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Survival, Recovery, and Inactivation of Human Norovirus Surrogates, Feline Calicivirus and Murine Norovirus, on Carpets

David Buckley
Clemson University, buckleyd07@gmail.com

SURVIVAL, RECOVERY, AND INACTIVATION OF HUMAN NOROVIRUS SURROGATES, FELINE CALICIVIRUS AND MURINE NOROVIRUS, ON CARPETS

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David Buckley
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Accepted by:
Xiuping Jiang, Committee Chair
Simon Scott, Co-Chair
Angela Fraser
Charles Pettigrew

ABSTRACT

Worldwide, enteric viruses are the main cause of acute gastroenteritis (AGE). Among these viruses, human noroviruses (HuNoV) are leading cause of AGE and account for ca. 20% of all diarrheal cases, a top-five cause of death worldwide. In humans, these viruses spread via person-to-person contact, food, water, and/or the environment. Person-to-person contact is the most common mode of HuNoV transmission. Yet, environmental transmission has been linked to several outbreaks and prolonged others. HuNoV survival and inactivation on hard environmental surfaces have been extensively studied. However, nonlaunderable soft surfaces, such as carpet, have received little attention despite epidemiological evidence suggesting their role in transfer and transmission of HuNoV. Currently there are no commercially available products for sanitizing these surface after a contamination event. Documenting the efficacy of sanitizers intended for virally contaminated soft surfaces is also compounded by no standardized method for the recovery of viruses. Therefore our aims for this study, using the environmentally relevant soft surface, carpet, were to (i) determine factors that influence the survival and inactivation of enteric viruses on nonlaunderable soft surfaces (ii) determine survival of HuNoV surrogates on an carpet, (iii) compare sampling methods to determine their ability to recover HuNoV surrogates from carpet, and (iv) to assess two sanitizing technologies, silver dihydrogen citrate (SDC) and steam vapor, against a HuNoV surrogate, FCV, on carpet.

A systematic review of the literature was conducted to determine factors that influence the survival and inactivation of enteric viruses on nonlaunderable soft surfaces.

EBSCO and Web of Science were searched for experimental studies published between 1965 and 2015 using Preferred Reporting Items for Systematic Reviews and Meta-Analyses methods. Titles and abstracts were screened using 3 eligibility criteria. The quality of all study methods was also assessed. Our search yielded 12 articles. Viruses survived between 0 hours and 140 days depending on surface and environment conditions. Virus survival was influenced by temperature, relative humidity, organic content, and deposition method. A variety of chemistries were tested across studies and were shown to have a varied effect on enteric viruses. Chlorine, glutaraldehyde, vaporous ozone, and hydrogen peroxide were the most efficacious against enteric viruses (> 3-log reduction). The efficacy of liquid and vaporous chemistries are associated with surface and virus type

The survival profile of HuNoV surrogates, FCV and murine norovirus (MNV), as studied on carpet. First, we measured the zeta potential and absorption capacity of wool and nylon carpet fibers, developed a mini-spin column elution method (MSC), and characterized the survival of HuNoV surrogates, FCV and MNV over 60 days under 30 and 70% relative humidity (RH) on carpets and a glass surface. Carpet surface charge was negative at a typical buffer pH while wool could absorb ca. 2X more liquid than nylon. Percent recovery efficiency with the MSC ranged from 4.34 to 20.89% and 30.71 to 54.14% for FCV and MNV on carpet fibers, respectively. Moreover, elution buffer type did not significantly affect recovery of either surrogate virus. Infectious FCV or MNV survived between <1 and 15 or 3 and 15 days, respectively. However, MNV survived longer under some conditions and at significantly higher titers compared to

FCV. Albeit, surrogates followed similar survival trends, i.e. both survived longest on wool followed by nylon and glass while 30% RH provided a more hospitable environment compared to 70% RH. qRT-PCR signals for both surrogates were detectable for the entire study but FCV genomic copies experienced significantly higher reductions (<3.80 $\log_{10}$ copies) on all surfaces compared to MNV (<1.10 $\log_{10}$ copies).

Virus recovery methods were compared to evaluate their ability to recover FCV and MNV from carpet. Specifically, we assessed and compared three recovery methods, i.e. bottle extraction (BE), macrofoam-tipped swabbing (MS), and the microbial vacuum (MVAC), using HuNoV surrogates, FCV and MNV, inoculated on wool and nylon. We also investigated detection issues for FCV after environmental recovery, i.e. inhibition. Infectious FCV and MNV percent recovery efficiency (% RE) of BE ranged from 0.44 to 48.44 and 40.77 to 68.83%, respectively, compared to MS % RE, which was 0.02 to 0.82% and 1.54 to 2.87%, respectively. The MVAC % RE of infectious FCV and MNV ranged from 7.30 to 18.29% and 52.58 to 74.67%, respectively. Percent RE of genomic copies of FCV and MNV with BE ranged from 0.36 to 2.53% and 3.34 to 14.97%, respectively, while MS % RE ranged from 1.03 to 2.24 and 2.02 to 4.25%, respectively. The MVAC % RE of genomic copies of FCV and MNV ranged from 2.49 to 23.72% and 28.78 to 79.15%, respectively. Significantly more plaque-forming units and genomic copies were recovered using BE and MVAC compared to MS, while buffer type played a significant role in recovery of infectious FCV. Additionally, qRT-PCR analysis indicated recovery from tested carpet types inhibited amplification of FCV RNA and required dilution after nucleic acid extraction

Two sanitizing technologies, SDC and steam vapor, were evaluated against FCV on wool and nylon carpet carriers. First, we evaluated both technologies effect on aesthetic appearance on carpet, developed a neutralizer for SDC, evaluated SDC's efficacy in suspension with and without 5% fetal bovine serum (FBS), SDC and steam vapor's efficacy on glass, each with and without 5% FBS, and finally tested both sanitizers on carpets. Wool and nylon carpet carriers exhibit no obvious color changes or abrasions after both treatments, however SDC treatment left a residue while steam left minor abrasions to the surface fibers. A sodium thioglycolate-based solution was found to adequately neutralize and eliminate SDC cytotoxicity. SDC in suspension and on glass reduced FCV by 4.65 and >4.66 $\log_{10}$ pfu, respectively, but demonstrated reduced efficacy in the presence of serum. However, SDC was only efficacious against FCV on nylon (3.62 $\log_{10}$ pfu reduction). Steam vapor reduced FCV by >4.93 $\log_{10}$ pfu on glass in 10 sec, with no observed difference among serum treatments, and >3.68 $\log_{10}$ pfu on wool and nylon carpet carriers in 90 sec. There was limited reduction to FCV RNA under both sanitizer treatments, but RNA reductions were higher in treatments with serum.

In this Ph.D. dissertation, we characterized wool and nylon carpet fibers based on their absorptive capacity and zeta potential while demonstrating that HuNoV surrogates, feline calicivirus (FCV) and murine norovirus (MNV), can survive for at least 15 days on carpets under some conditions. Additionally, we evaluated three methods' recovery efficiency with FCV and MNV on wool and nylon carpets that provides key data and analysis of methods intended for efficacy testing and environmental monitoring. Finally, we assessed two sanitizing technologies, silver dihydrogen citrate (SDC) and steam-

vapor with thermo-accelerated nano-crystal sanitation (TANCS) technology, against FCV, in suspension, glass, and wool and nylon carpet carriers of an experimental design for assessing efficacy of sanitizer intended for viruses on carpets. Results suggest SDC and steam-vapor with TANCS are efficacious against FCV but steam-vapor provides the highest level of inactivation. Ultimately, this is the first comprehensive study of HuNoV on carpet, an understudied fomite. Specifically, these studies estimate the survival characteristic of HuNoV on carpet, provide a comprehensive comparison of potential virus recovery methods from carpet, demonstrate the efficacy of two acceptable and reasonable virucidal sanitizers on carpet, and establish a much-needed experimental design for assessing virucidal sanitizers on carpets.

DEDICATION

I dedicate this work to my father, Michael Buckley, my grandparents, Jim and Shirley Norman, and my wife, Anna Buckley. I believe this dissertation would not have been possible without your love and support. Dad, thank you for sacrificing your job and time to provide a better life for your sons. Your love and commitment to me has always made me aim higher. Grandma and Grandpa, thank you for your constant encouragement and believing in me when others did not. And to Anna, your unconditional love, patience, and support throughout my education has been a blessing. I cannot thank you enough for your sacrifices and understanding while I pursued one my most important dreams.

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CHAPTER ONE

LITERATURE REVIEW – A BREIF OVERVIEW OF *NOROVIRUS*, ENTERIC VIRUS RECOVERY METHODS, AND SOFT SURFACES

INTRODUCTION

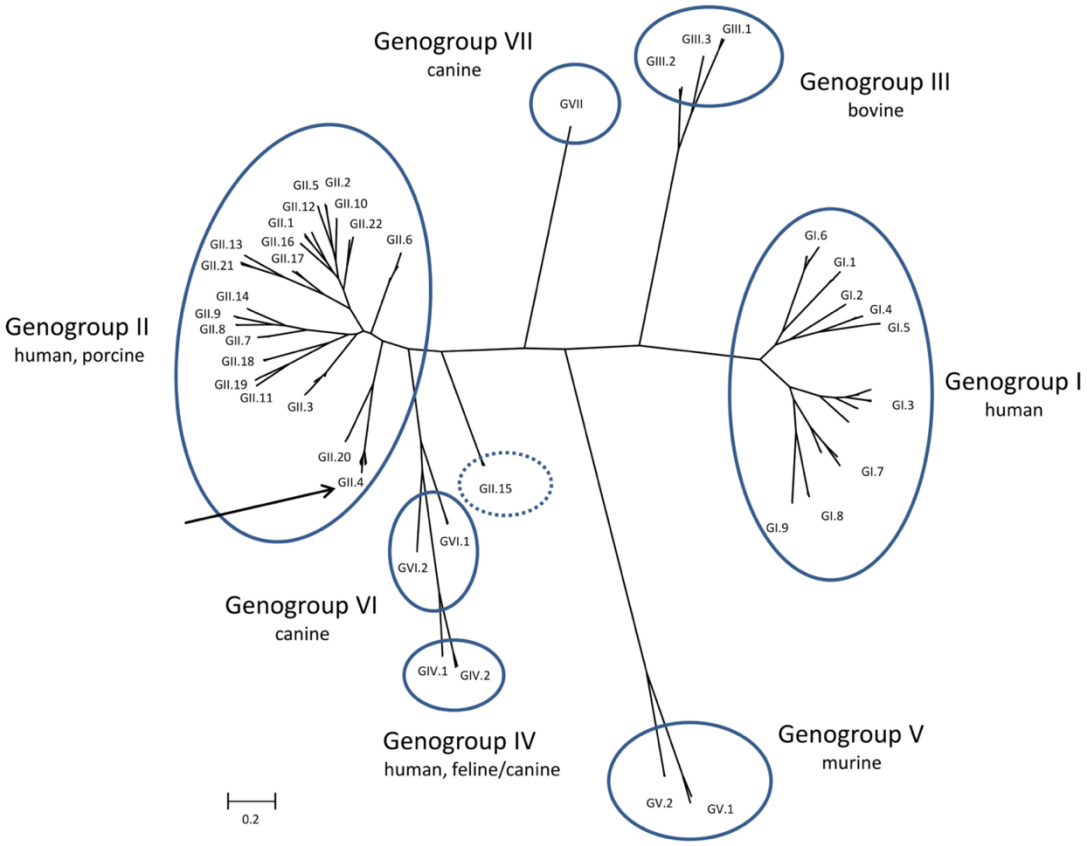
Norovirus (NoV) is a large and diverse genus of icosahedral enteric viruses belonging to the *Caliciviridae* (1). Human noroviruses (HuNoV), a subgroup of norovirus, are associated with acute gastroenteritis (AGE). AGE is a top-5 cause of death among humans while HuNoV account for ca. 20% of all diarrheal cases worldwide (2, 3). Additionally, HuNoV's economic burden worldwide is estimated to be $4.2 billion in health care costs and $60.3 billion in societal costs annually. In 2011, the Centers for Disease Control and Prevention (CDC), estimated that HuNoV accounted for 58% of the population afflicted by known foodborne disease in the United States (4, 5).

HuNoV were first discovered in 1972 using immune electron microscopy (IEM) to examine isolates from an elementary school outbreak that occurred 4 years earlier in Norwalk, Ohio (6). Over the years, HuNoV have been colloquially known as: winter vomiting disease, hyperemesis emesis, the stomach flu, stomach bug, and Norwalk virus (6). Symptoms of HuNoV infection include diarrhea, vomiting, nausea, dehydration, low-grade fever, muscle pains, and malaise. HuNoV are known to be shed in both vomit and diarrhea of infected patients. Diarrhea is due, in part, to malabsorption of carbohydrates and histological changes to the intestine, whereas delayed gastric emptying and motility are likely responsible for nausea and vomiting (2).

Culturing of NoV is not routinely available, except for murine strains precluding classification of NoV into serogroup or types. As such, NoV are classified into six

distinct genogroups (GI-GVI) consisting of 40 known genotypes based on genetic analysis (7). However, a new genogroup (GVII) has been adopted based on the genetic analysis of new 9 genotypes (7). HuNoV are located within GI, GII, and GIV, but the majority of HuNoV outbreaks are associated with GII.4 (**Figure 1.1**) (8).

Figure 1.1 Norovirus classification tree for nomenclature



Source: figure from Vinjé, Jan. "Advances in laboratory methods for detection and typing of norovirus." *Journal of clinical microbiology* 53.2 (2015): 373-381. (7).

PATHOGENESIS

Until recently, HuNoV were not cultureable, which hindered our ability to answer key questions surrounding their pathogenesis. The use of HuNoV-like particles have assisted with modeling infection. Working with these particles has demonstrated that HuNoV's capsid binding motifs likely attach to human blood group antigens prior to cell entry and replication within the intestinal milieu (9). In 2016, an *ex vivo* human intestinal enteroid (HIE) culturing system was shown to support HuNoV replication (10). Results of culturing HuNoV with the HIE system suggests HuNoV are capable of replicating in enterocytes from different segments of the small intestine. Furthermore, results also indicate that some strains require bile, whereas bile only enhances replication of other strains. With further work, this system may answer several key questions regarding intracellular replication and dissemination. At present the system is technically demanding and only available in two laboratories. But, additional studies are needed to make this culturing system more widely. Historically, human volunteer studies were conducted to answer key questions, such as infectious dose, viral shedding, and titer. The estimated infectious dose of HuNoV is 10 – 100 virions, which may be affected by strain type and host susceptibly (11). NoV's non-enveloped structure allows for its passage through harsh environments, such as the gastrointestinal track of animals, ambient environmental conditions, and a variety of disinfecting chemistries.

HUMAN NOROVIRUS SURROGATES

The lack of a routine culturing method has hampered our ability to study HuNoV. Even with the new HIE culture system, HuNoV research remains at a disadvantaged compared to other groups studying enteric viruses with adaptable cell culture models, e.g. rotavirus and hepatitis A virus (10). Some studies, e.g. disinfection and survival, require the use of infectious viruses because molecular assays, such as qRT-PCR, cannot differentiate between infectious and non-infectious particles. Accordingly, the investigators are forced to use infectious surrogates that mimic HuNoV both structurally and genetically. Common HuNoV surrogate viruses not infectious to humans are feline calicivirus (FCV), murine norovirus (MNV), tulane virus (TV), and porcine sapovirus (PSaV), among others (**Table 1.1**) (12).

FCV is the most recognizable surrogate used in HuNoV studies and has been selected based on its genetic similarities to HuNoV. Although FCV belongs to the *Caliciviridae* family, widely known for enteric viruses, it infects the upper respiratory track of cats (13). Many laboratories elect to use FCV because it demonstrates tropism for Crandell Rees kidney cells, and some strains, such as F9, have been cell culture adapted to form plaques. While there have been several recent studies that demonstrated FCV's susceptibility to some environmental factors and disinfectants, such as low pH and moderate levels of chlorine, FCV strain F9 remains the U.S. Environmental Protection Agency (EPA) designated surrogate for disinfectant efficacy studies (12).

MNV was discovered in 2003 within immunocompromised mice deficient in recombination-activating gene 2 (RAG2) and signal transducer and activator of

transcription 1 (STAT-1) and successfully cultured in a murine macrophage cell line (RAW 264.7) (14). Further work demonstrated murine microglial cells are susceptible to MNV infection. This marked the first successful cultivation of a NoV. Comparative studies using MNV illustrated similar qualities to HuNoV, such as size, shape, buoyant density, and biochemical features (15). For instance, MNV has a similar response to pH, temperature, and chlorine (12). An observed chemical response difference is MNV's susceptibly to ethanol and isopropanol treatments compared to HuNoV and FCV (12). Moreover, these viruses differ both clinically and genetically. Although found in stool, MNV does not cause AGE or vomiting in mice. In addition, HuNoV are known to bind with human blood group antigens (HBGA) and infect enterocytes, whereas MNV binds with sialic acid to infect macrophages and dendritic cells (14). Regardless of these differences, MNV is a more suitable surrogate compared to FCV for environmental and disinfectant studies.

Tulane virus (TV) is the newest virus to be used as a surrogate for the study of HuNoV and still not recognized by International Committee of Taxonomy of Viruses (ICTV). Discovered in 2008 in the stools of rhesus macaques, TV belongs to the *Caliciviridae* family and is included in a unique own genus: *Recovirus* (16). Benefits for using TV, include similarities to HuNoV, such as size, shape, buoyant density, and biochemical features (16). TV was successfully cell culture adapted and readily infects African monkey kidney cells in a plaque assay format. Like HuNoV, symptoms of TV infection include diarrhea and TV also recognizes HBGA antigens (15). TV is a

promising surrogate for HuNoV, but because of its novelty, more studies are needed as many technologies have not been extensively evaluated against TV.

PSaV, a surrogate commonly used for the uncultureable human sapovirus, has been proposed as a surrogate for HuNoV. PSaV was first cultured in 1980 within pig kidney cells (17). As a calicivirus, PSaV shares similar structural and genetic features to HuNoV, FCV, MNV, and TV, while also sharing similar biochemical features to MNV and TV (15). Furthermore, and like the HuNoV HIE, PSaV infects intestinal cells of pigs and requires additional supplements for infection, such as bile. The downsides to this surrogate are the low titer in cell culture compared to other surrogates and its inability to form plaques in culture (17).

Overall, these 4 surrogates (FCV, MNV, TV, and PSaV) have features that allow us, in the absence of a reliable culturing system, to estimate the effect of interventions and measure environmental stability of HuNoV. But each has drawbacks that limit our ability to make informed decisions regarding prevention and control strategies. At present, the best method for the study of HuNoV is human challenge studies, but these studies are expensive and not warranted in many cases. It is imperative that investigators select the most resilient surrogates for their application, e.g. do not select MNV for ethanol sanitizer tests, and, if possible, test multiple surrogates within the same study. Until, HIE or another culturing method is developed, infectious surrogates are the safest options for evaluating HuNoV.

Table 1.1 Overview of human norovirus surrogates

	FCV	**MNV**	**TV**	**PSaV**	**NoV VLP**	**AiV**
Host	Feline	Murine	Primate	Porcine	None	Human
Family	*Caliciviridae*	*Caliciviridae*	*Caliciviridae*	*Caliciviridae*	None	*Picornaviridae*
Genus	*Vesivirus*	*Norovirus*	*Recovirus*	*Sapovirus*	None	*Kobuvirus*
Symptoms	No diarrhea	No diarrhea*	Diarrhea	Diarrhea	None	Diarrhea
Virus titer	10^6-10^8	10^6-10^8	10^6-10^7	10^5-10^6	$>10^8$	10^6-10^8
Cell line	CRFK	RAW 264.7	LLC-MK2	LLC-PK2	None	Vero
Assay	Plaque Assay	Plaque Assay	Plaque Assay	TCID 50	--	Plaque Assay

*virus shed in stool but not apparent AGE symptoms.

Source: Table composed of information from (7, 9, 14, 16–18)

TRANSFER AND TRANSMISSION OF HUMAN NOROVIRUSES

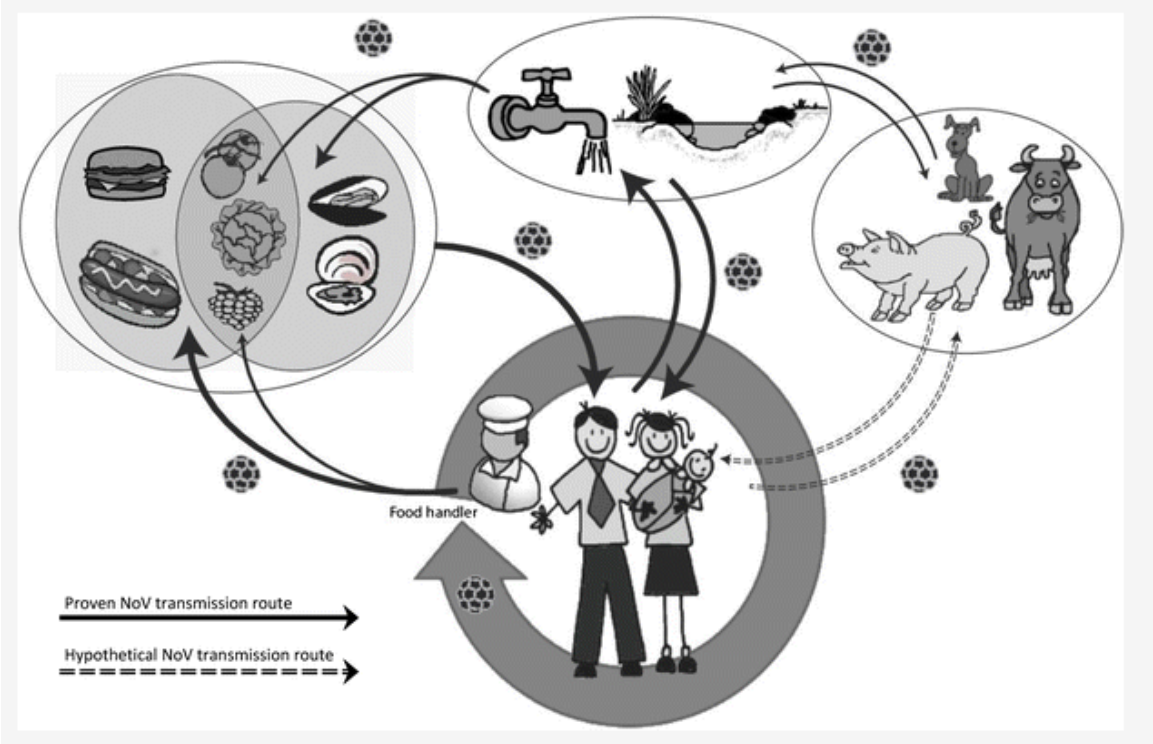
HuNoV are transmitted via the fecal oral route or vomitus oral route. The most common exposure pathways are person-to-person contact (66%), contaminated foods (25%), water (0.2%) and environmental transmission (0.3%) (**Figure 1.2**) (19, 20). Symptoms of HuNoV include involuntary vomiting and diarrhea that can harbor 10^5-10^9 HuNoV particles/g and 10^7 particles/30 ml, respectively (21, 22). These symptoms, among other reasons, contribute to the spread of HuNoV. HuNoV from both vomit and diarrhea can be found on hands and deposited onto surrounding surfaces, such as food and both hard and soft surface fomites, where they can survive for extended periods of time (23–25).

Transmission infers the passage of an infectious pathogen to a competent host causing disease, whereas transfer means the movement of a pathogen from one surface to another. Episodes of transmission are often based on epidemiological reports. Person-to-person and foodborne transmission are estimated to be the top causes of HuNoV transmission (19). Although there are several epidemiological investigations suggesting environmental transmission of HuNoV (**Table 1.2**). For instance, two carpet fitters exhibited HuNoV-like symptoms after removing carpet from a room used to cohort patients with HuNoV-like symptoms 16 days after the room had been vacated and decontaminated (25). Authors highlighted that the only intervention used on the carpet was a dry vacuum and the two carpet fitters had no other contact while working in the hospital ward. Another retrospective study found that 300 students visiting a concert hall were infected with HuNoV (26). A guest from the previous night experienced HuNoV

infection symptoms in the carpeted hallway. The highest reported attack rates (75%) were observed in students seated near the guest's seat and students using the same hallway (30-50%). Based on the negative results from food and water, and infection onset, investigators believed environmental transmission played a critical role in the outbreak, which was compounded by ineffective decontamination strategies and the environmental stability of the virus.

Evidence of virus transfer has been evaluated under controlled conditions. Several laboratory-based studies have documented the transfer of enteric viruses between surfaces (**Table 1.3**). For example, poliovirus is capable of being transferred between both natural, e.g. wool, and synthetic, e.g. nylon, surfaces (27). By the same token, and in separate studies, poliovirus, rotavirus, human adenovirus, and MS2 phage were found to be transferable between a variety of both natural and synthetic soft surfaces when washing (28–30). More importantly, investigators have demonstrated that contact between some soft surfaces can transfer a HuNoV surrogate, MS2 phage, to other surfaces, including hands (31). Taken together, both epidemiological reports of transmission and controlled transfer studies with soft surfaces suggest they may be important and overlooked fomites contributing to HuNoV outbreaks.

Figure 1.2 Overview of known and hypothetical human norovirus transmission routes



Source: Figure from Mathijs, E., et al. "A review of known and hypothetical transmission routes for noroviruses. Food Environ Virol 4: 131–152." (2012). (32).

