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Persistence of Enteric Viruses on Surfaces under Varying Environmental Conditions

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Persistence of Enteric Viruses on Surfaces under Varying Environmental Conditions

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Abstract

Human enteric viruses such as human norovirus (hNoV) and Aichivirus A (AiV) are common foodborne viruses with hNoVs being identified as the leading causative agent of foodborne illnesses in the U.S. Moreover, hNoVs have been identified as the leading cause of nonbacterial acute gastroenteritis in the U.S. and worldwide. Fomite surface contamination is a major transmission route for enteric viruses. The application of an optimized virus recovery method from fomites is essential for better understanding of virus persistence under varying environmental conditions (EC). This study aimed to optimize a surface sampling method for virus recovery from nonporous food contact surfaces (FCS) for further application in environmental persistence studies under varying temperature and relative humidity (RH) combinations. Initially, feline calicivirus (FCV), hNoVs (GI.1; GII.17), AiV, and Tulane virus were selected for FCS sampling optimization. FCS selected for analysis included plastic chopping board, acrylic-based solid surface, and stainless steel. Sampling methods selected for evaluation included 3 implements (cell scraper, macrofoam swab, repeated pipetting) and 2 eluents (1×Phophaste buffered saline solution (PBS), 1×PBS+0.1%Tween80 (PBST; 1:1 v/v)). The repeated pipetting method with PBST eluent was selected for persistence studies though no significant differences were observed compared to other methods. Overall, mean recovery efficiencies using repeated pipetting with PBST ranged from 2.0% ± 0.6% to 82.36% ± 38.6% depending on virus and FCS type. For persistence studies, temperature (22°C, 15°C, 6°C) and RH (60%, 90%) combinations appropriate to food processing and storage were chosen for evaluation. AiV was stable on all FCS with about a 3 $\log_{10}$ titer reduction for 22°C/60% RH and 15°C/60% RH and about a 1 $\log_{10}$ titer reduction for 15°C/90% RH and 6°C/90% RH over 14d. Generally, higher RH (90%) displayed more stability for GI.1 and AiV over time than lower RH

(60%), which is consistent with previous studies. However, lower temperatures may not be a major influencing factor of GI.1 and AiV persistence, which differed from previous studies. Furthermore, the impact of surface type was inconsistent which is similar to the variability seen across studies. This is the first study to demonstrate AiV persistence on nonporous FCS under varying EC.

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Dedication

        I would like to dedicate this thesis to my family and friends for their support and encouragement throughout my education.

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List of Abbreviations

ABSS	Acrylic-based solid surface
AiV	Aichivirus A
CDC	Centers for Disease Control and Prevention
C_T	Cycle threshold value
CRFK	Crandell Rees feline kidney cells
D-value	Decimal reduction value
EBSS	Earle's buffered saline solution
EC	Environmental conditions
FBS	Fetal bovine serum
FCS	Food contact surface
FCV	Feline calicivirus
GC	Genomic copies
HAV	Hepatitis A virus
HDPE	High density polyethylene
hNoV	Human norovirus
MNV	Murine norovirus – strain type 1
NIFA	National Institute of Food and Agriculture
NORS	National Outbreak Reporting System
NPF	Nonporous formic
PA	Plaque assay
PBS	Phosphate buffered saline solution
PBST	1×Phosphate buffered saline solution + 0.1% Tween80
PCRu	Polymerase chain reaction amplifiable unit
PE	Polyethylene
PF	Porous formic
PFU	Plaque forming units
RB	Rubberized surface
RH	Relative humidity
RT	Room temperature
RT-qPCR	Reverse transcription, real time quantitative polymerase chain reaction
STEC	Shiga toxin producing *Escherichia coli*
SS	Stainless steel
TGBE	100mM Tris – HCl – 50 mM glycine – 1.5% beef extract
TuV	Tulane virus
USDA	U.S. Department of Agriculture

List of Published Articles

1. Chapter 2: Turnage, N.L., & Gibson, K.E. (2017). Sampling Methods for Recovery of Human Enteric Viruses from Environmental Surfaces. *Journal of Virological Methods, 248*, 31-38. Published.

Chapter 1: Literature Review

    1. **Burden of Disease related to foodborne pathogens**

1.1 Brief introduction

With approximately 1.45 million deaths per year, acute gastroenteritis causes the second greatest infectious disease burden globally (Ahmed et al. 2014). Moreover, acute gastroenteritis accounts for 178.8 million illnesses, 473,832 hospitalizations, and 5,072 deaths in the United States alone (Scallan et al. 2011). An estimated 31 pathogens are known to be causative agents of acute gastroenteritis and/or foodborne illness, and these include astrovirus, norovirus, rotavirus, *Vibrio cholera*, *Camplyobacter* spp, *Escherichia coli* (Shiga toxin producing *Escherichia coli* (STEC) 0157, STEC non-0157, and enterotoxigenic *Escherichia coli* strains), Hepatitis A virus, and others (Scallan et al. 2011). Of these pathogens, noroviruses have been identified as a leading cause of acute nonbacterial gastroenteritis outbreaks in the U.S. and worldwide (Green 2007). Noroviruses account for 56,000-71,000 hospitalizations and 570-800 deaths per year in the U.S., and causes illness in approximately 1 in every 15 Americans each year (Hall et al. 2014). From 2009-2012, 1,008 foodborne norovirus outbreaks were reported to the U.S. Centers for Disease Control and Prevention's (CDC) National Outbreak Reporting System (NORS) representing approximately 48% of foodborne outbreaks with one known pathogenic agent (Hall et al. 2014). Scallan et al. (2011) reported similar numbers with noroviruses estimated as being the causative agent for 59% of foodborne illnesses.

1.2 Burden of disease related to human noroviruses

Generally, human noroviruses are associated with about one-fifth of all acute gastroenteritis cases worldwide for all age groups, and this group of viruses has been identified as the leading cause of foodborne illnesses in the U.S. (Lopman et al. 2016). Reported illnesses attributed to norovirus could be underestimated in the U.S., possibly related to underreporting, the short duration of the norovirus illnesses, and prolonged transmission through viral shedding of both asymptomatic and symptomatic individuals. The full extent of norovirus illnesses is not known especially with unspecified foodborne agents being estimated to cause 38.4 million (80%) of foodborne illnesses, 71,878 (56%) of hospitalizations, and 1,686 (56%) of deaths in the U.S. per year (Scallan et al. 2011). Typically, norovirus has a short incubation period of approximately 24 hours with most symptoms resolving after 1 to 3 days (Green 2007; Hall et al. 2011). The duration of transmission can be extensive since norovirus shedding can occur up to an estimated 4 weeks after initial infection with the highest amounts of norovirus shedding often happening after symptoms cease (Atmar et al. 2008; Rockx et al. 2002; Ronnqvist and Maunula 2016). Additionally, 32% of adults exposed to norovirus in a volunteer study displayed asymptomatic shedding of norovirus at similar concentrations to those with symptoms (Atmar et al. 2008; Sukhrie et al. 2012). Consequently, a combination of these factors along with many people not seeking medical services due to the short incubation period and moderate severity of symptoms could contribute to an underestimation of norovirus illnesses in the U.S.

Regardless of low mortality rates in foodborne illnesses attributable to norovirus in comparison to other foodborne diseases, the overall impact of the disease contributes to a disruption of services and economic losses with an estimated loss of $2 billion USD per year in the U.S. alone (Arias et al. 2013). Lopman et al. (2016) reported norovirus outbreaks cause

approximately $60.3 billion USD in societal costs and $4.2 billion USD in direct healthcare costs per year worldwide. Therefore, studies understanding transmission and environmental stability could potentially lead to better preventive measures and for control of this important foodborne pathogen.

2. Transmission of foodborne pathogens

2.1 Brief introduction of transmission modes of foodborne enteric viruses

The most relevant foodborne viral infections are viruses that can be spread by vomiting or shedding into the stool and infects cells in the lining of the intestinal tract (Koopmans and Duizer 2004). Foodborne viruses have general features and important differences that distinguish them from foodborne bacterial infections. These general characteristics include having a low infectious dose, high viral shedding in stools, host specificity, resistant in pH extremes, cannot replicate outside of the host, and is relatively stable in the environment (Koopmans and Duizer 2004; Rzezutka and Cook 2004). Generally speaking, a virus has a greater chance of transmission the longer it is able to survive outside its host, and this ability is influenced by various environmental conditions including moisture, temperature, and pH (Rzezutka and Cook 2004).

Common foodborne viruses are separated into three distinct categories based on the type of illnesses they cause: (1) gastroenteritis (e.g. norovirus, rotavirus, coronavirus, Aichivirus A, and others), (2) enterically transmitted hepatitis viruses (e.g. hepatitis A and E), and (3) viruses that replicate in the human gut but migrate and cause illness in other organs (e.g. poliovirus) (Koopmans and Duizer 2004). The most common human enteric viruses that cause foodborne illnesses have been shown to be hepatitis A and noroviruses (Cliver 1997; Koopmans and Duizer

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2004). Generally, acute gastroenteritis outbreaks due to viruses are transmitted through food and water contamination, contaminated environmental sources, direct person-to-person contact, and other unknown sources (Wikswo et al. 2015). Typically, human enteric viruses are known to spread by fecal-oral contamination, and there is growing evidence of viral transmission occurring through contaminated fomite surfaces in a variety of ways and settings inclusive of food preparation environments (Boone and Gerba 2007; Rzezutka and Cook 2004). Overall, there are few studies that focus on assessing the role of fomites and environmental contamination in the chain of transmission of human enteric viruses (Rzezutka and Cook 2004). Moreover, very few acute gastroenteritis outbreaks reported to NORS during 2009-2013 were attributed to environmental contamination. This lack of environmental source attribution is due to many factors such as the difficulty in differentiating between environmental contamination and direct person-to-person contact transmission, multiple modes of transmissions often involved in most outbreaks, unclear evidence of contamination, and underreporting due to lack of understanding the definition of environmental contamination (Wikswo et al. 2015).

2.2 Transmission modes of human norovirus

As indicated in the previous section, norovirus transmission occurs through fecal-oral contamination specifically by consumption of contaminated food and water, contact with contaminated environmental surfaces, and direct person-to-person contact (Hall et al. 2014; Karst and Baric 2015; Lopman et al. 2012). During 2009-2012, NORS reported that 23% of the norovirus outbreaks were related to foodborne transmission whereas person-to-person, environmental sources, and waterborne caused 69, 0.35, and 0.26%, respectively (Hall et al. 2014). Furthermore, 16% of foodborne norovirus outbreaks were caused by secondary

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transmission through environmental, waterborne, person-to-person contact, or other unknown sources (Hall et al. 2014).

A large human reservoir, chemical disinfectant resistance, environmental stability, prolonged and copious shedding in feces, rapid and widespread distribution by vomit, and the diverse range of fomite surfaces that can become contaminated are factors that promote the environmental transmission of noroviruses (Lopman et al. 2012). Norovirus is also spread via ingestion of aerosolized droplets from vomiting episodes through which an estimated 30 million or more viral particles can be dispersed during a single vomiting event leading to the contamination of a multitude of surfaces within the immediate vicinity (Caul 1994; Hall et al. 2014; Sukhrie et al. 2012). Furthermore, noroviruses have a low infectious dose of 18 to 2,800 viral particles and remain communicable at low and high temperatures and on surfaces for two weeks or longer (Hall et al. 2014). Overall, the various transmission modes along with the environmental stability of noroviruses provide challenges for norovirus preventions and controls.

2.3 Primary surfaces and settings implicated in outbreaks due to environmental transmission

Fomite surfaces play an important role in enteric viral outbreaks and transmission in a variety of settings. Enteric viruses have been shown to maintain infectivity on fomites over prolonged periods (Rzezutka and Cook 2004). Specifically, echovirus, coxsackievirus, and poliovirus have been shown to remain infectious from 2 to >12 days on household representative surfaces such as glass, cotton fabric, and painted wood especially with an enhanced survival in the presence of coliform bacteria, protein, dust, and fat particles (Kiseleva 1968). Additionally, there is evidence of prolonged norovirus survival in porous fomites such as carpets. For example, two carpet fitters extensively removed a carpet from a hospital ward's side room after 12 days of an outbreak on

the ward, and both men came down with norovirus symptoms with the carpet as their common source of exposure (Cheesbrough et al. 1997). Noroviruses have been detected on surfaces such as televisions, cellular phones, bathroom light switches, public phones, microwave ovens, chairs, keyboards, computer mice, toilet light switches, bed frames, and chairs (Gallimore et al. 2006, 2008).

Transmission and the spread of norovirus occurs in a variety of settings such as long-term facilities, childcare facilities, summer camps, schools, social events with catered meals, restaurants, airplanes, military barracks, and cruise ships (Green 2007; Matthews et al. 2012). For example, year round prevalence of noroviruses on environmental surfaces of catering facilities without a recently reported outbreak of acute gastroenteritis have been reported (Boxman et al, 2011). Boxman et al. (2011) further showed that norovirus prevalence on surfaces correspond to the seasonality of norovirus and dominant strains circulating in the population. Most importantly, noroviruses were detected on environmental surfaces in 61.1% of catering settings with recent outbreaks displayed divergent to only 4.2% of catering settings without a recent outbreak (Boxman et al. 2011). The authors also reported pension/hotels catering company types and elderly homes as having the greatest prevalence of positive norovirus environmental swab samples at 33.3% and 22.2% respectively (Boxman et al. 2011).

From 2009-2012, 90% of foodborne norovirus outbreaks reported occurred in a food preparation setting. Overall, restaurants accounted for 64%, banquet or catering facilities accounted for 17%, schools for 13%, and long-term care facilities for 12% of norovirus foodborne outbreaks (Hall et al. 2014). During 2009-2012 in the U.S., 52% of foodborne norovirus outbreaks were due to food contamination where 70% of these cases identified infectious food service employees as the source. Furthermore, bare-hand contact of ready-to-eat

foods was implicated in 54% of these outbreaks (Hall et al. 2014). Hall and co-authors (2014) cited 92% of foods were contaminated during food preparation with 75% being from consumption of uncooked or raw foods such as leafy vegetables, fruits, and deli meats. Institutional settings such as lunchrooms and long-term care facilities were more likely to have noroviruses on surfaces in contrast to commercial settings (Boxman et al. 2011; Verhoef et al. 2013). Other settings such as private residences, schools, hospitals, day cares, and other/multiple settings account for a range of 0.1-13% of norovirus outbreaks (Hall et al. 2014).

Numerous factors such as improper cleaning of surfaces, lack of handwashing compliance, working while ill, lack of knowledge and awareness about foodborne viruses, and other workplace culture practices, have been shown to contribute to environmental surface contamination and potentially norovirus transmission (Carpenter et al. 2013; Verhoef et al. 2013). Additionally, studies have shown food establishments with employees citing the intention to work while sick to significantly contribute to the prediction of the existence of noroviruses on environmental surfaces in these facilities (Verhoef et al. 2013). In an observational study of food service employee in restaurants, Green et al. (2006) reported the occurrence of proper hand washing in only 27% of recommended activities and less frequent when wearing gloves at 16%. Even in healthcare settings, compliance rates of proper hand washing are approximately 50% (Kampf and Kramer 2004). Noroviruses have been shown to survive on unwashed hands for at least 2 hours (Liu et al. 2009). Barker et al. (2004) reported the possible transmission of noroviruses to up to seven clean surfaces by contaminated hands. Improper cleaning could lead to the further spread of noroviruses as displayed in a study of a college summer camp where a norovirus outbreak occurred, and improper cleaning caused the spread of noroviruses from 40 rooms and additional 73 rooms after cleaning (Fankem et al. 2014). Another incident occurred

where hotel employees cleaned hard surfaces and carpets without any disinfectants due to a concern about damaging the furnishings while the hotel was closed for disinfection after an outbreak; however, these actions led to a prolonged outbreak with new cases of illness after the hotel reopened (Cheesbrough et al. 2000). Moreover, improper hand washing along with improper disinfecting and cleaning could contribute to the persistence and transmission of noroviruses on environmental surfaces in food preparation settings.

3. Research relevant to norovirus and its surrogates on surfaces

3.1 Norovirus and the use of surrogates in research

Traditionally, surrogates including feline calicivirus (FCV), murine norovirus (MNV), hepatitis A virus (HAV), MS2 bacteriophage, Tulane virus (TuV), and Aichivirus A (AiV) have been used for norovirus infectivity studies (Ronnqvist and Maunula 2016). This is due to the fact that no *in vitro* cell culture system for norovirus was available until recently (Ettayebi et al. 2016). Until reproducible and readily available infectivity assays for noroviruses are developed, norovirus surrogates still provide much needed information on norovirus infectivity. Also, HAV and AiV are known human pathogens that cause foodborne illnesses in addition to being norovirus surrogates (Koopmans and Duizer 2004). Norovirus surrogates such as FCV, TuV, and MNV are still important for understanding norovirus infectivity and persistence due to their relatedness to noroviruses and the diversity among genotypes (Arthur and Gibson 2015; Cannon et al. 2006; Yeargin et al. 2015).

3.2 Factors impacting viral persistence for norovirus and its surrogates

Kramer et al. (2006) examined literature about persistence of nosocomial pathogens on inanimate fomite surfaces. The authors reported enteric viruses such as HAV, astrovirus, poliovirus, and rotavirus are able to survive on surfaces for approximately two months (Kramer et al. 2006). Furthermore, environmental conditions such as relative humidity and temperature have shown to play a role in viral persistence on fomite surfaces. Low humidity (<70%) persistence is associated with enteric viruses such as HAV. Low temperatures of 4 or 6°C are associated with longer persistence for most viruses (Kramer et al. 2006). Other factors that can impact viral persistence include higher inoculum levels and the co-presence of fecal suspension or organic matter in general (Abad et al. 1994; Faix 1987). Kramer et al. (2006) concluded that for longer viral persistence, a high virus inoculum on a surface in a cold room with high relative humidity would be ideal. Overall, virus persistence—including norovirus and its surrogates—on fomite surfaces is influenced by surface type, temperature, relative humidity, and amount of virus.

Fomite surfaces are generally categorized as either porous or nonporous where examples of porous surface types are wood, carpets, lettuce, deli meats, and fruits, and examples of nonporous surfaces are stainless steel, ceramic, glass, and acrylic, and surface type have been shown to have some effect on norovirus viral persistence. D'Souza et al. (2006) reported the ease of transfer of norovirus and FCV from stainless steel to lettuce, which confirmed a potential transmission role for environmental contamination. Kim et al (2012) reported MNV (strain type 1) as being more stable longer on wood than stainless steel. Another study found MNV stability on surfaces in the following descending order: plastic, rubber, glass, ceramic, wood, and stainless steel (Kim et al. 2014). However, Kim et al. (2012) found the role of relative humidity in the

persistence of MNV to be inconsistent within the range of 30-70%. Overall, relative humidity was found to not significantly change the inactivation rate of MNV (Kim et al. 2012).

Additionally, temperature has been shown to be the main influencing factor on viral persistence and survival for noroviruses on environmental surfaces (Ahmed et al. 2014). Mormann et al. (2015) also reported 1-log reduction for MNV at room temperature on stainless steel after 7-15 days; however, a reduction of virus was not observed at 7°C. Furthermore, the study confirmed that MNV may be a better suited surrogate under dry conditions than FCV since MNV was more stable at 7°C (Mormann et al. 2015). Further study on relative humidity and temperature combinations are needed on other human enteric viruses and their surrogates such as AiV to better understand the role of humidity on norovirus persistence. Generally, the potential transmission of enteric viruses through contaminated fomite surfaces rely on their ability to maintain viral infectivity especially since viruses are obligate parasites that require a host to replicate (Boone and Gerba 2007). Typically, pH and UV exposure have little effects on viral persistence and survival on surfaces in indoor settings. Noroviruses displayed a persistence of over 40 days in simulated vomit-like gastric fluid with a pH of 2.5 with only a 1.1-1.3 log viral reduction within the time period suggesting the potential role of pH and vomit droplets in environmental transmission (Tung-Thompson et al. 2015). Also, the presence of microbes may or may not influence viral persistence on environmental surfaces while increasing the numbers of microbes may provide protection for viruses from disinfection and desiccation; however, the virus may experience harmful effects from fungal enzymes or microbial proteases (Schwartz et al. 2003; Sobsey and Meschke 2003). In conclusion, there are many factors contributing to viral persistence on surfaces under environmental conditions in food preparation settings.

4. Where are the gaps in the literature?

As stated previously, food preparation settings, food contamination through fomites and infectious food service employees, and person-to-person contact are key modes of transmission for noroviruses. Understanding noroviruses and its surrogates' persistence on nonporous fomite food preparation surfaces in relation to various environmental conditions could provide insight on ways to limit and prevent norovirus outbreaks and transmission. Appropriate surface sampling techniques are needed to properly evaluate the viral persistence of norovirus on surfaces in laboratory settings. Evaluation of surface sampling techniques are typically limited to swabs for application in environmental sampling during norovirus outbreaks, and information is lacking on evaluating tools used in laboratory sampling techniques for recovery optimization of viruses. Due to the recent cultivability and discovery of AiV, there is limited data in the literature on AiV persistence on any surfaces in relation to relative humidity and various temperatures over time. This study aimed to address the gaps in knowledge related to the persistence of human enteric viruses and their surrogates on food preparation surfaces and optimization of laboratory surface techniques. The objectives of this study were (1) to optimize surface sampling methods for recovery of human enteric viruses and their surrogates, and (2) to evaluate the different surfaces and environmental conditions for viral persistence.

References

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

Ahmed, S. M., Hall, A. J., Robinson, A. E., Verhoef, L., Premkumar, P., Parashar, U. D., Lopman, B. A. (2014). Global prevalence of norovirus in cases of gastroenteritis: A systematic review and meta-analysis. *The Lancet Infectious Diseases, 14*, 725-730.

