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Evaluation of Tulane Virus as a Surrogate for the study of Human Norovirus

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Evaluation of Tulane Virus as a Surrogate for the Study of Human Norovirus

Evaluation of Tulane Virus as a Surrogate for the Study of Human Norovirus

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

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University of Cape Coast
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This thesis is approved for recommendation to the Graduate Council.

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Abstract

Among all known causes of acute gastroenteritis, human noroviruses (HuNoV) are the primary cause (68%) of outbreaks and are associated with 78% of illnesses, 46% of hospitalizations, and 86% of deaths. The main obstacle to studying the pathogenesis of HuNoV is the lack of cell culture system and small animal model. Murine norovirus (MNV) and feline calicivirus (FCV) have been utilized as model surrogate viruses to study HuNoV. In this research, a more recent surrogate virus, Tulane virus (TV), was evaluated for physicochemical stability and environmental persistence. The primary goal was to determine the suitability of TV as a surrogate for HuNoV by comparing its environmental persistence and physicochemical stability to previously published results for MNV and FCV. Physicochemical profiles suggest that TV is more stable at 56, 63 and 72°C. When exposed to 60 and 70% ethanol concentrations at room temperature (RT), TV is more tolerant, but at 90% ethanol, TV is less tolerant. Tulane virus is also stable at acidic (2 and 3), neutral (7) and basic (9 and 10) pH levels though after 90 min there was a 2.25-log reduction in TV at pH 10. Last, TV is stable on a solid surface when exposed to 200 and 1000ppm chlorine for 10 min resulting in 0.63- and 2.22-$\log_{10}$ reduction, respectively. For environmental persistence, TV can survive in surface water for 28 d with less than 0.3-$\log_{10}$ reduction at RT under diurnal variations. Conversely, TV is not persistence in groundwater in the dark at 4°C with a complete loss of infectivity after day 14. Tulane virus is also stable at RT on non-porous fomite surfaces (acrylic based solid surface and stainless steel surface) with only a 1-$\log_{10}$ reduction at day 14. Comparing these observations and data published on TV elsewhere to previously published studies on MNV and FCV, it can be concluded that TV is likely a more conservative surrogate to study HuNoV though experimental differences make direct comparisons difficult.

Acknowledgement

My first and special appreciation goes to the Almighty God for how far He has led me. Second, I extend my profound gratitude to Dr. Kristen Gibson, my advisor, and all my committee members for their wonderful contribution and support to the success of my study. Third, a special thank you goes to all my lab mates (Giselle, Tun-Yun, Anisha and Danielle). Finally, to all my friends and loved ones, I appreciate you being a part of my experience in the course of my education.

Dedication

Dedicated to the Arthur family, and my home country, Ghana.

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1. Chapter 2. Arthur, S.E. and Gibson, K.E. (2015). Comparison of methods for evaluating the thermal stability of human enteric viruses. *Food and Environmental Virology,* 7, 14-26.

CHAPTER 1: LITERATURE REVIEW

I.   **Foodborne Pathogens**

Foodborne disease is any disease acquired through the consumption of contaminated food. Although viruses, bacteria, fungi, parasites, toxins, and prions may all cause foodborne diseases, the major causative agents of foodborne disease outbreaks are viruses (Li et al., 2012). Thirty-one major pathogens are known to cause 9.4 million episodes of foodborne illness each year in the U.S. with human norovirus (HuNoV) as the primary etiologic agent (Scallan et al., 2011). Public health officials at the Center for Disease Control and Prevention (CDC) estimate that 48 million individuals, about 17%, of the population, fall sick each year due to foodborne illness (i.e. due to both known and unknown pathogens) causing about 128,000 hospitalizations and 3,000 fatalities (CDC 2011). An earlier estimate by Mead et al. (1999) attributed over 67% of foodborne illnesses to viruses in general. However, over a decade later, Scallan et al. (2011) estimated that HuNoV specifically causes 58% of illnesses (5.4 million) due to known pathogens with 26% leading to hospitalization and 11% leading to death (Scallan et al., 2011). Based on these findings, HuNoV maintains a status as the primary foodborne virus of concern.

II.   **Human Norovirus**

i.   **Introduction**

Human noroviruses are in the family *Caliciviridae*. They are a group of single-stranded RNA viruses, nonenveloped and classified into the genus *Norovirus*. Other recognized genera of the *Caliciviridae* family include *Sapovirus*, *Nebovirus, Lagovirus*, and *Vesivirus* (Green et al., 2000). Moreover, two additional genera have been proposed including *Recovirus* and *Valovirus*. Based on at least 60% identity in the amino acid sequence in the major capsid protein (VP1), NoV

strains are classified into the same genogroup (Hutson et al., 2004). Five genogroups, assigned GI, GII, GIII, GIV and GV are identified based on genetic similarity in highly conserved areas of the genome such as the RNA-dependent RNA polymerase (RdRp) and the shell domain or the VP1 (Green 2007). Human noroviruses are found in GI, GII, and GIV whereas genogroups III and V infect bovine and murine species, respectively (Zheng et al., 2006). The genogroups have further been classified into clusters. For example, a HuNoV designated as GI.1, indicates that the strain belongs to genogroup I and genotype or genocluster 1 (Atmar 2010). Thirty-one genoclusters have been identified (Figure 1) (Wang et al., 2005; Zheng et al., 2006).
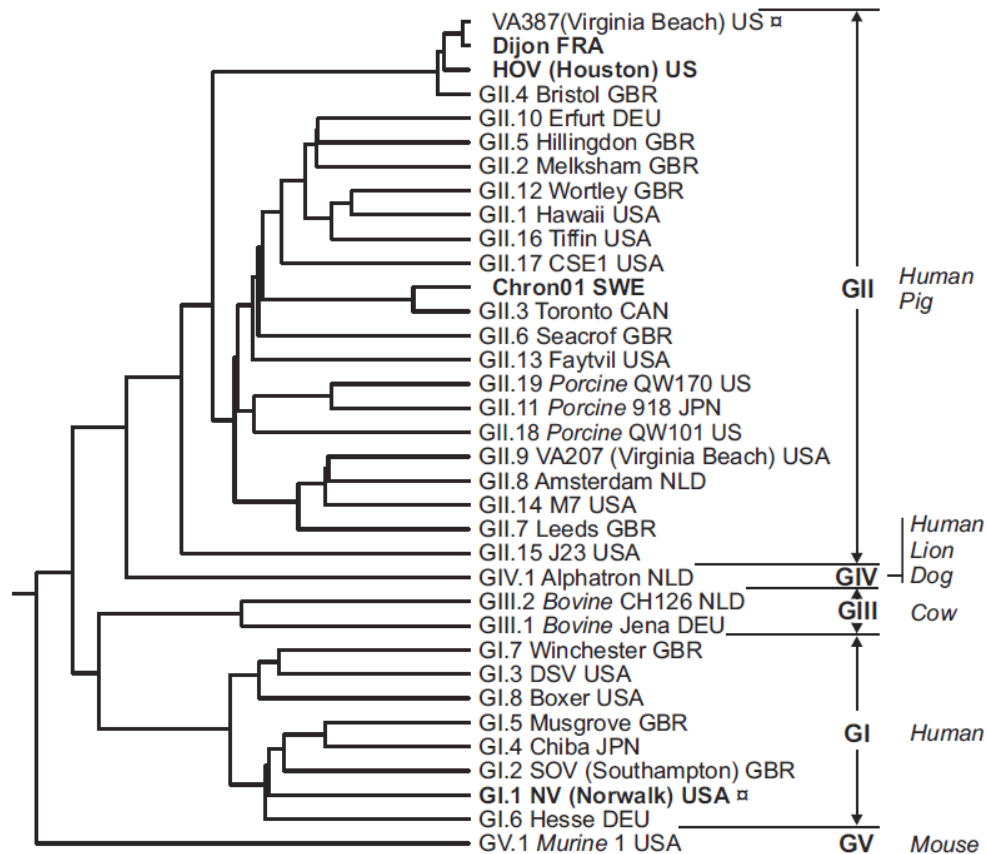
Figure 1. Genoclusters of norovirus with the strain name and country of isolation. Source: Gustaf E. Rydell (2009). (¤) indicates strains with the structure of the capsid protein determined. Host species are indicated and those not humans are italicized.

Human noroviruses are the primary etiological agent of viral gastroenteritis worldwide (Siebenga et al., 2009) as well as the chief cause of foodborne disease in Europe (Kroneman et al., 2008) and the U.S. (Scallan et al., 2011). Estimated number of 5.5 and 2 million cases of viral gastroenteritis caused by HuNoV are recorded annually in the U.S. and Europe, respectively (Scallan et al., 2011; Phillips et al., 2010). According to Lopman et al. (2002), HuNoVs are not significantly associated with mortality; however, in neonates, HuNoVs have been associated with serious health problems such as seizures (Chen et al., 2009) and necrotizing enterocolitis (Turcios-Ruiz et al., 2008).

Clinical features of HuNoV infection include vomiting, diarrhea, abdominal cramping, nausea, headache, chills, low-grade fever and dehydration (Weber et al., 2010). The virus has a 1–3 day incubation period with about 2–3 days of persistent symptoms (Koopmans 2008). For every gram of feces from the stool of a norovirus-infected patient, an estimated number of 100 billion virus particles are shed (CDC 2011).

ii.   **Molecular Structure and Function**

Human noroviruses are nonenveloped viruses – one of the primary characteristics enabling its persistence in the environment. Noroviruses can persist in the environment due to their tolerance of a wide range of temperatures from freezing to warmer temperatures (Weber et al., 2010). These viruses can persist on fomites, in drinking water or in other various water sources, within food items such as bivalve mollusks, and on vegetables that are irrigated with sewage contaminated water and are consumed fresh or partially cooked (Weber et al., 2010).

The outer shell of HuNoV is a highly stable protein capsid composed of 180 capsid protein monomers (90 dimers) (Tresset et al., 2013) and with symmetry of the icosahedron (Prasad et al., 1999). Three open reading frames (ORFs) are encoded by the approximately 7.7-kb genome (Figure 2) (Xi et al., 1990; Jiang et al., 1993). Open reading frame 1 encodes for a nonstructural polyprotein split into six nonstructural proteins including N-terminal protein (designated p48 for Norwalk virus), NTPase, 3A-like protein (designated p22 for Norwalk virus), viral protein genome-linked (VPg) which serves as a primer during RNA synthesis, viral protease (3CLpro), and RdRp, an essential enzyme that catalyzes the replication of RNA (Belliot et al., 2003; Green 2007). The second, ORF2, encodes for Viral Protein 1 (VP1), a major capsid protein, and ORF3 encodes VP2, the minor structural capsid protein (Jiang et al., 1993; Jiang et al., 1990; Hardy 2005). The VP1 has a length ranging between 530 and 555 amino acids, approximately 58–60 kDa molecular weight. The viral capsid is made up of VP1 and has several functions in the life cycle of the virus. Viral protein 1 binds to the presumed functional receptor, the histo-blood group antigen (HBGA), on the surface of host cells and mediates virus entry (Tan et al., 2003; Hutson et al., 2002) as well as determines the antigenicity and strain specificity of norovirus (Huston et al., 2004; Katayama et al., 2002; Prasad et al., 1999). Also, VP1 is the host-protective antigen that elicits neutralizing antibody as well as mucosal and cellular immunities (Ball et al., 1998). Finally, VP1 probably plays many other roles such as uncoating, genome release, and assembly in the life cycle of the virus (Hardy 2005). The minor capsid protein, VP2, plays a role in RNA packaging and regulation of the synthesis of VP1 (Glass et al., 2000; Bertolotti-Ciarlet et al., 2003). Moreover, VP2 functions in the increment of VP1 stability and protection from disassembly and protease degradation (Bertolotti-Ciarlet et al., 2003).

Histo-blood group antigens (HBGA) have been identified as the presumed functional receptors for most HuNoV (Tan et al., 2003). These HBGA receptors are carbohydrate complexes present on the surface of erythrocytes as well as intestinal, respiratory, and genitourinary epithelia (Li et al., 2012). The HBGA are also free oligosaccharides found in saliva, milk, blood, and in intestinal contents. Three major families of HBGAs – Lewis, secretor, and ABO – are recognized by different strains of HuNoV. From the study of the process involved in HuNoV virus-like particles (VLPs) interactions with HBGA receptors, amino acid residues in the P domain of the VP1 protein have been demonstrated as being responsible for the specificity of receptor binding (Tan et al., 2003; Tan et al., 2008).

	ORF1		ORF2	ORF3	
5' —	p48 NTPase p22 VPg 3CLpro RdRp		VP1	VP2	— $(A)_n$ 3'
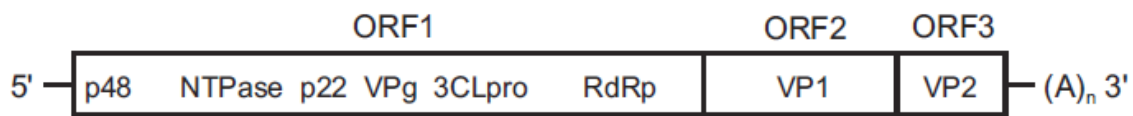
Figure 2: Norovirus genome organization (Hardy, 2005)

iii.   Transmission and Outbreaks

Human norovirus transmission can occur via numerous routes but on many occasions, it is transmitted through food and water. As a result of limited, long-term immunity against HuNoVs, institutions such as nursing homes, hospitals, and schools, which have populations in semi-confined settings, are more susceptible to outbreaks unless proper control and preventive measures are implemented (CDC 2011). An outbreak can involve different transmission routes at the same time. For example, an outbreak at one point from exposure of food can be transmitted through person-to-person within an institutional setting. Fecal-oral route is the well-documented mode for HuNoV transmission (Atmar and Estes 2001; Koopmans and Duizer 2004). In a populated setting, transmission of the virus through feces or vomit of an infected person can occur via indirect contact

through aerosolized droplets or direct contact through the mouth, hands, or both (Marks et al., 2004). Person-to-person contact and ingestion of contaminated water or food are the primary routes of transmission that lead to the outbreak of the disease (Becker et al., 2000). Data reported by Zheng et al. (2010) indicated that most of the HuNoV outbreaks that occurred from 1994 to 2006 happened in school communities, followed by institutions such as hospitals and nursing homes, restaurants, and catered events with cruise ships coming last. In addition, GII.4 noroviruses were implicated in most of the outbreaks followed by other GII genotype members and then GI members.

Virus transmission by contaminated water and fomites is influenced by persistence of viruses in the environment (Lopman et al., 2012). Human noroviruses along with other human enteric viruses (e.g., adenovirus, hepatitis A virus, hepatitis E virus, and rotavirus) have been detected in a variety of water sources and implicated globally in outbreaks associated with contaminated recreational waters, drinking water, and treated and untreated groundwater (Pusch et al., 2005; Wyn-Jones et al., 2011; Aw and Gin 2011; Kishida et al., 2012).

Prevalence of HuNoV and other human enteric viruses (i.e. enteroviruses and Hepatitis A virus) in environmental waters have been linked with a number of waterborne viral gastroenteritis outbreaks reported each year (Gibson 2014). From 2003 to 2010, thirty-eight outbreaks of waterborne disease, primarily due to HuNoV, were reported in the United States (Gibson 2014). One hundred and forty-eight waterborne disease outbreaks due to human enteric viruses were reported in the European Union from 2000 to 2007 (WHO 2009). These data together with sporadic outbreaks reported in other publications (Ashbolt 2004; Hoebe et al., 2004; Fretz et al., 2005; Werber et al., 2009; Koh et al., 2011) indicate that the morbidity due to water contaminated with human enteric viruses is quite significant.

Fomite surfaces are another significant environmental reservoir for HuNoV. Fecal material and droplets from aerosolized vomit containing HuNoV can contaminate various surfaces—both porous and non-porous—and the viruses can persist on these surfaces for several days or even weeks (Cheesbrough et al., 1997; Green et al., 1998; Liu et al., 2003; D'Souza et al., 2006; Clay et al., 2006; Lamhoujeb et al., 2009). Persistence of HuNoV on surfaces and fomites often leads to continued transmission of HuNoV during outbreaks (Donaldson et al., 2008). For instance, Widdowson et al. (2004) partially attributed HuNoV persistence in the environment to the occurrence of HuNoV outbreaks on cruise ships. Human norovirus has also been detected on surfaces around patients in a hospital ward during a gastroenteritis outbreak (Green et al., 1998). Additionally, an acute gastroenteritis outbreak due to HuNoV occurred at long-term care facility in 2003, and various fomite surfaces were contaminated with HuNoV, possibly causing the prolonged outbreak (Wu et al., 2005). Considering these examples, it is certain that HuNoV transmission via contaminated surfaces is of significant concern.

III.   Human noroviruses and primary food of concern

i.   Introduction

Fresh produce such as tomatoes, lettuce, melons, green onions, strawberries, raspberries, and blueberries is considered high risk for HuNoV contamination since it usually goes through minimal or no processing prior to consumption. These characteristics allow for contamination at any stage from pre-harvest to post-harvest (Lynch et al., 2009). Along with fresh produce, bivalve mollusks including oysters, clams, mussels, scallops and cockles are also a high risk food for HuNoV contamination primarily due to their feeding patterns (Li et al., 2012). Bivalve mollusks are filter feeders that feed by sieving large amounts of water daily. By so doing, contaminants

present in the water, including HuNoV, may be concentrated in bivalve mollusks through the filtration process and thus, the mollusks become contaminated.

ii.   Fresh produce

The presence of carbohydrate moieties that mimic HBGAs in vegetables and fruits might account for the possible attachment mechanism of HuNoV. Unlike the internalization and dissemination of bacterial pathogens in fresh produce (Lynch et al., 2009; Doyle and Erickson 2008), the mechanism involved in the virus attachment, uptake, and persistence in plants such as lettuce, spinach, celery, green onions, clover sprouts, and raspberries is not well understood. According to Wei et al. (2010), HuNoVs are also likely to be taken in through leaves and/or the roots of romaine lettuce since water and/or soil contaminated with sewage may contain the virus. There have been very few studies that have actually investigated internalization of HuNoV in produce.

Before food arrives at the consumer's plate, contamination might have already occurred because individuals handling the food through processing to preparation may be infected with HuNoV. Different food products classified as ready-to-eat have been involved in HuNoV outbreak investigations (CDC 2010). In general, food handled by infected food service workers, especially minimally processed and raw foods, is the outstanding factor that contributes to foodborne HuNoV outbreaks worldwide (Lynch et al., 2006; Hall 2010). Pre-harvest practices such as the use of sewage-contaminated water to irrigate fresh produce or the application of municipal biosolids as fertilizer are ways by which HuNoV can be transmitted at the source of production (Falkenhorst et al., 2005). Post-harvest practices may also lead to contamination and transmission of the virus. Malek et al. (2005) indicated that contamination with HuNoV can occur during processing of food,

in this instance pre-sliced, packaged deli meats. In a 2006 HuNoV outbreak, a food handler vomited at work and roughly 500 gastroenteritis cases were reported, suggesting that large quantities of food can easily be contaminated a single food handler owing primarily to the presumed low infectious dose of HuNoV (CDC 2010).

iii.   Bivalve Mollusks

Seafood such as bivalve mollusks is at risk for HuNoV contamination. Le Guyader et al. (2000) reported that HuNoV-contaminated mussels collected in France were harvested from areas that are frequently impacted with sewage from humans. Costantin et al. (2006) also reported the presence of animal and human enteric caliciviruses in oysters found in U. S. markets. In the United Kingdom, Lowther et al. (2008) reported that most of the oyster samples collected from two different sites during October and November tested positive for HuNoV. These data suggest that HuNoV contamination in bivalve mollusks depends on location and season of cultivation.

Additional research conducted on oysters demonstrated that HuNoV VLPs bind specifically to the midgut, main and secondary ducts as well as tubules presenting molecules that look similar to HBGA in humans (Le Guyader et al., 2006). However, VLPs from different genogroups of HuNoV showed different affinity of binding to tissues in oyster (Maalouf et al., 2010). It has also been documented that differences in strains together with seasonality greatly influence the bioaccumulation of HuNoV in oysters (Maalouf et al., 2011).

IV.   Human norovirus surrogates

i.   Introduction

An *in vitro* cell culture system for HuNoV has been developed recently (Jones et al., 2014); however, the developed system has not been applied or reproduced by other researchers. Therefore, studying HuNoV transmission and development of control strategies still remains difficult. Gastrointestinal epithelial cells of humans as well as other animal and human tissues have been examined and proven to be resistant to successful virus cultivation (Duzier et al., 2004). For this reason, researchers utilize several surrogates to aid in the study of HuNoV. Viral surrogates have been extensively used to study HuNoV, but since none of these surrogates has 100% resemblance to HuNoV (Richards 2011), further research is being conducted to discover the most suitable surrogate for a given research question. Primary surrogates include feline calicivirus (FCV), murine norovirus (MNV), porcine sapovirus (PoSV), and Tulane virus (TV). These surrogates are used to study HuNoV survival, transmission, infectivity, susceptibility to disinfectants, and stability in the environment. Table 1 summarizes the evaluations completed on the properties of surrogates to determine which is more suitable to aid in HuNoV studies.

ii.   Feline Calicivirus

Though non-infectious to humans, FCV causes upper respiratory tract infection in cats (Doultree et al., 1999). Feline calicivirus is a poor surrogate for HuNoV studies in that 1) it has different biochemical properties from HuNoV, 2) it is not an enteric virus, and 3) it does not belong to the genus *Norovirus* but rather *Vesivirus* in the family *Caliciviridae*. It also has a reduced stability and persistence in the environment (Cannon et al., 2006). However, it was used widely until 2003 when MNV was discovered since FCV has a desirable *in vitro* growth system (Slomka

and Appleton 1998) and can also be easily manipulated genetically (Sosnovtsev et al., 1995). Feline calicivirus is adapted to CRFK (Crandell Reese Feline Kidney) cells for *in vitro* cultivation.

iii.   Murine Norovirus

Murine norovirus was first identified in highly immunocompromised mice that lacked recombination-activating gene 2 (RAG 2) as well as signal transducer and activator of transcription 1 (STAT-1) (Karst et al., 2003). Murine norovirus is adapted to cell lines with a hematopoietic lineage such as RAW 264.7 (Wobus et al., 2006) and has become the desired surrogate for HuNoV studies due to the following reasons: 1) the existence of a cell culture system; 2) similarity in genetic identity; 3) availability of a small animal model; 4) thermal and pH resistance as well as high stability and persistence in the environment (Chaudhry et al., 2007; Cannon et al., 2006; Wobus et al., 2006; Taube et al., 2009). However, according to Karst et al. (2003), clinical signs of gastroenteritis caused by MNV are different from that caused by HuNoV. Also, sialic acid is the functional receptor for MNV (Wobus et al., 2006) whereas HBGAs are the presumed and dominant receptors for HuNoV (Tan and Jiang 2005).

iv.   Porcine Sapovirus

Porcine sapovirus is a member of the family *Caliciviridae* under the genus *Sapovirus* (Wang et al., 2005; 2007). Porcine sapovirus was adapted to cell culture by passaging serially in a continuous cell line (LLC-PK) (Flynn and Saif 1988). It has potential to be one of the most suitable surrogates for HuNoV in the sense that it is enteric, genetically related to HuNoV, and causes gastroenteritis in pigs with similar symptoms as humans (Flynn et al., 1988; Guo et al., 2001). Wang et al. (2012) compared PoSV and HuNoV and reported that PoSV was stable at room

temperature after exposure to a pH range of 4 to 8 with an observation of less than 1.0 $\log_{10}$ reduction in virus titer at pH 3 demonstrating that the virus is almost as stable as HuNoV. The authors also showed that PoSV and HuNoV displayed approximately equal resistance to chlorine treatment and heat (Wang et al., 2012). However, aside from the studies by Wang et al. (2012), very little research has been published on PoSV.

v.   Tulane Virus

Farkas et al. (2008) first isolated and characterized TV from the stools of *Macaca mulatta*, juvenile rhesus macaques held in the Tulane National Primate Research Center. *Recovirus* has been proposed as the genus name of a new group of caliciviruses represented by TV—known as the monkey calicivirus (rhesus enteric *Calicivirus*) (Farkas et al., 2008). Tulane virus and HuNoV have similar characteristics such as genetic identity and HBGA receptor recognition allowing TV to be considered an appropriate surrogate for HuNoV studies (Farkas et al., 2010). Additionally, TV is robustly replicable in monkey kidney cells (LLC-MK2)—an ideal characteristic for a suitable surrogate for HuNoV studies *in vitro* (Li et al., 2012).