Table 1.2 Suspected soft surface-mediated transmission of human noroviruses

Setting	Surface	Cases	Duration of Outbreak	Disinfection methods	Outcomes	Reference
Concert Hall	Carpet	>300	5 days	"emergency spillage compound", vacuuming	High attack rate associated with seating near patient-zero seat (75%) and with patrons using carpeted corridor (30-50%)	Evans, M.R. et al. (26)
Airplane	Carpet, Upholstered seats, curtains	27	5 days	Soft surfaces within 3 rows of incident removed. Other carpeted areas received steam cleaning	All hard surface negative for HuNoV, suggesting survival within carpets after treatment	Thornley, C. et al. (33)
Hotel	Carpet	942	5 months	Vacuuming, water, detergents	62% samples from carpets positive after cleaning	Cheesbrough, J. et al. (34)
Hospital	Carpet	2	N/A	Vacuuming	HuNoV likely transmitted while removing carpets 13 days outbreak cleared and 12 days after cleaning.	Cheesbrough, J. et al. (25)
Soccer Tournament	Reusable grocery bag	10	N/A	N/A	Soft surface contaminated via aerosolized HuNoV transferred to other surfaces	Repp and Keene (35)

Source: Yeargin, Thomas, "The role of human norovirus surrogates, feline calicivirus and murine norovirus, on non-porous and soft porous surfaces" (2014). *All Theses*. 1882. http://tigerprints.clemson.edu/all_theses/1882. (36)

Table 1.3 Transfer of enteric viruses and surrogates from soft surfaces

Surfaces	Virus[a]	Study design[b]	Significant Results	Reference
Cotton and wool fabrics: Dull nylon jersey Dacron/Cotton Shirting	PV	**Time**: 16 h **DM**: direct contact and aerosol **Temp:** 25˚C **RH:** 35% RH **Treatment:** inoculated carriers tumbled dried with sterile carriers.	Infectious PV transferable (3 $\log_{10}$) between surface. Wool showed highest transfer rate. poliovirus within 10 min.	Sidwell et al. (27)
Cotton and wool fabrics: Dull nylon jersey Dacron/Cotton Shirting	PV	**Time**: N/A **DM**: direct contact and aerosol **Temp:** 21-27, 38-43, and 54-60°C **RH:** N/A **Treatment:** Inoculated carriers washed with sterile carriers	PV reduced by washing treatment but transfer did not differ significantly between treatments and surface type	Sidwell et al. (28)
Cotton	RV HAV ADV	**Time**: 12/3 min cycle **Temp:** 20-23°C, 55°C **RH:** N/A **Treatment:** Washed and rinsed with detergent	RV, HAV, and ADV transferred 3.54, 3.18, and 3.4 $\log_{10}$ pfu/carrier, respectively, to sterile carrier. Transfer after drying: RV, HAV, and ADV 3.35, 3.43, and 3.4 log pfu/carrier, respectively	Gerba and Kennedy (29)

Hands, cotton/polyester blended knit weave, cotton toweling, cotton knit weave	MS2	**Time**: 16/10 min rinse/spin cycle **Temp:** N/A **RH:** N/A **Treatment:** Carriers washed in 69 L with sterile carriers. After, transferred to finger pad.	Up to 3.77 $\log_{10}$ pfu/carrier MS2 transferred between carriers. MS2 could be transferred to finger pad after washing with average transfer rate of 0.19%	O'toole et al. (30)
Cellulose/cotton cloths, microfiber cloth, nonwoven cloth, cotton terry towel	FCV PRD1 MS2	T**ime**: 12/3 min cycle **Temp:** RT **RH:** N/A **Treatment:** sterile surfaces wiped contaminated cloths	Surrogates transferred between 0.41 and 2.91 $\log_{10}$ pfu/ml to hard surfaces. Nonwoven and terry cloth transferred more virus to hard surfaces.	Gibson et al. (37)
Cotton, polyester, paper currency	MS2	**Time:** 10s **Temp:** RT **RH:** 15-32%, 40-65% **Treatment:** Index, middle, and ring finger pressed against inoculated carriers	% MS2 transfer efficiency ranged from 0.03 to 0.4% under low RH and 0.3 to 2.3% high RH	Lopez et al. (31)

[a]PV: poliovirus; RV: rotavirus; HAV: hepatitis A virus; ADV: human adenovirus; MS2: MS2 bacteriophage; FCV: feline calicivirus; PRD1: PRD1 bacteriophage.
[b]DM: deposition method; RH: relative humidity

DETECTION METHODS

A variety of tools have been used for qualitative and quantitative detection of HuNoV and their surrogates: visualization, i.e. microscopy, cell culture-based, immunological-based, and molecular-based (**Table 1.3**) (7). Electron microscopy (EM) was a popular tool in the 1970s through 1980s for visualization and confirmation (via immune EM). Currently, EM is a popular tool for visualizing the effect of disinfectant treatments against HuNoV's capsid. Since the sequencing of HuNoV in 1990, real-time polymerase chain reaction (RT-PCR) has replaced EM as a diagnostic tool because EM requires expensive equipment and training with low throughput and sensitivity (2, 6).

Cell culturing is the most desired method for observation and characterization of enteric viruses. The most common cell culture formats for quantifying enteric viruses are the plaque assay and 50% tissue culture infectious dose (TCID50). Plaque assays are the gold standard for infectious viral detection (15). Another infection detection assay is TCID50 that is typically used for high throughput analysis but results develop slower and in some cases not as sensitive as plaque assays (38). Both techniques rely upon the use of a virally competent cell line. But TCID50 is considered an endpoint dilution assay that provides qualitative results per well, which collectively can be used for quantification. On the other hand, plaque assays provide quantitative results per well by the development of individual plaques. These plaques theoretically represent a single virus. With this, investigators can isolate and purify clonal population, unlike the TCID50 assay. The downsides to both plaque assays and TCID50 are time, skills, and cost. Plaque assays also take 24-72 hours for completion compared to up to 1 week for TCID50 not including

the time it takes to prepare the assay. For example, propagating cells may take up to 1.5 weeks before there are an adequate number of cells for an assay. Some cells are also delicate, heterogeneous, or may activate after numerous passages, which may create batch-to-batch variation. Furthermore, special care is needed when passaging the cells and proper neutralizers are needed when conducting disinfection studies to achieve a successful infection and avoid erroneous results.

Currently, only murine stains are routinely available for cell culture. On the other hand, the HIE system for culturing HuNoV strains can be completed but HuNoV replication requires three separate media types for passage and differentiation of HIEs. Not to mention, the assay for cultivation of HuNoV appears to be particularly sensitive to bile type for the replication of some strains. HIEs are a promising culture-based method for studying HuNoV, but this method was only recently published and has yet to be replicated. Future studies should attempt to replicate this work by improving its limit of detection, ease of use, and burden of cost.

Molecular-based technologies, in part, have sustained HuNoV research. Some common molecular tools for analyses of HuNoV include: RT-PCR, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, and HBGA assays (39). RT-PCR, focused on here, is the most commonly used tool for HuNoV detection. To detect and differentiate HuNoV genotypes, investigators rely on differences between their plus sense, single stranded RNA genome, which is divided into 3 open reading frames (ORF). Primers used to detect HuNoV focus on the ORF 1 and 2 junction (**Figure 1.3**) (11). The 3' end of ORF 1 contains a gene sequence for the highly-

conserved RNA-dependent RNA polymerase among the family *Norovirus*, which is required for replication. ORF 2 is considered the hyper-variable region as it codes for the capsid protein which frequently changes due to antigenic drift (40). Investigators used ORF 2 regions C and D to differentiate genotype and strains, respectively. Drawbacks to this method include a post-amplification step for confirmation of amplification unless quantitative RT-PCR (qRT-PCR) is used. This technique allows for real-time amplification via fluorescent dyes or probes and allows for quantification, if desired. Downsides to this method are the inability to distinguish between viable and non-viable nucleic acid, although enzyme-based protocols can be used to lyse unstable capsids and cleave exogenous RNA to amplify stable and presumably intact and infectious virions (41, 42). Moreover, other disadvantages of PCR are false-positive and false-negative results. This can be attributed to non-specific amplification and PCR inhibitors. The overriding issue with PCR, regardless of treatment, is its inability to differentiate between infectious and non-infectious virus.

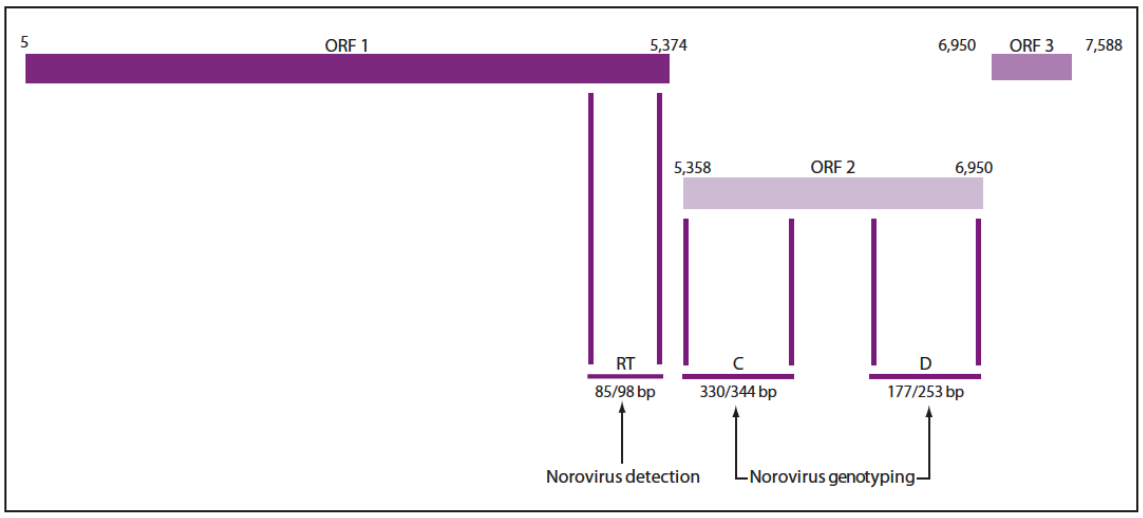
Table 1.4 Overview of laboratory assays for norovirus detection

Laboratory test(s)	Advantage	Disadvantage	Time (sample to result)	FDA (510k)-cleared test	Market
Electron microscopy	Ability to detect multiple viral pathogens	Expensive equipment and training; low throughput; insensitive	15 min		Reference laboratories
Immunological					
Enzyme immunoassay	High specificity, high throughput	57–76% sensitivity	60–90 min	R-Biopharm	Public health, clinical laboratories
Immunochromatographic	High specificity, no special equipment Single sample can be tested	35–52% sensitivity	15 min		Point of care
Molecular					
Conventional RT-PCR	PCR amplicons can be sequenced and used for typing	Results must to be confirmed by sequencing or hybridization	5–6 h[a]		Reference laboratories
Real-time RT-PCR	High specificity, sensitivity and throughput; possibility to multiplex mulitple targets	PCR equipment required; reduced clinical specificity	3 h[a]	Tests in pipeline	Public health, clinical laboratories
Molecular multiple enteric pathogen					
xTAG GPP	High sensitivity, high throughput; detects 11 different enteric pathogens	Expensive equipment and kit format	5 h[a]	Luminex Corporation	Public health, clinical laboratories
FilmArray GI Panel, Verigene Enteric Pathogens Test	Includes nucleic acid extraction; detects 23 (FilmArray) and 9 (Verigene) different enteric pathogens; single sample can be tested	Expensive equipment and kit format	2 h	Biofire Diagnostics Inc.; Nanosphere Inc.; tests from other companies pending 510k clearance	Clinical laboratories

[a] Without nucleic acid extraction.

Source: Data from Vinjé, Jan. "Advances in laboratory methods for detection and typing of norovirus." *Journal of clinical microbiology* 53.2 (2015): 373-381. (7).

Figure 1.3 Genomic regions targeted by reverse transcription-polymerase chain reaction (RT-PCR) assays used for norovirus detection and genotyping



Abbreviations: ORF = open reading frame; RT = TaqMan real-time RT-PCR region for norovirus detection (*87*); C = region C for norovirus genotyping (*93*); D = region D for norovirus genotyping (*92*); and bp = base pairs.

Source: Hall, Aron J., et al. "Updated norovirus outbreak management and disease prevention guidelines." *Morbidity and Mortality Weekly Report: Recommendations and Reports* 60.3 (2011): 1-15. (11). Data adapted from (43–45)

RECOVERY METHODS

Environmental transmission of HuNoV is estimated to be low (19). However, ample epidemiological evidence suggests transmission from fomites with one controlled laboratory study documenting the transfer of HuNoV between surfaces and skin (31). Many disinfection processes have been evaluated in several studies to prevent and control outbreaks. However, as stated previously, there is a knowledge gap regarding the correlation between risk of infection and level of contamination that may influence the

efficacy of these disinfection processes (46). To elucidate this correlation comprehensive comparative sampling studies are needed.

The bedrock of pathogen detection methods are effective sampling methods. Viral recovery from surfaces is dependent on a variety of factors, such as virus type, surface type, implement type, and eluent type. Traditionally, the implement type used for detection of HuNoV on environmental surfaces is swabs. Typical methods include the swab rinse methods, antistatic wipes, or cotton swabs as recommend by ISO 15216 (47) for both hard and soft surface. In fact, Julian et al. (48) completed a meta-analysis of recovery methods used to elute viruses from environmental samples. A subset, focusing on enteric viruses only, are annotated in **Table 1.5**. Consistent with ISO 15216, the majority (n=12) of studies used cotton-tipped swabs, while some (n=5) used other swab materials, i.e. antistatic cloth and polyester swabs. The investigators followed up their meta-analysis with a controlled study evaluating antistatic cloth and cotton and polyester-tipped swabs with MS2 phage, a HuNoV surrogate. Their results indicate that polyester-tipped swabs perform better than antistatic and cotton when assessed via infectivity assay. This is likely due to the irregular shaped fibers of cotton and inhibitory effect associated with the antistatic cloth (48). Although it should be mentioned that higher amounts of MS2 RNA were recovered from the antistatic cloth compared to polyester and cotton-tipped swabs.

Controlled laboratory studies measuring survival or inactivation of enteric viruses on hard and soft surfaces can vary. An ASTM International standard mandates the use of cell scrapers and a neutralizing/recovery broth to elute enteric viruses from hard surfaces

(49). Although, there are published variations of this method including vigorous pipetting or vortexing the virus film for desorption and resuspension (50, 51). Correspondingly, these methods are also used to recover HuNoV from foods. Conversely, comparisons of methods used for recovery of enteric viruses from soft surfaces varied widely (**Table 1.6**). But, generally, these studies used a mixture of destructive sampling methods, e.g. agitating, vortexing, sonicating, macerating, and stomaching. For instance, one study found ca. 3.5 to 6 $\log_{10}$ pfu/ml of FCV and MNV could be recovered from soft surfaces when using a combination of sonication and stomaching depending upon the surface type (52).

Eluent type is another critical factor to consider when developing and evaluating recovery methods intended for enteric viruses. Julian et al. (48)'s combined meta-analysis and controlled laboratory study suggests that eluent type did not play a significant role in the recovery of enteric viruses. To the contrary, other studies have indicated that eluent type could play a critical role in the improvement of viral recovery and stabilization (53, 54). For example, Taku et al. (54) suggested that the eluent type significantly influenced recovery of FCV, indicating that using solutions with low ionic strength and a pH above a virus's isoelectric point (pI) recover more non-enveloped viruses. Another study supports claims made by Taku et al. (54) by suggesting that important components of a recovery buffer are: pH, ionic strength, and amino acids (55). Moreover, Fowler (56) found that a larger foam-topped swab could recover significantly more HuNoV compared to smaller swabs. This suggests that surface size could influence the recovery rate based on adsorption capacity (56). Unfortunately, some studies do not invest time in elution buffer

development and simply use a phosphate buffered-saline (PBS) solution, modified PBS solutions, or a complex cell culture media (37, 53).

Overall, more advanced methods, such as destructive sampling, can be implemented to increase the amount of virus recovered (57). These methods may improve recovery but are not feasible when sampling from immovable soft surfaces, such as carpet and upholstery in the natural environment (52, 58, 59). The two overriding issues surrounding virus recovery from soft surfaces are the lack of consistency between studies and the lack of internationally recognized recovery methods. Furthermore, to our knowledge, there are no comparative studies aimed at investigating recovery methods intended for relevant soft surfaces i.e. carpets (58), contaminated with viruses. Therefore, we suggest, based on scant literature, a comprehensive comparative study should investigate methods intended for recovery of HuNoV from soft surfaces.

Table 1.5 Recovery of enteric viruses from environmental surfaces

Author	Virus	Assay	Implement	Eluent	Surface	Positive	Total	Location
Boxman et al. (60)	HuNoV	qRT-PCR	Antistatic	Ringer's	Toilet seat Knife grips	3	6	Ship
Boxman et al. (61)	HuNoV	qRT-PCR	Antistatic	Ringer's	Cash desk Telephone Handrail Elevator button Door	48	119	Ship
Bright et al. (62)	HuNoV	RT-PCR	Rayon	Amies	Desks Computers Doorknobs Handles Counters Towel dispensers	9	55	Class
Butz et al. (63)	RoV	RT-PCR	Cotton	PBS	Telephone Fountain Toilet handle Sink handle Plastic toys	14	91	DCC
Carducci et al. (64)	HuNoV RoV HCV AdV	qRT-PCR PCR	Cotton	BE	General surgery	1 0 0 1	114	Hospital Hospital Hospital Hospital
Cheesbrough etal. (34)	HuNoV	qRT-PCR	Cotton	VTM	Carpets Toilets	61	144	Hotel

					Tables Phones Cushions			
Gallimore et al. (65)	AsV HuNoV RoV	qRT-PCR	Cotton	Saline	Game Console Toilet taps	6 28 24	154 154 155	Hospital Hospital Hospital
					Phone Medical equip			
Gallimore et al. (66)	AsV HuNoV RoV	qRT-PCR	Cotton	Saline	Toilet tap Light switch	3 12 28	242 242 242	Hospital
Green et al. (67)	HuNoV	qRT-PCR	Cotton	VTM	Lockers Curtains Commodes	11	36	Ward
Jones et al. (68)	HuNoV	qRT-PCR	Rayon	Amies Gel	Bathroom surfaces Kitchen surfaces Doorknobs	11	14	Boat
Keswick et al. (69)	HuNoV	Antigen	Cotton	MEM	Diaper pail Doorknob Sink Hands	4	25	DCC
Kuusi et al. (70)	HuNoV	qRT-PCR	Cotton	PBS	Ultrasound handle Bathroom door handle, toilet seat	4	30	Hotel
Lyman et al.	AdV	qRT-PCR	NR	NR	NR	16	27	DCC

(71)	AsV					9	45	DCC
	HuNoV					11	40	DCC
	RoV					38	38	DCC
Morter et al. (72)	HuNoV	qRT-PCR	Cotton	Water	Blood pressure machine Computer Hand rails Lockers Soap dispenser	75	239	Hospital
Ramani et al. (73)	RoV	qRT-PCR	Cotton	MEM	Bedclothes	30	30	Hospital
	RoV				Cradle Toys	28	30	Hospital
Sandora et al. (74)	HuNoV	qRT-PCR	Polyester	VTM	Computer mouse Desk Water fountain	59	294	DCC
Soule et al. (75)	RoV	qRT-PCR	Cotton	MEM	Handles Playmats Cleaning cloths Tables Medical equip Washbasins	22	45	Hospital

Source: data adapted from Julian, Timothy R., et al. "Comparison of surface sampling methods for virus recovery from fomites." *Applied and environmental microbiology* 77.19 (2011): 6918-6925. (47).

Table 1.6 Enteric virus soft surface recovery methods

Author	Virus[a]	Assay[b]	Implement	Eluent[c]	Surface[d]
Dixon et al. (76)	PV	CCID50	Maceration	BME	**Fabric**: WB, WG, CS, CTC, CJK
Sidwell et al. (77)	PV	CCID50	Maceration	BME	**Fabric**: Cotton "wash-and wear" with Triazone resin
Sattar et al. (78)	RoV	PA	10 min Sonication	NR	**CM:** Poster card, Paper currency, Paper **Fabric:** Cotton-polyester
Abad et al. (79)	ADV B40-8 HAV PV RoV	MPNCU	Vigorous pipetting 20X	3% BE	**CM:** Paper **Fabric:** Cotton
Abad et al. (80)	ADV B40-8 HAV PV RoV	IFT MPNCU RT-PCR	Vigorous pipetting 20X	3% BE	**CM:** Paper
Malik et al. (81)	FCV	TCID50	Agitation with rotary shaker at 150 rpm	3% BE-0.05M glycine pH 8.5	**Fabric:** cotton, polyester, cotton polyester blend. **Carpets:** olefin, polyester, nylon/olefn blend
Hudson et al. (82)	FCV HuNoV	PA qRT-PCR TCID50	NR	NR	**Fabric**-- Cotton, fabric (undefined) **Carpet--** Undefined
Fijan et al. (83)	RoV	RT-PCR	Swab	MEM w/ supplements	**Fabric:** cotton textile fabric
Lee et al. (84)	MNV	PA qRT-PCR	Sonication Vortexing	0.3% BE	**CM:** Diapers **Fabrics:** Gauze
Fisher et	MS2	PA	Vortexing	271	**CM:** FFR

al. (85)				medium	
Tuladhar et al. (86)	PV RoV MNV	PA TCID50	Rayon Swab	DMEM	**Fabric**: Gauze
Yeargin et al. (52)	FCV MNV	PA qRT-PCR	Sonication Stomaching	0.01M PBS w/ 0.02% Tween 80	**Fabric:** Cotton, Polyester

a: FCV: feline calicivirus, HuNoV: human norovirus, PV: poliovirus, RoV: rotavirus, MNV: murine norovirus
b: $CCID_{50}$: 50% cell culture infectious dose, $TCID_{50}$: 50% tissue culture infectious dose; PA: plaque assay: qRT-PCR: quantitative polymerase chain reaction
b: BME: Eagle's basal medium; NR: not reported; BE: beef extract; MEM: minimal essential medium;
d: WB: wool blanket, WG: wool gabardine, CS: cotton sheeting, CTC: cotton, terry cloth, CJK: cotton jersey knit, CM: complex matrix, FFR: filter face piece respirator

SOFT SURFACE PROPERTIES

Soft and hard surfaces are differentiated based on porosity of the surface. Generally, soft surfaces are porous, whereas hard surfaces are not. Although, there are exceptions, such as wood and some polymers, which can be categorized as hard-porous surfaces. Furthermore, soft surfaces, for sanitizing or disinfecting purposes, can be separated into two categories: launderable and non-launderable (58). Launderable surfaces include linens and textiles, whereas non-launderable surfaces are immovable, such as carpets and upholstery. Moisture retention, moisture regain, and wettability can be used to characterize a soft surface's interaction with aqueous liquids (87, 88). Moisture retention, i.e. absorptive capacity, is described as the volume of liquid that a specific weight of fiber can retain. However, moisture regain is determined by the fiber's ability to absorb air moisture under ambient conditions. Finally, wettability is defined by the time required for a surface to absorb and wick a liquid. These factors can change depending upon the surface type and construction. For instance, a single fiber may perform differently than a woven fabric of the sample material due to differences in geometry created by fabrication (88).

Identifying a soft surface's characteristics is an important step to characterizing the overall relationship with viruses (58). For instance, hydrophobic surfaces absorb aqueous solutions poorly compared to hydrophilic surfaces. The increased absorption observed in hydrophilic surfaces will theoretically provide more moisture retention, regain, and wettability. This may positively affect the virus survival during the desiccation process and survival thereafter based on data that suggests adsorbed viruses

survive longer as compared to free, unbound viruses (89). An additional surface characteristic is the electrokinetic potential, i.e. zeta potential (90). This intermediate value can be used to estimate a surface charge under various solution characteristics, e.g. pH and ionic strength. Because non-enveloped viruses behave like zwitterions they possess a pI (91), which may change based on the solution pH and ionic strength. Consequently, the zeta potential of the surface under a given condition, in addition to knowledge of a virus's pI, may assist with explaining surface interactions and difference in recovery of viruses. Unfortunately, investigators studying the relationship between enteric virus and soft surfaces often fail to characterize the surface, making comparisons between studies difficult (58). Future studies should incorporate detailed descriptions of the surfaces and seek surfaces previously used.

CARPETS

Carpets can be found within homes, businesses, and most importantly, long-term care facilities where over 60% of HuNoV outbreaks occur in the United States annually (92). Additionally, epidemiological reports have suggested soft surfaces, such as carpets, may harbor and transmit HuNoV. As early as 1850, the risks associated with carpets were understood. As Florence Nightingale once wrote, *"For a sick room a carpet is perhaps the worst expedient could by any possibility have been invented…A dirt carpet literally infects the room"* (93). Carpets can harbor a variety of unwanted contaminants including allergens, mites, bedbugs, mold, bacteria, and viruses harmful to human health (94). Because of this association, the carpet industry has estimated a $2 million annual loss in

revenue from schools and hospitals between 1999 and 2003 (94). Regardless of the contaminants carpets may hold, they remain commonplace in a variety of other settings.

To combat these contaminants, a variety of technologies have been used to clean and sanitize the surfaces. Popular interventions include steam-cleaning, stain-resistant finishes, and antimicrobial finishes. Recently, ASTM International developed a standard method for evaluating the efficacy of liquid sanitizers intended for carpets (59). However, this method is only recognized for bacterial use, not viruses. Currently, the Occupational Safety and Hazard Administration (OSHA) and Centers for Disease Control and Prevention (CDC) recommend steam-cleaning carpets for 5 min at 70˚C or 1 min at 100˚C after a suspected HuNoV contamination event. However, efficacy and effectiveness of steam-cleaning has not been validated against viruses on soft surfaces. Furthermore, there is a lack of standards for assessing the efficacy of disinfection interventions against viruses contaminated on carpets.

Carpets can be difficult to assess compared to hard surfaces. In addition to what is stated above in “soft surface properties”, carpets can be characterized based on their construction gauge/pitch, pile height, stitches/wires, face weight, finish, backing, yarn type, ply, material, and fiber twist, all of which contribute to their complexity. Secondly, carpets can also be divided into natural, e.g. wool, and synthetic, e.g. nylon, and blended, categories which may affect performance in the presence of soils. Bradbury et al. (95) underscores issues surrounding some textiles, such as wool, and our lack of knowledge regarding modification performed during processing. Sanitizers used on soft surfaces have also demonstrated limited efficacy based on the fiber’s absorptive capacity. For

example, gauze can remove 21.1 mg/ml of a quaternary ammonium compound (QUAT) while wool has been shown to remove up to 98% of an 800 ppm chlorine solution (96, 97). The complexity of these surfaces, lack of knowledge regarding processing, and number of structural facets of carpets makes comparisons between studies difficult.

CONCLUSION

In summary, results from both epidemiological investigation and controlled studies have demonstrated that HuNoV (i) can be transferred between surfaces and hands and (iii) soft surfaces, such as carpets, may be a route of transmission for HuNoV. Furthermore, literature on HuNoV recovery and recovery efficiency is scant and presents a knowledge gap, which suggests the need for a comprehensive comparative study investigating a variety of methods for recovery of HuNoV from relevant soft surface.

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CHAPTER TWO

THE SURVIVAL AND INACTIVATION OF ENTERIC VIRUSES ON SOFT SURFACES: A SYSTEMATIC REVIEW OF THE LITERATURE

ABSTRACT

Worldwide, enteric viruses are the main cause of acute gastroenteritis. In humans, these viruses spread via person-to-person contact, food, water, and/or the environment. Their survival and inactivation on hard surfaces has been extensively studied; however, nonlaunderable soft surfaces, such as upholstery and carpet, have received little attention. The aim of this systematic review was to determine factors that influence the survival and inactivation of enteric viruses on nonlaunderable soft surfaces. EBSCO and Web of Science were searched for experimental studies published between 1965 and 2015 using Preferred Reporting Items for Systematic Reviews and Meta-Analyses methods. Titles and abstracts were screened using 3 eligibility criteria. The quality of all study methods was also assessed. Our search yielded 12 articles. Viruses survived between 0 hours and 140 days depending on surface and environment conditions. Virus survival was influenced by temperature, relative humidity, organic content, and deposition method. A variety of chemistries were tested across studies and were shown to have a varied effect on enteric viruses. Chlorine, glutaraldehyde, vaporous ozone, and hydrogen peroxide were the most efficacious against enteric viruses (> 3-log reduction). Environmental factors, such as temperature and relative humidity, can influence survival of enteric viruses on nonlaunderable soft surfaces. The efficacy of liquid and vaporous chemistries are associated with surface and virus type

INTRODUCTION

Acute gastroenteritis (AGE) is among the top-5 causes of death worldwide (1). Most cases occur in young children in resource-poor countries, whereas in industrialized countries, such as the United States and in Europe, AGE sickens individuals across all age groups with the most common symptoms being vomiting and diarrhea. AGE etiologies include both microorganisms (bacteria, viruses, and parasites) and chemical compounds (toxins and pharmaceutical drugs). Enteric viruses are the most common etiology with caliciviruses, such as human noroviruses, and rotaviruses, causing most cases of illness (2–4).