Arias, A., Emmott, E., Vashist, S., & Goodfellow, I. (2013). Progress towards the prevention and treatment of norovirus infections. *Future Microbiology, 8*, 1475.

Arthur, S., & Gibson, K. (2015). Physicochemical stability profile of tulane virus: A human norovirus surrogate. *Journal of Applied Microbiology, 119*, 868-875.

Atmar, R. L., Opekun, A. R., Gilger, M. A., Estes, M. K., Crawford, S. E., Neill, F. H., & Graham, D. Y. (2008). Norwalk virus shedding after experimental human infection. *Emerging Infectious Diseases, 14*, 1553-1557.

Barker, J., Vipond, I., & Bloomfield, S. (2004). Effects of cleaning and disinfection in reducing the spread of norovirus contamination via environmental surfaces. *Journal of Hospital Infection, 58*, 42-49.

Boone, S. A., & Gerba, C. P. (2007). Significance of fomites in the spread of respiratory and enteric viral disease. *Applied and Environmental Microbiology, 73*, 1687-1696.

Boxman, I.L., Verhoef, L., Dijkman, R., Hagele, G., Te Loeke, N.A., & Koopmans, M. (2011). Year-round prevalence of norovirus in the environment of catering companies without a recently reported outbreak of gastroenteritis. *Applied and Environmental Microbiology, 77*, 2968−2974.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L., & Vinjé, 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*, 2761-2765.

Carpenter, L., Green, A. L., Norton, D. M., Frick, R., Tobin-D'Angelo, M., Reimann, D. W., Everstine, K. (2013). Food worker experiences with and beliefs about working while ill. *Journal of Food Protection, 76*, 2146-2154.

Caul, E. O. (1994). Small round structured viruses: Airborne transmission and hospital control. *The Lancet, 343*, 1240-1242.

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

Cheesbrough, J., Green, J., Gallimore, C., Wright, P., & Brown, D. (2000). Widespread environmental contamination with norwalk-like viruses (NLV) detected in a prolonged hotel outbreak of gastroenteritis. *Epidemiology and Infection, 125*, 93-98.

Cliver, D. O. (1997). Virus transmission via food. *Food Technology*, *51*, 71-78.

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*, 84-91.

Ettayebi, K., Crawford, S. E., Murakami, K., Broughman, J. R., Karandikar, U., Tenge, V. R., Estes, M. K. (2016). Replication of human noroviruses in stem cell-derived human enteroids. *Science, 353*, 1387-1393.

Faix, R. G. (1987). Comparative efficacy of handwashing agents against cytomegalovirus. *Infection Control, 8*, 158-162.

Fankem, S. L., Boone, S. A., Marlene Gaither, M., & ME, R. (2014). Outbreak of norovirus illness in a college summer camp: Impact of cleaning on occurrence of norovirus on fomites. *Journal of Environmental Health, 76*, 20-26.

Gallimore, C. I., Taylor, C., Gennery, A. R., Cant, A. J., Galloway, A., Iturriza-Gomara, M., & Gray, J. J. (2006). Environmental monitoring for gastroenteric viruses in a pediatric primary immunodeficiency unit. *Journal of Clinical Microbiology, 44*, 395-399.

Gallimore, C. I., Taylor, C., Gennery, A. R., Cant, A. J., Galloway, A., Xerry, J., Gray, J. J. (2008). Contamination of the hospital environment with gastroenteric viruses: Comparison of two pediatric wards over a winter season. *Journal of Clinical Microbiology, 46*, 3112-3115.

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, L. R., Selman, C. A., Radke, V., Ripley, D., Mack, J. C., Reimann, D. W., & Bushnell, L. (2006). Food worker hand washing practices: An observation study. *Journal of Food Protection, 69*, 2417-2423.

Hall, A. J., Wikswo, M. E., Pringle, K., Gould, L. H., & Parashar, U. D. (2014). Vital signs: Foodborne norovirus outbreaks - United States, 2009-2012. *Morbidity and Mortality Weekly Report, 63*, 491-495.

Kampf, G., & Kramer, A. (2004). Epidemiologic background of hand hygiene and evaluation of the most important agents for scrubs and rubs. *Clinical Microbiology Reviews, 17*, 863-893.

Karst, S. M., & Baric, R. S. (2015). What is the reservoir of emergent human norovirus strains? *Journal of Virology, 89*, 5756-5759.

Kim, A. N., Park, S. Y., Bae, S. C., Oh, M. H., & Ha, S. D. (2014). Survival of norovirus surrogate on various food-contact surfaces. *Food and Environmental Virology*, *6*, 182-188.

Kim, S. J., Si, J., Lee, J. E., & Ko, G. (2012). Temperature and humidity influences on inactivation kinetics of enteric viruses on surfaces. *Environmental Science & Technology, 46*, 13303-13310.

Kiseleva, L. (1968). Survival of enteric viruses in water and foodstuffs and on various surfaces. *Hygiene and Sanitation, 33*, 439-440.

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

Kramer, A., Schwebke, I., & Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infectious Diseases, 6*, 130.

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*, 141-147.

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

Lopman, B. A., Steele, D., Kirkwood, C. D., & Parashar, U. D. (2016). The vast and varied global burden of norovirus: Prospects for prevention and control. *PLoS Medicine, 13*, e1001999.

Matthews, J., Dickey, B., Miller, R., Felzer, J., Dawson, B., Lee, A., Moe, C. (2012). The epidemiology of published norovirus outbreaks: A review of risk factors associated with attack rate and genogroup. *Epidemiology and Infection, 140*, 1161-1172.

Mormann, S., Heißenberg, C., Pfannebecker, J., & Becker, B. (2015). Tenacity of human norovirus and the surrogates feline calicivirus and murine norovirus during long-term storage on common nonporous food contact surfaces. *Journal of Food Protection, 78*, 224-229.

Rockx, B., Wit, M. D., Vennema, H., Vinjé, J., Bruin, E. D., Duynhoven, Y. V., & Koopmans, M. (2002). Natural history of human calicivirus infection: A prospective cohort study. *Clinical Infectious Diseases, 35*, 246-253.

Ronnqvist, M., & Maunula, L. (2016). Noroviruses on surfaces: Detection, persistence, disinfection and role in environmental transmission. *Future Virology, 11*, 207-217.

Rzeżutka, A., & Cook, N. (2004). Survival of human enteric viruses in the environment and food. *FEMS Microbiology Reviews, 28*, 441-453.

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

Schwartz, T., Hoffmann, S., & Obst, U. (2003). Formation of natural biofilms during chlorine dioxide and UV disinfection in a public drinking water distribution system. *Journal of Applied Microbiology, 95*, 591-601.

Sobsey, M. D., & Meschke, J. S. (2003). Virus survival in the environment with special attention to survival in sewage droplets and other environmental media of fecal or respiratory origin. *Report for the World Health Organization,* 1-70.

Sukhrie, F. H. A., Teunis, P., Vennema, H., Copra, C., Thijs Beersma, M. F. C., Bogerman, J., & Koopmans, M. (2012). Nosocomial transmission of norovirus is mainly caused by symptomatic cases. *Clinical Infectious Diseases, 54*, 931-937.

Tung-Thompson, G., Gentry-Shields, J., Fraser, A., & Jaykus, L. (2015). Persistence of human norovirus RT-qPCR signals in simulated gastric fluid. *Food and Environmental Virology, 7*, 32-40.

Verhoef, L., Gutierrez, G. J., Koopmans, M., & Boxman, I. L. (2013). Reported behavior, knowledge and awareness toward the potential for norovirus transmission by food handlers in Dutch catering companies and institutional settings in relation to the prevalence of norovirus. *Food Control, 34*, 420-427.

Wikswo, M. E., Kambhampati, A., Shioda, K., Walsh, K. A., Bowen, A., & Hall, A. J. (2015). Outbreaks of acute gastroenteritis transmitted by person-to-person contact, environmental contamination, and unknown modes of transmission--United States, 2009-2013. *MMWR Rep: Surveillance Summaries, 64*, 1-12.

Yeargin, T., Fraser, A., Huang, G., & Jiang, X. (2015). Recovery and disinfection of two human norovirus surrogates, feline calicivirus and murine norovirus, from hard nonporous and soft porous surfaces. *Journal of Food Protection, 78*, 1842-1850.

Chapter 2: Sampling Methods for Recovery of Human Enteric Viruses from Environmental Surfaces

Abstract

Acute gastroenteritis causes the second highest infectious disease burden worldwide. Human enteric viruses have been identified as leading causative agents of acute gastroenteritis as well as foodborne illnesses in the U.S. and are generally transmitted by fecal-oral contamination. There is growing evidence of transmission occurring via contaminated fomite including food contact surfaces. Additionally, human enteric viruses have been shown to remain infectious on fomites over prolonged periods of time. To better understand viral persistence, there is a need for more studies to investigate this phenomenon. Therefore, optimization of surface sampling methods is essential to aid in understanding environmental contamination to ensure proper preventative measures are being applied. In general, surface sampling studies are limited and highly variable among recovery efficiencies and research parameters used (e.g., virus type/density, surface type, elution buffers, tools). This review aims to discuss the various factors impacting surface sampling of viruses from fomites and to explore how researchers could move towards a more sensitive and standard sampling method.

1. Introduction

Acute gastroenteritis causes the second highest infectious disease burden worldwide with an estimated 1.45 million deaths per year (Ahmed et al., 2014). In the United States alone, acute gastroenteritis causes 178.8 million illnesses, 473,832 hospitalizations, and 5,072 deaths (Scallan et al., 2011). There are approximately 31 major pathogenic agents known to cause acute gastroenteritis and/or foodborne illness including human enteric viruses such as astrovirus, rotavirus, hepatitis A virus (HAV), and human norovirus (hNoV) (Scallan et al., 2011). The most common enteric viruses that cause foodborne illnesses are hNoVs and HAV (Cliver, 1997; Koopmans and Duizer, 2004).

Generally, viral acute gastroenteritis is transmitted through food and water contamination, contaminated environmental surfaces, direct person-to-person contact, and other unknown sources (Wikswo et al., 2015). Furthermore, enteric viruses are spread by fecal-oral contamination, and there is growing evidence of viral transmission occurring through contaminated fomites in a variety of ways and settings including food preparation environments (Boone and Gerba, 2007; Rzezutka and Cook, 2004). Enteric viruses have been shown to maintain infectivity on fomites over prolonged periods of time (Escudero et al., 2012). For instance, seminal research by Kiselva et al. (1968) reported on the survival of echovirus, coxsackievirus, and poliovirus on representative surfaces (painted wood, glass, cotton fabric) in households and showed that these viruses maintained infectivity for two to more than 12 days. Human norovirus survival for up to 12 days has also been reported on carpets subject to vomiting episodes after an initial outbreak in a hospital ward (Cheesbrough et al., 1997). There are some studies focusing on the role of fomites and environmental contamination in the transmission of

enteric viruses; however this specific route of transmission is difficult to determine during outbreaks (Rzezutka and Cook, 2004).

To better understand the role of environmental surface transmission during outbreaks due to human enteric viruses, the persistence of viruses on various surface types must be investigated. To do this, a surface sampling method must be applied for recovery of viruses. For instance, understanding the persistence of human enteric viruses on inanimate fomite surfaces in relation to various environmental conditions could provide insight on ways to limit and prevent virus transmission and subsequent outbreaks. However, studies on surface sampling techniques are typically limited to swabs for application in environmental sampling during foodborne outbreaks or for investigation of baseline virus prevalence. As a result, information is lacking on evaluating tools used in laboratory sampling studies for the optimal recovery of viruses. Thus, this review aims to: (1) discuss and compare evaluations of surface sampling methods for optimal recovery of human enteric viruses from inanimate fomite surfaces and (2) explore how researchers could move towards one standard methodology for surface sampling of human enteric viruses and their surrogates.

2. Background

The most common foodborne viruses are categorized based on the type of disease they cause: (1) gastroenteritis (e.g. rotavirus, hNoV, Aichi virus A, coronavirus, and others), (2) enterically transmitted hepatitis viruses (e.g. hepatitis E and A), and (3) viruses that replicate in the human gut then migrate to other organs to cause disease (e.g. poliovirus) (Koopmans and Duizer, 2004). Enteric viruses are typically spread by vomiting or shedding into the stool and have a greater chance of transmission the longer the virus is able to survive outside the host. This survival is

impacted by various environmental conditions such as pH, moisture, and temperature (Koopmans and Duizer, 2004; Rzezutka and Cook, 2004).

2.1 Enteric virus transmission due to environmental surface contamination

As indicated previously, enteric viruses have been shown to maintain infectivity on surfaces over prolonged periods. Human noroviruses have been detected on a variety of surfaces including cellular phones, public phones, televisions, chairs, keyboards, microwave ovens, bathroom light switches, various handles and knobs of kitchen and bathroom items, bed frames, and chairs (Boxman et al. 2011; Gallimore et al., 2006. 2008). Boxman et al. (2011) reported year round prevalence of hNoVs on environmental surfaces of catering facilities even without a recently reported outbreak of acute gastroenteritis. The authors reported that hNoV was recovered from 61.1% of catering settings with recent outbreaks in contrast to only 4.2% of catering settings without a recent outbreak. Elderly homes and pension/hotels catering company types had the highest prevalence of positive swab samples for hNoVs (Boxman et al., 2011). Moreover, multiple studies have shown institutional settings such as cafeterias and long-term facilities are more likely to have hNoVs on surfaces compared to food service settings (Boxman et al., 2011; Hall et al., 2014; Verhoef et al., 2013).

2.2 Current standard methods for surface sampling and analysis

For environmental surface sampling, the International Organization of Standardization (2017) recommends swabbing with a sterile cotton swab presoaked in PBS followed by RNA extraction and reverse transcription, real time PCR (RT-qPCR) analysis for HAV and hNoV sampling and detection on nonporous FCS. In the U.S., there is not a standardized method

available. However, the Centers for Disease Control and Prevention (CDC; 2012) does recommend the use of swabs for obtaining norovirus from environmental surfaces; however, the CDC has also reported that swabbing is highly variable and that the interpretation of results should be conducted with caution.

Currently, hNoVs are most often detected by RT-qPCR due to its high sensitivity and low detection limits using measurements such as PCR amplifiable units (PCRU/ml). These PCRUs are determined by a standard curve produced from a 10-fold dilution series of the virus where one PCRU corresponds to the highest dilution with a quantifiable RT-qPCR value (or cycle threshold [C_T] value) (Knight et al. 2013; Tung et al. 2013). However, Knight et al. (2013) pointed out that the determination of PCRUs in correspondence to specific C_T values is dependent on the sample matrix and the standard used. Moreover, the cut-off C_T values (i.e. endpoint of detection) for hNoVs also vary across studies ranging from 32 to 40 (Knight et al., 2013). The presence of inhibitory components within some sample matrices could impact amplification efficiencies especially in contaminated food and environmental samples that typically have low viral loads (Knight et al., 2013; Sair et al., 2002). Regardless, RT-qPCR is primarily chosen for the analysis of viruses in environmental and food samples to allow for increased sensitivity to detect low viral concentrations that are typically present (Knight et al. 2013). However, as the authors of the review indicated, this method cannot determine infectivity since it may recognize intact or degraded viral nucleic acid, nonviable viruses, or defective viral particles (Knight et al. 2013). Consequently, the use of surrogates and other infectivity assays remain important in investigating enteric viral viability and infectivity in lab-based studies as further discussed in Section 2.3.2.

2.3 Factors impacting recovery of viruses from surfaces

Virus density, the rate of positive environmental samples of total samples collected, and exposure magnitude provide information about virus contamination on surfaces (Julian et al. 2011). However, these factors are impacted by the surface sampling method and detection assay selected. Subsection 2.2.1 to 2.2.5 will examine the variability among the many factors impacting recovery of viruses from surfaces, specifically surface type, virus type/density, drying time, elution buffers, and implement/recovery tool selection.

2.3.1 Surface type

Fomites are generally categorized as either nonporous or porous. Examples of nonporous surfaces are ceramic, glass, acrylic, and stainless steel, and examples of porous surfaces include carpets, lettuce, deli meats, wood, latex, and fruits. Surface type has been shown to have some effect on surface sampling recovery efficiencies (Table 1). Tung-Thompson et al. (2017) swabbed foods (cheese, apple, green pepper, tomato) and hard surfaces (stainless steel and ceramic) with wipes that were inoculated with 10 µl of varying PCR-units (PCRU)/ml of hNoV GII.4. The study obtained a mean range recovery efficiency of 74% to approximately 100% for all surfaces except for cheese, which was significantly different from the other surfaces with 29% to 69% recovery for high inoculum levels (10^4 to 10^6 PCRU) and no detection at low inoculum levels (10^2 to 10^3 PCRU) (Tung-Thompson et al., 2017). The authors were not able to determine if the lipid content of the cheese contributed to the possible absorption and recovery of the virus samples even though a previous study suggested this possibility for hNoVs (Fumain et al., 2009; Tung-Thompson et al., 2017).

Furthermore, surface properties can also impact recovery efficiencies in a variety of ways. For instance, stainless steel is a hydrophilic(contact angle of 58.2° in water, surface energy of 50.3 mJ/m^2) and negatively charged surface in which microorganisms have been shown to develop irreversible attachment within one minute potentially making surface recovery more difficult (Mafu et al., 1990; Mafu et al., 1991). The orientation of a surface could interfere with adequate surface sampling and collection as seen in a study involving vertical and horizontal stainless steel surfaces. Taku et al. (2002) determined that greater recovery efficiency could be obtained by allowing the elution buffer to sit on the surface for 15 min—something that cannot be performed on a vertical surface. The mean recovery for horizontal surfaces and sinks using the cell scraper-aspiration method ranged from 32% to 71% while vertical stainless steel surfaces only obtained a mean recovery of 11% since the buffer was not in contact with the surface long enough to facilitate virus recovery (Taku et al., 2002). Scherer et al. (2009) suggested physical properties of nonporous and porous could reduce virus recovery via trapping virus particles within the matrix/crevices or facilitate enhanced virus recovery by smooth/porous surfaces. Mattison et al. (2007) suggested the low mean recovery of feline calicivirus (FCV) from strawberries might be due to its surface texture and how the crevices may shield viruses against environmental conditions. Furthermore, the authors observed a pH change in the elution buffer from 7.2 to 5.5 when strawberries were immersed, which could impact virus recovery by either partial viral inactivation or interference with FCV recovery (Mattison et al., 2007). Overall, physical and chemical properties of nonporous and porous food and food contact surfaces could impact recovery efficiencies of enteric viruses. This review will focus on surface sampling techniques for enteric viruses from nonporous, inanimate surfaces.

2.3.2 Virus type and density

Virus type and density may have varying effects on surface sampling techniques and recovery efficiencies. Traditionally, surrogates including murine norovirus strain type 1 (MNV-1), MS2 bacteriophage, Tulane virus (TuV), and FCV have been used for infectivity studies related to hNoVs (Rönnqvist and Maunula 2016). There has not been an *in vitro* cell culture system for hNoVs available until recently (Ettayebi et al., 2016), and until reproducible and readily available infectivity assays are developed, surrogates still provide much needed information on infectivity of hNoVs. Multiple surrogates are important for understanding infectivity due to variations in their genetic relatedness to hNoVs and the diversity among hNoV genotypes. Other cultivable viruses utilized in environmental persistence research include Aichivirus A (AiV) and HAV—both known human enteric pathogens (Cannon et al. 2006; Koopmans and Duizer 2004; Yeargin et al. 2015). Diversity among hNoV genotypes could impact the recovery efficiency from surfaces; however, studies focus mainly on hNoV GII.4 (Table 1). This focus is a result of GII.4 being the pandemic genotype of hNoV and accounting for over 80% of all hNoV outbreaks in the U.S. since 1996 (Glass et al. 2009). Surrogates provide essential information on hNoV infectivity in relation to viral persistence on food contact surfaces (FCS), and numerous studies have shown FCV, MNV, and TuV to remain infectious on multiple surfaces for at least 7 days or more (Arthur and Gibson 2015; Fallahi and Mattison 2011; Mattison et al. 2007).

Some studies have compared the recovery efficiency between different types of enteric viruses. Scherer et al. (2009) compared hNoV GII.3 and rotavirus recovery efficiencies using a cotton swab from various porous and nonporous FCS. Table 1 shows the recovery varied between virus types for a given surface. For instance, Scherer et al. (2009) reported the highest

percentage of hNoV was recovered on ceramic (31-52%) while rotavirus was recovered at a slightly higher percentage (46-58%) on the same surface. The authors suggested the varying recovery rates observed between the two enteric viruses may be due to the abilities of the different viruses to adhere to the various surfaces as well as differences in virus properties affecting attachment (Scherer et al., 2009). A greater variety of surrogates and enteric viruses need to be evaluated for surface sampling to ensure accurate prevention and detection methods are being implemented.

Virus density could also impact the amount of virus recovered from a given surface. In general, higher starting densities of viruses equal greater recovery efficiencies—primarily due to the limit of detection of the downstream assay. Tung-Thompson et al. (2017) reported recovery efficiency variability by virus density when using wipes on food and nonporous food contact surfaces. The authors showed that recovery was consistent at high inoculum levels (10^4-10^6 PCRU/ml) of GII.4 while more variability was observed at lower inoculum levels (10^2-10^3 PCRU/ml). In contrast, Rönnqvist et al. (2013) also reported variability among lower concentrations of GII.4 with higher mean recoveries for hNoV GII.4 at 10^2 PCRU than 10^3 PCRU when evaluating four different swabs on environmental surfaces. For 10^3 PCRU of GII.4, there was no significance difference for recovery efficiency among the swabs evaluated except on latex surfaces with polyester swabs regardless of buffer type. Meanwhile, microfiber swabs combined with glycine buffer for elution was found to be a significantly better recovery method for 10^2 PCRU of GII.4 on all the surfaces (Rönnqvist et al. 2013). Scherer et al. (2009) reported that the mean recovery efficiencies for rotavirus and hNoV GII.3 were higher from various nonporous and porous surfaces using a cotton swab-rinse method at higher inoculum levels (2×10^5 PCRU for hNoV; 2×10^4 PCRU for rotavirus) than lower inoculum levels (2×10^4 PCRU

for hNoV; 2×10^3 PCRU for rotavirus). The authors also mentioned how reverse transcription became less efficient at low inoculum levels resulting in an increase in statistical errors. Overall, the higher the inoculum level for all enteric viruses, the higher the mean recovery rate regardless of the variability among methods, virus type, and high standard deviations of the mean recovery rates.

