestinal tract and has therefore been used as a surrogate to study behaviour of HNoV under different environments *(35)*. Furthermore, it has been reported to survive for up to 50 days at 4 and 8°C, with a reduction of less than 1 log PFU/mL, and for up to 9 days (>1 log PFU/mL reduction) at 22°C on fresh produce such as leafy greens, carrot, tomato, strawberries, and raspberries *(35)*.

1.2.6 Virus like particles (VLPs)

They are non-infectious HNoV particles which can be expressed in baculovirus-infected insect cells, and can bind to HBGA receptors on mucosal cell surfaces and are known to be acid and heat stable *(62)*. They can be assembled by expression of the capsid protein in insect cells such as Spodopterafrugiperda Sf21 cells *(12)*.

1.3 AiV

The emerging foodborne enteric Aichi virus (AiV) was first isolated from stool specimens of patients suffering with acute gastroenteritis due to the consumption of

contaminated raw oysters *(135)*. This virus has a morphologically distinct round-structure, ~ 20 to 40 nm in diameter that belongs to the *Kobuvirus* genus in the same *Picornaviridae* family as Hepatitis A virus (HAV). It has three known species A, B and C and is cultivable in lab using African green monkey kidney Vero cell line and infectivity can be determined through plaque assays *(133, 135)*. The viral genome consists of a single-stranded, positive-sense RNA molecule of 8,280 nucleotides and a poly (A) tail *(5, 133)*. AiV is transmitted through the faecal oral route and has been known to spread through contaminated water and shellfish *(72, 97, 133, 134)*. It has been thus far isolated from stool samples over the world including Japan, France, Pakistan, Netherlands, Tunisia, Spain and South America *(72, 85, 98, 133, 134)*. Symptoms of AiV infection include nausea, vomiting and diarrhea within 12 to 48 h after ingestion, while faecal shedding of AiV can last up to 5-7 days after infection and the infectious dose is currently not known *(30, 116)*. Currently there are no antivirals described for AiV infection treatment *(14)*.It has been shown to be highly resistant than other HNoV surrogates under different environmental conditions of inactivation including heat, pH, high pressure processing, chlorine bleach and alcohol *(33)*.

The unavailability of antiviral treatments and low infectious dose of HNoVs *(119)* and a number of methods of transmission necessitates the prevention of spread and contamination of HNoV through sanitation and hygienic practices or by using inactivation techniques.

2 INACTIVATION TECHNIQUES

There have been various studies on novel techniques for inactivation of HNoV and its surrogates.

2.1 Chemical

2.1.1 Ethanol

Ethanol alters the structural integrity of viral capsid proteins and increases the permeability while in the case of enveloped viruses, it dissolves the envelope lipids *(82)*. Ethanol at 70% for 1min was reported to result in a 3.54 average log reduction in genomic copies/ml (gc/ml) of HnoV GII.4 *(2)*.Treatment of HNoV GII.4 in 1.5 ml micro-

centrifuge to an ethanol concentration of 70% for 20s at room temperature reduced the log of RCVGS from 2.5 (untreated) to 0.05 (treated) *(127)*.

FCV-F9 when treated with 75% ethanol for 1min in pollyalomer ultracentrifuge tubes showed 1.25 log $TCID_{50}$/ml reduction *(40)*. MNV-1 has been reported to show 5 log PFU/ml reduction in viral titer using 70 and 90% ethanol or isopropanol for 1 min, while TV and AiV had <1 log PFU/ml reduction under the same conditions *(33)*. Whereas, FCV-F9 showed ~1.5 log PFU/ml reduction with 70% ethanol for 1 min *(33)*.

2.1.2 Trisodium phosphate (TSP)

TSP is a common cleaner which has an alkaline pH of 12. It has been approved for beef carcass decontamination by Food Safety Inspection Services. It is known to inactivate virus by destabilizing the viral capsid *(9)*. HNoV GI.1 (initial titer of 8 log genomic equivalents/ml) on exposure to TSP at 5% for 5min showed 1.6 log reduction in binding *(68)*.Kim et al, 2015 mixed MNV-1 with ethanol to a final concentration of 30%, 5% and 70% treated with TSP at 1%, 2% and 5% up to 30min at 25°C on SS discs at 4°C for 0, 1, 5, 15, and 30 min (using RT-PCR), as well as tested on lettuce and bell peppers *(65)*. The researchers found that in the case of ethanol, there were persisting RT-PCR products of MNV-1 after using 70% ethanol. Whereas, MNV-1 was inactivated after 1min by using 2% and 5% TSP, as was also shown earlier *(34)* that there was 7 log reduction after 15 s using 5% TSP on MNV-1 (34). They also showed that in case of combined effect, MNV-1 was completely reduced after 5 mins in solution whereas, for lettuce and bell pepper matrices, there was complete inactivity after 15 and 1min on exposure to 1% TSP and 30% ethanol *(65)*.

FCV-F9 and MNV-1 (at 7 log PFU/ml) spiked on lettuce and jalapeno peppers have been studied for treatment with 2 and 5% TSP and 200mg/L sodium hypochlorite for 15 and 30 s at room temperature *(111)*. FCV-F9 was reduced by 5 and 1.4 log PFU/mL with 2% TSP and 200 mg/L sodium hypochlorite, respectively while MNV-1 was decreased by ~2-3.4 and 1.3 log PFU/mL with 2% TSP; and sodium hypochlorite, respectively, while both viruses, FCV-F9 and MNV-1 were reduced to undetectable levels using 5% TSP*(111)*.MS2 was reported to be reduced by >6 and 4.5 log PFU/ml by 5 and 1% TSP, respectively in 30s *(34)*.

2.1.3 Organic Acids

The proposed mode of action of an organic acid is through damage of the viral capsid and by protein denaturation through low pH *(99)*.Tannic acid at 0.2 mg/mL was shown to reduce FCV-F9 by 1.95 log PFU/ml and gallic acid at 0.1, 0.2, and 0.4 mg/mL was shown to reduce FCV-F9 by 2.50, 2.36, and 0.86 log PFU/mL, respectively in cell culture media after 2 h at room temperature *(34)*.TV and MNV-1 (both at 7 log PFU/ml titer initially) in cell culture media showed only 0.2 log PFU/ml reduction in infectivity using 100mM citric acid (pH 2) after 30 min at 37°C, while FCV (at 9 log PFU/ml initially) was reduced to undetectable levels initial (*33*).

Levulinic Acid (LV), an acid anionic sanitizer that has been approved by FDA as a food additive (21 CFR 172.515), at 0.5% with 0.1% sodium dodecyl sulfate (SDS) for 1min was effective in decreasing HNoV GI.1 and GII.4 by 8.97 and 8.13 average relative differences with and without RNAse A in RT-qPCR quantification cycle (Cq) values, respectively (initial titer of 3-5 log gc/ml) *(2)*. When stainless steel (SS) coupons were inoculated with 6-8 log PFU/ml of MNV-1 and dried for 50min by using a carrier method at room temperature *(16)*, average reductions of 0.85, 2.71 and 1.16 log PFU/ml with sodium dodecyl sulfate (SDS), levulinic acid (LEV) with SDS and free chlorine, respectively were obtained after application with a conventional hydraulic spray apparatus*(16)*. Use of electrostatic sprays of these sanitizers caused MNV-1 reduction of 0.31, 1.16 and 1.66 log PFU/ml with SDS, LEV with SDS and free chlorine, respectively. When a robotic wiping device was used, reductions of 3.53, 7.05 and 7.05 log PFU/ml for MNV-1 with SDS, LEV with SDS and free chlorine, respectively were reported*(16)*.No reduction of HNoV GI.1 was reported with 0.5 % levulinic Acid (LV)/0.01 % Sodium Dodecyl Sulfate Treatments (SDS) (low), or 0.5 % LV/0.1 % SDS (high) for 1min *(2)*.When FCV-F9 and MNV-1 at an initial titer of 6 log PFU/ml (partially purified cell lysates) were treated with 3% levulinic acid or 2% SDS, no reduction of MNV-1 or FCV-F9, with only 0.43 log PFU/ml reduction of FCV titer using 3% levulinic acid was obtained. However, both MNV-1 and FCV-F9 were reduced below detection limits of 2.7 log PFU/ml using 0.5% LVA with 0.5% SDS and 2% LVA with 1% SDS *(26)*.

2.1.4 pH

The changes in the hydrogen ion concentration that leads to changes in the pH have been proposed to cause alteration in the viral capsid and hence denaturation of

nucleic material *(99)*. Both TV and MNV-1 in cell culture media show 0.2 and 2 log PFU/ml reduction in infectivity using 100mM citric acid (pH 2) and 100mM carbonate buffer (pH 10) for 30 min at 37°C, whereas FCV-F9 is completely inactivated to non-detectable levels at both pH 2 and pH 10 under the same conditions, while AiV showed <0.5log PFU/ml reduction *(33)*. SaV has shown <1 log $TCID_{50}$ reduction at pH 3 and 8 using 0.2 M dibasic sodium phosphate or 0.1M citric acid *(130)*. Therefore, TV and AiV showed highest resistance at pH 2 and 10, followed by MNV, while FCV-F9 showed least resistance at both pH.

2.1.5 Chlorine

It is one of the common sanitizers and is approved by FDA for use as an antimicrobial agent in wash water for fruits and vegetables up to 3 ppm limit of residual aq. chlorine dioxide (US FDA 21 CFR Part 173.30). Mode of action is through alteration in the polypeptide backbone of viral capsid proteins *(68)*. Hypochlorous acid (HOCl) is known to have antimicrobial activity against spore-forming bacteria *(31)*. It is used for water purification, electrolysed water and chemical wash at 200ppm levels as a disinfectant for raw produce such as tomato, dairy and food processing equipment cleaning (USDA-Agricultural Marketing Services, 2011). There are two ways in which a sanitizer such as the chlorine based sanitizers HOCl and free chlorine could inactivate a non-enveloped virus, by damaging the capsid sufficiently to prevent binding to host cell receptors, or secondly, damaging the RNA template within the target sequences preventing replication *(108)*.

Aqueous chlorine treatment with free chlorine levels showed reduction in the log receptor-captured viral genomic signal (RCVGS) of HNoV GII.4 (measured by in-situ capture qRT-PCR) from 3.20 to 2.92, 2.68 and 2.34 at 2, 4 and 8 ppm of free chlorine, respectively after 10 min *(127)*. HNoV GI.1 at an initial titer of 8 log genomic equivalents/ml (ge/ml) after treatment with 8.5% sodium hypochlorite at 33, 173 and 189 ppm aqueous chlorine for 1 min at room temperature using PGM-MB binding assay showed 1.48, 3.65 and 4.14 log reduction in binding activity *(68)*. Aqueous chlorine dioxide at 240 ppm for 10, 30 and 60min was also reported to result in 0.8, 1.5 and 2.8 log reduction in binding of HNoV GI.1, respectively *(68)*. Free chlorine has also been used to decontaminate fecally contaminated surfaces to control HNoVs *(91)*. Using RT-PCR, HNoV GII.4 was shown to be reduced by 0.1 log in viral RNA after

120min using 0.1 mg/ml free chlorine at room temperature, whereas 0.5mg/ml chlorine further reduced HNoV GII.4 RNA by 3.21 log after 30min *(69)*. Using RT-PCR, HNoV GII.4 was shown to be reduced by 0.6 log RNA copy number/ml after 10 min exposure to 500ppm NaOCl, whereas 5000 ppm NaOCl for 4 min showed a reduction of 1.4 log RNA copy number/ml *(91)*.

On treating MNV-1 with free chlorine (prepared using sodium hypochlorite) at concentration 0-20 ppm for 3min, average infectious titer were reduced to by approx. 2 and 3.5 log PFU/ml at 0.2 and 5 ppm, respectively and the log genomic copies/ml decreased by 0.2 and 0.4 units at 0.2 and 5 ppm, respectively *(103)*. When MNV-1 was spiked on lettuce (final concentration of 3×10^6 $TCID_{50}$/ml) a peroxyacetic acid-based biocide and chlorine prepared from sodium hypochlorite solution at 100 and 15 ppm, respectively caused reduction by 1.4 and 2.3 log $TCID_{50}$/ml *(51)*. The researchers showed that peroxyacetic based biocide was more efficient for reducing the level of infectious MNV-1 on lettuce than bleach, with a reduction of 1.4 log and 0.4 log $TCID_{50}$/ml, respectively *(51)*. MNV-1 reductions on spiked on plates, forks and drinking glasses after simple washing treatment were 2.6, 1.3 and 0.7 log PFU/ml, respectively (45), with mean reductions after washing and chlorine sanitation of 3.2, 1.5 and 1.4 log PFU/ml, respectively whereas after washing and sanitizing with the QAC sanitizer, caused reductions on the ceramic plates by 2.7 log PFU/ml and the mean reduction for both the forks and glasses were 1.6 and 1.4 log PFU/ml, respectively *(47)*.

For a 1 log $TCID_{50}$/coupon reduction of MNV-1, 3.96 to 2.21 min were needed for treatment with 1,000 and 5,000 ppm of aqueous chlorine respectively whereas for 3 log$TCID_{50}$/coupon reduction required 4.98 to 2.25 min at 1,000 and 5,000 ppm respectively and for 5 log$TCID_{50}$/coupon reduction the treatment time was 5.26 to 3.3 min at 1,000 and 5,000 ppm, respectively *(66)*. For FCV-F9, the treatment time was reduced from 2.77 to 1.33 min at 1,000 ppm aqueous chlorine for 1 log $TCID_{50}$/coupon reduction and from 5.15 to 3.15 min at 5,000 ppm aqueous chlorine for 5 log $TCID_{50}$/coupon reduction *(66)*.

The D-values at 1, 1.5, and 2 mg/liter chlorine dioxide gas for MNV-1 on inoculated stainless steel surfaces were 2.79, 2.07 and 1.87 min, respectively *(138)*. The researchers stated that ClO_2 gas after 1 at 2.5 mg/l reduced MNV-1 by ~3 log PFU/coupon and after 2min there was reduction to non-detectable levels.

Furthermore, 2, 2.5, and 4 mg/liter ClO_2 reduced MNV-1 by 3 log PFU/coupon after 5, 2, and 1 min, respectively *(138)*.

HNoV GII.4 after treatment with hypochlorous acid solution (HAS) having free chlorine concentrations of 188, 38 and 18.8 ppm at a pH of 5.5 to 6.2 which was prepared electrolytically from dilute NaCl in Sterilox system, for different time points on ceramic and stainless steel tiles and in suspension showed 3 log reduction of viral RNA at room temperature after 20 min *(89)*. MNV-1 was reduced by only 1 log PFU/ml at 2ppm aq. chlorine dioxide after 5min, whereas there was a 3 log PFU/ml reduction at 20 ppm after 5min using aq. chlorine dioxide *(54)*. In the case of peroxyacetic acid based disinfectants, a 3, 4 and 4 log PFU/ml reduction of MNV-1 titer after 5 min at 20, 85 and 250 ppm, respectively was reported *(54)*. Further analysis of three disinfectants individually and in combination with Feclone on ready to eat foods such as blueberries, strawberries, parsley and lettuce with sodium hypochlorite at 50 ppm, showed 2 log PFU/ml reduction of MNV-1 in blueberries and less than 1 log PFU/ml reduction on strawberries and lettuce *(54)*. MNV-1 was reduced by approx. 3, 3.5 and 4.5 log PFU/ml on blueberries, strawberries and lettuce, respectively with PAA and Felcone at 85 ppm of peroxyacetic acid for 1min, whereas there was a reduction of about 3, 2.5 and 2.5 log PFU/ml when only PAA at 85 ppm was used on blueberries, strawberries and lettuce, respectively. The researcher**s** did not show a significant reduction of MNV-1 (<0.5 log PFU/ml) with aq. chlorine dioxide at 20ppm of free chlorine on any of the food matrices, whereas there was a slight reduction (~1 log PFU/ml) on using chlorine dioxide with Feclone *(54)*. When FCV-F9 was treated with hypochlorite solution for 1min at room temperature in pollyalomer ultracentrifuge tubes, 1.75, 0.75, 1.5, 2.5 and 5 log $TCID_{50}$/ml reduction at 5000ppm was observed *(40)*. Therefore, though chlorine and chlorinated compounds can reduce viral titers on surfaces, they are known to form carcinogenic by-products with organic matter *(37)* and hence alternative techniques need to be further studied.