V.   Research Objectives

Because there is no reproducible method to culture HuNoV in the lab, the search for an ideal surrogate virus still continues. To enable an understanding of how to compare physicochemical profiles of various enteric virus surrogates, the first part of this research was to review the methods used in evaluating the thermal stability profiles of human enteric viruses. The primary aim was to understand how differences in experimental methods and specific parameters influence the ability to compare both surrogate viruses and human enteric viruses, not only thermal

stability but also other physicochemical properties that are evaluated and compared between human enteric viruses. The hypothesis of this research is that TV is the most suitable surrogate for HuNoV studies when compared to traditional surrogates (MNV and FCV). The main objectives are 1) to establish the physiochemical properties of TV and 2) to evaluate the environmental persistence of TV. These results for TV will be compared to the previously established profiles for the traditional surrogates (MNV and FCV) to establish the most conservative surrogate to study HuNoV stability and persistence *in vitro*.

REFERENCES

Ashbolt, N. J. (2004). Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology, 198*(1), 229-238.

Atmar, R. L. (2010). Noroviruses: State of the art. *Food and Environmental Virology, 2*(3), 117-126.

Atmar, R. L., & Estes, M. K. (2001). Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. *Clinical Microbiology Reviews, 14*(1), 15-37.

Bae, J., & Schwab, K. J. (2008). Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Applied and Environmental Microbiology, 74*(2), 477-484.

Becker, K. M., Moe, C. L., Southwick, K. L., & MacCormack, J. N. (2000). Transmission of Norwalk virus during a football game. *New England Journal of Medicine, 343*(17), 1223-1227.

Belliot, G., Sosnovtsev, S. V., Mitra, T., Hammer, C., Garfield, M., & Green, K. Y. (2003). In vitro proteolytic processing of the MD145 norovirus ORF1 nonstructural polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells. *Journal of Virology, 77*(20), 10957-10974.

Bertolotti-Ciarlet, A., Crawford, S. E., Hutson, A. M., & Estes, M. K. (2003). The 3′ end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: A novel function for the VP2 protein. *Journal of Virology, 77*(21), 11603-11615.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L., & Vinje, J. (2006). Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus. *Journal of Food Protection, 69*(11), 2761-2765.

Centers for Disease Control and Prevention (CDC). (2010). Surveillance for foodborne disease outbreaks --- United States, 2007. *Morbidity and Mortality Weekly Report, 59*(31), 973-979.

Centers for Disease Control and Prevention. (2011). Updated norovirus outbreak management and disease prevention guidelines. *Morbidity and Mortality Weekly Report. Recommendations and Reports, 60*, 1–18.

Chaudhry, Y., Skinner, M. A., & Goodfellow, I. G. (2007). Recovery of genetically defined murine norovirus in tissue culture by using a fowl pox virus expressing T7 RNA polymerase. *Journal of General Virology, 88*(Pt 8), 2091-2100.

Cheesbrough, J., Barkess-Jones, L., & Brown, D. (1997). Possible prolonged environmental survival of small round structured viruses. *Journal of Hospital Infection, 35*(4), 325-326.

Chen, S., Tsai, C., Lai, M., Chen, C., Lin, K., Lin, T., & Chiu, C. (2009). Norovirus infection as a cause of diarrhea-associated benign infantile seizures. *Clinical Infectious Diseases, 48*(7), 849-855.

Clay, S., Maherchandani, S., Malik, Y. S., & Goyal, S. M. (2006). Survival on uncommon fomites of feline calicivirus, a surrogate of noroviruses. *American Journal of Infection Control, 34*(1), 41-43.

Costantini, V., Loisy, F., Joens, L., Le Guyader, F. S., & Saif, L. J. (2006). Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. *Applied and Environmental Microbiology, 72*(3), 1800-1809.

Donaldson, E. F., Lindesmith, L. C., Lobue, A. D., & Baric, R. S. (2008). Norovirus pathogenesis: Mechanisms of persistence and immune evasion in human populations. *Immunological Reviews, 225*(1), 190-211.

Doultree, J., Druce, J., Birch, C., Bowden, D., & Marshall, J. (1999). Inactivation of feline calicivirus, a Norwalk virus surrogate. *Journal of Hospital Infection, 41*(1), 51-57.

Doyle, M., & Erickson, M. (2008). Summer meeting 2007–the problems with fresh produce: An overview. *Journal of Applied Microbiology, 105*(2), 317-330.

D'Souza, D. H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C., & Jaykus, L. (2006). Persistence of caliciviruses on environmental surfaces and their transfer to food. *International Journal of Food Microbiology, 108*(1), 84-91.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F., & Koopmans, M. (2004). Inactivation of caliciviruses. *Applied and Environmental Microbiology, 70*(8), 4538-4543.

Duizer, E., Schwab, K. J., Neill, F. H., Atmar, R. L., Koopmans, M. P., & Estes, M. K. (2004). Laboratory efforts to cultivate noroviruses. *Journal of General Virology, 85*(1), 79-87.

Esseili, M. A., Wang, Q., Zhang, Z., & Saif, L. J. (2012). Internalization of Sapovirus, a surrogate for norovirus, in romaine lettuce and the effect of lettuce latex on virus infectivity. *Applied and Environmental Microbiology, 78*(17), 6271-6279.

Falkenhorst, G., Krusell, L., Lisby, M., Madsen, S. B., Bottiger, B., & Molbak, K. (2005). Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005. *Euro Surveillance: Bulletin Europeen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin, 10*(9), E050922.2.

Fallahi, S., & Mattison, K. (2011). Evaluation of murine norovirus persistence in environments relevant to food production and processing. *Journal of Food Protection, 74*(11), 1847-1851.

Farkas, T., Sestak, K., Wei, C., & Jiang, X. (2008). Characterization of a rhesus monkey calicivirus representing a new genus of caliciviridae. *Journal of Virology, 82*(11), 5408-5416.

Farkas, T., Cross, R. W., Hargitt, E.,3rd, Lerche, N. W., Morrow, A. L., & Sestak, K. (2010). Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses. *Journal of Virology, 84*(17), 8617-8625.

Flynn, W. T., & Saif, L. J. (1988). Serial propagation of porcine enteric calicivirus-like virus in primary porcine kidney cell cultures. *Journal of Clinical Microbiology, 26*(2), 206-212.

Flynn, W., Saif, L., & Moorhead, P. (1988). Pathogenesis of porcine enteric calicivirus-like virus in four-day-old gnotobiotic pigs. *American Journal of Veterinary Research, 49*(6), 819-825.

Fretz, R., Svoboda, P., Lüthi, T., Tanner, M., & Baumgartner, A. (2005). Outbreaks of gastroenteritis due to infections with norovirus in Switzerland, 2001–2003. *Epidemiology and Infection, 133*(03), 429-437.

Gibson, K. E. (2014). Viral pathogens in water: Occurrence, public health impact, and available control strategies. *Current Opinion in Virology, 4*, 50-57.

Gibson, K. E., & Schwab, K. J. (2011). Thermal inactivation of human norovirus surrogates. *Food and Environmental Virology, 3*(2), 74-77.

Glass, P. J., White, L. J., Ball, J. M., Leparc-Goffart, I., Hardy, M. E., & Estes, M. K. (2000). Norwalk virus open reading frame 3 encodes a minor structural protein. *Journal of Virology, 74*(14), 6581-6591.

Green, J., Wright, P., Gallimore, C., Mitchell, O., Morgan-Capner, P., & Brown, D. (1998). The role of environmental contamination with small round structured viruses in a hospital outbreak investigated by reverse-transcriptase polymerase chain reaction assay. *Journal of Hospital Infection, 39*(1), 39-45.

Green, K. Y. (2007). Caliciviridae: The noroviruses. In D. M. Knipe & P. M. Howley (Eds.), *Fields virology* (5th ed., pp. 950–979). Philadelphia, PA: LWW.

Green, K., Ando, T., Balayan, M., Berke, T., Clarke, I., Estes, M., et al., (2000). Taxonomy of the caliciviruses. *Journal of Infectious Diseases, 181*(Supplement 2), S322-S330.

Guo, M., Hayes, J., Cho, K., Parwani, A., Lucas, L., & Saif, L. (2001). Comparative pathogenesis of tissue culture-adapted and wild-type cowden porcine enteric calicivirus (PEC) in gnotobiotic pigs and induction of diarrhea by intravenous inoculation of wild-type PEC. *Journal of Virology, 75*(19), 9239-9251.

Hall, A. (2010). Foodborne norovirus outbreaks in the United States: Burden and attribution [presentation]. *4th International Conference on Caliciviruses, Santa Cruz, Chile,*

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

Hewitt, J., Rivera-Aban, M., & Greening, G. (2009). Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies. *Journal of Applied Microbiology, 107*(1), 65-71.

Hirneisen, K. A., & Kniel, K. E. (2013). Comparing human norovirus surrogates: Murine norovirus and Tulane virus. *Journal of Food Protection, 76*(1), 139-143.

Hoebe, C. J., Vennema, H., de Roda Husman, A. M., & van Duynhoven, Y. T. (2004). Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain. *The Journal of Infectious Diseases, 189*(4), 699-705.

Hutson, A. M., Atmar, R. L., & Estes, M. K. (2004). Norovirus disease: Changing epidemiology and host susceptibility factors. *Trends in Microbiology, 12*(6), 279-287.

Hutson, A. M., Atmar, R. L., Graham, D. Y., & Estes, M. K. (2002). Norwalk virus infection and disease is associated with ABO histo-blood group type. *Journal of Infectious Diseases, 185*(9), 1335-1337.

Jiang, X., Wang, M., Wang, K., & Estes, M. K. (1993). Sequence and genomic organization of Norwalk virus. *Virology, 195*(1), 51-61.

Jones, M.K., Watanabe, M., Zhu, S., Graves, C.L., Keyes, L. R., Grau, K.R., et al., (2014). Enteric bacteria promote human and mouse norovirus infection of B cells. *Science,* 346, 755-759.

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., & Virgin, H. W. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science, 299*(5612), 1575-1578.

Katayama, K., Shirato-Horikoshi, H., Kojima, S., Kageyama, T., Oka, T., Hoshino, F. B., et al., (2002). Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. *Virology, 299*(2), 225-239.

Kingsley, D. H., Vincent, E. M., Meade, G. K., Watson, C. L., & Fan, X. (2014). Inactivation of human norovirus using chemical sanitizers. *International Journal of Food Microbiology, 171*, 94-99.

Kishida, N., Morita, H., Haramoto, E., Asami, M., & Akiba, M. (2012). One-year weekly survey of noroviruses and enteric adenoviruses in the tone river water in Tokyo metropolitan area, japan. *Water Research, 46*(9), 2905-2910.

Koh, S., Cho, H. G., Kim, B. H., & Choi, B. Y. (2011). An outbreak of gastroenteritis caused by norovirus-contaminated groundwater at a waterpark in Korea. *Journal of Korean Medical Science, 26*(1), 28-32.

Koopmans, M. (2008). Progress in understanding norovirus epidemiology. *Current Opinion in Infectious Diseases, 21*(5), 544-552.

Koopmans, M., & Duizer, E. (2004). Foodborne viruses: An emerging problem. *International Journal of Food Microbiology, 90*(1), 23-41.

Kroneman, A., Verhoef, L., Harris, J., Vennema, H., Duizer, E., Van Duynhoven, Y., et al., (2008). Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the foodborne viruses in Europe network from 1 July 2001 to 30 June 2006. *Journal of Clinical Microbiology, 46*(9), 2959-2965.

Lamhoujeb, S., Fliss, I., Ngazoa, S., & Jean, J. (2009). Molecular study of the persistence of infectious human norovirus on food-contact surfaces. *Food and Environmental Virology, 1*(2), 51-56.

Lamhoujeb, S., Fliss, I., Ngazoa, S. E., & Jean, J. (2008). Evaluation of the persistence of infectious human noroviruses on food surfaces by using real-time nucleic acid sequence-based amplification. *Applied and Environmental Microbiology, 74*(11), 3349-3355.

Le Guyader, F., Haugarreau, L., Miossec, L., Dubois, E., & Pommepuy, M. (2000). Three-year study to assess human enteric viruses in shellfish. *Applied and Environmental Microbiology, 66*(8), 3241-3248.

Le Guyader, F. S., Loisy, F., Atmar, R. L., Hutson, A. M., Estes, M. K., Ruvoën-Clouet, N., et al., (2006). Norwalk virus–specific binding to oyster digestive tissues. *Emerging Infectious Diseases, 12*(6), 931.

Lee, J., Zoh, K., & Ko, G. (2008). Inactivation and UV disinfection of murine norovirus with TiO_2 under various environmental conditions. *Applied and Environmental Microbiology, 74*(7), 2111-2117.

Li, J., Predmore, A., Divers, E., & Lou, F. (2012). New interventions against human norovirus: Progress, opportunities, and challenges. *Annual Review of Food Science and Technology, 3*, 331-352.

Liu, B., Maywood, P., Gupta, L., & Campbell, B. (2003). An outbreak of Norwalk-like virus gastroenteritis in an aged-care residential hostel. *New South Wales Public Health Bulletin, 14*(6), 105-109.

Liu, P., Chien, Y., Papafragkou, E., Hsiao, H., Jaykus, L., & Moe, C. (2009). Persistence of human noroviruses on food preparation surfaces and human hands. *Food and Environmental Virology, 1*(3-4), 141-147.

Lopman, B., Brown, D., & Koopmans, M. (2002). Human caliciviruses in Europe. *Journal of Clinical Virology, 24*(3), 137-160.

Lopman, B. A., Reacher, M. H., Vipond, I. B., Sarangi, J., & Brown, D. W. (2004). Clinical manifestation of norovirus gastroenteritis in health care settings. *Clinical Infectious Diseases, 39*(3), 318-324.

Lopman, B., Gastañaduy, P., Park, G. W., Hall, A. J., Parashar, U. D., & Vinjé, J. (2012). Environmental transmission of norovirus gastroenteritis. *Current Opinion in Virology, 2*(1), 96-102.

Lowther, J. A., Henshilwood, K., & Lees, D. N. (2008). Determination of norovirus contamination in oysters from two commercial harvesting areas over an extended period, using semiquantitative real-time reverse transcription PCR. *Journal of Food Protection, 71*(7), 1427-1433.

Lynch, M., Tauxe, R., & Hedberg, C. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. *Epidemiology and Infection, 137*(3), 307.

Maalouf, H., Schaeffer, J., Parnaudeau, S., Le Pendu, J., Atmar, R. L., Crawford, S. E., & Le Guyader, F. S. (2011). Strain-dependent norovirus bioaccumulation in oysters. *Applied and Environmental Microbiology, 77*(10), 3189-3196.

Maalouf, H., Zakhour, M., Le Pendu, J., Le Saux, J., Atmar, R. L., & Le Guyader, F. S. (2010). Distribution in tissue and seasonal variation of norovirus genogroup I and II ligands in oysters. *Applied and Environmental Microbiology, 76*(16), 5621-5630.

Malek, M., Barzilay, E., Kramer, A., Camp, B., Jaykus, L., Escudero-Abarca, B., et al., (2009). Outbreak of norovirus infection among river rafters associated with packaged delicatessen meat, Grand Canyon, 2005. *Clinical Infectious Diseases, 48*(1), 31-37.

Marks, P., Vipond, I., Regan, F., Wedgwood, K., Fey, R., & Caul, E. (2003). A school outbreak of Norwalk-like virus: Evidence for airborne transmission. *Epidemiology and Infection, 131*(1), 727-736.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., et al., (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases, 5*(5), 607.

Pusch, D., Oh, D., Wolf, S., Dumke, R., Schröter-Bobsin, U., Höhne, M., et al., (2005). Detection of enteric viruses and bacterial indicators in German environmental waters. *Archives of Virology, 150*(5), 929-947.

Phillips, G., Tam, C. C., Conti, S., Rodrigues, L. C., Brown, D., Iturriza-Gomara, M., et al., (2010). Community incidence of norovirus-associated infectious intestinal disease in England: Improved estimates using viral load for norovirus diagnosis. *American Journal of Epidemiology, 171*(9), 1014-1022.

Prasad, B. V., Hardy, M. E., Dokland, T., Bella, J., Rossmann, M. G., & Estes, M. K. (1999). X-ray crystallographic structure of the Norwalk virus capsid. *Science, 286*(5438), 287-290.

Richards, G. P. (2012). Critical review of norovirus surrogates in food safety research: Rationale for considering volunteer studies. *Food and Environmental Virology, 4*(1), 6-13.

Rydell, G. E. (2009). *Norovirus, causative agent of winter vomiting disease, exploits several histo-blood group glycans for adhesion* Institute of Biomedicine. Department of Clinical Chemistry and Transfusion Medicine.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M., Roy, S. L., et al., (2011). Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases, 17*(1), 7-15.

Seitz, S. R., Leon, J. S., Schwab, K. J., Lyon, G. M., Dowd, M., McDaniels, M., et al., (2011). Norovirus infectivity in humans and persistence in water. *Applied and Environmental Microbiology, 77*(19), 6884-6888.

Siebenga, J. J., Vennema, H., Zheng, D., Vinjé, J., Lee, B. E., Pang, X., et al., (2009). Norovirus illness is a global problem: Emergence and spread of norovirus GII. 4 variants, 2001–2007. *Journal of Infectious Diseases, 200*(5), 802-812.

Slomka, M., & Appleton, H. (1998). Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish. *Epidemiology and Infection, 121*(02), 401-407.

Tan, M., Fang, P., Chachiyo, T., Xia, M., Huang, P., Fang, Z., et al., (2008). Noroviral P particle: Structure, function and applications in virus–host interaction. *Virology, 382*(1), 115-123.

Tan, M., Huang, P., Meller, J., Zhong, W., Farkas, T., & Jiang, X. (2003). Mutations within the P2 domain of norovirus capsid affect binding to human histo-blood group antigens: Evidence for a binding pocket. *Journal of Virology, 77*(23), 12562-12571.

Tan, M., & Jiang, X. (2005). Norovirus and its histo-blood group antigen receptors: An answer to a historical puzzle. *Trends in Microbiology, 13*(6), 285-293.

Taube, S., Perry, J. W., Yetming, K., Patel, S. P., Auble, H., Shu, L., et al., (2009). Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses. *Journal of Virology, 83*(9), 4092-4101.

Tresset, G., Le Coeur, C., Bryche, J., Tatou, M., Zeghal, M., Charpilienne, A., et al., (2013). Norovirus capsid proteins self-assemble through biphasic kinetics via long-lived stave-like intermediates. *Journal of the American Chemical Society, 135*(41), 15373-15381.

Turcios-Ruiz, R. M., Axelrod, P., St John, K., Bullitt, E., Donahue, J., Robinson, N., & Friss, H. E. (2008). Outbreak of necrotizing enterocolitis caused by norovirus in a neonatal intensive care unit. *The Journal of Pediatrics, 153*(3), 339-344.

Wang, D., & Tian, P. (2014). Inactivation conditions for human norovirus measured by an in situ capture-qRT-PCR method. *International Journal of Food Microbiology, 172*, 76-82.

Wang, Q., Costantini, V., & Saif, L. J. (2007). Porcine enteric caliciviruses: Genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology. *Vaccine, 25*(30), 5453-5466.

Wang, Q., Han, M. G., Cheetham, S., Souza, M., Funk, J. A., & Saif, L. J. (2005). Porcine noroviruses related to human noroviruses. *Emerging Infectious Diseases, 11*(12), 1874.

Wang, Q., Zhang, Z., & Saif, L. J. (2012). Stability of and attachment to lettuce by a culturable porcine Sapovirus surrogate for human caliciviruses. *Applied and Environmental Microbiology, 78*(11), 3932-3940.

Wang, Q., Hirneisen, K. A., Markland, S. M., & Kniel, K. E. (2013). Survival of murine norovirus, Tulane virus, and hepatitis A virus on alfalfa seeds and sprouts during storage and germination. *Applied and Environmental Microbiology, 79*(22), 7021-7027.

Weber, D. J., Rutala, W. A., Miller, M. B., Huslage, K., & Sickbert-Bennett, E. (2010). Role of hospital surfaces in the transmission of emerging health care-associated pathogens: Norovirus, clostridium difficile, and Acinetobacter species. *American Journal of Infection Control, 38*(5), S25-S33.

Wei, J., Jin, Y., Sims, T., & Kniel, K. E. (2010). Manure-and biosolids-resident murine norovirus 1 attachment to and internalization by romaine lettuce. *Applied and Environmental Microbiology, 76*(2), 578-583.

Werber, D., Laušević, D., Mugoša, B., Vratnica, Z., Ivanović-Nikolić, L., Žižić, L., et al., (2009). Massive outbreak of viral gastroenteritis associated with consumption of municipal drinking water in a European capital city. *Epidemiology and Infection, 137*(12), 1713-1720.

WHO: Outbreaks of Waterborne Diseases, Fact Sheet 1.1. 2009, RPG1_WatSan_E1

Widdowson, M. A., Cramer, E. H., Hadley, L., Bresee, J. S., Beard, R. S., Bulens, S. N., et al., (2004). Outbreaks of acute gastroenteritis on cruise ships and on land: Identification of a predominant circulating strain of norovirus--United States, 2002. *The Journal of Infectious Diseases, 190*(1), 27-36.

Wobus, C. E., Thackray, L. B., & Virgin, H. W. (2006). Murine norovirus: A model system to study norovirus biology and pathogenesis. *Journal of Virology, 80*(11), 5104-5112.

Wu, H. M., Fornek, M., Schwab, K. J., Chapin, A. R., Gibson, K., Schwab, E., et al., (2005). A norovirus outbreak at a long-term-care facility: The role of environmental surface contamination. *Infection Control, 26*(10), 802-810.

Wyn-Jones, A. P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., et al., (2011). Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Research, 45*(3), 1025-1038.

Xi, J., Graham, D. Y., Wang, K., & Estes, M. K. (1990). Norwalk virus genome cloning and characterization. *Science, 250*(4987), 1580-1583.

Zheng, D., Ando, T., Fankhauser, R. L., Beard, R. S., Glass, R. I., & Monroe, S. S. (2006). Norovirus classification and proposed strain nomenclature. *Virology, 346*(2), 312-323.

Zheng, D., Widdowson, M., Glass, R. I., & Vinjé, J. (2010). Molecular epidemiology of genogroup II-genotype 4 noroviruses in the united states between 1994 and 2006. *Journal of Clinical Microbiology, 48*(1), 168-177.

Figure legends

Figure 1: Genoclusters of norovirus with the strain name and country of isolation. Source: Gustaf E. Rydell (2009). (¤) indicates strains with the structure of the capsid protein determined. Host species are indicated and those not humans are italicized.

Figure 2: Norovirus genome organization showing the three open reading frames and the proteins they encode. Source: Hardy (2005).

CHAPTER 2: COMPARISON OF METHODS FOR EVALUATING THE THERMAL

STABILITY OF HUMAN ENTERIC VIRUSES

Abstract

Human enteric viruses have been identified as one of the predominant causative agents of food-borne illnesses in developed countries, and it is estimated that human norovirus (HuNoV) accounts for a majority of these illnesses each year. Not all of these viruses can be cultured and hence, relatively little is known about their pathogenesis and physicochemical properties. To overcome this, researchers have utilized different virus surrogates for the study of non-cultivable human enteric viruses. In this review, we discuss various methods utilized for the evaluation of the thermal stability of human enteric viruses; compare the results of these methods; and examine how researchers may move towards a single standard approach (i.e. temperatures, virus concentrations, volume/weight of matrices, etc.) for determining thermal inactivation profiles of human enteric viruses and their surrogates. Based on the review, we found that temperature, time of exposure, type of matrix, analysis type, type of heat application, and the concentration and volume of virus used in the experiments were highly variable across virus surrogates even for the same surrogates. Because of these differences—along with the inherent limitations of using surrogate viruses—comparison of these methods and how the results may be extrapolated to human enteric viruses is quite challenging. As a result, we discuss how researchers may move towards a single standard approach for determining thermal inactivation profiles of human enteric viruses and their surrogates.