Additionally, organic matter such as coagulated food and other debris while on environmental surfaces may impact the effect of virus density on recovery efficiency. For instance, fatty foods such as cheese have been known to contribute to absorption and recovery of virus samples for hNoVs due to lipid content (Fumain et al. 2009). Furthermore, Abad et al. (1994) studied the effect of fecal matter on the persistence of enteric viruses and reported varying results between virus types and fomites. The authors found no effect on the persistence of HAV and human rotavirus with the exception of longer persistence of HAV on latex surfaces. Overall, Abad et al. (1994) observed longer persistence for adenovirus and poliovirus on nonporous fomites (china, glazed tile, aluminum, and latex, and a decrease in persistence of adenovirus and poliovirus on porous fomites (cotton cloth and paper).

For hNoVs, the preparation of stool samples (i.e. because hNoV does not have a routine culture method) is not always specifically stated in studies on virus persistence and recovery from surfaces. For example, Park et al. (2015) include a clarification step—a brief centrifugation to separate the large particulates from the viruses in 10% fecal suspensions—while others (De Keuckelaere et al. 2014; Ronnqvist et al. 2013) use hNoVs in the original 10% fecal suspension for their studies. The presence or absence of organic matter can certainly impact both virus persistence and recovery; however, it should also be noted that the presence of organic matter could also impact downstream analysis such as RT-qPCR via inhibition (Wilson 1997), also

indicated in Section 2.2. Even though virus persistence and recovery from food matrices are not within the scope of this review, enteric virus recovery from nonporous environmental surfaces as a function of particle association (e.g., food and debris) is lacking and does need further study.

2.3.3 Drying time

Drying time for enteric virus surface sampling is highly variable and dependent on factors including volume of virus suspension and desiccation (Table 1). Drying times range from 15 min to overnight at ambient conditions with volumes ranging from 5 µl to 100 µl. Drying time impacts the recovery efficiencies of surface sampling methods, and generally, the longer a virus is on a surface, the harder it is to recover the virus from the surface. Mattison et al. (2007) tested recovery of FCV from stainless steel surfaces using vortexing at 30 min post inoculation versus immediate recovery after inoculation of 3.0×10^5 FCV in 10 µl. The difference in recovery between elution immediately following and after 30 min of drying was 33 and 11%, respectively—a three-fold difference. While this review is focused on FCS and not food, the authors did note that the difference between viral recovery from lettuce and stainless steel may be due to viruses being more influenced by the effects of air drying when on a flat nonporous surface. Park et al. (2015) observed a reduction in the recovery efficiency of hNoV GII.4 from stainless steel and toilet representative surfaces as a function of drying time. On stainless steel surfaces using macrofoam swabs, the recovery efficiency was 43.5% ± 21.4% without drying, 25.7% ± 10.6% at 1 h, 18.2% to 25.7% ≤ 24 h, and 10.0% ± 2.3% after 48 h (Park et al. 2015). Based on the evidence presented above, there is a need for uniformity among studies and standardization in drying time and inoculum amount in order to properly evaluate virus recovery and surface sampling methods.

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2.3.4 Type of elution buffers

The recovery efficiencies for the numerous eluent-tool combinations are variable and often impacted by both intrinsic factors related to the actual tool and eluent types as well as the extrinsic factors already introduced (Sections 2.3.1-2.3.3). The differences in eluent formulations such as pH, salinity, and use of a surfactant can impact the recovery efficiency of viruses from surfaces. Ionic strength and pH of eluents have been known to impact the net charge of viral particles (Gerba, 1984). Rönnqvist et al. (2013) obtained slightly higher recovery efficiencies using an alkaline glycine buffer (pH 9.5) than eluting with PBS (pH 7.2). Conversely, Taku et al. (2002) recovered more FCV from stainless steel surfaces using a slightly acidic glycine buffer (pH 6.5) with a mean recovery of 42% compared to 28 and 10% recovery using glycine buffer (pH 9.5) or culture medium (pH 7.2), respectively.

Surfactants are another common component added to elution buffers. These are known to increase the water content of the surface, assist in solubilization of proteins and cells from the surface, and can disrupt hydrophobic interactions between charged viruses and surfaces thus enhancing virus recovery (Farrah 1982; Lukasik et al. 2000; Moore and Griffith 2007). Park et al. (2015) suggested that adding a surfactant (0.02% Tween 80) to the PBS elution buffer of a swab rinse protocol enhanced viral recovery efficiency of hNoV GII.4 even though no significance was observed. Meanwhile, another study found higher recovery of hNoV GII.7 and mengovirus from laminated wooden surfaces when using lysis buffer compared to 100 mM Tris-HCl – 50 mM glycine -1.5% beef extract (TGBE, pH 9.5); however, again no significance difference was observed (Ibfelt et al. 2016).

For MS2 recovery, two separate studies found the eluent type to not be significantly different (Casanova et al. 2009; Julian et al 2011). Furthermore, eluent type for MS2 recovery was

suggested to be selected based on experimental design such as considering eluents compatible

with nucleic acid extraction for molecular detection-based sampling studies or with tissue culture

for infectivity-based studies (Julian et al. 2011). Moreover, Rönnqvist et al. (2013) suggested an

elution buffer be selected based on the specific situation with the consideration of factors such as

the time elapsed between swabbing and sample analysis. Overall, eluent type can impact viral

recovery, and thus eluent-tool combinations must be chosen with consideration of surface, virus,

and eluent interactions for efficient surface sampling and recovery. Therefore, a matrix of elution

buffers and when to apply given a certain situation or parameters would be a valuable resource.

2.3.5 Recovery tool options

The majority of tools used in laboratory-based studies for evaluation of surface sampling

methods have focused on various types of swabs (Table 1). This finding comes as no surprise

since swabbing is known as the gold standard for hNoV sampling and detection on FCS (ISO,

2017). Evaluation of swabs has shown varying recovery rates for enteric viruses; however, while

the swab itself may be the primary driver in recovery, numerous other factors can play a role as

indicated previously. More specifically, the material and properties of the recovery tool can

impact recovery efficiencies. For example, the dying process of microfiber cloths can change its

net surface charge, which could impact viral attachment and detachment from surfaces

(Rönnqvist et al. 2013). Taku et al. (2002) suggested the selection of swabs are due to the ease of

operation over small surface areas even though swabs yield consistently poor results in

comparison to other methods evaluated, possibly due to surface area of the swab head and

smearing virus over surfaces. Macrofoam, polyester-tipped, and/or cotton swabs have been

shown to be more efficient among swabs tested in viral recovery from fomites depending on a

given study's conditions and parameters (Ibfelt et al. 2016; Julian et al. 2011; Scherer et al. 2009). For instance, Julian et al. (2011) reported that polyester-tipped swabs recovered a greater amount of infectious MS2 than antistatic cloths. However, as indicated in Section 2.3.4, the elution buffer and tool combination complicates matters. For instance, Rönnqvist et al. (2013) reported that elution buffer type only impacted the recovery efficiency of microfiber cloths composed of polyester and polyamide materials where 50 mM glycine buffer (pH 9.5) performed better than PBS. Additionally, the authors reported better recovery of low inoculum hNoV GII.4 on latex surfaces when using polyester swabs, though it is unclear why. Unfortunately, it is difficult to compare swab types across studies due to differences among surface types, virus types, virus volume, and virus concentrations used for the evaluations of the swab sampling protocols.

3. Methods for recovery of enteric viruses from surfaces

As evidenced by Table 1, surface sampling methods used in the recovery of enteric viruses are highly variable and diverse. A majority of studies focus on swabbing for a variety of reasons. In fact, the International Organization of Standardization (2017) recommends hNoV sampling and detection on nonporous FCS to be collected with a cotton swab moistened with PBS followed by RNA extraction and reverse transcription – quantitative PCR (RT-qPCR) analysis. Other tools and methods such as repeated pipetting, cell scraping, and sonication/stomaching have been used for viral persistence and disinfection studies (Arthur and Gibson 2015; Fallahi and Mattison 2011; Yeargin et al. 2015).

3.1 Outbreak sampling techniques – Swabbing

Studies involving environmental surface sampling for applications in detecting viruses during outbreaks can be used as a baseline for standard surface sampling techniques for enteric viruses. Swabbing is the technique typically used for enteric virus studies involving applications in detection of viruses during outbreaks. Thus, studies have focused on evaluating swab protocols on surfaces associated with outbreaks such as on cruise ships and FCS (Table 1). Rönnqvist et al. (2013) evaluated four swab types (e.g. flocked nylon, cotton wool, microfiber, and polyester) in either PBS or glycine buffer at pH 9.5 for collecting hNoV GII.4 from stainless steel and plastic surfaces. Park et al. (2015) evaluated five swab types (e.g. cotton, rayon, polyester, antistatic cloth, and macrofoam) using hNoV GII.4 from stainless steel and toilet representative surfaces with macrofoam swabs producing the highest recovery efficiencies. During comparison of these two studies, microfiber performed better than macrofoam swabs with 79.0% ± 10.2% and 25.7% ± 10.6% recovery efficiency, respectively, when elution buffer (glycine buffer) and surface type (stainless steel) were the same. However, the amount and concentration of hNoV GII.4 varies between the two studies, and this could also impact recovery efficiencies as reviewed in Section 2.3.2. Rönnqvist et al. (2013) also provides information on using swabs on plastic surfaces. Overall, there is a need for more studies involving more viruses and nonporous surfaces to properly determine a standardized approach for surface sampling of enteric viruses during outbreaks.

3.2 Laboratory-based techniques for persistence and surface disinfection studies

Several different methods have been used to optimize recovery of enteric viruses from inanimate fomites in laboratory-based persistence studies. Furthermore, differences among the

studies include virus types, volume and concentration of virus as well as tools, FCS, and type of analysis. In this subsection, we will further examine these differences and how they could contribute to the varying results of surface sampling method evaluation studies. Summaries of these studies are available in Table 1.

3.2.1 Swabbing

As stated in Section 3.1, swabbing has traditionally been the focus in studies on virus detection and persistence (Table 1). A few studies focused on evaluating one swab implement for use in recovering enteric viruses from a variety of surface types and virus inoculum levels. Scherer et al. (2009) evaluated a cotton swab with PBS (pH 7.2) elution buffer for collecting hNoV GII.3 and rotavirus from different FCS (i.e. stainless steel, ceramic, high-density polyethylene, and wooden chopping board) with recovery efficiencies ranging from $10.3 \pm 13.0\%$ (wood, 10^4 PCRU) to $51.9 \pm 38.5\%$ (ceramic, 10^5 PCRU) for GII.3 and $5.4 \pm 1.5\%$ (wood, 10^2 $TCID_{50}$) to $57.7 \pm 25.9\%$ (ceramic, 10^3 $TCID_{50}$) for rotavirus. The authors found recoveries for both hNoV and rotavirus to be higher from FCS than food surfaces at both inoculum concentrations (Scherer et al., 2009). Additionally, Ganime et al. (2015) evaluated the recovery rates of MNV-1 and bacteriophage PP7 from porous formic, non-porous formic, and rubberized surfaces using a rayon swab with culture media with recovery efficiencies ranging from 0.6 to 11.5% (PP7) and 12.2 to 77.0% (MNV-1). While these two studies evaluate how one particular swab performs, other studies expand their evaluations to provide a better comparison of different swabs and tools and their recovery of particular enteric viruses.

For example, Ibfelt et al. (2016) evaluated three different swabs (i.e. cotton, foamed cotton, and polyester) and two elution buffers (i.e. direct lysis or alkaline TGBE – pH 9.5) for recovery

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of hNoV GII.7 and mengovirus from 100 cm^2 laminated wooden surfaces. The authors found a significantly better virus recovery using polyester swabs with the direct lysis in comparison to other combinations tested; however, recovery efficiencies were ≤13% for all combinations. Ibfelt and others (2016) suggested their low recovery rates may be due to the size of the surface or differences in experimental design in comparison to other swab studies. Furthermore, Julian et al. (2011) also recommended the use of polyester swabs pre-moistened in either Ringer's or 0.85% saline solution for MS2 recovery from plastic and stainless steel surfaces following evaluation of three tools (cotton swab, polyester swab, and antistatic cloth) and four elution buffers (saline, Ringer's solution, viral transport media, and acid/base). Based on a meta-analysis of MS2 surface sampling, the authors noted that polyester swabs obtained significantly higher positive MS2 rates in comparison to rayon and cotton (Julian et al., 2011).

Conversely, De Keuckelaere et al. (2014) found cotton and polyester swabs to not be significantly different in their recovery efficiencies of hNoVs GI.4 and GII.4 from nitrile gloves, polyethylene, or neoprene rubber surfaces. Park et al. (2015) reported a similar result when evaluating the recovery efficiencies of four swab types (macrofoam, rayon, cotton, and polyester). The authors applied the different swabs for recovery of hNoV GII.4 from stainless steel and toilet representative surfaces and found that rayon, cotton, and polyester were not significantly different. However, macrofoam swabs obtained significantly higher recovery efficiencies of hNoV GII.4 in comparison to the other three swabs after 8 h of drying on a given surface (Park et al., 2015). Additionally, some studies found other tools and methods such as biowipes and cell scraper-aspiration methods to be potentially more efficient for enteric virus recovery from surfaces in comparison to cotton and/or polyester swabs. These studies are further examined in Sections 3.2.2 and 3.2.3 (De Keuckelaere et al., 2014; Taku et al., 2002).

3.2.2 Cloths and wipes

Cloths and wipes have also been introduced as possible alternatives to swabbing methods for obtaining higher recovery efficiencies of enteric viruses from surfaces. De Keuckalaere et al. (2014) evaluated two swabs (cotton and polyester) along with biowipes (Biomérieux, Lyon, France) composed of a mixture of fibers and microfibers (cotton, polyester, and polyamide fibers) moistened in PBS (pH 8.0) by recovering GI.4 and GII.4 hNoVs from FCS (high-density polyethylene, nitrile gloves, and neoprene rubber). There was no significant difference among any of the three tools evaluated based on recovery efficiency from polyethylene surfaces and nitrile gloves for hNoV GI.4. Meanwhile, the authors found significantly higher recovery efficiencies using biowipes (41.3 ± 12.4%) compared to cotton swabs (13.2 ± 5.2%) on the coarser rubber surface (De Keuckelaere et al., 2014). The authors also found that the mean recovery efficiency of biowipes for GI.4 from rubber surfaces was higher than using polyester swabs even though no significant difference was observed. For hNoV GII.4, there was no significant difference in recovery observed between all three tools tested on polyethylene surfaces and nitrile gloves even though the biowipes had significantly higher recovery efficiency (56.1 ± 12.5%) on rubber surfaces compared with both polyester (22.5 ± 8.7%) and cotton (16.9 ± 6.6%) swabs (De Keuckelaere et al., 2014). Another study further confirmed the effectiveness of these biowipes in collecting hNoV GII.4 at various inoculum concentrations (10^2 to 10^6 PCRU) from stainless steel and ceramic FCS (Tung-Thompson et al., 2017). The authors reported a range of mean recovery efficiencies of GII.4 using biowipes (bioMerieux SA, Grenoble, France): 76.8 to 99.3% (stainless steel) and 42.4 to 96.6% (ceramic). It should be noted that recovery efficiencies reported by Tung-Thompson et al. (2017) were generally much higher than other studies included in Table 1.

However, a few studies showed certain swabs to be more efficient for recovery of enteric viruses than cloths. For example, macrofoam swabs had a higher recovery efficiency of hNoV GII.4 (7.08 ± 2.21%) from large (161.3 cm^2) stainless steel surfaces than antistatic cloths (0.33 ± 0.21%) (Park et al., 2015). Additionally, Julian et al. (2011) determined that polyester swabs obtained higher recoveries of infectious MS2 than antistatic cloths as well. Overall, cloths and wipes may be a valuable tool for collecting enteric viruses from FCS, and there is a need for further studies using cloths and wipes involving a greater variety of virus types, cloth types, surface types, and infectivity analyses.

3.2.3   Alternative methods for laboratory-based studies

Other surface sampling methods such as vortexing, repeated pipetting, stomaching/sonication, and cell scraping have been used for baseline information for viral persistence studies and disinfection studies (Table 1). The studies summarized in the Table 1 use different surrogates, initial drying times, and elution buffers making it difficult to adequately compare the studies. Fallahi and Mattison (2011) recovered 37% of MNV-1 from stainless steel after a 20 min drying time using a repeated pipetting method with EBSS eluent. Mattison et al. (2007) recovered 11% of FCV from stainless steel after a 30 min drying time by vortexing for 30 s in EBSS eluent. Arthur and Gibson (2015) obtained recovery efficiencies of 10% and 30% for TuV from acrylic and stainless steel surfaces, respectively, after a drying time of 1 h using a cell scraping techniques. The cell scraping technique was confirmed as possible with TuV and has also been evaluated using FCV previously (Taku et al. 2002). Taku et al. (2002) found consistently better mean virus efficiencies for FCV using 50 mM glycine (pH 6.5) from stainless steel surfaces in comparison to 50 mM glycine (pH 9.5) and Modified Eagle's medium (pH 7.2)

using the scraping-aspiration method. The mean FCV recovery efficiencies for the scraping-aspiration method from stainless steel were reported to be 42% (glycine pH 6.5), 28% (glycine pH 9.5), and 10% (Modified Eagle's medium). The authors suggested the modified Eagle's medium complex composition may have played a role in being less efficient than the glycine buffers (Taku et al., 2002). Taku et al (2002) added cell scraping to the aspiration method for better recovery efficiencies speculating that cell scraping may facilitate release of virus from surface. In addition, Yeargin et al. (2015) recovered a range of 0.15% (cotton) to 35.22% (glass) for FCV and 0.85% (cotton) to 24.27% (glass) for MNV-1 from three surface types (i.e. polyester, cotton, and glass) using a stomaching/sonication method. The authors also found the recovery efficiencies to be highest for glass and lowest for polyester and cotton for both virus types. The recovery efficiencies were also reported to be significantly different among all surface types for the same virus type while only cotton swab recoveries showed a significant difference between MNV-1 and FCV (Yeargin et al., 2015). Similar to other techniques, more studies with inclusion of more virus types and standardized drying times are needed to provide information on using these alternative techniques for future persistence and environmental sampling studies.

4. Conclusions and recommendations

Surface sampling of enteric viruses varies across studies throughout the literature. This variability in results may exist due to varying human behavior, the tool used, and/or the elution buffer type used to recover the virus from the surface as well as numerous other factors outlined in the present review. Most surface sampling evaluations have focused on various swab types while there are limited studies focused on evaluation of other possible tools and techniques such as repeated pipetting and cell scraper application, historically used in a laboratory setting. As a

result, food and environmental virology researchers may have difficulty in selecting the most appropriate surface sampling method for a particular study. Additionally, we found that no single standard approach to recover enteric viruses from FCS exists.

The following suggestions are based on our review to assist researchers in moving towards one standard methodology for optimizing the recovery of enteric viruses from fomite surfaces:

- Eluent buffer used to recover sample needs to be standardized.

- Concentrations and volumes of virus need to be more consistent and include standard low and high inoculum levels.

- The impact of organic materials on enteric virus recovery from surfaces needs further investigation.

- Infectivity assays such as plaque assays are highly recommended for the analysis of surface sampling optimization in order to distinguish infectious particles from non-infectious viral particles. However, this is currently only possible with cultivable viruses and hNoV surrogates.

- Results need to be reported in one standard form of measurement.

- More techniques and tools need to be evaluated along with the swab protocols and these evaluations should include of a variety of human enteric viruses and their surrogates.

Acknowledgments

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References

Abad, F.X., Pinto, R.M., Bosch, A., 1994. Survival of enteric viruses on environmental fomites. App. Environ. Microbiol. 60, 3704-3710.

Ahmed, S.M., Hall, A.J., Robinson, A.E., Verhoef, L., Premkumar, P., Parashar, U.D., Koopmans, M., Lopman, B.A., 2014. Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. Lancet Infect. Dis. 14, 725−730.

Arthur, S.E., Gibson, K.E., 2015. Environmental persistence of Tulane virus: a surrogate for human norovirus. Can. J. Microbiol. 62, 449−454.

Boone, S.A., Gerba, C.P., 2007. Significance of fomites in the spread of respiratory and enteric viral disease. Appl. Environ. Microbiol. 73, 1687−1696.

Boxman, I.L., Verhoef, L., Dijkman, R., Hagele, G., Te Loeke, N.A., Koopmans, M., 2011. Year-round prevalence of norovirus in the environment of catering companies without a recently reported outbreak of gastroenteritis. Appl. Environ. Microbiol. 77, 2968−2974.

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

Casanova, L., Rutala, W.A., Weber, D.J., Sobsey, M.D., 2009. Methods for the recovery of a model virus from healthcare personal protective equipment. J. Appl. Microbiol. 106, 1244−1251.

Cheesbrough, J.S., Barkess-Jones, L., Brown, D.W., 1997. Possible prolonged environmental survival of small round structured viruses. J. Hosp. Infect. 35, 325−326.