2.1.6 NaCl

The antimicrobial effects of fermented foods are attributed to the low pH produced by lactic acid and bacteriocins such as niacin as well as to other factors such as salt concentration, temperature, and curing time. Salt lowers the pH and is supposed to denature capsid protein, and inactivates the virus through binding and subsequent denaturation of nucleic acid *(110)*. When MNV-1 (at initial titer of 6.39-6.43 log PFU/g)

was spiked on oysters using 5 and 10% NaCl and fermented for 18 hours up to 15 days, the MNV-1 titers decreased from 6.44 log PFU/g to 5.76 log PFU/g at 3 days post fermentation (DPF) and reached a minimum of 4.84 log PFU/g at 15 DPF (108). Whereas 10% NaCl reduced MNV-1 titers from 6.44 log PFU/g at 0 DPF to 5.90 log PFU/g at 3 DPF and 5.45log PFU/g at 15 DPF while the LAB populations increased from 3.11 log CFU/g to 6.29 log CFU/g at 7 DPF and 6.96 log CFU/g at 15 DPF *(104)*. FCV-F9 was reduced by 0.5 log $TCID_{50}$/ml at 0.1% lactic acid (pH 6) after 3 hours at both 4 and 20°C, while this reduction increased to 1.5 log $TCID_{50}$/ml at 0.4% lactic acid (pH 3.2) after 3 hours at 20°C, and 0.9 log $TCID_{50}$/ml reduction at 4°C (114). When NaCl was used for treatment of FCV-F9, reduction of 0.2 log $TCID_{50}$/ml at 2% NaCl at both temperatures was reported, while around 0.6 and 0.2 log $TCID_{50}$/ml reduction at 20% NaCl at 20 and 4°C, respectively after 3 h was obtained *(110)*. Therefore, change in pH or salt concentration shows reduction in titers of HNoV surrogates such as MNV-1 and FCV.

2.2 Ultraviolet light (UV)

UV light has been approved by the US FDA for treatment of food at 2,537 A intensity of radiation for control of surface microorganisms and for sterilization of water used in food production (US FDA 21 CFR Part 179.39). UV at higher doses of 1,000 mW s/cm^2 is proposed to affect the viral capsid proteins, thereby making the genome susceptible to RNase enzymes *(49, 106)*. Although it has minimal detrimental effect on nutrient content in food and has low installation cost, but it has a disadvantage of shallow penetration depth on food surfaces *(79)*.

UV treatment of 320µl aliquots of PBS-diluted HNoV GII.4 virus stock in 90 mm Petri dishes resulted in the RCVGS reduction from 3.26 to 2.96, 2.45, 1.54, −0.37 and −0.37 log at 250, 500, 750, 1000, and 1500 mJ/cm^2 UV radiation, respectively *(127)*. When MNV-1 at 50 µL was deposited on either the skin or the calyx tissue of blueberries and treated with UV directly (dry UV treatment) or immersed in agitated water during the UV treatment (water-assisted UV treatment) for 1–5 min at 10 mW/cm^2, MNV-1 titers were reduced from 7 log PFU/sample to non-detectable levels *(79)* with water-assisted UV and 10-ppm chlorine treatment after 2-min treatment. Water wash alone for 5 min was reported to be achieve only a 1.73 log reduction of MNV-1. MNV-1 was reduced by 2.43, 2.48 and 3.04 log PFU/sample after 1, 2 and 5min respectively using dry UV and using water assisted UV, reductions were 3.23,

4.32 and 4.36 log PFU/sample after 1, 2 and 5min respectively, respectively (81). When wash water with added blueberry juice (2%) for 2min UV+chlorine (10ppm) treatment, a reduction greater than 3.51 log PFU/sample was obtained *(79)*. UV exposure reduced MNV-1by 0, 0.1, 0.3, 2, 2.3, 2.5, 2.9, 3.7, 3.9, 4.2 and 4.4 log at 10, 20, 30, 40, 50, 60, 90, 120, 180, 240 and 300 mWs/cm^2 of UV exposure, respectively *(93)*. UV treatment at 254nm at room temperature showed 99% inactivation of FCV-F9 at 16 and 13 mJ/cm^2 UV dosage in buffered demand free (BDF) and ground water respectively, whereas 55mJ/cm^2 UV dose was required for 99% inactivation of MS2 in BDF water *(122)*. MNV-1, FCV-F9 and MS2 at an initial titer of 8, 8.5 and 10 log PFU/ml respectively could be reduced by 4 log using 25, 29 and 70 mJ/cm^2, for MNV-1, FCV-F9 and MS2, respectively (Table 1.3-7) *(90)*. Lettuce, strawberries and green onions infected with FCV-F9 at an initial titer of 7 to 9 log$TCID_{50}$/ml treated with UV at 240 mW s/cm^2 *(49)*, showed reduction of 4.5 to 4.6, 2.5 to 5.6 and 1.9 to 2.6 log $TCID_{50}$/ml on lettuce, green onions and strawberries, respectively *(49)*.

2.3 Ozone

Ozone is attractive for use in the food environment because it naturally decomposes to elemental oxygen, is a very powerful oxidant, and has high penetrability and reactivity *(95)*. Ozone (O_3) is a triatomic oxygen molecule that exists as a bluish gas with a strong characteristic odour with antimicrobial activity against bacteria, fungi, spores, protozoa, and viruses *(4, 95)*. It is known to directly disrupt various cellular constituents such as proteins, nucleic acids and virus capsid components *(95, 129)*. Buffered-demand free water inoculated with FCV-F9 and treated with 1mg/ml ozone for 0.25 and 1.2min at pH 7 at 5°C.showed 4.28 and >4.7 log $TCID_{50}$/ml reduction in titer, respectively whereas 1.85 and 2.77 log $TCID_{50}$/ml reduction was obtained at 0.06 mg/ml ozone after 0.25 and 5min, respectively *(121)*. Alfalfa seeds (1g per sample) inoculated with 500 µl MNV-1 at an initial titer of 6.66 log PFU/ml and dried for 60 min at 22°C in a biosafety cabinet,after 0.5 min of aqueous 6.25 ppm ozone treatment, showed an immediate loss of >4 log PFU, with increased reduction of 5.6 log PFU after 30 min, and reduction of 4.04, 4.27, 4.56, 4.90 and 5.60 log PFU/g of seed after 0.5, 1, 5, 15 and 30 min, respectively *(129)*. Treatment of liquid MNV-1 stocks with gaseous ozone at 6% wt/wt ozone in oxygen created by corona discharge from extra dry compressed purified (99.6%) caused reduction of 4.1 log

PFU/ml after 10 min and reduction to non-detectable levels after 40min, while glass and stainless steel surfaces inoculated MNV-1 still had 2.5 log PFU/ml viral titer left after 40 min treatment *(95)*. Inoculated onions treated with bubbling gaseous ozone from an ozone generator at 6.25 ppm for 10min caused MNV-1inactivation of 1.5 and 2.5 log PFU/plant after 10min at 20°C for internalized and external MNV-1*(60)*. Ozone treatment shows effective inactivation of HNoV surrogates, but with a long exposure time, which necessitates the search for alterative inactivation techniques.

2.4 High hydrostatic pressure

High hydrostatic pressure (HHP) is used for non-thermal processing of food products such as oysters, guacamole, fruit jams, ready-to-eat meats, salsa, and orange juice (76). A possible mode of action is the loss of receptor-binding function of the virus*(80, 118)*. High hydrostatic pressure controls or reduces contamination from foodborne pathogens with minimal impact on the taste, texture, appearance and nutritional value of food, *(76)*. The efficacy of high hydrostatic pressure (HHP) on virus inactivation depends on pressure level (directly related), treatment time (directly related), treatment temperature, pH of substrates and the virus type *(76)*.

Previously, Lou et al, *(80)* showed that a 600-MPa treatment was able to destroy MNV-1 capsid integrity using transmission electron microscopy Strawberry purees (one-gram samples) inoculated with 10 µl of the HNoV GI.1 or GII.4 treated at 450, 500 and 550MPa high hydrostatic pressure (HHP) for 2min at initial sample temperatures of 0, 4 and 20°C, showed reduction of approx. 1.5, 2.5 and 3 log genomic copies/g (gc/g), respectively at 0°C; 1.25, 2.2 and 3 log gc/g, respectively at 4°C and 0.75, 1 and 1.5 log gc/g, respectively at 20°C (63). HNoV GI.1 strain on blueberries was shown to be reduced by 3.2 log gc/g at 550 MPa (HHP) after 2 min, while HHP treatment at 650 MPa caused only 1.7 and 2.5 log gc/g reductions of GI.1 strain on strawberry quarters and raspberries, respectively while for HNoV GII.4 strain on strawberry quarters, 650 MPa caused reduction of 3.1 log gc/g *(61)*.

HNoV GII.4 and GI.1 treated with HHP at 200-550 MPa at 1, 4, 10, 21 and 35°C for 2min *(76)*, showed no reduction at 21 and 35°C, but >3 and 1.8 log RNA level reduction at 1 and 4°C, respectively was obtained for GI.1 *(76)*. Similarly, for GII.4 strain at 250 MPa at 21 and 35°C showed no reduction, but caused >2 log RNA level reduction at 1 and 4°C *(76)*. HNoV GII.4 at 1°C was shown to be reduced by 2.9 log RNA level reduction with 250 MPa HHP while GI.1 had 1.7 log RNA level reduction

with 400 MPa HHP *(76)*. HHP at 500 MPa and 21°C for 2 min was reported to result in reduction of 2.3-log RNA level of GI.1 RNA at neutral pH and a 0.4-log RNA level reduction at pH 4 while there was 3 and 1.5 log RNA level reduction of GI.1 strain at 550 MPa at neutral pH and pH 4, respectively *(76)*. While 350 MPa at 21°C for 2 min was shown to result in 3.8 log RNA level reduction of GII.4 at neutral pH and a 1.2-log reduction at pH 4, and 550MPa reduced GI.1 by 3.75 and 1.25 log RNA level at neutral pH and at pH 4, respectively *(76)*. Inoculated HNoV GI.1 in blueberries in dry state at 1°C was shown to be reduced by 0.5 and 0.9 log RNA level at 500 and 600 MPa, respectively. While in wet state 0.5 and 2.7 log RNA level reduction at 500 and 600 MPa, respectively at 1°C was obtained while there was 0.5 and >3 log RNA level reduction in wet state at 21°C at 500 and 600 MPa. This shows that the efficacy of HHP increased with decreasing temperature for both GII.4 and GI.1 strain *(76)*.

HNoVGI.1 and GII.4 inoculated into oysters and treated with HHP at 350 and 500 MPa at 0°C were reported to be reduced by 4 log RNA level as determined by PGM-MB RT-PCR, while there was no significant change colour or texture of oyster tissue *(136)*. Using RT-PCR, 3.2 and >4 log reduction in RNA level of HNoV GII.4 at 300 and 350 MPa, respectively at 0°C for 2 min was reported, while HNoV GI.1 showed 3.2 and >4 log RNA reduction at 450 and 500 MPa, respectively at 0°C for 2 min *(137)*. In another study, HNoV GI.1 and GII.4 HNoV inoculated into oyster homogenates showed >4 log reduction in RNA level of both GI.1 and GII.4 at 600MPa at 6°C, and 0.7 and 1.3 log reduction in RNA level of GI.1 at 300 and 400MPa, respectively at 6°C and 2.9 and 3.6 log reduction in RNA level of GII.4 at 300 and 400MPa, respectively at 6°C. At 25°C, they found similar reduction of 1 and 3.6 log in RNA level of GI.1 and GII.4, respectively at 400 MPa *(136)*. HNoV GI.1 at initial concentration of $7.2X10^8$ genomic equivalent copies (GEC) was inoculated at $1.0X10^4$ GEC in oysters *(73)* and treated with HHP at different pressures and subjects were challenged with the treated oysters and controls (76). Using RT-PCR none of the 10 subjects challenged with HNoV-seeded oysters treated by 600 MPa at 6°C for 5 min were shown to be infected with HNoV, whereas 400MPa at both 4 and 25°C resulted in positive infection of subjects *(73)*. MNV-1 min (at an initial titer of 5.8±0.2 log PFU/blueberry) when pressurized at 350 and 400 MPa at 21°C for 2 min in wet state showed >5.6 log reductions of MNV-1, but almost no reduction was achieved at 35°C when treated at 400 MPa, while MNV-1 was below detection limit when treated at 350MPa and 400MPa for 2min at 4°C *(77)*.

FCV-F9 was shown to be reduced by 5 and 4 log PFU/ml at 200 MPa at -10 and 50°C, respectively after 4 min, with a 4 log PFU/ml reduction after 7 min at 59.3°C and a reduction of 2.8 log PFU/ml at 200 MPa for 20 min at 21°C *(67)*. MNV-1 in cell culture media when subjected to pressure range of 350 to 450 MPa for 5-min at 20°C *(67)* showed that 450-MPa treatment was sufficient to inactivate 6.85 log PFU of MNV-1. Furthermore, a 5-min pressure treatment of 350 MPa at 30°C inactivated 1.15 log PFU/ml of virus titer, while the same treatment at 5°C resulted in a reduction in virus titer of 5.56 log PFU/ml *(67)*.

2.5 Irradiation

Irradiation causes nucleotide degradation through production of oxygen and hydroxyl radicals which interact with cellular genetic material *(92)*. Doses of up to 4.0 kGy to control foodborne pathogens in fresh iceberg lettuce and spinach and 7 kGy for frozen meat is approved by FDA *(50)*.

At a dose of 2.8 kGy, VLPs were found to be clumped together, along with altered morphology (45). With an increased dose of 5.6 kGy, large concentration of protein debris was reported to be obtained, and the structure of VLPs was destroyed and particles could not be seen after 22.4 kGy irradiation, concluding the absence of any small spherically structured VLPs *(48)*. On analysis of irradiated VLPs by SDS-PAGE, approx. 40 and 25% of HNoV VP1 protein remained after treatment at doses of 2.8 and 5.6 kGy, respectively *(48)*.

When MNV-1 was inoculated in 5.0 g of green algae, fulvescene sample to get a final titer of 5-6 log PFU/ml and exposed to 0, 3, 5, 7 or 10 kGy of gamma rays using a cobalt-60 gamma irradiator at 10 kGy/h, titters of 1.78 (2.46 log reduction), 2.30 (1.94 log reduction), 2.83 (1.41 log reduction), and 3.08 (1.16 log reduction), respectively were reported *(92)*. On treatment of FCV-F9 and MS2 with gamma irradiation at 200 Gy, the researchers observed reduction in titer of 1.6 and 5-7 log $TICD_{50}$/ml in case of FCV-F9 and MS2, respectively *(36)*. Hence, irradiation is not as effective in reduction of titers of HNoV surrogates and alternate strategies need to be studied.

E-beam: It is proposed to inactivate non-enveloped viruses by disrupting the viral structure, degrading the viral proteins, and degrading genomic RNA. Although it can be used for high-throughput products, but it has the disadvantage of having less

penetration effect as compared to gamma irradiation *(140)*. It uses machine accelerated electrons for production of irradiation as opposed to gamma rays *(96)*. The permissible limit provided by FDA is up to 4.0 kGy to control foodborne pathogens in fresh iceberg lettuce and spinach *(50)*.