Keywords

I.   Introduction

Human enteric viruses that have been well studied belong to the families *Adenoviridae* (adenoviruses), *Caliciviridae* (noroviruses, sapoviruses), *Picornaviridae* (Hepatovirus: Hepatitis A virus; the enteroviruses: polioviruses, coxsakieviruses, and echoviruses), and *Reoviridae* (rotaviruses). These viruses are mostly associated with acute, self-limiting gastroenteritis and diarrhea; however, in immunocompromised persons, these viruses can also be associated with respiratory infections, conjunctivitis, hepatitis, and deadly diseases, such as aseptic meningitis, encephalitis, and paralysis (Kocwa-Haluch 2001). Some of the enteric viruses such as coxsackievirus B have also been related to chronic diseases such as myocarditis and insulin-dependent diabetes (Kocwa-Haluch 2001; Griffin et al., 2003). Additionally, human enteric viruses have been identified as one of the dominant causative agents of food-borne illnesses (Lynch et al., 2006; Scallan et al., 2011). It is estimated that human noroviruses (HuNoV) account for a majority (58%, or 5.5 million) of all foodborne illnesses in the United States each year (Scallan et al., 2011). Also, HuNoVs are the primary etiological agents of viral gastroenteritis worldwide and the chief cause of foodborne diseases in the European Union (Kroneman et al., 2008; Siebenga et al., 2009). These viruses are carried in the feces or vomitus of infected persons and readily transmitted to others via contamination of food and beverages (Marks et al., 2004). In addition, places that are highly populated or have semi-confined settings are more susceptible to outbreaks caused by these viruses (CDC 2011).

Not all of these enteric viruses can be cultivated *in vitro* (Duzier et al., 2004b; Straub et al., 2007) on a routine basis, and therefore, relatively little is known about them with respect to their various physicochemical properties. Researchers have utilized different viral surrogates for the study of non-cultivable human enteric viruses, most notably human noroviruses. The most

common HuNoV surrogates are feline calicivirus vaccine strain (FCV-F9), murine norovirus Type 1 (MNV), and Hepatitis A virus HM-175 (HAV) (Cannon et al., 2006; Hewitt and Greening 2006; Bae et al., 2008; Gibson and Schwab 2011; Laird et al., 2011). Tulane virus (TV), porcine sapovirus (PoSV)—also known as porcine enteric calicivirus (PEC)—poliovirus type 1 (PV-1), and canine calicivirus (CaCV) have been studied as well (Duzier et al., 2004a; Nuanualsuwan and Cliver 2002; Wang et al., 2012; Tian et al., 2013; Hirneisen and Kniel 2013) though there are not as much data available when compared to the so-called traditional surrogates. Some of the limitations associated with using surrogates, specifically MNV and feline calicivirus (FCV), are noted here. First, there is no surrogate that is identical to HuNoV in both structure and function (Richards 2012). Second, although in the same family as HuNoV, FCV has properties that are biochemically different from HuNoV (Cannon et al., 2006). In addition, even though MNV has been shown to be more similar to HuNoV, clinical signs of gastroenteritis caused by MNV are different from that caused by HuNoV according to Karst et al. (2003). Last, sialic acid is the functional receptor for MNV (Wobus et al., 2006) and FCV whereas histo-blood group antigens (HBGAs) are the presumed receptors for HuNoV (Tan and Jiang 2005). Here, it is important to note that a cell culture system for HuNoV was very recently developed using human B cells in the presence of free HBGA or HBGA-expressing bacteria (Jones et al., 2014). However, as of this publication, no additional research has been published to show reproducibility and application.

In an attempt to identify a suitable surrogate for the study of HuNoV, as well as for the study of other human enteric viruses, physicochemical properties and environmental persistence of the surrogate viruses are often evaluated. Since heat treatment is a traditional and industrial way of processing and keeping food safe, most studies on physicochemical stability profiles include the evaluation of thermal inactivation (Slomka and Appleton 1998; Hewitt and Greening 2006;

Gibson and Schwab 2011). In these experiments, viruses are exposed to temperature and time points that are used in food industries such as during processing (i.e. pasteurization) and at retail (i.e. food service settings, restaurants) and subsequently evaluated for stability under such conditions. However, there is no standard approach for determining the thermal stability of surrogate viruses used in studies designed to better understand non-cultivable human enteric viruses. Therefore, this review aims to 1) discuss the various methods employed for evaluation of the thermal stability of human enteric virus surrogates; 2) compare the results of these methods; and 3) examine how researchers may move towards a single standard approach for determining thermal inactivation profiles of human enteric viruses and their surrogates.

II.   Thermal Stability Methods *In Vitro*

Different methods have been used for *in vitro* thermal inactivation studies. These differences include the choice of temperature and time point, the type of medium or buffer (matrix), the type of heat (dry vs. wet-based), the type of infectivity analysis as well as the volume and concentration of virus analyzed. Here, we will explore how these parameters may influence the results of *in vitro* thermal stability assays. Summaries of the studies discussed here are shown in Table 1.

Temperature choice, as previously mentioned, plays an important role in heat inactivation studies. Lower temperatures ($\leq 37°C$) used in heat inactivation studies of human enteric viruses and their surrogates are supposed to mimic the normal environment where they can remain infectious (Fields et al., 2007). Research findings have repeatedly shown that MNV, FCV, and CaCV are very stable over long periods of time at 37°C (Duizer et al., 2004a; Buckow et al., 2008; Gibson and Schwab 2011; Tian et al., 2013). At temperatures greater than 37°C, 5 min to 2 h is

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required to achieve higher $\log_{10}$ reductions in infectivity due to the inability to destroy the capsid proteins (Croci et al., 2012). Estes et al. (1979) reported reduction of rotavirus (RV) by 2-logs after 30 min at 50°C while O'Mahony et al. (2000) observed reduction in RV by at least 7-logs in 10 min at 60°C. Gibson and Schwab (2011) also showed that heat treatment at 60°C was more effective than 50°C for inactivation of surrogate viruses including FCV, MNV, and HAV. However, at elevated temperatures (>60°C), virus inactivation is consistently reported as being more rapid than exposure to temperatures below 60°C (Volkin et al., 1997; Auser et al., 2006). At 72°C, most human enteric viruses or their surrogates rapidly lose their infectivity (Nuanualsuwan and Cliver 2002; Lamhoujeb et al., 2008; Bozkurt et al., 2013; Tian et al., 2013; Wang et al., 2014) (Table 1). Occasionally, some studies have reported that wild-type human enteric viruses (e.g., HuNoV) are markedly more resistant to elevated temperatures than the viral surrogates (Lamhoujeb et al., 2008; Topping et al., 2009; Escudero-Abraca et al., 2014). For instance, Topping et al. (2009) observed a 13°C difference in affective temperature (i.e. maximal exposure with RNA degradation) between FCV and a clinical isolate of HuNoV GII.4. More specifically, the authors demonstrated rapid degradation of FCV RNA at 63.3°C after 2 min whereas the same degree of degradation was achieved for HuNoV GII.4 RNA at 76.6°C (Topping et al., 2009). Similar findings between FCV and HuNoV were also reported by Lamhoujeb et al. (2008). In addition, Escudero-Abarca et al. (2014) analyzed Snow mountain virus—reference strain for HuNoV GII—stability at 77°C and obtained D-values of 25.6 ± 2.8 and 16.4 ± 0.4 min. Overall, these higher D-values may be the result of the type of detection method applied, RT-qPCR, which targets the RNA of the virus as opposed to the capsid. Heat treatment may cause little to no damage to the genome of the virus (Pecson et al., 2009) and therefore non-infectious virus may still be detected by RT-qPCR. Although discussed later, some of the thermal inactivation studies

involving HuNoV also used small volumes (i.e. < 200 μl) and thin-walled PCR tubes which will impact the rate of heat transfer to the sample affecting the ability to directly compare the results.

Another important factor in thermal stability studies is the buffer selection. Estes et al. (1979) compared tris-buffered saline (TBS) and phosphate-buffered saline (PBS) to distilled (DI) water and reported that RV infectivity decreased at the same rate in the buffers and in DI water. However, when comparing buffers used in studies of FCV, Nuanualsuwan and Cliver (2002) and Lamhoujeb et al. (2008) both used PBS for thermal inactivation studies and reported 1-log reductions at 72°C after approximately 7.69 s and 19.5 s, respectively. Meanwhile at the same temperature, Bozkurt et al. (2013) reported 3.6 s to reduce the FCV titer by 1-log when inoculated in Dulbecco's Modified Eagle's Medium (DMEM) (Table 1). Conversely, in studies using TV as a surrogate, Cromeans et al. (2014) reported that at 56°C only 4 min (estimated D-value) were required to reduce TV titer by 1-log in PBS whereas Tian et al. (2013) observed 11.8 min to reduce TV by 1-log at 56°C in medium 199 (M199). Though it is not immediately apparent what role PBS— or salt-based buffers in general—and cell culture medium play in the kinetics of thermal inactivation, it is clear that the buffer selected can impact the results depending on the virus surrogate. Grausgruber (1963) showed that virus resistance to heat inactivation was enhanced by the presence of salt in pickling sausage batter. Volkin et al. (1997) also showed that thermoresistance of viruses may be induced by salt concentrations.

When comparing the types of heat (dry vs. wet) used in thermal inactivation experiments, not much has been explored. Wang et al. (2012) recorded $2.38 \pm 0.18 \log_{10} \text{TCID}_{50}$ reduction after 30 min and greater than $4 \pm 0.53 \log_{10} \text{TCID}_{50}$ reduction after 2 h for PoSV at 56°C with wet heat in PBS + FBS. On the other hand, with dry heat at 56°C in PBS, Cromeans et al. (2014) recorded a 2-log reduction of PoSV after only 20 min. However, the difference in buffer may be a potential

confounding factor leading to the difference in results between wet and dry heat. There was also a striking difference in FCV reduction rates reported by Buckow et al. (2008) and Lamhoujeb et al. (2008) (Table 1). The former recorded 6-log reduction after 1.5 min with dry heat at 70°C and the latter recorded approximately 6.5-log reduction after 4 min with wet heat at 72°C. When MNV were treated with wet heat at temperatures above 70°C, D-values of 0.17, 1.03 and 0.11 min were recorded by Cannon et al. (2006), Tuladhar et al. (2012) and Bozkurt et al. (2013), respectively. On the contrary, Hirneisen and Kniel (2013) observed inactivation beyond the limit of detection for MNV at both 70°C and 75°C after 2 min when dry heat was applied; therefore, complete inactivation of the initial 6-log PFU was achieved in ≤ 2 min. For thermal inactivation of TV, Wang et al. (2014) reported that no viruses (i.e. 100% reduction of the initial 7.71×10^4 TCID$_{50}$ or 4.89-log reduction) were detected after 2 min at 72°C using wet heat. In contrast, for TV at the same temperature with dry heat, Tian et al. (2013) reported only a 1-log reduction in virus titer after 4.3 min. Based on the limited studies that can be compared, the type of heat applied may or may not be of importance since other confounding factors such as buffer type and volume could have also played a role in the differences in results reported.

Another important parameter is the type of analysis performed to detect infectious virus particles. Heat treatment may cause damage to the receptor binding sites on the surface of the virus and therefore the virus would be non-infectious (Wang et al., 2012). However, little to no damage to the genome of the virus may be caused by the treatment (Pecson et al., 2009) and therefore molecular methods such as reverse transcription PCR (RT-PCR) can still detect the viral genome and give false positive results for infectivity. For instance, RT-PCR may detect naked viral RNA that is still intact even though the capsid is destroyed and infectivity is lost (Richards 1999; Knight et al., 2013). Furthermore, if the target amplification sequence remains intact, molecular methods

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such as RT-PCR cannot detect single strand breaks in the genome of the virus, although these breaks may render the virus non-infectious (Knight et al., 2013). Last, since PCR based methods are normally preceded by recovery and concentration of viruses from the matrix of interest (i.e. food or water), PCR inhibitors may also be concentrated and thus inhibit the detection of the virus resulting in false negatives or inaccurate quantification if using qPCR methods (Julian and Schwab 2012). For example, Lamhoujeb et al. (2008) reported a D-value of approximately 0.62 min for FCV at 72°C using real time nucleic acid sequence-based amplification (NASBA) while Cannon et al. (2006) reported a D-value of 0.12 min at the same temperature for FCV using the conventional plaque assay method. With the same concentration and volume of TV treated at 56°C for 2 min, Wang et al. (2014) reported approximately 1.6 and 0.8-log reduction in virus titer when assayed with *in situ* capture (based on human blood group antigens [HBGA]) quantitative PCR and TCID$_{50}$ assay, respectively.

Preheating the buffer prior to virus inoculation may also influence the results of thermal inactivation studies such as reported by Gibson and Schwab (2011), Buckow et al. (2008), Lamhoujeb et al. (2008) and others. Buckow et al. (2008) and Duizer et al. (2004a) reported a 6-log reduction in FCV titer in 1.5 min at 70°C (estimated D-value = 0.25 min) and 3-log reduction in 1 min at 71.3°C (estimated D-value = 0.3 min), respectively. Though temperatures were slightly different at 70 and 71.3°C, all other conditions were kept constant so it is conceivable that preheating may have accounted for the differences in FCV reduction rates seen by Buckow et al. (2008) who used pre-heating and Duizer et al. (2004a) who did not apply pre-heating.

Total volume of sample and the concentration of virus used are also important. Nuanualsuwan and Cliver (2002) tested FCV at a concentration of about 10^3 PFU/ml and observed a complete reduction at 72°C in less than 1 min whereas Lamhoujeb et al. (2008) tested the same

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virus at 6×10^5 PFU/ml and observed approximately 6.5-log reduction after 4 min at the same temperature (Table 1). In this instance, the difference in the initial concentration of the virus could be impacting what is considered "complete reduction" since Lamhoujeb et al. (2008) also reported a 3-log reduction between 60 to 72 s. Cannon et al. (2006) tested MNV at about 10^6 PFU/ml and recorded a D-value of 0.116 min at 72°C whereas Tuladhar et al. (2012) used 10^7 PFU/ml (estimated) and recorded a D-value of approximately 1 min (62 s) at 73°C (Table 1). With respect to Cannon et al. (2006) and Tuladhar et al. (2012), an additional factor that may affect the results of the study is the material of the tube that is used to incubate the virus suspension as well as the volume—50 vs. 100 µl, respectively. For instance, glass tubes used in thermal inactivation studies (Cannon et al., 2006; Bozkurt et al., 2013) might influence the results obtained. Due to the unlike charges between viruses and glass surfaces, there is a possibility of adsorption of virus to the inside surfaces of the glass (Gerba 1984). Conversely, viruses adsorb weakly to organic surfaces such as polypropylene and polystyrene (Murray 1980; Al-Kaissi and Mostratos 1982).

III.   Thermal Stability Methods in Food Matrices

The various methods used in thermal stability experiments are expected to differ by the type of virus and food matrix. However, the methods employed often differ when the same viruses and same food groups are being evaluated. These differences may greatly influence the results that are obtained. Bozkurt et al. (2014c) and Slomka and Appleton (1998) studied the thermal inactivation profile of FCV-F9 in bivalve mollusks. Bozkurt et al., (2014c) treated FCV-contaminated mussel homogenate at 50 to 72°C and found corresponding D-values of 5.20 to 0.07 min, respectively (Table 2). Meanwhile, Slomka and Appleton (1998) examined FCV inactivation profile in live, intact cockles (Table 2). Here, the authors inoculated artificial seawater containing

cockles with 10^{11} TCID$_{50}$ FCV per 20 L and allowed for FCV uptake in the presence of yeast over a 24 h period. After 24 h, the cockles were boiled (presumably at a temperature of $\geq 100°C$) up to 3 min reaching an internal temperature of at least 78°C. After 0.5 min of boiling, Slomka and Appleton (1998) observed a 1.7-log reduction (estimated D-value = 0.29 min). The method used here was different from that used by Bozkurt et al. (2014b) in that they inoculated seawater allowing for natural uptake of the virus as opposed to inoculating the food matrix directly with viruses. From the results obtained in the different experiments, the time to reduce FCV by 1-log differed by approximately 4-fold with Slomka and Appleton (1998) reporting a longer time than that of Bozkurt et al. (2014c), most likely owing to the difference in methodology. The effect of differences in methodology can also be observed in the experiments conducted by Croci et al. (1999) and Hewitt and Greening (2006). Croci et al. (1999) observed more than 2-log reduction after a 10 minute treatment of HAV in mussel homogenate at 60°C whereas Hewitt and Greening (2006) observed 1.5-log reduction after a 3 minute steam treatment at 63°C of whole mussel samples injected with HAV in their gut region.

Similar to thermal inactivation methods conducted *in vitro*, the sample volume and the initial concentration of virus are critical, and specific to thermal inactivation in foods, the amount of food matrix is also important. The final concentration of FCV inoculated in seawater containing cockles by Slomka and Appleton (1998) was lower than that inoculated by Bozkurt et al. (2014c) in mussel homogenate. This might be another possible reason for the differences in their results. Of course, it is to be expected that different viruses will have different thermal inactivation profiles; however, this cannot be confidently concluded if the sample volume and concentrations of viruses tested differ for the same food type evaluated. For instance, Baert et al. (2008) inoculated 10 g of raspberry with 5.75 ± 0.34 log MNV followed by heat treatment. Deboosere et al. (2004) mixed

mashed strawberry with HAV to a final concentration of 10^7 PFU/ml. Glass trial tubes (100 mm long and 0.5 mm thick) were filled with one gram of the total HAV- contaminated mashed strawberry and immersed simultaneously in glycerol bath (Table 2). In this instance, both studies by Baert et al. (2008b) and Deboosere et al. (2004) evaluated fleshy aggregate (i.e. soft-fleshed) fruits such as berries, but the amount of each fruit analyzed and the concentration of virus used differed by approximately 10-fold. This difference in volume could certainly impact the thermal kinetics in the experiment allowing for perceived differences in virus inactivation both for the same virus and between virus types. Moreover, Deboosere et al. (2004) also considered sucrose concentrations and pH in the thermal inactivation of HAV and showed variability with respect to pH.

Another example can be seen for studies involving heat inactivation of viruses in vegetables. Bozkurt et al. (2014b) inoculated 25 g of homogenized spinach with 5 ml of MNV (9.39 log PFU total) (Table 1) followed by heat treatment. However, in the experiment by Laird et al. (2011), 1.9 pieces of onion samples were inoculated with 10 to 20 µl of HAV to give approximately 3 to 5-log PFU per onion sample followed by thermal inactivation in a household dehydrator. Because vegetables are not often subjected to thermal treatment prior to consumption, there are not many peer-reviewed studies that address thermal inactivation of enteric viruses in vegetables. However, the studies by Bozhurt et al. (2014a) and Laird et al. (2011) demonstrate the issues related to the lack of a standardized protocol for the amount of each food type to be used for thermal inactivation studies in food matrices.

For "liquid" food matrices, differences in volumes used were also identified. For instance, Strazynski et al. (2002) mixed 10^5 to 10^6 PFU/ml of PV with milk and yogurt followed by heat treatment, and Hewitt et al. (2009) heat-treated 100 µl of milk inoculated with MNV and HAV at

final concentrations of 3×10^4 and 5×10^4 PFU/ml respectively (Table 2). Here, the difficulties in comparing the results from these studies lies in the omission of the volume of each food matrix that was utilized by Strazynski et al. (2002). Therefore, even though similar temperature ranges, virus concentrations, and food groups were evaluated, the volume of the food matrix might have influenced the results observed in all of the experiments (Table 2).

Additional important factors in food matrix-related thermal inactivation studies include temperature and time points selected for analysis. In general, temperatures around 50 to 75°C for short time periods are chosen in these experiments (Strazynski et al., 2002; Baert et al., 2008b; Hewitt et al., 2009; Barnaud et al., 2012). At around 50°C, cell receptor binding sites of the viruses can be destroyed while the capsids themselves are rarely affected, enabling the virus to protect the nucleic acid (Croci et al., 2012). Conversely, at higher temperatures (greater than 60°C), viral capsid protein can rapidly unfold, leading to nucleic acid deterioration and the loss of infectivity (Volkin et al., 1997; Auser et al., 2006). At mild temperatures (between 47.8 and 55.1°C), very little log reduction was observed over a long period of treatment (Laird et al., 2011; Bozhurt et al. 2014a, b). In comparison, with elevated temperatures (> 70°C) used by Hewitt et al. (2009) and Deboosere et al. (2004), greater log reductions were observed over very short treatment times (Table 2). However, clearly temperature influence on log reduction depends on the virus type as well as food group. Croci et al. (1999) observed a >2-log reduction after 3 min of treatment of HAV in mussel homogenate at 80°C whereas Hewitt and Greening (2006) observed 1.5-log reduction after 3 min of heat treatment at 63°C. Also, Slomka and Appleton (1998) and Bozkurt et al., (2014b) studied FCV in mussels at different temperatures and reported different results (Table 2). Although the data reported in all of these studies are crucial to understanding thermal inactivation of viruses, it is obvious that there is a need to define standard temperatures to be

evaluated for specific food commodities and virus types, thus allowing for direct comparison of data as opposed to relying on inappropriate extrapolations and inferences by the research community.

Within the same food types, it is also expected that different viruses will have different thermal inactivation profiles. Deboosere et al. (2004) treated HAV in mashed strawberries at 80, 85 and 90°C and Baert et al. (2008b) studied MNV in strawberries as well at 65 and 75°C. These authors observed different results which may be expected as it has consistently been shown that HAV is more heat stable (Croci et al., 1999; Hewitt and Greening 2006). However, the primary observation here is that because the temperatures evaluated are different, it is still difficult to compare the thermal inactivation profiles of the two different viruses within the same food type. Di-Girolamo et al. (1970) and Hewitt and Greening (2006) studied thermal stability of PV and HAV in bivalve mollusks, respectively. The former recorded a 2-log reduction after 30 min of steaming (93.7°C internal temperature), and the latter observed a 1.5-log reduction after 3 min of steaming (63°C internal temperature). These results cannot be compared because of different temperatures. For virus inactivation in dairy products, Hewitt et al., (2009) heat treated HAV inoculated in milk at 72°C and observed a 1-log reduction after ≤ 0.3 min whereas Strazynski et al. (2002) mixed PV with milk and heat treated at 72°C and reported reductions of 0.56 and >5 logs after 0.25 and 0.5 minutes, respectively. Even though different viruses were evaluated, Hewitt et al. (2009) and Strazynski et al. (2002) used the same temperatures allowing for a much easier comparison of results between virus types. However, according to Duizer et al. (2004a), slight differences in temperature do not really influence log reduction values in viruses analyzed in the same matrix for the same time. This may be confirmed in the results reported by Barnaud et al. (2012) which show no difference in log reduction of Hepatitis E virus at 68 and 71°C (Table 2)

though molecular analysis was utilized to determine log reduction as opposed to an infectivity assay. Overall, this may not necessarily hold true in all scenarios and for all virus types.

As discussed in the *in vitro* section, the different analysis methods (RT-PCR, RT-qPCR, TCID$_{50}$, and plaque assay) are also likely to influence the results obtained from thermal stability experiments in food matrices. As stated earlier, heat treatment may cause damage to the receptor binding site on the surface of the virus with little or no effect on the genome (Pecson et al., 2009; Wang et al., 2012). Therefore, molecular detection assays can still detect the viral genome housed in a noninfectious viral particle. Laird et al. (2011) alluded to the impracticality of using molecular detection assays for determining exact levels of infectious viruses contained in processed foods. Perhaps the recent publication of a culture system for HuNoV may help to alleviate the issues related to molecular detection of HuNoV in foods. With that said, separate studies evaluating the same viruses in the same food groups have yielded different results (controlling for temperature), probably owing to the different methods of analysis. Slomka and Appleton (1998) used RT-PCR to analyze FCV reduction in cockles and reported a 1-log reduction after 0.29 min of boiling (assuming 100°C). On the contrary, when Bozkurt et al. (2014c) analyzed the infectivity of FCV in mussels with conventional plaque assay they observed a 1-log reduction after 0.07 min of treatment at 72°C. Laird et al. (2011) analyzed HAV in onion samples after 20 h of heat treatment at 60.7°C and with plaque assay they observed an overall virus reduction of 2.64-log and with RT-qPCR, no reduction was observed.