The primary mode of transmission (direct or indirect) of all enterics, including viruses, is the fecal-oral or vomitus-oral route, which occurs via person-to-person contact, food, water, and/or environmental surfaces (5). Although most cases of illness are due to person-to-person transmission, a growing body of evidence suggests contaminated environmental surfaces play an important role in spreading viruses (6–8). Surfaces in the environment can become contaminated by direct contact with vomit or feces, soiled hands, aerosolized virus generated by vomiting, or airborne virus that settles after disturbance of a contaminated surface (e.g., walking on contaminated carpeting). However, just because a surface becomes contaminated with an enteric pathogen does not mean the pathogen survives, illustrating the importance of examining their survival and persistence on surfaces. Survival of enteric bacteria and viruses has been studied on hard surfaces but little attention has been given to studying enterics, particularly viruses, on soft surfaces. It is presumed that intrinsic factors, such as surface properties or virus

characteristics, and extrinsic factors, including temperature and relative humidity of the environment, influence survival just as they do with enterics on hard surfaces.

Studying survival of enteric viruses on soft surfaces, particularly nonlaunderable soft surfaces, is essential for 2 reasons. First, semienclosed environments, such as long-term-care facilities, restaurants, and schools are common settings for AGE outbreaks, particularly norovirus outbreaks. These settings often include many soft surfaces (eg, carpeted floors, upholstered furniture, and draperies), which epidemiologic evidence suggests the surfaces may play a role in transmission of enteric viruses (9–13). Secondly, laboratory studies investigating transfer efficiency have demonstrated the transfer of infectious viral surrogates from soft surfaces to hands and inanimate objects (14, 15). These studies conclude that virus surface interactions influencing survival and transmission may be much more complex on soft surfaces than hard surfaces, presumably because of the porous and 3-dimensional nature of soft surfaces, so knowledge regarding influencing factors on hard surfaces cannot necessarily be used in relation to soft surfaces (16, 17).

Identifying factors associated with survival on soft surfaces is the underpinning of the design of inactivation treatments, which are essential to prevent as well as control the spread of enteric viruses. Published literature reviews have focused primarily on the environmental contamination of hard surfaces by enteric viruses with limited attention given to soft surfaces (18–20).

Although validated procedures have been developed to inactivate enteric viruses, such as human noroviruses (HuNoV), on hard surfaces, such procedures are not available

for nonlaunderable soft surfaces. Understanding which inactivation chemistries are efficacious (> 3-log reduction) against enteric viruses is also critical to understanding survival and persistence. To our knowledge, this has not been reviewed in detail. Our aim was to review published studies to answer the following 2 research questions: What factors influence the survival of enteric viruses on nonlaunderable soft surfaces? and, What chemistries are associated with the inactivation of enteric viruses on nonlaunderable soft surfaces?

METHODS

We used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses principles to create a transparent (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) PRISMA, valid review (**Figure 2.1**). The PRISMA statement, an evidence-based set of 27 items used to conduct systematic reviews and meta-analyses, is an international standard. To be included in our analysis, each article had to meet 3 eligibility criteria: peer-reviewed in a scientific journal, published in English, and used an experimental study design that examined survival and/or inactivation of AGE-associated enteric viruses on nonlaunderable soft surfaces. A nonlaunderable soft surface material was defined as a porous material that could absorb and wick liquid. Hereafter these surfaces are referred to as soft surfaces. Please note that raw soft surface materials, in general, can be used to create both launderable and nonlaunderable surfaces. Studies in which filters, membranes, and laundry practices were analyzed for their intended purpose

were excluded. Moreover, studies that examined both hard and soft surfaces were included but only the soft surface findings were used in our analysis.

Two online databases—EBSCO and Web of Science—were searched for articles published between 1965 and 2015. EBSCO and Web of Science were selected as searches of multiple databases; for example, Academic Search Complete and MEDLINE could be simultaneously performed. Each database was searched using the search string presented in **Table 1.2**.

Study selection. After the initial search, duplicates were removed then titles and abstracts screened to determine eligibility. A full-text article was retrieved if the title or abstract met our 3 eligibility criteria, as described above. Two trained reviewers analyzed all full-text articles to determine eligibility. Articles meeting the 3 eligibility criteria were then analyzed and summarized by surface type, country, inoculation concentration, detection method, virus, treatment, and significant findings.

Quality assessment. To our knowledge no quality assessment tool exists to evaluate the quality of laboratory-based microbiologic studies. Therefore, we identified 5 criteria to assess the quality of study methods and reporting: methods clearly described, adequate controls, studies performed at least in duplicate, appropriate detection methods, and statistical analysis of data (**Table 2.2**). Two reviewers rated each article using a pass or fail scoring system for each of the 5 criteria. Articles that met the criteria received a 1, whereas those that did not received a 0. The maximum points per article was 5.

RESULTS

Literature search strategy and selection. The 2 electronic databases yielded 860 articles (**Figure 2.1**). Results were cross-referenced and 261 duplicates were removed. The title and abstracts of the remaining articles (n = 601) were then screened. After screening, 526 articles were excluded based on title and abstract alone—8 were not published in English and 7 full-text articles were not available. Two reviewers screened full-text versions of the 73 remaining articles. In addition, the reference lists of these 73 articles were checked for related articles and yielded 2 new articles. A total of 61 full-text articles were excluded: nonenteric virus (n = 24), hard surfaces (n = 15), laundering (n = 4), related to food (n = 5), review articles (n = 5), transmission not fomite-mediated (n = 3), membrane filters (n = 3), and transmission (n = 2). A total of 12 articles were eligible for our review.

Study characteristics. The 12 eligible studies were divided into 2 groups to help us answer our 2 research questions about survival (n = 7) and inactivation (n = 5). All studies were published between 1966 and 2015. Eligible studies were conducted in 6 countries, with most conducted in the United States (n = 5) followed by Spain (n = 2) and Canada (n = 2). Five virus families of importance to human health were represented in the studies we reviewed: *Calicivirdae* (n = 3), *Picornaviridae* (n = 2), *Reoviridae* (n = 1), *Adenoviridae* (n = 1), and *Astroviridae* (n = 1). In addition, 2 bacteriophage families, frequently used as surrogates when studying human enteric viruses, were also included: *Leviviridae* (n = 1) and *Siphoviridae* (n = 1). A variety of methods were used to detect viruses with most investigators using cell culture methods (n = 5) followed by molecular

based methods (n = 2). Type of soft surface varied greatly across studies. To simplify the presentation of our findings, we assigned the type of material examined in each study to 1 of 3 categories: fabric (n = 8), complex-matrix (n = 6), and carpet (n = 4). Materials were classified as complex if the authors did not identify the composition of the material, such as diapers.

Quality assessment. All articles had sound research methods (**Table 2.1**). Nearly all used appropriate controls, quantified viruses appropriately (given the time published), and completed ≥ 2 experiments in duplicate. One deficiency across studies was that 4 of 10 studies did not perform statistical analysis of data.

Significant findings

Factors associated with survival of enteric viruses. Thirteen types of material and 8 types of viruses spanning 7 families were evaluated across the studies (**Table 2.3**). To further complicate our analysis, no standard protocol was used across the studies. Surface size, study duration, inoculum volume and concentration, and recovery method varied widely. As such, many results could not be aggregated for comparison. Those factors that could be compared because of homogeneity include temperature, relative humidity (RH), organic content, and deposition method.

Virus survival was assessed at low (−20°C – 4°C), ambient (18°C – 27°C), and/or high (≥30°C) temperatures but only 4 studies modified temperatures to compare its effects on survival (21–24). In addition to measuring temperature, RH was also

measured. The RH ranged from 25%-90%, and its effect on survival was only measured in 3 studies (21, 22, 25) (**Table 2.3**). All enteric viruses, except human rotavirus (RoV), tested in the 7 studies, followed a temperature trend of low > ambient > high on survival. Survival of RoV contradicted this temperature trend. The findings in 2 studies published by Abad et al (22, 23) showed that RoV titers at a low temperature (4°C) were similar to those at an ambient temperature (20°C). RoV survival also differed from other enteric viruses in the relationship between RH and enteric survival (22). For example, viruses, such as poliovirus (PV), adenovirus (ADV), and hepatitis A virus (HAV), had similar survival results at 50% and 85% RH, whereas RoV did not survive as well (~1.0-log reduction) at 50% RH compared with 85% RH. These findings are in conflict with RoV results from Sattar et al (21) and PV results from Dixon et al. (25) Sattar et al. (21) reported that RoV survival was lower at higher RH (85%) compared with low RH (25% and 50%). Dixon et al. (25) reported survival of PV was lower at 78% compared with 35% RH. The data from Dixon et al. (25) also demonstrated PV titers decreased less rapidly at higher RH. Deposition method (ie, direct contact, droplet nuclei formation via aerosolization, and virus-containing dust) was studied with 2 viruses (PV and MS2). The level of survival across studies was direct contact > aerosolization > virus-containing dust (25, 26). Dixon et al. (25) showed that PV survived < 7 days on wool fabric compared with ~84 and ~140 days when deposited via aerosol and direct contact, respectively, whereas Fisher and Shaffer (26) reported infectious MS2 bacteriophage deposited on to filter face piece respirators material via direct contact survived with a higher titer (~2.5 log) compared with aerosolization.

The authors of 3 studies reported adding a soiling component, such as fecal matter, in their viral inoculum (21–23). The effect of fecal matter was not consistent across the 5 viruses tested. Sattar et al. (21) observed that RoV maintained a higher titer when deposited with a fecal suspension (FS) compared with tryptose phosphate buffer. FS did not provide a protective matrix or sustain a higher titer for HAV and RoV when compared with phosphate buffered saline (PBS) (23). In the case of some viruses (ADV, PV, and astrovirus (AsV), FS had a significantly lower titer compared with PBS (22, 23).

Survival differed among enteric viruses when survival rates were compared on the same soft surface under the same conditions (22, 23). Generally, HAV, RoV, and bacteriophage B40-8 were less susceptible to desiccation (0.6- to 1.6-log reduction) compared with PV and ADV (1.5- to 3.7-log reduction) under all conditions assessed (22). AsV was shown to survive significantly longer ($P < .05$) and at a higher level (> 2 log) on porous surfaces compared with PV and ADV, suggesting PV and ADV are less environmentally stable on porous surfaces when compared with AsV, bacteriophage B40-8, RoV, and HAV (22, 23).

Type of soft surface affected the ability of enteric viruses to survive. The type of surface providing the least and most hospitable environment for virus survival were poster card (0 hour) (21) and wool blanketing (140 days), (25) respectively (**Table 2.3**). For example, Abad et al. (22) reported HAV, contained in PBS, survival rates were significantly less (−1.19 log most probable number of cytopathogenic units/mL reduction) on nonlaunderable soft surfaces compared with hard surfaces, whereas the survival of other viruses tested were not significantly different. Other viruses, such as

ADV and PV, had higher infectivity losses (> 2 log) than HAV, but these results were consistent across the type of surface tested. Fabrication and fiber type appeared to influence survival (21, 24). Dixon et al. (25) reported PV could survive up to 56 days longer on wool than on cotton at 35% RH. Similarly, Lee et al. (24) found a ~3-log reduction difference between diapers and gauze over 40 days. The effect of fabrication was also apparent. Dixon et al. (25) observed PV could be recovered from wool blankets for up to 140 days but only 42 days on wool garments (gabardine) when deposited via direct contact and held at 35% RH. Likewise, PV survived on cotton knitted material for 14 and 28 days less than on cotton terry cloth and cotton sheeting, respectively, although these results were not reported as statistically significant.

Inactivation of enteric viruses. Chemical inactivation (liquid, vapor, and a premodified antimicrobial textile) was the only method studied with 10 chemicals assessed (**Table 2.4**). Viral inactivation was always measured by log reduction with efficacy defined as a 3-log reduction (27). Unfortunately, authors did not comment on the aesthetics of the surfaces after application of the chemical.

Application method, chemical, contact time, and surface types were all important factors associated with the inactivation of feline calicivirus (FCV), murine norovirus (MNV), HuNoV, PV, and RoV. The least effective chemical application was an antimicrobial (triazone with formaldehyde) impregnated into a cotton fabric, which showed <1-log reduction of PV within 2 hours.28 Vaporous sanitization methods, such as ozone and hydrogen peroxide, achieved the highest recorded reduction on the viruses

they assessed (3 log->5 log) (28, 29). RoV was the only virus tested that did not achieve a 3-log reduction when using a vaporous chemicals (hydrogen peroxide > 2 log). A liquid version of this chemistry, accelerated hydrogen peroxide, had an effect on FCV (>3 log) but a limited effect on MNV (0.17 and 0.57 log) on cotton and polyester, respectively (30). This same study found that sodium hypochlorite (5,000 ppm) was effective at reducing infectious FCV and MNV by > 3 log on both surfaces within 5 minutes. Malik et al. (31) extensively investigated the effect of 5 liquid chemicals against FCV on a variety of fabrics and carpets. Only 1 liquid chemical (activated 2.6% glutaraldehyde) achieved a >3-log reduction of FCV on all test surfaces, excluding blended carpet. FCV was easier to inactivate on fabrics than carpets with the exception of 100% polyester fabric. Their data demonstrated a positive linear relationship between contact time and inactivation.

DISCUSSION

The aim of this systematic review was to determine factors that influence the survival and inactivation of enteric viruses on nonlaunderable soft surfaces.

Findings from the articles presented in our review were derived from well-designed research studies as determined by our quality assessment tool. Our analysis suggests temperature and RH are important extrinsic factors associated with virus survival in a manner similar to that of bacterial survival. We also determined, from the current literature, that deposition method and organic content are contributing factors to enteric virus survival on nonlaunderable soft surfaces. Furthermore, enteric viruses were

resistant, under controlled conditions, to a variety of chemistries that are otherwise efficacious against other microorganisms, such as enveloped viruses and vegetative bacteria (32, 33). Other published reviews reported similar conclusions for survival of viruses on a variety of surfaces, such as porous, hard nonporous surfaces, and produce (18–20). However, these reviews did not address the collective knowledge regarding enteric virus survival and inactivation on nonlaunderable soft surfaces. Controlling transmission through the elimination of enteric viruses on environmental surfaces, including nonlaunderable soft surfaces, is an important (and overlooked) step in reducing the burden of illness, especially in semienclosed indoor environments, a common setting for outbreaks of AGE. Results must be interpreted with caution because the duration of survival studies varied and viruses may survive longer than the reported time. For example, the longest low-temperature survival study was conducted for 90 days compared with 140 days for ambient temperature (23, 25). As such, the authors of ambient temperature studies reported longer survival times.

Factors associated with survival. As expected, temperature is, generally, inversely related to enteric virus survival on nonlaunderable soft surfaces. This survival trend is similar to results reported about survival on hard nonporous surfaces, (18–20) which might explain the seasonality of outbreaks attributed to enteric viruses. Lower temperatures, a common method of preservation for enteric viruses and other microorganisms, can slow or halt biological and chemical activities in the liquid in which they are contained. However, nonlaunderable surfaces of concern, such as mattresses or

carpets, are not typically found at low temperatures (−20°C – 4°C). The most alarming result was how long enteric viruses survived (60-140 days) at ambient temperatures. This is of concern given that nonlaunderable soft surfaces are commonly found at ambient temperatures and in indoor settings so individuals can come in contact with contaminated surfaces repeatedly. Some viruses, such as RoV, demonstrated enhanced survival at ambient temperatures. One plausible explanation for the increased survival of RoV is that their genome and structure differ from other common enteric viruses because it is composed of an intermediate and inner capsid containing a double-stranded RNA segmented genome (34). These structural features may explain the ability of RoV to have higher survival rates at ambient temperatures
compared with other enteric viruses. Although this trait is worrisome this should not lessen our concern of other enterics.

The effect of RH on virus survival varied, particularly by virus type and temperature, based on the literature included in our literature analysis. As described elsewhere, (35) nonenveloped viruses survive better at higher RH compared with enveloped viruses that favor lower RH. Typically, indoor environments range from 40% to 70% RH, which was measured in several studies in this review (36). Theoretically, high RH could limit evaporation and desiccation and positively influence the survival of nonenveloped viruses. Evaporation of water molecules from airborne bacteria has been shown to decrease survival by removing essential water molecules (37). The studies investigating deposition method support this idea (25, 26). Moreover, this trend is directly related to the amount of liquid present during deposition. Aerosolized particles

can form droplet nuclei that stay suspended for extended periods of time so have less liquid than

do droplets or virus directly applied to a surface (26). The reduced liquid content associated with droplet nuclei also makes the particles more susceptible to desiccation. As postulated previously (38), the displacement of water essential to the viral capsid could increase inactivation.

These forms of deposition can be represented by vomiting, a common method of soft surface contamination by enteric viruses (9–12). The act of vomiting has been shown to deposit enteric viruses via direct contact and aerosolization at distances > 3 meters (39). The hypothesis

that higher liquid volume content equals longer survival rate could explain the increased survival at higher RH. However, there are exceptions to this trend. The discrepancies in survival across studies within this review (21, 23, 25) may be attributed to experimental factors, such as low RH percentages ranging from 35% to ~50% RH, inoculation composition, surface type, and strain type. Thus, it is difficult to state a common trend for RH effects on enteric viruses

contaminated on soft surfaces with the current literature.

Mixing viruses with solutions containing organic matter, such as fetal bovine serum or FS, is a common practice because it can mimic the fecal-oral or vomitus-oral route. Therefore, it is assumed that soft surfaces contaminated with an enteric virus will also be contaminated with organic matter (typically from vomitus or diarrhea). Organic matter provides a protective matrix for bacterial and viral pathogens, making it more

difficult to eliminate them (18, 24, 40, 41). The varied effect of organic content presented in this review is unknown. However, speculations point to the adsorption state of the virus on a particular surface. When viruses are deposited onto a soft surface they are subjected to a variety of factors, such as adsorption ability of the material, electrostatic interactions, and can be complicated by the solution characteristics; that is, ionic strength (41, 42). Outside of their host, adsorbed enteric viruses are more stable under environmental conditions than free, unbound, virus (42). Gerba (42) stated that due to the low isoelectric point, organic matter may compete with viruses for available adsorption sites and as a result the adsorption ability of a particular virus can aid in predicting its inactivation. The organic component of the FS used by Abad et al (22, 23) may have competed for adsorption sites, resulting in shorter survival. Moreover, it is conceivable that fecal constituents may have interfered with the cell culture system, inhibited elution for AsV, PV, and ADV, or had a synergistic effect across fecal components, virus, and the soft surface, although no data support these hypotheses (22, 23). Nevertheless, these results are in contrast to the accepted theory that organic material-containing solutions provide a protective environment for enteric viruses. Because the sources of enteric viruses contaminated on soft surfaces are usually vomitus or diarrhea, this subject warrants further investigation.

The relationship between surface type and virus is inconclusive because too many surfaces were used to aggregate the findings. Inferences could only be made in regard to some specific surface types and the survival of some enteric viruses. Results were as expected for the natural hygroscopic fibers, wool, and cotton. Wool retained PV up to 56

days longer than cotton, presumably because of its ability to absorb more liquid than cotton (43), a factor not studied when assessing hard nonporous surfaces. Materials that have a higher adsorption capacity have been shown to positively influence virus survival compared to surfaces with low absorption capacity as seen by the differences in MNV and RoV survival (21, 24).

Interestingly, the higher PV survival rate (98 days) on wool blanketing compared with wool garments suggests the construction of a material influences virus survival (25). To explain this phenomenon, the authors of a single study (14) found that loose fabrics transfer more virus than denser fabrics and speculate that loose fabrics may not adsorb virus as readily compared to denser fabrics which creates higher amounts of free, unbound virus susceptible to desiccation.

As stated previously (14, 20, 41, 44), viruses are more likely to remain infectious if adsorbed. This is due to viruses being immobilized and protected from environmental factors such as temperature and solution characteristics (eg pH, ionic strength) that would otherwise inactivate the virus (45).

The use of different elution buffers was also mentioned by Dixon et al (25) and may explain the higher survival rates between surface types. Recovery efficiencies may decrease depending upon the eluent characteristics (eg, pH). Buffer type and the method used for viral recovery were shown to produce different elution efficiencies as they relate to MNV (24). For example, Abad et al (22) reported their elution buffer to be pH ~7.4. As a result, under those conditions, RoV would theoretically be positively charged (pI, ~8.0), whereas HAV would be negatively charged (pI, ~ 2.8) (45) These differences

could explain why some enteric viruses survived longer or at higher levels on surfaces, such as the ability of HAV to survive significantly longer on hard nonporous surfaces than on soft surfaces while other viruses, in the same study, survived longer and at higher titers on soft surfaces compared to hard nonporous surfaces. To support this hypothesis, a more recent study (30) reported a significantly different recovery efficiency of enteric viruses from cotton compared with other surfaces (ie, glass and polyester fabric), even when destructive sampling methods were used, such as sonication and stomaching. One important factor not mentioned in the studies reviewed was the material's surface charge. Knowing the charge of a virus and the surface are mutually important for understanding elution patterns. It is possible that these differences could affect elution and adsorption to the soft surface and may account for the difference in reported survival times. Overall, the results of this review were based on contact time and did not account for all human enteric viruses discussed in this review. Moreover, we do not believe these results can be generalized to all enteric viruses because the resiliency differs among them. The current literature underscores the need to study survival profiles of multiple enteric viruses under the same conditions to determine environmental resilience. Furthermore, investigating multiple enteric viruses can aid in determining potential surrogates for nonculturable viruses, such as human noroviruses. Lastly, knowledge of survival time can improve retrospective epidemiologic investigations and aid in selection of better chemistries to remove enteric viruses from nonlaunderable soft surfaces.

Inactivation. To reduce the burden of illness associated with enteric viruses we must understand the risks associated with contaminated surfaces in the environment by establishing survival profiles. The next logical step is to develop an effective pathogen inactivation treatment that could reduce or eliminate the risks. Enteric viruses of public health importance, such as HuNoV, HAV, RoV, PV, and AsV, lack an envelope, making them environmentally stable and resistant to much of the common chemistry used for inactivation (46, 47). For instance, triazone resin containing formaldehyde, the least efficacious reduction method tested on porous surfaces, was designed to make fabrics quick-dry so in theory the resin could lead to greater inactivation during viral attachment (48). The resin demonstrated some virucidal activity against PV when compared with a control. However, the reduction of PV was too slow to be considered as a sanitizer or disinfectant (<99% in 1 day). These data suggest that active ingredients, if any, present in the resin are not efficacious against PV or concentrations were too low to have a quicker effect.

Other chemicals tested, like those from Malik et al. (31) did not reduce the surrogate for HuNoV adequately due to their lipophilic mode of action. The most efficacious chemicals tested were liquid and vaporous hydrogen peroxide, vaporous ozone, and sodium hypochlorite. These compounds are strong oxidizing agents that damage the viral capsid, nucleic acid, or a combination of both resulting in virucidal efficacy (49). Liquid hydrogen peroxide, although efficacious against FCV, may cause superficial damage to soft surfaces and would not be an appropriate treatment. In addition, hydrogen peroxide was shown to be ineffective against MNV depending on

concentration and contact time, which may discredit its efficacy against HuNoV (30). Unlike liquids, vaporous chemicals, such as ozone, may be an important tool for sanitizing soft surfaces. Vaporous chemicals can penetrate deeper into a surface compared with a liquid surfactant product alone. These products are also ideal for large institutional settings, such as long-term-care facilities and hospital wards, but are not recommended for household use due to chemical hazard concerns. Manufacturers and consumers should not consider only the pathogen-chemical interaction, but also the soft surface-chemical interaction.

An observation noted by Malik et al. (31) was the difference in inactivation when using the same chemical compound on different types of surfaces. The limited efficacy of these products may be linked to the surface to which they are applied due to chemical adsorption. Some studies have characterized the degree to which soft surfaces interact with the active ingredient of a disinfectant solution (50–52). For example, cotton, wool, and polyester were shown to remove quaternary ammonium compounds, sodium hypochlorite, and benzalkonium chloride, respectively, from solutions until the adsorptive capacity of the fibers was met (50–52). Due to the large variety of both soft surfaces and disinfectants, these relationships are not universally defined. However, they are an essential aspect in regard to sanitization of soft surfaces because efficacy can vary based on the soft surface. The most effective liquid chemical in this study, tested by Malik et al. (31) was 2.6% activated glutaraldehyde, a widely used disinfectant in hospitals that has been shown to induce alkylation of key functional groups and amino acids on a variety of bacteria (49). These reactions are capable of damaging the integrity

of the viral capsid and genome. Glutaraldehyde has also been shown to resist adsorption by fibers, which may be another reason for its broad effectiveness (52).

At present, bleach is the only chemical for removal of enteric viruses of importance, such as HuNoV, recommended by the Centers for Disease Control and Prevention. Bleach has been shown to be effective at reducing infectious MNV and FCV, 2 surrogates for HuNoV, on both hard and soft surfaces and is the current recommended tool for eliminating HuNoV from hard nonporous surfaces (30, 53). However, bleach cannot be used for contaminated nonlaunderable soft surfaces as bleach will adversely affect the surfaces aesthetic appearance by, oxidizing the surface and damaging the fibers. These same adverse effects are also true for other chemistries, such as hydrogen peroxide, tested on soft surfaces. Another commonly recommended method is steam cleaning at a minimum of 70°C or 100°C for 5 minutes or 1 minute, respectively, but the efficacy of this method has not been determined under controlled conditions (54). Therefore, a limited number of tools are available to inactivate enteric viruses on nonlaunderable soft surfaces.

FUTURE RESEARCH

Because experimental evidence is limited in regard to survival and inactivation of enteric viruses on soft surfaces, this review suggests the need for additional studies. Future research should consult previously used methods and experimental designs. Investigators should also assess ≥ 2 enteric viruses under environmentally relevant conditions with surfaces that can easily be characterized and procured. Inactivation

studies should investigate chemistries that are efficacious and known to not affect the aesthetic appearance.

CONCLUSIONS

The literature shows that several key factors influence survival: temperature, RH, organic content, deposition method, and virus type. Of the studies that evaluated different chemistries, it was shown that chlorine, glutaraldehyde, and oxidizing vaporous products demonstrated the best efficacy against enteric viruses on soft surfaces. Because the EPA currently does not have a standard method for assessing virucidal activity of product on nonlaunderable soft surfaces, there are no registered products that can be reported to inactivate enteric viruses contaminated on nonlaunderable soft surfaces.