Lettuce inoculated with FCV-F9 at initial titer of 7.6 log $TICD_{50}$/ml was reported to show 90% reduction of FCV-F9 titer on lettuce required 2.95 kGy dose at 4°C *(140)*. MNV-1 virus stock (at 6 log PFU/ml) showed a reduction of 1.6 and 1.2 log PFU/ml of MNV-1 in phosphate buffered saline (PBS) and Dulbecco's Modified Eagle Medium (DMEM) with e-beam doses of 4 kGy *(96)*. MNV-1 was also shown to have 0.5 log PFU/ml of virus remaining in PBS at 16.9 kGy, 0.8 log PFU/ml of virus remaining in DMEM at 26.5 kGy, whereas it was not detectable in media or PBS after treatment of 26.5 to 32.7 kGy *(96)*.

TV (initially at 6.6 log PFU/ml) in solution in cell culture media was reported to be reduced to 3.2 and 1.8 log PFU/ml after treatment with 8.6 and 16.9 kGy e-beam. In case of lettuce and strawberries inoculated with TV (at initial titer of 3.7 and 4.4 log PFU/ml, respectively), the final titer after treatment with 8.7 kGy e-beam was reported to be non-detectable for lettuce and 1.8 log PFU/ml for strawberries *(96)*. This shows that e-beam was more effective in reducing the virus in food matrix as compared to cell culture media, but the dosage required for reduction of TV to non-detectable level was higher (8.7 kGy for lettuce and 16.9 kGy for strawberries) than permissible limits approved by FDA *(96)*.

2.5.1 Pulsed light

Pulsed light treatment has shown to be effective for inactivating bacteria, fungi, and yeasts in water and foods and in diagnostic laboratories *(125)*. Besides, the advantages include speed and cost-effectiveness, as it does not require the addition of any chemical product *(125)*. This technology is based on very short, high-intensity pulses of white light, from UV-C to near infrared, and according to the FDA, pulsed light may be safely used for the decontamination of food and food contact surfaces by using a xenon lamp emitting wavelengths between 200 and 1,000 nm, pulse durations not exceeding 2 milliseconds, and cumulative intensity less than 12 J/cm^2 *(50)*. On pulsed light inactivation of MNV-1 on polyethylene disks, the reduction increased from around 0.5 to 4 log PFU/ml as fluence increased from 0.69 to 8.98 J/cm^2 at 80mm from xenon lamp *(125)*. While on polyvinyl chloride disks, the reduction of MNV-1

increased from 0.5 to 3.5 log PFU/ml and on stainless steel disks it increased from 0.5 to 2.5 log as fluence increased from 0.69 to 8.98 J/cm^2 *(125)*.

2.6 Cold Plasma

Cold atmospheric pressure plasma (CAPP) generated by applying an electrical field to an initially electrically neutral gas consisting of nitric oxide and reactive oxygen species is known to have anti-bacterial anti-fungal properties, and also has been shown to inactivate HNoV GII.4 virus particles *(3)*. It is proposed that the oxidation of viral capsid proteins by plasma-produced reactive oxygen and nitrogen species could be responsible for antiviral action of CAPP *(1)*. It is used for preservation in meat, poultry, fruits and vegetables industry as it shows reductions of greater than 5 logs for bacterial pathogens with treatment up to 120s *(83)*. Although it shows antimicrobial action at short treatment time, it has several disadvantages such as complexity of equipment and cost, unexplored sensory and nutritional impact on food products *(83)*.

HNoV GII.4 (at an initial load of 2.4X10^7 virus particles per ml) after exposure to cold plasma was found to be reduced by1.23 and 1.69 log after 10 and 15 min, respectively *(3)*. Furthermore, at an initial titer of 1.1X10^3 genomic equivalents/ml and 3.67X10^1 genomic equivalents/ml, they showed 10 fold reduction in viral load after CAPP exposure for 2 min and 1 min, respectively *(3)*. To analyze the effect of cold gaseous plasma, FCV-F9 was exposed to Argon atmospheric plasma generated at 1, 1.5, 2, 2.5 and 3 W up to 180 s *(1)*, that led to gradual reductions in the FCV-F9 titer ranging from 0.33 to 2.66, 0.66 to 4.00, 0.88 to 4.66, 0.99 to 5.55, and 1.11 to 5.55 log$TCID_{50}$/0.1 ml, respectively while they showed complete reduction after exposure to 2.5 and 3W plasma for 120 s *(1)*. Although CAPP treatment shows titer reduction of HNoV surrogates, it has certain disadvantages such as high cost, uncertainty of sensory and nutritional impact in food matrix, complexity of equipment. Therefore, there is a need to search for alternative inactivation techniques.

2.7 Ultrasound

Ultrasound are sound waves with frequency higher than 20 kHz whereas high intensity ultrasound has frequency range of 20 kHz - 2 MHz and causes microbial inactivation by shearing the cell wall, disrupting the cell membrane and damaging the DNA through the production of free radicals *(42, 114)*. FCV-F9, MNV-1 and MS2 at an initial titer of 4 log PFU/ml in PBS when subjected to ultrasound treatment at 20 kHz was reported

to show reduction to non-detectable levels of FCV-F9, MS2 and MNV-1 after 5, 10 and 30 min, respectively *(114)*. Whereas, in orange juice, FCV-F9 showed complete reduction after 15 min while MNV-1 showed only 1.55 log reduction after 30 min with what treatment time, temperature *(114)*. Therefore, ultrasound only showed minimal reduction of HNoV surrogates even at long exposure time, necessitating the need for alternative inactivation technique.

2.8 Antimicrobials

Grape seeds, the by-products of wine and the grape juice industries, contain large quantities of phenolic compounds as well as dimeric, trimeric, and proantho-cyanidins (PAC) or condensed tannins *(64)*. It has been considered as a generally regarded as safe (GRAS) by the U.S. Food and Drug Administration (sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act). It inhibits viral replication for enveloped and non-enveloped virus by blocking the virus binding to the host cell receptors or by coating the virus to prevent attaching to the host-cell or by causing clumping of viral particles *(64)*.

HNoV in lettuce extract was found to be reduced by approx. 0.5 and 1.5 log genomic copies/ml after treatment with 0.2 and 2 mg/ml GSE *(76)*. Similarly in 0.01% milk, reduction of approx. 1 and 1.25 log genomic copies/ml of HNoV GII.4 with 0.2 and 2 mg/ml GSE was reported, while in the case of 0.1% milk there was no reduction with 0.2 mg/ml GSE but a reduction of 1.25 log genomic copies/ml HNoV GII.4 for 2mg/ml GSE *(76)*. In PBS, HNoV GII.4 was reduced by 1 and 2 log genomic copies/ml at GSE concentrations of 0.2 and 2 mg/ml, respectively *(75)*.

GSE at 0.25, 0.5, and 1.0 mg/ml GSE has been shown to reduce MNV-1 (at 5 log PFU/ml) by 1.49, 1.72, and 1.97 log PFU/ml at 37°C for 2 h, whereas the reduction at same concentrations at room temperature were 1.37, 1.48, and 1.67 log PFU/ml *(112)*. At 37°C, 0.25 mg/ml GSE reduced FCV-F9 titers by 0.26, 0.31, 0.34, 0.46, 1.73 log PFU/ml after 5, 10, 15, 30 min and 1 h, respectively, and to undetectable levels after 2, 6, and 24 h (66). GSE at 1 and 2 mg/ml in apple juice (AJ) was reported to reduce FCV-F9 titers to undetectable levels after 5 min, while GSE at 2, 4and 8 mg/ml in 2% milk at 37°C showed 0.92, 0.96 and 1.07 log PFU/ml reduction of FCV-F9 respectively. MNV-1after 30min treatment with GSE at 1, 2 and 4mg/ml in AJ at pH 3.6 was reduced to undetectable levels, while GSE with 2% milk showed no reduction

in MNV-1 titer even after 24 h *(64)*. Hence, it can be seen that grape seed extract has the potential for reduction of HNoV surrogates but with long treatment time.

Natural extracts such as essential oils or their main compounds, categorized as Generally Recognised as Safe (GRAS), are potential alternatives to chemical preservatives, that can possess antibacterial, antifungal, insecticidal, pesticidal, antitoxigenic, antiviral and anti-parasitic activity *(102)*. They are extracted by hydro-distillation of plants that are rich in sources of biologically active compounds, such as phenolic acids and terpenoids *(101)***.** Carvacrol, a monoterpenic phenol, is the primary component of oregano essential oil, with reported antimicrobial activity against a wide range of food spoilage or pathogenic fungi, yeast and bacteria *(101)*. It shows antiviral activity by altering the capsid integrity and by preventing adsorption of the virus to host cells *(52)*. Carvacrol at 0.25, 0.5 and 1% was shown to reduce MNV-1 (initial titer 5.7 log $TCID_{50}$/ml) by 1.86, >3.57 and >3.57 log$TCID_{50}$/ml, respectively and FCV-F9 by 3.41, >4.53 and >4.53 log $TCID_{50}$/ml, respectively after 2 h at 37°C in suspension *(101)*. When MNV-1 (initial titer 10^6 $TCID_{50}$/ml) was treated with 4% oregano oil or 0.5% carvacrol (v/v) for up to 24 hours, >3.27 log reduction in MNV-1 titer after 1 and 2 h with carvacrol at 0.5 and 0.25%, respectively was obtained *(52)*. Treatment of MNV-1-1 with 0.5, 1 and 2% oregano oil for 2h at 37°C was reported to show 1.04, 1.17 and 1.62 log $TCID_{50}$/ml reduction while there was 1.38, 2.46 and 3.75 log $TCID_{50}$/ml reduction for FCV-F9 *(44)*. MNV-1 (at initial titer of 6.07 $TCID_{50}$/ml) with thymol, one of the main components of oregano, at 0.5, 1 and 2% showed a 0.5, 1.66 and 2.45 log $TCID_{50}$/ml reduction in suspension at 37°C after 2 h *(102)*. Treatment with clove essential oil (EO) at 0.1, 0.5 and 1% at 37°C was reported to cause reductions of MNV-1 by 0.42, 0.5 and 0.67 log $TCID_{50}$/ml at 37°C, respectively and 0.96, 0.58 and 0.83 log $TCID_{50}$/ml reduction at 4°C, whereas for FCV-F9 reduction of 0.92, 2.26 and 3.75 log $TCID_{50}$/ml at 37°C and 0, 0.25 and 0.04 log $TCID_{50}$/ml at 4°C was obtained *(44)*.

Zataria essential oil at 0.01, 0.04, 0.08 and 0.1%, caused MNV-1 reductions of 0, 0, 0.55 and 1.01 log $TCID_{50}$/ml at 4°C respectively, and reduction of 0.04, 0.54, 0.29 and 0.25 log $TCID_{50}$/ml at 37°C, whereas for FCV-F9 reduction of 0.13, 1.04, 1.91, 4.17 and 4.51 log $TCID_{50}$/ml at 37°C and no reduction at 4°C was reported *(44)*. Lemongrass oil, citral (one of the main components of lemongrass oil) and allspice oil (68.6% eugenol *(41)*) at 2 and 4% (vol/vol) treatment of MNV-1 (at initial titer of 6 log $TCID_{50}$/ml) was reported to cause reduction of 0.38, 0.74 and 2.19 log $TCID_{50}$/ml after

0.5, 6 and 24 hours with 2% lemongrass oil, respectively, whereas 4% lemongrass oil reduced MNV-1 by 0.59, 0.9 and 2.74 log, respectively after 0.5, 6 and 24 hours *(53)*. Citral contains two isomeric acyclic monoterpene aldehydes, geranial and neral. Citral at 2% was able to reduce MNV-1 by 0.67, 1.4 and 2.4 log $TCID_{50}$ after 0.5, 6 and 24 h, respectively and 4% showed reductions of 0.7, 1.88 and 3 log after these time intervals, respectively *(53)*.

Seeds and pericarp of *Zanthoxylum schinifolium*, which belongs to the *Rutaceae* family, are widely consumed in Korea, China, and Japan as a spice and in folk medicine as an antimicrobial and antioxidant for the treatment of vomiting, diarrhea, and abdominal pain *(44)*. Post treatment with *Z. schinifolium* seed oil at 0.01% after 1 h at 37°C, showed 70% and 20% inhibition in plaque formation ofFCV-F9 and MNV-1, respectively *(87)*. Thyme EO, which is extracted from *Thymus vulgaris* of the mint family, at 2% has been reported to reduce MNV-1 by 0.5 log $TCID_{50}$/ml after 1 h at 37°C, whereas 2% oregano EO and Mint EO were shown to reduce MNV-1 by 0.75 and 0.87 log $TCID_{50}$/ml at the same time temperature conditions *(43)*.

Cranberry has historically been used by native Americans for treating bacterial infections. MNV-1 was shown to be reduced by 1.90, 1.66, 2.24 and 2.94 log PFU/ml after treatment for 1 h at room temperature with cranberry juice (CJ) at pH 2.6, CJ at pH 7.0, 0.15 mg/ml polymeric proanthocyanidins (PAC) and 0.30 mg/ml PAC, respectively *(113)*. Pomegranate juice and 6 mg/ml pomegranate polyphenols (PP), has been reported to reduce MNV-1 by 1.32 and 3.61 log PFU/ml, respectively with one hour at room temperature *(113)*. Black raspberry juice at 3% concentration has been reported to show 43% and 58% inhibition in viral replication of MNV-1 at co-treatment and posttreatment for one hour at 37°C *(86)*. Therefore, natural extracts have the potential for inactivation of HNoV surrogates, but a long treatment time is required.

2.9 Heat

One of the most successful inactivation strategy/approach is thermal inactivation. Heat disrupts the hydrogen bonding and destroys the spatial relationships necessary to maintain the structural integrity of viral proteins *(22)* thereby leading to denaturation of viral proteins as well as destruction of virus particles into non-infectious viral subunits and single proteins and releasing RNA or denaturing RNA *(107)*. During

thermal processing, heat inactivation kinetics (D- and z-values) are used to determine adequate processing to ensure food safety.

The D-value is defined as the time at a given temperature necessary to reduce a microbial population present in a defined medium by 90% and is indicative of the thermal resistance of a microorganism at a constant temperature *(19)*. The z-value is the change in temperature required to increase or decrease the D-value by 90% and is indicative of the temperature dependence of microbial inactivation *(19)*. Industrially, often six log reduction is used for processes such as pasteurization. This is represented by the 6D- value *(19)*. HNoVGI.1 samples heated in a thermocycler at 99°C for 5 min showed 1.77 and 1.73 log reduction in genomic copies/ml (gc/ml) after thermal inactivation with and without RNase A treatment respectively. Similar reductions of 1.71 and 1.56 log gc/ml were obtained with and without RNase A treatment for what HNoV GII.4 *(6)*.

Artificially contaminated oysters with HNoV GII.4 VLPs at 4log RNA copies/ml were investigated for HNoV ability to bind to porcine gastric mucin conjugated to magnetic beads (PGM-MBs), after heating at 80°C for 10, 30, 60s, or 5min, or at 100°C for 5s in a capillary tube sealed with a vinyl plastic cover followed by rapid cooling in ice *(6)*. The researchers found that at 80°C after 5min there was a slight reduction in protein density of the VLPs binding to the PGM-MBs as compared to original input VLPs and they completely lost their ability to bind after treatment at 100°C for 5s *(6)*.

Wang et al treated HNoV at different conditions including heat treatment of 300 µl aliquots of PBS-diluted virus stock in 1.5 ml microcentrifuge tubes at 56, 63, 72, and 100°C up to 60 min *(127)*. They showed by using in situ capture qRT-PCR that treatment for 2 min at 100°C resulted in the complete loss of HNoV RCVGS whereas treatment after 2min at 72°C showed only 0.60 to 1.04 log reductions, while a complete loss of the RCVGS could be observed at 4 min and longer. Treatment at 63°C also resulted in a time-dependent loss of HNoV RCVGS, but a 60-min treatment only achieved a 1.37 log reduction *(127)*.