IV.   Conclusions

Virus inactivation varies among virus types, matrix, and type of treatment. Also, there are a limited number of matrices and inactivating parameters that have been studied, and hence,

researchers in the field of food and environmental virology may have difficulty in determining the most suitable virus for a particular treatment in a specific matrix. In addition, there are not common heat treatment methods for viruses in every type of matrix (Bertrand et al., 2012).

In this review, we discussed the various methods employed in studying human enteric viruses *in vitro* (cell culture media or buffer) and in various food matrices. Based on the review, we found that, no standard method has been used to study thermal stability. Temperature, time points, type of matrix, analysis type, heat type, and concentration and volume of virus used in the experiments were mostly different across the research studies included in this review. This together with result presentation (i.e. log remaining, log reduction, decimal reduction value, etc.) makes comparison quite challenging. However, we realized that certain temperatures and analysis types are very important. Here, we must again emphasize that formation of a standardized approach to thermal inactivation studies for human enteric viruses does not necessarily eliminate the limitations associated with the use of surrogate viruses as outlined in the introduction.

The following are suggestions based on this review to help researchers move toward a single standard approach for determining thermal inactivation profiles of human enteric viruses and their surrogates:

- Standard temperatures used in food production environments -56, 63, 72 and 100°C- should be evaluated to determine inactivation profiles of established and novel surrogate viruses with the most heat-resistant surrogate considered the best indicator of HuNoV infectivity under the experimental conditions used.

- When possible, plaque assay or $TCID_{50}$ should be used in the analyses of virus reduction. Otherwise, a modified molecular method (Nuanualsuwan and Cliver 2002; Lamhoujeb et al., 2008; Nowak et al., 2011) should be used over conventional methods (Laird et al.,

2012; Barnaud et al., 2012) as enzyme treatment can enable discrimination of infectious from non-infectious viral particles though this should be validated for each virus being studied.

- Starting concentration of viruses should be higher ($\geq 10^5$ PFU/ml) and consistent across studies. Moreover, the virus inoculation procedure should be the same for both *in vitro* and food matrix experiments. Different methods employed by Croci et al. (1999) and Hewitt and Greening (2006) might have influenced the variation in their results.

- To be sure that inactivation is done at a consistent starting temperature, preheating of media or buffer is recommended.

- To efficiently compare results between experiments, the D-value system (first order kinetics) or a best fit model (e.g., Weibull distribution) should be used to report results for the same virus-matrix pair.

- When employed, molecular methods should include a host of controls to guard against false positives and negatives that might be caused by inhibitory compounds which are commonly co-extracted with viruses found in environmental samples such as food and water (Julian and Schwab 2012)

REFERENCES

Al-Kaissi, E. N., & Mostratos, A. (1982). Some reactions of influenza viruses adsorbed topolystyrene for enzyme immunoassay. *Journal of Virological Methods, 4*(6), 353-358.

Ausar, S. F., Foubert, T. R., Hudson, M. H., Vedvick, T. S., & Middaugh, C. R. (2006). Conformational stability and disassembly of Norwalk virus-like particles. Effect of pH and temperature. *The Journal of Biological Chemistry, 281*(28), 19478-19488.

Bae, J., & Schwab, K. J. (2008). Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Applied and Environmental Microbiology, 74*(2), 477-484.

Baert, L., Wobus, C. E., Van Coillie, E., Thackray, L. B., Debevere, J., & Uyttendaele, M. (2008a). Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Applied and Environmental Microbiology, 74*(2), 543-546

Baert, L., Uyttendaele, M., Van Coillie, E., & Debevere, J. (2008b). The reduction of murine norovirus 1, B. fragilis HSP40 infecting phage B40-8 and E. coli after a mild thermal pasteurization process of raspberry puree. *Food Microbiology, 25*(7), 871-874.

Barnaud, E., Rogee, S., Garry, P., Rose, N., & Pavio, N. (2012). Thermal inactivation of infectious hepatitis E virus in experimentally contaminated food. *Applied and Environmental Microbiology, 78*(15), 5153-5159.

Bertrand, I., Schijven, J., Sánchez, G., Wyn-Jones, P., Ottoson, J., Morin, T., et al., (2012). The impact of temperature on the inactivation of enteric viruses in food and water: A review. *Journal of Applied Microbiology, 112*(6), 1059-1074.

Bozkurt, H., D'Souza, D. H., & Davidson, P. M. (2013). Determination of the thermal inactivation kinetics of the human norovirus surrogates, murine norovirus and feline calicivirus. *Journal of Food Protection, 76*(1), 79-84.

Bozkurt, H., D'Souza, D. H., & Davidson, P. M. (2014a). Determination of thermal inactivation kinetics of hepatitis A virus in blue mussel (mytilus edulis) homogenate. *Applied and Environmental Microbiology, 80*(10), 3191-3197

Bozkurt, H., D'souza, D. H., & Davidson, P. M. (2014b). Thermal inactivation of human norovirus surrogates in spinach and measurement of its uncertainty. *Journal of Food Protection, 77*(2), 276-283.

Bozkurt, H., Leiser, S., Davidson, P. M., & D'Souza, D. H. (2014c). Thermal inactivation kinetic modeling of human norovirus surrogates in blue mussel (mytilus edulis) homogenate. *International Journal of Food Microbiology, 172*, 130-136.

Buckow, R., Isbarn, S., Knorr, D., Heinz, V., & Lehmacher, A. (2008). Predictive model for inactivation of feline calicivirus, a norovirus surrogate, by heat and high hydrostatic pressure. *Applied and Environmental Microbiology, 74*(4), 1030-1038.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L., & Vinje, J. (2006). Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus. *Journal of Food Protection, 69*(11), 2761-2765.

Centers for Disease Control and Prevention. (2011). Updated norovirus outbreak management and disease prevention guidelines. *Morbidity and Mortality Weekly Report. Recommendations and Reports,* 60, 1–18.

Croci, L., Ciccozzi, M., De Medici, D., Di Pasquale, S., Fiore, A., Mele, A., & Toti, L. (1999). Inactivation of hepatitis A virus in heat-treated mussels. *Journal of Applied Microbiology, 87*(6), 884-888.

Croci, L., Suffredini, E., Di Pasquale, S., & Cozzi, L. (2012). Detection of Norovirus and feline Calicivirus in spiked molluscs subjected to heat treatments. *Food Control, 25*(1), 17-22.

Cromeans, T., Park, G. W., Costantini, V., Lee, D., Wang, Q., Farkas, T., et al., (2014). Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Applied and Environmental Microbiology,* 80(18), 5734-5751.

Deboosere, N., Legeay, O., Caudrelier, Y., & Lange, M. (2004). Modeling effect of physical and chemical parameters on heat inactivation kinetics of hepatitis A virus in a fruit model system. *International Journal of Food Microbiology, 93*(1), 73-85.

DiGirolamo, R., Liston, J., & Matches, J. R. (1970). Survival of virus in chilled, frozen, and processed oysters. *Applied Microbiology, 20*(1), 58-63.

Doultree, J., Druce, J., Birch, C., Bowden, D., & Marshall, J. (1999). Inactivation of feline calicivirus, a Norwalk virus surrogate. *Journal of Hospital Infection, 41*(1), 51-57.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F., & Koopmans, M. (2004a). Inactivation of caliciviruses. *Applied and Environmental Microbiology, 70*(8), 4538-4543.

Duizer, E., Schwab, K. J., Neill, F. H., Atmar, R. L., Koopmans, M. P., & Estes, M. K. (2004b). Laboratory efforts to cultivate noroviruses. *Journal of General Virology, 85*(1), 79-87.

Escudero-Abarca, B., Rawsthorne, H., Goulter, R., Suh, S., & Jaykus, L. (2014). Molecular methods used to estimate thermal inactivation of a prototype human norovirus: More heat resistant than previously believed? *Food Microbiology, 41*, 91-95.

Estes, M. K., Graham, D. Y., Smith, E. M., & Gerba, C. P. (1979). Rotavirus stability and inactivation. *The Journal of General Virology, 43*(2), 403-409.

Fields, B., Knipe, D., & Howley, P. (2007). Fields virology (5th ed.,). Philadelphia, PA: Lippincott Williams and Wilkins.

Gerba, C. P. (1984). Applied and theoretical aspects of virus adsorption to surfaces. *Advances in Applied Microbiology, 30*, 133-168.

Gibson, K. E., & Schwab, K. J. (2011). Thermal inactivation of human norovirus surrogates. *Food and Environmental Virology, 3*(2), 74-77.

Grausgruber,W., (1963), Investigations of the inactivation of infectious swine paralysis virus in scalded sausages. *Wiener Tierärztliche Monatsschrift* 50:678–685.

Griffin, D. W., Donaldson, K. A., Paul, J. H., & Rose, J. B. (2003). Pathogenic human viruses in coastal waters. *Clinical Microbiology Reviews, 16*(1), 129-143.

Hewitt, J., Rivera-Aban, M., & Greening, G. (2009). Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies. *Journal of Applied Microbiology, 107*(1), 65-71.

Hewitt, J., & Greening, G. E. (2006). Effect of Heat Treatment on Hepatitis A Virus and Norovirus in New Zealand Greenshell Mussels (*Perna canaliculus*) by Quantitative Real-Time Reverse Transcription PCR and Cell Culture. *Journal of Food Protection, 67*(8), 1743-1750.

Hirneisen, K. A., & Kniel, K. E. (2013). Comparing human norovirus surrogates: Murine norovirus and Tulane virus. *Journal of Food Protection, 76*(1), 139-143.

Jones, M. K., Watanabe, M., Zhu, S., Graves, C. L., Keyes, L. R., Grau, K. R. et al., (2014). Enteric bacteria promote human and mouse norovirus infection of B cells. *Science (New York, N.Y.), 346*(6210), 755-759.

Julian, T. R., & Schwab, K. J. (2012). Challenges in environmental detection of human viral pathogens. *Current Opinion in Virology, 2*(1), 78-83.

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., & Virgin, H. W. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science, 299*(5612), 1575-1578.

Knight, A., Li, D., Uyttendaele, M., & Jaykus, L. (2013). A critical review of methods for detecting human noroviruses and predicting their infectivity. *Critical Reviews in Microbiology, 39*(3), 295-309.

Kocwa-Haluch, R. (2001). Waterborne enteroviruses as a hazard for human health. *Polish Journal of Environmental Studies, 10*(6), 485-488.

Koopmans, M., & Duizer, E. (2004). Foodborne viruses: An emerging problem. *International Journal of Food Microbiology, 90*(1), 23-41.

Kroneman, A., Verhoef, L., Harris, J., Vennema, H., Duizer, E., Van Duynhoven, Y., et al., (2008). Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the foodborne viruses in Europe network from 1 July 2001 to 30 June 2006. *Journal of Clinical Microbiology, 46*(9), 2959-2965.

Laird, D. T., Sun, Y., Reineke, K. F., & Carol Shieh, Y. (2011). Effective hepatitis A virus inactivation during low-heat dehydration of contaminated green onions. *Food Microbiology, 28*(5), 998-1002.

Lamhoujeb, S., Fliss, I., Ngazoa, S. E., & Jean, J. (2008). Evaluation of the persistence of infectious human noroviruses on food surfaces by using real-time nucleic acid sequence-based amplification. *Applied and Environmental Microbiology, 74*(11), 3349-3355.

Lopman, B., Gastañaduy, P., Park, G. W., Hall, A. J., Parashar, U. D., & Vinjé, J. (2012). Environmental transmission of norovirus gastroenteritis. *Current Opinion in Virology, 2*(1), 96-102.

Lynch, M., Painter, J., Woodruff, R., & Braden, C. (2006). *Surveillance for foodborne: Disease outbreaks: United states, 1998-2002* US Department of Health and Human Services.

Marks, P., Vipond, I., Regan, F., Wedgwood, K., Fey, R., & Caul, E. (2003). A school outbreak of Norwalk-like virus: Evidence for airborne transmission. *Epidemiology and Infection, 131*(01), 727-736.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., et al., (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases, 5*(5), 607.

Murray, J. P. (1980). Physical chemistry of virus adsorption and degradation on inorganic surfaces: Its relation to waste water treatment.

Nowak, P., Topping, J., Fotheringham, V., Gallimore, C., Gray, J., Iturriza-Gomara, M., & Knight, A. (2011). Measurement of the virolysis of human GII. 4 norovirus in response to disinfectants and sanitisers. *Journal of Virological Methods, 174*(1), 7-11.

Nuanualsuwan, S., & Cliver, D. O. (2002). Pretreatment to avoid positive RT-PCR results with inactivated viruses. *Journal of Virological Methods, 104*(2), 217-225.

O'Mahony, J., O'Donoghue, M., Morgan, J. G., & Hill, C. (2000). Rotavirus survival and stability in foods as determined by an optimised plaque assay procedure. *International Journal of Food Microbiology, 61*(2), 177-185.

Pecson, B. M., Martin, L. V., & Kohn, T. (2009). Quantitative PCR for determining the infectivity of bacteriophage MS2 upon inactivation by heat, UV-B radiation, and singlet oxygen: Advantages and limitations of an enzymatic treatment to reduce false-positive results. *Applied and Environmental Microbiology, 75*(17), 5544-5554.

Richards, G. P. (1999). Limitations of molecular biological techniques for assessing the virological safety of foods. *Journal of Food Protection, 62*(6), 691-697.

Richards, G. P. (2012). Critical review of norovirus surrogates in food safety research: Rationale for considering volunteer studies. *Food and Environmental Virology, 4*(1), 6-13.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M., Roy, S. L et al., (2011). Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases, 17*(1), 7.

Siebenga, J. J., Vennema, H., Zheng, D., Vinjé, J., Lee, B. E., Pang, X., et al., (2009). Norovirus illness is a global problem: Emergence and spread of norovirus GII. 4 variants, 2001–2007. *Journal of Infectious Diseases, 200*(5), 802-812.

Slomka, M., & Appleton, H. (1998). Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish. *Epidemiology and Infection, 121*(02), 401-407.

Straub, T. M., Honer zu Bentrup, K., Orosz-Coghlan, P., Dohnalkova, A., Mayer, B. K., Bartholomew, R. A., et al., (2007). In vitro cell culture infectivity assay for human noroviruses. *Emerging Infectious Diseases, 13*(3), 396-403.

Strazynski, M., Krämer, J., & Becker, B. (2002). Thermal inactivation of poliovirus type 1 in water, milk and yoghurt. *International Journal of Food Microbiology, 74*(1), 73-78.

Teunis, P. F., Moe, C. L., Liu, P., E Miller, S., Lindesmith, L., Baric, R. S., et al., (2008). Norwalk virus: How infectious is it? *Journal of Medical Virology, 80*(8), 1468-1476.

Thurston-Enriquez, J. A., Haas, C. N., Jacangelo, J., Riley, K., & Gerba, C. P. (2003). Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. *Applied and Environmental Microbiology, 69*(1), 577-582.

Tan, M., & Jiang, X. (2005). Norovirus and its histo-blood group antigen receptors: An answer to a historical puzzle. *Trends in Microbiology, 13*(6), 285-293.

Tian, P., Yang, D., Quigley, C., Chou, M., & Jiang, X. (2013). Inactivation of the Tulane virus, a novel surrogate for the human norovirus. *Journal of Food Protection, 76*(4), 712-718.

Topping, J., Schnerr, H., Haines, J., Scott, M., Carter, M., Willcocks, M., et al., (2009). Temperature inactivation of feline calicivirus vaccine strain FCV F-9 in comparison with human noroviruses using an RNA exposure assay and reverse transcribed quantitative real-time polymerase chain reaction—a novel method for predicting virus infectivity. *Journal of Virological Methods, 156*(1), 89-95.

Tuladhar, E., Bouwknegt, M., Zwietering, M., Koopmans, M., & Duizer, E. (2012). Thermal stability of structurally different viruses with proven or potential relevance to food safety. *Journal of Applied Microbiology, 112*(5), 1050-1057.

Volkin, D. B., Burke, C. J., Marfia, K. E., Oswald, C. B., Wolanski, B., & Middaugh, C. R. (1997). Size and conformational stability of the hepatitis A virus used to prepare VAQTA, a highly purified inactivated vaccine. *Journal of Pharmaceutical Sciences, 86*(6), 666-673.

Wang, D., & Tian, P. (2014). Inactivation conditions for human norovirus measured by an in situ capture-qRT-PCR method. *International Journal of Food Microbiology, 172*, 76-82.

Wobus, C. E., Thackray, L. B., & Virgin, H. W. (2006). Murine norovirus: A model system to study norovirus biology and pathogenesis. *Journal of Virology, 80*(11), 5104-5112.

Table 1. Thermal inactivation of virus surrogates *in vitro*

Virus Type	Method	Heat Type	Volume and Concentration	Buffer Matrix	Analysis Type	Results[a]	Reference
RV	Virus were heat treated in 50°C water bath	WH	3.5×10^7 PFU/ml in 0.2 ml	TBS	PA	50°C, 30 min = 2 log	Estes et al. (1979)
RV	Virus was heated in thermal cycler	DH	1.2×10^7 PFU/ml in 500 μl total	DMEM	PA	60°C, 10 min ≥7 log	O'Mahony et al. (2000)
FCV, HAV, PV-1	Viruses diluted in preheated PBS	WH	10^3 PFU/ml	PBS	RT-PCR	D-value at 72°C: • FCV = 7.39 s • HAV = 18.35 s • PV-1 = 5.44 s	Nuanualsuwan and Cliver (2002)
FCV, CaCV	Samples heated followed by transfer to -20°C until analysis	WH	280 μl portions of 2×10^5 to 1×10^6 TCID$_{50}$/ml	DMEM	TCID$_{50}$ Assay (T$_{50}$A)	Time required for 3 log reduction: • 20°C = 1 week • 37°C = 24 h • 56°C = 8 min • 71.3°C = 1 min	Duizer et al. (2004a)
FCV, MNV	Capillary tubes filled with virus, heat sealed and submerged in water	WH	50 μl portions of $5 \times 10^5 - 1 \times 10^6$ PFU/ml	MEM	PA	D-values: • 56°C = 3.473 and 6.715 min for	Cannon et al. (2006)

	bath set at specified temperatures.					MNV and FCV, respectively • 63°C = 0.435 and 0.406 min for MNV and FCV, respectively • 72°C = 0.166 and 0.118 min for MNV and FCV, respectively	
MNV	Microcentrifuge tubes filled with virus and placed in heating block for varying amounts of time	DH	400 µl portions of 10^7 PFU/ml	DMEM	PA, RT-qPCR	PA and RT-qPCR, respectively: • 80°C, 150 s = 6.5 log and no reduction	Baert et al. (2008a)
FCV	Virus added to 0.2 ml tubes containing preheated culture media and heat treated in a thermal cycler	DH	100 µl portions of 10^7 to 10^8 PFU/ml	DMEM	PA	70°C, 1.5 min = 6 log	Buckow et al. (2008)
FCV	Virus diluted in preheated PBS	WH	500 µl portion of 6×10^5 PFU/ml	PBS	Real-time NASBA	72°C, 4min = approx. 6.5 log	Lamhoujeb et. al (2008)

FCV, MNV, HAV	Virus added to PBS preheated to the specified temperature	WH	5 ml of PBS containing 4.2×10^4 to 1.5×10^5 PFU	PBS	PA	D-values: • 37°C =769 and 599 min for MNV and FCV, respectively • 50°C = 106, 50.6, and 385 min for MNV, FCV, and HAV, respectively • 60°C =13.7, 14.1 and 74.6 min for MNV, FCV, and HAV, respectively • 70°C = 3.84 min for HAV	Gibson and Schwab (2011)
Porcine SaV, FCV, MNV	Viruses were incubated in water bath at 56°C, placed immediately on ice, and then held at 4°C until analysis	WH	5.7 log $TCID_{50}$/ml of each virus	PBS + 10% FBS (SaV, FCV), DMEM (MNV)	$T_{50}A$	Log reductions by at 56°C: • 30 min = >4.92 ± 0.16, 2.42 ± 0.67 and 2.38 ± 0.18 log for FCV, MNV and SaV, respectively • 2 h = >3.9 ± 0.43, >4 ± 0.53 and >5.01 ± 0.25 log for FCV, MNV,	Wang et al. (2012)

						and SaV, respectively	
MNV-1, PC-1, PV, AD 5	The viral suspensions were preheated to 30°C; 400ul of DMEM preheated to 69 or 94.5°C was added to achieve temperatures of 56 or 73°C, respectively.	WH	100ul portions: $PV = 6.3 \times 10^8$ TCID$_{50}$/ml, AD5 $= 6.3 \times 10^7$ TCID$_{50}$/ml, PC-1$= 1.3 \times 10^8$ TCID$_{50}$/ml , MNV1$= 1.7 \times 10^7$ TCID$_{50}$/ml,	DMEM	PA, T$_{50}$A	D-values (best fit model): • 56°C: PC-1 (27 min), MNV-1 (4.21 min), PV (18 s), AD5 (9.6 s) • 73°C: MNV-1 (1.06 min), AD5 (24 s), PC-1 (21 s), PV (14.4 s)	Tuladhar et al. (2012)
MNV-1, FCV	Glass capillary tubes (100 µl) filled with virus stock by capillary force, heat-sealed, and immersed in a water bath with a circulator to maintain a constant temperature (±0.1°C).	WH	50 µl portions of MNV-1 $= 4 \times 10^7$ PFU/ml and FCV $= 5.8 \times 10^8$ PFU/ml	DMEM	PA	D-values (first order kinetics) for MNV and FCV, respectively: • 50°C = 34.49 and 20.23 min • 56°C = 3.65 and 6.36 min • 60°C = 0.57 and 0.56 min • 65°C = 0.30 and 0.32 min • 72°C = 0.15 and 0.11 min	Bozkurt et al. (2013)

MNV, TV	Heat treatment was performed in a thermal cycler in 0.2-ml PCR tubes.	DH	200 µl aliquots of 6 $\log_{10}$ PFU/ml	DMEM and M199	PA	Log PFU/ml reduction after 2 min for MNV and TV, respectively: • $50°C = 0.81 \pm 0.68$ and 1.79 ± 0.42 • $55°C = 1.69 \pm 0.26$ and 1.83 ± 0.29 • $60°C = 3.11 \pm 0.78$ and 2.90 ± 1.16 • $65°C = 3.55 \pm 0.63$ and 3.07 ± 1.12 • $70°C$ and $75°C =$ LOD	Hirneisen et al. (2013)
TV	Virus in micro-centrifuge tubes were incubated in heat blocks at select temperatures	DH	150 µl of virus stock; 9.6×10^7 $TCID_{50}$ total based on back calculations	M199	$T_{50}A$	D-values: • $56°C = 11.8$ min • $63°C = 2.6$ min • $72°C = 4.3$ min	Tian et al., (2013)
TV, AiV, FCV, MNV, PEC	Virus in PBS and incubated at $56°C$ in a digital dry bath	DH	Total volume of 1 ml; 10^7 to 10^9 PFU/ml for TV, AiV, FCV, and MNV; PEC $= 3.8 \times 10^5$ $TCID_{50}$/ml	PBS	PA, $T_{50}A$	Log reductions at $56°C$ for 20 min: TV = 5, FCV = 5, MNV = 5, PEC = 2, AiV = 4 D-value(estimated)	Cromeans et al. (2014)

						- 4 min for TV,FCV, MNV - 10 min for PEC - 5 min for AiV	
SMV	Virus suspensions in 0.2 ml microcentrifuge tubes heated in thermal cycler followed by propidium monoazide (PMA) or RNase treatment.	DH	50ul virus suspension	PBS	RT-qPCR	D-values (PMA and RNase, respectively): - $77°C = 25.6 \pm 2.8$ and 16.4 ± 0.4 min - $80°C = 3.1 \pm 0.1$ and 3.9 ± 0.2 min - $82°C = 0.7 \pm 0.04$ and 0.9 ± 0.3 min - $85°C = 0.2 \pm 0.07$ and 0.12 ± 0.0 min	Escudero-Abarca et al. (2014)
TV	Virus incubated in heat blocks for 2 minutes.	DH	300 μl portions of 2.57×10^5 $TCID_{50}$/ml	M199/E BSS	ISC-RT-qPCR,T_{50}A	% reduction by ISC-RT-qPCR: - $72°C = 100\%$ - $56°C = 97.6\%$ % reduction by T_{50}A: - $72°C = 100\%$ - $56°C = 84.2\%$	Wang et al. (2014)

WH = Wet heat; DH = Dry heat; $TCID_{50}$ Assay (T_{50}A); Plaque Assay (PA);

RV = rotavirus; FCV = Feline calicivirus; MNV = Murine norovirus; MNV-1 = murine norovirus type 1; SMV = Snow-mountain virus; HAV = Hepatitis A virus; CaCV = Canine calicivirus; TV = Tulane virus; SaV = Sapovirus; PV = Polio virus; AiV = Aichi virus; PEC = porcine enteric calicivirus; PC-1 = Parechovirus 1, PV-1 = poliovirus Sabin 1, AD5 = adenovirus type 5

DMEM=Dulbecco's Modified Eagle's Medium; MEM=Minimum Essential Medium; PBS=Phosphate buffered saline, TBS=Tris-buffered saline

RT-qPCR = reverse transcription, quantitative PCR; ISC = *in situ* capture; LOD = limit of detection

[a] All results expressed in $\log_{10}$ represent log reduction, unless otherwise stated.