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Table 2.1 Search strings used for online databases

Research questions	Virus		Surface		Role
Research question 1	Virus	AND	Fabric Or Textile OR soft surface OR porous surface OR fomite OR upholstery OR carpet OR rug OR draper	AND	Persist OR surviv OR recover OR transmission
Research question 2	Virus	AND	Fabric OR textile OR soft surface OR porous surface OR fomite OR upholstery OR carpet OR rug OR draper	AND	disinfect OR sanitize OR clean

Table 2.2 Quality assessment of eligible articles (n=12) based on 5 criteria.

Quality Assessment Criteria	Pass	Fail	%Pass
Used adequate controls	12	0	100%
Clear experimental design	10	2	83%
Appropriate quantification technique employed	10	2	83%
Experiment done in duplicate and more than 1 trial completed	10	2	83%
Statistical analysis completed	8	4	67%
Total	50	10	Avg: 83%

Table 2.3 Survival of enteric viruses on soft surfaces.

Surfaces[A]	Inoculation Level[B]	Detection Method[C]	Virus[D]	Treatment[F]	Significant findings[F]	Reference
Fabric: WB, WG, CS, CTC, CJK	~5~10 log $CCID_{50}$	•$CCID_{50}$	PV	**Temp:** 25 °C **RH:** 35 and 78% **DM:** Aerosol, direct, VCD **SD:** 140 d	**35% RH:** Survived on wool for 140 d & on cotton 84 d. **78% RH:** Survived on wool 84 d & on cotton 42 d. Rapid decrease in titer on cotton under both 35 and 78% RH. Better survival via direct contact > aerosol > VCD. Better survival on WG > CTC > CKJ > CS	Dixon et al. (25)
CM: Poster card, Paper currency, Paper **Fabric:** Cotton-polyester	~5.3 pfu	•Plaque assay	RoV	**Temp:** 4 and 22˚C **RH:** 25, 50 and 85% **IC:** FS **SD:** 12 d	RoV survived for 2 and 10 d at 22 and 4˚C on cotton-polyester. No RoV detected on poster card due to inhibition. Titers too low & variable to include for paper currency results. 50-80% recovery on paper after 3 h & no long-term samples taken.	Sattar et al. (21)
CM: FFR	7 log pfu	•Plaque assay	MS2	**Temp:** 22˚C **RH:** 30% **DM:** Aerosol & direct **SD:** 10 d	MS2 detected up to 10 d. Direct contact showed higher (5.7 log pfu) terminal survival than aerosol (3.2 log).	Fisher and Shaffer (26)
CM: Paper **Fabric:** Cotton	Not described	•MPNCU	ADV B40-8 HAV PV RoV	**Temp:** 4 and 20˚C **RH:** 90 (-4˚C), 85 and 50% (20˚C) **IC:** PBS or 20% FS **SD:** 60 d	RoV>HAV>B40-8>PV>ADV. PV/ADV sig. inactivated by FS. Excluding RoV, all survival sig. enhanced at 4˚C. Viruses' survived for 60 d in all conditions, except when suspended in FS (7-60 d). RoV survived longer in high RH. HAV and B40-8 decreased survival on soft surfaces compare to hard surfaces.	Abad et al. (22)
CM: Paper	5~5.7 log MPN	•IFT •MPNCU •RT-PCR	AsV	**Temp:** 4 and 20˚C **RH:** 90% **IC:** PBS or 20% FS **SD:** 90 d	**4°C:** AsV survived up to 90 d in PBS & FS. **20°C:** AsV survived up to 60 & 7 d in PBS & FS.	Abad et al. (23)
			ADV AsV HAV PV RoV	**Temp:** 4 and 20˚C at **RH:** 90% **IC:** 20% FS or PBS **SD:** 7 d	RoV/HAV > AsV > PV/ADV. AsV not sig. inactivated by FS at 4 & 20˚C. No sig increase for RoV survival at 4˚C. RoV/HAV similar decay rates. PV & ADV decreased survival at 20˚C in FS.	
CM: Diapers **Fabrics:** Gauze	8 log pfu	•Plaque assay •qRT-PCR	MNV	**Temp:** -20, 4, 18 and 30 °C **SD:** 40 d	**-20°C:** <2 & <1 log reduction on gauze & diapers after 40 d, respectively. **4°C:** 2 & <2 log reduction on gauze & diapers after 30 & 40 d, respectively. **18 and 30°C:** 3 logs after 1 d on both surfaces.	Lee et al. (24)
Fabric: cotton textile fabric	~7~4 log RT-PCR units	•RT-PCR	RoV	**Temp:** Room temp **SD:** 24 h	After 24 h RoV RNA detected on cotton after traditional nested PCR for all inoculation levels, except disinfection control.	Fijan et al. (55)

A: WB: wool blanket, WG: wool gabardine, CS: cotton sheeting, CTC: cotton, terry cloth, CJK: cotton jersey knit, CM: complex matrix, FFR: filter face piece respirator

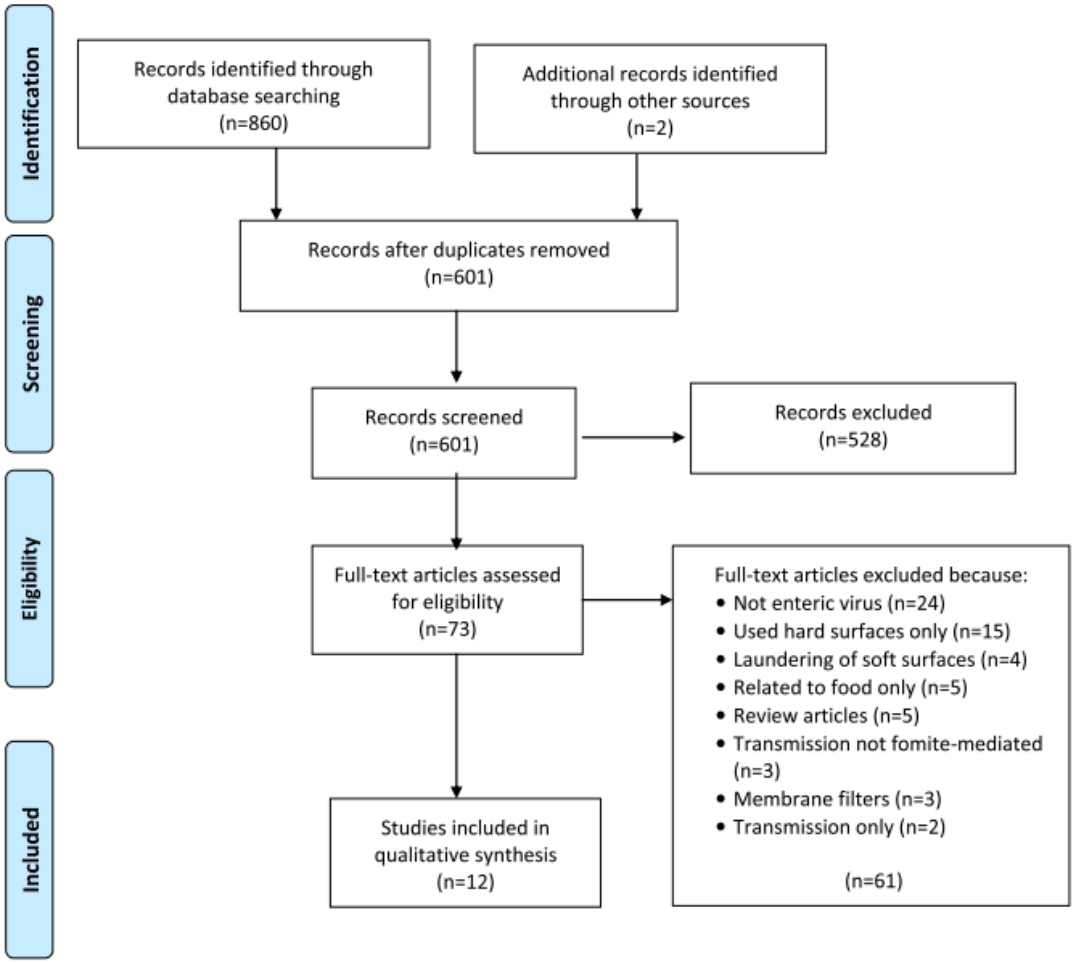
B: pfu: plaque-forming units, $CCID_{50}$: 50% cell culture infectious dose, RT-PCR: reverse transcriptase-polymerase chain reaction.
C: $TCID_{50}$: 50% tissue culture infectious dose, MPNCU: most probable number cytopathogenic units, IFT: indirect immunofluorescences test, qRT-PCR: quantitative reverse transcriptase-polymerase chain reaction
D: PV: poliovirus, RoV: human rotavirus, MNV: murine norovirus, HAV: hepatitis A virus, ADV: enteric adenovirus, AsV: astrovirus
E: Non human enteric viruses: MS2, B40-8, and MNV
F: RH: relative humidity, DM: deposition method, SD: study duration, IC: inoculum composition, FS: fecal suspension

Table 2.4. Inactivation of enteric viruses on soft surfaces.

Surface	Inoculum level[A]	Detection method[B]	Virus[C]	Treatment[D]	Significant Results	Reference
Fabric: Cotton "wash-and wear" with Triazone resin	10 log $CCID_{50}$	•$CCID_{50}$	PV	**Chemical:** Material impregnated with Triazone resin **Contact time:** 10 d **RH:** 35 and 78%	PV survived up to 7 d on impregnated cotton.	Sidwell et al. (48)
Fabric: cotton, polyester, cotton polyester blend. **Carpets:** olefin, polyester, nylon/olefn blend	8 log $TCID_{50}$	•$TCID_{50}$	FCV	**Chemical:** Activated 2.6% glutaraldehyde, 4.75% o-benzyl p-chlorophenol + 4.75% o-phenylphenol, 10% $NaHCO_3$ + 10% quaternary ammonium compound, 70% isopropanol, 2.5% $NaHCO_3$ + 1.3% glutaraldehyde **Contact time—**1, 5, and 10 min.	Activated dialdehyde product achieved 99.9% reduction on all surfaces, except blended carpet. All other products <99.86. Polyester least amenable fabric.	Malik et al. (31)
Fabric-- Cotton, fabric (undefined) **Carpet--** Undefined	**FCV:** 5.7~6.3 log pfu **HuNoV:** Not described	•Plaque assay, •qRT-PCR •$TCID_{50}$	FCV HuNoV	**Chemical:** 20-25 ppm ozone with 5 min vapor burst **Contact time:** 20 min	>3 log reduction in infectivity for FCV and RNA for HuNoV.	Hudson et al. (28)
Fabric: Gauze	**PV:** 7.7 $TCID_{50}$ **RoV:** 5.5 $TCID_{50}$ **MNV:** 6 log pfu	•Plaque assay •$TCID_{50}$	PV RoV MNV	**Chemical:** 127 ppm hydrogen peroxide vapor **Contact time:** 1 h	>5, >3, >2 log reduction of PV, MNV, RoV	Tuladhar et al. (29)
Fabric: Cotton, Polyester	**FCV:** ~6.6 log pfu **MNV:** ~6.3 log pfu	•Plaque Assay •qRT-PCR	FCV MNV	**Chemical:** 5000 ppm NaOCl, 2656 ppm AHP **Contact time:** 5 min	NaOCl >3 log reduction of FCV, MNV on both surfaces. AHP 0.57, 0.17 log reduction of MNV on polyester & cotton. >3 & >2 log reduction of FCV on polyester & cotton	Yeargin et al. (30)

A: $CCID_{50}$: 50% cell culture infectious dose, $TCID_{50}$: 50% tissue culture infectious dose, pfu: plaque forming units, FCV: feline calicivirus, HuNoV: human norovirus, PV: poliovirus, RoV: rotavirus, MNV: murine norovirus
B: qRT-PCR: quantitative reverse transcriptase-polymerase chain reaction
C: non-human enteric viruses: FCV and MNV
D: AHP: activated hydrogen peroxide, RH: relative humidity, ppm: parts per million

Figure 2.1 Preferred Reporting Items for Systematic Reviews and Meta-Analysis flow Chart describing the literature search procedure



CHAPTER THREE

RECOVERY OPTIMIZATION AND SURVIVAL OF HUMAN NOROVIRUS SURROGATES, FELINE CALICIVIRUS AND MURINE NOROVIRUS ON CARPET

ABSTRACT

Human noroviruses (HuNoV) remain the leading cause of acute gastroenteritis worldwide, in part, because of their ability to survive for extended periods of time on environmental surfaces. Furthermore, viral recovery from environmental surfaces, e.g. soft surfaces, remains undeveloped. Our aim was to determine survival of HuNoV surrogates on an understudied environmental surface, carpet. First, we measured the zeta potential and absorption capacity of wool and nylon carpet fibers, developed a mini-spin column elution method (MSC), and characterized the survival of HuNoV surrogates, feline calicivirus (FCV) and murine norovirus (MNV), over 60 days under 30 and 70% relative humidity (RH) on carpets and a glass surface. Carpet surface charge was negative at a typical buffer pH while wool can absorb ca. 2X more liquid than nylon. Percent recovery efficiency with the MSC ranged from 4.34 to 20.89% and 30.71 to 54.14% for FCV and MNV on carpet fibers, respectively. Moreover, elution buffer type did not significantly affect recovery of either surrogate virus. Infectious FCV or MNV survived between <1 and 15 or 3 and 15 days, respectively. However, MNV survived longer under some conditions and at significantly higher titers compared to FCV. Albeit, surrogates followed similar survival trends, i.e. both survived longest on wool then nylon and glass while 30% RH provided a more hospitable environment compared to 70% RH. qRT-PCR signals for both surrogates were detectable for the entire study but FCV genomic copies

experienced significantly higher reductions (<3.80 $\log_{10}$ copies) on all surfaces compared to MNV (<1.10 $\log_{10}$ copies).

INTRODUCTION

Human noroviruses (HuNoV) are recognized as the leading cause of acute gastroenteritis worldwide as well as the most common cause of foodborne disease in the United States (1). Symptoms may include both diarrhea and vomit, which can contain up to 10^{11} viruses/g and 10^{7} viruses/30 mL, respectively (2, 3). This, coupled with their environmental stability and low infectious dose, makes HuNoV highly contagious. Primary modes of transmission are fecal-oral or vomitus-oral and can occur via person-to-person or spread via food, water, and environmental surfaces (4). Although environmental transmission of HuNoV is estimated to be low, environmental surfaces may serve as a temporary reservoir and act as a secondary source of transmission (5). These temporary reservoirs allow a person to become infected without direct contact with the primary source of infection, leading to prolonged and reoccurring outbreaks. This thinking is supported by epidemiological investigations attributing HuNoV outbreaks (6, 7) to contaminated environmental fomites and laboratory-based studies that documented transfer between surfaces and hands (8). For example, a hotel in England experienced a 5-month HuNoV outbreak (6). Outbreak investigators attributed this to the ability of the virus to survive for prolonged periods on both hard and soft surface along with ineffective decontamination strategies. Additionally, another research group demonstrated that enteric viruses and infectious HuNoV surrogates, MS2 coliphage, are

transferable between environmental surfaces, such as glass, cotton, and polyester, and hands (8).

Understanding survival profiles under various conditions could improve epidemiologic investigations and microbial risk assessment in addition to answering key questions surrounding environmental stability, decontamination strategies, and seasonality of HuNoV. Most studies investigating enteric virus survival used hard surfaces, whereas few used soft surfaces (9, 10). These hard surface studies demonstrated the resiliency of HuNoV, especially under low temperature conditions, i.e. 4˚C. For example, Escudero et al. (11) detected HuNoV via qRT-PCR on 3 hard surfaces for up to 42 days and infectious murine norovirus (MNV), a surrogate for HuNoV, was detectable for at least 14 days. Likewise, Lamhoujeb et al. (12) demonstrated a GII HuNoV genome was detectable for up to 56 days on PVC and stainless steel. In the absence of laboratory-based evidence, ample epidemiological evidence suggests non-launderable, soft surfaces, such as carpet, may also be a HuNoV fomite. Currently, only 2 laboratory-based studies have documented HuNoV and their surrogate survival on soft surfaces (13, 14). Moreover, no Environmental Protection Agency (EPA)-registered commercial products are available to sanitize these fomites (15). Taken together, these findings illustrate a significant public health concern, especially in settings where HuNoV outbreaks and soft surfaces are common, such as long-term care facilities and daycare facilities.

To our knowledge, no published studies have reported the survival of HuNoV, or their surrogates, on carpet. Therefore, our three specific aims were: (i) study the carpet characteristics, i.e. zeta potential and absorption capacity, of wool and nylon carpet

fibers, (ii) develop and assess the recovery efficiency of a mini-spin column based virus elution method, and (iii) determine the survival of HuNoV surrogates, feline calicivirus (FCV) and MNV, on wool and nylon carpet fibers and a glass surface (as a hard surface control) over 60 days under 2 relative humidities (RH), 30 and 70%, at 25˚C. As a first-generation study, the intent was to develop an experimental model to produce infectious estimates for microbial risk assessments while providing an analysis of these complex surfaces.

MATERIALS AND METHODS

Virus propagation, cells, and plaque assay. A stock of murine norovirus (MNV) strain CW3 (kindly provided by Dr. Herbert Virgin at the University of Washington, St. Louis) was propagated by infecting 60 – 80% confluent monolayers of RAW 264.7 cells (ATCC TIB-71. American Type Culture Collection, Manassas, VA) at a multiplicity of infection (MOI) of 0.05 in a supplemented medium described elsewhere (16). Feline calicivirus (FCV) strain F9 (kindly provided by Dr. Jan Vinje at the Centers for Disease Control and Prevention, Atlanta, GA) was propagated by infecting 90% confluent monolayers of Crandell-Rees kidney cell (CRFK) (ATCC CCL-94, American Type Culture Collection, Manassas, VA) at a MOI of 0.01 in Complete Eagles Modified Essential Media (Corning, Corning, NY) supplemented with 10% low-endotoxin heat inactivated fetal bovine serum (FBS) (Seradigm, VWR International, Randor, PA), 100 U/liter penicillin (HyClone, GE, Boston, MA), and 100 µg/liter streptomycin (HyClone, GE, Boston, MA). Both cell lines were incubated at 37˚C and 5% CO_2 (Symphony, VWR International, Randor, PA) until

complete cytopathic effect was observed (1 – 3 days). Both surrogate viruses were harvested from cell lysates by three cycles of freeze-thawing followed by centrifugation for 10 min at 5,000 x g and 4˚C then extracted with chloroform as previously described (16). MNV (ca. 7 log PFU/ml) and FCV (ca. 8 log PFU/ml) stocks were aliquoted and stored at -80˚C.

Infectious MNV and FCV were quantified by standard plaque assays as previously described with modifications (16, 17). Briefly, MNV plaque assays were completed by seeding 6-well dishes with RAW 264.7 cells at 1 x 10^6 viable cells/well and incubated until 60 – 80% confluent (4 – 8 hr). MNV experimental samples were diluted, if needed, in MNV infection medium, described elsewhere, containing 5% FBS (CDMEM-5) to improve plaque formation (16). FCV plaque assays were based on previous work with significant modifications (17). CRFK cells were seeded in 6-well dishes at 2.5 x 10^5 viable cells/well and incubated until ca. 90% confluent (2 days). FCV samples were serially diluted in 1X phosphate buffered saline (PBS) if needed. After a 1 hr absorption phase, 2 ml of 1:1 mixtures of 3% seaplaque agarose (Lonza, Switzerland) and 2X Temin's Modified Eagle Medium (MEM) were added to each well incubated until visible plaque formation (1 – 3 days). The 2X MEM was supplemented with 10% low-endotoxin heat inactivated FBS, 100 U/liter penicillin, 100 µg/liter streptomycin, 10mM HEPES (HyClone, GE, Boston, MA), and 1 mM non-essential amino acids (HyClone, GE, Boston, MA). MNV and FCV plaques were visualized by staining agarose plugs with a 0.03% neutral red solution (Carolina Biological, Burlington, NC) mixed with 1X PBS and enumerated on a light box (Futura light box, Logan Electric,

Bartlett, IL). Plaque assays for both MNV and FCV contained a stock suspension of virus and CDMEM-5 or PBS as a positive and negative control, respectively, to test for cell line permissiveness and contamination. Cell lines were not passaged >25 times.

RNA extraction and qRT-PCR. Viral extraction was performed as previously described with minor modifications (18). Viral RNA was extracted from 0.15 ml of a sample or virus stock with E.N.Z.A Viral RNA Kit (OMEGA Bio-Tek, Norcross, GA) per manufacturer instructions. Viral RNA was extracted on the day of recovery experiments and stored at -80˚C prior to use. qRT-PCR for FCV and MNV was completed with KAPA SYBR Fast Universal One-Step qRT-PCR Kit (Kapa Biosystems, Wilmington, MA) on a Realplex2 Mastercycler platform (Eppendorf, Hauppauge, NY). Forward and reverse primer sequences for FCV qRT-PCR analysis were GCCATTCAGCATGTGGTAGTAACC and GCACATCATATGCGGCTCTG, respectively whereas MNV qRT-PCR forward and reverse primer sequences were TGATCGTGCCAGCATCGA and GTTGGGAGGGTCTCTGAGCAT, respectively (19). The standard curves for both viruses were prepared by performing an 8-step 10-fold dilution of virus stocks. Log reductions (**Equation 1**) of virus RNA were performed as previously described (19).

$$RNA\ log\ reduction = \frac{(C_{T,t} - C_{T,c})}{K} \quad \textbf{(Equation 1)}$$

where $C_{T,t}$ is the cycle threshold (C_T) for the experiment group, $C_{T,c}$ is the cycle threshold for the control recovered at time 0, and k is the slope obtained from plotting the C_T values vs. the $\log_{10}$ of the RNA copy number used for presenting the standard curve (19).

Carpet and carpet fiber preparation. Wool and nylon carpet panels (SDL-ATLAS, Rock Hill, SC) were selected from ASTM standard F655-13 (20). Carpet materials had no finishes, e.g. antimicrobial or stain-resistant finishes. Carpet fibers were prepared by shaving nylon and wool fibers from their polypropylene backings with a 22-blade scalpel and autoclaving prior to use in all experiments. Carpet fibers were prepared from the same carpet panel for the entire study, autoclaved on a 30-min dry cycle, then used for the entire study.

Electrokinetic potential. The zeta potential (ζ) of sterile wool and nylon fibers was measured as previously described with modifications to surface only (21). Wool and nylon fibers (1 g) were packed into a SurPASS electrokinetic analyzer cylinder (Anton Paar GMBH, Graz, Austria). The ζ was calculated using VisioLab software from Streaming Potential Measurements (SPM) using the Fairbrother-Mastin equation (**Equation 2**). Flow of the electrolyte (0.001 M KCl) was directed through cells by linearly ramping pressure from 0 to 300 mbar in both directions. Electrodes on either side measured the streaming current. Two cycles of pressure ramping in each direction were performed and the average ζ reported. An HCl (0.1 M) and NaOH (0.1 M) titration were used to measure the ζ between pH 2 and 9. The pH conditions were adjusted by increments of 0.2 with an auto titration unit. The system was rinsed with nanopure water between titrations and between each trial (n=3) to reduce ionic strength build up.

$$\zeta = \frac{dU}{dp} \times \frac{\eta}{\varepsilon \times \varepsilon_O} \times k_B \times R \quad \textbf{(Equation 2)}$$

where dU/dp is the slope of streaming potential versus pressure, η is the electrolyte viscosity, ε is the dielectric constant of elect, ε_0 this vacuum permittivity, κ_B is the electrical conductivity of electrolyte outside the capillary cell, and R is the Ohm resistance inside the measuring cell (21).

Carpet absorption capacity. To test the absorption capacity of the carpet, wool and nylon fibers (0.1 g) were packed into 2 ml microcentrifuge tubes (VWR International, Randor, PA) and autoclaved on a 30-min dry cycle. Wool and nylon fibers were saturated with 0.1% safranin and 0.2% crystal violet solution, respectively, in increments of 0.05 ml up to 1 ml. After application of the indicator liquid, samples were vortexed for 30 sec, carpet fibers were removed, and empty microcentrifuge tubes were weighed (X564, Mettler-Toledo, Switzerland) for residual liquid. Absorption capacities were tested in triplicate in 3 separate experiments at room temperature.

Recovery efficiency. Four elution buffers were assessed for their ability to elute FCV and MNV from wool and nylon carpet fibers using a newly designed MSC method. Virus samples were recovered after desiccation, i.e. 6 and 12 h for saturated nylon and wool, respectively, at room temperature. The buffers assessed were deionized water (DI) (buffer 1), Phosphate buffer (Butterfield's buffer) (buffer 2), DI water + 0.01 M sodium bicarbonate + 0.02% Tween 20 (buffer 3), and 0.01 M phosphate buffered saline + 0.02% Tween 80 (buffer 4).

Wool and nylon carpet fibers were prepared, packed, and autoclaved as described above. Aliquots of FCV and MNV containing a known concentration of virus were thawed in a 37˚C water bath (IR35 New Brunswick Scientific, New Brunswick, NJ) and diluted in CDMEM-5 cell culture media. Separate virus inocula were prepared for each carpet type based on their absorption capacity, i.e. 0.8 ml/0.1 g wool carpet and 0.4 ml/0.1 g nylon carpet. Samples were vortexed for 30 sec followed by the removal of carpet fibers and placement into 60 mm dishes (Corning, Corning, NY). Residual liquid, if any, was pipetted back onto the carpet fibers. Samples were then placed into an open chamber (480 HP, VWR International, Randor, PA), with lids ajar to expose samples to the atmosphere.

To recover viruses, carpet fibers were packed into empty mini spin columns (USA Scientific, Orlando, FL) with sterile forceps and eluted 2 times by applying 0.5 ml aliquots of respective elution buffers and centrifuged (Model 5424, Eppendorf, Germany) at 2,000 x g for 1 min at room temperature. Each 0.5 ml fraction was combined into a microcentrifuge tube, vortexed, weighed, and stored at -80˚C. All samples were assayed via plaque assay. Recovery efficiency (RE) was tested in duplicate in 3 separate experiments. RE is defined as the number of pfu recovered and divided by the number of pfu initially seeded (22).

Survival study design. Prior to characterizing the survival of surrogate viruses artificially contaminated on carpet fibers and a glass surface, environmental chambers (480 HP, VWR International, Randor, PA) were established with 30 and 70% RH

chambers relative humidity (RH) by maintaining with a saturated $MgCl_2$ and 1:1 mixture of NaCl + KCl solution, respectively (23).

Carpets fibers and virus inocula were prepared as described above. Glass cover slips (25 mm^2) (VWR International, Randor, PA), included as a hard surface control, were rinsed in sterile DI water, 100% ethanol, and sterile DI again prior to autoclaving on a 30-min dry cycle. Carpet fibers and glass samples were stored at room temperature under ambient conditions until used.