The protruding (P) domain of VP1 (major structural protein) of NoV capsid when expressed in *Escherichia coli* forms subviral particles, the P particles *(74)*. P particles from several HNoV strains (GII.4, GII.9, GI.4, GI.1) were subjected in parallel to critical heat treatment (60°C for 30 min and 70°C for 2 min) and harsh heat treatment (85°C for 2 min) *(74)*. GII.4 was found to be the most resistant to critical heat treatment and GII.9 was found to be the most resistant to harsh heat treatment while HNoV GI.1 was

found to be the most sensitive one to all the tested heat treatments of the four norovirus strains studied *(74)*. HNoV GII.4 extracted from positive stool samples at initial concentration of 4 to 5 log RT-QPCR copies was subjected to heat inactivation for 2 min at different temperatures from 20 to 90°C in thin walled PCR tubes in a thermal cycler *(124)*. On measuring % survival of Taqman RT-QPCR target following RNAse treatment HNoV showed 60% survival at 60°C for 2min and was reduced to 0% after 80°C for 2min *(124)*.

Brie et al, 2016 showed that when MS2 was exposed to 72°C, the particles were disrupted and the genome became susceptible to degradation by RNases *(25)*. When MS2 at an initial concentration of 10 log PFU/ml was heated at 72°C in thin-walled, 500-µl tubes in a PCR thermocycler and 30 W UV for 1 to 4 min *(94)*, the first-order rate for enzymatic treatment qPCR was 5.2 ± 1.3 min^{-1} and 2.5 ± 0.04 min^{-1} for heat and UV treatment respectively giving 8.6 and 8.7 log PFU/ml loss in infectivity *(94)*.

The D-values calculated from the first-order model (50 to 72°C) ranged from 0.21 to 19.75 min for FCV-F9, 0.25 to 36.28 min for MNV-1in 2-ml GLASS vials in cell culture media using a circulating water bath *(18)*. There have been various studies for determination of D and Z-values of HNoV surrogates in food matrices (Table1.7-1.12). In blue mussels, the D-value at 50 to 72°C in 2ml vials was reported to be 0.07 - 5.2 min for FCV-F9 and 0.18 – 20.9 min for MNV-1 using the first order model *(19)*. Whereas in turkey deli meats, the D-values calculated using first order model ranged from 0.1 – 9.9 min for FCV-F9 and 0.2 – 21 min for MNV-1 in vacuum bags at a temperature range of 50 to 72°C *(22)*.For spinach, the D values at 50 to 72°C in 2ml vials ranged from 0.15 – 17.39 min for FCV-F9 and 0.16 – 14.57 min for MNV-1 *(18)*. However, HAV showed the most resistance to heat when compared to MNV-1 or FCV-F9, with D-values of 56.2, 8.4 and 2.67 min in 2-ml glass vials with cell culture media *(19)* and 42, 20.6 and 5.9 min for turkey deli meat in vacuum bags *(21)*, 34.4, 8.43 and 4.55 min for spinach in vacuum bags *(24)* at 50, 56 and 60°C.

TV has reported D-values of 11.8, 2.6 and 5.3 min in cell culture media in microcentrifuge tubes using tissue culture infectious dose *(123)* and 4.03, 1.18 and 0.24 min using plaque assay *(7)* at 56, 63 and 72°C, respectively. Others have shown, 1.79, 1.83, 2.9 and 3.07 log PFU/ml reduction in TV titer when heated at 50, 55, 60 and 65°C, respectively in 0.2 ml PCR tubes for 2 min *(59)* (Table 1.13). AiV (initially at 7 log PFU/ml) is reported to be more resistant than MNV-1 or FCV-F9 showing 4 and

5 log reduction after 10 and 20 min in microcentrifuge tube in cell culture media at 56°C *(33)*.

Based on the information available to-date on the thermal inactivation kinetics of foodborne viruses (Table 1.7-12), research is on-going in the search for potential surrogates for HNoV. Furthermore, there have been only a few studies done for determining thermal inactivation behavior of AiV in cell culture media and to our knowledge no reported studies are present on thermal inactivation kinetics of TV in food matrices have been reported to date.

Therefore, the objectives of this research were to:

1) To determine heat inactivation kinetics of Tulane virus (as a cultivable human norovirus surrogate) in cell culture media in 2 ml glass vials and spinach in vacuum bags.

2) To determine heat inactivation kinetics of Aichi virus in cell culture media in 2 ml glass vials.

Based on these results, potentially appropriate thermal processing parameters to inactivate foodborne viruses can be designed.

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4 APPENDIX

Tables

Table 1.1 Recent outbreaks of HNoV worldwide

Year	State	Deaths	Food Vehicle/ Contamination source	Reference
2015	Kansas	1	Not specified	CDC, 2016
2015	Ohio	3	Homemade potato salad, Beef	CDC, 2016
2015	North Carolina	1	Pork	CDC, 2016
2015	Florida	1	Raw oysters	CDC, 2016
2015	Utah	1	Not specified	CDC, 2016
2015	US (multistate)	13	Pre-packaged lettuce, cucumber	CDC, 2016
2015	Belgium	-	Healthcare facility	*(100)*
2014	New York	2	Not specified	CDC, 2016
2014	US (multistate)	2	Mung bean sprouts	CDC, 2016
2014	US (multistate)	7	Caramel apple	CDC, 2016
2013	Rhode Island	2	Not specified	CDC, 2016
2012	California	4	Soup, mushroom	CDC, 2016
2012	US (multistate)	3	Cantaloupe	CDC, 2016
2012	US (multistate)	5	Ricotta salata cheese	CDC, 2016
2011	Rhode Island	2	Not specified	CDC, 2016
2011	Oregon	2	Strawberries	CDC, 2016
2011	US (multistate)	33	Cantaloupe	CDC, 2016
2011	Norway	-	Not specified	*(55)*
2011	France	-	Pasta, raw vegetables	*(81)*

Table 1.2 Non-thermal inactivation of MNV

Treatment	*Matrix*	*Conditions*	*Log Reduction*	*Units*	*Ref*
UV	Cell culture	10 mW/cm^2, 2min	ND	PFU/ml	*(79)*
	Blueberries	10 mW/cm^2, 5min	1.73		
		10 mW/cm^2, 1min	2.43		
		10 mW/cm^2, 2min	2.48		
		10 mW/cm^2, 5min	3.04		
Ozone	Alfalfa seeds	6.25 ppm, 22°C, 0.5 min	4.04	PFU/g	*(129)*
	Cell culture	6.25 ppm, 22°C, 1 min	4.27		
	Green onions	6.25 ppm, 22°C, 5 min	4.56		
	Internalized	6.25 ppm, 22°C, 15 min	4.9		
	Green onions	6.25 ppm, 22°C, 30 min	5.6		
	external	6% wt/wt, 10 min	4.1	PFU/ml	*(95)*
		6.25 ppm, 20°C, 10 min	1.5	PFU/plant	*(60)*
			2.5		

Table 1.2 Continued

Treatment	*Matrix*	*Conditions*	*Log Reduction*	*Units*	*Ref*
Pulsed light	Cell culture	polyethylene disks, 0.69 J/cm^2	0.5	PFU/ml	*(125)*
		polyethylene disks, 8.98 J/cm^2	4		
		polyvinyl chloride disks, 0.69 J/cm^2	0.5		
		polyvinyl chloride disks, 8.98 J/cm^2	3.5		
		stainless steel disks, 0.69 J/cm^2	0.5		
		stainless steel disks, 8.98 J/cm^2	2.5		
Ultrasound	Orange juice	20kHz, 30 min	4	PFU/ml	*(114)*

Table 1.3 Non-thermal inactivation of HNoV

Strain	*Treatment*	*Conditions*	*Reduction*	*Units*	*Ref*
HNoV	UV	250 mJ/cm^2	3.26	log RCVGS	*(127)*
GII.4		500 mJ/cm^2	2.96		
		750 mJ/cm^2	2.45		
		1000 mJ/cm^2	1.54		
		1500 mJ/cm^2	-0.37		
NV	Ozone	0.37-mg/liter, pH 7, 5°C, 0s	>4	genomic RNA	*(105)*
8FIIa		0.37-mg/liter, pH 7, 5°C, 20s	>4.5		
HNoV	Cold	cold atmospheric pressure plasma, 10 min	1.23	genomic	*(3)*
GII.4	plasma	cold atmospheric pressure plasma, 15 min	1.69	equivalents/ml	

Table 1.4 Non-thermal inactivation of FCV

Treatment	*Matrix*	*Conditions*	*Log Reduction*	*Units*	*Ref*
UV	Cell culture	29 mJ/cm^2	4	PFU/ml	*(90)*
	Lettuce	40 mW s/cm^2	3.48	$TCID_{50}$/ml	*(49)*
	Green onions		2.46		
	Strawberries		1.13		
	Lettuce	240 mW s/cm^2	4.62		
	Green onions		3.88		
	Strawberries		2.28		
Ozone	Buffered	1 mg/ml, 0.25 min	4.28		*(121)*
	demand free	1 mg/ml, 1.2 min	>4.7		
	water	0.06 mg/ml, 0.25 min	0.85		
		0.06 mg/ml, 5 min	2.77		
Cold plasma	Cell culture	Ar atmospheric plasma, 180s, 1W	2.66	$TCID_{50}$/0.1ml	*(1)*
		Ar atmospheric plasma, 180s, 1.5W	4		
		Ar atmospheric plasma, 180s, 2W	4.66		
		Ar atmospheric plasma, 180s, 2.5W	5.55		
		Ar atmospheric plasma, 180s, 3W	5.55		
Ultrasound	Orange juice	20kHz, 5 min	4	PFU/ml	*(114)*

Table 1.5 Non-thermal inactivation of TV

Treatment	*Matrix*	*Conditions*	*Log Reduction*	*Units*	*Ref*
UV	Cell culture	30 mJ/cm^2	3	$TCID_{50}$/ml	*(132)*
		60 mJ/cm^2	5.4		
Ozone	Cell culture	6% wt/wt, 10 min	0.5	PFU/ml	*(95)*
		6% wt/wt, 40 min	ND		
	Alfalfa seeds	6.25 ppm, 22°C, 0.5 min	1.66	PFU/g	*(129)*
		6.25 ppm, 22°C, 1 min	2.03		
		6.25 ppm, 22°C, 5 min	3		
		6.25 ppm, 22°C, 15 min	3.45		
		6.25 ppm, 22°C, 30 min	3.83		

Table 1.6 Non-thermal inactivation of AiV

Treatment	*Matrix*	*Conditions*	*Log Reduction*	*Units*	*Ref*
UV	Cell culture	10 mW s/cm^2	2 to 3	$TCID_{50}$/ml	*(49)*
		50 mW s/cm^2	5 to 6		
		75 mW s/cm^2	6 to 7		
	Lettuce	40 mW s/cm^2	3.96		
	Green onions		2.35		
	Strawberries		1.53		
	Lettuce	120 mW s/cm^2	4.41		
	Green onions		3.66		
	Strawberries		1.6		
	Lettuce	240 mW s/cm^2	4.59		
	Green onions		2.49		
	Strawberries		1.87		

Table 1.7 Heat inactivation parameters of HNoV in different matrices at different temperatures.

Virus	Temp (°C)	Matrix	Vessel	Time (min)	Log Reduction	Unit	Reference
NoV GII.4	56	Cell culture	1.5 ml microcentrifuge tube	2	0.02	RCVGS	*(127)*
	63				0.08		
	72				0.6		
	100				2.8		
NoV GII.4	60	Viral suspension (FCV+NoV GII.4)		15	0.6	RT-PCR	*(32)*
NoV GII.4	80	Viral suspension (FCV+NoV GII.4)			0.9	RT-PCR	
NoV GII.4	60	Viral suspension (FCV+NoV GII.4) in spiked mussels			0.3	RT-PCR	
NoV GII.4	80	Viral suspension (FCV+NoV GII.4) in spiked mussels			0.5	RT-PCR	

Table 1.8 Heat inactivation parameters of MNV in different matrices at different temperatures.

Temp (°C)	Matrix	Vessel	Time	D-value (min)	Log Reduction	Unit	Reference
50	Cell culture	Glass capillary tube		34.49			*(18)*
56				3.65			
60				0.57			
65				0.3			
72				0.15			
63	Water	0.2 ml PCR tube	10 min	0.9	3.28	PFU/ml	*(57)*
72				<0.3	>3.5		
63	Milk			0.7	>3.5		
72				0.5	>3.5		
50	Cell culture	0.2 ml PCR tube	2 min		0.81	PFU/ml	*(59)*
55					1.69		
60					3.11		
65	Raspberry (pH	Stomacher bag	30s		1.86	PFU/g	*(11)*
75	3.1)		15s		2.81		
50	Blue mussels	2ml glass vials	6 min	20.19	2.11	PFU/ml	*(23)*
56			3 min	6.12	1.76		
60			60 s	2.64	2.15		

Table 1.8 Continued

Temp (°C)	Matrix	Vessel	Time	D-value (min)	Log Reduction	Unit	Reference
50	Spinach	2ml glass vials	6 min	14.57	0.47	PFU/ml	*(17)*
56			3 min	3.29	1.82		
60			3 min	0.98	3.61		
65			40 s	0.4	3.66		
72			20 s	0.16	3.21		
56	Cell culture	microcentrifuge tube	20 min		>5	TCID50/ml	*(33)*
			10 min		5.5		
50	Cell culture	2ml glass vials		36.28			*(19)*
56				3.74			
60				1.09			
65				0.77			
72				0.25			
50	Turkey deli meat	Vacuum bag		21			*(21)*
56				7.3			
60				2.7			
65				0.9			
72				0.2			

Table 1.9 Heat inactivation parameters of FCV in different matrices at different temperatures.

Temp (°C)	Matrix	Vessel	Time	D-value (min)	Log Reduction	Unit	Reference
50	Cell culture	Glass capillary tube		20.23			*(18)*
56				6.36			
60				0.56			
65				0.32			
72				0.11			
50	Blue mussels	2ml glass vials	6 min	5.2	1.81	PFU/ml	*(23)*
56			3 min	3.33	2.75		
60			60 s	0.77	4.93		
65			15 s	0.33	5.62		
72			10 s	0.07	6.41		
50	Spinach	2ml glass vials	6 min	17.39	0.68	PFU/ml	*(17)*
56			3 min	5.83	2		
60			3 min	0.78	4.64		
65			40 s	0.27	5.76		
72			20 s	0.15	3.17		
56	Cell culture	microcentrifuge tube	20 min		>5	TCID50/ml	*(33)*
			10 min		4.5		

Table 1.9 Continued

Temp (°C)	Matrix	Vessel	Time	D-value (min)	Log Reduction	Unit	Reference
60	Viral suspension (FCV+NoV GII.4)		15 min		0.4	RT-PCR	*(32)*
	Cell culture				>3.5	TCID50/ml	
80	Viral suspension (FCV+NoV GII.4)				0.6	RT-PCR	
	Cell culture				>3.5	TCID50/ml	
60	Viral suspension (FCV+NoV GII.4)				0.8	RT-PCR	
	Spiked mussels				2.2	TCID50/ml	
80	Viral suspension (FCV+NoV GII.4)				1.2	RT-PCR	
	Spiked mussels				2.2	TCID50/ml	
50	Cell culture	2ml glass vials		19.95			*(19)*
56				6.37			
60				0.94			
65				0.72			
72				0.21			
50	Turkey deli meat	Vacuum bag		9.9			*(21)*
56				3			
60				0.8			
65				0.4			
72				0.1			

Table 1.10 Heat inactivation parameters of HAV in different matrices at different temperatures.