Table 2. Thermal inactivation of virus surrogates in food matrices

Virus Type	Method	Heat Type	Volume and Concentration	Food Matrix	Analysis Type	Results[a]	Reference
PV	Oysters placed in contaminated sea water for 48 h, moved to metal pans and held under flowing steam for 30 min; cooled to 25°C prior to assay	WH	10^5 PFU/ml; 3.5 L seawater	Oysters	PA	• Steaming, 30 min = 2 log	Di Girolamo et al. (1970)
FCV	Virus contaminated cockles immersed in boiling water for up to 3 min	WH	1×10^{11} TCID$_{50}$/20 L	Cockles	RT-PCR	• 0.5 min boiling = 1.7 log • > 1 min = LOD	Slomka and Appleton (1998)
HAV	Virus suspension and mussel homogenates immersed in water bath at various temperatures and then cooled at -20°C	WH	4×10^5 TCID$_{50}$ total in 225 ml of mussel homogenate	Mussel	T$_{50}$A	• 60 °C, 10 min = >2 log • 80 °C, 3 min = >2 log	Croci et al. (1999)
PV-1	Viruses were mixed with milk or yoghurt followed by heat treatment	WH	10^5–10^6 PFU/ml	Milk, yoghurt, water	PA	Milk: • 55°C = ≥ 5 (30 min) • 62°C = ≥ 5 (30 min)	Strazynski et al. (2002)

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	at various temperatures.					• 72°C = 0.56 ± 0.30 (15 s);≥ 5 (30 s) • 95°C = ≥ 5 (15 and 30 s) Yoghurt: • 42°C = 0.30 ± 0.25 (30 min); 0.41 ± 0.23 (3 h) • 55°C = ≥ 5 (30 min)	
HAV	Glass trial tubes, 100 mm long and 0.5 mm thick (Fisher Bioblock Scientific) containing virus in synthetic media were simultaneously immersed in a glycerol bath at various temperatures for selected time points	WH	10^7PFU/ml in 1g	Mashed strawberries	PA	D-values at 80, 85, and 90°C, respectively, with varying sucrose concentrations: • 28°Brix = 1.22, 0.96, and 0.32 min • 52°Brix = 8.94, 4.98, and 3.00 min	Deboosere et al. (2004)
HAV	Mussels injected at gut region with virus, placed at 4°C for up to 1 h,	WH	50µl virus at 5.0×10^5 $TCID_{50}$/10µl	Mussels	RT-qPCR, $T_{50}A$	Log reduction based on $T_{50}A$:	Hewitt and Greening (2006)

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	then subjected to steaming, boiling, or a combination					• 63°C, steam (3 min) = 1.5 log • 92°C, boiling (37s) + 63°C, steam (3 min) = 2.1 log • 92°C, boiling (180s) = 3.5	
MNV-1	Virus stock inoculated in pre-heated raspberry puree in stomacher bag and held in water bath	WH	5.75 ± 0.34 log PFU/ml in 10g	Raspberry	PA	65°C, 0.5 min = 1.86 ± 0.32 log 75°C, 0.25 min = 2.81 ± 0.39 log	Baert et al. (2008b)
HAV, MNV	Milk was seeded with viruses and heated for various time points in thermal cycler	DH	10^4 PFU/ml in 100 µl	Milk	PA, $T_{50}A$	D-values in milk: MNV: 63°C = 0.7 min 72°C = 0.5 min HAV: 63°C = 1.1 min 72°C ≤ 0.3 min	Hewitt et al. (2009)
HAV	Onion samples inoculated with virus and allowed to air dry for 30 min. Virus inoculated onion	DH	10-20 µl (3-5 logs PFU) virus per onion sample	Onions	PA, RT-qPCR	Reduction after 20 h by: PA assay • 47.8°C = 1 log • 55.1°C = 2 log	Laird et al. (2011)

	samples were then placed in a household dehydrator for 20 h.					• 62.4°C = 3 log RT-qPCR • 47.8, 55.1, and 62.4°C = no reduction	
HEV	Virus-contaminated liver pâté heated at various temperatures for selected time points.	WH	2.24×10^7 copies per g in 25g	Liver pâté	RT-qPCR	Reduction after 5, 10, and 20 min treatment, respectively: • 71°C = 4.4, 4.8, and 4.7 • 68°C = 5.1, 5.1, and 5.0 Reduction after 5, 20, and 120 min treatment, respectively: • 62°C = 6.2, 5.5, and 5.2	Barnaud et al. (2012)
HAV	Homogenized mussels inoculated with virus were placed in 2 ml glass vials and held in water bath.	WH	5ml of 7.04 ± 1.34 log PFU/ml in 25g	Mussels	PA	D-values (first order kinetics): • 50°C = 54.17 min • 56°C = 9.32 min • 60°C = 3.25 min • 65°C = 2.16min • 72°C = 1.07 min	Bozkurt et al. (2014a)

MNV-1, FCV	Homogenized frozen, chopped spinach were inoculated with virus and then portions were placed in 2 ml glass vials and held in water bath.	WH	5ml of each virus (FCV at 8.19 ± 0.97 log PFU/ml; MNV-1 at 7.40 ± 1.12 log PFU/ml) in 25g	Frozen, chopped spinach	PA	D-values (first order kinetics) for MNV-1 and FCV, respectively: • 50°C = 14.57 and 17.39 min • 56°C = 3.29 and 5.83 min • 60°C = 0.98 and 0.78 min • 65°C = 0.40 and 0.27 min • 72°C = 0.16 and 0.15 min	Bozkurt et al. (2014b)
FCV, MNV-1	Virus-contaminated mussels heated at various temperatures for selected time points.	WH	5ml of each virus (FCV at 8.06 ± 1.24 log; MNV-1 at 7.14 ± 1.12 log PFU/ml) in 25g	Mussels	PA	D-values (first order kinetics) for MNV-1 and FCV, respectively: • 50°C = 20.19 and 5.20 min • 56°C = 6.12 and 3.33 min • 60°C = 2.64 and 0.77 min • 65°C = 0.41 and 0.33 min • 72°C = 0.18 and 0.07 min	Bozkurt et al. (2014c)

WH = Wet heat; DH = Dry heat; $TCID_{50}$ Assay ($T_{50}A$); Plaque Assay (PA);

FCV = Feline calicivirus; MNV = Murine norovirus; MNV-1 = murine norovirus type 1; HAV = Hepatitis A virus; HEV = Hepatitis E

virus; PV = Polio virus; PV-1 = poliovirus Sabin 1

RT-qPCR = reverse transcription, quantitative PCR; LOD = limit of detection

[a] All results expressed in $\log_{10}$ represent log reduction, unless otherwise stated.

CHAPTER 3: PHYSICOCHEMICAL STABILITY PROFILE OF TULANE VIRUS —A HUMAN NOROVIRUS SURROGATE

Abstract

Human norovirus (HuNoV) is estimated to cause 19 to 21 million illnesses each year in the U.S. A major limitation in HuNoV research is the lack of an *in vitro* culture system; therefore, surrogate viruses including murine norovirus (MNV) and feline calicivirus (FCV) are used to study HuNoV. Here we aim to establish the physiochemical properties of Tulane virus (TV)—a newer HuNoV surrogate. For thermal inactivation, TV was exposed to 37°C for 2h, and 56, 63 and 72°C for 30 min. For ethanol tolerance, TV was treated with 60, 70 and 90% ethanol at room temperature (RT) for 5 min. Tulane virus pH stability at pH 2, 3, 7, 9 and 10 was performed at RT for 90 min. At 37°C, there was no significant reduction of TV after 2 h. However, at 56, 63 and 72°C, D-values of 1.71, 1.29 and 1.25 min, were calculated, respectively. The D-values obtained for TV ethanol tolerance were 1.78, 2.42 and 0.31 min at 60, 70 and 90%, respectively. Less than 1 $\log_{10}$ reduction was observed for TV at all pH levels except pH 10 where about a 2-$\log_{10}$ reduction was observed. Tulane virus was also tolerant to chlorine disinfection on a solid surface with D-values of 15.82 and 5.42 min at 200 and 1000 ppm, respectively. Tulane virus is likely a suitable surrogate to study HuNoV thermal stability as well as ethanol tolerance below 90%. Tulane virus also is a promising surrogate to study HuNoV pH stability and chlorine tolerance on solid surfaces. Based on current work, *in vitro* studies demonstrate that TV is overall a conservative and suitable surrogate for the study of HuNoV physicochemical properties.

Keywords *Norovirus, surrogates, thermal inactivation, Tulane virus, disinfectants*

I.   Introduction

Human noroviruses (HuNoV) belong to the family *Caliciviridae*. Classified into the genus *Norovirus*, HuNoVs are a group of nonenveloped, single-stranded RNA viruses. In the United States, it is estimated that HuNoV accounts for 19 to 21 million gastrointestinal illnesses each year and 58% (5.5 million) of foodborne illnesses due to major pathogens (Scallan et al., 2011). Also in the European Union, 2 million cases of foodborne viral gastroenteritis caused by HuNoV are reported annually (Phillips et al., 2010); although, the actual incidence is likely much higher. The virus is mainly transmitted by person-to-person and by ingestion of contaminated food and water (Becker et al., 2000; Koopmans and Duzier 2004).

A key limitation to HuNoV research has been the inability to culture the virus *in vitro* and the absence of a small animal model to study its pathogenesis. Although a HuNoV cell culture system was recently developed utilizing human B cells in the presence of free histo-blood group antigens (HBGA) or HBGA-expressing bacteria (Jones et al., 2014), nothing has been subsequently published to show reproducibility and application. Therefore, the use of surrogate viruses to study HuNoV is still important. Murine norovirus (MNV) and feline calicivirus (FCV) have been widely used as surrogates for the study of HuNoV (Richards 2012). Human norovirus and FCV are in the same family, but they differ in some biochemical properties (Cannon et al., 2006). Even though MNV is more similar to HuNoV (Cannon et al., 2006), symptoms characteristic of HuNoV gastroenteritis in humans are different from MNV infection in mice (Karst et al., 2003). In addition, MNV uses sialic acid as the functional receptor (Wobus et al., 2006), whereas human noroviruses presumably use HBGAs (Tan and Jiang 2005). Based on these limitations associated with MNV and FCV, researchers are currently studying alternative surrogates such as Tulane virus (TV)

Farkas et al. (2008) first isolated and characterized TV from the stools of *Macaca mulatta,* juvenile rhesus macaques captured in the Tulane National Primate Research Center. Tulane virus and HuNoV have similar characteristics including genetic identity as they are both caliciviruses and also recognize HBGA receptors which make TV an appropriate surrogate for HuNoV studies (Farkas et al., 2010). Additionally, TV is robustly replicable in cell culture – an ideal characteristic for a suitable surrogate for HuNoV studies *in vitro* (Li et al., 2012)—adapting successfully to monkey kidney cells (LLC-MK2 cells).

A recent study by Cromeans et al. (2014) evaluated the difference in physicochemical properties of TV, MNV, FCV, porcine enteric calicivirus, and Aichi virus. However, aside from the studies by Cromeans et al. (2014), only three other studies have been published on the inactivation of TV using infectivity assays (Tian et al., 2013; Hirneisen and Kniel 2013; Wang et al., 2014), and these applied various methodologies for evaluation of these physicochemical properties. Therefore, the objective of the present research was to determine and verify the physicochemical stability profiles of TV and compare them to previously published profiles for MNV and FCV. The overall aim of this research is to provide more supporting information to determine the most suitable surrogates for the study of HuNoV.

II.   Materials and Methods

i.   Virus propagation

Tulane virus was kindly provided by Dr. Jason Jiang (Cincinnati Children's Hospital Medical Center, Cincinnati, OH) and was propagated in LLC-MK2 cells (provided by Dr. Kalmia Kniel at the University of Delaware, Newark, DE) as previously described by Cromeans et al. (2014) with slight modification. Briefly, LLC-MK2 cells were grown in medium 199 (M199)

(Hyclone, Logan, UT) supplemented with 1% 100× penicillin–streptomycin (Cellgro, Mediatech Inc., Corning, NY), 1% Amphotericin B (Corning, Mediatech Inc., Manassas, VA) and 10% fetal bovine serum (FBS) (Hyclon Logan, UT). For plaque assay, 6-well plates seeded with $8×10^5$ LLC-MK2 cells per well were incubated for 24 h at 37°C under 5% CO_2. Serial dilution of virus stock was prepared in Opti-MEM (Gibco Life Technologies, Grand Island, NY) + 2% FBS. Confluent cells in 6-well plates were inoculated with 100 µL of virus dilution per well and incubated at 37°C under 5% CO_2 with gentle rocking for 1 h. Following incubation, 2 mL agarose overlay containing Opti-MEM + 2% FBS and 1.5% low-melting agarose (Lonza, Rockland, ME) in the ratio 1:1 was added to each well, followed by incubation at 37°C under 5% CO_2 for 72-96 h. To visualize virus plaques, cells were subsequently stained with 2 mL of 0.1% neutral red (Sigma, St. Louis, MO) in 1×PBS per well and plaques were counted after 3 to 5 h. Tulane virus stock concentrations ranged from 10^5 to 10^6 PFU/mL.

ii. Thermal Inactivation Assay

For thermal stability experiments, 900µL of 1×PBS (pH 7.4) in 1.5 mL microcentrifuge tubes was preheated in the respective water bath for approximately 15 min to achieve a temperature of 37, 56, 63, and 72°C. One hundred microliters of virus stocks were added to the preheated 1×PBS in the microcentrifuge tubes to reach a final concentration of 10^5 PFU/mL for TV. Tubes were vortexed briefly, immersed in a water bath, and held at 37°C for up to 2 h and at 56, 63, and 72°C for up to 30 min. At each designated time point, tubes were immediately transferred to ice, followed by 10-fold sample dilutions in Opti-MEM + 2% FBS. The plaque assays were performed as described for the determination of virus stock concentrations. Positive controls included virus stocks diluted in 1× PBS and incubated at room temperature as well as virus stock diluted in Opti-MEM + 2% FBS, and negative controls included 1× PBS and Opti-MEM + 2% FBS without virus.

These controls were included for each inactivation trial. At least 4 experimental replicates were performed, and all samples were analyzed in duplicate.

iii.   Ethanol Inactivation Assay

Ethanol inactivation studies were performed at 60, 70, and 90% concentrations for up to 10 min at room temperature (RT) in 1.5-mL microcentrifuge tubes with 10^4 total PFU. For virus treatment at 60%, 300 µL of 1× PBS was mixed with 100 µL of virus and then added to 600 µL lab grade (95-100%) ethanol. At 70%, 200 µL of 1× PBS was mixed with 100 µL of virus and then diluted in 700 µL lab grade ethanol. At 90%, 100 µL of the virus was diluted in 900 µL lab grade ethanol. At each designated time point, serial dilutions were immediately prepared in Opti-MEM + 2% FBS to dilute the ethanol. The plaque assays were performed as described for the determination of virus stock concentrations. As an experimental control, sterile distilled water inoculated with virus was used in place of ethanol for each experiment. In addition, virus stocks diluted in only 1× PBS were used as a positive control, Opti-MEM + 2% FBS and 1× PBS without viruses were used as negative controls. At least three independent experiments were performed in duplicates.

iv.   pH stability assay

Virus stocks were diluted to 10^5 PFU/mL in M199 adjusted with 0.5 M NaOH and 0.5 M HCl to a pH of 2, 3, 7, 9, or 10 followed by incubation at RT for various time points up to 90 min. At each designated time point, serial dilutions were immediately prepared in Opti-MEM + 2% FBS to stop the inactivation. Virus stability profile in M199 at pH 7 was used as the positive control. Virus stock diluted in 1× PBS was used as an additional positive control. Uninoculated

M199 was used as a negative control. The plaque assays were performed as described for the determination of virus stock concentrations. Three experimental replicates were performed, and all samples were analyzed in duplicate.

v. Chemical disinfection assay

Chemical disinfection assay was performed as described previously by Cromeans et al. (2014) with modification. Briefly, 50 µL of TV stock were pipetted on to 3-in^2 (7.6-cm^2) 100% acrylic-based, nonporous solid surface samples (13-mm-thick Wilsonart laminate; Wilsonart International, Inc., Temple, TX) and allowed to dry for 30 min. After drying, 150 µL of commercial bleach (Arctic White™ Bleach; KIK Custom Products, Bentonville, AR) was added at concentrations of 200 and 1000 ppm and incubated for up to 10 min at RT. At each time point reached, the free chlorine activity was quenched by the addition of 450 µL of 0.2 M sodium thiosulfate (Sigma-Aldrich, St. Louis, MO). Viruses were then recovered from the solid surface using a cell scraper, and the solution containing viruses was pipetted into a microcentrifuge tube, followed by serial dilutions in Opti-MEM + 2% FBS. The plaque assays were performed as described for the determination of virus stock concentrations. Positive control was set up with lab-grade sterile Milli Q water (Millipore, Billerica, MA) used in the place of bleach. Dilution of virus stock served as a control for assay performance. Uninoculated Opti-MEM + 2% FBS was used as a negative control. Three independent experiments were performed, with 6 replicates total (n=6).

vi. Statistical analysis

Decimal reduction values (*D*-value) – the time required to achieve a 1-log$_{10}$ reduction in infectious virus titer – were determined for both the thermal and ethanol inactivation experiments.

This was obtained by the plotting linear regression line in Excel 2010 (Microsoft Corporation, Redmond, WA) and finding the negative reciprocal of the slope. The mean $\log_{10}$ remaining virus titer after the entire treatment time of 90 min was used to describe inactivation during pH treatment. Analysis of variance (ANOVA) was used to compare the D-values calculated between treatments. The student t-test was used to compare D-values between pairs of treatment. Student t-tests and ANOVA were applied using JMP® Pro 11 statistical software (SAS, Inc., Cary, NC).

III. Results

i. Thermal Inactivation

Tulane virus thermal stability was evaluated at 37°C (n= 12) at various time points up to 2 h and 56 (n= 4), 63 (n=4), and 72°C (n=6) at various time points up to 30 min.

At 37 °C, TV was very stable with no reduction in virus concentration after 2 h (Figure 1). Conversely, TV was completely inactivated at 30, 10, and 5 min at 56, 63, and 72°C, respectively (Figure 1). Virus reduction at 37°C was significantly different from all other temperatures evaluated ($p< 0.05$). The mean $\log_{10}$ PFU remaining at 56 and 72°C were significantly different at 5 min ($p >0.05$). However, overall, there was no significant difference in virus reduction between 63°C and 56 or 72°C. The D-values obtained by plotting the best fit line for TV were 500, 4.03, 1.18 and 0.24 min for 37, 56, 63 and 72°C, respectively. No plaques were observed for negative controls. On the positive control plates, results were comparable to treatment at time point zero (estimated 5-$\log_{10}$PFU/mL) at all temperatures.

ii. Ethanol Inactivation

Tulane virus was exposed to three different concentrations (60, 70, and 90%) of ethanol at RT for up to 10 min. Virus reduction occurred rapidly in 90% ethanol with total inactivation of infectious virus by 45 s whereas in 60% and 70%, TV remained stable until approximately 1 and

3 min, respectively, when there was a 1-$\log_{10}$ PFU reduction. There was no significant difference in reduction of infectious virus titer at 60 and 70% ethanol ($p > 0.05$); however, there was a significant difference in reduction between 90% and 60 and 70% concentrations ($p < 0.05$) (Table 1). No plaques were observed on negative controls. On the positive control plates, results were comparable to treatment at time point zero (an average of ~3.5-$\log_{10}$PFU/mL) at all ethanol concentration. No cytotoxicity due to ethanol activity was observed, showing effective dilution of the ethanol after each treatment.

iii.   pH stability

The pH stability profile for TV was evaluated by exposing the virus to pH 2, 3, 7, 9 and 10 at RT. Three experimental replicates were performed for each pH value. Tulane virus was fairly stable after 90 min of treatment at all pH levels tested. As shown in Figure 2, less than 1 $\log_{10}$ reduction was achieved at pH 2, 3, and 9 and a 2.25-$\log_{10}$ PFU reduction was observed at pH 10. There was no significant difference in the log reductions reported at pH 2, 3, 9, and 10 ($p > 0.05$) except at 90 min where log reduction observed at pH 10 was significantly different ($p < 0.05$) from all other pH levels. In addition, $\log_{10}$ PFU reduction at pH 7 was also significantly different from all other pH values ($p < 0.05$). Compared to results at time 0 (estimated 4.4-$\log_{10}$PFU/mL) for all pH levels, PFU counts on positive controls indicated that the assay was working optimally. No plaques were observed on negative controls.

iv.   Chemical disinfection

Tulane virus tolerance to commercial bleach on a non-porous surface was evaluated at 200ppm and 1000ppm for up to 10 min at RT. After the 10 min exposure on a solid surface, TV exhibited greater tolerance to 200ppm than 1000ppm with <1-$\log_{10}$ PFU/mL and <2.5-$\log_{10}$

PFU/mL reduction, respectively. Tulane virus log reduction at 200 and 1000ppm was significantly different ($p>0.05$). Table 1 shows the D-values for TV at each concentration of bleach. Virus stock positive controls indicated that the assay was performed and worked correctly. Positive control TV inoculated with lab-grade sterile Milli Q water—replacement for bleach—were comparable to results at time zero (estimated 3-log_{10}PFU/mL). Results for negative controls were as expected with no plaques observed and no cytotoxicity. Based on these results, bleach activity was effectively quenched by sodium thiosulfate volume and concentration.

IV. Discussion

As indicated previously, human norovirus (HuNoV) is the leading cause of viral gastroenteritis related to person-to-person transmission as well as foodborne gastroenteritis, and a major impediment to HuNoV research is the lack of an *in vitro* culture system and a small animal model to study the pathogenesis of HuNoV. Recently, a cell culture system was developed for HuNoV but its reproducibility and application has not yet been confirmed, hence the use of surrogate viruses still remains important to study HuNoV. In this study, we aim to establish the physicochemical differences between well-characterized surrogates (MNV and FCV) and a more recent surrogate—Tulane virus—by comparing our results (and other's results) for TV with those determined for MNV and FCV in previously published papers.

The temperature points for thermal inactivation considered in this study are important in the food production environment. Physiological temperature (37°C) was used as a positive control and as such it was expected that TV would remain stable over long periods of exposure just as most enteric viruses (Fields et al., 2007). In this study, our data demonstrates that TV is very stable at 37°C (D-value of 500 min). A temperature of 56°C represents the temperature used in food

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buffets whereas 63 and 72°C represent common pasteurization temperatures. These temperatures are expected to inactivate a wide range of bacteria and viruses; however, HuNoV are reported to persist at these elevated temperatures longer than the traditional surrogates (Wang and Tian 2014) though these results are based on molecular detection as opposed to infectivity. In the present study, less than 3 $\log_{10}$ reduction was observed for TV after 5 min at 56°C, and no virus was detected after 30 min of treatment. Based on an estimated D-value of 4 min, TV stability at 56°C as reported by Cromeans et al. (2014) was not different from that observed in the present study (D-value = 4.03 min). Additional studies by Wang et al. (2014) and Tian et al. (2013) also report log reduction over time at 56°C for TV, and these values are both lower (i.e. 0.8 - 1.6-$\log_{10}$ PFU reduction in 2 min) and higher (D-value = 11.8 min) than what was observed here.