Wool and nylon fibers were prepared and inoculated as described above. Glass coverslips were inoculated with the same inoculum as nylon, i.e. 0.4 ml/25mm^2. Samples were recovered on day 0, 1, 2, 3, 7, 15, 30, and 60. Viruses contaminated on carpets were recovered with the MSC method as described above using 0.01 M PBS + 0.02% Tween 80 and 4 separate washes. Using the sample recovery buffer, glass coverslips were recovered as previously described (24). After elution fractions were collected, microcentrifuge tubes were vortexed, weighed, and stored at -80˚C. Prior to storage, an aliquot was removed for qRT-PCR analysis. All samples were assayed via plaque assay and qRT-PCR.

Statistical analysis. Statistical analysis was performed using one-way multiple comparisons t-test. A power analysis was performed prior to the survival study with a 95% confidence interval. All results are expressed as mean ± standard deviation. Statistical significance was defined as $P \leq 0.05$. Statistical analyses were conducted using JMP (JMP 11.2.1, SAS Inc., Cary, NC).

RESULTS

Electrokinetic potential. Both wool and nylon fibers were negatively charged and the values inversely proportional to pH between pH 2.7 and 9 based on their electrokinetic potential analysis (**Figure 3.1**). Between pH 2.7 and 9, nylon fiber's ζ ranged from -0.33 to -68.20 mV, whereas wool fiber's ζ ranged from -27.55 to -58.78 between pH 2.7 and 8.3. Comparatively, nylon fibers maintained a higher ζ than wool fibers until pH 8.5 when the nylon fiber's ζ decreased to ca. -68 mV. During the HCl titration, wool and nylon fibers differed by ca. 25 mV between pH 2.7 and 4 then progressively grew closer. However, between pH 7.5 and 8.2, both fiber types showed little difference in ζ.

Carpet absorption capacity. Wool absorbed up to 0.8 ml/0.1 g of saffarin solution whereas nylon only absorbed up 0.4 ml/0.1 g of crystal violet solution (**Table 3.1**). Although not compared statistically, because of different indicator liquids were used, wool fibers were capable of absorbing 2 times more liquid than nylon fibers.

Recovery efficiency. Table 3.2 shows the RE percentage of FCV and MNV after desiccation when recovered using the MSC method with 4 different elution buffers. Desiccation of wool took 12 hr compared to 6 hr for nylon. For FCV, RE percentages from wool and nylon carpet fibers ranged from 11.44 to 20.89% and 4.34 to 8.05%, respectively. The recovery of MNV from wool and nylon carpet fibers ranged from 30.71 to 38.34% and 40.89 to 54.14%, respectively. Elution buffers did not significantly affect the recovery of FCV and MNV when using the MSC method under ambient conditions.

Statistical comparisons were not conducted between carpet types due to the difference in absorption capacity and drying time. Though not shown, more infectious FCV and MNV were recovered from wool fibers compared to nylon when both surrogates were recovered from both fiber types at 6 hr of drying.

Survival of surrogate viruses. Figure 3.2 shows the survival characteristics of FCV and MNV inoculated onto carpet fibers and glass under 30 and 70% RH at 25˚C over a 60-day period. Infectious FCV survived up to 15, 3, and 3 days at 30% RH, whereas FCV only survived for 7, 1, and <1 days at 70% RH on wool, nylon, and glass, respectively. Infectious MNV survived for up to 15, 7, and 7 days at 30% RH while MNV held at 70% RH survived for 7, 3, and 3 days on wool, nylon, and glass, respectively. Overall, FCV and MNV survived longer and at significantly higher infectious levels when held at 30% RH compared to 70% RH. Additionally, surface type played a significantly role in the survival of both surrogates with wool providing a more hospitable environment. Generally, under each RH condition survival for both surrogate viruses was wool>nylon>glass. Comparatively, MNV survived longer and at a significantly higher titer on each surface after the first day compared to FCV.

Figure 3.3 illustrates the reduction of FCV and MNV genomic copies contaminated on wool, nylon, and glass under 30 and 70% RH at 25˚C over a 60-day period. FCV and MNV were detected for up to 60 days on all surfaces. The maximum $\log_{10}$ copy reductions for FCV after 60 days were <1.30, <3.10, and <3.80 $\log_{10}$ copies, whereas MNV exhibited a maximum $\log_{10}$ copy reduction of <0.70, <1.10, and <0.80 on

wool, nylon, and glass, respectively, all at 70% RH. FCV genomic copies recovered from all surfaces were significantly different, whereas no significant difference was observed between MNV genomic copies among surfaces tested. Comparatively, after day 3, significantly more MNV genomic copies were detected compared to FCV.

DISCUSSION

Historically, FCV and MNV have been used as surrogates to study HuNoV on both hard and soft surfaces (24). To date, no studies have investigated the survival of HuNoV or their surrogates on carpet despite ample epidemiological evidence suggesting soft surfaces, such as carpet, may be a mode of transmission for HuNoV (6, 7). In this study, we characterized the carpet fiber's ζ and absorption capacities, developed and assessed a new virus elution method for carpet fibers, and provided evidence that infectious HuNoV surrogates, FCV and MNV, can survive for at least 15 days, depending on environmental conditions and on type of carpet fiber. These findings provide laboratory-based evidence to support published epidemiological evidence regarding the prolonged survival of viruses on soft surfaces, e.g. carpet.

It is important to note that soft surface studies can be challenging, especially when drawing conclusions between studies with limited information regarding surface characteristics (10). ζ is a useful intermediate value for estimating surface charge, which knowing this information may aid in a better understanding of the virus-soft surface interaction. Our ζ results support previous findings that suggest an inverse relationship with pH (25, 26). ζ is considered pH dependent because functional groups at the surface

can become ionized under varying pH conditions. For example, carboxylate groups, commonly found on wool and nylon fibers, can contribute to an increased negative ζ when the pH of a solution is increased (26). The difference can be attributed to absorption qualities of the fibers. Due to limited absorption, synthetic fibers, such as nylon, have a higher ζ compared to wool. Furthermore, swelling of fibers can affect a surface's ζ (25, 26). The SurPASS electrokinetic analyzer, used here, conducts the acid titration immediately after saturation, whereas the base titration is completed ca. 30 min after saturation. This may further explain the ζ differences between these fibers observed between pH 3 and 5. Carpet ζ's reported here differ in the range of ca. 5 and 30 mV between pH 3 and 7 from previous findings with wool and nylon surfaces (26). Although values were distinct between studies, the wool and nylon trends reported were similar. Difference are expected because ζ measurements can be affected by many experimental factors, such as surface type, fiber aging and processing, porosity, dyes, electrolyte solution, surface treatments, and cleaning procedures (25, 26).

Fibers used in this study were autoclaved prior to use. Common laboratory procedures for cleaning fibers prior to ζ measurements include scouring by washing with detergents, petroleum ethers, or via Soxhlet extraction (25). Our intent was to measure the behavior of these fibers under their natural conditions and how they interact with FCV and MNV, not to assess the ζ of pure wool or nylon. Taken together, these results suggest that buffers, intended for the elution of HuNoV or their surrogates from wool and nylon fibers, should be > pH 7.25 because of reported isoelectric points of FCV (4.9) and

MNV (5.0 – 6.0) and fiber charge. Under pH conditions >7.25 both viruses and fibers would be negatively charged leading to increased repulsion and better virus recovery.

As expected, wool and nylon fibers are capable of absorbing different amounts of liquid. Saffarin and crystal violet solutions were selected over traditional inocula to enable direct observation of liquid coverage, in addition to weighing residual liquid. Unfortunately, these characteristics were not reported in studies investigating the enteric virus-soft surface relationship (10). Like all condense-phase material, wool and nylon are hydrophilic in nature, but, the magnitude of hydrophilicity can vary between surfaces (27). By the same token, each surface's magnitude of hydrophilicity is directly related to its absorption capacity. Our results are supported by observed and calculated sorption isotherms previously investigated (28). Hailwood and Horrobin (28) demonstrated that wool's percent regain could be >4 times that of nylon, depending on the RH. The absorption capacity of a soft surface is a critical factor to consider. Higher absorption capacities may allow for higher adsorption of viruses, and, as stated previously, viruses in the environment are less susceptible to desiccation and inactivation when adsorbed to a surface (29). These relationships may explain the longer virus survival observed on wool compared to nylon fibers and glass surface. Previous work has demonstrated that natural fibers provide a more protective environment for enteric viruses, such as poliovirus, when inoculated at the same volume (30, 31). Because these fibers absorb different amounts of liquid, we chose to inoculate the carpet fibers based on their maximum absorptive capacity to mimic a natural contamination event but maintained the same level of

inoculation of each virus. Therefore, natural surfaces with high absorption capacity may facilitate longer survival times compared to synthetic, low absorbing surfaces.

The above discussions highlight the importance of assessing the characteristics of a soft surface, such as ζ and absorption capacity. These characteristics can be used in microbial risk assessments to assist in predicting the fate of a virus along with developing more efficient recovery buffers. As previously stated, the lack of consistency between soft surface studies and limited descriptions of surfaces leaves little room for adequate comparisons (10). Further, studies should consider characterizing soft surfaces prior to analysis or use the same materials from previous studies to broaden our knowledge regarding virus-soft surface interactions.

To evaluate the survival of a virus inoculated onto carpet, improved recovery methods and buffer optimization were needed. Previous recovery methods designed for carpet are time-consuming and resource intense (32, 33). Some methods, e.g. orbital shaking and bottle extraction, require high buffer volumes that may lower the RE and increase detection limits. The MSC method, developed in this study, allows for a simplistic, volume adaptable, and resource light approach to assess survival of non-enveloped viruses on soft surfaces. Our results indicate that the MSC method is efficient at eluting both FCV and MNV from wool and nylon carpet fibers. Additionally, buffer type does not significantly influence % RE when using the MSC approach for both viruses and carpet fiber type, among tested buffers. The mechanism of recovery is likely a result of fiber rehydration that changes the surface charge and assists with resuspending the virus in solution while the centrifugal force pulls the solution into the collect tube.

Ultimately, the Tween 80-based solution was selected for follow-up experiments, i.e. virus survival assessment, as it has been used previously and is a safe storage medium for both virus surrogates (18).

Virus survival can be affected by many factors, such as temperature, RH, organic content, deposition method, and adsorption (10). Temperature remains the most important environmental factor affecting virus survival. Typically, virus's survival is inversely proportional to temperature on both hard and soft surfaces (10, 34). For instance, Lee et al. (14) reported that MNV survived longer and at higher titers on cotton gauze and diapers at low (4˚C) temperatures compared to higher (18 and 30˚C) temperatures. However, carpet is generally found indoors with climate control where temperature varies little. In contrast, RH can vary indoors (40 – 70%) and has been shown to significantly affect the survival of enteric viruses (10). Equally important are the discrepancies among studies investigating effect of RH on virus survival. As described previously, non-enveloped viruses tend to survive longer under high RH conditions (34). However, other studies countered this trend by demonstrating that enteric viruses and their surrogates, such as rotavirus, poliovirus, MNV, and MS2 phage, favor low RH (10). Reasons behind these conflicting results are unclear, but can be attributed to difference in interactions of the follow factors: temperature, surface type, virus type, experimental design. In our study, FCV and MNV favored low RH on all surfaces types. Ideally, low RH provides a quicker time to desiccation and adsorption. Moreover, an adsorbed non-enveloped virion is more stable compared to a free, unbound, virion. This protection is thought to be garnered by aggregation and reduced accessibility (35).

Longer times to desiccation observed under high RH conditions permits virions to stay free and unbound where they are more vulnerable to environmental conditions and solution characteristics, e.g. ionic strength and pH (36).

The difference between survival profiles of FCV and MNV were not surprising as previous studies have demonstrated that FCV has a higher susceptibility to pH, temperature, and some environmental conditions compared to other HuNoV surrogates, such as MNV (18, 24, 37). For instance, D'Souza et al. (37) found that FCV could survive on 3 hard surfaces for 7 days but experienced up to 4 and 7 log pfu reduction after 2 and 7 days, respectively. Similarly, when compared under wet conditions, MNV's survival was significantly enhanced compared to FCV inoculated onto hard surfaces (24). Our results, in conjunction with previously published findings, suggest that FCV may not be a suitable surrogate for estimating the survival of HuNoV on carpet with MNV being the more appropriate option.

Previous studies have documented the divergence among infectious and molecular data, i.e. qRT-PCR (11, 14). qRT-PCR should still be used in surrogate studies as HuNoV culturing is not routinely available. Additionally, qRT-PCR can be useful in determining the mode of inactivation of a virus along with assessing the fidelity of a recovery method after infectious virus falls below the limit of detection. Our results assist with confirming this trend that the presence of a viral genome does not signify infectious virus. Furthermore, our MNV qRT-PCR results demonstrated a limited reduction of genomic copies suggesting that our MSC recovery method is not affected by wetting or study duration. However, the reduction of FCV genomic copies over 60 days suggest that

capsid integrity and binding motifs may be more susceptible to environmental factors compared to MNV.

CONCLUSION

In summary, results presented here demonstrated that characterizing a soft surface can improve our understanding of virus-soft surface interactions. Furthermore, infectious HuNoV surrogates, FCV and MNV, can survive for extended periods of time on carpet fibers. This survival can be affected by at least 2 factors: RH and surface type. Specifically, low RH favors FCV and MNV survival while natural fibers, such as wool, may provide a more protective environment compared to synthetic fibers and hard surfaces.

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Table 3.1 Absorptive capacity of carpet fibers

Sample[a]	Volume Added (ml)	Residual Weight[b] (g)
Wool	0.650	0.009 ± 0.004 A[c]
	0.700	0.015 ± 0.003 AB
	0.750	0.018 ± 0.003 BC
	0.800	0.021 ± 0.003 C
	0.850	0.051 ± 0.013 D
Nylon	0.300	0.003 ± 0.002 A
	0.350	0.003 ± 0.003 A
	0.400	0.005 ± 0.002 A
	0.450	0.019 ± 0.005 B

[a] Carpet fiber samples were 0.1 grams each.
[b] Residual weight based on remaining liquid after carpet fiber removal.
[c] Data are expressed as log means ± standard deviation. Means with different letters in the same column and surface type are significantly different ($P<0.05$).

Table 3.2 Recovery efficacy of FCV and MNV from wool and nylon fibers using a mini spin column extraction method with 4 buffer types

	RE% (log recovery [PFU])[a]							
	FCV				MNV			
Material	Buffer 1 A[b]	Buffer 2 A	Buffer 3 A	Buffer 4 A	Buffer 1 A	Buffer 2 A	Buffer 3 A	Buffer 4 A
Wool	15.38 (5.06 ± 0.31)[c]	11.44 (4.92 ± 0.32)	13.47 (5.02 ± 0.23)	20.89 (5.19 ± 0.28)	38.34 (5.35 ± 0.13)	32.63 (5.28 ± 0.10)	30.71 (5.24 ± 0.17)	32.25 (5.27 ± 0.18)
Nylon	4.34 (4.52 ± 0.12)	6.22 (4.67 ± 0.24)	8.05 (4.80 ± 0.10)	7.23 (4.75 ± 0.13)	45.13 (5.46 ± 0.17)	40.98 (5.42 ± 0.19)	50.27 (5.47 ± 0.18)	54.14 (5.36 ± 0.20)

[a]Samples were recovered at 12 and 6 hr for wool and nylon, respective, for each surrogate

[b]Buffers with different letters in the same row are significantly different ($P<0.05$), for each surrogate.

[c]Data are expressed as percent recovery (log mean ± standard deviation).

Figure 3.1 Electrokinetic potential analysis of wool and nylon via SurPASS titration. One gram of each surface, wool and nylon, was packed into a cylindrical cell to estimate each surface's electrokinetic potential. Hydrochloric acid (□) titrations for nylon were followed by a NaOH (◇) titration. Similarly, HCl (△) titrations for wool were followed by a NaOH (✕) titration. Parameters were set to assess electrokinetic potential between pH 2 – 9. Error bars indicated standard deviation among 12 replicates from 3 independent experiments.

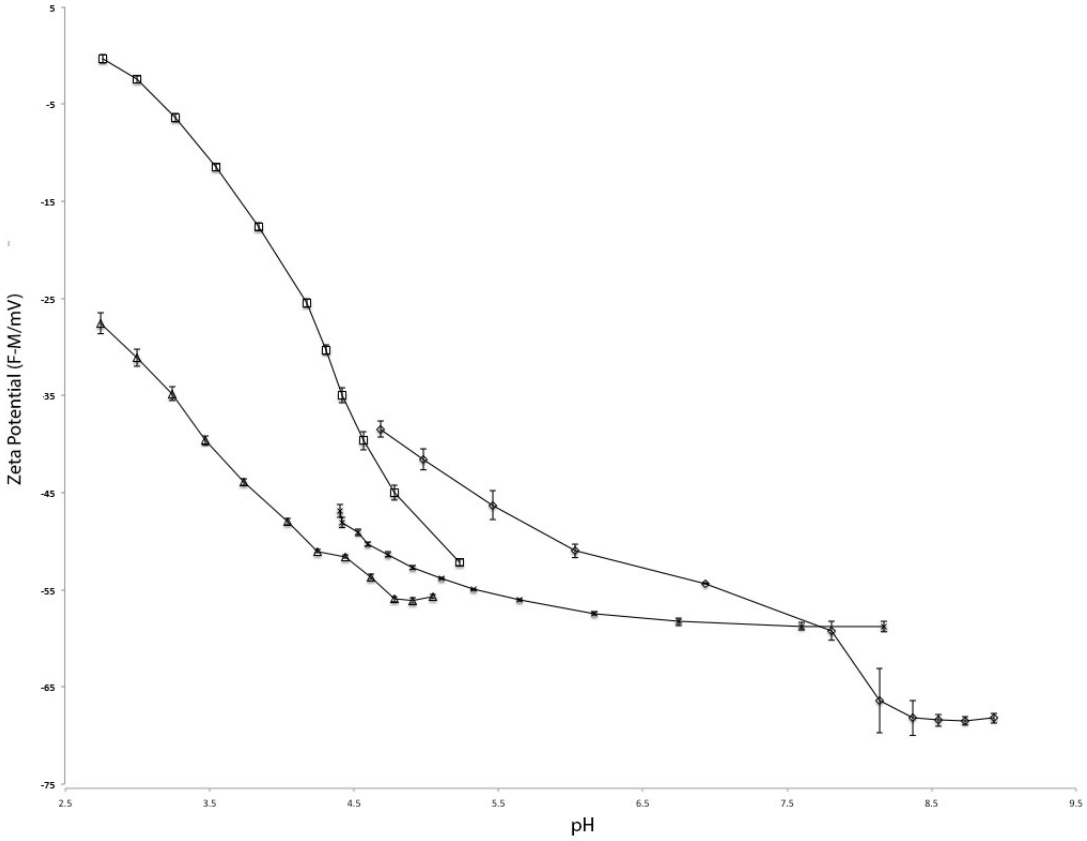


Figure 3.2 Survival analysis of FCV and MNV assessed via plaque assay on nylon, wool, and glass surfaces at 30% RH (□) and 70% RH (△). The dotted line indicates detection limits. Surfaces were individually inoculated with ca. 6 log PFU of FCV and MNV and recovered with the MSC method using 4 washes with five hundred microliters of buffer on days 0, 1, 2, 3, 7, 15, 30, and 60. Data were expressed as the mean ± standard deviation of 6 replicates from two independent experiments

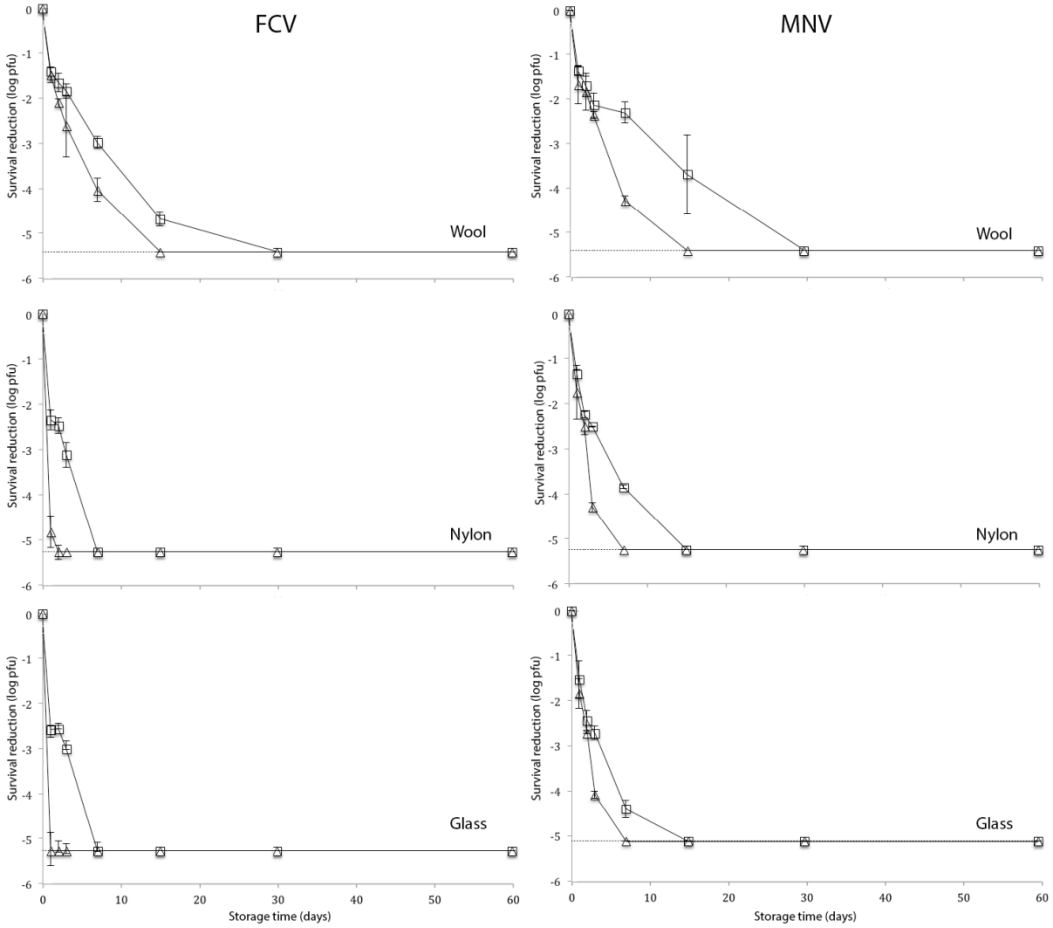
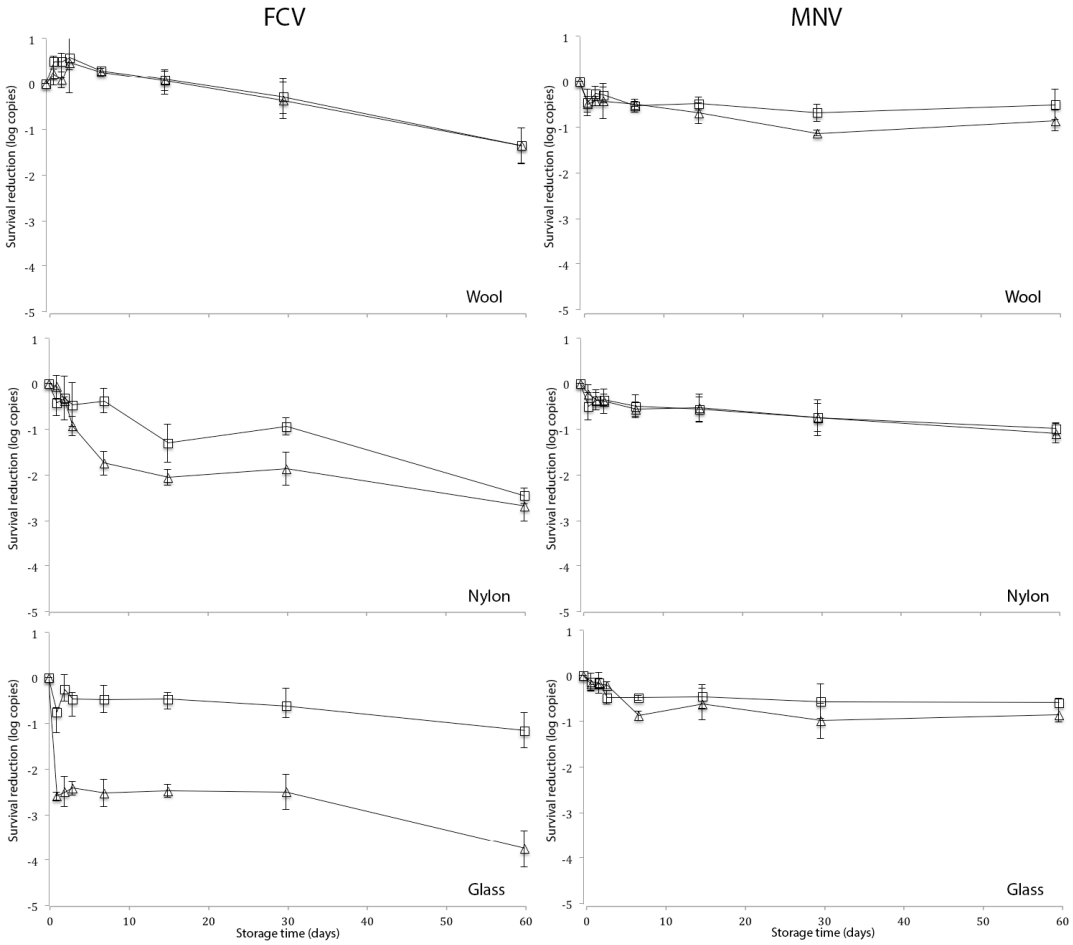


Figure 4.3. Survival analysis of FCV and MNV assessed via qRT-PCR on wool, nylon, and glass surfaces at 30% RH (☐) and 70% RH (△). Surfaces were individually inoculated with ca. 6 log PFU of FCV and MNV and recovered with the MSC method using 4 washes with five hundred microliters of buffer on days 0, 1, 2, 3, 7, 15, 30, and 60. Data were expressed as the mean ± standard deviation of 6 replicates from two independent experiments.



CHAPTER FOUR

COMPARATIVE RECOVERY OF HUMAN NOROVIRUS SURROGATES, FELINE CALICIVIRUS AND MURINE NOROVIRUS, FROM CARPET WITH THREE RECOVERY METHODS

ABSTRACT

Human noroviruses (HuNoV) are the leading cause of acute gastroenteritis worldwide as well as the most common cause of foodborne disease in the United States. Prevention and control of HuNoV contaminated on soft surfaces, such as carpet, remains a challenge due, in part, to a lack of robust recovery methods. Our aim was to compare recovery methods to determine their ability to recover HuNoV surrogates from carpet. Specifically, we assessed and compared three recovery methods, i.e. bottle extraction (BE), macrofoam-tipped swabbing (MS), and the microbial vacuum (MVAC), using HuNoV surrogates, feline calicivirus (FCV) and murine norovirus (MNV), inoculated on wool and nylon carpet carriers. We also investigated qRT-PCR detection issues for FCV after environmental recovery, i.e. inhibition. Infectious FCV and MNV percent recovery efficiency (% RE) of BE ranged from 0.44 to 48.44% and 40.77 to 68.83%, respectively, compared to % RE of MS, which was 0.02 to 0.82% and 1.54 to 2.87%, respectively. The % RE of MVAC for infectious FCV and MNV ranged from 7.30 to 18.29% and 52.58 to 74.67%, respectively. Percent RE of genomic copies of FCV and MNV with BE ranged from 0.36 to 2.53% and 3.34 to 14.97%, respectively, while % RE of MS ranged from 1.03 to 2.24 and 2.02 to 4.25%, respectively. Percent RE of genomic copies of FCV and MNV with MVAC ranged from 2.49 to 23.72% and 28.78 to 79.15%, respectively. Significantly more plaque-forming units and genomic copies were recovered using BE

and MVAC compared to MS, while buffer type played a significant role in recovery of infectious FCV. Additionally, qRT-PCR analysis indicated recovery from tested carpet types inhibited amplification of FCV RNA and required dilution after nucleic acid extraction.