Temp (°C)	Matrix	Vessel	Time (min)	D-value (min)	Log Reduction	Unit	Reference
50	Mussels	2ml glass vials		54.17			*(20)*
56				9.32			
60				3.25			
65				2.16			
72				1.07			
42.7	Green onions	Dehydration for	20 hours		0.84	PFU/ml	*(115)*
50.6		20h at target			0.94		
56.4		temp			1.61		
60.7					2.64		
65.9					3.91		
63	water	0.2 ml PCR tube	10 min	0.6	>3.5	IU/ml	*(58)*
72				<0.3	>3.5		
63	milk			1.1	>3.5		
72				<0.3	>3.5		
50	Spinach	Vacuum bag		34.4			*(24)*
56				8.43			
60				4.55			
65				2.3			

Table 1.10 Continued

Temp (°C)	Matrix	Vessel	Time (min)	D-value (min)	Log Reduction	Unit	Reference
56	Cell culture	2ml glass vial		8.4			(19)
60				2.67			
65				1.73			
72				0.88			
50	Turkey deli meat	Vacuum bag		42			*(21)*
56				20.6			
60				5.9			
65				2.3			
72				1			

Table 1.11 Heat inactivation parameters of TV in different matrices at different temperatures.

Temp (°C)	Matrix	Vessel	Time (min)	D-value (min)	Log Reduction	Unit	Reference
50	Cell culture	0.2 ml PCR tube	2 min		1.79	PFU/ml	*(59)*
55					1.83		
60					2.9		
65					3.07		
56	Cell culture	microcentrifuge tube	20 min 10 min		~3.5 ~4	TCID50/ml	*(33)*
56	Cell culture	microcentrifuge tubes		11.8			*(123)*
63				2.6			
72				4.3			
37	Cell culture	microcentrifuge tubes		500			*(7)*
56				4.03			
63				1.18			
72				0.24			

Table 1.12 Heat inactivation parameters of AiV in different matrices at different temperatures.

Temp (°C)	Matrix	Vessel	Time (min)	Log Reduction	Unit	Reference
56	Cell culture	microcentrifuge tube	20 min 10 min	4 5	$TCID_{50}$/ml	*(33)*

CHAPTER II

THERMAL INACTIVATION KINETICS OF TULANE VIRUS IN MEDIA AND SPINACH

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1 ABSTRACT

Human noroviruses (HNoVs) cause gastrointestinal diseases worldwide. Tulane virus (TV) is recognized as a cultivable HNoV surrogate used to determine control measures against HNoVs. The objective of this study was to determine the heat inactivation kinetics (D- and z-values) of TV in cell-culture media in 2ml glass vials and spinach in vacuum bags using the first-order and Weibull models. TV in cell-culture media at ~7 log PFU/ml in 2-ml glass vials was heated at 52, 54, 56 and 60^{o}C for up to 10 min in a circulating water-bath. Survivors were enumerated by plaque assay using confluent LLC-MK2 cells in 6-well plates. Each treatment was replicated thrice, assayed in duplicate and data were statistically analyzed. The D-values by the first order model for TV in cell culture media at 52, 54, 56 and 60^{o}C were 4.59±0.02, 2.91±0.01, 1.74±0.41 and 0.58±0.36 min, respectively, with a z-value of 9.09±0.01^{o}C (R^2 = 0.997). The Weibull model showed $t_{d=1}$ values of 2.53±0.08, 1.99±0.10, 0.57±0.64 and 0.22±0.25 min, respectively for TV at the same temperatures. The D-values for TV in spinach were 7.94±0.21, 4.09±0.04 and 1.43±0.02 min and a z-value of 10.74±0.01^{o}C (R^2 = 0.98) by the first order model and 4.89±0.02, 3.21±0.45 and 0.25±0.38 min for the Weibull model at 50, 54 and 58^{o}C, respectively. In comparison to MNV-1, TV in cell-culture media and spinach showed lower D- and z-values. TV may not be as suitable a surrogate as MNV-1 for HNoV heat inactivation studies in cell culture media in 2-ml vials or spinach in vacuum bags.

2 INTRODUCTION

Human noroviruses (HNoVs) are the leading causative agents of acute gastroenteritis responsible for illness of 1 in every 15 people each year in the United States alone (22). Fever, diarrhea, vomiting, chills, and severe dehydration are some of the common symptoms of HNoV infection (21). Human noroviruses pose a potential risk to public health as it has a low infectious dose and can spread through water, food, and person-to-person contact (17). Consumption of HNoV-contaminated minimally processed food and fresh produce, shellfish, ready- to-eat meals, sandwiches, baked products, water, and ice pose health risks to humans (20, 26). Human noroviruses belong to the *Caliciviridae* family and contain a single-stranded positive-sense RNA of 7.5 to 8.5 kilobases in length enclosed in a capsid (14). Currently there are six known genogroups of HNoVs (with one additional genogroup proposed) out of which genogroup I (GI), GII, and GIV affect humans (15). Due to the difficulty in assessing infectivity and current unavailability of animal models or cell culture infectivity assays for HNoVs, animal caliciviruses such as murine norovirus (MNV-1), feline calicivirus (FCV-F9), porcine enteric sapovirus (PEC), and Tulane virus (TV) are used as surrogates for current research (12, 13, 17).

Tulane virus was recently isolated from the stool samples of rhesus monkeys that belongs to the genus *Recovirus* in the *Caliciviridae* family (25). Similar to HNoVs, it binds to histo-blood group antigens and is closely related to HNoV genogroup II (17). It is small, non-enveloped, and icosahedral with positive-sense, single-stranded RNA genome which is 6714 nt in length and 40 nM in diameter (14). Both, HNoV and TV have three open reading frames (ORF) wherein ORF1 encodes non-structural protein, ORF2 encodes the capsid protein (VP1), and ORF3 encodes minor structural proteins (VP2) (14). The 90 dimers of capsid protein are divided into two domains, the shell domain (S) which is an eight-folded jellyroll structure and forms the icosahedral shell and the protruding domain (P) with subdomains P1 and P2, which emanate from S and form the dimeric protrusion and is responsible for the viral entry into the host cell (27). The VP1 (capsid protein) comprises of 534 amino acids (aa) with a molecular mass of 57.9 kilodaltons (kDa), whereas VP2 (minor structural protein) contains 218 aa and has a molecular mass of 22.8 kDa (14). Hence, this cultivable virus is used to determine appropriate inactivation methods against HNoV.

When comparing HNoV surrogates for their stability under different environmental conditions, TV and MNV-1 in cell culture media were reported to be more pH resistant than FCV-F9. Both, TV and MNV-1 showed <0.5 log PFU/ml reduction in infectivity with 100 mM citric acid (pH 2) and only ~ 2 log PFU/ml reduction with 100mM carbonate buffer (pH 10) after 30 min at 37°C, while FCV-F9 titers are reduced to non-detectable levels at both pH 2 and pH 10 under the same conditions (12). TV showed reduction in infectivity of 1.2 log PFU/ml when treated with commercial bleach (1,000 ppm chlorine) for 5 min at room temperature on stainless steel discs, while MNV-1 showed a slightly higher reduction of 1.4 log PFU/ml (11). FCV-F9 was the most sensitive among the three tested viruses that showed 5.3 log PFU/ml reduction under the same treatment conditions (12). TV is also known to be highly resistant to high hydrostatic pressure processing showing 6 log PFU/ml reduction at 600 MPa for 1 min at 4°C, as compared to 300 and 400 MPa pressure required for FCV-F9 and MNV-1, respectively (12). These results suggest that TV is most resistant to change in pH, pressure or chlorine concentration as compared to FCV-F9 and MNV-1 under the given conditions, and is therefore a suitable surrogate for HNoV for the above-mentioned processing and inactivation approaches.

Thermal inactivation has been used traditionally and most-widely to inactivate pathogens in food and thus preserve the food. Heat disrupts the hydrogen bonding of proteins and destroys the spatial relationships necessary to maintain the structural integrity of bacterial and viral proteins thereby leading to their denaturation as well as the destruction of virus particles into non-infectious viral subunits and single proteins (8, 23). Above 60°C, after the inactivation of host-cell recognition and binding receptors, an alteration of the tertiary protein structure is reported to occur, which can facilitate the access of thermal energy to the nucleic material (3). For industrial purposes and to understand the heat inactivation kinetics of microorganisms, D- and z-values are determined. The D-value is defined as the time at a given temperature necessary to reduce a microbial population present in a defined medium by 90% (or one-log) and is indicative of the thermal resistance of a microorganism at a constant temperature (4, 6). The z-value is the change in temperature required to increase or decrease the D-value by 90% and is indicative of the temperature dependence of microbial inactivation (4, 6). Inversing the slope of log D versus temperature is used to obtain the z-value. Earlier research has been carried out on cultivable HNoV surrogates, FCV-F9, MNV-1 as well as hepatitis A virus (HAV) in buffer and various

model food systems (6-8, 25). Although studies have been performed to analyze thermal inactivation kinetics of TV in media, only 1.5 ml centrifuge tubes (2, 24) or 0.2 ml PCR tubes (17) or capillary tubes (1) were used. The heat-inactivation kinetics of TV in 2-ml glass vials as well as the Z- values have not been reported to date. The container type and size can play a role in heat-inactivation due to differences associated with the heat transfer rate and come-up times (7). Moreover, the heat-inactivation or persistence of HAV, MNV-1 and FCV-F9 have been previously reported in leafy vegetables including spinach (6, 8), lettuce (18), basil and parsley (11). To the best of our knowledge, there are no reported studies on thermal inactivation of TV in spinach.

The objectives of this study were to (i) determine the D- and z-values of TV in cell-culture media contained in 2-ml glass vials, (ii) determine the thermal inactivation behavior of TV in spinach in vacuum bags, and (iii) compare the first order and Weibull models to describe the heat inactivation trends of TV in terms of selected statistical parameters. The suitability of using TV as a relevant surrogate for heat inactivation of HNoV would be thus determined.

3 MATERIALS AND METHODS

3.1 Virus and Host

Tulane virus (TV) was obtained as a gift from Dr. J. Jiang (Cincinnati Children's General Hospital, Cincinnati, Ohio) and LLCMK-2 host cells were used for virus infectivity and propagation. TV stocks were prepared by infecting confluent LLC-MK2 cells in 175 cm^2 flasks with Opti-MEM (minimum essential media; Opti-MEM® I Reduced Serum Medium, GlutaMAX™ Supplement, Grand Island, NY) containing 1 X Anti-anti (Antibiotic Antimycotic; Invitrogen, Grand Island, NY), supplemented with 2% heat inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and incubating at 37°C under 5% CO_2 until lysis for 3 days as reported before (*11, 16*). The virus was recovered by centrifugation at 5000×g for 10 min, followed by filtration through 0.2 µm (2×10^{-7}m) filters, aliquoted, and stored at -80°C until use.

3.2 Heat Treatment of TV with cell-culture media in 2-ml glass vials

Heat treatment was carried out in a circulating water bath (Isotemp 2150, Fisher Scientific, Pittsburgh, PA) in 2-ml screw-capped glass vials as reported earlier (7). Briefly, autoclaved (121°C, 15 min) vials were carefully filled with 1.8 ml TV by using sterile micro pipettes in a biosafety cabinet. The vials containing TV were surface rinsed in 70% ethanol before immersion in a thermostatically controlled water-bath. The temperature was confirmed by placing one end of type-T thermocouple (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water-bath and another thermocouple probe was placed at the geometric center of a vial through the lid to monitor the temperature of the buffered media as reported earlier (7). These thermocouples were connected to MMS3000-T6V4 type portable data recorder (Commtest Inc., New Zealand) to monitor temperature. The 2-ml glass vial containing samples were held at 52, 54, 56 and 60°C for varying treatment times of 0 to 10 min. The treatment time began (recorded as come-up time) when the target internal temperature reached the target temperature. The come-up times were 120, 140, 172 and 163 s for 52, 54, 56 and 60°C. After the thermal treatment, sample vials were surface rinsed with 70% ethanol and immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. Un-heated virus suspensions in 2-ml glass vials were used as controls. Ten-fold serial dilutions of the virus were carried out in 1.5 ml Opti-MEM (containing 2% FBS, and 1% antibiotic-antimycotic) and enumerated by plaque assays.

3.3 Inoculation of spinach with TV

Frozen chopped spinach samples from local grocery stores were mildly heated in the microwave (700 Watts/120 Volts/15 Amps) for 5 min and blended using a blender for homogenization. Five-milliliters of TV stock with initial titers of 7.25 ± 0.05 log PFU/ml was aseptically added to 25 g of spinach sample in a sterile beaker, covered with foil in a biosafety hood and stirred overnight under refrigeration at 4°C.

3.4 Thermal treatment of spinach inoculated with Tulane virus

Six-milliliter samples of homogenized TV-inoculated spinach were added to moisture barrier plastic bags (13 cm × 19 cm) and were vacuum sealed in to -100 kPa (Multivac

A300/16 vacuum packaging unit, Sepp Haggemuller KG, Wolfertschwenden, Germany), and were then flattened and placed in a holding unit as described before (10). Briefly, the holding unit was immersed in a thermostatically controlled circulating water bath (± 1°C) whose temperature was confirmed with by placing one type-T thermocouple (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water-bath and another thermocouple probe at the geometric center of an uninoculated bag of spinach. As described above for the 2-ml vials, the thermocouples were connected to a MMS3000-T6V4 type portable data recorder (Commtest Inc., New Zealand) to monitor temperature. The spinach samples containing TV were held at 50, 54 and 58ºC for varying treatment times of 0-8 min. The treatment time began (and was recorded as come-up time) when the target internal temperature reached the desired temperature. The come-up time for target temperature in vacuum bags were 44.5, 79.5 and 168.7 s for 50, 54 and 58°C respectively. The bags were rinsed with ethanol and cooled in an ice bucket after the set-time at target temperature. Un-heated virus suspensions from uninoculated spinach bags were used as controls.

3.5 Tulane virus extraction from spinach

Virus extraction was performed as described by Bozkurt et al. (7) with some modifications. In a biosafety cabinet, the spinach inoculated with TV bags were aseptically cut with sterilized scissors to transfer the contents to sterile beakers. For virus elution form the spinach, 15-ml elution buffer containing 0.1 M Tris-HCl, pH 9.5 (to elute the virus particles from the spinach sample in the presence of an alkaline environment), 3% beef extract paste and 0.05 M glycine (to reduce non-specific virus adsorption to the food matrix during extraction) was used. After adjusting the pH to 9.5 using 10 M NaOH, the samples were kept rotated on a shaking platform at 120 rpm for 20 min at 4°C. These samples were then transferred to sterile stomacher bags with filter compartments, and stomached twice at high speed for 40 s intervals. The obtained filtrate was centrifuged at 10,000×g for 15 min at 4°C, and the pH of the supernatant was adjusted to 7.2-7.4 using 6 N HCl to facilitate the polyethylene glycol precipitation of the virus particles as reported earlier (6). Polyethylene glycol 6000 (PEG) (to precipitate virus at high ionic concentrations without precipitation of other organic materials) and NaCl was added to obtain a final concentration of 10% PEG and 0.3 M NaCl. These samples were placed on a shaking platform (120 rpm)

overnight at 4°C and then centrifuged at 10,000×g for 30 min at 4°C. After discarding the supernatant, the remaining pellet, containing the virus, was dissolved in 1 ml of phosphate-buffered saline. Virus extracts were stored at -80°C until enumeration of plaques using the TV plaque assay.