Cliver, D.O., 1997. Virus transmission via food. World Health Stat. Q. 50, 90−101.

Centers for Disease Control and Prevention, 2012. Norovirus: Specimen collection. http://www.cdc.gov/norovirus/lab-testing/collection.html (accessed 31.5.17).

De Keuckelaere, A., Stals, A., Uyttendaele, M., 2014. Semi-direct lysis of swabs and evaluation of their efficiencies to recover human noroviruses GI and GII from surfaces. Food Environ. Virol. 6, 132−139.

Escudero, B.I., Rawsthorne, H., Gensel, C., Jaykus, L.A., 2012. Persistence and transferability of noroviruses on and between common surfaces and foods. J. Food Prot. 75, 927−935.

Ettayebi, K., Crawford, S.E., Murakami, K., Broughman, J.R., Karandikar, U., Tenge, V.R., Neill, F.H., Blutt, S.E., Zeng, X.L., Qu, L., Kou, B., Opekun, A.R., Burrin, D., Graham, D.Y., Ramani, S., Atmar, R.L., Estes, M.K., 2016. Replication of human noroviruses in stem cell-derived human enteroids. Science 353, 1387−1393.

Fallahi, S., Mattison, K., 2011. Evaluation of murine norovirus persistence in environments relevant to food production and processing. J. Food Prot. 74, 1847−1851.

Farrah, S.R., 1982. Chemical factors influencing adsorption of bacteriophage MS2 to membrane filters. Appl. Environ. Microbiol. 43, 659−663.

Fumian, T.M., Leite, J.P.G., Marin, V.A., Miagostovich, M.P., 2009. A rapid procedure for detecting noroviruses from cheese and fresh lettuce. J. of Virol. Methods 155, 39-43.

Gallimore, C.I., Taylor, C., Gennery, A.R., Cant, A.J., Galloway, A., Iturriza-Gomara, M., Gray, J.J., 2006. Environmental monitoring for gastroenteric viruses in a pediatric primary immunodeficiency unit. J. Clin. Microbiol. 44, 395−399.

Gallimore, C.I., Taylor, C., Gennery, A.R., Cant, A.J., Galloway, A., Xerry, J., Adigwe, J., Gray, J.J., 2008. Contamination of the hospital environment with gastroenteric viruses: comparison of two pediatric wards over a winter season. J. Clin. Microbiol. 46, 3112−3115.

Ganime, A.C., Leite, J.P.G., de Abreu Corrêa, A., Melgaço, F.G., Carvalho-Costa, F.A., Miagostovich, M.P., 2015. Evaluation of the swab sampling method to recover viruses from fomites. J. Virol. Methods 217, 24–27.

Gerba, C.P. 1984. Applied and theoretical aspects of virus adsorption to surfaces. Adv. Appl. Microbiol. 30, 133−168.

Glass, R.I., Parashar, U.D., Estes, M.K., 2009. Norovirus gastroenteritis. New Eng. J. Med. 361, 1776−1785.

Hall, A.J., Wikswo, M.E., Pringle, K., Gould, L.H., Parashar, U.D., Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, CDC, 2014. Vital signs: foodborne norovirus outbreaks - United States, 2009-2012. MMWR Morb. Mortal. Wkly. Rep. 63, 491−495.

Ibfelt, T., Frandsen, T., Permin, A., Andersen, L.P., Schultz, A.C., 2016. Test and validation of methods to sample and detect human virus from environmental surfaces using norovirus as a model virus. J. Hosp. Infect. 92, 378−384.

ISO, 2017. Microbiology of food and animal feed—horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR—Part 1: Method for Quantification. *International Organization for Standardization, Geneva. CEN ISO/TS*, 15216-1.

Julian, T.R., Tamayo, F.J., Leckie, J.O., Boehm, A.B., 2011. Comparison of surface sampling methods for virus recovery from fomites. Appl. Environ. Microbiol. 77, 6918−6925.

Kiseleva, L.F., 1968. Survival of enteric viruses in water and foodstuffs and on various surfaces. Hyg. Sanit. 33, 439−444.

Knight, A., Li, D., Uyttendaele, M., Jaykus, L.A., 2013. A critical review of methods for detecting human noroviruses and predicting their infectivity. Crit. Rev. in Microbiol. 39, 295-309.

Koopmans, M., Duizer, E., 2004. Foodborne viruses: an emerging problem. Int. J. Food Microbiol. 90, 23−41.

Lukasik, J., Scott, T.M., Andryshak, D., Farrah, S.R., 2000. Influence of salts on virus adsorption to microporous filters. Appl. Environ. Microbiol. 66, 2914−2920.

Mafu, A. A., Roy, D., Goulet, J., Magny P., 1990. Attachment of Listeria monocytogenes to stainless steel, glass, polypropylene, and rubber surfaces after short contact times. J. Food Prot. 53, 742-746.

Mafu, A. A., Roy, D., Goulet, J., Savoie, L., 1991. Characterization of physicochemical forces involved in adhesion of *Listeria monocytogenes* to surfaces. Appl. Environ. Microbiol. 57, 1969−1973.

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. J. Food Prot. 70, 500−503.

Moore, G., Griffith, C., 2007. Problems associated with traditional hygiene swabbing: the need for in-house standardization. J. Appl. Microbiol. 103, 1090−1103.

Park, G.W., Lee, D., Treffiletti, A., Hrsak, M., Shugart, J., Vinje, J., 2015. Evaluation of a new environmental sampling protocol for detection of human norovirus on inanimate surfaces. Appl. Environ. Microbiol. 81, 5987−5992.

Rzezutka, A., Cook, N., 2004. Survival of human enteric viruses in the environment and food. FEMS Microbial. Rev. 28, 441−453.

Rönnqvist, M., Maunula, L. 2016. Noroviruses on surfaces: detection, persistence, disinfection and role in environmental transmission. Future Virol. 11, 207−217.

Rönnqvist, M., Rättö, M., Tuominen, P., Salo, S., Maunula, L., 2013. Swabs as a tool for monitoring the presence of norovirus on environmental surfaces in the food industry. J. Food Prot. 76, 1421−1428.

Sair, A.I., D'Souza, D.H., Moe, C.I., Jaykus, L.A., 2002. Improved detection of human enteric viruses in foods by RT-PCR. J Virol. Methods, 100, 57-69.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. Emerg. Infect. Dis. 17, 7−15.

Scherer, K., Mäde, D., Ellerbroek, L., Schulenburg, J., Johne, R., Klein, G., 2009. Application of a swab sampling method for the detection of norovirus and rotavirus on artificially contaminated food and environmental surfaces. Food Environ. Virol. 1, 42−49.

Taku, A., Gulati, B.R., Allwood, P.B., Palazzi, K., Hedberg, C.W., Goyal, S.M., 2002. Concentration and detection of caliciviruses from food contact surfaces. J. Food Prot. 65, 999−1004.

Tung, G., Macinga, D., Arbogast, J., Jaykus, L.A., 2013. Efficacy of commonly used disinfectants for inactivation of human noroviruses and their surrogates. J. Food Prot. 76, 1210-1217.

Tung-Thompson, G., Escudero-Abarca, B.I., Outlaw, J., Ganee, A., Cassard, S., Mabilat, C., Jaykus, L.A., 2017. Evaluation of a surface sampling method for recovery of human noroviruses prior to detection using reverse transcription quantitative PCR. J. Food Prot. 80, 231−236.

Verhoef, L., Gutierrez, G.J., Koopmans, M., Boxman, I.L.A., 2013. Reported behavior, knowledge and awareness toward the potential for norovirus transmission by food handlers in Dutch catering companies and institutional settings in relation to the prevalence of norovirus. Food Cont. 34, 420−427.

Wikswo, M.E., Kambhampati, A., Shioda, K., Walsh, K.A., Bowen, A., Hall, A.J., Centers for Disease Control and Prevention, 2015. Outbreaks of acute gastroenteritis transmitted by person-to-person contact, environmental contamination, and unknown modes of transmission--United States, 2009-2013. MMWR Surveill. Summ. 64, 1−16.

Wilson, I., 1997. Inhibition and facilitation of nucleic acid amplification. App. Environ. Microbiol. 62, 3741-3751.

Yeargin, T., Fraser, A., Huang, G., Jiang, X., 2015. Recovery and disinfection of two human norovirus surrogates, feline calicivirus and murine norovirus, from hard nonporous and soft porous surfaces. J. Food Prot. 78, 1842−1850.

Table 1: Surface sampling methods for human enteric viruses – nonporous and porous environmental surfaces (not food based).

Virus type	Method(s) evaluated	Surface type(s)	Conditions	Volume and concentration	Buffer Matrix(s)	Analysis	Recovery results	Reference
hNoV GII.4	Biowipes (cotton, polyester, poly-amide fibers)	SS, ceramic	40 min drying time	10 µl of virus of varying PCRU (10^2 to 10^6)	Biowipes moistened in PBS (pH 8.0)	RT-qPCR	SS: 76.8% ± 19.7% (10^4) to 99.3% ± 1.1% (10^3) Ceramic: 42.4% ± 50.8% (10^3) to 96.6% ± 3.4% (10^5)	Tung-Thompson et al. (2017)
hNoV GII.7; mengo-virus (MV)	Swabs (3 types) - Cotton - Foamed cotton - Polyester	Laminated wooden fibreboard	1 h drying time	100 µl of either 10^5 PFU/ml MV or 10^3 GC/ml hNoV in PBS	1) Direct Nuclisens lysis buffer 2) Tris-HCl (100mM) – glycine (50mM)- beef extract buffer	RT-qPCR	Highest recovery = polyester swab with direct lysis Recovery rates of ≤ 13.7% dependent on concentration, virus type, and method used	Ibfelt et al. (2016)

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Table 1: Surface sampling methods for human enteric viruses – nonporous and porous environmental surfaces (not food based).

Virus type	Method(s) evaluated	Surface type(s)	Conditions	Volume and concentration	Buffer Matrix(s)	Analysis	Recovery results	Reference
TuV	Scraping – aspiration	SS, ABSS	Ambient conditions	50 µl of 5×10^4 PFU/ml	450 µl of PBS	PA	SS ~ 30% ABSS ~ 10%	Arthur and Gibson (2015)
Bacterio-phage PP7; MNV-1	Swab (rayon) – rinse protocol	RB, PF, NPF	3 h drying time at ambient conditions (RT; 50-80% RH)	50 µl of 10^3 - 10^6	Culture medium	RT-qPCR	0.6% to 11.5% (PP7) and 12.2% to 77.0% (MNV-1) No significant difference between viruses	Ganime et al. (2015)
hNoV GII.4	Swab (4 types) – rinse protocol	SS	Ambient conditions (RT; 45-60% RH), 1-48 h drying time	50 µl virus suspension	2.5 ml PBST	RT-qPCR	SS (no drying): 16.6% ± 2.3% (polyester) to 43.5% ± 21.4% (macrofoam) - decrease with drying time and surface area	Park et al. (2015)

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Table 1: Surface sampling methods for human enteric viruses – nonporous and porous environmental surfaces (not food based).

Virus type	Method(s) evaluated	Surface type(s)	Conditions	Volume and concentration	Buffer Matrix(s)	Analysis	Recovery results	Reference
FCV, MNV	Stomaching - sonication	Glass, cotton, polyester	Ambient conditions, 40 min drying time	200 µl 6.6-7 log PFU/ml FCV or 5.9-6.3 log PFU/ml MNV	10 ml PBST	PA	FCV: 0.15% (cotton) to 35.22% (glass) MNV: 0.85% (cotton) to 24.27% (glass)	Yeargin et al. (2015)
hNoV GI.4, GII.4	Swab (cotton) elution – extraction	HDPE	45 min drying in biosafety cabinet	100 µl of 10^4 GC of each virus	PBS (3 strategies differ when/how often swab moistened)	RT-qPCR	For both hNoVs, strategy 2 and 3 were significantly different from 1. Strategy 2 (Highest): 27.0% ± 26.5%	De Keuckelaere et al. (2014)
hNoV GII.4	Swab/cloth with semidirect lysis method	Low density PE, SS	Dry overnight at RT	100 µl of 10^{-4} to 10^{-6} GII.4 particles	2 ml of either PBS or 50 mM glycine buffer	RT-qPCR	PE: (highest) microfiber cloth 1 = 88.7% ± 2.7% (glycine) SS: (highest) microfiber cloth 1 = 79.0% ± 10.2% (glycine)	Rönnqvist et al. (2013)

Table 1: Surface sampling methods for human enteric viruses – nonporous and porous environmental surfaces (not food based).

Virus type	Method(s) evaluated	Surface type(s)	Conditions	Volume and concentration	Buffer Matrix(s)	Analysis	Recovery results	Reference
MNV	Repeated pipetting (25x)	SS	20 min drying at RT	10 µl of 2×10^5 virus	1 ml EBSS	PA	37% recovery after 20 min drying	Fallahi and Mattison (2011)
Bacterio-phage MS2	Swab – elution extraction - cotton swab - polyester swab antistatic cloth	PVC, SS	45 min drying time (RT; 45-60% RH)	3.7 $\log_{10}$ in 5 µl	1) 0.85% saline 2) Ringer's solution 3) viral transport media 4) acid/base eluent	PA, RT-qPCR	Implement significantly influenced recovery - lowest recovery = antistatic cloth - highest recovery = swabs <0.3 to 97% recovery for infective MS2	Julian et al. (2011)
hNoV GII.3; rotavirus	Swab protocol	HDPE, SS, ceramic, wood	15 min drying in laminar flow hood	100 µl of 10^5 - 10^7 PCRU/ml of each virus	PBS	RT-qPCR	hNoV: 10.3-25.5% (wood) to 31-51.9% (ceramic) rotavirus: 5.4-10.2% (wood) to 45.9-57.7% (ceramic)	Scherer et al. (2009)

Table 1: Surface sampling methods for human enteric viruses – nonporous and porous environmental surfaces (not food based).

Virus type	Method(s) evaluated	Surface type(s)	Conditions	Volume and concentration	Buffer Matrix(s)	Analysis	Recovery results	Reference
FCV	Vortex for 30s in scintillation vials	SS	30 min drying in hood	10 µl of 3.0×10^7 PFU/ml	990 µl of EBSS	PA	33 and 11% after 0 and 30 min drying time, respectively	Mattison et al. (2007)
FCV	1) Swabbing 2) flooding –aspiration 3) scraping –aspiration	SS	15 min drying time	Not specified	1) 0.05 M glycine buffer, pH 6.5 2) 0.05 M glycine buffer, pH 9.5 3) Modified Eagle's medium, pH 7.2	RT-qPCR	Highest recoveries with 0.05 M glycine at pH 6.5 for FCV Scraping – aspiration method best Cell scraper mean recovery range on SS surfaces: 11% to 71%	Taku et al. (2002)

ABSS = acrylic-based solid surface; EBSS = Earle's buffered saline solution; FCV = feline calicivirus; GC = genomic copies; HDPE = high density polyethylene; hNoV = human norovirus; MNV = murine norovirus; NPF = non-porous formic; PA = plaque assay; PBS = phosphate buffered saline; PBST = PBS + 0.02% Tween 80; PCRU = polymerase chain reaction units; PE = polyethylene; PF = porous formic; PFU = plaque forming units; RH = relative humidity; RB = rubberized surface; RT-qPCR = reverse transcription quantitative PCR; RT = room temperature; SS = stainless steel

Chapter 3: Optimization of surface sampling methods for human noroviruses and its surrogates

Abstract

Human enteric viruses, specifically human noroviruses (hNoVs), are the leading causes of acute nonbacterial gastroenteritis and foodborne illnesses in the United States. Fomite surface contamination is a major route of transmission. The application of an optimized virus recovery method is essential to better understand human enteric virus persistence on fomites under various environmental conditions. This study aimed to evaluate three surface sampling methods and two elution buffers for human enteric viruses and their surrogates from nonporous food contact surfaces for application in environmental persistence studies. First, feline calicivirus (FCV) was selected for surface sampling optimization. 100 µl of virus (10^4-10^6 PFU/ml) was inoculated onto duplicate surfaces (plastic chopping board, stainless steel, acrylic) and dried under ambient conditions for one hour. FCV was recovered using 1 of 3 implements (cell scraper, repeated pipetting, or macrofoam swab) combined with 1 of 2 eluents ($1\times$ phosphate buffered saline (PBS) or $1\times$PBS + 0.1%Tween80 (PBST, 1:1 v/v)). The repeated pipetting with PBST method was selected though no significant differences were observed compared to other methods. Then, hNoVs (GI.1; GII.17), Aichivirus A, and a hNoV surrogate—Tulane virus—were optimized from nonporous food contact surfaces using repeated pipetting with PBST in a similar manner as to FCV optimization. Overall, mean recovery efficiencies using repeated pipetting with PBST ranged from 2.0% ± 0.6% to 82.36% ± 38.6% depending on virus and food contact surface type. The repeated pipetting with PBST method was chosen for further studies on viral persistence on nonporous food contact surfaces over a two-week period under varying environmental conditions.

1. Introduction

Human noroviruses (hNoV), a human enteric virus, have been identified as a leading cause of acute nonbacterial gastroenteritis in the U.S. and are estimated to cause 48% to 59% of foodborne illnesses (Green 2007; Hall et al. 2014; Scallan et al. 2011). The transmission of human enteric viruses typically occurs through fecal-oral contamination. However, there is growing evidence of environmental transmission of hNoV through contaminated fomite surfaces in a variety of settings including food preparation environments (Boone and Gerba 2007; Rzezutka and Cook 2004; Wikswo et al. 2015). There is a need for studies focusing on viral persistence on food contact surfaces to provide better understanding of the role of fomites in environmental transmission of hNoV and other human enteric viruses.

Surface sampling of human enteric viruses and their surrogates of fomites have highly variable results throughout the literature. This variability in recovery could exist in part due to varying human behavior, the tool used, or even the type of elution buffer used to recover the sample from the surface or the sampling tool. Most environmental sampling evaluations have focused on various swab types to be used for applied environmental sampling during hNoV outbreaks. Very few studies focus on evaluating other tools and techniques used, such as cell scraper and repeated pipetting, for use in the laboratory to study viral persistence under varying environmental conditions. Furthermore, most viral persistence and surface sampling studies focus on nonporous fomites such as stainless steel and glass while there is less information on surface sampling of hNoV and its surrogates on plastic chopping board and acrylic-based surfaces. Moreover, there is limited information in general on Aichivirus A (AiV) and Tulane virus (TuV)—a hNoV surrogate—surface sampling and viral persistence on food preparation surfaces under food storage and other appropriate environmental conditions in the literature. The

objective in this study was to evaluate three surface sampling methods for human enteric viruses and their surrogates and two elution buffers to improve the recovery of viruses from nonporous surfaces in order to further study viral persistence on fomites under varying environmental conditions.

2. Materials and Methods

Objective 1 was spilt into two parts. The first part focused on the evaluation of three surface sampling methods and two elution buffers using the hNoV surrogate, feline calicivirus (FCV). Part 2 involved the optimization of the most effective surface sampling method from Part 1 using hNoVs (GI.1 and GII.17) and the surrogate Tulane virus (TuV) as well as Aichivirus A (AiV)—a cultivable human pathogenic, enteric virus.

2.1 Part 1: Evaluation of three surface sampling methods using FCV

2.1.1   Virus propagation of FCV

FCV- F9 (a gift from Dr. Kellogg Schwab at Johns Hopkins School of Public Health, Baltimore, MD) were propagated in CRFK cells (Crandell Rees feline kidney; ATCC CCL94) in complete growth media, which contained MEM 1× (Corning, catalog #10-010-CV, Corning, NY), 10% fetal bovine serum (FBS; Hyclone™, catalog #SH 30396.03, Logan, UT), 1% 100× penicillin/streptomycin (Gibco, catalog #15140-122, Rochester, NY), and 1% non-essential amino acids solution (Gibco, catalog #11140-050) at 37°C and 50% CO_2. FCV concentrations were determined through a plaque assay as described previously by Hsueh and Gibson (2015). Briefly, six well plates were seeded with 7×10^5 cells per well for CRFK and grown to 90-100% confluency in 2 ml of complete growth medium within 24 h. Cell monolayers were inoculated

with 500 µl of virus sample per well, and continuously rocked at about 18 oscillations per min

for 1 h at 37°C and 5% CO_2 followed by aspiration of the inocula. Cells were covered with 2 ml

per well of overlay medium containing 4% NuSieve™ low melting point agarose (Lonza, catalog

#50080, Rockland, ME) and incubated for 48 h. After 48 h, plaques were visualized by adding 2

ml of 0.01% neutral red (Sigma-Aldrich Corp., catalog #N2889, St. Louis, MO) in 1×PBS to

each well. The plates were incubated for 1 h at 37°C and 5% CO_2, and after incubation, plaques

were counted after aspirating the neutral red solution from each well.

2.1.2   Inoculation of surfaces

Three nonporous surfaces were used for testing the surface sampling methods for virus

recovery. A set of 7.6 cm^2 100% acrylic-based surface samples (13 mm thick Wilsonart

laminate; Wilsonart International Inc., Temple, TX), 10.16 cm^2 stainless steel coupons (type

304/16 gauge, unpolished; Rose Metal products, Springfield, MO), and 10.16 cm^2 plastic

chopping board were prepared by washing sequentially with 0.1% Tween80, sterile DI water,

70% ethanol, and 10% bleach, and were allowed to air dry under ambient conditions in between

washes and sanitizer treatments. The surfaces were also placed under a UV light in a biosafety

cabinet for 30 minutes prior to use.