INTRODUCTION

Worldwide, human noroviruses (HuNoV) account for ca. 20% of all diarrheal cases (1,2). Its economic burden is estimated to be $4.2 billion in direct health care costs and $60.3 billion in societal costs per year. Control of HuNoV within the environment remains a challenge due to their stability and low infectious dose.

Environmental transmission and transfer of viral pathogens has been documented in both epidemiological and laboratory-based studies on hard and soft surfaces (1–3). For example, two carpet fitters exhibited HuNoV-like symptoms after removing carpet from a room used to cohort patients with HuNoV-like symptoms 16 days after the room had been vacated and decontaminated (1). Likewise, carpet samples from a hotel, associated with a 5-month HuNoV outbreak, had the highest swab positivity rates (2). These high positivity rates were attributed to the environmental stability of HuNoV along with the use of ineffective decontamination strategies. However, as stated previously, there is a knowledge gap regarding the correlation between risk of infection and level of contamination that may affect the effectiveness of these disinfection processes (4). To elucidate this correlation comprehensive comparative sampling studies are needed.

The efficacy of sanitizers and disinfectants against HuNoV on hard surfaces is well documented (5). However, only 3 studies have tested soft surfaces, such as carpet (6). Currently, the U.S. Environmental Protection Agency (EPA) does not recognize a standard test method for evaluating the efficacy of an antiviral sanitizer intended for carpets. But, the EPA does have methods for other types of soft surfaces in order to make claims against bacteria (7). This is, in part, because of inadequate recovery methods for carpets, making it difficult to quantify the effect of control strategies.

Adequate recovery methods are the underpinning of environmental detection methods. Improving and characterizing these methods would provide higher recoveries of pathogens that would increase their detection and lead to higher positivity rates. To improve our understanding and better detect and quantify enteric viruses, such as HuNoV, deposited on soft surfaces, current recovery methods should be assessed and compared. A meta-analysis of the literature conducted by Julian et al. (8) showed that only 2 of 59 studies sampled soft surfaces for HuNoV with both using cotton-tipped swabs (2, 9). Moreover, the laboratory-based studies investigating HuNoV and soft surfaces relied on destructive recovery methods, e.g. sonication, stomaching, vortexing, and orbital shaking (10–12), which may not be appropriate for some types of carpet.

Common methods to recover pathogens from complex surfaces, such as carpet, include bottle extraction (BE), microbial vacuum (MVAC), and swabbing. BE uses both sonication and handshaking to recover microorganisms of interest and is the American Society of Testing and Materials (ASTM) recommended method for recovery of bacteria from carpet carriers (13). On the other hand, the MVAC, which was designed to recover

pathogens from food matrices, has recently been used in the forensic science field to recover a variety of body fluids from complex surfaces, e.g. soft surfaces (14). Even so, swabbing is still the most frequently used method based on a previous review of the literature and ISO 15216's recommendation to use cotton-tipped swabs for recovery of hepatitis A virus and noroviruses from foodstuffs and food surfaces (8, 15).

This study's aim was to compare viral recovery methods and improve detection of viruses contaminated on carpet. To our knowledge, no published studies have assessed recovery methods for any enteric viruses, including HuNoV, from carpet. Therefore, our objectives were: (i) compare applicable surface recover methods for two HuNoV surrogates, feline calicivirus (FCV) and murine norovirus (MNV), on wool and nylon carpets, with BE, MVAC, and a macrofoam-tipped swab (MS), and (ii) improve detection via qRT-PCR by clarifying samples prior to analysis.

MATERIALS AND METHODS

Virus propagation and cell culture. Feline calicivirus (FCV) strain F9 (kindly provided by Dr. Jan Vinje at the Centers for Disease Control and Prevention, Atlanta, GA) stocks were made by infecting 90% confluent monolayers of Crandell Rees feline kidney (CRFK) (ATTC CCL-94, American Type Culture Collection, Manassas, VA) at a multiplicity of infection of 0.01 in Complete Eagles Modified Essential Media (Corning, Corning, NY) supplemented with 10% low-endotoxin heat inactivated fetal bovine serum (FBS) (Seradigm, VWR International, Randor, PA), 100 U/liter penicillin (HyClone, GE, Boston, MA), and 100 µg/liter streptomycin (HyClone, GE, Boston, MA). Murine

norovirus (MNV) CW3 (kindly provided by Dr. Herbert Virgin at the University of Washington, St. Louis) stocks were made by infecting 60 – 80% confluent monolayers of RAW 264.7 cell (ATCC TIB-71. American Type Culture Collection, Manassas, VA) at a multiplicity of infection (MOI) of 0.05. The medium used to grow RAW 264.7 cells and propagate MNV was previously described (16). CRFK and RAW 264.7 cells were grown at 37˚C and 5% CO_2 (Symphony, VWR International, Randor, PA) until complete cytopathic effect (CPE) was observed (24 – 72 hr). After CPE, both viruses were harvested by three cycles of freeze-thawing followed by centrifugation for 10 min at 5,000 x g and 4˚C. Finally, viral suspensions were chloroform extracted based on previous methods (16). Stocks of FCV (ca. 9 log PFU/ml) and MNV (ca. 8 log PFU/ml) were aliquoted and stored at -80˚C.

Plaque assays. Infectious MNV and FCV were quantified by standard plaque assay as previously described with modifications (16, 17). Briefly, MNV plaque assay was completed by seeding 6-well dishes with RAW 264.7 cells at 1 x 10^6 viable cells/well and incubated until 60 – 80% confluent (4 – 8 hr). MNV experimental samples were diluted, if needed, in MNV infection medium, described elsewhere (16), containing 5% FBS (complete Eagles Modified Essential medium) to improve plaque formation. FCV plaque assay was based on previous work with significant modifications (17). CRFK cells were seeded in 6-well dishes at 2.5 x 10^5 viable cells/well and incubated until ca. 90% confluent (48 hr). FCV samples were serially diluted in CDMEM-5 if needed. After a 1 hr absorption phase, 2 ml of 1:1 mixtures of 3% sea plaque agarose (Lonza, Switzerland)

and 2X Temin's Modified Eagle Medium (MEM) were added to each well and incubated until visible plaque formation (24 – 72 hr). The 2X MEM was supplemented with 10% low-endotoxin heat inactivated FBS, 100 U/liter penicillin, 100 µg/liter streptomycin, 10mM HEPES (HyClone, GE, Boston, MA), and 1 mM NEAA (HyClone, GE, Boston, MA). MNV and FCV plaques were visualized by staining agarose plugs with a 0.03% neutral red solution (Carolina Biological, Burlington, NC) mixed with 1X PBS and enumerated on a light box (Futura light box, Logan Electric, Bartlett, IL). Plaque assays for both MNV and FCV contained a stock suspension of virus and CDMEM-5 as a positive and negative control, respectively, to test for cell line permissiveness and contamination. Cell lines were not passaged >25 times.

Preparation of carpet carriers. Wool level loop and nylon multi-level loop carpets (SDL-ATLAS, Rock Hill, SC) were selected from a list provided in ASTM standard F655-13 ((18). The carpet fiber characteristics, e.g. absorption capacity and zeta potential, are described elsewhere (Buckley et al. unpublished). Carpet samples contained no finishes, e.g. antimicrobial or soil retardant, and were cut into 5 x 5 cm carriers with a mechanical cutting die (Model #1500, Freeman Schwabe, Batavia, OH) (courtesy of Dr. Daniel Price, Interface Inc., Atlanta, GA). After cutting, carpets were shaken by hand to remove loose fibers, wrapped in aluminum foil, and autoclaved on a 30-min dry cycle.

Sampling experiments. Wool and nylon carpet carriers (5 x 5 cm), each contained in a petri dish, were individually inoculated with 0.1 ml of FCV or MNV. Inocula were

diluted in CDMEM-5 and contained ca. 7 log PFU/ml of FCV or MNV. Carpet carriers were dried for 1 hr in a 30% relative humidity (RH) chamber (480 HP, VWR International, Randor, PA) maintained with a saturated $MgCl_2$ solution. After drying, three recovery procedures, i.e. BE, MS, and MVAC, were assessed for their ability to elute FCV and MNV from wool and nylon carpets. Additionally, two separate elution buffers, 0.01 M phosphate buffered saline (PBS) + 0.02% Tween 80 (T80) and ¼ strength Ringer's solution (RS) were tested using the BE and MS. Due to design limitations, only one buffer was used for the MVAC, i.e. sterile collection solution (SRS) (SRS 1000, M-VAC Systems, Sandy, UT).

The MVAC was used in accordance with required training provide by M-Vac Systems, Incorporated, Sandy, UT. To recover viruses from carpet carriers using the MVAC, carriers were moved to a 150 mm Kirby Bauer dish (VWR International, Randor, PA) to adequately collect residual liquid. The M-VAC head has two functions: dry vacuum and wet vacuum extraction. During dry vacuum extraction, the edge of the circular head aspirates whereas during wet vacuum extraction the head aspirates while simultaneously spraying SRS from the head's center in a horizontal plane. During recovery, the MVAC head was passed over three separate areas of the carrier three times, both horizontally and vertically. On each initial pass, the wet vacuum was on, whereas during the remaining two passes, in each area, only the dry vacuum was on.

Recovery of FCV and MNV from carpet carriers were consistent with BE methods outlined in ASTM 2966-14 (13). Dried inoculated carpet carriers were each placed in separate 500 ml wide mouth polypropylene bottles (Fisher Scientific)

containing 100 ml of buffer. Bottles were then sonicated (FS110, Fisher Scientific International, Pittsburg, PA) for 1 min at 40 kHz and hand shaken for l min. After shaking, carriers were removed with sterile forceps.

Due to high sample volumes associated with BE and MVAC, a concentration step was applied. Immediately after recovery, BE and MVAC samples were concentrated via ultrafiltration (Amicon Ultra-15 30K, Millipore, Billerica, MA) at 4,000 x g for 15 min at 4˚C (Allegra X-30R, Beckman Coulter, Brea, CA). Supernatants were pooled, vortexed, weighed, and stored at -80˚C prior to infectivity and qRT-PCR analysis.

The MS was designed by Puritan Medical Supply (NC1213065 ENVIROMAX PROTOTYPE, Medical Supply, Guilford, ME) and purchased dry. Swabs were pre-moistened by filling sleeves with sterile buffers, mentioned above, then gently rolled for 3 minutes. Carpet carriers were swabbed horizontally, vertically, and diagonally, twice. Next, the swab head was pressed and twisted within its sleeve to elute any remain liquid. After eluting, the swab head was discarded. Samples were vortexed, weighed, aliquoted, and stored at -80˚C prior to infectivity and qRT-PCR analysis.

Sample clarification. To elucidate issues surrounding recovery and detection with qRT-PCR recovery of FCV from wool carpet with BE and 0.01 M + 0.02% Tween 80 was used. Virus, recovery method, and carpet were selected based on the least efficacious RNA recovery rates reported in the comparison of sample recovery experiments (**Table 2**). Carpet samples and viral inocula were prepared and executed as described above. After recovery samples were clarified by either filtering with 0.2 or 0.4 μm syringe filters

(Sterile Syringe Filter, VWR International, Randor, PA), centrifuging at 4,000 x g for 15 mins at 4˚C (Allegra X-30R, Beckman Coulter, Brea, CA), 10^{-1} dilution before FCV RNA extraction in diethyl pyrocarbonate (DEPC) water (OMEGA Bio-Tek, Norcross, GA), or 10^{-1} dilution after FCV RNA extraction in DEPC water. The control samples were recovered and concentrated as stated above with no additional treatments.

Real time qRT-PCR. Viral extraction was performed as previously described with minor modifications (11). QRT-PCR assays were completed with the E.N.Z.A Viral RNA Kit (OMEGA Bio-Tek, Norcross, GA), per manufacturer instructions, by extracting viral RNA from 0.15 ml of sample or stock. MS samples were extracted directly, whereas BE and MVAC samples were extracted after concentration via ultrafiltration and stored at -80˚C prior to assessment. qRT-PCR of both viruses were completed with KAPA SYBR Fast Universal One-Step qRT-PCR Kit (Kapa Biosystems, Wilmington, MA) on a Realplex2 Mastercycler platform (Eppendorf, Hauppauge, NY). Forward and reverse primer sequences for FCV analysis were GCCATTCAGCATGTGGTAGTAACC and GCACATCATATGCGGCTCTG, respectively, whereas MNV forward and reverse primer sequences were TGATCGTGCCAGCATCGA and GTTGGGAGGGTCTCTGAGCAT, respectively (19). A standard curve was generated for both viruses by performing a 7-step 10-fold diluting of a previously titered and RNA extracted virus stock. Each dilution's threshold cycle (C_T) values was used to plot against its corresponding plaque forming unit (PFU) and calculated as previously described (19).

Data Analysis. All experiments were performed in triplicate with three replicates per experiment. Percent recovery efficiency (% RE) was defined as the number of PFU or genomic copies recovered divided by the number of PFU or genomic copies initially seeded (8). One-way multiple comparisons to determine significance among treatments. All results were expressed as mean ± standard deviation. Statistical significance was defined as $P \leq 0.05$. Statistical analyses were conducted using JMP (JMP Pro 12.2.0, SAS Inc., Cary, NC).

RESULTS

Virus recovery efficiency via plaque assay. Table 4.1 shows the % RE and $\log_{10}$ PFU recovery of FCV and MNV from wool and nylon carpet carriers as measured by plaque assay. Percent RE and $\log_{10}$ PFU recovery of infectious FCV ranged from 0.37 to 48.44% and 3.24 to 5.51 $\log_{10}$ PFU on wool carrier, respectively, compared to 0.02 to 16.24% and 2.10 to 5.03 $\log_{10}$ PFU on nylon carriers, respectively. The % RE and $\log_{10}$ recovery for infectious MNV ranged from 1.87 to 68.83% and 4.48 to 6.10 $\log_{10}$ PFU, respectively, on wool, compared to 1.54 to 74.67% and 4.30 to 6.13 $\log_{10}$ PFU, respectively, on nylon carpet carriers. The recovery method and eluent used significantly affected the recovery of infectious FCV whereas only the recovery method affected the recovery of MNV. BE with T80 and RS, and the MVAC achieved significantly higher recovery rates for FCV and MNV than did MS, excluding recovery of FCV using BE with RS from nylon carpet carriers. Carpet type significantly affected recovery of FCV when using BE and MS with the elution buffer RS with more FCV was recovered from wool than from nylon.

Comparatively, significantly more infectious MNV was recovered from both carpet types than was FCV, regardless of method and eluent.

Virus recovery efficiency via qRT-PCR. Table 4.2 shows the % RE and $\log_{10}$ genomic copy recovery of FCV and MNV from wool and nylon carpet carriers measured with qRT-PCR. Percent RE and $\log_{10}$ genomic copy recovery of FCV ranged from 0.36 to 4.95% and 2.63 to 3.81 $\log_{10}$ genomic copies, respectively, on wool carpet carriers compared to 0.69 to 23.72% and 2.78 to 4.49 $\log_{10}$ genomic copies, respectively, on nylon carpet carriers. To the contrary, % RE and $\log_{10}$ genomic copy recovery of MNV ranged from 2.42 to 38.78% and 4.36 to 5.40 $\log_{10}$ genomic copies, respectively, on wool carpet carriers compared to 2.02 to 79.15% and 4.13 to 5.68 $\log_{10}$ genomic copies. Recovery method and eluent type significantly affected the recovery of both FCV and MNV on both carpet types when assessed via qRT-PCR. MVAC exhibited significantly higher recoveries of genomic copies for FCV and MNV compared to all other methods, excluding FCV recovery using MS with RS on nylon and MNV recovery using BE with T80 and RS. Carpet type did not play a significant role in the recovery of FCV and MNV genomic copies, excluding recovery of MNV using BE T80 with RS. Overall, significantly more MNV genomic copies were detected compared to FCV, regardless of method and eluent. Additionally, visible wool and nylon carpet debris was accumulated when using BE in both the recovery suspension and within the RNA extraction columns, whereas with the MVAC, only wool debris appears in the recovery suspension and RNA extraction columns.

FCV detection after sample clarification. Sample clarification results are presented in **Table 4.3**. Due to low RNA % RE from wool, FCV was further tested for optimization of recovery and detection. Syringe filtering with 0.2 and 0.4 μm resulted in volumes too low for plaque assay and qRT-PCR analysis, as such no analysis was completed for this treatment. Recovery of FCV genomic copies from the control treatments and centrifuged treatments ranged from 4.14 to 4.89 $\log_{10}$ copies and 3.88 to 4.90 $\log_{10}$ copies, respectively. In contrast, recovered infectious FCV from the control was 5.76 $\log_{10}$ PFU, whereas, centrifugation yielded 5.61 $\log_{10}$ PFU. Recovery of infectious FCV and genomic copies were significantly reduced with centrifugation alone compared other clarification methods. At the same time, amplification of the PCR target from the 10^{-1} dilution of both control and centrifuged samples after RNA extraction was significantly higher compared to all other clarification processes.

DISCUSSION

FCV and MNV are commonly used as surrogates to study the behavior of HuNoV in the environment (20). Recovery methods are the underpinning of environmental detection procedures. However, there is scant literature regarding recovery efficiency of methods used to detect HuNoV as well as other enteric viruses on complex soft surfaces, such as carpet, and of those studies, many use different recovery procedures. In this study, we evaluated three recovery methods' ability to recover two HuNoV surrogates, FCV and MNV, from wool and nylon carpet carriers. Additionally, we attempted to improve detection via qRT-PCR by clarifying samples prior to analysis. Our findings

demonstrate the efficiency and limitations these three methods may have when used to recover and detect HuNoV surrogates from complex soft surfaces.

The findings from previously published studies indicated that both recovery method and eluent type could significantly affect the recovery of viruses with some study authors suggesting that optimization of methods may improve recovery (21, 22). In our study, data from plaque assays, i.e. infectious data, indicated that recovery method and eluent type significantly affected the recovery of HuNoV surrogates, FCV and MNV. Among the carpet samples tested, BE and MVAC were the most efficacious methods for recovering infectious FCV and MNV from wool and nylon carpets. The mode of action for each recovery method tested relied upon fiber rehydration, which changes the zeta potential of the fiber and the virus surface charge. However, each recovery method differed in the physical removal and recovery of the virus after the elution buffer application. For instance, the mechanism of action for MVAC relies upon the dual use of a spray, which assists in desorption of virus from the fibers, while simultaneously aspirating the virus containing suspension. In contrast, BE uses sonication which weakens virus surface attachment, providing higher recovery than traditional handshaking (11). However, both methods are expensive and rely upon the use of high volumes to recover virus, which lowers the detection limit and necessitates the use of a concentration step. Swabs are the most frequently used tool for environmental recovery method because they are simple to use, economical, and low-volume, i.e. no addition concentration step. Nevertheless, the problem with swabbing is that it is usually accompanied by a significant loss in recovery efficiency. Ultimately, each method may

be useful based on – time, funding, technical expertise, and study type, i.e. efficacy study or environmental monitoring.

Recovery of infectious virus from environmental samples can vary based on surface type. The surface hydrophilicity, absorption capacity, zeta potential, and chemical surface interactions in addition to a virus's characteristics, i.e. isoelectric point, can influence both survival and recovery of the virus. For example, Yeargin et al. (11) found that after drying, recovery of both FCV and MNV was significantly different across cotton, polyester, and glass when using the same recovery method. Another study demonstrated higher recovery efficiency of MNV from inoculated diapers compared to cotton gauze (10). Unpublished data from M-Vac Systems, Incorporated, suggested some biological material, such as DNA, has higher recovery rates on natural surfaces, such as wool and cotton, compared to synthetic material, nylon and rayon (14). Consistent with these findings, we found that higher titers of FCV and MNV were recoverable from wool compared to nylon. This is likely a result of wool's higher absorption capacity and ability to provide a more protective environment during desiccation (Buckley et al. chapter 3). This assertion is supported by a published study that examined the same fibers, suggesting both FCV and MNV can survive longer on wool carpet fibers than on nylon carpet fibers. Fabrication of soft surfaces has also been shown to influence the recovery and survival of enteric viruses, such as poliovirus (23). The additional fiber twist that accompanies a tuft carpet construction, observed in nylon carpet but not in wool carpets, may further prevent elution of viruses.

When selecting a recovery method, eluent type is a critical factor to consider based on recovery type, downstream assays, and storage conditions, especially when a concentration step is required for detection. Concentration methods can also concentrate inhibitors cytotoxic to cell culture systems or may interfere with PCR, e.g. degradation of target nucleic acid or reduced extraction efficiency from heterogeneous samples (5, 24). However, a meta-analysis of virus surface sampling literature suggests eluent type does not significantly influence recovery of viruses (8). Although, the meta-analysis conducted in that study did not specifically target non-enveloped viruses and most recovery methods used swabs. Ultimately, the authors suggested using ¼ strength Ringer's solution with a polyester-tipped swab. Our results indicated eluent type can significantly influence virus recovery of FCV, when using BE and MS methods. Typically, eluents range from pH 6 – 9 to buffer the solution, reduce cytotoxicity, and create a negative viral surface charge (25). Often, surfactants are included to assist with recovery via reduced surface tension and micelles formation. In this study, the addition of Tween 80 to a simple, low ionic strength buffer, significantly improved FCV recovery compared to using ¼ strength Ringer's solution, which contains no surfactants. On the other hand, another high efficiency recovery method for soft surfaces using a mini-spin column-based approach had similar recovery rates of FCV and MNV as the BE and MVAC methods from the same carpet fibers but did not require high volumes or a concentration step (Buckley et al. chapter 3). Additionally, and consistent with the findings of Julian et al. (8), the mini-spin column method results indicated eluent type did not significantly affect recovery of FCV and MNV (Buckley et al. chapter 3). Albeit, this method was not incorporated to

our study because it requires packable fibers, e.g. shaved carpet fibers or swatches, and does not mimic the scale or surface complexity needed to assess virus recovery of intact carpet carriers. Collectively, these findings suggest that eluent type may affect recovery of some viruses and is dependent upon both recovery method and virus type.

Several published papers have detailed the inactivation profiles of HuNoV surrogates (6, 26). Not surprisingly and consistent across previous studies, significantly more infectious and genomic copies of MNV were detected compared to FCV. MNV has been demonstrated to be more environmentally resilient on both hard and soft porous surfaces and is more resistant to some chemical inactivation treatments compared to FCV (20, 27). Although a calicivirus, FCV is also classified as a respiratory pathogen and cannot survive harsh climates like the gastrointestinal system. We believe the difference is primarily due to capsid integrity and higher receptor sensitivity to environmental factors, although other structural or solution characteristics cannot be eliminated.

The divergence between infectivity assays and molecular data, i.e. qRT-PCR, has been previously documented (10, 28). Traditionally, unless samples are enzymatically pre-treated, concentrations of RNA as measured by qRT-PCR, are higher compared to infectious counts. This is due to capsids becoming inactivated but not lysed, allowing for temporary preservation of its genome. However, there is evidence to dispute this. For instance, an extensive recovery comparison study using another HuNoV surrogate, MS2 phage, showed that fractions of MS2 phage RNA recovered from environmental surfaces were lower than recovered infectious MS2 phage (8). Consistent with those findings, we found that recovery of FCV and MNV genomic copies were less than the recovery of

infectious FCV and MNV among undiluted, RNA extracted samples. We believe an inhibitor impacted our qRT-PCR amplification and/or extraction efficiency, whereas Julian et al. (8) suggested the lower fractional recovery may be a result of exogenously seeded RNA or viral aggregates (8). Degradation or lysis of capsids can occur leading to higher exogenous genomic copies compared to estimates of infectious counts in stocks and inocula. Commonly, when seeded, the exogenous RNA would degrade more quickly than capsid-protected RNA, leading to an inflated initial copy count, and resulting in lower calculated RE (28). Be that as it may, viral aggregates, may also reduce the recovery of RNA as they are not distinguishable within a plaque assay and may decrease extraction efficiency.

In our study, the sample matrices were wool and nylon, and the fibers in recovered samples may have affected qRT-PCR. Therefore, sample clarification was studied to improve detection by removing contaminants that may interfere with the assay. Attempts to elucidate possible interferences with qRT-PCR via sample clarification both positively and negatively affected genomic copy recovery. We believed carpet fibers may reduce extraction efficiency based on accumulated carpet debris observed within RNA extraction columns. Our attempts to remove the carpet fibers via syringe filtering (data not shown) and centrifugation significantly lowered volume and both PFU and copy number, respectively. This suggests some viruses may be attached or trapped within pelleted fibers. At any rate, both syringe filtering and centrifuging are not recommended as sample clarification techniques for recovery of FCV from carpets. Dilution is also a simple but effective method for removing inhibitors. Our qRT-PCR results of samples

diluted prior to RNA extraction suggested a filterable PCR inhibitor was concentrated with FCV genomic RNA, prompting us to dilute the extracted RNA samples. Under these conditions, the inhibitor was reduced, which allowed for significantly more target amplification. The specific inhibitor is unknown but, as stated previously, PCR can be inhibited by numerous factors, such as complex polysaccharides, organic material, and metal ions (29). The inhibitor could have originated from a variety of sources but likely is due to the carpet or extraction column. Future studies designing or optimizing recovery of viruses from environmental samples are encouraged test for qRT-PCR for inhibition with specificity, i.e. fluorogenic probe, and incorporate an external standard.

CONCLUSIONS

The assessment of recovery methods is a critical first step to improve the detection and quantification of viruses. Here, we tested 3 mechanistically different recovery methods intended for HuNoV recovery from carpets. BE and MVAC exhibited higher recovery efficiencies compared to MS. Furthermore, detection of FCV and MNV via qRT-PCR can be inhibited when recovered from wool and nylon carpets. Ultimately, each method demonstrates merit for use under different conditions, e.g. standard testing and environmental monitoring.