3.6 Tulane Virus Plaque Assay

TV survivors were enumerated by infecting 500 µl of aliquots of dilutions of heat-treated or untreated virus on to confluent LLC-MK2 host cells in 6-well plates for 3 h at 37°C with 5% CO_2 before overlaying with 2X media comprising of Opti-MEM (containing 2% FBS, and 1% antibiotic-antimycotic) in 1.5% Noble agar (Difco Agar Noble, BD, Sparks, MD). Plates were then incubated at 37°C for 3 days followed by staining with neutral red and 1:1 Opti-MEM (containing 2% FBS, and 1% antibiotic-antimycotic) in 1.5% Noble agar and plaques were counted. Viral survivors or infectious particles were enumerated as plaque forming units/ml (PFU/ml).

3.7 Statistical Analysis

Each experiment at each temperature was repeated thrice and assayed in duplicate and data was analyzed using SAS 9.4 (SAS Institute, Cary, NC, USA) with PROCREG command for linear model and NLIN command for Weibull model.

3.8 Modeling of inactivation kinetics

First-order kinetics: The traditional approach to describe the change in number of survivors over time for first-order kinetic model can be written using the following equation (as described earlier (4, 6):

$$-\frac{dN}{dt} = kN$$

$$\frac{N(t)}{N_0(t)} = e^{-kt} \qquad (6)$$

This is an equation of a straight line with a slope –k, where N(t) is the number of survivors after an exposure time (t) in PFU/ml and the initial population is N_0 (PFU/ml). D-value is the decimal reduction time in min (time required to inactivate 90% of viruses at a given temperature) and t is the treatment time (min).

Z-value is defined as the change in temperature necessary to cause a 90% change in the decimal reduction time (D-value) (4, 6). The D-values for different temperatures were plotted on semilog coordinates, and the temperature increase for a one log-cycle change in D- values was calculated as the z- value. Based on the definition, z- value has been expressed by the following equation:

$$Z = \frac{T_2 - T_1}{\log D_2 - \log D_1}$$

Weibull Model:

$$\log\left(\frac{N}{N_0}\right) = -b * (t^{\beta})$$

$$b = \left(\frac{1}{2.303}\right) * \left(\alpha^{-\beta}\right)$$

From this we get value of alpha after substituting values of b and β.

$$T_d = \alpha * \left(-\ln\left(10^{-d}\right)\right)^{1/\beta}$$

Where d is the targeted decimal reduction.

After iteration of b and β for best fit, we get value of $T_{d=1}$, for that data set.

Here α and β are scale and shape parameters, respectively. β< 1 implies that the surviving particles are infectious, whereas β> 1 implies that particles are damaged and are non-infectious (7, 16). The Weibull distribution corresponds to a concave upward (tailing) survival curve if β is <1, concave downward (shoulder) if β is >1, and linear if β equals 1 (19). Concave upward or tailing behavior indicates that some population can survive the applied heat, while sensitive members of the population are destroyed relatively quickly, whereas a concave downward behavior shows that the population is increasingly damaged (6).

4 RESULTS AND DISCUSSION

TV at an initial titer of 7.22±0.18 log PFU/ml (±standard error) was used for heat inactivation, where significant differences in the number of survivors at 52, 54, 56 and 60°C at all tested time points were obtained (P<0.05). Figure 2.1 shows the average recovered titer of TV at the different time points for each inactivation temperature. Unheated TV at room temperature (22°C) was used as positive control. At 52°C, the average TV titer decreased from 6.37±0.41 log PFU/ml at 0 min to 3.84±0.35 log PFU/ml after 10 min. Similarly, at 54°C, the average titer of TV decreased from 6.38±0.21 log PFU/ml at 0 min to 3.02±0.14 log PFU/ml after 10min. Similarly, at 56°C,

the average number of viral survivors decreased from 6.08±0.3 log PFU/ml to undetectable after 10 min, and at 60°C it decreased from 4.49±0.06 log PFU/ml to undetectable level after 1.5 min, respectively, with limit of detection as 1 log PFU/ml. While there was a decrease of about only 3 log PFU/ml in TV titers after heating at 52°C for 10 min, an increase in temperature resulted in TV titer reduction to non-detectable levels by plaque assay after 10 and 1.5 min at 56 and 60°C, respectively (Figure 2.1). This shows that both increasing time and temperature have a direct relationship to TV titer reduction. The D-values for TV in 2-ml glass vials at 52, 54, 56 and 60°C calculated from first order model were 4.59 ± 0.05, 2.91 ± 0.05, 1.74 ± 0.07 and 0.58 ± 0.02 min, respectively with z- value as 9.09°C (R^2 = 0.997) (Figure 2.2B).

Previously in literature, a D-value of 1.09 min at 55°C for TV has been reported by Hirneisen and Kniel using 0.2 ml PCR tubes (17). However, Tian et al. (23) and Arthur and Gibson (2) reported higher D-values at 56°C of 11.8 and 4.03 min, respectively while at 63°C D- values were 2.6 and 1.18 min, respectively in 1.5 ml microcentrifuge tubes using the first order model (2, 24). These results were similar to the results reported by Araud et al. (1) who showed a D-value of 2.38 min at 62°C in cell culture media in capillary tube with first order model (1, 2, 24). However, in this current study, a D-value of 0.58 min at 60°C in 2-ml glass vials was obtained. The difference in the D-values obtained could be due to the difference in the type of container and heat-transfer rate. Microcentrifuge tubes or 0.2 ml thin walled PCR tubes, made from polypropylene, can reach the desired temperature almost instantly when heated in a thermocycler, while there was a short come-up time required to achieve the desired temperature in the case of the glass 2-ml vials in a circulating water bath (8). The come-up times were 120, 140, 172 and 163 s for 52, 54, 56 and 60°C. During the come-up time, which was indicated by the 0 min time point, the reduction in number of survivors varied from 0.41 to 3.05 log PFU/ml from 52 to 60°C, respectively, and hence can lead to differences in the D-values when compared to that obtained by using polypropylene microcentrifuge tubes. Thus, the type and shape of the container played an important role in determining the heat inactivation kinetics of these viruses as also reported earlier (5). The difference in the methods of detection such as tissue culture infectious dosage (24) or plaque assay can also contribute towards difference in D-values.

The shape and scale parameters for the Weibull model for the different temperatures were used to calculate $T_{d=1}$ value as an analogue to D-value from first

order model (Table 2.1). The results revealed that the value of scale parameter, α ranged from 0.64 to 0.06 min when temperature increases from 52 to 60°C. This shows that as temperature increases and value of α decreases, there is a faster reduction in the number of survivors over a shorter duration of time. In comparison, MNV-1 and FCV-F9 also showed a reduction in α with an increase in temperature. For MNV-1, α decreased from 7.42 to 0.14 min from 50 to 72°C, while for FCV-F9 it decreased from 5.17 to 0.14 min from 50 to 72°C (6). The values obtained for β show a tailing behavior (concave upward) (Table 2.1). These findings are similar to Bozkurt et al. (6) which show that both MNV-1 and FCV-F9 had tailing and shoulder behavior which was irrespective of the heating temperature.

As a change in the scale factor described a change in the heating environment, as opposed to a nearly constant shape factor, a second order polynomial was derived to measure the influence of temperature on scale factor and hence, $T_{d=1}$ values:

$$\alpha = 0.005[T(°C)\text{^}2] - 0.61\,T(°C) + 19.66 \qquad R^2 = 0.80$$

On using an average β value of 0.65, the dependence of $T_{d=1}$ on scale parameter can be expressed as:

$$T_{d=1} = 1.72\alpha$$

$T_{d=1}$ values obtained from the Weibull model were 2.53 ± 0.08, 1.99 ± 0.1, 0.57± 0.41 and 0.22 ± 0.25 min for the same temperatures, respectively with z- value as 7.14°C (R^2 = 0.95) (Figures 2.2B). This indicates over-processing for one log reduction in case of first order model as compared to Weibull model. Moreover, as one log reduction is rarely used in industry, time required to achieve 6 log reduction was calculated, which is represented by 6D and $T_{d=6}$ for first order and Weibull model, respectively as described before by Bozkurt et al. (5, 6, 7) (Table 2.3). The results indicate that for the studied temperature range (52-60°C), under-processing may occur for a targeted six log reduction when first order model is applied, instead of Weibull model. Similar observation has been reported for FCV-F9, MNV-1 and HAV with 6D values of 38.22, 22.44 and 50.4 min and $T_{d=6}$ values of 43.73, 29.66 and 58.57 min, respectively at 56°C, showing under processing in case of first order model as compared Weibull model (9). TV had a much lower $T_{d=1}$ value at 60°C of 0.22 min as compared to MNV-1 or FCV-F9 in 2-ml vials in cell culture media, which had $T_{d=1}$ values at 56°C of 1.11 min and 0.91 min, respectively (6).

The D-value of TV at 56°C (1.74±0.07 and 0.57±0.41 min, using first order and Weibull model, respectively and a z-value of 9.09°C), is lower than that of various

cultivable HNoV surrogates reported in literature. MNV-1 in cell-culture media had a D-value of 3.74 ± 0.68 and 2.34±0.43 min at 56°C in 2-ml glass vials and a z-value of 9.31 and 9.19°C using first order and Weibull model respectively (5). On the other hand, FCV-F9 had a D-value at 56°C of 6.37±0.59 and 4.05±0.09 min, and a z-value of 9.36°C and 9.31°C using first order and Weibull model, respectively (6). For HAV, the D-value at 56°C was 8.4±0.43 and 11.11±8.73 min and the z-value was 12.49°C and 14.05°C, using first order and Weibull model respectively (6, 7). At 56°C, HAV appears to be the most resistant to heat followed by FCV-F9, MNV-1 and TV. HNoV GII.4 had a reported D-value of 100 min at 56°C and z-value of 20.61°C in 1.5 ml microcentrifuge tubes as measured by in situ capture quantitative reverse transcription PCR (25). Therefore, it appears that at 56°C HNoV could be most resistant as compared to any of its surrogates and HAV. However, this data should be interpreted with caution because infectious assays were not used during this study due to unavailability of cell-culture systems for infectious HNoVs at that time.

TV titers from unheated spinach (control) ranged from 6.95 ± 0.13 to 5.28 ± 0.10 log PFU/ml, indicating a recovery of 73 to 96% which was similar to that observed by Bozkurt et al. (5) showing 97 and 89% recovery for MNV-1 and FCV-F9 in spinach. This suggests that there is greater adsorption of foodborne viruses on the food matrices that can lead to outbreaks when consumed raw or under-cooked. The come-up time for target temperature in vacuum bags were 44.5, 79.5 and 168.7 s for 50, 54 and 58°C respectively. In the current study, the first order model D-value of TV in spinach in vacuum bags at 50°C was 7.94 min, which is lower than the D-value of 14.57 and 17.39 min for MNV-1 and FCV-F9, respectively in 2-ml glass vials and 34.4 min for HAV in vacuum bags at 50°C. The z-value calculated for TV in spinach was 10.74°C (R^2 = 0.98) using the first order model as shown in Figure 2.3. Lower D-values of TV in cell culture matrix in 2-ml glass vials compared to D-values in spinach in vacuum bags were observed such as 2.91±0.05 and 4.09±0.04 min, respectively at 54°C using first order model. Similar trends have been reported previously in literature for HAV at temperatures above 56°C showing lower D-values in cell culture media in 2-ml glass vials (2.67±0.42 min at 60°C) as compared to D-values in spinach in vacuum bags (4.55±0.82 min at 60°C) (10).

As β ranges from 0.17, 0.78 and 0.43 at 50, 54 and 58°C, the Weibull model shows that the heating temperature did not influence the shape parameter, whereas α decreased from 2.24, 1.09 and 0.04 min (Table 2.2). $T_{d=1}$ value for TV in spinach in

vacuum bags (4.89 min at 50°C) was lower than those reported for MNV-1, FCV-F9 of 15.26 and 20.71 min, respectively in spinach using 2-ml glass vials at 50°C (5). The calculated 6D values at 50°C were 206.4 and 47.64 min for HAV and TV in spinach in vacuum bags and 104.34 and 87.42 min for MNV-1 and FCV-F9 in spinach in 2 ml glass vials (5, 10).

On analysis of heat treatment on the capsid integrity of TV, it was reported that at 80°C after the shortest time point of 5 s, the appearance of the heated virus changed with the loss of normal round structure which was accompanied with appearance of rough edges (1). It is known that the protruding domain of the capsid protein is responsible for viral entry into the host cell (27), therefore, the loss of capsid integrity can lead to reduction in host cell binding. The decrease in infection ability due to loss of capsid integrity could explain the greater reduction of TV at high temperature of 60°C. Further, unfolding of capsid protein and loss of capsid integrity could lead to nucleic acid degradation and therefore loss of infectivity (2).

In summary, based on the regression co-efficient that was comparatively higher for the Weibull model than the first order model, the Weibull model seems to better represent the heat-inactivation behavior of TV. When comparing the heat-inactivation behavior of HNoV surrogates, it appears that overall MNV-1 (except at 56^{o}C) is more heat-resistant than FCV-F9 or TV. However, HAV is more heat-resistant than all the currently known cultivable HNoV surrogates. Thus, based on the data from this study with TV in 2-ml vials and inoculated spinach in vacuum bags, TV does not appear to be as suitable as MNV-1 as a HNoV surrogate for use in heat-inactivation studies. Based on these results and that an ideal surrogate should be more resistant to treatments than the target pathogens, be non-pathogenic, easily cultivable in the laboratory and industrial settings, and easy to handle, further studies are on-going and needed to determine a suitable cultivable HNoV surrogate for future validation studies.

5 ACKNOWLEDGEMENTS

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7 APPENDIX

Figures

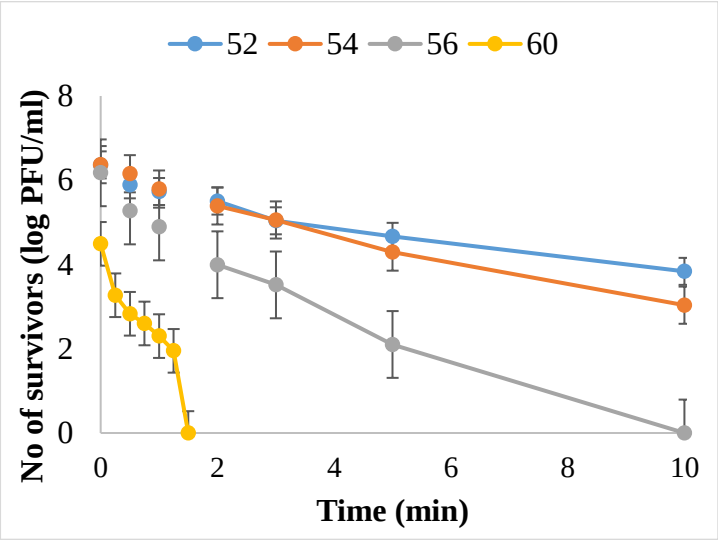

Figure 2.1. Thermal treatment of Tulane virus (TV) in cell culture media after heating for various times at different temperatures of 52, 54, 56 and 60°C in 2-ml glass vials using first order model.

Error bars represent the standard errors (SE) from the three replicates of plaque assays carried out in duplicate for evaluating inactivation of the virus.