For all protocols, 100 µl of FCV (10^4-10^6 PFU/ml) suspension was inoculated onto each

surface. Each surface was placed in a 150 ×15mm Petri dish, and the samples were allowed to

dry at ambient conditions (16-22°C, 45-60% RH) for approximately 1 h. Subsequently, the virus

was recovered from the surfaces by a surface sampling method protocol as described below

(macrofoam swab, cell scraper, or repeated pipetting). Two different elution buffers specifically

1× phosphate buffered saline (PBS) only or 1xPBS+0.1%Tween80 (PBST, 1:1 v/v) were

evaluated for each surface sampling method technique. Also, one surface was inoculated with

100 µl of deionized water for each environmental surface type per experiment as a negative control. After viruses were recovered from the surfaces, FCV concentrations were obtained through plaque assay as described previously by Hsueh and Gibson (2015).

2.1.3   Macrofoam swab protocol

The macrofoam swab protocol was conducted based on the procedure previously described by Park et al. (2015) with slight modifications. After allowing the samples to dry on the surfaces for approximately 1 h under ambient conditions, a sterile macrofoam swab (ITW Texwipe, Kernersville, NC, tip size 19×26.7 mm) was dipped into a 15 ml sterile centrifuge tube containing 2.5 ml of swab elution buffer ($1 \times$PBS or PBST). Subsequently, the swab was pressed against the side of the tube to remove excess liquid. The entire surface was swabbed for 20 s horizontally and 20 s vertically in a back-and-forth motion while rotating the swab tip. Next, the swab was placed back into the tube containing the elution buffer and mixed by vortexing for 10 s. The swab was then pressed against the side of the tube to remove excess buffer again. The recovered elution volume for each sample was measured and recorded.

2.1.4   Cell scraper protocol

The cell scraper protocol was conducted based on the procedure as described by Arthur and Gibson (2015) with some modifications. After approximately 1 h drying time under ambient conditions, the virus sample was removed from the surfaces by adding 500 µl of elution buffer ($1 \times$PBS or PBST) followed by physical removal with a cell scraper (Greinerbio-one, 40 cm length, catalog #541080, Monroe, NC) applied in a back-and-forth motion across the entire surface for 20 s horizontally and 20s vertically. The elution buffer was recovered by pipetting

into a microcentrifuge tube. The recovered elution buffer volume for each sample was measured and recorded.

2.1.5   Repeated pipetting protocol

The repeated pipetting protocol was conducted in a similar manner as described by Cannon et al. (2006) with some modifications. After approximately 1 h of drying under ambient conditions, the virus sample was eluted from the surface by pipetting 500 µl of elution buffer (1×PBS or PBST) back-and-forth 25 times. The elution buffer with recovered viruses was collected into a 1.5 ml microcentrifuge tube, and the recovered volume for each sample was measured and recorded.

2.2 Part 2: Optimization of surface sampling for hNoVs and its surrogates

2.2.1   Virus propagation and quantification: AiV and TuV

AiV (kindly provided by Dr. Pierre Pothier at Dijon University Hopsital, Dijon, France) were propagated in Vero cells (ATCC CCL-81) in complete growth media, which contained MEM 1× (Corning, catalog #10-010-CV), 10% FBS (Hyclone™, catalog #SH 30396.03), 1% 100× penicillin/streptomycin (Gibco, catalog #15140-122), and 1% non-essential amino acids solution (Gibco, catalog #11140-050) at 37°C and 5% CO_2. A cell density of $2×10^6$ Vero cells per well were used to seed six-well plates, and grown to 100% confluency in 2 ml of complete growth medium within 24 h. Ten-fold serial dilutions of virus sample was prepared in maintenance media, which contained MEM 1× (Corning, catalog #10-010-CV), 2% FBS, 1% 100× penicillin/streptomycin, and 1% non-essential amino acids solution. Cell monolayers were inoculated with 500 µl of virus sample per well, and continuously rocked for 3 h at 37°C and 5%

CO_2. The inocula were subsequently aspirated from each well, and the cells were covered with 2 ml per well of agarose overlay containing 1.5% analytical grade agarose (Promega Corp., catalog #V3121, Madison, WI) and maintenance media in a 1:1 ratio, and was incubated for 72 h at 37°C and 5% CO_2. After 72 h, the plaques were visualized by adding 2 ml of 0.01% neutral red and PBS staining solution to each well and incubating for 2 h at 37°C and 5% CO_2. Plaques were enumerated after incubation.

TuV (kindly provided by Dr. Jason Jiang of Cincinnati Children's hospital, Cincinnati, OH) were propagated in LLC-MK2 cells (ATCC CCL-7) in complete growth media. MK2 complete growth media contained M199/EBSS (Hyclone™, catalog #SH 30253.01), 10% FBS (Hyclone™, catalog #SH 30396.03), 1% 100× penicillin/streptomycin (Gibco, catalog #15140-122), and 1% amphotericin B (Corning, catalog #30-003-CF) at 37°C and 5% CO_2. A cell density of 8×10^5 MK2 cells per well was used to seed six-well plates, and grown to 100% confluence in 2 ml of complete growth medium within approximately 24 h. Ten-fold serial dilutions of virus sample were prepared in MK2 maintenance media. MK2 maintenance media contained Opti-MEM (Gibco), 2% FBS, 1% 100× penicillin/streptomycin, and 1% Amphotericin B. Cell monolayers were inoculate with 500 µl of virus sample per well, and continuously rocked for 1 h at 37°C and 5% CO_2. The inocula were then aspirated from each well, and the cells were covered with 2 ml per well of overlay medium containing 3% NuSieve™ low melting point agarose and MK2 maintenance media in a 1:1 ratio. The plates were incubated for 72-96 h at 37°C and 5% CO_2. After approximately 72 h, plaques were visualized by adding 2 ml of 0.01% neutral red and PBS staining solution to each well and incubated for 3-5 h at 37°C and 5% CO_2. Plaques were counted after incubation.

2.2.2   Reverse transcription, real time PCR detection of hNoVs

Human norovirus concentrations were obtained through RT-qPCR as described

previously by Kageyama et al. (2003) with slight modifications. Additionally, hNoV clarification

of GII.17 was conducted in a similar manner as described previously by Gibson et al. (2012).

First, hNoV GII.17 stool samples in 10% suspension were clarified with the following steps: (1)

vortexing the samples, (2) centrifuging the samples at $3,000 \times g$ (~4,000 rpm) for 10 min at 4°C,

and (3) removing the supernatants carefully and placing into new microcentrifuge tubes for

storage at -80°C. Also, hNoV GI.1 samples in 10% suspension were used for all experiments.

Next, the hNoVs were inoculated onto surfaces and removed from surfaces as described in the

next section 2.2.3 "Inoculation of surfaces."

RNA extraction from hNoVs recovered from surfaces was completed using a simple heat

release protocol as described previously by Gibson et al. (2012) in which the virus samples were

heated at 95°C for 5 minutes, cooled on ice for 2 minutes, and then, processed immediately

afterwards. For each sample, a 25 µl reaction mixture was prepared containing the following: 20

µl of RT-PCR master mix, 5 µl of sample, and DEPC H_2O for the remaining volume. The RT-

PCR master mix contained QuantiTect probe PCR kit mixes (Qiagen, Germantown, MD),

custom primers (Integrated DNA Technologies, Coralville, IA), and TaqMan probes (Biosearch

Technologies, Petaluma, CA). The primers and probes used for hNoVs of genotype I were Cog1

primers and Ring1a and Ring 1b probes, and for hNoVs of genotype II were Cog2 primers and

Ring 2 probe. Real-time PCR amplification was conducted under the following thermacycler

conditions: (1) reverse transcription for 30 min at 50°C, (2) denaturation for 15 min at 95°C, and

(3) 45 cycles of denaturation at 95°C for 15s and primer annealing/extension at 60°C for 60 s.

A standard curve was produced based on estimated RT-PCR units (PCRu) of a 10-fold

dilution series of hNoVs GI.1 and GII.17 through RT-qPCR. A standard curve was developed for

each hNoV genotype (Figure 1). Each viral amplification sample contained the same

composition mixture as the sample master mix. PCR amplification was conducted under the

same thermal cycling profile conditions as indicated above.

2.2.3   Inoculation of surfaces

The most effective surface sampling method and elution buffer combination using FCV

was selected for further evaluation to confirm if applicable to use for the other surrogates and

hNoV on the environmental surfaces. After the virus sample was collected from the surfaces,

viral concentrations of TuV and AiV were obtained through plaque assay as described previously

Arthur and Gibson (2015) for TuV and D'Souza et al. (2016) for AiV with slight modifications.

For all assays, plates with 5 to 50 plaque forming units (PFU) were used to determine the virus

titer as PFU per milliliter. For hNoVs GI.1 and GII.17 samples, viral concentrations were

obtained from RT-qPCR as described above in the "Reverse transcription, real time PCR

detection of hNoVs" Section 2.2.2. The equation of the fit line from each standard curve was

used to determine RT-PCRu and relative log reductions (Figure 1). The cut-off C_T value (i.e.

limit of detection) was determined to correspond to 40 for GI.1 and GII.17 based on the highest

dilution with a quantifiable RT-qPCR from the standard curves.

2.3 Statistical Analysis

All experiments were repeated at least twice as independent replicates with duplicates in each

experiment. Descriptive statistics including means and standard deviations were determined. For

comparison of virus recovery across methods and surfaces types, two-way analysis of variance

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(ANOVA) was performed. All analysis were completed using SAS® 9.4 (SAS Institute Inc., Cary, NC).

3. Results

3.1 FCV Recovery

Overall, FCV recovery from the surfaces ranged from 3.9% ± 5.3% (stainless steel) to 56.5% ± 15.5% (plastic chopping board) depending on the recovery method used (Figure 2a and 2b). The range for the plastic chopping board was determined to be 12.3% ± 7.6% with the macrofoam swab and 1×PBS elution buffer to 56.46% ± 15.49% with the repeated pipetting method and 1×PBS elution buffer (Figure 2a and 2b). The viral recovery range for acrylic-based surface was 17.6% ± 6.8% with the cell scraper method and 1×PBS elution buffer to 44.4% ± 16.9% with the repeated pipetting method and PBST elution buffer (Figure 2a and 2b). The FCV recovery range for the stainless steel surface was found to be from 3.9% ± 5.3% with the repeated pipetting method and 1×PBS elution buffer to 30.4% ± 6.3% with the repeated pipetting method and PBST elution buffer. It should be noted that the repeated pipetting method with PBST was found to have the second highest viral recovery for the plastic chopping board surface at 45.2% ± 12.1% (Figure 2a). Based on these results, the repeated pipetting sampling method with PBST was determined to be the most effective surface sampling technique to recover FCV from all three nonporous surfaces after one hour drying time under ambient conditions (Figure 2a and 2b). No significance differences were detected among methods and elution buffers for each surface type ($p > 0.05$).

3.2 AiV and TuV Recovery

Generally, AiV and TuV were successfully recovered from surfaces using the repeated

pipetting method with PBST buffer under ambient conditions and 1h drying time (Figure 3).

TuV recovery ranges were from 36.2% ± 11.5% (stainless steel) to 82.6% ± 38.6% (plastic

chopping board) (Figure 3). AiV recovery ranges were 30.9% ± 2.3% (stainless steel) to 82.5% ±

31.9% (acrylic-based solid surface) (Figure 3). No significant differences were observed among

surrogates on each surface type or among surface types except between TuV on plastic to both

AiV and FCV on stainless steel ($p<0.05$).

3.3 hNoV Recovery

Human noroviruses GI.1 (unclarified) and GII.17 (clarified) were recovered from

nonporous food contact surfaces using both the cell scraper method with PBST and the repeated

pipetting method with PBST under ambient conditions for one hour drying time (Table 1 and

Figure 4). For mean log loss, theortical log loss between a 10-fold dilution series were calculated

using the linear regression lines from standard curves produced for each hNoV (Figure 1). Mean

log reductions for hNoV GI.1 (unclarified) ranged from 0.78 ± 0.11 (acrylic; repeated pipetting)

to 1.59 ± 0.98 (acrylic; cell scraper) while for hNoV GII.17 (clarified) mean log reductions

ranged from 0.97 ± 0.62 (stainless steel; cell scraper) to the limit of detection, 1.61 ± 0.00

(acrylic; repeated pipetting). Mean percentage ranges from hNoV GI.1 were from 8.1% ± 5.0%

(stainless steel; cell scraper) to 17.0% ± 4.3% (acrylic; repeated pipetting), and for hNoV GII.17

recovery ranges were 2.0% ± 0.6% (stainless steel; repeated pipeting) to 19.7% ± 17.5%

(stainless steel; cell scraper) (Figure 4). Mean C_T values for hNoV GI.1 ranged from 33.43 ±

0.34 (acrylic; repeated pipetting) to 36.02 ± 3.15 (acrylic; cell scraper) while the range for hNoV

GII.17 was from 36.66 ± 3.22 (stainless steel; cell scraper) to the limit of detection, 40 (acrylic; repeated pipetting) (Table 1). Generally, repeated pipetting with PBST method recovered more hNoV GI.1 from nonporous food contact surfaces while the cell scraper method with PBST recovered more hNoV GII.17 though no significant differences were observed between the two methods for each virus for all surfaces (p>0.05).

4. Discussion

As stated previously, hNoV is the leading cause of foodborne illnesses in the United States. Enteric viruses such as hNoVs and AiV are spread through the fecal-oral route with growing evidence of environmental contamination through fomites. Investigating viral persistence on various environmental surface types is essential in understanding the role of environmental surface transmission during outbreaks due to enteric viruses. Moreover, selection of an optimized virus recovery method is critical for application in viral persistence studies. Typically, evaluation of surface sampling methods is limited to swabs for environmental sampling during hNoV outbreaks and baseline studies of virus prevalence. For example, multiple studies evaluate several swab protocols (flocked nylon, cotton wool, microfiber, macrofoam swab, etc.) on surfaces associated with outbreaks such as food contact surfaces, bathroom surfaces, and cruise ships (Ibfelt et al. 2016; Julian et al. 2011; Park et al. 2015; Ronnqvist et al. 2013; Scherer et al. 2009). However, it is difficult to determine the best protocol for laboratory-controlled persistence studies based on the aforementioned studies. Overall, there is a need for more studies involving additional viruses, tool types, and surfaces to adequately determine a standardized approach for virus surface sampling for application in viral persistence studies.

Initially, FCV was used for the evaluation of three implements (macrofoam swab, cell scraper, repeated pipetting) and two elution buffers (PBS, PBST). For all surfaces except for

plastic chopping board, the repeating pipetting method with PBST elution buffer was found to have the highest mean recovery efficiencies though no significant difference was found among methods and elution buffers for all surfaces. Previous studies have also found varying recovery efficiencies for FCV on various surfaces using different tools (Mattison et al. 2007; Taku et al. 2002; Yeargin et al. 2015). Taku et al. (2002) reported recovery efficiencies for FCV ranging from 11% to 71% using the cell scraper method with 0.05M glycine buffer (pH 6.5) from stainless steel surfaces after 15 min drying time. The mean recovery efficiencies for this study on stainless steel using cell scraper method ranged from 4.7% ± 1.6% (PBST) to 19.6% ± 12.8% (PBS) after 1 h drying time. The lower mean recovery efficiencies reported here could be impacted by drying time. For example, Mattison et al. (2007) showed a decrease in FCV recovery efficiency from stainless steel surfaces using vortexing method with Earle's buffered saline solution eluent from 0 to 30 min with efficiencies of 33% and 11%, respectively.

Furthermore, surface type could play a role in recovery efficiencies. Physical properties of nonporous and porous environmental surfaces have been suggested to impact virus recovery by enhancing virus recovery by smooth surfaces or hindering recovery through entrapment of virus particles within cervices (Scherer et al. 2009). Yeargin et al. (2015) reported better recovery of FCV from nonporous surface (glass) than porous surfaces (cotton, polyester) with mean recovery efficiencies ranging from 0.15% (cotton) to 35.22% (glass). In this study, plastic chopping board and acrylic-based solid surfaces tended to have higher mean recovery efficiencies for FCV than stainless steel for all methods. Even though all three surfaces are classified as nonporous, this study confirmed the impact of surface type on virus recovery.

Additionally, eluent formulations could impact virus recovery efficiencies from surfaces through factors such as pH, use of a surfactant, and salinity (Gerba 1984; Ronnqvist et al. 2013;

Taku et al. 2002). This study evaluated the recovery efficiency of FCV from surfaces using an addition of a surfactant (0.01% Tween80) to a common saline solution (PBS). The addition of a surfactant (0.02% Tween80) to PBS eluent of a swab rinse protocol has been suggested to enhance hNoV GII.4 viral recovery efficiency though no significant difference was observed in the study (Park et al. 2015). In this study, mean recovery efficiencies of FCV from nonporous surfaces were higher for all methods and surfaces using PBST as the eluent except for the cell scraper method for all three surfaces and the repeated pipetting method on plastic though no significant differences were detected between the two elution buffers evaluated for all methods and surfaces. Overall, the addition of a surfactant may increase virus recovery from fomites.

Next, the repeated pipetting method with PBST elution buffer was evaluated for recovery of hNoVs (GI.1, GII.17), AiV, and TuV from nonporous surfaces. This study is the first to evaluate hNoVs (GI.1, GII.17), AiV, and hNoVs surrogates (FCV and TuV) using the repeated pipetting method. For cultivable viruses (FCV, AiV, TuV), mean recovery efficiencies varied among surface and virus types though no significant difference was shown, which could be impacted by surface properties (as indicated above with FCV recovery) and virus properties. For example, Scherer et al. (2009) showed variation in recovery from various nonporous and porous fomites between hNoV GII.3 and rotavirus using a cotton swab. The authors suggested that the variation in recovery from fomites between the two human enteric viruses may be due to differences in virus properties affecting attachment and their abilities to adhere to different surface types (Scherer et al. 2009). In this study, hNoVs GII.17 and GI.1 showed variation in recovery efficiencies between two sampling methods (cell scraper vs. repeated pipetting), which may also be influenced by virus and surface properties. Overall, the variability between surface and virus

types highlights the need for more studies involving a variety of surfaces and enteric viruses to move towards a more standardized and consistent method of virus recovery from fomites.

Here, it is important to highlight some of the limitations of the present study. First of all, hNoV GI.1 was not clarified prior to surface inoculation while hNoV GII.17 was clarified. The presence of organic matter in unclarified hNoV GI.1 may have an impact on virus recovery efficiency depending on the surface type (Figure 5). However, the preparation of hNoV stool samples in previous studies investigating virus recovery is not always stated. Therefore, the impact of organic matter on virus recovery from nonporous fomites is lacking in the literature and needs further investigation. Along with the potential impact on virus recovery, the presence of organic matter can also impact RT-qPCR and other downstream analysis through the introduction of inhibitory compounds (Knight et al. 2013; Wilson 1997). Another aspect related to inhibition of RT-qPCR is the method selection for RNA extraction. For instance, the simple heat release method of RNA extraction for hNoVs used in the present study could also impact RT-qPCR analysis via inhibition (Schwab et al. 1997). However, as indicated earlier, hNoV GI.1 RT-qPCR signal is likely not limited by inhibitory compounds based on the standard curve but rather impacted more by surface recovery (Figures 4 and 5). Furthermore, the preparation of GII.17 and surface recovery may have impacted its recovery efficiencies (Figure 4). Overall, surface recovery techniques may be the most influencing factor for virus recovery.

5. Conclusions

Surface sampling studies have been primarily limited to evaluation of swab protocols with limited data on other hNoVs besides GII.4 and other surrogates. This study showed repeated pipetting with PBST elution buffer displayed the highest mean recovery efficiency for FCV on nonporous surfaces even though no significant differences were found among methods and

elution buffers for all surfaces. This method was also successful in obtaining mean recovery efficiencies for hNoVs (GI.1, GII.17), AiV, and TuV. However, this study also found variability among hNoVs in tool selection since GII.17 recovery efficiencies fared better with the cell scraper method and PBST elution buffer unlike GI.1. This study is the first to evaluate repeated pipetting for AiV, TuV, and hNoVs (GI.1, GII.17). Overall, this study demonstrated the potential impact of recovery on viral persistence studies due to its variability among surface types and viruses. Surface sampling techniques need to be evaluated for more hNoVs and surrogates to provide more adequate information on the appropriate method to choose for a study's parameters including viral persistence studies.

References

Arthur, S. E., & Gibson, K. E. (2015). Environmental persistence of tulane virus—a surrogate for human norovirus. *Canadian Journal of Microbiology, 62*(5), 449-454.

Arthur, S., & Gibson, K. (2015). Physicochemical stability profile of tulane virus: A human norovirus surrogate. *Journal of Applied Microbiology, 119*, 868-875.

Boone, S. A., & Gerba, C. P. (2007). Significance of fomites in the spread of respiratory and enteric viral disease. *Applied and Environmental Microbiology, 73*, 1687-1696.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L., & Vinjé, 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*, 2761-2765.

D'Souza, D. H., Dice, L., & Davidson, P. M. (2016). Aqueous extracts of *Hibiscus sabdariffa* Calyces to control aichi virus. *Food and Environmental Virology, 8*, 112-119.

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

Gibson, K.E., Crandall, P.G., & Ricke, S.C. (2012). Removal and transfer of viruses on food contact surfaces by cleaning cloths. *Applied and Environmental Microbiology*, *78*, 3037-3044.

Gibson, K. E., & Schwab, K. J. (2011). Tangential-flow ultrafiltration with integrated inhibition detection for recovery of surrogates and human pathogens from large-volume source water and finished drinking water. *Applied and Environmental Microbiology, 77*, 385-391.

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.

Hall, A. J., Wikswo, M. E., Pringle, K., Gould, L. H., & Parashar, U. D. (2014). Vital signs: Foodborne norovirus outbreaks - United States, 2009-2012. *Morbidity and Mortality Weekly Report, 63*, 491-495.