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Table 4.1 Recovery efficiency percentages of FCV and MNV via plaque assay from carpet

		%RE (log recovery [PFU])[a]			
		FCV		MNV	
Recovery Method[b]	Buffer[c]	Wool	Nylon	Wool	Nylon
BE	T80	48.44 (5.51 ± 0.13)^{A}	16.24 (5.03 ± 0.16)^{BC}	68.83 (6.10 ± 0.09)^{A}	60.47 (6.07 ± 0.06)^{A}
BE	RS	10.41 (4.88 ± 0.07)^{BC}	0.44 (3.42 ± 0.30)^{DE}	38.87 (5.88 ± 0.09)^{A}	40.77 (5.91 ± 0.10)^{A}
MS	T80	0.82 (3.76 ± 0.16)^{D}	0.54 (3.62 ± 0.01)^{D}	1.87 (4.50 ± 0.17)^{B}	2.87 (4.58 ± 0.29)^{B}
MS	RS	0.37 (3.24 ± 0.34)^{E}	0.02 (2.10 ± 0.22)^{F}	2.16 (4.48 ± 0.26)^{B}	1.54 (4.30 ± 0.29)^{B}
MVAC	SRS	18.29 (5.16 ± 0.04)^{AB}	7.30 (4.75 ± 0.01)^{C}	52.58 (5.99 ± 0.08)^{A}	74.67 (6.13 ± 0.15)^{A}

[a]Data are expressed as percent recovery (log means ± standard deviation). Within each surrogate, $\log_{10}$ values with different letters are significantly different ($P<0.05$), among both surfaces.

[b]BE: bottle extraction; MS: macrofoam-tipped swab; MVAC: microbial vacuum;

[c]T80: 0.01 M phosphate buffered saline + 0.02% Tween 80; RS; ¼ strength Ringer's solution; SRS: sterile rinse solution

Table 4.2. Recovery efficiency percentage of FCV and MNV via qRT-PCR from carpet

		%RE (log recovery [genomic copies])[a]			
		FCV		MNV	
Recovery Method[b]	Buffer[c]	Wool	Nylon	Wool	Nylon
BE	T80	0.81 (2.95 ± 0.19)^{DE}	2.53 (3.46 ± 0.21)^{CD}	3.37 (4.44 ± 0.19)^{CD}	8.04 (4.83 ± 0.15)^{ABC}
BE	RS	0.36 (2.63 ± 0.19)^{F}	0.69 (2.78 ± 0.36)^{EF}	3.34 (4.47 ± 0.12)^{CD}	14.97 (5.15 ± 0.06)^{AB}
MS	T80	1.03 (2.95 ± 0.33)^{DE}	1.31 (3.18 ± 0.25)^{CDE}	4.25 (4.59 ± 0.07)^{BCD}	3.08 (4.34 ± 0.25)^{D}
MS	RS	1.70 (3.23 ± 0.24)^{CDE}	2.24 (3.45 ± 0.12)^{BC}	2.42 (4.36 ± 0.03)^{CD}	2.02 (4.13 ± 0.29)^{D}
MVAC	SRS	4.95 (3.81 ± 0.05)^{AB}	23.72 (4.49 ± 0.09)^{A}	38.78 (5.40 ± 0.36)^{A}	79.15 (5.68 ± 0.27)^{A}

[a]Data are expressed as percent recovery (log means ± standard deviation). Within each surrogate, $\log_{10}$ values with different letters are significantly different ($P<0.05$), among both surfaces.

[b]BE: bottle extraction; MS: macrofoam-tipped swab; MVAC: microbial vacuum;

[c]T80: 0.01 M phosphate buffered saline + 0.02% Tween 80; RS; ¼ strength Ringer's solution; SRS: sterile rinse solution

Table 4.3. Recovery of FCV from wool carpet with bottle extraction method after sample clarification

Clarification Process[b]	Recovery[a]	
	qRT-PCR log copies ± SD	Plaque Assay log PFU ± SD
Control	4.14 ± 0.29^{B}	5.76 ± 0.10^{A}
10^{-1} control before RNA extraction	4.36 ± 0.08^{B}	-
10^{-1} control after RNA extraction	4.89 ± 0.12^{A}	-
Centrifugation treatment	3.88 ± 0.17^{C}	5.61 ± 0.10^{B}
10^{-1} before RNA extraction	4.27 ± 0.21^{B}	-
10^{-1} after RNA extraction	4.90 ± 0.12^{A}	-

[a]Log_{10} values with different letters in the same column are significantly different ($P<0.05$).

[b]Control: unclarified FCV recovery from wool carpet; 10^{-1} dilution before FCV RNA extraction in diethyl pyrocarbonate (DEPC) water; 10^{-1} dilution after FCV RNA extraction in DEPC water; Centrifugation at 4,000 x g for 15 mins; 10^{-1} dilution of centrifuged samples before FCV RNA extraction in DEPC water; 10^{-1} dilution of centrifuged samples after FCV RNA extraction in DEPC water.

[c] – not tested as there was no suspected interference.

CHAPTER FIVE

EFFICACY OF SILVER DIHYDROGEN CITRATE AND STEAM VAPOR AGAINST A HUMAN NOROVIRUS SURROGATE, FELINE CALICIVIRUS, IN SUSPENSION, ON GLASS, AND CARPET

ABSTRACT

Human noroviruses (HuNoV) are responsible for 19-21 million illnesses each year in the United States while also accounting for ca. 20% of all diarrheal cases worldwide. Effective environmental hygiene programs are important to prevent and control of HuNoV outbreaks. However, despite our increasing knowledge of the role of soft surfaces in the transmission of HuNoV, no commercially available disinfection technologies have been evaluated on carpets. Our aim was to assess two disinfection technologies, silver dihydrogen citrate (SDC) and steam vapor, against one HuNoV surrogate, feline calicivirus (FCV), on wool and nylon carpet. First, we evaluated the effect of both technologies on the aesthetic appearance of carpet. After developing a neutralizer for SDC, we evaluated the efficacy of SDC in suspension with and without 5% fetal bovine serum (FBS) and the efficacy of SDC and steam vapor on glass, each with and without 5% FBS. Lastly, we tested both technologies on carpets. Wool and nylon carpet carriers exhibited no obvious color changes after both treatments, however, SDC treatment left a residue while steam resulted in minor abrasions to surface fibers. A sodium thioglycolate-based solution was found to adequately neutralize and eliminate SDC cytotoxicity. SDC in suspension and on glass reduced FCV by 4.65 and >4.66 $\log_{10}$ pfu with in 30 mins, respectively, but demonstrated reduced efficacy in the presence of serum. However, SDC was only efficacious against FCV on nylon (3.62 $\log_{10}$ pfu

reduction) as compared with 1.82 $\log_{10}$ reduction on wool carpet. Steam vapor reduced FCV by >4.93 $\log_{10}$ pfu on glass in 10 sec, with no observed difference among serum treatments, and >3.68 $\log_{10}$ pfu on wool and nylon carpet carriers in 90 sec. There was limited reduction to FCV RNA under both treatments regardless of treatment compared to infectivity assays but RNA reductions were higher in sample that contained 5% serum.

INTRODUCTION

Human noroviruses (HuNoV) are the leading causes of acute gastroenteritis worldwide as well as the most common cause of foodborne disease in the United States. Worldwide, its economic burden is estimated to be US$4.2 billion in health care costs alone illustrating its significance as a public health problem (1, 2). Common symptoms include both diarrhea and vomiting, which aid in its spread. Transmission occurs two ways -- the fecal-oral or vomitus-oral route through person-to-person contact, food, water, or environmental surfaces (1).

Environmental transmission of HuNoV is estimated to be low (3). Even so, several epidemiological investigations and laboratory-based studies suggest the environment plays an important role in transmission as both hard and soft surfaces may initiate and prolong HuNoV outbreaks (4). For example, a hotel in the United Kingdom experienced a 5-month long HuNoV outbreak (5). Outbreaks investigators suggested, in addition to HuNoV environmental stability, ineffective decontamination of soft surfaces assisted in prolonging the outbreak.

Focusing on environmental sanitation is a recommended strategy to prevent and control HuNoV outbreaks. The challenge to effective environmental sanitation is that HuNoV can be shed in high titers from infected individuals. This, coupled with its environmental stability, low infectious dose, and resistance to many commonly used disinfectants, e.g. phenolic and quaternary ammonium compounds, makes HuNoV outbreaks difficult to control. The current recommendation for HuNoV clean-up includes using1,000–5,000 ppm bleach or an Environmental Protection Agency (EPA)-registered disinfectant (6). However, these were validated for hard surfaces not soft surfaces. Moreover, their use may damage the appearance of soft surfaces, such as carpet and soft furnishings. Furthermore, carpet and other soft furnishings can absorb toxic active ingredients causing irritation of the skin, eyes, and respiratory tract (7). Fogging with certain chemistries, e.g. ozone and H_2O_2, has demonstrated efficacy against some enteric viruses but is impractical in some settings, e.g. long-term care facilities and residential homes, due to temporary removal of residents and cost (8, 9). Taken together, the disinfectant shortcomings of current disinfectant procedures present a gap in evidence-based control strategies for disinfecting soft surfaces contaminated with viruses suggesting the need to evaluate safe and practical technologies for use on soft surfaces.

To our knowledge, only one previous study has investigated the use of disinfection technologies against viruses on carpets, all of which are liquid-based (10). Investigators found that a glutaraldehyde-based solution could achieve EPA efficacy standards for disinfection on soft surfaces against a HuNoV surrogate, feline calicivirus (FCV) (3.0 $\log_{10}$ reduction) but was linked to negative health effects, such as skin rashes

and respiratory irritation (11). Other chemistries tested were salt and quaternary ammonium-based products, which have shown little efficacy against HuNoV (10). Recently, transitional metal ions, such as silver, have demonstrated broad-range efficacy against microorganisms although they are more stable when generated in the presence of citric acid to form silver dihydrogen citrate (SDC). The benefit of SDC is that it is less toxic compared to other chemistries, i.e. glutaraldehyde, and may be more gentle on delicate soft surfaces. Previous studies with SDC have demonstrated its efficacy against HuNoV but these studies lack infectious data required by the EPA to be registered (12, 13).

The Centers for Disease Control and Prevention (CDC) and the Occupational Safety and Hazard Administration (OSHA) have both recommended steam cleaning for carpets after a suspected HuNoV contamination event (14). Steam has been shown to be efficacious against non-sporeforming bacteria on hard surfaces, but there is scant literature of its efficacy against viruses. A dry-steam vapor system with thermos accelerated nano-crystal sanitation (TANCS) technology (Advanced Vapor Technologies, Seattle, WA) is a promising tool for disinfecting virally contaminated soft surfaces. Previous work with MS2 phage and FCV on hard surfaces demonstrated a >6 log and >4 log reduction in less than 5 and 10 sec, respectively (15, 16). However, the efficacy of steam or moist heat against virus contamination on soft surfaces has not been measured under controlled conditions (17). This is due, in part, to a lack of standardized test methods for quantitative assessment of disinfecting solutions for carpets contaminated with viruses.

The gap in knowledge about the efficacy of soft surface disinfection technologies and the lack of standardized test methods for carpets warrants further exploration. To our knowledge, no published studies have investigated either technology against infectious HuNoV surrogates on carpet. The aim of this study was to assess these two disinfection technologies against FCV on wool and nylon carpet. Specific objectives were to: (i) assess these technologies in suspension, (ii) on a hard surface with current American Society for Testing and Materials (ASTM) International standards, and (iii) on carpets by adapting a current ASTM International standard.

MATERIALS AND METHODS

Virus propagation, cell culture, and plaque assay. A stock of feline calicivirus (FCV) strain F9 (kindly provided by Dr. Jan Vinje at the Centers for Disease Control and Prevention, Atlanta, GA) was propagated by infecting 90% confluent monolayers of Crandell-Rees kidney cell (CRFK) (ATCC CCL-94, American Type Culture Collection, Manassas, VA) at a multiplicity of infection (MOI) of 0.01 in Complete Eagles Modified Essential Media (Corning, Corning, NY) supplemented with 10% low-endotoxin heat inactivated fetal bovine serum (FBS) (Seradigm, VWR International, Randor, PA), 100 U/liter penicillin (HyClone, GE, Boston, MA), and 100 µg/liter streptomycin (HyClone, GE, Boston, MA). CRFK was incubated at 37˚C and 5% CO_2 (Symphony, VWR International, Randor, PA) until complete cytopathic effect was observed (1 – 3 days). FCV was harvested from cell lysates by three cycles of freeze-thawing followed by centrifugation for 10 min at 5,000 x g and 4˚C then extracted with chloroform as

previously described (18). FCV (ca. 9 log PFU/ml) stocks were aliquoted and stored at -80˚C.

Infectious FCV was quantified by standard plaque assays as previously described with modifications (19). Briefly, CRFK cells were seeded in 6-well dishes at 2.5 x 10^5 viable cells/well and incubated until ca. 90% confluent (2 days). FCV samples were serially diluted in an infection medium described elsewhere, containing 5% FBS (CDMEM-5) if needed. During the plaque assay 0.2 ml of sample was added to each well containing 0.3 ml of CDMEM-5 and immediately swirled. After a 1 hr absorption phase, 2 ml of 1:1 mixtures of 3% seaplaque agarose (Lonza, Switzerland) and 2X Temin's Modified Eagle Medium (MEM) were added to each well incubated until visible plaque formation (1 – 3 days). The 2X MEM was supplemented with 10% low-endotoxin heat inactivated FBS, 100 U/liter penicillin, 100 μg/liter streptomycin, 10 mM HEPES (HyClone, GE, Boston, MA), and 1 mM NEAA (HyClone, GE, Boston, MA). FCV plaques were visualized by staining agarose plugs with a 0.03% neutral red solution (Carolina Biological, Burlington, NC) mixed with 1X PBS and enumerated on a light box (Futura light box, Logan Electric, Bartlett, IL). FCV plaque assays contained a stock suspension of virus and CDMEM-5 as a positive and negative control, respectively, to test for cell line permissiveness and contamination. CRFK was passaged fewer than 25 times.

RNA extraction and qRT-PCR. Viral extraction was performed as previously described with minor modifications (20). Viral RNA was extracted from 0.15 ml of a sample or

virus stock with ENZA Viral RNA Kit (OMEGA Bio-Tek, Norcross, GA) per manufacturer instructions. Viral RNA was extracted on the day of recovery experiments and stored at -80˚C prior to use. KAPA SYBR Fast Universal One-Step qRT-PCR Kit (Kapa Biosystems, Wilmington, MA) was used to detect FCV on a Realplex2 Mastercycler platform (Eppendorf, Hauppauge, NY). Forward and reverse primer sequences for FCV qRT-PCR analysis were GCCATTCAGCATGTGGTAGTAACC and GCACATCATATGCGGCTCTG, respectively (21). The standard curve for FCV was prepared by performing an 7-step 10-fold dilution of virus stocks. Log reductions (**Equation 1**) of virus RNA were performed as previously described (21).

$$RNA\ log\ reduction = \frac{(C_{T,t} - C_{T,c})}{K} \quad \textbf{(Equation 1)}$$

where $C_{T,t}$ is the cycle threshold (C_T) for the experimental group, $C_{T,c}$ is the cycle threshold for the control recovered at time 0, and k is the slope obtained from plotting the C_T values vs. the $\log_{10}$ of the RNA copy number used for presenting the standard curve (21).

Preparation of surface samples. Wool-level loop and nylon multi-level loop carpets (SDL-ATLAS, Rock Hill, SC) were selected according to ASTM standard F655-13 (22). The carpet fiber characteristics, e.g. absorption capacity and zeta potential, are described elsewhere (Buckley et al. Chapter 3). Carpets contained no finishes, e.g. antimicrobial or soil retardant, and were cut into 5 x 5 cm carriers with a mechanical cutting die (Model #1500, Freeman Schwabe, Batavia, OH) (courtesy of Dr. Daniel Price, Interface Inc.,

Atlanta, GA). After cutting, carpets were dusted by hand to remove loose fibers, wrapped in aluminum foil, and autoclaved on a 30-min dry cycle.

Disinfection technologies. We tested two disinfection technologies: SDC and steam-vapor (2300SB with TANCS, Advanced Vapor Technologies, Seattle, WA). SDC contained 0.003% silver ion stabilized in 4.846% citric acid to form SDC. The steam-vapor device with a TANCS processer was filled with tap water and connected to a hose with a ca. 2.5 cm diameter cleaning head. A standard cotton terry cloth was autoclaved, folded to yield 4 layers, wrapped around the cleaning head, held with a rubber band, and changed between each sample. Before each use the system was pre-heated by saturating the hose line and cleaning head with steam (ca. 20 sec). During application, the cleaning head was vertically guided across a surface while providing a temperature between 85 to 104˚C and a pressure of 0.83 to 1.38 bar per the manufactures specifications.

Cytotoxicity and neutralization testing. SDC cytotoxicity testing, validation of neutralization, and assessment of neutralized test substance interference with FCV infectivity were conducted in accordance with methods outlined in ASTM 2197-11 (23). Two SDC neutralizers were tested: neutralizer 1: 2.2 g/l $NaHCO_3$, 1 g/l $Na_2S_2O_3$, 0.01 M PBS, and 0.02% Tween 80, pH = 8.5; and neutralizer 2: 4.4 g/l $NaHCO_3$, 3 g/l $Na_2S_2O_3$, 10 mM HEPES, 0.01 M PBS, and 0.02% Tween 80, pH = 8.5. Briefly, CRFK monolayers were observed under a microscope (ACCU-SCOPE, Commack, NY) for apparent cytotoxicity after a 1 hr contact time with dilutions (1:10 and 1:20) of SDC. Next, dilution with no cytotoxicity and a control, CDMEM-5, were spiked with 10 to 100

PFU of FCV and measured for infectivity with plaque assay. Infectivity interference was measured by incubating the dilutions of the SDC with CRFK monolayers for 1 hr followed by a standard plaque assay with similarly spiked samples and controls mentioned above. Neutralizers were considered suitable if 80% of the spiked FCV titer was recovered (23).

Quantitative suspension test. Efficacy of SDC, with and without 5% FBS, was tested in accordance with ASTM standard E1052-11 with minor modifications (24). Briefly, a FCV stock was diluted in CDMEM-5 or 1X phosphate buffered saline (PBS) to yield a ca. 1 x 10^7 pfu/ml concentration. A 100 µl volume of each inocula were combined with 900 µl of SDC for 1, 5, 10, and 30 min contact times. Samples were neutralized by mixing 100 µl of sample with 900 µl of a neutralizer containing 4.4 g/l $NaHCO_3$ + 3 g/l $Na_2S_2O_3$ + 10 mM HEPES + 0.01 M PBS + 0.02% Tween 80. To verify neutralization, neutralization and cytotoxicity controls were incorporated. Separate aliquots for each sample were prepared for infectivity and qRT-PCR analysis and frozen at -80˚C.

Quantitative disk carrier test. The efficacy of SDC and steam-vapor against FCV on a hard surface, with and without 5% FBS, were performed in accordance with ASTM standard E1053-11 with modifications (25). Briefly, glass coverslips (25 mm x 25 mm) contained in a glass petri dish (Corning, Corning, NY) were inoculated with 25 µl (ca. 1 x 10^7 pfu/sample) and dried for 1 hr in a 30% relative humidity (RH) chamber (480 HP, VWR International, Randor, PA) then maintained with a saturated $MgCl_2$ solution.

After drying, virus films were inoculated with 200 µl of SDC for 1, 5, 10, and 30 min contact times. At each time point, 1.8 ml of a neutralizing broth, mentioned above, were pipetted on the cover slip. On the other hand, steam-vapor was applied for 10, 30, 60, and 90 sec. Samples treated with steam-vapor were neutralized by applying 2 ml of a 4˚C chilled neutralizer (0.01 M PBS + 0.02% Tween 80) to the glass surface. To verify neutralization, virus, neutralization, and cytotoxicity controls were incorporated. In both experiments, samples were recovered as previously described, aliquoted for infectivity and qRT-PCR analysis, and frozen at -80˚C (26).

Quantitative carpet carrier test. The efficacy of SDC and steam-vapor against FCV inoculated onto carpets was performed in accordance with ASTM E2966-14 with modifications (**Figure 5.1**) (27). Wool and nylon carpet carriers (5 x 5 cm), each contained in a petri dish, were inoculated with 100 µl of FCV, and dried for 1 hr at 30% RH. Inocula were diluted in CDMEM-5, contained ca. 7 log pfu/ml of FCV. For SDC treatment, carpet carriers were sprayed 5 times (6.85 ± 0.21 ml) and scrubbed clockwise and counter-clockwise for 30 sec each with a saturated surgical scrub brush (1.23 ± 0.41 ml) (Becton Dickinson, Franklin Lakes, NJ) without detergent and left at ambient conditions for a 1 hr contact time. In contrast, steam-vapor was applied to the carpet carriers for a 90 sec contact time.

To recover FCV, dried inoculated carpet carriers were aseptically transferred to a 500 ml bottle (Thermo Fisher Scientific, Waltham, MA) containing 100 ± 1 ml of their respective neutralizing broth, mentioned above, sonicated for 1 min at 40 KHz (FS110, Fisher Scientific International, Pittsburg, PA), and hand shaken for 1 min. Next, carpet

carriers were aseptically removed, and samples frozen at -80˚C. On a separate day, samples were thawed in a water bath at 37˚C (IR35 New Brunswick Scientific, New Brunswick, NJ), transferred to 50 ml conical tubes (VWR International, Randor, PA), centrifuged at 4,000 x g for 15 min at 4˚C (Allegra X-30R, Beckman Coulter, Brea, CA), and concentrated via ultrafiltration (Amicon Ultra-15 30K, Millipore, Billerica, MA) at 4,000 x g for 15 min at 4˚C. Supernatants were pooled, vortexed, weighed, aliquoted, and stored at -80˚C prior to infectivity and qRT-PCR analysis.

Qualitative appearance test. Wool and nylon carpet carriers were treated with SDC and steam vapor as described above. Carriers were photographed with a camera (AX53, Sony, Minato, Tokyo, Japan) at time 0, 60 min, and 24 hr.

Statistical analysis. All experiments were performed in triplicate with three replicates per experiment except for the carpet experiment, which had 5 replicates in 3 independent experiments. Log reductions were calculated by Log N/N_0 where N is the average of treatment samples and N_0 is the average of control samples. Statistical analysis was performed using one-way multiple comparisons. All results were expressed as mean ± standard deviation. Statistical significance was defined as $P \leq 0.05$. Statistical analyses were conducted using JMP (JMP Pro 12.2.0, SAS Inc., Cary, NC).

RESULTS

Cytotoxicity and neutralization of SDC. Neutralizers 1 and 2 were evaluated based on their ability to prevent cytotoxicity and neutralize SDC. SDC showed no apparent cytotoxicity toward CRFK cells after 1 hr incubation and at the 1:10 and 1:20 dilutions in both neutralizers. Furthermore, during validation of neutralization both neutralizers achieved >80% recovery of FCV compared to the controls. However, FCV was unable to form plaques when using neutralizer 1 as all results indicated 100% cytopathic effect at both 1:10 and 1:20 dilutions of SDC during the assessment of neutralized SDC interference with infectivity testing, whereas neutralizer 2 did not show signs of cytotoxicity and yielded >84% recovery of FCV with a 1:10 and 1:20 dilution of SDC. As a results neutralizer 2 was used in the following studies.

Efficacy of SDC in suspension. Table 5.1 shows the efficacy of SDC against infectious FCV with and without 5% serum between a 1 and 30 min contact time in suspension. With the addition of serum, infectious FCV was reduced by 4.29 $\log_{10}$ pfu within 1 min and continued to inactivate FCV up to 4.65 $\log_{10}$ pfu after 30 min. Conversely, SDC treatments of FCV with no serum were reduced by 4.51 $\log_{10}$ pfu within 1 min but no additional inactivation was observed after 5 min. Comparatively, between 1 and 10 min serum significantly reduced SDC's ability to inactivate FCV compared serum-free treatments. Likewise, contact time significantly affected SDC in the presence of serum but not for treatments without serum. However, overall there was no significant difference observed between serum treatments after a 30 min contact time.

Table 5.1 shows the efficacy of SDC against FCV evaluated via qRT-PCR. Log_{10} reduction of FCV RNA ranged between 1.85 to 1.93 log_{10} copies and 1.72 to 1.84 log_{10} copies for treatments with and without serum, respectively. Contact time did not significantly affect FCV's RNA, regardless of serum presence. However, there was a significant difference observed among samples treated with and without serum after 5 mins of SDC exposure, albeit, the difference was only 0.12 log_{10} copies. Similarly, other serum-free treatments exhibited lower copy reductions compared to treatments with serum.

Efficacy of SDC and dry-steam on glass carriers. Table 5.2 shows the efficacy of SDC against FCV on a glass surface with and without serum between 1 and 30 min of contact time. On glass the initial level of FCV recovered from controls during SDC testing for samples with and without serum were 5.68 ± 0.24 log_{10} pfu and 4.50 ± 0.04 log_{10} pfu, respectively. SDC reduced FCV by >4.66 and >3.46 log_{10} pfu within 30 and 10 min with and without serum, respectively. Inactivation of FCV by SDC was significantly affected by time. The presence of serum demonstrated a higher log reduction compared to serum-free treatments. However, there was significant difference (1.18 log_{10} pfu) among recovered control samples from serum and serum-free carriers. SDC qRT-PCR results exhibit a maximum reduction of 1.01 log_{10} copies over 30 min. And, like suspension tests, serum-free samples exhibited a lower reduction in RNA compared to serum treated samples.

Table 5.3 shows the efficacy of dry-steam vapor with TANCS against FCV on a

glass surface with and without serum between a 10 and 90 sec contact time. On glass the initial level of FCV recovered from controls during dry-steam vapor testing for samples with and without serum were 5.99 ± 0.20 $\log_{10}$ pfu and 5.17 ± 0.16 $\log_{10}$ pfu, respectively. Dry-steam reduced infectious FCV by >4.93 and >4.11 $\log_{10}$ pfu within 10 sec, both with and without serum, respectively. These values represent the method's limit of detection for each treatment. As such, no further inactivation of FCV was detected beyond the 10 sec treatment. There was no observed time effect between 10 and 90 sec. However, treatments with serum exhibited significantly higher reduction compared to serum-free samples. However, this was due to a difference in recoverable FCV from controls after drying. Analysis via qRT-PCR shows steam-vapor could reduce FCV's RNA between 1.92 and 2.31 $\log_{10}$ copies. Unlike samples treated with serum, serum-free samples were significantly affected by time. Furthermore, qRT-PCR analysis shows a similar serum trend to infectious FCV treated with steam-vapor, i.e. lower reductions among serum-free samples.

Efficacy of SDC and steam-vapor on carpets. Table 5.4 shows the efficacy of SDC and steam-vapor with TANCS against FCV on wool and nylon carpet carriers treated for 60 min and 90 sec, respectively. On carpet, initial levels of FCV recovered from wool and nylon carpet during SDC testing were 5.11 ± 0.06 and 5.20 ± 0.22 $\log_{10}$ pfu. SDC reduced FCV by 1.82 and 3.62 $\log_{10}$ pfu on wool and nylon carpet carriers within 60 min, respectively. On the other hand, initial levels of FCV recovered from wool and nylon carpet during dry-steam testing were 5.38 ± 0.19 and 5.26 ± 0.07 $\log_{10}$ pfu. Steam-vapor

reduced FCV by 3.80 and 3.68 $\log_{10}$ pfu on wool and nylon carpet carriers, respectively. Mixed carpet type effects were observed between treatments assessed via plaque assay. The efficacy of SDC significantly affected by the carpet type, whereas no significant surface effect was observed across steam-vapor treatments. Analysis by qRT-PCR demonstrated little reduction among SDC and steam-vapor treatments. However, there was a surface type effect among both treatments. Specially, significantly more $\log_{10}$ copy reductions were observed across nylon carpet carriers compared to wool carpet carriers.