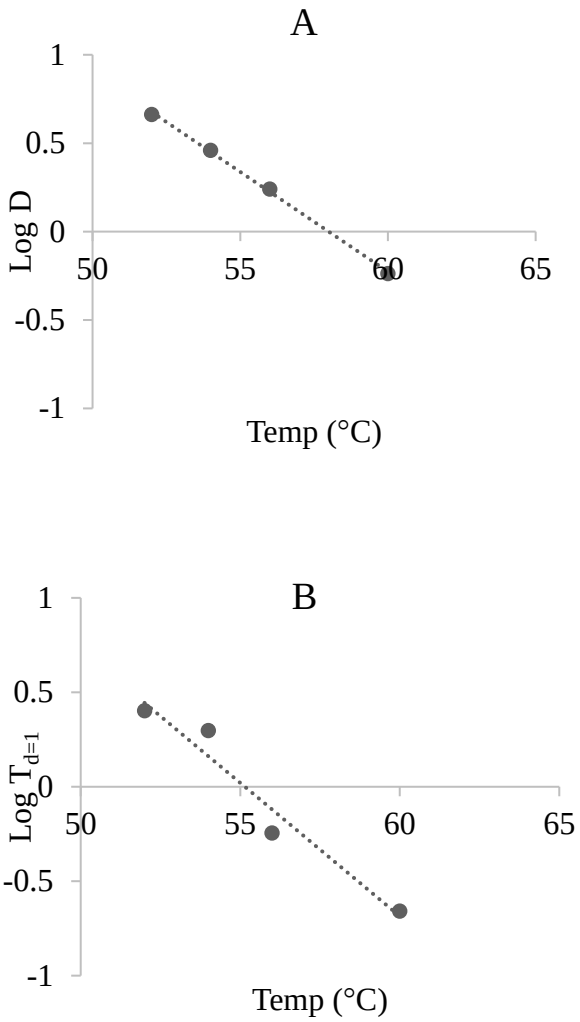

Figure 2.2. Comparison of thermal inactivation curves of Tulane virus (TV) in cell culture media in 2-ml glass vials using the first order and Weibull models. The data points represent Log_{10} D (A) or Td=1(B) (min) values for three replicates at 52, 54, 56 and 60°C used for computing Z-values for (A) first order model (R^2 = 0.997) and (B) Weibull model (R^2 = 0.95).

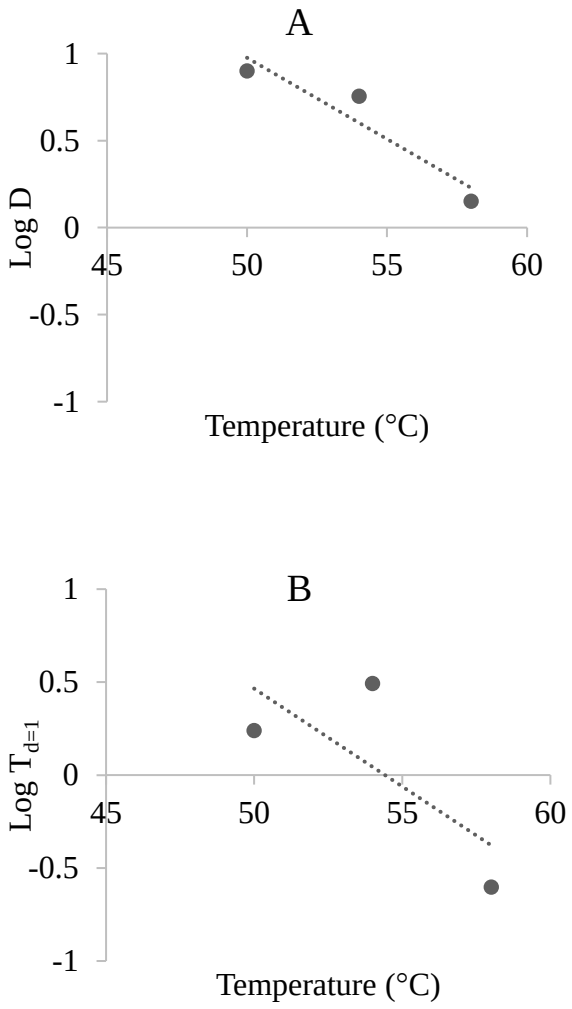

Figure 2.3. Thermal inactivation curves of Tulane virus (TV) in spinach in vacuum bags for (A) first order model (R^2 = 0.98) and (B) Weibull model (R^2 = 0.86).

Tables

Table 2.1. Coefficients of the first-order and Weibull models for the survival curves of Tulane virus (TV) during thermal inactivation in cell culture media using 2-ml vials.

	Weibull distribution				First order kinetics		
T (°C)	α(min)	$T_{d=1}$(min)	β	R^2	D (min)	R^2	Z (°C)
52	0.64±0.06	2.53±0.08	0.61±0.06	0.96	4.59±0.05	0.84	
54	0.68±0.05	1.99±0.10	0.78±0.09	0.97	2.91±0.05	0.90	9.09
56	0.15±0.09	0.57±0.41	0.64±0.03	0.98	1.74±0.07	0.92	
60	0.06±0.09	0.22±0.25	0.61±0.05	0.97	0.58±0.02	0.85	

**Thermal death time ($T_{d = 1,}$ D- values* ± SE*), shape and scale factors (β and α, respectively) and coefficient of determination (R^2). The data is the average of three replicates assayed in duplicate for each temperature.*

Table 2.2. Coefficients of the first-order and Weibull models for the survival curves of Tulane virus (TV) during thermal inactivation in spinach using vacuum bags.

	Weibull distribution				First order kinetics		
T (°C)	α (min)	$T_{d=1}$ (min)	β	R^2	D (min)	R^2	Z (°C)
50	2.24±0.06	4.89±0.02	0.17±0.04	0.99	7.94±0.21	0.60	
54	1.09±0.15	3.11±0.45	0.78±0.21	0.83	4.09±0.04	0.60	10.74
58	0.04±0.02	0.25±0.38	0.43±0.10	0.96	1.43±0.02	0.81	

**Thermal death time ($T_{d=1}$, D- values ± SE), shape and scale factors (β and α, respectively) and coefficient of determination (R^2). The data is the average of three replicates assayed in duplicate for each temperature.*

Table 2.3. Calculated process time (min) to achieve 6-log reduction by the first-order and Weibull models of Tulane virus (TV) during thermal inactivation in cell culture media in 2-ml glass vials and in spinach in vacuum bags.

Model	Temperature (°C)			
	52	54	56	60
Cell-culture	Time (min)			
First order (6D)	27.55	17.28	10.44	3.48
Weibull ($T_{d=6}$)	48.94	20.06	9.46	3.98
Spinach	50	54	58	
First order (6D)	47.64	24.54	8.58	
Weibull ($T_{d=6}$)	55.87	32.05	15.94	

CHAPTER III

THERMAL INACTIVATION KINETICS OF AICHI VIRUS IN CELL-CULTURE MEDIA USING 2-ML GLASS VIALS

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Key words: Aichi Virus, heat-inactivation, D-value, z-value

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1 ABSTRACT

Aichi virus (AiV) is an emerging, non-enveloped, single-stranded RNA virus that causes human gastrointestinal disease associated with the consumption of contaminated water and shellfish. The current unavailability of vaccines and resistance of AiV to commonly-used inactivation techniques, makes it necessary to evaluate its thermal inactivation kinetics. The objective of this study was to determine the heat inactivation kinetics (D- and z-values) of AiV in cell-culture media using 2-ml glass vials by the first-order and Weibull models. AiV in cell-culture media at ~7.4 log plaque forming units (PFU)/ml in 2-ml glass vials was heated at 50, 54, 58°C for up to 90 min in a circulating water-bath. Survivors (infectious particles) were enumerated by plaque assay using confluent Vero host cells in 6-well plates. Each treatment was replicated thrice, assayed in duplicate and data were statistically analyzed. D-values for AiV in 2-ml glass vials at 50±1°C (±=standard error) (come-up time=68s), 54±0.7°C (130s), and 58±0.6°C (251s) by the first order model were 47.62±1.2(R^2=0.94, RMSE=0.19), 7.14±1.13(R^2=0.70, RMSE=0.34) and 2.12±0.04min (R^2=0.70, RMSE=0.31), respectively, with a z-value of 5.92°C (R^2=0.98, RMSE=0.25). The Weibull model showed $T_{d=1}$ of 34.53±0.03 (R^2=0.99, RMSE=0.16, α (scale parameter) =10.27, β (shape parameter) =0.69), 2.59±0.05 (R^2=0.96, RMSE=0.23, α=0.22, β=2.59) and 0.91±0.06 min (R^2=0.96, RMSE=0.25, α=0.14, β=0.91) for the same temperatures, respectively with a z-value of 5.26°C (R^2=0.94, RMSE=0.11). The Weibull model showed a better fit with higher R^2 and lower RMSE values. These data should help in the industrial design of thermal processes to inactivate AiV and prevent AiV transmission and outbreaks.

2 INTRODUCTION

The emerging foodborne enteric Aichi virus (AiV) was first isolated from stool specimens of patients suffering from acute gastroenteritis due to the consumption of contaminated raw oysters (29). This virus has a morphologically distinct round-structure, ~ 20 to 40 nM in diameter and belongs to the genus *Kobuvirus* in same *Picornaviridae* family as Hepatitis A virus (HAV), which has three known species A, B, and C (27, 29). The viral genome consists of a single-stranded, positive-sense RNA molecule of 8,280 nucleotides and a poly(A) tail (1, 27).

AiV is transmitted through the fecal oral route and has been known to spread through contaminated water and shellfish (17, 20, 27). It has thus far been reported to be isolated from stool samples over the world including Japan, France, Pakistan, Netherlands, Tunisia, Spain and South America (17, 19, 21, 22, 24, 27, 28). Symptoms of AiV infection include nausea, vomiting and diarrhea that can occur within 12 to 48 h after ingestion, while fecal shedding of AiV can last for up to 5-7 days after infection (9, 24). Currently there are no available antivirals described for AiV infection treatment and its infectious dose remains unknown (4).

There have been few studies on the persistence and survival of AiV in different environments. AiV has shown to be stable in 10% chloroform for 3 h (27) and can survive without any significant reduction after high hydrostatic pressure processing treatment at 600 MPa for 5 min (15). However, AiV was reduced by 5 log $TCID_{50}$/ml upon exposure to UV at 16 mW/s^2 (14). AiV was reported to be more resistant than porcine enteric virus (PEC) and human norovirus (HNoV) surrogates such as murine norovirus (MNV-1), feline calicivirus (FCV-F9), and Tulane virus (TV) to inactivation treatments like pH, high hydrostatic pressure, heat and ethanol (10). Furthermore, recently our lab showed that AiV (at initial titer 5 log PFU/ml) had no significant reduction with aqueous hibiscus extracts at 100 mg/ml or 40 mg/ml at 37°C after 6 h, but was reduced to non-detectable levels after 24 h by both concentrations (11).

One of the most widely used techniques for the inactivation of foodborne pathogens is thermal inactivation. Heat has been known to alter the structural integrity of microorganisms by disrupting hydrogen bonding, denaturing the viral proteins and causing them to become non-infectious subunits (7, 23). Above 60°C, after the inactivation of host-cell recognition and binding receptors can occur, an alteration of the tertiary protein structure is reported to occur (3, 7). This is turn facilitates the access

of thermal energy to the nucleic material, causing its degradation as well as exposes the nucleic acid material to environment damage, enzymes such as nucleases, extreme pH, etc (3). For industrial purposes and to understand the heat inactivation kinetics of microorganisms, D- and z-values are determined. As previously described, the D-value is defined as the time at a given temperature necessary to reduce a microbial population present in a set medium by 90% and is indicative of the thermal resistance of a microorganism at a constant temperature (7). The z-value is the change in temperature required to increase or decrease the D-value by 90% and is indicative of the temperature dependence of microbial inactivation (7). Inversing the slope of log D versus temperature is used to obtain the z-value.

Given the high prevalence of AiV around the globe and its high resistance to many treatment and disinfection techniques, it becomes necessary to evaluate thermal inactivation kinetics of AiV to prevent its transmission and disease outbreaks. Hence, the objectives of this study were to (i) Determine the thermal inactivation kinetics of AiV in cell culture media using 2-ml glass vials and (ii) Compare the first order and Weibull models to describe the heat inactivation of AiV in cell culture media. The data obtained would be useful in designing appropriate heat inactivation parameters to prevent AiV transmission.

3 MATERIALS AND METHODS

3.1 Virus and Host

AiV was obtained as a gift from Dr. David Kingsley (USDA ARS, Delaware) along with its host cell line, Vero cells. For preparation of virus stocks, AiV was propagated in 175 cm^2 flasks containing confluent Vero cells in Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12; HyClone Laboratories, Logan, UT) containing 1 X Anti-anti (Antibiotic Antimycotic; Invitrogen, Grand Island, NY), supplemented with 2% heat inactivated fetal bovine serum (FBS) (Fetal Bovine Serum HyClone Laboratories, Logan, UT) and incubated at 37°C under 5% CO_2 until lysis for 3 days as reported earlier (11, 14). The virus was recovered after freeze-thawing thrice, followed by centrifugation at 5000×g for 10 min, and filtering through 0.2 µm filters. The obtained filtrate was aliquoted and stored at -80°C until use.

3.2 Heat Treatment of AiV in cell-culture media contained in 2-ml glass vials

Heat treatment was carried out in a circulating water bath (Isotemp 2150, Fisher Scientific, Pittsburgh, PA) in 2-ml screw-capped glass vials as reported earlier (7). Briefly, autoclaved (121°C, 15 min) vials were carefully filled with 1.8 ml AiV stock at 7.41± 0.25 (± standard error) log PFU/ml initial titer, by using sterile micro pipettes in a biosafety cabinet. The filled vials were surface rinsed in 70% ethanol and immersed in a thermostatically controlled water-bath, whose temperature was monitored with thermocouples connected to MMS3000-T6V4 type portable data recorder (Commtest Inc., New Zealand). As previously reported, one end of the type-T thermocouple (Omega Engineering, Inc., Stamford, CT) was placed in the geometric center of the water-bath and another thermocouple probe end was placed in the geometric center of a vial through the lid to monitor the temperature of the buffered media (7). Samples were heated at 50, 54 and58°C for varying treatment times up to 10 min. The treatment time began (come-up time was recorded) when the target internal temperature reached the target temperature. The come-up times were 68, 130 and 251 s at 50, 54 and 58°C, respectively. After the thermal treatment, sample vials were surface rinsed with 70% ethanol and immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. Samples were aseptically serially diluted 10-fold first in DMEM-F12 containing 10% FBS followed by subsequent 10-fold serial dilutions in 1.5 ml DMEM-F12 (containing 2% FBS) followed by enumeration of recovered infectious AiV using plaque assays. Un-heated virus suspensions were used as controls.

3.3 AiV Plaque Assay

AiV survivors were enumerated by infecting confluent Vero host in 6-well plates with 500 µl of aliquots of dilutions of heat-treated or untreated virus cells and incubating at 37°C with 5% CO_2 for 3 h before overlaying with complete DMEM (containing 2% FBS, and 1% antibiotic-antimycotic) in 1.5% Noble agar (Difco Agar Noble, BD, Sparks, MD). Plates were then incubated at 37^{o}C for 3 days followed by staining with neutral red contained in complete DMEM (containing 2% FBS, and 1% antibiotic-antimycotic) in 1.5% Noble agar and plaques were counted and reported as plaque forming units/ml (PFU/ml). Each experiment at each temperature was repeated thrice and assayed in

duplicate and data was analyzed using SAS 9.4 (SAS Institute, Cary, NC, USA) with PROCREG and NLIN commands for linear and Weibull models, respectively.

3.4 Modeling of inactivation kinetics:

First-order kinetics: The first order change in the number of survivors with time at a constant temperature, and as reported earlier, can be expressed as:

$$-\frac{dN}{dt} = kN$$

$$\frac{N(t)}{N_0(t)} = e^{-kt} \qquad (4)$$

This is an equation of a straight line with a slope –k, where N(t) is the number of survivors after an exposure time (t) in PFU/ml and the initial population is N_0 (PFU/ml). D is the decimal reduction time in min (time required to kill 90% of viruses) and t is the treatment time (min). The log-cycle change in D-values over temperature can be plotted on semi-log coordinates to obtain the z- value:

$$Z = \frac{T_2 - T_1}{\log D_2 - \log D_1}$$

Where D_2 and D_1 are the different D-values at temperatures T_2 and T_1, respectively.