At temperatures above 56°C (i.e. 63 and 72°C), virus infectivity reduced rapidly after 1 min in the present study. At similar temperatures, Hirneisen and Kniel (2013) observed 2.90 ± 1.16- and 3.07 ± 1.12-$\log_{10}$ PFU reductions in TV after 2 min of treatment at 60 and 65°C, respectively. At 70 and 75°C, reduction in TV observed by the authors was beyond the limit of detection after 2 min (Hirneisen and Kniel, 2013). Tian et al. (2013) recorded D-values of 2.6 and 4.3 min at 63 and 72°C, respectively. Similar to the present study, Cromeans et al. (2014) observed a rapid decline in TV infectivity at 60 and 63°C. Wang et al. (2014) reported 100% reduction in TV (i.e. >5-$\log_{10}$ PFU reduction based on estimate) at 72°C after 2 min. Capsid integrity can possibly explain the dramatic difference in the rate of inactivation at temperatures below 60°C compared to those above 60°C. Croci et al., (2012) stated that at about 50°C, the capsid is not affected and therefore the nucleic acids remain intact, with possibly only the receptor binding sites of the virus being destroyed (Croci et al., 2012). On the other hand, temperatures greater than 60°C induce rapid viral capsid protein unfolding, leading to nucleic acid degradation and hence no

infectivity (Volkin et al., 1997; Auser et al., 2006). Overall, the minor differences in the thermal stability of TV determined in this experiment and that in previously published experiments might be due to differences in the type of heat (dry against wet heat), amount and concentration of virus analyzed, and the buffer used as described by Arthur and Gibson (2015).

As mentioned previously, one of the aims of this study was to compare our results for TV to previous studies evaluating the thermal stability of the so-called traditional HuNoV surrogates—MNV and FCV. In previous studies below 60°C (i.e. 50 to 56°C), MNV and FCV have been reported to be stable (Doultree et al., 1999; Duizer et al., 2004; Cannon et al., 2006; Gibson and Schwab 2011; Wang et al., 2012; Tuladhar et al., 2012; Bozkhurt et al., 2013; Cromeans et al., 2014). In general, when the data from this study as well as previous studies on TV stability at 56°C (Tian et al., 2013; Hirneisen and Kniel 2013; Cromeans et al., 2014) are compared to the results reported for MNV and FCV, it seems that all three surrogates are suitable for the study of HuNoV thermal stability at 56°C. However, at approximately 63°C, studies have reported D-values of less than 1 min for both FCV and MNV (Cannon et al., 2006; Baert et al., 2008; Hewitt et al., 2009; Barnaud et al., 2012; Hirneisen and Kniel 2013). Studies have also shown D-values less than 0.2 min for MNV and FCV at 72°C (Nuanualsuwan and Cliver 2002; Cannon et al., 2006; Bozkhurt et al., 2013; Hewitt et al., 2009). In the present study at 63 and 72°C, we reported D-values of 1.18 and 0.24 min for TV, making it only slightly more stable than the traditional surrogate at these temperatures though probably not significant.

Along with thermal stability, we also evaluated the stability of TV at varying concentrations of ethanol. Generally, alcohol concentrations of 60 to 95% are most often included as the active ingredients in alcohol-based hand sanitizers (WHO 2009), and these concentrations are also regularly used to disinfect fomite surfaces that are possibly contaminated with pathogens

including viruses and bacteria (Cromeans et al., 2014). Stability of TV at 70% ethanol determined in the present study is consistent with the data published by Cromeans et al. (2014) but contrary to data reported by Wang et al. (2014) and Tian et al. (2013). In the study by Wang et al. (2014), complete inactivation of TV exposed to 70% ethanol occurred after 30 seconds while Tian et al. (2013) reported 100% inactivation at 70% ethanol after just 20 seconds. However, this could be a result of the selected sampling times. In the present study, complete reduction was observed by 5 min and 10 min with 60% and 70% ethanol, respectively, and the D-values were 1.46 and 1.93 min, respectively. These data are similar to Cromeans et al. (2014) who reported an estimated D-value of $\leq$ 2 min during treatment with 70% ethanol. An interesting finding in the present study is the ability of 60% ethanol to inactivate TV more effectively than 70% concentrations though again the difference was not statistically significant (p>0.05). This phenomenon was also observed in the data reported for FCV by Cromeans et al. (2014) and Park et al. (2010).

With respect to the traditional surrogates and tolerance to ethanol, published studies have reported that FCV is less sensitive to alcohols than MNV (D'Souza and Su 2010; Park et al., 2010; Wang et al., 2012; Cromeans et al., 2014). Other studies have also shown that MNV is very sensitive to alcohols relative to other non-enveloped viruses such as FCV and HuNoV genogroup II strains (GII.2 and GII.4) (Belliot et al., 2008; Park et al., 2010; Sattar et al., 2011; Tung et al., 2013). Compared to the results in our study and Cromeans et al. (2014), TV is more stable at 60 and 70% ethanol than MNV and FCV (D- values of less than 1min) (Belliot et al., 2008; Wang et al., 2012). This may indicate that TV would be the more conservative choice for evaluating ethanol-based sanitizers at levels below 90%. Moreover, FCV has been reported to be less susceptible to higher ethanol concentration (i.e. 90%) (Gehrke et al., 2004; Park et al., 2010;

Cromeans et al., 2014) and hence, may be a more conservative surrogate to study HuNoV stability at higher concentrations.

In this study, we also evaluated the persistence of TV exposed to varying levels of pH since HuNoV is presumed to be highly stable at very low pH values equivalent to stomach pH (1.5-3.5) (Dolin et al., 1972; Pallansch and Roos 2001; Koopmans and Duizer 2004). This is an important characteristic of all enteric viruses since virions must first survive the acidic environment in the stomach to get to their target intestinal cells (Cannon et al., 2006). Here, TV was very stable at all pH values with less than 1-$\log_{10}$ reduction after 1.5 h at pH 2, 3 and 9. At pH 10, there was an estimate 2- $\log_{10}$ PFU reduction after 1.5 h. These results are similar to those previously published for TV at pH ≥ 2 and <10 (Hirneisen and Kniel 2013; Tian et al., 2013; Cromeans et al., 2014). However, contrary to our results at pH 10, Tian et al. (2013) reported complete reduction of TV after only 10 minutes. Slight differences in pH stability data reported in our study and previously published data are likely to be due to temperature conditions, cell culture media or buffer used, concentration and volume of virus analyzed, and analysis type ($TCID_{50}$ vs. plaque assay). For MNV and FCV, previously published reports indicate that MNV is more stable than FCV at all pH levels (Duizer et al., 2004; Cannon et al., 2006; Hirneisen and Kniel 2013; Li et al., 2013; Cromeans et al., 2014). In comparison to the data in our study as well as others, TV and MNV seem to be the more conservative surrogates to study the stability of HuNoV at all pH levels as opposed to FCV.

Chlorine concentrations of 1000 to 5000ppm are recommended to disinfect a non-food contact surface contaminated with HuNoV (CDC 2011) and 200ppm of chlorine concentration is required to sanitize food contact surfaces (FDA 2013). Therefore, a suitable surrogate to study HuNoV tolerance to chlorine should tolerate up to a concentration of 5000ppm. Here, we evaluated

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TV tolerance to 200 and 1000ppm of chlorine. Less than one log reduction was observed at 200ppm while less than 2.5-$\log_{10}$ PFU reduction was observed at 1000ppm. The D-values calculated in our study taken together with the estimated D-values—16.7 min and 4.2 min at 200 and 1000ppm, respectively—calculated based on Cromeans et al. (2014) shows that TV is very tolerant to chlorine. On the contrary, Hirneisen and Kniel (2013) and Tian et al. (2013) reported that TV is less stable to chlorine treatment; however, these authors performed their virus inactivation in solution as opposed to on a surface as was done in our study and that of Cromeans et al. (2014). In general, viruses are more resistant to disinfectants when on fomite surfaces, owing possibly to the inability to expose the entire virus particle to the disinfectant (Russell et al., 1999; Park et al., 2007). This may account for the high resistance of TV to chlorine in our experiment. Comparing chlorine tolerance of TV to MNV and FCV on surfaces, data from previous experiments (Whitehead and McCue 2010; Park and Sobsey 2011; Cromeans et al., 2014) show that TV and MNV are more tolerant than FCV and hence are the conservative surrogates to study HuNoV tolerance to chlorine.

V. Conclusion

Overall, it is very important to note that the minor differences in environmental stability profiles of the different HuNoV genogroups and the inability of the proposed surrogate viruses to wholly mimic HuNoV makes it difficult to use only a single surrogate virus to study HuNoV (Richards 2012; Hirneisen and Kniel 2013). Although MNV and FCV have been the traditional surrogates, based on data in the present study as well as three previous studies, TV seems to be a good surrogate to study HuNoV thermal stability *in vitro* at temperatures relevant in the food industry (i.e. 56, 63, and 72C°). To study the tolerance of HuNoV at lower ethanol concentrations

(i.e. <90%), the data from this study and previously published data show that TV is also likely the better surrogate. However, FCV may be more useful for HuNoV tolerance studies at 90% ethanol. Furthermore, it was found from the present study and previously published data that TV and MNV will be the most promising viral surrogates to study HuNoV pH tolerance at all pH levels. Finally, this current study together with already existing data shows that TV and MNV are more tolerant to chlorine disinfection on surfaces. Based on the data reported here and studies published elsewhere, it can be concluded that TV is likely a more conservative surrogate virus to study HuNoV physicochemical stability.

REFERENCES

Arthur, S.E. and Gibson, K.E. (2015). Comparison of methods for evaluating the thermal stability of human enteric viruses. *Food and Environmental Virology,* 7, 14-26.

Ausar, S.F., Foubert, T.R., Hudson, M.H., Vedvick, T.S. and Middaugh, C.R. (2006). Conformational stability and disassembly of Norwalk virus-like particles effect of pH and temperature. *The Journal of Biological Chemistry,* 281, 19478-19488.

Baert, L., Wobus, C.E., Van Coillie, E., Thackray, L.B., Debevere, J. and Uyttendaele, M. (2008). Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Applied and Environmental Microbiology,* 74, 543-546.

Barclay, L., Park, G.W., Vega, E., Hall, A., Parashar, U., Vinjé, J. and Lopman, B. (2014). Infection control for norovirus. *Clinical Microbiology and Infection,* 20, 731-740.

Barnaud, E., Rogee, S., Garry, P., Rose, N. and Pavio, N. (2012). Thermal inactivation of infectious hepatitis E virus in experimentally contaminated food. *Applied and Environmental Microbiology,* 78, 5153-5159.

Becker, K.M., Moe, C.L., Southwick, K.L. and MacCormack, J.N. (2000). Transmission of Norwalk virus during a football game. *New England Journal of Medicine,* 343, 1223-1227.

Belliot, G., Lavaux, A., Souihel, D., Agnello, D. and Pothier, P. (2008). Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants. *Applied and Environmental Microbiology,* 74, 3315-3318.

Bozkurt, H., D'Souza, D.H. and Davidson, P.M. (2013). Determination of the thermal inactivation kinetics of the human norovirus surrogates, murine norovirus and feline calicivirus. *Journal of Food Protection,* 76, 79-84.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L. and Vinje, J. (2006). Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus. *Journal of Food Protection,* 69(11), 2761-2765.

Centers for Disease Control and Prevention. (2011). Updated norovirus outbreak management and disease prevention guidelines. *Morbidity and Mortality Weekly Report. Recommendations and Reports,* 60, 1–18.

Croci, L., Suffredini, E., Di Pasquale, S. and Cozzi, L. (2012). Detection of norovirus and feline calicivirus in spiked molluscs subjected to heat treatments. *Food Control,* 25, 17-22.

Cromeans, T., Park, G.W., Costantini, V., Lee, D., Wang, Q., Farkas, T., et al., (2014). Comprehensive comparison of cultivable norovirus surrogates in response to different

inactivation and disinfection treatments. *Applied and Environmental Microbiology,* 80(18), 5734-5751.

Dolin, R., Blacklow, N.R., DuPont, H., Buscho, R.F., Wyatt, R.G., Kasel, J.A., Hornick, R. and Chanock, R.M. (1972). Biological properties of Norwalk agent of acute infectious nonbacterial gastroenteritis. *Experimental Biology and Medicine*, 140, 578-583.

Doultree, J., Druce, J., Birch, C., Bowden, D. and Marshall, J. (1999). Inactivation of feline calicivirus, a Norwalk virus surrogate. *Journal of Hospital Infection,* 41, 51-57.

D'Souza, D.H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C. and Jaykus, L. (2006). Persistence of caliciviruses on environmental surfaces and their transfer to food. *International Journal of Food Microbiology,* 108, 84-91.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F. and Koopmans, M. (2004). Inactivation of caliciviruses. *Applied and Environmental Microbiology,* 70, 4538-4543.

Farkas, T., Sestak, K., Wei, C. and Jiang, X. (2008). Characterization of a rhesus monkey calicivirus representing a new genus of *Caliciviridae*. *Journal of Virology,* 82, 5408-5416.

Farkas, T., Cross, R.W., Hargitt, E., Lerche, N.W., Morrow, A.L. and Sestak, K. (2010). Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses. *Journal of Virology,* 84, 8617-8625.

FDA. 2013. Food Code 2013. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration, College Park, MD.

Fields, B., Knipe, D. and Howley, P. (2007). *Fields virology* (5th ed.). Philadelphia, PA: Lippincott Williams and Wilkins.

Gehrke, C., Steinmann, J. and Goroncy-Bermes, P. (2004). Inactivation of feline calicivirus, a surrogate of norovirus (formerly Norwalk-like viruses), by different types of alcohol in *vitro* and *in vivo*. *Journal of Hospital Infection,* 56, 49-55.

Gibson, K.E. and Schwab, K. J. (2011). Thermal inactivation of human norovirus surrogates. *Food and Environmental Virology,* 3, 74-77.

Hewitt, J., Rivera-Aban, M. and Greening, G. (2009). Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies. *Journal of Applied Microbiology,* 107, 65-71.

Hirneisen, K.A. and Kniel, K.E. (2013). Comparing human norovirus surrogates: Murine norovirus and Tulane virus. *Journal of Food Protection,* 76, 139-143.

Jones, M.K., Watanabe, M., Zhu, S., Graves, C.L., Keyes, L. R., Grau, K.R., et al., (2014). Enteric bacteria promote human and mouse norovirus infection of B cells. *Science,* 346, 755-759.

Karst, S.M., Wobus, C.E., Lay, M., Davidson, J. and Virgin, H.W. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science,* 299, 1575-1578.

Koopmans, M. and Duizer, E. (2004). Foodborne viruses: An emerging problem. *International Journal of Food Microbiology,* 90, 23-41.

Li, J., Predmore, A., Divers, E. and Lou, F. (2012). New interventions against human norovirus: Progress, opportunities, and challenges. *Annual Review of Food Science and Technology,* 3, 331-352.

Nuanualsuwan, S. and Cliver, D.O. (2002). Pretreatment to avoid positive RT-PCR results with inactivated viruses. *Journal of Virological Methods,* 104, 217-225.

Pallansch, M., Roos, R., Fields, B. and Knipe, D. (2001). Enterovirus: Poliovirus, coxsackievirus, echovirus and newer enteroviruses. *Virology.*

Park, G.W., Barclay, L., Macinga, D., Charbonneau, D., Pettigrew, C.A. and Vinjé, J. (2010). Comparative efficacy of seven hand sanitizers against murine norovirus, feline calicivirus, and GII. 4 norovirus. *Jounal of Food Protection,* 73(12), 2232-2238.

Park, G.W. and Sobsey, M.D. (2011). Simultaneous comparison of murine norovirus, feline calicivirus, coliphage MS2, and GII. 4 norovirus to evaluate the efficacy of sodium hypochlorite against human norovirus on a fecally soiled stainless steel surface. *Foodborne Pathogens and Disease,* 8(9), 1005-1010.

Park, G.W., Boston, D.M., Kase, J.A., Sampson, M.N. and Sobsey, M.D. (2007). Evaluation of liquid- and fog-based application of sterilox hypochlorous acid solution for surface inactivation of human norovirus. *Applied and Environmental Microbiology,* 73, 4463-4468.

Phillips, G., Tam, C. C., Conti, S., Rodrigues, L.C., Brown, D., Iturriza-Gomara, M., et al., (2010). Community incidence of norovirus-associated infectious intestinal disease in England: Improved estimates using viral load for norovirus diagnosis. *American Journal of Epidemiology,* 171, 1014-1022.

Richards, G.P. (2012). Critical review of norovirus surrogates in food safety research: Rationale for considering volunteer studies. *Food and Environmental Virology,* 4, 6-13.

Russell, A.D., Hugo, W.B., and Ayliffe, G. A. J. (1999). Disinfection, preservation and sterilization, 3rd ed. Blackwell Science, Osney Mead, Oxford, United Kingdom.

Sattar, S.A., Ali, M., Tetro, J.A., (2011). *In Vivo* Comparison of Two Human Norovirus Surrogates for Testing Ethanol-Based Handrubs: The Mouse Chasing the Cat! *PLoS ONE* 6(2): e17340. doi:10.1371/journal.pone.0017340

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M., Roy, S.L., et al., (2011). Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases,* 17, 7-15.

Tan, M. and Jiang, X. (2005). Norovirus and its histo-blood group antigen receptors: An answer to a historical puzzle. *Trends in Microbiology,* 13(6), 285-293.

Tian, P., Yang, D., Quigley, C., Chou, M., and Jiang, X. (2013). Inactivation of the Tulane virus, a novel surrogate for the human norovirus. *Journal of Food Protection*, 76(4), 712-718.

Tree, J., Adams, M. and Lees, D. (2005). Disinfection of feline calicivirus (a surrogate for norovirus) in wastewaters. *Journal of Applied Microbiology,* 98, 155-162.

Tuladhar, E., Bouwknegt, M., Zwietering, M., Koopmans, M. and Duizer, E. (2012). Thermal stability of structurally different viruses with proven or potential relevance to food safety. *Journal of Applied Microbiology,* 112(5), 1050-1057.

Tung, G., Macinga, D., Arbogast, J. and Jaykus, L. (2013). Efficacy of commonly used disinfectants for inactivation of human noroviruses and their surrogates. *Journal of Food Protection,* 76(7), 1210-1217.

Volkin, D.B., Burke, C.J., Marfia, K.E., Oswald, C.B., Wolanski, B. and Middaugh, C.R. (1997). Size and conformational stability of the hepatitis A virus used to prepare VAQTA, a highly purified inactivated vaccine. *Journal of Pharmaceutical Sciences,* 86(6), 666-673.

Wang, D., Xu, S., Yang, D., Young, G.M., and Tian, P. (2014). New *in situ* capture quantitative (real-time) reverse-transcription PCR method as an alternative approach for determining inactivation of Tulane virus. *Applied and Environmental Microbiology*, 80(7), 2120-2124.

Wang, D. and Tian, P. (2014). Inactivation conditions for human norovirus measured by an in situ capture-qRT-PCR method. *International Journal of Food Microbiology,* 172, 76-82.

Whitehead, K., & McCue, K.A. (2010). Virucidal efficacy of disinfectant actives against feline calicivirus, a surrogate for norovirus, in a short contact time. *American Journal of Infection Control,* 38, 26-30.

Wobus, C.E., Thackray, L.B. and Virgin, H.W. (2006). Murine norovirus: A model system to study norovirus biology and pathogenesis. *Journal of Virology,* 80, 5104-5112.

WHO. 2009. CDC/WHO Hand Hygiene Guidelines crosswalk. *Jt Comm Perspect*, 28, 4–7.

Table 1: D-values for Tulane virus inactivation at various ethanol and chlorine concentrations

D-values (min) by Treatment				
Ethanol (n = 8)			Chlorine (n = 6)	
60%	70%	90%	200 ppm	1,000 ppm
1.46[a]	1.93[a]	0.35[b]	15.82[x]	5.86[y]

[a-b]Different letters indicate significant difference ($p<0.05$) between ethanol concentrations
[x-y]Different letters indicate significant difference ($p<0.05$) between chlorine concentrations

Figure Legends

Figure 1: Tulane virus stability profile at 37, 56, 63 and 72°C over time. Each data point represents the mean $\log_{10}$ remaining at each time point of at least 4 replicates. Error bars represent the standard deviations at each time point. Different letters (a-b) represent significance difference ($p<0.05$) at 5 minutes. Significant difference ($p<0.05$) at 37°C when compared to all other temperatures is represented by *.

Figure 2: Tulane virus stability profile at pH 2, 3, 7, 9 and 10 at RT over time. Each data point represents the mean $\log_{10}$ remaining at each time point of at least 6 replicates. Error bars represent the standard deviations at each time point. Different letters (a-c) represent significant difference at 90 min ($p<0.05$).

Figure 1. Tulane virus stability profile at 37, 56, 63 and 72°C over time.
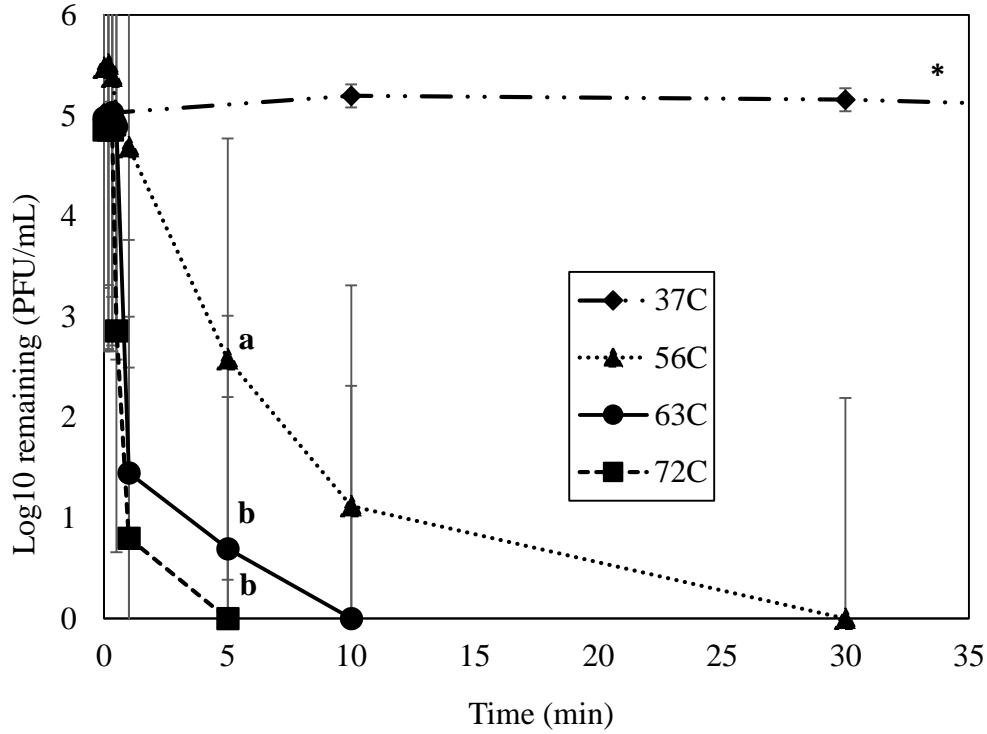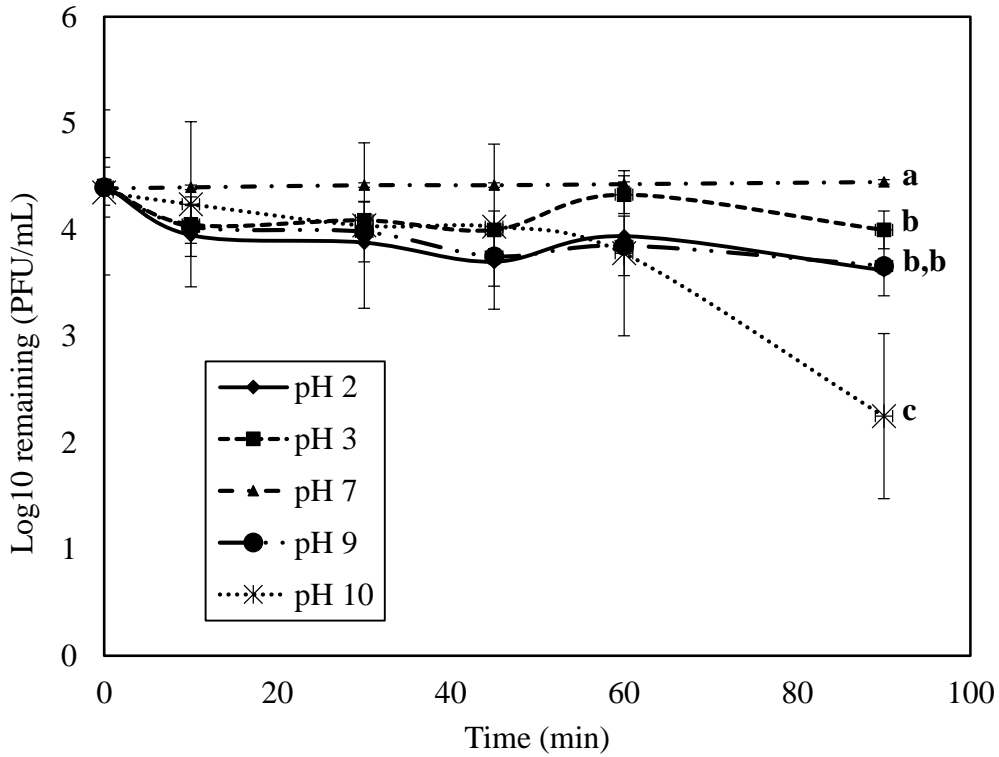

Figure 2. Tulane virus stability profile at pH 2, 3, 7, 9 and 10 at RT over time.