SDC and steam-vapor effect on carpet appearance. Figure 5.2 illustrates the effects of both SDC and steam vapor immediately after application and after 60 min and 24 hr of drying. After application of SDC and scrubbing, a white film appeared over wool and nylon carriers but dissipated within 60 min. After 24 hr no visual effects were observed although the carriers had a sticky residue. After 90 sec of steam treatment carriers appeared wet with minor abrasion to the carriers. After 60 mins, wool and nylon carriers were dry but surface still appeared to have minor abrasion to the surface fibers.

DISCUSSION

An effective environmental hygiene program is important for the prevention and control of HuNoV outbreaks (28). The efficacy of a variety of technologies and chemistries have been tested against HuNoV and their surrogates (29). However, a limited number of these interventions have been evaluated for their efficacy on carpets. In this study, we demonstrated the efficacy of a novel liquid disinfectant, SDC in

suspension, on glass, and carpets as well as the efficacy of steam-vapor on glass and carpets against the HuNoV surrogate FCV.

Cytotoxicity and neutralization tests are critical steps for the successful evaluation of chemistries intended for virucidal efficacy testing (23). A previous study investigating SDC against HuNoV used Dey/Engley neutralizing broth to quench silver ions and increase the solution pH (13). However, Dey/Engley broth is a complex medium and considered a universal neutralizing broth that contains a variety of neutralizers, such as sodium thiosulfate, sodium bisulfite, and sodium thioglycolate. Because this broth contains a variety of complex compounds and molecules this broth was not filterable via ultrafiltration, a tool essential for detection of viruses on carpet with our method, and necessitated the development of a targeted, filterable neutralizer. We successfully developed filterable sodium thioglycolate-based neutralizer based on work by Liau et al (30) who demonstrated silver ion's affinity for thiol-containing groups. While the sodium thioglycolate component was necessary for neutralization of silver ions, sodium bicarbonate and HEPES buffer were added to eliminate cytotoxicity caused by low pH, whereas the non-ionic surfactant, Tween 80, was used to assist with recovery of viruses.

In suspension, SDC could reach the EPA standard for antiviral efficacy against HuNoV (4 $\log_{10}$ reduction) within 1 min both with and without 5% serum present. This contrasts with previous results with SDC against HuNoV (31). Manuel et al. (13)'s results suggested 5 mins are needed to reach a 4 $\log_{10}$ reduction of HuNoV RNA under pristine condition whereas a 5% soil load only reduced HuNoV RNA copy number by ca.

2.5 $\log_{10}$ in 30 mins. This discrepancy can be attributed to FCV's documented susceptibility to low pH solutions compared to HuNoV (29).

During suspension testing, the efficacy of SDC was significantly lower in treatments with serum compared treatments without serum between 1 and 10 min. The reduced efficacy of liquid chemistries in the presence organic soil has been documented (13, 32, 33). For instance, Manuel et al. (13) while studying SDC's efficacy against HuNoV, found similar results with and without a soil component. Furthermore, the CDC recommends higher concentrations of bleach, i.e. 5,000 ppm, for soiled surfaces compared to 1,000 ppm for pre-cleaned surface and is why the EPA requires one-step cleaning products to incorporate a 5% soil load (6). In this study, the lower efficacy under 5% serum conditions is not surprising as SDC has an affinity for thiol-containing groups, which are present in FBS, i.e. amino acids cysteine and methionine.

On the other hand, SDC demonstrated little efficacy against FCV's RNA (<2 $\log_{10}$ copies) in 30 mins. However, unlike Manuel et al. (13), we chose to not treat samples with RNase, a method commonly used in studies that evaluate strains of HuNoV not cultureable. This method assist with remove exogenous nucleic acid that may inflate the copy number. By not applying RNase and measuring infectivity of surrogates we can gain a better understanding of the disinfectant technology's mode of action under certain conditions. The difference in $\log_{10}$ reduction between infectious and molecular data suggests the SDC primarily works against FCV's capsid compared to the RNA genome and is supported by previous work (13). Counter to the trend observed in infectious data, lower FCV RNA $\log_{10}$ reductions were reported in serum-free samples compared to

samples with serum. The serum-free samples were diluted in inert PBS, whereas serum-treated samples contain FBS and a variety complex molecules and compounds that may have reduced amplification or degraded exogenous genomic RNA but this has not been confirmed. However, this contrary to trends observed in infectious estimates where serum provides a protection against biocides.

Results for our glass carrier test with SDC demonstrated its efficacy against HuNoV, albeit SDC's efficacy is reduced compared to it efficacy in suspension. Generally, suspension tests overestimate the efficacy of a technology compared to hard surface testing. As postulated previously, this likely due to adsorption and aggregation of virions on the surface which provides less accessibility compared to free, unbound viruses in suspension with more exposure (34, 35). Regardless, hard surface testing simulates in-use conditions better than in suspension testing. Consistent with suspension tests, FCV inactivation showed a time-dependent response to SDC and limited FCV RNA reduction. However, inactivation was not as immediate as suspension testing because SDC tests took up to 30 min to achieve a >4 $\log_{10}$ reduction in the presence of serum. Nevertheless, serum-free samples treated with SDC met our limit of detection within 10 mins due to lower recovery after desiccation.

In contrast to SDC, steam-vapor demonstrated rapid inactivation of FCV by achieving a >4.11 $\log_{10}$ reduction in 10-sec on glass carriers. The inactivation time is a critical factor because typical contact times for liquid disinfectants are between 1 and 10 mins and surfaces must be thoroughly wet. Steam vapor with TANCS technology appears to provide synergism between heat and municipal tap water containing natural impurities,

such as calcium carbonate, that can be crystalized with heat (36). In addition to the steam vapor, these crystals are thought to provide an additional hurdle for microorganisms by interacting with cell membranes, although this has yet to be confirmed. Regardless, our results support previous finding that steam vapor can reduce FCV and MS2 phage beyond the EPA standard in short contact times, i.e. <10 sec (15, 16).

Currently, there are no standardized methods for evaluation of disinfectants intended for viruses contaminated on carpet but the EPA requires soft surface disinfectants to meet a minimum of a 3 $\log_{10}$ reduction (37). SDC could meet this requirement in 60 min on nylon but not wool carpet carriers with significantly higher reduction found on nylon carpets. To our knowledge, only one other study has evaluated liquid chemistries against viruses inoculated on carpet. Malik et al. (10) indicated that of the disinfectants tested only 2.6% activated glutaraldehyde was effective on synthetic carpets, i.e. olefin, polyester, nylon, and blended carpets. Although this may be true, activated glutaraldehyde, at that level, may not be safe for application on soft surfaces because glutaraldehyde is listed as a Category I and III for primary eye irritation and acute dermal exposure, respectively, according to the EPA's toxicity rating, whereas SDC falls within Category IV, EPA lowest toxicity rating (38).

It is especially important to consider toxicity of chemicals being applied to soft surface that can absorb liquids. Previous work with quaternary ammonium compounds (QUAT) and chlorine on soft surfaces demonstrated some soft surface can adsorb and sequester active ingredients (38). In addition to toxic residues, this ability may reduce the efficacy of some disinfectants. For instance, McNeil et al. (39) found that some soft

surfaces, e.g. gauze and yarn, could adsorb up to >40% of a QUAT while a similar study using chlorine determined another surface, e.g. cotton, could adsorb up to 98% of a 800 ppm chlorine solution. Taken together, these results may explain the difference in SDC's efficacy against FCV on wool and nylon carpets as wool has been demonstrated to absorb ca. 2 time more liquid than nylon (Buckley et al. from chapter 3). Because of this, it is likely more silver ions were sequestered by wool fibers than nylon, which decreased the availability of free silver ions.

Heat treatments are effective at inactivating viruses both in suspension and on hard surfaces and have no toxicity or residues. This may be why steam treatments have been recommended by multiple government agencies. Be that as it may, their efficacy has never been demonstrated on carpets. In this study, steam vapor with TANCS met the EPA's 3 $\log_{10}$ reduction standard for soft surfaces in 90 sec (>3.68 $\log_{10}$ pfu), although FCV was not inactivated below the limit of detection with dry-steam as it was when dried on a glass surfaces. Depending upon the environment, FCV has been shown to survive significantly better on both wool and nylon carpet fibers compared to glass (Buckley et al. chapter 3). It is possible these fibers do not transfer heat as efficiently as glass but more importantly FCV is adsorbed and trapped within the substrata of these multilayered fibers, which occludes the capsid and may prevent inactivation.

Consistent with suspension tests, both SDC and steam vapor had a limited effect on FCV RNA when tested on glass and wool and nylon carpets. This provides more evidence that SDC and steam vapor primarily target the capsid. More importantly, similar trends between serum and serum-free treatments were found among these testes, which

support our hypothesis that serum presence may reduce amplification or degrade exogenous genomic RNA compared to samples that did not contain 5% serum.

Determining a disinfection technologies mode of action is a basic component of any characterization study. Although not our study's aim, our results provide evidence of SDC and steam vapor's mode of action against FCV. The thermal heat provided by the steam vapor presumably denatures the capsid and eventually begins to degrade FCV RNA. Different from dry-steam vapor, the citrate in SDC may change the particles morphology while the silver ions attack cysteine residues important for capsid stabilization and formation. A previous study using a similar disinfecting technology found that citrate altered HuNoV virus-like particle's morphology and their ability to bind to human blood group antigens (HBGA) (12). But a follow-up study only found a 25% reduction in capsid protein and suggests silver ion either potentiates or synergistically impacts the efficacy of citrate (13).

Appearance is also a critical factor to consider when developing disinfectants intended for soft surface. Although some may be efficacious, many chemistries can be damaging to soft surface. Our qualitative data of treatments using both technologies demonstrated little effect to the aesthetic appearance of the carpet. This suggests, if efficacious, these technologies can be applied with limited damage to wool and nylon surfaces.

A limitation of our study is the recovery of FCV from control glass carriers. Regardless of disinfection technology, lower FCV titers were recovered from serum-free samples (ca. 1 $\log_{10}$ pfu and copies). Carriers for both treatments were dried, recovered,

and disinfected under the same conditions. But, it is commonly known that organic soils can provide protection to enteric viruses. It is likely the reduced organic load and 30% RH contributed to higher inactivation during desiccation compared to treatments deposited with serum. Although serum-free samples treated with both SDC and steam vapor were inactivated below our limit of detection, the difference between recovered controls represents a limitation to our study as we cannot statistically compare serum and serum-free treatments after they fallen below the limit of detection.

CONCLUSION

In summary, SDC was found to be efficacious against FCV in suspension, on glass, and nylon carpets. However, SDC is less efficacious against FCV in the presence of serum and wool. On the other hand, steam vapor with TANCS was efficacious on all surfaces tested and exhibited no loss to its efficacy in the presence of serum. Furthermore, treatments with these technologies do not affect the aesthetic appearance of the carpets. Taken together, these results suggest surfaces should be thoroughly pre-cleaned for SDC to become efficacious while steam vapor with TANCS demonstrated rapid inactivation and could be an appropriate disinfection technology for virally contaminated natural and synthetic carpets.

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Table 5.1 Virucidal efficacy of silver dihydrogen citrate against FCV in suspension measured by plaque assay and qRT-PCR

	Reduction*			
	Plaque assay (log PFU)		qRT-PCR (log copies)	
Contact time (min)	With 5% serum†	Without 5% serum control‡	With 5% serum†	Without 5% serum control‡
1	4.29 ± 0.12A[a]	4.51 ± 0.09A[b]	1.93 ± 0.07A[a]	1.84 ± 0.19A[a]
5	4.37 ± 0.06AB[a]	4.69 ± 0.28A[b]	1.85 ± 0.05A[a]	1.72 ± 0.17A[b]
10	4.41 ± 0.08B[a]	4.67 ± 0.01A[b]	1.89 ± 0.04A[a]	1.84 ± 0.10A[a]
30	4.65 ± 0.11C[a]	4.65 ± 0.05A[a]	1.88 ± 0.07A[a]	1.78 ± 0.08A[a]

*The plaque assay and qRT-PCR data are expressed as mean ± deviation of 9 replicates from 3 independent experiment. Values with different Letters in the same column are significantly different ($P<0.05$), whereas values with different superscript letters in the same row for each detection method are significantly different.

†FCV stocks concentrated via ultrafiltration were diluted in complete Dulbecco's modified eagle's medium with 5% low endotoxin (<10 EU/ml) fetal bovine serum.

‡FCV stocks concentrated via ultrafiltration were diluted in 1X phosphate buffered saline.

Table 5.2 Virucidal efficacy of silver dihydrogen citrate against FCV on glass measured by plaque assay and qRT-PCR

Contact time (min)	Reduction*			
	Plaque assay (log PFU)		qRT-PCR (log copies)	
	With 5% serum†	Without 5% serum control‡	With 5% serum†	Without 5% serum control‡
1	1.17 ± 0.30A[a]	0.77 ± 0.41A[a]	0.12 ± 0.25A[a]	-0.85 ± 0.09A[b]
5	3.71 ± 0.35B[a]	2.49 ± 0.08B[b]	0.53 ± 0.07AB[a]	-0.58 ± 0.26A[b]
10	3.84 ± 0.45B	>3.46A	0.45 ± 0.06B[a]	-0.66 ± 0.10A[b]
30	>4.66C	>3.46A	1.01 ± 0.28C[a]	-0.45 ± 0.38A[b]

*The plaque assay and qRT-PCR data are expressed as mean ± deviation of 9 replicates from 3 independent experiment. Values with different letters in the same column are significantly different ($P<0.05$) whereas values with different superscript letters in the same row for each detection method are significantly different.

†FCV stocks concentrated via ultrafiltration were diluted in complete Dulbecco's modified eagle's medium with 5% low endotoxin (<10 endotoxin units/ml) fetal bovine serum. Recovered control was 5.68 ± 0.24 $\log_{10}$ pfu

‡FCV stocks concentrated via ultrafiltration were diluted in 1X phosphate buffered saline. Recovered control was 4.50 ± 0.04 $\log_{10}$ pfu

Table 5.3 Virucidal efficacy of dry-steam vapor with TANCS against FCV on glass measured by plaque assay and qRT-PCR

Contact time (seconds)	Reduction*			
	Plaque assay (log PFU)		qRT-PCR (log copies)	
	With 5% serum†	Without 5% serum control‡	With 5% serum†	Without 5% serum control‡
10	>4.93A	>4.11A	2.31 ± 0.25A[a]	0.70 ± 0.32A[b]
30	>4.93A	>4.11A	1.92 ± 0.21A[a]	0.20 ± 0.10A[b]
60	>4.93A	>4.11A	1.94 ± 0.07A[a]	0.30 ± 0.38AB[b]
90	>4.93A	>4.11A	1.93 ± 0.32A[a]	1.03 ± 0.36B[b]

*The plaque assay and qRT-PCR data are expressed as mean ± deviation of 9 replicates from 3 independent experiment. Values with different letters in the same column are significantly different ($P<0.05$) whereas values with different superscript letters in the same row for each detection method are significantly different.

†FCV stocks concentrated via ultrafiltration were diluted in complete Dulbecco's modified eagle's medium with 5% low endotoxin (<10 EU/ml) fetal bovine serum. Recovered control was 5.99 ± 0.20 $\log_{10}$ pfu

‡FCV stocks concentrated via ultrafiltration were diluted in 1X phosphate buffered saline. Recovered control was 5.17 ± 0.16 $\log_{10}$ pfu.

Table 5.4 Virucidal efficacy of silver dihydrogen citrate and dry-steam vapor with TANCS against FCV on wool and nylon carpet measured by plaque assay and qRT-PCR

Treatment†	Contact time	Surface	Reduction*	
			Plaque assay (log PFU)	qRT-PCR (log copies)
SDC	60 minutes	Wool	1.82 ± 0.19A	-0.06 ± 0.26A
		Nylon	3.62 ± 0.32B	0.49 ± 0.27B
Dry-steam	90 seconds	Wool	3.80 ± 0.16B	0.03 ± 0.17A
		Nylon	3.68 ± 0.09B	0.39 ± 0.23B

*The plaque assay and qRT-PCR data are expressed as mean ± deviation of 15 replicates from 3 independent experiment. Values with different letters in the same column are significantly different ($P<0.05$).

†FCV stocks concentrated via ultrafiltration were diluted in complete Dulbecco's modified eagle's medium with 5% low endotoxin (<10 EU/ml) fetal bovine serum.

Figure 5.1 Flow chart for performing disinfection efficacy testing

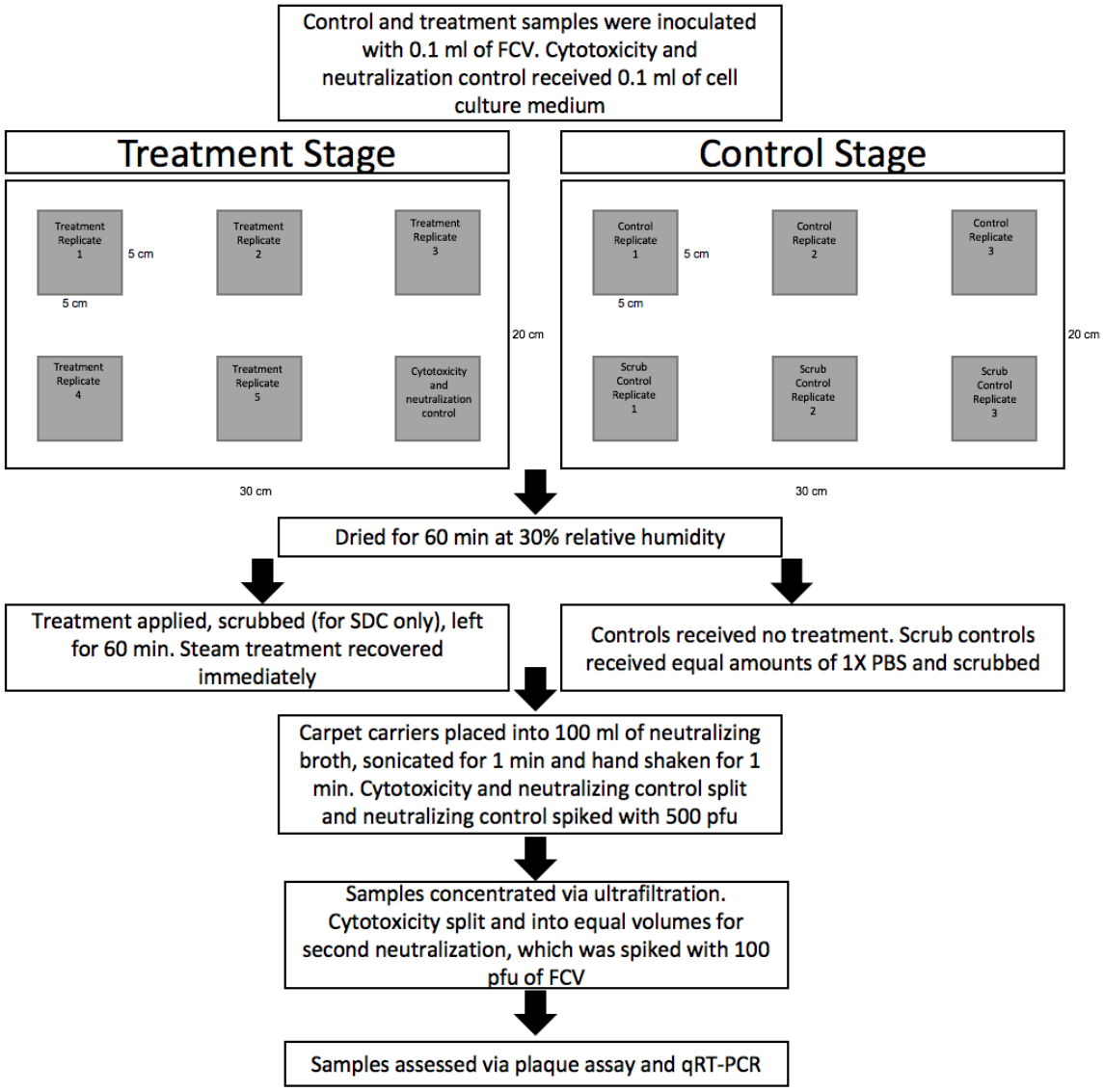
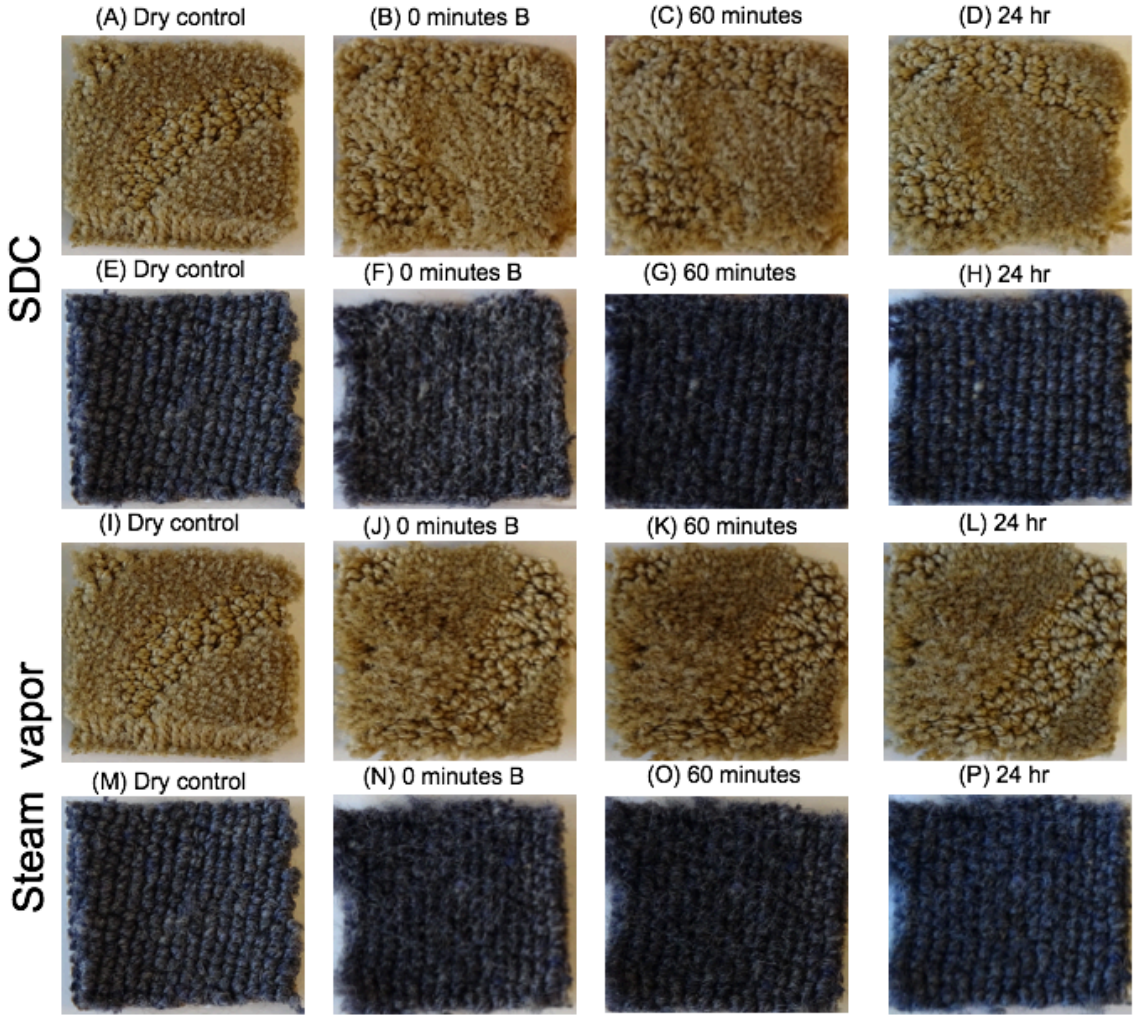


Figure 5.2. Effect of silver dihydrogen citrate on appear of wool and nylon carpet carriers between 0 and 24 hr (A – P)



CONCLUSIONS

HuNoV remain the leading cause of acute gastroenteritis worldwide, which can be transmitted via person-to-person, food, water, or the environment. Current literature shows that several key factors influence virus survival: temperature, RH, organic content, deposition method, and virus type. Of the studies that evaluated different chemistries, it was shown that chlorine, glutaraldehyde, and oxidizing vaporous products demonstrated the best efficacy against enteric viruses on soft surfaces. Be that as it may, some of these technologies may not be safe or practical on carpets. Furthermore, because the EPA currently does not have a standard method for assessing virucidal activity of product on nonlaunderable soft surfaces, to include recovery methods, there are no registered products that can be reported to inactivate enteric viruses contaminated on nonlaunderable soft surfaces.

Results presented here demonstrated that characterizing a soft surface can improve our understanding of virus-soft surface interactions. Furthermore, infectious HuNoV surrogates, FCV and MNV, can survive for extended periods of time on carpet fibers. This survival can be affected by at least 2 factors: RH and surface type. Specifically, low RH favors FCV and MNV survival while natural fibers, such as wool, may provide a more protective environment compared to synthetic fibers and hard surfaces. Additionally, the assessment of recovery methods is a critical first step to improve the detection and quantification of viruses. Here, we tested 3 mechanistically different recovery methods intended for HuNoV recovery from carpets. Bottle extraction and the microbial vacuum exhibited higher recovery efficiencies compared to

macrofoam-tipped swabs. Moreover, detection of FCV and MNV via qRT-PCR can be inhibited when recovered from wool and nylon carpets. Ultimately, each method demonstrates merit for use under different conditions, e.g. efficacy testing and environmental monitoring. Sanitization programs are essential for prevention and control measures against HuNoV. Our efficacy testing found that silver dihydrogen citrate (SDC) was efficacious against FCV in suspension, on glass, and nylon carpet. However, SDC is sensitive to serum. On the other hand, steam vapor with TANCS technology was efficacious on all surfaces tested and exhibited no loss to its efficacy in the presence of serum. Furthermore, treatments with these technologies do not affect the aesthetic appears of the carpets. Taken together, these results suggest that SDC, to remain efficacious, should be used as a two-step cleaner while steam vapor with TANCS demonstrated rapid inactivation and could be an appropriate sanitizing technology for virally contaminated natural and synthetic carpets.

Although our findings suggest HuNoV can survive for extended periods of time on carpet, future studies are still needed to validate the survival profiles of HuNoV. Also, to usher in a standard for assessing the efficacy of sanitizers against viruses intended for carpets, our ASTM International-adapted standard should be used as a platform in future studies. While surrogate testing is an acceptable alternative for the study of HuNoV, there are significant drawbacks. Based on SDC and steam-vapor's efficacy demonstrated here, these technologies warrant further testing with the human enteroid system that support HuNoV propagation.