Hsueh, T. Y., & Gibson, K. E. (2015). Interactions between human norovirus surrogates and *Acanthamoeba* spp. *Applied and Environmental Microbiology, 81*, 4005-4013.

Ibfelt, T., Frandsen, T., Permin, A., Andersen, L.P., & Schultz, A.C. (2016). Test and validation of methods to sample and detect human virus from environmental surfaces using norovirus as a model virus. *Journal of Hospital Infection, 92,* 378−384.

Julian, T.R., Tamayo, F.J., Leckie, J.O., & Boehm, A.B. (2011). Comparison of surface sampling methods for virus recovery from fomites. *Applied and Environmental Microbiology, 77,* 6918−6925.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., et al. (2003). Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology, 41*, 1548–1557.

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

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,* 500−503.

Mormann, S., Heißenberg, C., Pfannebecker, J., & Becker, B. (2015). Tenacity of human norovirus and the surrogates feline calicivirus and murine norovirus during long-term storage on common nonporous food contact surfaces. *Journal of Food Protection, 78*, 224-229.

Park, G. W., Lee, D., Treffiletti, A., Hrsak, M., Shugart, J., & Vinje, J. (2015). Evaluation of a new environmental sampling protocol for detection of human norovirus on inanimate surfaces. *Applied and Environmental Microbiology, 81*, 5987-5992.

Rönnqvist, M., Rättö, M., Tuominen, P., Salo, S., & Maunula, L. (2013). Swabs as a tool for monitoring the presence of norovirus on environmental surfaces in the food industry. *Journal of Food Protection, 76,* 1421−1428.

Rzeżutka, A., & Cook, N. (2004). Survival of human enteric viruses in the environment and food. *FEMS Microbiology Reviews, 28*, 441-453.

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

Scherer, K., Mäde, D., Ellerbroek, L., Schulenburg, J., Johne, R., & Klein, G. (2009). Application of a swab sampling method for the detection of norovirus and rotavirus on artificially contaminated food and environmental surfaces. *Food and Environmental Virology, 1,* 42−49.

Schwab, K. J., Estes, M. K., Neill, F. H., & Atmar, R. L. (1997). Use of heat release and an internal RNA standard control in reverse transcription-PCR detection of Norwalk virus from stool samples. Journal of Clinical Microbiology, *35*, 511–514.

Taku, A., Gulati, B.R., Allwood, P.B., Palazzi, K., Hedberg, C.W., & Goyal, S.M. (2002). Concentration and detection of caliciviruses from food contact surfaces. *Journal of Food Protection, 65,* 999−1004.

Wilson, I. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology, 62*, 3741-3751.

Wikswo, M. E., Kambhampati, A., Shioda, K., Walsh, K. A., Bowen, A., & Hall, A. J. (2015). Outbreaks of acute gastroenteritis transmitted by person-to-person contact, environmental contamination, and unknown modes of transmission--United States, 2009-2013. *MMWR Rep: Surveillance Summaries, 64*, 1-12.

Yeargin, T., Fraser, A., Huang, G., & Jiang, X. (2015). Recovery and disinfection of two human norovirus surrogates, feline calicivirus and murine norovirus, from hard nonporous and soft porous surfaces. *Journal of Food Protection, 78,* 1842−1850.

Virus type/method	Average C_T value		
	ABSS	P	SS
GI.I CS-PBST	$36.02 \pm 3.15^{A,X}$	$34.43 \pm 1.80^{A,X}$	$35.21 \pm 2.38^{A,X}$
GI.1 RP-PBST	$33.43 \pm 0.34^{A,Y}$	$33.66 \pm 0.57^{A,X}$	$33.89 \pm 1.17^{A,X}$
GII.17 CS-PBST	$39.13 \pm 2.14^{A,Z}$	$36.97 \pm 3.11^{A,Y}$	$36.66 \pm 3.22^{A,X}$
GII.17 RP-PBST	$LOD^{A,Z}$	$39.57 \pm 1.22^{A,Z}$	$40.56 \pm 0.98^{A,Y}$

Table 1: Average C_T values of human noroviruses GI.1 and GII.17 recovered from various nonporous food contact surfaces. Human noroviruses GI.1 (unclarified) and GII.17 (clarified) were optimized from three nonporous food surfaces (ABSS – -acrylic-based solid surface, P-plastic chopping board, SS – stainless steel) using either repeated pipetting (RP) or cell scraper (CS) method with PBS+0.1%Tween80 (PBST) elution buffer. Letters A-C indicated significant difference between surfaces within one virus type/method (rows) while letters X-Z indicated significant difference between virus types/methods for one surface type (columns; $p<0.05$). Limit of detection (LOD) was determined to be 40 from standard curves produced by RT-qPCR for both genotypes.
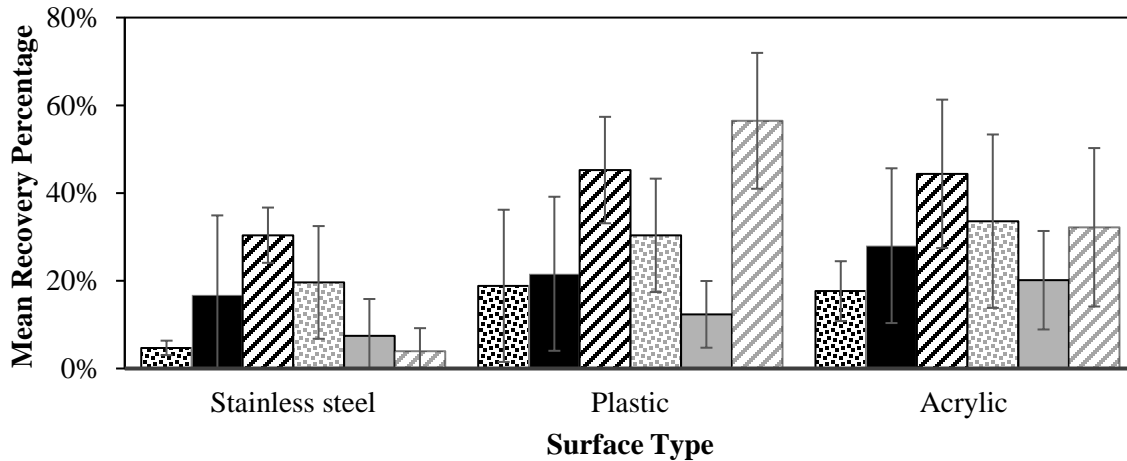
$$y = -1.40\ln(x) + 37.36$$
$$R^2 = 0.96$$

A.)

$$y = -2.25\ln(x) + 36.81$$
$$R^2 = 0.99$$
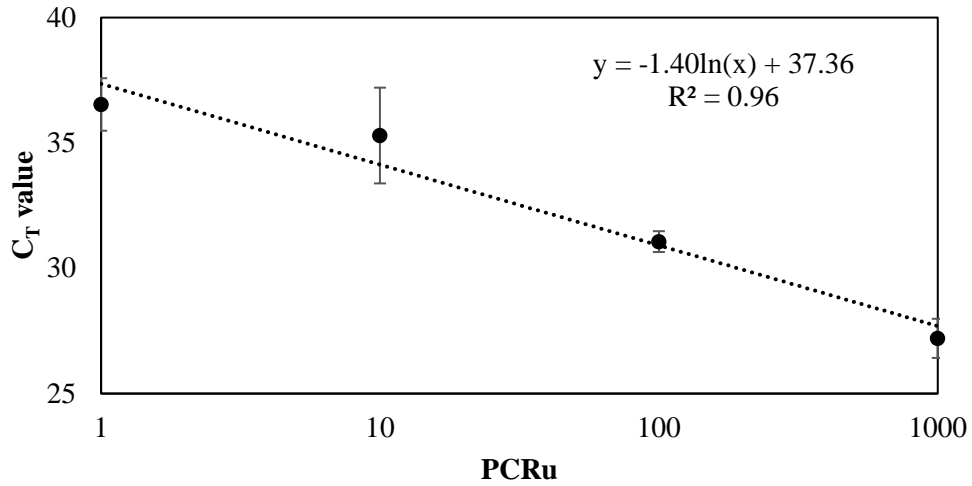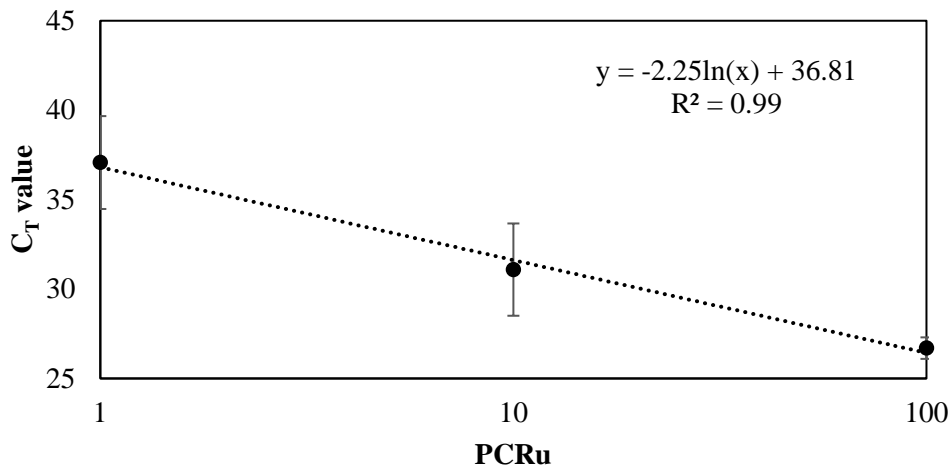
B.)

Figure 1: Standard curves for human noroviruses GI.1 and GII.17. Standard curves for human noroviruses (A) GI.1 (unclarified) and (B) GII.17 (clarified) were produced by estimated RT-PCR units (PCRu) from a 10-fold dilution series using RT-qPCR. PCRu were plotted on a $\log_{10}$ scale.

Figure 2: Evaluation of surface sampling methods and elution buffer combinations using feline calicivirus. The three surface sampling methods (cell scraper – dotted pattern, macrofoam swab – solids, and repeated pipetting – striped) were evaluated on the three surfaces (stainless steel, plastic chopping board, and acrylic-based) using either 1×PBS (gray) or PBST (black) elution buffer under ambinet conditions for one hour drying time.
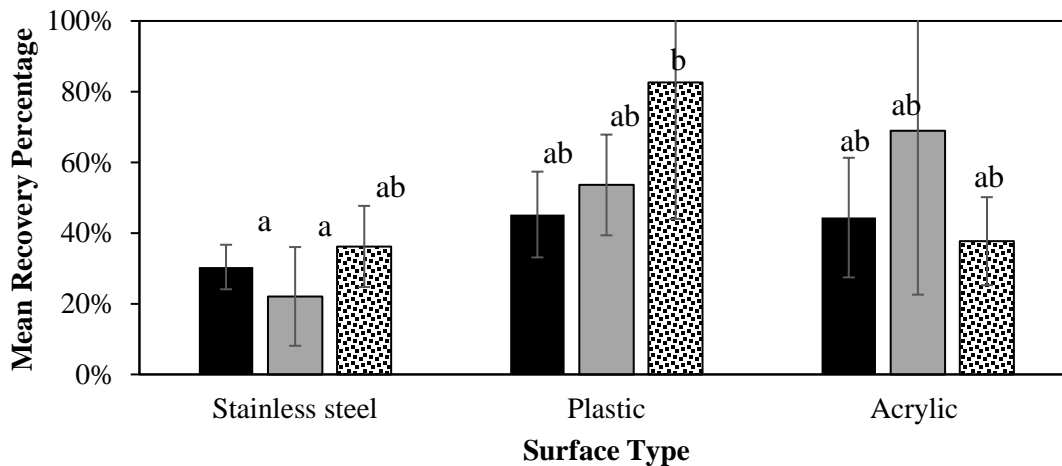
Figure 3: Optimization of human norovirus surrogate recovery from surfaces using repeated pipetting method and PBS+0.1% Tween 80 buffer. The surrogates (feline calicivirus- black solid, Aichivirus A – gray solid, and Tulane virus – black dotted) were optimized from three nonporous fomites (stainless steel, plastic chopping board, and acrylic-based solid surface) using the repeated pipetting method with PBST buffer under ambient conditions for one hour drying time. Letters a-b indicated significant differences ($p<0.05$).

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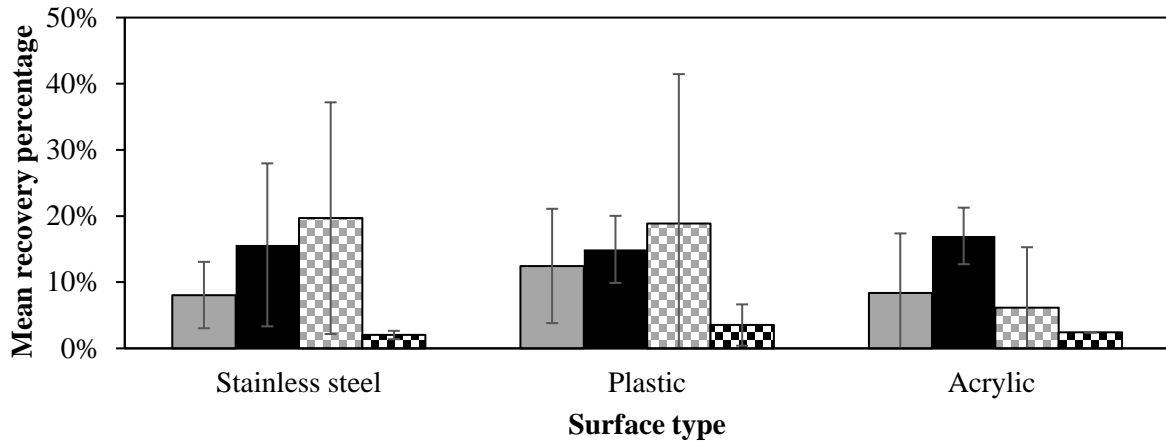
Figure 4: Comparison of two surface sampling methods for recovery of human noroviruses GI.1 and GII.17 from nonporous food contact surfaces. Human noroviruses GI.1 (unclarified; solids) and GII.17 (clarified; checker board patterns) were optimized from three nonporous food contact surfaces (stainless steel, plastic chopping board, and acrylic based solid surface) using repeated pipetting with 0.1%Tween80+PBS (black) or cell scraper with 0.1%Tween80+PBS (gray) under ambient conditions for one hour drying time. The results were displayed as mean recovery percentages.
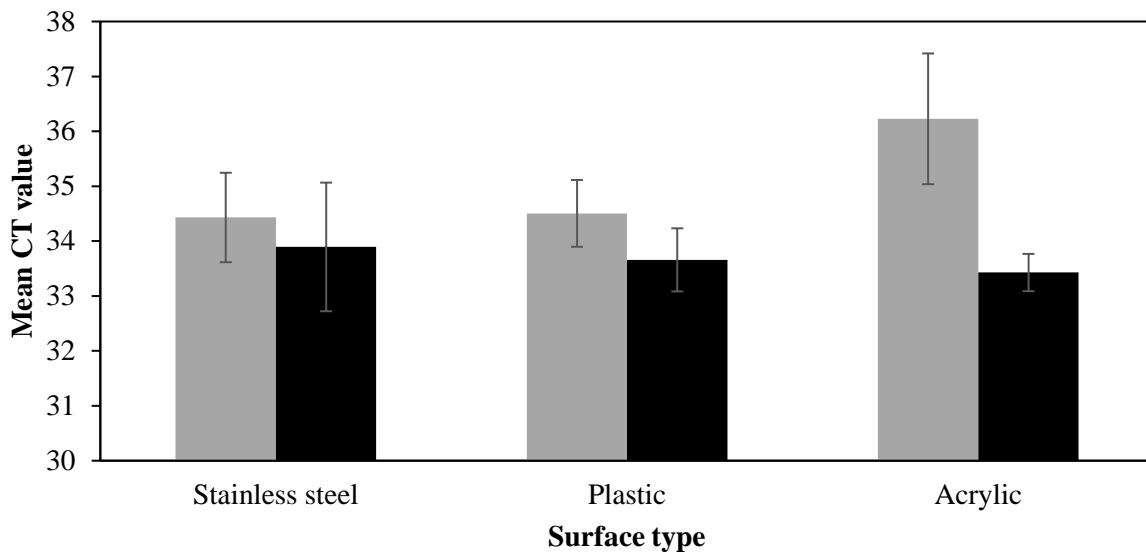
Figure 5: Comparison of the recovery of clarified and unclarified human norovirus GI.1 using repeated pipetting with PBS+0.1% Tween 80 buffer based on C_T values. Unclarified (gray) and clarified (black) human norovirus GI.1 was recovered from three nonporous food contact surfaces (stainless steel, plastic, acrylic-based) using repeated pipetting with PBS+0.1% Tween 80 elution buffer under ambient conditions for one hour drying time.

Chapter 4: Evaluation of different surfaces and environmental conditions for viral persistence

Abstract

Human enteric viruses such as human noroviruses (hNoVs) and Aichivirus A (AiV) have been identified as leading causative agents of acute gastroenteritis and foodborne illnesses in the U.S. There is growing evidence of contaminated fomites playing a role in the chain of transmission. Enteric viruses have been found to retain infectivity on fomites over prolonged periods of time. The aim of the study was to evaluate environmental persistence of hNoVs (GI.1, GII.17) and AiV on nonporous food contact surfaces (FCS; stainless steel, plastic chopping board, acrylic-based solid surface) under varying environmental conditions appropriate to food processing and storage over two-weeks. A cocktail containing 100 µl of each virus was inoculated onto duplicate FCS, and then FCS were placed into an environmental chamber at varying temperatures (6°C, 15°C, 22°C) and relative humidity (RH; 60%, 90%). Viruses were recovered from FCS at specified time points over two-weeks using the repeated pipetting method with 1×PBS+0.1%Tween80 elution buffer. Virus concentrations were determined through either plaque assay or RT-qPCR. Decimal reduction values (D-values) ranged from 3.19 ± 1.13 (22°C/60% RH, plastic) to 47.62 ± 0.10d (6°C/90% RH, plastic) for AiV. Mean $\log_{10}$ reductions for hNoV GI.1 on surfaces ranged from 1.33 ± 0.30 (15°C/60% RH, stainless steel) to 2.76 ± 0.23 (22°C/60% RH, plastic) over 14d. There were variabilities in significant differences across days among FCS types and temperature/RH combinations for hNoV GI.1 and AiV. Generally, higher RH (90%) resulted in greater stability and persistence for GI.1 and AiV over two-weeks than lower RH (60%). This is the first study to demonstrate AiV persistence on nonporous FCS. Overall, hNoV GI.1 and AiV remained stable on a variety of nonporous fomites over a two-week time period.

1. Introduction

Human noroviruses (hNoVs) have been determined as the leading cause of foodborne illnesses in the U.S., and there is growing evidence of enteric viral transmission through contaminated fomite surfaces in settings such as food preparation environments (Boone and Gerba 2007; Rzezutka and Cook 2004; Scallan et. 2011). HNoVs have been shown to survive and maintain infectivity on fomites over a prolonged period of time (Kramer et al. 2006). Many factors such as relative humidity, temperature, co-presence of organic matter, and higher inoculum levels have been shown to impact viral persistence on environmental surfaces (Abad et al. 1994; Faix 1987; Kramer et al. 2006). Of these factors, temperature has been shown to be a major influencing factor on viral persistence for hNoVs on environmental surfaces (Ahmed et al. 2014).

Understanding hNoVs and its surrogates' persistence on environmental surfaces could lead to better controls and prevention of hNoV outbreaks. Traditionally, hNoVs (primarily GII.4) and its surrogates including feline calicivirus (FCV) and murine norovirus-1 (MNV) have been used to study hNoV persistence on nonporous and porous surfaces and foods under varying environmental conditions including temperature and relative humidity (D'Souza et al. 2006; Kim et al.2012). Additionally, many studies focus on temperature with fewer focusing on relative humidity and temperature combinations as factors for viral persistence on fomites. Further studies focusing on viral persistence on environmental surfaces in relation to relative humidity and temperature for other hNoVs—emergent GII.17— are needed to better understand hNoV persistence. This study aimed to evaluate viral persistence of human enteric viruses, hNoVs (GI.1 and GII.17) and AiV, on nonporous fomite surfaces using environmental condition

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combinations of temperature and relative humidity that represent food preparation and food storage conditions over a 14-day period.

2. Methods and materials

2.1 Propagation and quantification of AiV

As described in Chapter 3, AiV (a gift from Dr. Pierre Pothier at Dijon University Hopsital, Dijon, France) were propagated in Vero cells (ATCC CCL-81) in complete growth media, which contained MEM 1× (Corning, catalog #10-010-CV, Corning, NY), 10% FBS (Hyclone™, catalog #SH 30396.03, Logan, UT), 1% 100× penicillin/streptomycin (Gibco, catalog #15140-122, Rochester, NY), and 1% non-essential amino acids solution (Gibco, catalog #11140-050) at 37°C and 5% CO_2. Virus concentrations for AiV were obtained through plaque assay as previously described by D'Souza et al. (2016) with some modifications. A cell density of 2×10^6 Vero cells per well was used to seed six-well plates. Cells were grown to 100% confluence in 2 ml of complete growth medium per well within 24 h. Ten-fold serial dilutions of virus sample were prepared in Vero maintenance media, which contained MEM 1× (Corning, catalog #10-010-CV), 2% FBS, 1% 100× penicillin/streptomycin, and 1% non-essential amino acids solution. Cell monolayers were inoculated with 500 µl of virus sample per well. Plates were continuously rocked for 3 h at 37°C and 5% CO_2, and then, the inocula was removed from each well. After aspiration of the inocula, the cells were covered with 2 ml per well of agarose overlay containing 1.5% analytical grade agarose (Promega Corp., catalog #V3121, Madison, WI) and Vero maintenance media in a 1:1 ratio. The plates were incubated for 72 h at 37°C and 5% CO_2. After approximately 72 h, 2 ml of 0.01% neutral red and PBS staining solution were added to each well for visualization of plaques, and then, the plates were incubated for

approximately 2 h at 37°C and 5% CO_2. Plaques were counted after incubation and removal of the staining solution. Plates with 5 to 50 plaque forming units (PFU) were used to determine the virus titer as PFU per milliliter for the AiV assay.