CHAPTER 4: ENVIRONMENTAL PERSISTENCE OF TULANE VIRUS – A SURROGATE FOR THE STUDY OF HUMAN NOROVIRUS

Abstract

In this study, the environmental persistence of a novel HuNoV surrogate, Tulane virus (TV), was evaluated. Survival of TV in surface water (SW) and ground water (GW) was investigated. Each water type was inoculated with TV and incubated at either $4 \pm 2°C$ in the dark (GW) or at room temperature (RT) subjected to diurnal variations (SW). After 28 d, TV remained very stable in SW as well as the tap water control with less than 1-log_{10} reduction in titer. Conversely, TV was completely inactivated in GW by day 21. Virus survival in GW and sterile distilled water control was significantly different ($p < 0.05$) from SW and tap water control. Survival of TV on fomite surfaces over a 14 day period at RT was also evaluated using an acrylic-based solid surface material and stainless steel. Decimal reduction values of 18.5 ± 0.34 and 13.1 ± 0.36 d on solid and stainless steel surfaces were determined, respectively. There was no significant difference in virus persistence on solid and stainless steel surfaces from day 0 to 7, but significant differences ($p < 0.05$) were detected from day 10 to 14. This is the first study to demonstrate persistence of TV in environmental water sources and fomite surfaces. Overall, based on these results TV is a suitable surrogate to study HuNoV persistence in the environment.

I. Introduction

Persistence of human enteric viruses in the environment is assumed to play an important role in transmission of viruses by contaminated water and fomites (Lopman et al., 2012). With respect to contamination of water sources, HuNoVs along with other human enteric viruses (e.g., adenovirus, hepatitis A virus, hepatitis E virus, and rotavirus) have been both detected in a variety of water sources and implicated worldwide in outbreaks linked to contaminated recreational waters, drinking water, and treated and untreated groundwater (Pusch et al., 2005; Wyn-Jones et al., 2011; Aw and Gin 2011; Kishida et al., 2012). Pusch et al. (2005) detected human enteric viruses in 76% of surface water (i.e. river, lake) samples analyzed in Germany while Kishida et al. (2012) reported 44 to 63% prevalence of HuNoV and human adenoviruses in river water samples collected over a one-year period. Based on the high prevalence level of human enteric viruses reported in these studies along with numerous others as reviewed by Gibson (2014), it is important to understand enteric virus persistence in various water sources.

Along with studies on the prevalence of human enteric viruses in environmental waters, numerous outbreaks are reported each year related to water sources contaminated with viral pathogens—most often HuNoV, enteroviruses, and Hepatitis A virus (Gibson 2014). In the U. S., thirty-eight waterborne disease outbreaks (i.e. both drinking water and recreational water) were reported from 2003 to 2010—mostly due to HuNoV (Gibson 2014). In the European Union, 148 waterborne disease outbreaks due to human enteric viruses—42% of outbreaks—were reported from 2000 to 2007 (WHO 2009). Based on these data and sporadic outbreaks reported in the published literature (Ashbolt 2004; Hoebe et al., 2004; Fretz et al., 2005; Werber et al., 2009; Koh et al., 2011), the morbidity due to water contaminated with human enteric viruses is quite significant.

Another important environmental reservoir for HuNoV is fomite surfaces. Various surfaces—both porous and non-porous—can become contaminated with fecal material and droplets from aerosolized vomit containing HuNoV. Various studies have shown that HuNoV can persist on a variety of environmental surfaces for more than seven days (Cheesbrough et al., 1997; Green et al., 1998; Liu et al., 2003; D'Souza et al., 2006; Clay et al., 2006; Lamhoujeb et al., 2009). Persistence of HuNoV on surfaces and fomites often leads to continued transmission of the virus during outbreaks (Donaldson et al., 2008). For example, Widdowson et al. (2004) suggested that HuNoV persistence in the environment was a partial contributing factor to HuNoV outbreaks in cruise ships. Green et al. (1998) reported that HuNoV was detected on surfaces around patients in a hospital ward during a gastroenteritis outbreak. Wu et al. (2005) suggested that environmental surfaces contributed to a HuNoV gastroenteritis outbreak at a long-term care facility in 2003. Based on these examples, it is clear that HuNoV transmission via contaminated surfaces is also a significant concern.

The major limitation in determining the persistence and survival of HuNoV in the environment is the non-cultivability of the virus and the absence of a small animal model to study pathogenesis of HuNoV (Duizer et al., 2004; Straub et al., 2007). In lieu of a cell culture system, environmental persistence of HuNoV has been studied with murine norovirus (MNV) and feline calicivirus (FCV) as surrogates (Bae and Schwab 2008; Cannon et al., 2006; D'Souza et al., 2006; Belliot et al., 2008). However, MNV and FCV have several limitations as surrogates for HuNoV. Though in the same family as HuNoV, FCV has properties that are biochemically different from HuNoV (Cannon et al., 2006). In addition, even though MNV has been shown to be more similar to HuNoV with respect to physicochemical properties (Cannon et al., 2006), clinical signs of gastroenteritis presented by humans infected with HuNoV are different than those presented by

mice infected with MNV (Karst et al., 2003). Depending on the strain of HuNoV, functional receptors may include histo-blood group antigens (HBGA), heparin sulfate, or sialic acid (Tamura et al., 2004; Le Pendu et al., 2006; Estes et al., 2006; Tan and Jiang 2007; Rydell et al., 2009; Taube at al., 2010). Conversely, MNV and FCV recognize only sialic acid as the functional receptor (Wobus et al., 2006; Stuart and Brown 2007; Taube et al., 2009; 2010) and therefore may not represent all the HuNoV strains.

Tulane virus (TV) is a more recently discovered calicivirus and has not been utilized extensively as surrogate for the study of HuNoVs; however, it has similar genetic identity to HuNoV and recognizes HBGA receptors (Farkas et al., 2010). This suggests that TV may be a more suitable surrogate to study the environmental persistence of HuNoV. In the present study, the persistence of TV in ground water (GW) and surface water (SW) as well as on fomite surfaces (acrylic-based solid surface and stainless steel) was evaluated. The main aim of the study is to understand TV persistence in the environment in order to determine its appropriateness as a surrogate to study HuNoV persistence in the environment *in vitro*.

II.   Materials and Methods

i.   Virus propagation

As described in Chapter 3, TV was propagated in LLC-MK2 cells. LLC-MK2 cells were grown in medium 199 (M199) (Hyclone, Logan, UT) supplemented with 1% 100× penicillin–streptomycin (Cellgro, Mediatech Inc., Corning, NY), 1% Amphotericin B (Corning, Mediatech Inc., Manassas, VA) and 10% fetal bovine serum (FBS) (Hyclon Logan, UT). For plaque assay, 6-well plates seeded with 8×10^5 LLC-MK2 cells per well were incubated for 24 h at 37°C under

5% CO_2. Serial dilution of virus stock was prepared in Opti-MEM (Gibco Life Technologies, Grand Island, NY) + 2% FBS. Confluent cells in 6-well plates were inoculated with 100 µL of virus dilution per well and incubated at 37°C under 5% CO_2 with gentle rocking for 1 h. Following incubation, 2 mL agarose overlay containing Opti-MEM + 2% FBS and 1.5% low-melting agarose (Lonza, Rockland, ME) in the ratio 1:1 was added to each well, followed by incubation at 37°C under 5% CO_2 for 72-96 h. To visualize virus plaques, cells were subsequently stained with 2 mL of 0.1% neutral red (Sigma, St. Louis, MO) in 1×PBS per well and plaques were counted after 3 to 5 h. Tulane virus stock concentrations ranged from 10^5 to 10^6 PFU/mL.

ii.   Evaluation of Water Persistence

Ground water and SW were collected from a private well and Beaver Lake drinking water reservoir, respsectively, in Northwest Arkansas in Fall 2014. These waters were analyzed for turbidity and pH using a HydroLab Quanta Multiparameter Water Quality Meter (Hydrolab, Loveland, CO). Based on preliminary testing indicating cell toxicity and microbial contamination of natural (i.e. unsterile and untreated) SW and GW, water was sterilized by autoclaving at 121°C and 15 psi. Similar to Bae and Schwab (2008), 20-mL portions of each water sample were seeded with 100 µL of virus stock containing approximately 10^5 PFU total. Water samples were incubated for 28 days with GW under refrigeration temperature about 4°C ± 2°C (i.e. the low temperature was 2.3°C and the high was 5.9°C with occasional spikes to 10°C) in the dark and SW at room temperature (RT) subjected to ambient light and diurnal variations. At least two experimental replicates were performed with samples analyzed in duplicate. Tap water and Laboratory sterile Milli-Q water (EMD Millipore, Billerica, MA) inoculated with the same amount of viruses as environmental samples was used as positive control and non-inoculated lab sterile Milli-Q water

and culture media served as negative controls. At specific time points, 1.5-mL subsamples were taken and analyzed for TV infectivity by plaque assay as described for the determination of TV stock concentrations.

iii.   Survival on Fomites

Virus survival on fomite surfaces was performed as described by Cannon et al. (2006) with modifications. Briefly, 50 µL of virus stock containing approximately 5×10^4 PFU total was inoculated at the center of 3-in^2 (7.6-cm^2) 100% acrylic-based, nonporous solid surface samples (13-mm-thick Wilsonart laminate; Wilsonart International, Inc., Temple, TX) and stainless steel sheets (type 304/16 gauge, unpolished; Rose Metal Products, Springfield, MO) cut into 4-in^2 (10.16-cm^2) coupons. Fomite surface samples were incubated at RT for up to 14 days under ambient light and relative humidity (RH). At specific time points, TV was eluted from the surface with 450 µL of 1×PBS by scrapping with a cell scraper. The viral suspensions were transferred to microcentrifuge tubes, and TV concentration was determined by plaque assay. Two experimental replicates with duplicate surfaces per sampling time point were completed and samples were also analyzed in duplicate. Virus survival at day 0 was used as the positive control to determine recovery efficiency and virus reduction over time. Fifty microliters of virus stock inoculated into 400 µL 1× PBS and Opti-MEM +2% FBS were used as additional positive controls. Unnioculated 1× PBS and Opti-MEM +2% FBS were used as negative controls. The average recovery efficiency of virus from stainless steel and solid surfaces was 10% and 29.7%, respectively. Throughout the experiment, the RH of the lab was measured with an Acurite Humidity Monitor (Acurite, Lake Geneva, WI).

iv.   Statistical analysis

Decimal reduction values (D-value) – the time required to achieve a 1-$\log_{10}$ reduction in infectious virus titer – were determined. These were obtained by plotting the linear regression line on Excel 2010 (Microsoft Corporation, Redmond, WA) to determine the negative reciprocal of the slope. Analysis of variance (ANOVA) was used to compare the D-values calculated between treatments and samples. The student t-test was used to compare D-values between pairs of treatments. Student t-tests and ANOVA were applied using Excel 2010.

III.   Results

i.   Persistence in Water

Prior to evaluation of TV persistence in various water sources, the pH of the SW and GW was measured as 6.9 and 8.4 and turbidity of 21.4 and 6.7 NTU was recorded, respectively. After 28 d incubation, TV remained very stable in SW as well as the tap water control with less than 1-$\log_{10}$ reduction in titer (Table 1). Conversely, TV was completely inactivated in GW by day 21. Virus survival in GW was significantly different ($p < 0.05$) from SW. Tulane virus was not as stable in lab sterile Milli-Q water control with approximately 2.1-$\log_{10}$ reduction after 28 d (Table 1). No virus plaques were observed on the non-inoculated sterile Milli-Q water controls as well as the culture media negative control. Positive controls using only TV stock resulted in the expected number of PFU.

ii. Survival on Fomite Surfaces

Tulane virus survival on fomite surfaces was evaluated on a 100% acrylic, solid surface and stainless steel under RT for 14 d. Tulane virus remained stable on both surfaces with about 1-$\log_{10}$ reduction in infectious titer at the end of the 14 d period (Figure 1). Decimal reduction values of 18.5 ± 0.34 and 13.1 ± 0.36 d were determined for solid and stainless steel surfaces, respectively. There were no significant differences in TV persistence between the two surface types ($p > 0.05$) from day 0 to 7; however, at day 10 and 14, there were significant differences between the two surfaces ($p < 0.05$). All positive controls were comparable with an estimated 4.3-$\log_{10}$ PFU/mL. There were no plaques observed on negative controls. The relative humidity (RH) over the period of the study ranged from 16% and 22.2% for all experiments.

IV. Discussion

As stated earlier, HuNoV is implicated in the majority of acute gastroenteritis outbreaks reported worldwide. The persistence of infectious virus in the environment is important to its transmission. Human noroviruses have been shown to persist in the environment for prolonged periods. For example, Ngazoa et al. (2008) reported that at 4 and 25°C HuNoV can be detected in mineral water for up to 100 days. Charles et al. (2009) showed that at 12°C HuNoV can persist in groundwater for 728 days. Bae and Schwab (2008) also calculated the nucleic acid reduction rate for Norwalk virus (GI.1, prototype strain of HuNoV) at 0.08 ± 0.02 $\log_{10}$/day and 0.01 ± 0.05 $\log_{10}$/day in SW and GW, respectively, at 25°C. Additionally, Seitz et al. (2011) detected Norwalk virus RNA within intact capsids in GW after 1,266 days with only a 1.10-$\log_{10}$ reduction. However, it is important to note that all of these analyses were based on molecular detection methods and do

not necessarily provide information about infectivity of the virus. The inability to determine infectivity is the primary reason HuNoV surrogates are also used in environmental persistence studies. In the present study, TV persistence in the environment was evaluated. This is the first study to establish TV persistence and survival in various water sources (GW and SW) as well as on fomite surfaces. These data provide much needed information on the persistence of TV in these different matrices.

First, the survival of TV in SW and GW was evaluated. In the present study, TV remained infectious for at least 28 days in SW incubated at RT under ambient light and diurnal variations. On the contrary, TV infectivity was reduced by >3-$\log_{10}$ in GW stored at 4°C ± 2°C in the dark, and by day 21, there was a complete loss of virus infectivity. The only other published study on TV persistence in water involved the evaluation of tap water at 4°C. This study by Hirneisen and Kniel (2013) reported a decline in TV infectivity after 15 days, and by day 30, a complete loss of infectivity was observed. The authors concluded that TV is less stable at 4°C with a D-value of 1.78 ± 0.21 days. These results are consistent with the present study showing that TV is less stable at 4°C; however, it is not possible to compare the results since Hirneisen and Kniel (2013) evaluated TV in tap water as opposed to GW evaluated in the present study. Thus it is unclear whether just temperature impacts TV survival or whether the type of water and inherent water quality properties (pH, chemical composition, etc.) play a more important role. Aside of survival of TV in tap water, the present study is the first to characterize TV survival in GW and SW. In comparison to published studies on survival of MNV and FCV, the present study indicates that TV is more stable at RT in SW and less stable at 4°C in GW than FCV and MNV. For example, Bae and Schwab (2008) reported MNV and FCV survival in SW at RT with estimated D-values of 6.25 and 2.5 days, respectively. The authors also reported estimated D-values of 5.3 and 16.7 days for

FCV survival in SW and GW at 4°C, respectively. However, MNV survival at 4°C was not reported (Bae and Schwab 2008). Hirneisen and Kniel (2013) reported that MNV was reduced by an estimated 4-log after 25 days with complete loss of infectivity by day 30 in tap water at RT. Conversely, at 4°C MNV remained stable with an estimated 1.5-log reduction in tap water by day 30 (D-value of 19.04 ± 5.70 days). Allwood et al. (2003) reported a D-value of 5.7 days and 7.3 days for FCV in dechlorinated water at 25°C and 4°C, respectively. Based on these studies, it is likely that temperature does have an effect on virus survival in water. However, various studies have stated that several factors in water including less predatory activities, absence of ultraviolet irradiation from ambient light (conditions typical to ground water) as well as low temperatures may increase the survival of viruses (Madson et al., 1991; Pedley et al., 2006). Moreover, it has been reported numerous times that low temperatures (i.e. refrigeration) support virus survival better when compared to high temperatures at or above RT (Allwood et al., 2003; Bae and Schwab 2008; Ngazoa et al., 2008; Charles et al., 2009; Hirneisen and Kniel 2013). However, this claim is contrary to the observations in the present study where lower persistence and higher persistence of infectious TV in GW at 4°C and in SW at RT were reported, respectively.

As indicated previously, fomite surfaces serve an important role in transmission of HuNoV. Several factors (e.g., temperature, RH, and surface type) may influence the differences in viral persistence on various surfaces (Sattar et al., 1986; Mbithi et al., 1991; Abad et al., 1994). In the present study, TV persistence on fomite surfaces—acrylic-based solid surface and stainless steel— was evaluated at RT for up to 14 days. Statistical analysis showed that the difference in $\log_{10}$ reduction from day 0 to day 7 between the two surfaces was not significant but significantly different at day 10 and day 14. The difference in survivability at day 10 and day 14, as well as D-values may be due to the recovery efficiency—10 and 29.7% for solid and stainless steel surfaces,

respectively. Although the surfaces used in this study were of similar texture, different surface types (i.e. porous and non-porous) have been shown to impact virus survivability (Sattar et al., 1986; Abad et al., 1994; Mattison et al., 2007). For instance, Abad et al. (1994) reported that poliovirus type 1 (Strain LSc 2ab) and human enteric adenovirus type 40 survived better on porous surface than non-porous surface. Also, Mattison et al. (2007) observed that lettuce and stainless steel surface which had a smooth-textured surface did not absorb as much virus (FCV) as a strawberry surface which had crevices that could provide protection to the virus. Therefore, FCV tended to have a higher survival rate on strawberries when compared to stainless steel and lettuce surfaces.

Aside from surface type, RH can also impact the survival of human enteric viruses on fomite surfaces. More specifically, HuNoVs have been shown to survive better at elevated RH (>80%) (Lamhoujeb et al., 2009; Girard et al., 2010). Abad et al. (1994) reported that the survival rates of non-enveloped viruses including poliovirus and hepatitis A virus (HAV) are higher at elevated RH (>80%). Results reported by Mbithi et al. (1991) on the survival of poliovirus (Sabin strain) on fomite surfaces confirms this claim by showing that virus survival was significantly better at RH of 95 ± 5% when compared to RH of 25 ± 5%. Conversely, Satter et al. (1986) and Mbithi et al. (1991) reported that virus survival at RH less than 50% seems to be specific to the enteric virus studied—in these instances rotavirus and HAV—and cannot be generalized to all non-enveloped, enteric viruses. In the present study, the high persistence rate of TV on fomite surfaces may be in part due to the low RH of the environment; therefore, evaluation of TV survival on fomites at various RH levels is warranted.

Although RH does influence the survival of human enteric viruses, the relationship between RH and virus survival on fomite surfaces can also be influenced by lower temperature.

For instance, keeping RH constant (86% ± 4%), Lamhoujeb et al. (2009) reported that a temperature of 7°C favored the survival of HuNoV when compared to 20°C. Mbithi et al. (1991) reported that at 95% RH, HAV survived better at 5°C than 35°C. Similarly, Abad et al. (2010) reported that at 90 ± 5% RH, astrovirus survived better at 4°C than 20°C. Last, Ansari et al. (1991) indicated that colder seasons and low RH (≤50%) favored rotavirus survival. Human norovirus outbreaks have been reported to occur year round; however, outbreaks peak during the winter season (Hall et al., 2011), possibly due to low temperatures coupled with low RH.

Several studies have utilized FCV and MNV as surrogates to study HuNoV persistence on surfaces. Doultree et al. (1999) observed complete reduction of FCV titer after 28 days on glass coverslips. D'Souza et al. (2006) reported that FCV could persist on fomite surfaces (ceramic coupon, stainless steel, and Formica) at ambient temperature for at least 7 days post inoculation (estimated D-value of 1.4 days). Cannon et al. (2006) observed a complete reduction of MNV infectivity after 5 days and near complete reduction of FCV by day 7 on stainless steel surface at RT. Conversely, Fallahi and Mattison (2011) recorded a D-value of approximately 15 days for MNV survival on stainless steel at RT. Based on these studies and the present study, TV is more likely to serve as a better surrogate to study HuNoV persistence on surfaces at RT and low RH. However, future experiments on survival of TV on surfaces at lower temperatures as well as various RH levels should be considered.

There are several limitations in the present study. First, it was not possible to analyze virus survival in unsterilized environmental waters due to bacterial and fungal contamination in the cell culture system. In a similar study by Bae and Schwab (2008), environmental water samples were not sterilized prior to experiments on MNV and FCV survival as well as HuNoV. Therefore, impact of endogenous environmental water components on TV survival should be considered in

future experiments if possible. Second, in the current study, different temperatures were considered for the water samples (i.e. RT for SW and 4°C for GW), making comparison between the two types of water challenging due to the unknown effect of temperature on virus survival. Previous experiments analyzed different water sources at the same temperatures (Bae and Schwab 2008). Future studies should consider evaluating TV survival in various water sources at the same temperatures. Also, only room temperature was used for evaluations of TV persistence on fomite surfaces. Meanwhile, research has shown that human enteric viruses—specifically HuNoV— persist longer at lower temperatures (i.e. 4°C). Since the present study did not evaluate TV persistence on fomites at lower temperatures, it is difficult to make conclusions about virus persistence at all temperatures. However, it may be crucial to consider the scenarios where HuNoV would be introduced to a fomite surface in such low temperatures such as commercial refrigerators used in the food industry or processing environments. A final limitation is related to the low recovery efficiency of TV on solid surface and stainless steel. Although both surfaces were non-porous, virus recovery efficiencies were highly variable between surfaces which likely impacts the difference in TV persistence on the two surfaces. In the future, a method to increase and maintain consistency in recovery should be considered.

V.   Conclusions

In the absence of a reproducible cell culture system, studying the environmental persistence of HuNoV is a challenge. Detection of viral nucleic acid by molecular methods is the only way to detect HuNoV in environmental matrices, and these methods do not give any information on infectivity (Hirneisen and Kniel 2013) therefore, cultivable surrogates are used for *in vitro* studies. Murine norovirus and FCV have been utilized as HuNoV surrogates most often; however, more

recently discovered surrogates such as TV may be better suited for *in vitro* studies. The present study showed that TV is more persistent in water and on fomite surfaces than MNV and FCV, depending on the experimental variables. Specifically, these results demonstrate that TV survives in SW at RT for 28 days with only a 0.2-$\log_{10}$ reduction. On the contrary, TV survival rate in GW was low with complete loss of infectivity after day 14 at refrigerated temperatures. This study also investigated the persistence of TV on solid and stainless steel surfaces for 14 days. Tulane virus was very persist on both surfaces with D-values of 18.5 ± 0.34 and 13.1 ± 0.36 days on solid and stainless steel surfaces, respectively. This is the first study to evaluate the persistence and survival of TV in environmental waters and on fomite surfaces. In comparison to environmental stability profiles for MNV and FCV in previously published studies, it can be concluded that TV is likely a more conservative surrogate to study HuNoV persistence in the environment.