Weibull Model: The Weibull model takes into consideration the scale and shape parameters which are α and β, respectively. The Weibull distribution corresponds to a concave upward survival curve if β is <1, concave downward if β is >1, and linear if β equals 1 (16). β<1 (concave upward or tailing behavior) implies that the surviving cells have retained the ability to be infectious, whereas β> 1 (concave downward) implies that the cells are damaged and are no longer infectious (7, 13).

$$\log\left(\frac{N}{N_0}\right) = -b * (t^{\beta})$$

$$b = \left(\frac{1}{2.303}\right) * \left(\alpha^{-\beta}\right)$$

After iteration of b and β for best fit, we get value of $T_{d=1}$, for that data set.
From this we get value of alpha after substituting values of b and β.

$$T_{d=1} = \alpha * \left(-\ln\left(10^{-D}\right)\right)^{1/\beta}$$

Where D is the targeted decimal reduction (7).

4 RESULTS AND DISCUSSION

AiV at an initial titer of 7.41± 0.25 (± standard error) log PFU/ml was used for heat inactivation, where significant difference between the number of survivors at 50, 54, and 58°C at all tested time points were obtained ($P<0.05$). Unheated AiV at room temperature (22°C) was used as a positive control. AiV showed reduction of 0.46 and 3.82 log PFU/ml after 10 min at 50 and 54°C, respectively. While at 58°C, AiV showed reductions of 4.19 to5.87 log PFU/ml after 0 and 3 min, respectively. This shows that both increasing time and temperature have a direct relationship in AiV titer reduction. The D-values for AiV in 2-ml glass vials at 50, 54, and 58°C calculated from first order model were 47.62± 1.12, 7.14±1.13 and 2.12±0.04 min, respectively (Table 3.1). The z-value calculated from first order model for AiV in 2-ml glass vials was 6.94°C (R^2 = 0.998) (Figure 3.1).

In comparison to AiV, HAV was reported to have higher D-values of 56.22 min at 50°C, followed by 8.4, 2.67, 1.73 and 0.88 min at 56, 60, 65 and 72°C in cell culture media using similar 2-ml glass vials (7). It is known that the thermal stability of viruses depends on the ionic composition of media and this effect is different for different viruses. Although HAV and AiV have similarities as they belong to the same *Picornoviridae* family, such as being similar size (27-30 nm), non-enveloped icosahedral structure, positive single stranded RNA (7.5-8 kb length), they have differences in guanine+cytosine (G+C) content (59% for AiV and 38% for HAV). In addition, the amino acid (aa) sequence length and composition of the viral protein 1 (VP1) (278 aa for AiV and 300 aa for HAV) might be another reason for the differences between the D-values of AiV and HAV (7, 29). HAV may have a more heat-stable capsid due to the protein structure arising from different amino acids and protein size. However, AiV with a D-value of 47.62 min in 2-ml glass vials at 50°C showed higher heat resistance than MNV-1 or FCV-F9 that had reported D-values of 36.28 and 19.95 min at 50°C, respectively (7). TV has been previously reported to show D-values of 500, 4.03, 1.18 and 0.24 min at 37, 56, 63 and 72°C in microcentrifuge tubes in cell culture media (2). MNV-1, FCV-F9, TV, SAV and AiV have similar non-enveloped icosahedral capsid, and a positive-sense single-stranded RNA (7.5-8 kb). Another reason for the differences in thermal resistance of these viruses could be attributed to the differences in isoelectric points (pH of 10 for TV (12), pH of 7.15 for HAV (26), pH of 4.1 for MNV-1 (18) and 4.27 for FCV-F9 (18) and 5 for porcine sapovirus (25)) that

may increase stability in the environment, associated with its different amino acid sequence and length composition as reported for VP1 (530-550 aa for MNV-1, 668 aa for FCV-F9, 534 aa for TV) (12).

In comparison to earlier heat inactivation reports, AiV has been found to show 4 log PFU/ml reduction after 20 min at 56°C in microcentrifuge tubes (10). Differences in the D-values are attributed to the difference in type and shape of container composition, as polypropylene microcentrifuge tubes or PCR tubes, can reach the desired temperature almost instantly in a thermocycler, while in the case of the 2-ml glass vial, there is a short come-up time required to achieve the desired temperature in a circulating water bath (6). The come-up times for the 2-ml glass vials were 124, 130 and 251s for 50, 54 and 58°C. The reduction in number of survivors during the come-up time is indicated by the 0 min time point. The reduction at 0 min varied from 0.13 to 4.19 log PFU/ml from 50 to 58°C, respectively. Moreover, on extrapolating for AiV in cell culture media in 2-ml glass vial at 56°C, a D-value of 4.17 min is calculated, which accounts for a 4-D value of 16.68 min. Extrapolating a come-up time of approx. 3.4 min from come up time versus temperature graph, it is seen that 4 log reduction in AiV titer was observed in 20.08 min, which is similar to previously reported near complete (4 log PFU/ml) reduction of AiV titers at 56°C in 1.8 ml microcentrifuge tube in a circulating water bath (10). This is also comparable to the previously reported D-value of 4.03 min obtained on thermal treatment of TV in cell culture media in microcentrifuge tube at 56°C (2).

Table 3.1 shows the shape and scale parameters for Weibull model for the different temperatures used to calculate $T_{d=1}$ value that was used as an analogue to D-value from first order model. The results revealed that the value of scale parameter, α ranged from 10.22 to 0.14 min when temperature increases from 50 to 58°C which is indicative of a shift in the graph of log (N/N_o) versus time towards the left implying that as temperature increases and value of α decreases, there is a faster reduction in the number of survivors over a shorter duration of time. Furthermore, it can also be seen that the values obtained for β show a tailing behavior (concave upward) at 50, 54 and 58°C with β as 0.69, 0.34 and 0.45, respectively. This suggests that at lower temperature of 50°C, only the sensitive members of the population are destroyed but the remaining cells are infectious, while as the temperature is increased, the cells become damaged and start losing the ability to infect.

As opposed to the shape parameter, the scale factor decreased almost linearly with temperature, which can be expressed as:

$$\alpha = -1.26[\mathrm{T}(°\mathrm{C})] + 71.92 \qquad R^2 = 0.76$$

On using an average value of shape parameter of β = 0.49, the dependence of $T_{d=1}$ values on α can be expressed as:

$$T_{d=1} = 5.45\alpha$$

$T_{d=1}$ values were 34.53 ± 0.03, 2.59 ± 0.05 and 0.91 ± 0.06 min for 50, 54 and 58°C, respectively. The difference in D-values using the linear model and $T_{d=1}$ values using the Weibull model can be ascribed to the pronounced curvature seen for number of survivors with respect to time owing to the low value of β of 0.3 at 50°C. This further indicates that there is a greater frequency of inactivation of the number of virus particles at shorter time points than that at higher time points (Table 3.2). For comparison, AiV with $T_{d=1}$value of 34.53 min in 2-ml glass vials at 50°C was more heat resistant than MNV-1 or FCV-F9 with $T_{d=1}$values of 26.78 and 13.27 min, respectively, but less heat resistant than HAV ($T_{d=1}$of 39.91 min) under the same conditions (7).

The first order model shows over processing for one log reduction as compared to the Weibull model. For industrial purposes, six log (6-D values) reduction is often used, which can be calculated by multiplying the D-value by a factor of six for first order model and substituting $T_{d=6}$ for Weibull model (8). The results show that for the studied temperatures (50-58°C), the first order model shows under processing for the targeted six log reduction as compared to Weibull model (Table 3.3). Similar observation has been reported for FCV-F9, MNV-1 and HAV with 6-D values of 38.22, 22.44 and 50.4 min and $T_{d=6}$ values of 43.73, 29.66 and 58.57 min, respectively at 56°C, showing under processing in case of first order model as compared Weibull model (5). The high regression coefficient suggests that the Weibull model is better representation of inactivation kinetics of AiV in cell culture media in 2-ml glass vials compared to first order model.

Based on these results, AiV is shown to have higher resistance to heat than cultivable HNoV surrogates (MNV-1 and FCV-F9) when tested in cell-culture within 2-ml glass vials at 50, 54 and 58^{o}C. This data will be useful to the food industry in selecting optimum process conditions to obtain the desired level of inactivation, to prevent foodborne illnesses and outbreaks. Further studies are needed to determine the thermal inactivation behavior of AiV in food matrices such as shellfish.

5 ACKNOWLEDGEMENTS

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7 APPENDIX

Figures

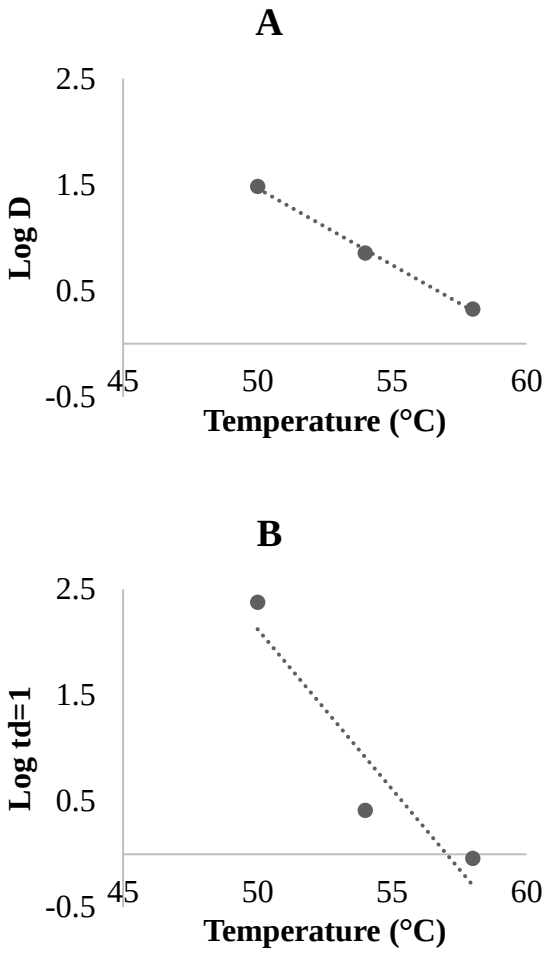

Figure 3.1. Comparison of thermal inactivation curves of Aichi virus (AiV) in cell culture media in 2 ml glass vials for (A) first order model (R^2 = 0.98) and (B) Weibull model (R^2 = 0.94). The data points represent Log_{10} D (A) or $T_{d=1}$(B) (min) values for three replicates at 50, 54 and 58°C used for computing Z-values.

Tables

Table 3.1. Coefficients of the first-order and Weibull models for the survival curves of Aichi virus (AiV) during thermal inactivation in cell culture media using 2-ml glass vials.

	Weibull distribution				First order kinetics		
T (°C)	α(min)	$T_{d=1}$(min)	β	R^2	D (min)	R^2	Z (°C)
50	10.27	34.53±0.03	0.69	0.99	47.62±1.12	0.94	
54	0.22	2.59±0.05	0.34	0.96	7.14±1.13	0.70	5.92
58	0.14	0.91±0.06	0.45	0.961	2.12±0.04	0.70	

*Thermal death time ($T_{d=1,}$ D- values ± SE), shape and scale factors (β and α, respectively) and coefficient of determination (R^2). The data is the average of three replicates assayed in duplicate for each temperature.

Table 3.2. Effect of thermal treatment on Aichi virus in cell culture media using 2-ml glass vials

Temp (°C)	Treatment	Recovered titer log (PFU/ml)
50	Control	7.30 ± 0.02
	0 min	7.17 ± 0.03
	0.5 min	7.03 ± 0.03
	1 min	6.97 ± 0.02
	2 min	6.93 ± 0.05
	3 min	6.88 ± 0.03
	5 min	6.85 ± 0.04
	10 min	6.80 ± 0.03
54	Control	7.41 ± 0.06
	0 min	5.33 ± 0.25
	0.5 min	4.89 ± 0.41
	1 min	4.58 ± 0.23
	2 min	4.31 ± 0.15
	3 min	4.16 ± 0.05
	5 min	3.93 ± 0.16
	10 min	3.59 ± 0.07
58	Control	7.44 ± 0.33
	0 min	3.24 ± 0.10
	0.25 min	2.67 ± 0.13
	0.5 min	2.52 ± 0.07
	0.75 min	2.30 ± 0.16
	1 min	2.23 ± 0.20
	1.25 min	2.14 ± 0.27
	1.5 min	1.82 ± 0.13
	2 min	1.74 ± 0.07
	3 min	1.57 ± 0.10

Each treatment was replicated thrice and assayed in duplicate for evaluation of inactivation of viruses (limit of detection was 1 log PFU/ml)

Table 3.3. Calculated process time (min) to achieve 6-log reduction of Aichi virus (AiV) during thermal inactivation in cell culture media in 2-ml glass vials by the first-order and Weibull models.

Model	Temperature (°C)		
	50	54	58
	Time (min)		
First order (6D)	285.72	42.84	12.72
Weibull ($T_{d=6}$)	466.59	396.63	51.79

CONCLUSION

Foodborne enteric viruses have been known to cause several outbreaks and illnesses. The current absence of available vaccines necessitates the development of alternative control strategies to control their spread. Thermal inactivation is one of the most widely used methods traditionally and in industry.

This study determined the thermal inactivation kinetics of Tulane virus (TV) (a cultivable human norovirus surrogate) and Aichi virus (AiV). Based on the regression coefficients, the Weibull model showed a better fit for thermal inactivation kinetics of TV in cell culture media in 2-ml glass vials as well as for inoculated spinach in vacuum bags and for AiV in cell culture media in 2-ml glass vials. The study also showed that as the targeted temperature increased, the value for the scale parameter for the Weibull model decreased, showing that there were more number of viruses inactivated during a shorter frequency of time.

At 56°C in cell culture media in 2-ml glass vial, the D-value of TV was 2.91 min while murine norovirus (MNV-1) had D-value of 3.74 min *(19)*. Similarly, at 50°C, D-value of TV was 7.94 min in spinach in vacuum bags while MNV-1 had D-value of 14.57 min in spinach in 2-ml glass vials *(17)*. Furthermore, hepatitis A virus (HAV) had D-values of 8.4 min in cell culture media in 2-ml glass vial and 8.43 min in spinach in vacuum bags *(24)*(Bozkurt et al, 2015), while the D-value obtained for AiV was 47.62 min at 50°C in cell culture media in 2-ml glass vials.

Based on these results, AiV is shown to have greater resistance to heat in comparison to MNV-1, feline calicivirus (FCV-F9) and TV in cell culture media in 2-ml glass vials with D-values of 47.62 min at 50°C for AiV in 2-ml glass vials, as compared to TV of 7.94 min, MNV-1 of 36.28 min and FCV-F9 of 19.95 min.

The overall results from this study show that TV did not appear to be a suitable HNoV surrogate when compared to murine norovirus (MNV-1) with regards to heat-inactivation kinetics, as MNV-1 had higher heat resistance than TV in both cell-culture media and spinach. From the current results, it appears that for heat-inactivation, the lab-adapted HAV and even MNV-1 are more heat resistant than TV.

Based on these results and with the knowledge that an ideal cultivable surrogate should have a similar structure and size to the target virus, be slightly more resistant to treatments, non-pathogenic, and easy to use in laboratory and industrial

settings, further studies are needed to determine a suitable HNoV surrogate for use in heat inactivation studies.

These results will be useful to the food industry in selecting optimum process conditions to obtain the desired level of inactivation, to prevent foodborne viral illnesses and outbreaks. Further studies are needed to determine thermal inactivation behavior of AiV in food matrices such as shellfish. However, sensory and nutritional attributes need to be studied for industrial application using these thermal processing conditions in industry.

VITA

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