2.2 Detection of hNoVs by real time PCR

Human norovirus concentrations were obtained through RT-qPCR as described previously by Kageyama et al. (2003) with slight modifications. Additionally, hNoV clarification of GII.17 was conducted in a similar manner as described previously by Gibson et al. (2012). Initially, hNoV GII.17 stool samples in 10% suspension were clarified by: (1) vortexing the samples, (2) centrifuging the samples at $3,000 \times g$ (~4,000 rpm) for 10 min at 4°C, and (3) removing the supernatant carefully and placing into new microcentrifuge tube for storage at -80°C. Also, hNoV GI.1 samples in 10% suspension were used for all experiments. Next, the hNoVs were inoculated and removed from surfaces as described in the next Section 2.3 "Viral persistence on surfaces under varying environmental conditions."

Next, RNA extraction from hNoVs recovered from surfaces was completed using a simple heat release protocol as described previously by Gibson et al. (2012) which involved heating the virus samples at 95°C for 5 minutes, cooling on ice for 2 minutes, and processing the samples immediately. For each sample, a 25 µl reaction mixture was prepared containing: 20 µl of RT-PCR master mix, 5 µl of sample, and DEPC H_2O for the remaining volume. The RT-qPCR master mix contained QuantiTect probe PCR kit mixes (Qiagen, Germantown, MD), custom primers (Integrated DNA Technologies, Coralville, IA), and TaqMan probes (Biosearch Technologies, Petaluma, CA). The primers and probes used for hNoV genotype I were Cog1 primers and Ring1 probes, and for hNoV genotype II were Cog2 primers and Ring2 probe. Real-time PCR amplification was conducted under the following thermacycler conditions: (1) reverse

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transcription for 30 min at 50°C, (2) denaturation for 15 min at 95°C, and (3) 45 cycles of denaturation at 95°C for 15s and primer annealing/extension at 60°C for 60 s.

Standard curves for both hNoVs GI.1 and GII.17 were produced based on estimated RT-PCR units (PCRu) of a 10-fold dilution series of each genotype through RT-qPCR (Figure 1 in Chapter 3). Each viral amplification sample contained an analogous composition mixture to the sample mix above PCR amplification was conducted under the same thermal cycling profile conditions as indicated above. The equation of the fit line from each standard curve was used to determine RT-PCRu and relative log reductions (Figure 1 in Chapter 3). Based on the highest dilutions with a quantifiable RT-qPCR signal from the standard curves, the cut-off C_T value for GI.1 and GII.17 was determined to correspond to 40.

2.3 Viral persistence on surfaces under varying environmental conditions

Three nonporous surfaces were used for evaluating viral persistence under varying environmental conditions. A set of 7.6 cm^2 100% acrylic-based surface samples (13 mm thick Wilsonart laminate; Wilsonart International Inc., Temple, TX), 10.16 cm^2 stainless steel coupons (type 304/16 gauge, unpolished; Rose Metal products, Springfield, MO), and 7.6 cm^2 plastic chopping board are the surfaces that were used. The surfaces were prepared prior to each experiment by washing sequentially with 0.1% Tween80, sterile DI water, 70% ethanol, and 10% bleach, and were allowed to air dry under ambient conditions in between washes and sanitization treatments. Then, the surfaces were placed under a UV light in a biosafety cabinet for 30 min prior to use.

For all experiments, a cocktail containing 100 µl of each virus (10^6 PFU/ml of AiV; 10^2 PCRu of GI.1; 10^1 PCRu of GII.17) were inoculated onto each surface in droplets around the center using a micropipette. The virus samples were allowed to dry at ambient conditions on the

surfaces for approximately 2.5 h until completely dried. After drying, each virus-inoculated

surface was placed into a relative humidity (RH) and temperature controlled environmental test

chamber (Caron, Model # 700-10-1, Marietta, OH). Different temperatures (6°C, 15°C, and

22°C) and RH (60% and 90%) combinations were used to simulate various production,

processing, and storage conditions of food preparation environments. Viruses were recovered

from the surfaces at five different time points (2.5h, 3d, 6d, 10d, and 14d). Additionally, two

surfaces were inoculated with 300 µl of deionized water for each surface type per experiment as

a negative control to be collected at 2.5h and 14d.

Virus samples were recovered from the surfaces in a similar manner as optimized using the

repeated pipetting method with 1×PBS + 0.1%Tween80 (PBST) elution buffer as described in

Chapter 3. This protocol was conducted in a similar manner as described by Cannon et al. (2006)

with slight modifications. At the specified time points, 500 µl of PBST was added to the surfaces

and allowed to set for approximately 10 minutes. The virus sample and PBST were subsequently

eluted from the surface by pipetting back-and-forth 25 times. Next, the recovered virus sample

and elution buffer was collected into a 1.5 ml microcentrifuge tube. The recovered volume for

each sample was measured and recorded. Next, viral concentrations of AiV were acquired

through plaque assay as described previously (refer to Section 2.1 on "Propagation and

quantification of AiV"). Samples were stored at -80°C until further processing of hNoVs as

described previously (refer to Section 2.2 on "Detection of hNoVs by real time PCR").

2.4 Statistical Analysis

All experiments were repeated at least two times as independent replicates with

duplicates in each experiment. Descriptive statistics including means and standard deviations

were determined. A linear regression line were plotted using SAS® 9.4 (SAS Institute Inc., Cary,

NC) to be used to calculate the decimal reductive values (D-value, the negative reciprocal of the slope of the line), which is the time needed to achieve a 1-log reduction in infectious virus titer. A one-way analysis of variance (ANOVA) and two-way ANOVA were performed using SAS. A one-way ANOVA compared D-values between variables and samples while a two-way ANOVA test compared D-values between pairs of variables.

3. Results

3.1 AiV persistence on fomites

Aichivirus A persistence on nonporous fomites was evaluated on acrylic-based solid surface, plastic chopping board, and stainless steel under varying environmental conditions for 14d. For both 22°C/60% RH and 15°C/60% RH combinations, AiV was stable on all three surfaces with $\log_{10}$ titer reductions ranging from 0.61 ± 0.44 (6°C/90%RH, plastic) to 4.51 ± 1.30 (15°C/60%RH, acrylic) (Figure 1). Across days, significant difference varied among surface types and temperature/RH combinations (Figure 1). At day 14, there were significant differences between low humidity combinations (22°C/60% RH and 15°C/60% RH) and high humidity combinations (15°C/90% RH and 6°C/90% RH) for all surfaces ($p<0.05$). At day 14, there were no significant differences between any of the surfaces for 22°C/60% RH, 15°C/60% RH, and 15°C/90% RH at 14d ($p>0.05$). However, there were significant differences between plastic and all other surfaces for 6°C/90% RH at 14d ($p<0.05$).

Decimal reduction values (D-values) for AiV ranged from 3.19 ± 1.13 (22°C/60% RH, plastic) to 47.62 ± 0.10d (6°C/90% RH, plastic) (Table 1). Generally, the higher relative humidity (90% RH) had higher D-values than lower relative humidity (60% RH) (Table 1). There were significant differences between low humidity combinations (22°C/60% RH and

15°C/60% RH) and high humidity combinations (15°C/90% RH and 6°C/90% RH) for all surfaces ($p<0.05$). Also, there was a significant difference between 15°C/90% RH and 6°C/90% RH combinations for all surfaces ($p<0.05$). Furthermore, no significant difference was found for D-values (days) among all surfaces for 22°C/60% RH and 15°C/60% RH combinations ($p>0.05$). However, significant differences were found between all surfaces for 6°C/90% RH and between plastic and other surfaces for 15°C/90% RH ($p<0.05$; Table 1).

3.2 hNoV persistence on fomites

Human norovirus (GI.1, GII.17) persistence was evaluated on nonporous fomites under varying environmental conditions over 14d. GI.1 was found to be more stable than GII.17 on nonporous surfaces at 22°C/60% RH and 15°C/60% RH with GI.1 detection over $\geq$ 14d and no signal detected for GII.17 at 3d (Figure 2 –GI.1; data not shown – GII.17). GII.17 was only detected at the 2.5 h drying time under ambient conditions (Figure 3). GII.17 was discontinued at other storage conditions due to inconsistency and inability to efficiently recover from surfaces.

Human norovirus GI.1 on nonporous surfaces under varying environmental conditions over 14d had a mean $\log_{10}$ reduction ranging from 1.33 ± 0.30 (15°C/60% RH, stainless steel) to 2.76 ± 0.23 (22°C/60% RH, plastic) (Figure 2). Across days, significance differences varied among surface types and temperature/RH combinations (Figure 2). Similar to the trend seen with AiV persistence, higher relative humidity (90%RH) had lower mean $\log_{10}$ reductions than the lower relative humidity (60%RH) over 14d on nonporous fomites. Additionally, there were no significant differences among any of the surfaces for 22°C/60% RH, 15°C/60% RH, and 15°C/90% RH combinations at 14d ($p>0.05$). There were no significant differences among surfaces for 6°C/90% RH ($p>0.05$) except between acrylic-based and stainless steel at 14d for hNoV GI.1 ($p<0.05$). Furthermore, there were no significant differences between low humidity

combinations for all surfaces for hNoV GI.1 at 14d ($p>0.05$). For acrylic-based solid surface, there were no significant differences found for all temperature/RH combinations for GI.1 at 14d ($p>0.05$). For stainless steel, there were significant differences between low humidity combinations (60%RH) and high humidity combinations (90%RH) at 14d for hNoV GI.1 ($p<0.05$). For 14d, there were significant differences between low humidity combinations to 15°C/90% RH on plastic for hNoV GI.1 ($p<0.05$). There were no significant differences between 6°C/90% RH to all other temperature/RH combinations on plastic for hNoV GI.1 at 14d ($p>0.05$).

4. Discussion

As stated previously, fomites play a major role in human enteric virus transmission in a variety of ways and settings including food preparation environments (Boone and Gerba 2007; Rzezutka and Cook 2004). Human enteric viruses have been demonstrated to retain infectivity over prolonged periods of time on various types of fomites (Escudero et al. 2012; Rzezutka and Cook 2004). For example, echovirus, coxsackievirus, and poliovirus have been shown to remain infectious on household representative surfaces (e.g. painted wood, cotton fabric, and glass) from 2 to >12 days (Kiseleva 1968). Furthermore, Kramer et al. (2006) reported enteric viruses such as hepatitis A, astrovirus, poliovirus, and rotavirus being able to survive on inanimate fomite surfaces for an estimated two months. Overall, several factors such as surface type, temperature, presence of organic matter, and RH may also impact virus persistence on nonporous food contact surfaces.

Temperature may play a role in enteric virus persistence. Kramer et al. (2006) reported low temperatures of 4°C or 6°C are associated with longer persistence of viruses such as hepatitis A (HAV), poliovirus, astrovirus, and adenovirus. Furthermore, Mormann et al. (2015) observed 1

log reduction at room temperature and no log reduction at 7°C for MNV on stainless steel after 7-15 days. In this study, temperature may play a role in longer virus persistence of AiV and hNoV GI.1 though no significant differences were observed. In addition, temperature appears to matter more at lower RH (60%) than higher RH (90%) in the present study with lower temperatures displaying higher D-values for AiV and lower mean log reductions for GI.1 though again there were no significant differences detected. There also appears to be differences among surface types particularly as the RH increased. It should also be noted that Abad et al. (1994) found the effect of low temperature (4°C) on HAV and adenovirus was only significant after 2 months on nonporous fomites. Further studies need to be conducted in order to better understand the role of temperature in combination with other factors and variables on human enteric viruses.

Surface type could impact virus persistence even though the food contact surfaces used in this study are all nonporous. Kramer et al. (2006) reported inconsistent results within the literature with respect to the role of surface type on virus persistence with some authors reporting no impact on virus persistence for rotavirus, poliovirus, norovirus, and other enteric viruses while viruses such as FCV were persistent on nonporous surfaces (e.g. telephone buttons and receivers). Furthermore, surface texture and crevices could possibly protect viruses against environmental conditions. For instance, Mattison and co-authors (2007) found a low mean recovery of FCV from strawberries due to surface properties even though food matrices and porous fomites were not within the scope of this study. In this study, surface type may impact virus persistence. The significant differences between surface types varied for each virus across days and temperature/RH combinations. Generally, GI.1 was found to be most stable on stainless steel while AiV was found to be most stable on plastic over 14d under varying environmental conditions.

The presence of organic matter in hNoV GI.1 samples (fecal) and AiV samples (cell-lysate) may or may not impact virus persistence. The effect of fecal matter on the enteric virus persistence varies between fomites and virus types (Abad et al. 1994). Abad et al. (2001) also observed varying effects of fecal matter on astrovirus under varying environmental conditions and surface types. For instance, longer persistence for astrovirus was observed on nonporous toilet china at 4°C and not significantly affected at 20°C in the presence of fecal matter (Abad et al. 2001). The effect of cell-lysates from cell culture processes of cultivable viruses and surrogates could underestimate the persistence of these viruses due to the possible presence of reactive oxygen species, proteolytic enzymes, and other metabolic by-products released from cells that may damage viral capsid proteins (Esseili et al. 2015). Additionally, Esseili et al. (2015) indicated a lower survival rate of FCV, porcine saporvirus, and Tulane virus (TuV) in suspension with cell-lysates while lesser effects on MNV were observed. Overall, further investigation of the impact of organic matter on virus persistence is needed for a wider range of enteric viruses and hNoVs on nonporous fomites.

The main influencing factor on virus persistence in this study appears to be relative humidity. Kramer et al. (2006) suggested low temperatures (4°C or 6°C) and high humidity (>70%RH) were associated with longer persistence for most viruses. Abad et al. (1994) observed enhanced persistence of poliovirus and HAV at higher RH (>80%RH) on nonporous fomites. Also, Lamhoujeb et al. (2009) reported longer persistence of hNoV for about 28 days at high RH (86% ± 4%) compared to about 7 days at low RH (30% ± 10%) on stainless steel and polyvinyl chloride surfaces. However, the persistence of MNV was found to be inconsistent within the range of 30%-70% RH on nonporous surfaces with MNV persisting longer on sealed wooden chopping board than stainless steel with an average inactivation of 16 days and 3 days,

respectively (Kim et al. 2012). In this study, the results were consistent with enhanced persistence of enteric viruses at higher RH (>80%RH). Significant differences were observed for GI.1 and AiV between environmental conditions containing lower RH (60%RH) vs higher RH (90%RH).

There are several limitations in this study. First, GII.17 could be influenced by surface sampling and/or lack of particle association to fecal matter. Virus recovery efficiencies are highly variable between surface and virus types, which greatly impacts virus persistence. They may also impact different genotypes of hNoVs in different ways, and this question needs to be further investigated. Also, persistence of GI.1 may be overestimated due to the limitations of using RT-qPCR. Human noroviruses have been shown to display less in reduction of RT-qPCR signals than compared to reductions of infectivity of cultivable surrogates (Knight et al. 2016). This observation is due to the limitations of RT-qPCR in detecting infectious and noninfectious virus and virus particles (Knight et al. 2013). Until a more routine culture method is available, surrogates such as FCV, MNV, and TuV are necessary in understanding infectivity and persistence of hNoVs in a wide range of study parameters (Arthur and Gibson 2015; Cannon et al. 2006; Yeargin et al., 2015).

5. Conclusions

Persistence studies of human enteric viruses are essential in understanding fomite contamination for prevention and control of foodborne illnesses. Most current virus persistence studies focus on human enteric viruses and their surrogates such as FCV, MNV, hNoV GII.4, and HAV. This study aimed to address the gaps in knowledge of virus persistence of AiV and hNoVs (GII.17, GI.1) under varying environmental conditions on nonporous food preparation surfaces. This was the first study to report on AiV persistence on nonporous surfaces. Moreover,

this present study was successful in determining the persistence of AiV and GI.1 under varying environmental conditions. AiV was found to be persistent on all surfaces with about a 3 $\log_{10}$ titer reduction for low RH (60%RH) combinations and about a 1 $\log_{10}$ titer reduction for high RH (90%RH) combinations over 14d. GI.1 was found to be more persistent than GII.17 on surfaces at 22°C/60% RH and 15°C/60% RH with GI.1 detection over ≥ 14d and no signal detected for GII.17 at 3d. GII.17 instability may be due to surface sampling techniques, virus type, and the absence of fecal matter in GII.17 samples. Multiple factors such as surface type, virus type, the presence of fecal matter, temperature, and RH may impact AiV and GI.1 persistence on nonporous surfaces and contribute to the high variability among surface types. Relative humidity may be the main influencing factor of longer persistence for GI.1 and AiV with higher RH (90%) causing more persistence in virus concentrations than lower RH (60%) over 14d. The effect of lower temperature on longer persistence of GI.1 and AiV appeared in only the lower RH combinations though no significant difference was observed. Overall, future studies should focus on moving towards a more standardized surface sampling technique for a variety of human enteric viruses and their surrogates to better understand virus persistence from surfaces.

References

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

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

Ahmed, S. M., Hall, A. J., Robinson, A. E., Verhoef, L., Premkumar, P., Parashar, U. D., Lopman, B. A. (2014). Global prevalence of norovirus in cases of gastroenteritis: A systematic review and meta-analysis. *The Lancet Infectious Diseases, 14*, 725-730.

Arthur, S. E., & Gibson, K. E. (2015). Environmental persistence of tulane virus—a surrogate for human norovirus. *Canadian Journal of Microbiology, 62*, 449-454.

Arthur, S., & Gibson, K. (2015). Physicochemical stability profile of tulane virus: A human norovirus surrogate. *Journal of Applied Microbiology, 119*, 868-875.

Boone, S. A., & Gerba, C. P. (2007). Significance of fomites in the spread of respiratory and enteric viral disease. *Applied and Environmental Microbiology, 73*, 1687-1696.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L., & Vinjé, 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*, 2761-2765.

D'Souza, D. H., Dice, L., & Davidson, P. M. (2016). Aqueous extracts of *Hibiscus sabdariffa* Calyces to control aichi virus. *Food and Environmental Virology, 8*, 112-119.

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*, 84-91.

Esseili, M. A., Saif, L. J., Farkas, T., & Wang, Q. (2015). Feline calicivirus, murine norovirus, porcine sapovirus, and Tulane virus survival on postharvest lettuce. *Applied and Environmental Microbiology*, *81*, 5085-5092.

Escudero, B.I., Rawsthorne, H., Gensel, C., & Jaykus, L.A. (2012). Persistence and transferability of noroviruses on and between common surfaces and foods. *Journal of Food Protection, 75,* 927−935.

Faix, R. G. (1987). Comparative efficacy of handwashing agents against cytomegalovirus. *Infection Control, 8*, 158-162.

Gibson, K.E., Crandall, P.G., & Ricke, S.C. (2012). Removal and transfer of viruses on food contact surfaces by cleaning cloths. *Applied and Environmental Microbiology*, *78*, 3037-3044.

Hsueh, T. Y., & Gibson, K. E. (2015). Interactions between human norovirus surrogates and *Acanthamoeba* spp. *Applied and Environmental Microbiology, 81*, 4005-4013.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., et al. (2003). Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology, 41,* 1548–1557.

Kim, S. J., Si, J., Lee, J. E., & Ko, G. (2012). Temperature and humidity influences on inactivation kinetics of enteric viruses on surfaces. *Environmental Science & Technology, 46*, 13303-13310.

Kiseleva, L. (1968). Survival of enteric viruses in water and foodstuffs and on various surfaces. *Hygiene & Sanitation, 33*, 439-440.

Knight, A., Haines, J., Stals, A., Li, D., Uyttendaele, M., Knight, A., & Jaykus, L. A. (2016). A systematic review of human norovirus survival reveals a greater persistence of human norovirus RT-qPCR signals compared to those of cultivable surrogate viruses. *International Journal of Food Microbiology*, *216*, 40-49.

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

Kramer, A., Schwebke, I., & Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infectious Diseases, 6*, 1.

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

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,* 500−503.

Rzeżutka, A., & Cook, N. (2004). Survival of human enteric viruses in the environment and food. *FEMS Microbiology Reviews, 28*, 441-453.

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

Yeargin, T., Fraser, A., Huang, G., & Jiang, X. (2015). Recovery and disinfection of two human norovirus surrogates, feline calicivirus and murine norovirus, from hard nonporous and soft porous surfaces. *Journal of Food Protection, 78,* 1842−1850.

| RH/Temperature	Decimal reduction value (days)		
	ABSS	P	SS
22°C 60%RH	$4.13 \pm 0.66^{A,X}$	$3.19 \pm 1.13^{A,X}$	$4.29 \pm 0.74^{A,X}$
15°C 60%RH	$3.75 \pm 1.03^{A,X}$	$3.75 \pm 1.17^{A,X}$	$5.62 \pm 0.16^{A,X}$
15°C 90%RH	$15.63 \pm 0.84^{A,Y}$	$28.57 \pm 0.13^{B,Y}$	$16.39 \pm 0.77^{A,Y}$
6°C 90%RH	$11.76 \pm 0.21^{A,Z}$	$47.62 \pm 0.10^{B,Z}$	$9.43 \pm 0.16^{C,Z}$

Table 1: Decimal reduction values of Aichivirus A under varying environmental conditions on various nonporous food contact surfaces. Decimal reduction values (days) were calculated using linear regressions produced for AiV samples collected from each relative humidity (RH) and temperature combination over a two-week period. Virus samples were collected from three nonporous food contact surfaces (ABSS – acrylic-based solid surface, P- plastic chopping board, SS – stainless steel) using repeated pipetting method with PBS+0.1%Tween80 elution buffer. Letters A-C indicated significant difference between surfaces within one RH/Temperature combination (rows) while letters X-Z indicated significant difference between RH/Temperature combinations for one surface type (columns; $p<0.05$).