REFERENCES

Abad, F. X., Pinto, R. M., & Bosch, A. (1994). Survival of enteric viruses on environmental fomites. *Applied and Environmental Microbiology, 60*(10), 3704-3710.

Abad, F. X., Villena, C., Guix, S., Caballero, S., Pinto, R. M., & Bosch, A. (2001). Potential role of fomites in the vehicular transmission of human astroviruses. *Applied and Environmental Microbiology, 67*(9), 3904-3907.

Allwood, P. B., Malik, Y. S., Hedberg, C. W., & Goyal, S. M. (2003). Survival of F-specific RNA coliphage, feline calicivirus, and escherichia coli in water: A comparative study. *Applied and Environmental Microbiology, 69*(9), 5707-5710.

Ansari, S. A., Springthorpe, V. S., & Sattar, S. A. (1991). Survival and vehicular spread of human rotaviruses: Possible relation to seasonality of outbreaks. *Review of Infectious Diseases, 13*(3), 448-461.

Ashbolt, N. J. (2004). Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology, 198*(1), 229-238.

Aw, T., & Gin, K. (2011). Prevalence and genetic diversity of waterborne pathogenic viruses in surface waters of tropical urban catchments. *Journal of Applied Microbiology, 110*(4), 903-914.

Bae, J., & Schwab, K. J. (2008). Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Applied and Environmental Microbiology, 74*(2), 477-484.

Belliot, G., Lavaux, A., Souihel, D., Agnello, D., & Pothier, P. (2008). Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants. *Applied and Environmental Microbiology, 74*(10), 3315-3318.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L., & Vinje, J. (2006). Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus. *Journal of Food Protection, 69*(11), 2761-2765.

Charles, K., Shore, J., Sellwood, J., Laverick, M., Hart, A., & Pedley, S. (2009). Assessment of the stability of human viruses and coliphage in groundwater by PCR and infectivity methods. *Journal of Applied Microbiology, 106*(6), 1827-1837.

Cheesbrough, J., Barkess-Jones, L., & Brown, D. (1997). Possible prolonged environmental survival of small round structured viruses. *Journal of Hospital Infection, 35*(4), 325-326.

Clay, S., Maherchandani, S., Malik, Y. S., & Goyal, S. M. (2006). Survival on uncommon fomites of feline calicivirus, a surrogate of noroviruses. *American Journal of Infection Control, 34*(1), 41-43.

Cromeans, T., Park, G. W., Costantini, V., Lee, D., Wang, Q., Farkas, T., et al., (2014). Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Applied and Environmental Microbiology, 80,* 5734-5751.

Donaldson, E. F., Lindesmith, L. C., Lobue, A. D., & Baric, R. S. (2008). Norovirus pathogenesis: Mechanisms of persistence and immune evasion in human populations. *Immunological Reviews, 225*, 190-211.

Doultree, J., Druce, J., Birch, C., Bowden, D., & Marshall, J. (1999). Inactivation of feline calicivirus, a Norwalk virus surrogate. *Journal of Hospital Infection, 41*(1), 51-57.

D'Souza, D. H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C., & Jaykus, L. (2006). Persistence of caliciviruses on environmental surfaces and their transfer to food. *International Journal of Food Microbiology, 108*(1), 84-91.

Duizer, E., Schwab, K. J., Neill, F. H., Atmar, R. L., Koopmans, M. P., & Estes, M. K. (2004). Laboratory efforts to cultivate noroviruses. *Journal of General Virology, 85*(1), 79-87.

Estes, M. K., Prasad, B. V., & Atmar, R. L. (2006). Noroviruses everywhere: has something changed?. *Current Opinion in Infectious Diseases*, *19*(5), 467-474.

Fallahi, S., & Mattison, K. (2011). Evaluation of murine norovirus persistence in environments relevant to food production and processing. *Journal of Food Protection, 74*(11), 1847-1851.

Farkas, T., Cross, R. W., Hargitt, E.,3rd, Lerche, N. W., Morrow, A. L., & Sestak, K. (2010). Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses. *Journal of Virology, 84*(17), 8617-8625.

Fretz, R., Svoboda, P., Lüthi, T., Tanner, M., & Baumgartner, A. (2005). Outbreaks of gastroenteritis due to infections with norovirus in Switzerland, 2001–2003. *Epidemiology and Infection, 133*(3), 429-437.

Gibson, K. E. (2014). Viral pathogens in water: Occurrence, public health impact, and available control strategies. *Current Opinion in Virology, 4*, 50-57.

Girard, M., Ngazoa, S., Mattison, K., & Jean, J. (2010). Attachment of noroviruses to stainless steel and their inactivation, using household disinfectants. *Journal of Food Protection, 73*(2), 400-404.

Green, J., Wright, P., Gallimore, C., Mitchell, O., Morgan-Capner, P., & Brown, D. (1998). The role of environmental contamination with small round structured viruses in a hospital

outbreak investigated by reverse-transcriptase polymerase chain reaction assay. *Journal of Hospital Infection, 39,* 39-45.

Hall, A. J., Vinjé, J., Lopman, B., Park, G. W., Yen, C., Gregoricus, N., et al., (2011). *Updated norovirus outbreak management and disease prevention guidelines* US Department of Health and Human Services, Centers for Disease Control and Prevention.

Hirneisen, K. A., & Kniel, K. E. (2013). Comparing human norovirus surrogates: Murine norovirus and Tulane virus. *Journal of Food Protection, 76*, 139-143.

Hoebe, C. J., Vennema, H., de Roda Husman, A. M., & van Duynhoven, Y. T. (2004). Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain. *The Journal of Infectious Diseases, 189*(4), 699-705.

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., & Virgin, H. W. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science, 299*(5612), 1575-1578.

Kishida, N., Morita, H., Haramoto, E., Asami, M., & Akiba, M. (2012). One-year weekly survey of noroviruses and enteric adenoviruses in the tone river water in Tokyo metropolitan area, japan. *Water Research, 46*(9), 2905-2910.

Koh, S., Cho, H. G., Kim, B. H., & Choi, B. Y. (2011). An outbreak of gastroenteritis caused by norovirus-contaminated groundwater at a waterpark in Korea. *Journal of Korean Medical Science, 26*, 28-32.

Lamhoujeb, S., Fliss, I., Ngazoa, S., & Jean, J. (2009). Molecular study of the persistence of infectious human norovirus on food-contact surfaces. *Food and Environmental Virology, 1*(2), 51-56.

Le Pendu, J., Ruvoën-Clouet, N., Kindberg, E., & Svensson, L. (2006, December). Mendelian resistance to human norovirus infections. In *Seminars in Immunology*, *18*(6), 375-386. Academic Press.

Liu, B., Maywood, P., Gupta, L., & Campbell, B. (2003). An outbreak of Norwalk-like virus gastroenteritis in an aged-care residential hostel. *New South Wales Public Health Bulletin, 14*(6), 105-109.

Lopman, B., Gastanaduy, P., Park, G. W., Hall, A. J., Parashar, U. D., & Vinjé, J. (2012). Environmental transmission of norovirus gastroenteritis. *Current Opinion in Virology, 2*, 96-102.

Madsen, E. L., Sinclair, J. L., & Ghiorse, W. C. (1991). In situ biodegradation: Microbiological patterns in a contaminated aquifer. *Science (New York, N.Y.), 252*(5007), 830-833.

Mattison, K., Karthikeyan, K., Abebe, M., Malik, N., Sattar, S. A., Farber, J. M., & Bidawid, S. (2007). Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus. *Journal of Food Protection*, *70*(2), 500-503.

Mbithi, J. N., Springthorpe, V. S., & Sattar, S. A. (1991). Effect of relative humidity and air temperature on survival of hepatitis A virus on environmental surfaces. *Applied and Environmental Microbiology, 57*(5), 1394-1399.

Ngazoa, E., Fliss, I., & Jean, J. (2008). Quantitative study of persistence of human norovirus genome in water using TaqMan real-time RT-PCR. *Journal of Applied Microbiology, 104*(3), 707-715.

Pedley, S., Yates, M., Schijven, J.F., West, J., Howard, G. and Barrett, M.H. (2006) Chapter 3. Pathogens: health relevance, transport and attentuation. In Protecting Groundwater for Health: Managing the Quality of Drinking-water Sources ed . Schmoll, O., Howard, G., Chilton, J. and Chorus, I. pp. 49–80 London: IWA Publishing.

Pusch, D., Oh, D., Wolf, S., Dumke, R., Schröter-Bobsin, U., Höhne, M., et al., (2005). Detection of enteric viruses and bacterial indicators in German environmental waters. *Archives of Virology, 150*(5), 929-947.

Rydell, G. E., Nilsson, J., Rodriguez-Diaz, J., Ruvoën-Clouet, N., Svensson, L., Le Pendu, J., & Larson, G. (2009). Human noroviruses recognize sialyl Lewis x neoglycoprotein. *Glycobiology*, *19*(3), 309-320.

Sattar, S. A., Lloyd-Evans, N., Springthorpe, V. S., & Nair, R. C. (1986). Institutional outbreaks of rotavirus diarrhea: Potential role of fomites and environmental surfaces as vehicles for virus transmission. *Journal of Hygiene, 96*(2), 277-289.

Seitz, S. R., Leon, J. S., Schwab, K. J., Lyon, G. M., Dowd, M., McDaniels, M., et al., (2011). Norovirus infectivity in humans and persistence in water. *Applied and Environmental Microbiology, 77*(19), 6884-6888.

Straub, T. M., Honer zu Bentrup, K., Orosz-Coghlan, P., Dohnalkova, A., Mayer, B. K., Bartholomew, R. A., et al., (2007). In vitro cell culture infectivity assay for human noroviruses. *Emerging Infectious Diseases, 13*(3), 396-403.

Stuart, A. D., & Brown, T. D. K. (2007). α2, 6-Linked sialic acid acts as a receptor for feline calicivirus. *Journal of General Virology*, *88*(1), 177-186.

Tamura, M., Natori, K., Kobayashi, M., Miyamura, T., & Takeda, N. (2004). Genogroup II noroviruses efficiently bind to heparan sulfate proteoglycan associated with the cellular membrane. *Journal of Virology*, *78*(8), 3817-3826.

Tan, M., & Jiang, X. (2005). Norovirus and its histo-blood group antigen receptors: An answer to a historical puzzle. *Trends in Microbiology, 13*(6), 285-293.

Tan, M., & Jiang, X. (2007). Norovirus–host interaction: implications for disease control and prevention. *Expert Reviews in Molecular Medicine*, *9*(19), 1-22.

Taube, S., Perry, J. W., Yetming, K., Patel, S. P., Auble, H., Shu, L., et al., (2009). Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses. *Journal of Virology*, *83*(9), 4092-4101.

Taube, S., Jiang, M., & Wobus, C. E. (2010). Glycosphingolipids as receptors for non-enveloped viruses. *Viruses*, *2*(4), 1011-1049.

Werber, D., Laušević, D., Mugoša, B., Vratnica, Z., Ivanović-Nikolić, L., Žižić, L., et al., (2009). Massive outbreak of viral gastroenteritis associated with consumption of municipal drinking water in a European capital city. *Epidemiology and Infection, 137*(12), 1713-1720.

WHO: Outbreaks of Waterborne Diseases, Fact Sheet 1.1. 2009, RPG1_WatSan_E1

Widdowson, M. A., Cramer, E. H., Hadley, L., Bresee, J. S., Beard, R. S., Bulens, S. N., et al., (2004). Outbreaks of acute gastroenteritis on cruise ships and on land: Identification of a predominant circulating strain of norovirus--United States, 2002. *The Journal of Infectious Diseases, 190*, 27-36.

Wobus, C. E., Thackray, L. B., & Virgin, H. W. (2006). Murine norovirus: A model system to study norovirus biology and pathogenesis. *Journal of Virology, 80*(11), 5104-5112.

Wu, H. M., Fornek, M., Schwab, K. J., Chapin, A. R., Gibson, K., Schwab, E., et al., (2005). A norovirus outbreak at a long-term-care facility: The role of environmental surface contamination. *Infection Control, 26*(10), 802-810.

Wyn-Jones, A. P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., et al., (2011). Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Research, 45*(3), 1025-1038.

Table 1: Mean $\log_{10}$ reduction of Tulane virus over time in various water types

Time (day)	Mean Log$_{10}$ Reduction (PFU/ml)*			
	SW[a]	GW[b]	diH$_2$O[c]	TW
0	0.000± 0.42	0.000± 0.38	0.000± 0.29	0.000± 0.04
2	ND	1.318± 0.04	ND	ND
3	ND	2.417± 0.24	ND	ND
5	ND	3.362± 0.50	ND	ND
7	0.138± 0.26	0.420± 0.66	0.369± 0.26	0.062± 0.26
14	0.527± 0.40	3.445± 0.58	0.760± 0.12	0.306± 0.04
21	0.188± 0.29	LOD	1.180± 0.20	0.382± 0.01
28	0.235± 0.25	LOD	2.149± 0.00	0.533± 0.02

*mean $\log_{10}$ reduction titer (n= 4 or 8) after 28 days

SW = surface water; GW = ground water; diH$_2$O = sterile distilled water; TW = tap water; LOD = beyond limit of detection; ND = analysis was not done

[a-c] mean $\log_{10}$ reduction after day 28 significantly different ($p < 0.05$)

Figure legend

Figure 1: Tulane virus persistence profile on non-porous, acrylic solid surface and stainless steel surface, incubated at room temperature for up to 14 days. Each data point represents the mean log remaining at each time point of at least 4 replicates. Error bars represent the standard deviations at each time point.

Figure 1. Tulane virus persistence profile on non-porous, acrylic solid surface and stainless steel surface, incubated at room temperature for up to 14 days.
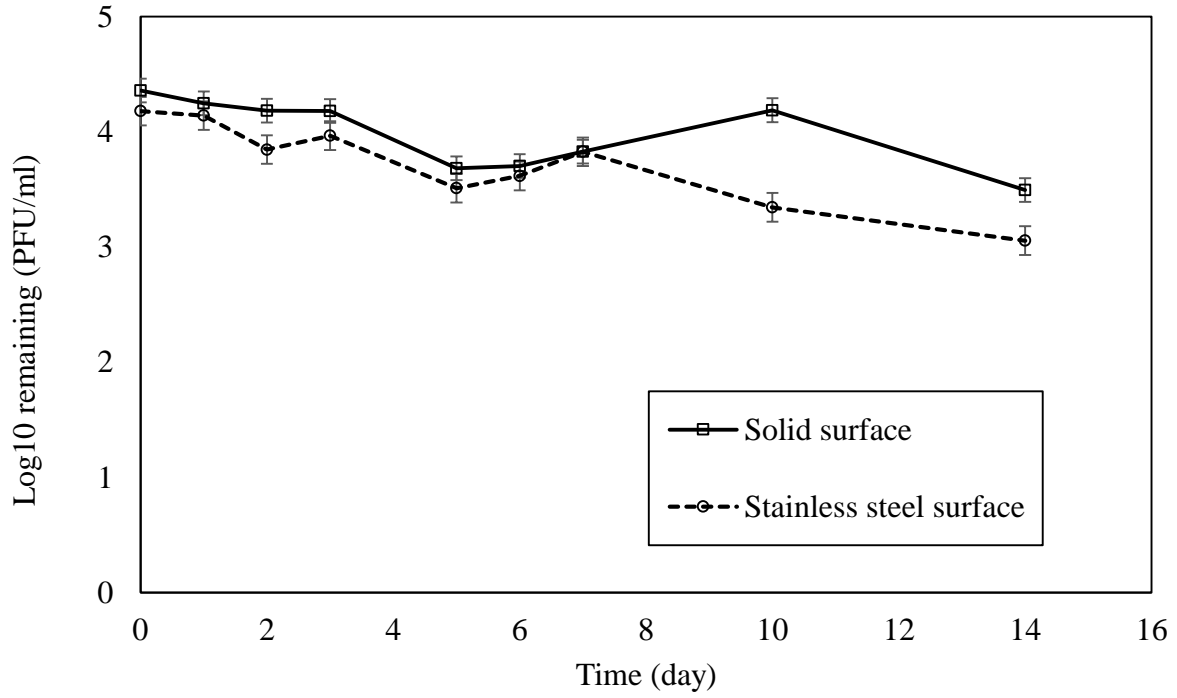
CHAPTER 5: CONCLUSION AND FUTURE DIRECTION

Human norovirus (HuNoV) is globally recognized as the chief cause of acute gastroenteritis, contributing to 50% of all epidemic gastroenteritis outbreaks in U.S. and Europe (Atmar and Estes 2006; Patel et al., 2009; Yen et al., 2011). Persistence of HuNoVs in the environment—built or natural—is a key factor for HuNoV transmission and associated outbreaks (Lopman et al., 2012; Donaldson et al., 2008). Due to the lack of a reproducible cell culture system or small animal model to study the pathogenesis of HuNoV, researchers have used viral surrogates—murine norovirus (MNV) and feline calicivirus (FCV) (Bae and Schwab 2008; Cannon et al., 2006; D'Souza et al., 2006; Belliot et al., 2008). However, these surrogates have several limitations as pointed out in the preceding chapters. In this research, it is hypothesized that the newly identified surrogate virus, Tulane virus (TV), is likely a more conservative virus to study HuNoV physicochemical stability and persistence in the environment.

My research focused on establishing TV physicochemical stability and environmental persistence profiles and comparing them to previously published data for MNV and FCV. Due to the comparative studies, prior to the current research, a review of the various methods used in evaluating the thermal stability profiles of human enteric viruses (Chapter 2). The review showed that differences in methods makes it difficult to effectively compare thermal stability profiles for different viruses and even the same viruses under a common parameter. Variability in concentrations of viruses analyzed, times of treatment, matrix type, type of analysis used, and type of heat application was identified. It must be noted that these experimental differences were not only present in viral thermal stability studies but also in the studies on other parameters such as pH stability, ethanol tolerance, bleach tolerance, and environmental stability. Based on the review,

it was suggested that a single standard approach for determining thermal inactivation profiles of human enteric viruses and their surrogates and provide some basic recommendations (Chapter 2).

The overall goal of my thesis is to determine if TV is a better surrogate to study HuNoV physicochemical stability and environmental persistence than traditional surrogates such as murine norovirus (MNV) and feline calicivirus (FCV). Based on this, the specific objectives were 1) to determine the physicochemical stability profile of TV and 2) to evaluate the environmental persistence of TV.

In the first objective (Chapter 3), the physicochemical stability profile of TV was determined by first evaluating the stability of the virus to different heat treatment treatments (37, 56, 63 and 72°C) for various time points. Second, the virus subjected to ethanol treatment at 60, 70 and 90% concentrations of the ethanol for up to 5 min at RT. Tulane virus was then exposed to various pH levels (2, 3, 7, 9, and 10) at room temperature (RT) for up to 90 min. Last, TV tolerance to different bleach concentrations (200 and 1000 ppm) was tested on solid surfaces at RT for up to 10 min. Data from these experiments were compared to MNV and FCV data in previously published studies and a conclusion that TV is more stable at 56, 63 and 72°C was made. Also, TV is more tolerant at lower ethanol concentrations (<90%) than MNV and FCV, and very stable at all pH levels tested. In addition, chlorine disinfection at 200 and 1000ppm was not effective against TV on solid surface.

The second objective (Chapter 4) evaluated the environmental persistence of TV. Specifically, the persistence of TV in environmental water (surface and ground water) samples was evaluated. The experiments were set up with SW at RT under ambient light and GW at refrigeration temperature in the dark for 28 days. Tulane virus survived in SW for the entire period analyzed with less than 0.5-log$_{10}$ reduction whereas TV survival in GW was low with complete

loss of infectivity by day 21. There was a significant difference between survival in SW and GW ($p < 0.05$). On fomite surfaces (acrylic-based solid surface and stainless steel) at RT, TV persisted for 14 days with estimated D-values of 18.5 ± 0.34 days and 13.1 ± 0.36 days on solid and stainless steel surfaces, respectively. There was no significant difference in TV persistence on both surfaces ($p < 0.05$). The data reported here show that TV can persist for even longer periods on fomites at RT.

Based on the results presented in Chapters 3 and 4, I can conclude that TV is generally a more conservative surrogate than MNV and FCV to study HuNoV physicochemical stability and environmental persistence. However, TV would not be the ideal surrogate to study HuNoV tolerance to higher ethanol concentrations ($\geq 90\%$). In GW, TV is also not the best surrogate to study HuNoV survival when molecular-based studies on HuNoV survival in GW are considered.

In the future, studies on TV tolerance to chlorine disinfection at much higher concentrations (>1000 ppm) should be considered as those concentrations have been shown to inactivate HuNoV on contaminated fomite surfaces (CDC 2011). In addition, analysis of TV persistence in water samples in this study did not consider the impact of temperature nor of endogenous environmental water components since the experiments were performed using sterile water. Future studies on the persistence of TV in unsterile environmental waters should considered. With respect to persistence on fomite surfaces, a method to increase virus recovery efficiency and reduce variability should be considered. Finally, since the current research only tested for TV survival on fomite surfaces at RT and low relative humidity (RH), evaluation of TV survival at lower temperatures (e.g., 4°C) and elevated RH (e.g., >80%) should be considered in future experiments to allow better comparison to other surrogate studies performed at lower temperatures and a wider range of RH.

REFERENCES

Atmar, R. L., & Estes, M. K. (2006). The epidemiologic and clinical importance of norovirus infection. *Gastroenterology Clinics of North America, 35*(2), 275-290.

Bae, J., & Schwab, K. J. (2008). Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Applied and Environmental Microbiology, 74*(2), 477-484.

Belliot, G., Lavaux, A., Souihel, D., Agnello, D., & Pothier, P. (2008). Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants. *Applied and Environmental Microbiology, 74*(10), 3315-3318.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L., & Vinje, J. (2006). Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus. *Journal of Food Protection, 69*(11), 2761-2765.

Centers for Disease Control and Prevention. (2011). Updated norovirus outbreak management and disease prevention guidelines. *Morbidity and Mortality Weekly Report. Recommendations and Report, 60*, 1–18.

Donaldson, E. F., Lindesmith, L. C., Lobue, A. D., & Baric, R. S. (2008). Norovirus pathogenesis: Mechanisms of persistence and immune evasion in human populations. *Immunological Reviews, 225*(1), 190-211.

D'Souza, D. H., Sair, A., Williams, K., Papafragkou, E., Jean, J., Moore, C., & Jaykus, L. (2006). Persistence of caliciviruses on environmental surfaces and their transfer to food. *International Journal of Food Microbiology, 108*(1), 84-91.

Lopman, B., Gastanaduy, P., Park, G. W., Hall, A. J., Parashar, U. D., & Vinjé, J. (2012). Environmental transmission of norovirus gastroenteritis. *Current Opinion in Virology, 2*(1), 96-102.

Patel, M. M., Hall, A. J., Vinjé, J., & Parashar, U. D. (2009). Noroviruses: A comprehensive review. *Journal of Clinical Virology, 44*(1), 1-8.

Yen, C., Wikswo, M. E., Lopman, B. A., Vinje, J., Parashar, U. D., & Hall, A. J. (2011). Impact of an emergent norovirus variant in 2009 on norovirus outbreak activity in the United States. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 53*(6), 568-571.

Appendix

UNIVERSITY OF
ARKANSAS

January 11, 2013

MEMORANDUM

TO: Dr. Kristen Gibson

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 13017

Protocol Title: "Understanding Environmental Reservoirs and Prevalence of Norovirus Surrogates to Reduce Impact on Public Health"

Approved Project Period: Start Date: January 10, 2013
Expiration Date: January 09, 2016

The Institutional Biosafety Committee (IBC) has approved Protocol 13017, "Understanding Environmental Reservoirs and Prevalence of Norovirus Surrogates to Reduce Impact on Public Health". You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.