Acrylic-based solid surface

A.)

Plastic chopping board

B.)

Stainless steel
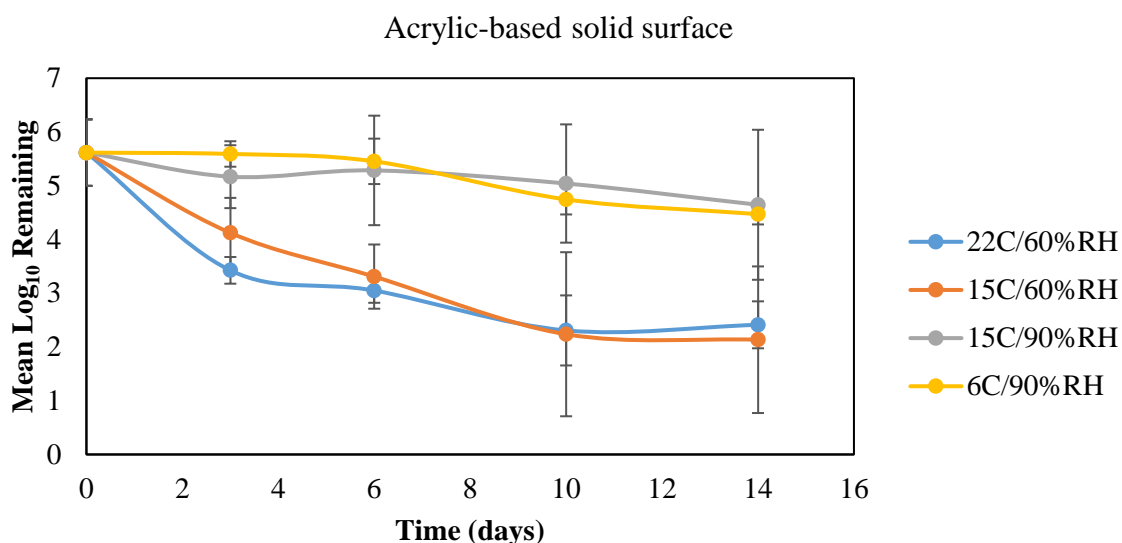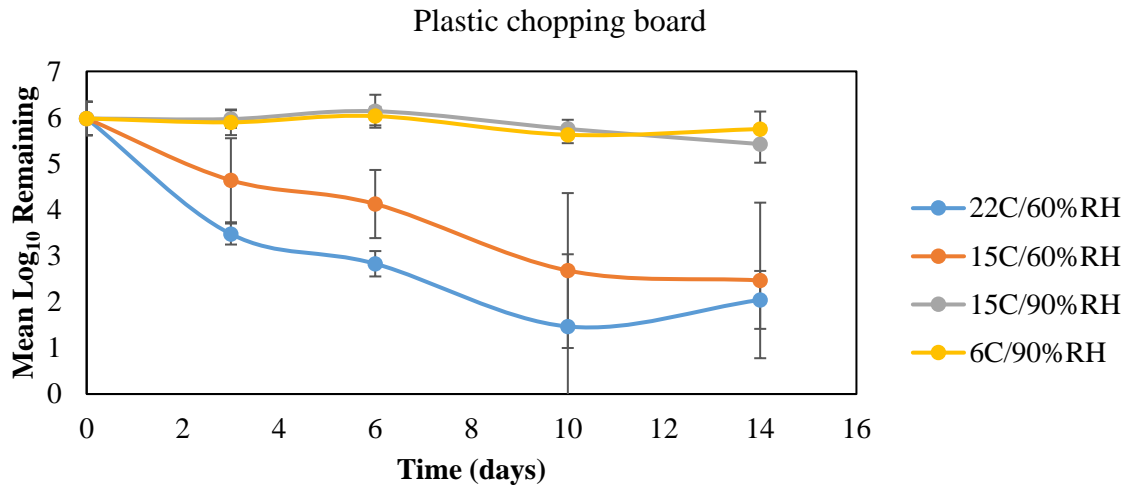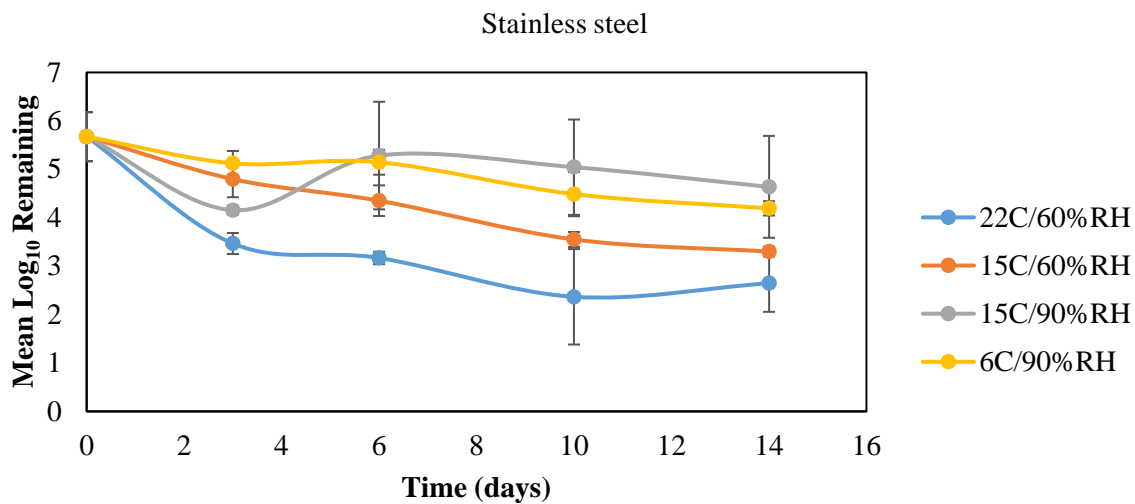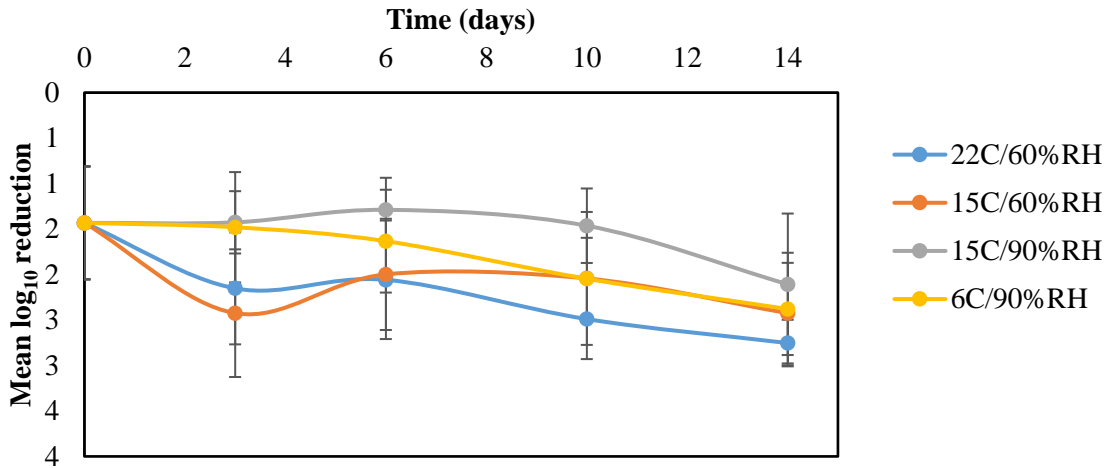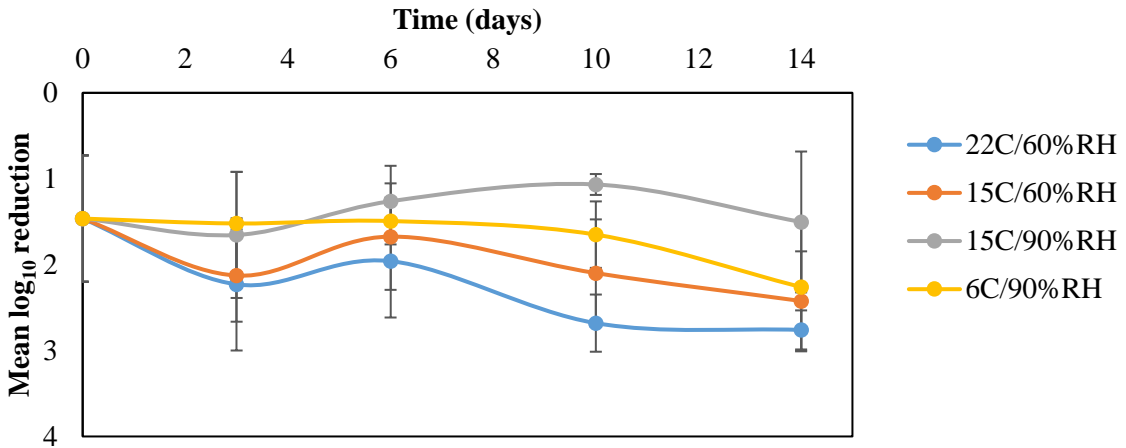

C.)

Figure 1: Comparison of Aichivirus A (AiV) persistence over two weeks on nonporous food contact surfaces under varying environmental conditions. AiV was inoculated onto three nonporous food surfaces (acrylic-based – A, plastic chopping board – B, stainless steel – C) and placed into an environmental chamber at various relative humidity and temperature combinations (22°C/60%RH – blue, 15°C/60%RH – orange, 15°C/90%RH – gray, 6°C/90%RH –yellow). Samples were collected using the repeated pipetting method with PBS+0.1%Tween80 elution buffer at various time periods (2.5h, 3d, 6d, 10d, 14d).
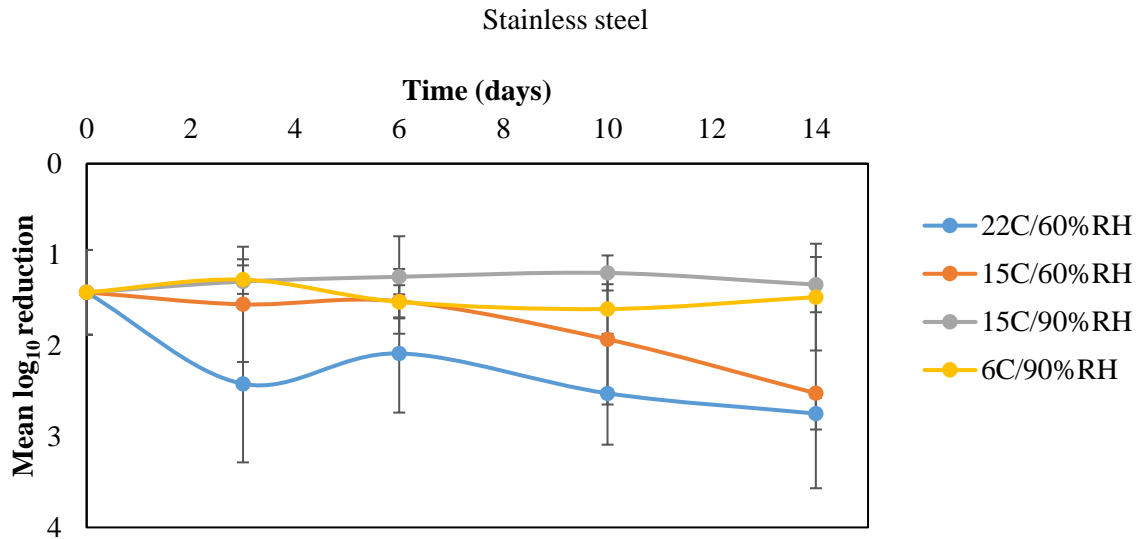
Acrylic-based solid surface

Time (days)

A.)

Plastic chopping board

Time (days)

B.)

Stainless steel


C.)

Figure 2: Comparison of human norovirus GI.1 persistence over two weeks on nonporous food contact surfaces under varying environmental conditions. hNoV GI.1 was inoculated onto three nonporous food surfaces (acrylic-based – A, plastic chopping board – B, stainless steel – C) and placed into an environmental chamber at various relative humidity and temperature combinations (22°C/60%RH – blue, 15°C/60%RH – orange, 15°C/90%RH – gray, 6°C/90%RH –yellow). Samples were collected using the repeated pipetting method with PBS+0.1%Tween80 elution buffer at various time periods (2.5h, 3d, 6d, 10d, 14d).
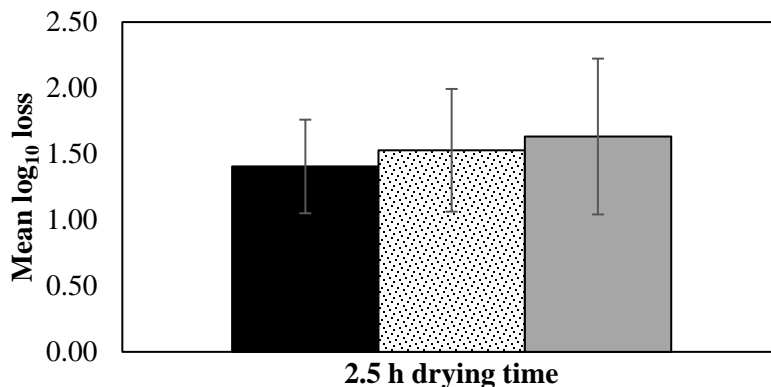
Figure 3: Human norovirus GII.17 recovery from nonporous food contact surfaces at 2.5 h drying time. hNoV GII.17 was recovered from three nonporous food contact surfaces (acrylic – black, plastic chopping board – dotted, stainless steel – gray) using the repeated pipetting method with PBST+0.1%Tween80 elution buffer after a 2.5 hour drying time under ambient conditions. Virus samples were analyzed using RT-qPCR, and relative log reductions were calculated using a standard curve produced by a 10-fold dilution series.

Chapter 5: Overall Conclusions

Foodborne human enteric viruses are known for causing diseases such as gastroenteritis, enterically transmitted hepatitis, and disease in other organs after replication in the gut (Koopmans and Duzier 2004). Specifically, human norovirus (hNoV) has been identified as the leading cause of acute viral gastroenteritis contributing to approximately 48% of foodborne outbreaks in the United States (Green 2007; Hall et al. 2014). Prolonged stability in the environmental is a major factor in human enteric virus transmission, and there is growing evidence of viral transmission via fomite contamination in a variety of ways and settings inclusive of food preparation environments (Boone and Gerba 2007; Koopmans and Duzier 2004; Rzezutka and Cook 2004). To better understand human enteric virus persistence on fomites under varying environmental conditions, the application of an optimized virus recovery method is essential.

Prior to the research presented here, a review of the various surface sampling methods used in the evaluation of laboratory-based virus recovery studies of human enteric viruses from nonporous fomites (Chapter 2). Generally, surface sampling studies were limited and varied among research parameters such as virus type/density, surface type, elution buffer used, tools, and drying time. This review demonstrated differences among methods, which contributed to the challenge to effectively compare surface sampling methods for various viruses under even similar parameters. Based on this review, no single standard approach to human enteric virus recovery from nonporous fomites was identified, and we provided some basic recommendations to move towards one methodology (Chapter 2).

Overall, this thesis aimed to optimize a surface sampling method for virus recovery from fomites with an application in environmental persistence studies of human enteric viruses and

their surrogates. The specific objectives for this study were to (1) optimize surface sampling methods for virus recovery of human enteric viruses and their surrogates, and (2) evaluate virus persistence of hNoVs and Aichivirus A (AiV) on three nonporous food contact surfaces under varying environmental conditions.

In Chapter 3, optimization of human enteric virus recovery from nonporous fomites was achieved by first evaluating three surface sampling techniques and two elution buffers using feline calicivirus (FCV). FCV was inoculated onto three nonporous fomites (stainless steel, plastic chopping board, and acrylic-based solid surface) under ambient conditions for one hour drying time. Then, virus samples were recovered using 1 of 3 implements (macrofoam swab, repeated pipetting, cell scraper) and 1of 2 eluents (1 × phosphate buffered saline solution (PBS) or PBS + 0.1% Tween80 (PBST, 1:1 v/v)). The variability among virus recovery for FCV was found to be consistent with other studies (Mattison et al. 2007; Taku et al. 2012; Yeargin et al. 2015). Then, the optimization of recovery of other human enteric viruses and their surrogates (hNoVs GI.1 and GII.17, Tulane virus (TuV), and AiV) from nonporous fomites occurred using the repeated pipetting with PBST method, which was demonstrated as the most efficient method for FCV recovery though no significant differences were found among all methods. The repeated pipetting with PBST method was successful in obtaining human enteric viruses and their surrogates from nonporous fomites, and this study was the first to evaluate repeated pipetting for hNoVs (GI.1 and GII.17), TuV, and AiV. However, tool selection was determined to vary among hNoVs since GII.17 fared better with the cell scraper method than repeated pipetting, unlike GI.1. Overall, this objective demonstrated the potential impact of recovery for persistence studies due to variability among tool selection, surface type, and virus type.

In Chapter 4, the environmental persistence of hNoVs (GI.1, GII.17) and AiV was evaluated under varying environmental conditions on nonporous food contact surfaces. A cocktail of the viruses were inoculated onto the three nonporous fomites and placed into an environmental chamber under varying temperatures (6°C, 15°C, 22°C) and relative humidity (RH; 60%, 90%). Virus was recovered using repeated pipetting with PBST at specified time points over a two-week time period. GII.17 recovery was found to be inconsistent at 2.5 h and no signal was detect at 3d for low humidity combinations (60%RH), which may be mainly impacted by the surface sampling technique selected as seen for GII.17 in Chapter 3. These results led to the discontinuation of GII.17 at other temperature/RH combinations. For AiV, decimal reduction values (D-values) ranged from 3.19 ± 1.13 (22°C/60% RH, plastic) to 47.62 ± 0.10d (6°C/90% RH, plastic). For GI.1, mean $\log_{10}$ reductions on surfaces ranged from 1.33 ± 0.30 (15°C/60% RH, stainless steel) to 2.76 ± 0.23 (22°C/60% RH, plastic) over 14d. There were variabilities in significant differences across days among surface types and temperature/RH combinations for GI.1 and AiV. Generally, higher RH (90%) caused more stability for GI.1 and AiV than lower RH (60%) for this objective, which is consistent with other studies on enteric virus persistence (Abad et al. 1994; Kramer et al. 2006; Lamhoujeb et al. 2009). Low temperature did not seem to be a major influencing factor in this study, which is inconsistent with some previous studies (Kramer et al. 2006; Mormann et al. 2015). Additionally, this is the first study to demonstrate AiV persistence on nonporous FCS. Overall, hNoV GI.1 and AiV remain stable on nonporous fomites over a two-week time period.

Further studies in surface sampling and virus persistence should move towards investigating such factors as the impact of organic matter (e.g. cell lysates, fecal matter) on a greater variety of human enteric viruses and their surrogates and particularly on both nonporous

and porous fomites. Furthermore, researchers should consider including time points over two-weeks to further investigate the role of temperature on virus persistence on nonporous fomites. Additionally, surface sampling appeared to be a major influencer in preventing a more accurate picture of virus persistence for some viruses. Researchers should move towards a single standard approach to surface sampling for laboratory-based studies by evaluating a variety of tools on a greater variety of human enteric viruses and their surrogates to reduce variability.

References

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

Boone, S. A., & Gerba, C. P. (2007). Significance of fomites in the spread of respiratory and enteric viral disease. *Applied and Environmental Microbiology, 73*, 1687-1696.

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.

Hall, A. J., Wikswo, M. E., Pringle, K., Gould, L. H., & Parashar, U. D. (2014). Vital signs: Foodborne norovirus outbreaks - united states, 2009-2012.*Morbidity and Mortality Weekly Report, 63*, 491-495.

Kramer, A., Schwebke, I., & Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infectious Diseases, 6*, 130.

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

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,* 500−503.

Mormann, S., Heißenberg, C., Pfannebecker, J., & Becker, B. (2015). Tenacity of human norovirus and the surrogates feline calicivirus and murine norovirus during long-term storage on common nonporous food contact surfaces. *Journal of Food Protection, 78*, 224-229.

Rzeżutka, A., & Cook, N. (2004). Survival of human enteric viruses in the environment and food. *FEMS Microbiology Reviews, 28*, 441-453. Taku, A., Gulati, B.R., Allwood, P.B., Palazzi, K., Hedberg, C.W., & Goyal, S.M. (2002). Concentration and detection of caliciviruses from food contact surfaces. *Journal of Food Protection, 65,* 999−1004.

Yeargin, T., Fraser, A., Huang, G., & Jiang, X. (2015). Recovery and disinfection of two human norovirus surrogates, feline calicivirus and murine norovirus, from hard nonporous and soft porous surfaces. *Journal of Food Protection, 78,* 1842−1850.

Appendix
IBC Approval Letter

UNIVERSITY OF ARKANSAS | **Research & Economic Development**
Research Compliance

March 8, 2016

MEMORANDUM

TO: Dr. Kristen Gibson

FROM: Dr. Ines Pinto
                                     Institutional Biosafety Committee

RE: IBC Protocol Approval

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, 2019

The Institutional Biosafety Committee (IBC) has approved the Renewal of Protocol 13017, with modification, "Understanding Environmental Reservoirs and Prevalence of Norovirus Surrogates to Reduce Impact on Public Health." You may continue 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.