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Developing Disinfection Strategies for Controlling Human Norovirus, Sars-Cov-2, and Clostridioides difficile Endospores In Long-term Care Facilities

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DEVELOPING DISINFECTION STRATEGIES FOR CONTROLLING HUMAN NOROVIRUS, SARS-COV-2, AND CLOSTRIDIOIDES DIFFICILE ENDOSPORES IN LONG-TERM CARE FACILITIES

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ABSTRACT

Long-term care facilities (LTCFs) provide an environment favorable for the transmission of three critical human pathogens: human norovirus (HuNoV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and *Clostridioides difficile*. Given residents in LTCFs are susceptible to infections due to their advanced ages and compromised immune systems, effective environmental surface disinfection plays a crucial role in controlling the spread of human pathogens within these settings and, therefore, mitigates the risk of infections caused by these pathogens. This dissertation aimed to assess the efficacy of various types of disinfectants against two HuNoV surrogates [feline calicivirus (FCV) and Tulane virus (TuV)], two SARS-CoV-2 surrogates [bovine coronavirus (BCoV) and human coronavirus (HCoV) OC43], and *C. difficile* endospores. The research encompasses surfaces commonly encountered in healthcare settings and public spaces, with a particular emphasis on the disinfection of these pathogens on soft porous surfaces in LTCFs.

First, nine chemical disinfectants on EPA's List G were selected using four criteria: 1) ready-to-use, 2) nonchlorine-based active ingredient, 3) commercially available, and 4) limited known health risks. Active ingredients of the products included hydrogen peroxide (H_2O_2), peracetic acid, quaternary ammonium compounds, or alcohols. The efficacy of the products against FCV, TuV and *C. difficile* spores was first screened using the American Society for Testing and Materials (ASTM) suspension test, and then the carrier test on stainless steel coupons for 1, 5 and 10 min (FCV, TuV) and 10

min (*C. difficile* spores). On stainless steel carriers, 8 of 9 products could reduce >3 $\log_{10}$ PFU of FCV within 5 min. One most efficacious product containing H_2O_2 as key active ingredient, reduced >5.1 $\log_{10}$ PFU of FCV and >3.1 $\log_{10}$ $TCID_{50}$ of TuV after 5 min, and >6.0 $\log_{10}$ CFU of *C. difficile* endospores after 10 min. Of the five products containing H_2O_2, no strong correlation (R^2=0.25, p=0.03) was observed between disinfection efficacy and H_2O_2 concentration. The addition of 0.025% ferrous sulfate to 1% H_2O_2 solution improved efficacy against all FCV, TuV, and *C. difficile*. Our results confirmed that both product formulation and the active ingredient concentration influence the efficacy. Additionally, TuV proved to be a more conservative surrogate for HuNoV than FCV.

Next, this dissertation evaluated the efficacy of chemical disinfectants (products A, B, and C) and steam vapor against HuNoV on nylon carpet with two different backings. Carpet coupons (5×5 cm^2) inoculated with a mixture of FCV and TuV were allowed to dry at room temperature under 30-50% relative humidity. The virus-inoculated carpet coupons were applied with three chemical disinfectants or steam vapor for different contact times. The viruses on the treated carpets were subsequently recovered and titrated. Additionally, the color and tensile strength of carpets were assessed after repeated disinfection 30 times to simulate long-term use for 1.5 years. Results suggested the efficacy of disinfectants was affected by the type of carpet backing. For carpet with a water-permeable backing (Color Accent®), products A, B, and C reduced 0.8, 3.1, and 0.9 $\log_{10}$ PFU of FCV, and 0.3, 2.5, and 0.4 $\log_{10}$ $TCID_{50}$ of TuV after a 30-min contact time, respectively. For the carpet with a waterproof backing (Highlight®), only product B

exhibited a substantial reduction of 5.0 $\log_{10}$ PFU for FCV and >3.0 $\log_{10}$ $TCID_{50}$ for TuV, while products A and C reduced 2.4 and 1.6 $\log_{10}$ PFU of FCV, and 1.2 and 1.2 $\log_{10}$ $TCID_{50}$ of TuV, respectively. Impressively, steam vapor achieved a ≥5.2 $\log_{10}$ PFU reduction of FCV and >3.2 $\log_{10}$ $TCID_{50}$ reduction of TuV in just 15 s on both types of carpet. Additionally, two H_2O_2-based disinfectants significantly impacted the tensile strength of carpet backings after repeated disinfection, with only product B causing cracks on nylon carpet fibers. The overall results highlighted the potential efficacy of steam vapor against HuNoV on both carpet types. Furthermore, one H_2O_2-based product (product B) exhibited efficacy on waterproof carpet, though the repeated use of disinfectants did affect some properties of the carpet.

To understand the transmission potential of SARS-CoV-2 via carpet, the persistence of SARS-CoV-2 surrogates, BCoV and HCoV OC43 on polyethylene terephthalate (PET) and nylon carpet was evaluated using both infectivity and RT-qPCR assays, and the efficacy of steam vapor treatment against BCoV and HCoV OC43 on nylon carpet was determined. After inoculation, the immediate recoveries were only 3.87% of HCoV OC43 from PET and 24.37% from nylon carpet. In contrast, the recovery rates of BCoV were 32.50% from PET and 34.86% from nylon carpet. Following a 1-h incubation at room temperature, BCoV and HCoV OC43 were reduced by 3.6 and >2.8 $\log_{10}$ $TCID_{50}$ on PET carpet but 0.6 and 1.8 $\log_{10}$ $TCID_{50}$ on nylon carpet, respectively. The reduction of total genomic RNA of BCoV and HCoV OC43 was also less on nylon carpet than on PET carpet, with first-order decay rates (k values) at 0.86 and 0.27 h^{-1} for nylon and 1.19 and 0.67 h^{-1} for PET carpet, respectively. These

findings suggest that both surrogates were more stable on nylon than on PET carpet. For carpet disinfection, steam vapor was demonstrated as an effective method for inactivating both surrogates on nylon carpet, by reducing >3.0 $\log_{10}$ $TCID_{50}$ of BCoV and >3.2 $\log_{10}$ $TCID_{50}$ of HCoV OC43 within 15 s.

In response to the absence of a validated disinfection method against *C. difficile* endospores on carpet, this dissertation undertook a two-step approach. First, the recovery method for *C. difficile* endospores from the carpet was optimized by experimenting with three concentrations of Tween-80 and different stomaching durations. Subsequently, the efficacy of three EPA-registered disinfectants (two H_2O_2-based and one chlorine-based) and steam vapor against *C. difficile* endospores was evaluated on nylon carpet with two types of backing. The incorporation of 0.2% Tween-80, followed by a 3-min stomaching and subsequent sonication, substantially enhanced the recovery rate of *C. difficile* endospores, exceeding 60%. Product B was the most efficacious of the three disinfectants tested, which achieved reductions of 5.8 and 4.9 $\log_{10}$ CFU of *C. difficile* endospores within a 30-min contact time on carpet Highlight® and Color Accent®, respectively. Additionally, steam vapor treatment for 120 s exhibited strong efficacy, reducing >6.0 and 4.9 $\log_{10}$ CFUof *C. difficile* endospores on carpet Highlight® and Color Accent®, respectively. Additionally, combining a 120-s steam vapor treatment with a less effective product A resulted in a 6.1 $\log_{10}$ CFU reduction of sensitized *C. difficile* endospores on carpet Highlight®.

Furthermore, the efficacy of an aqueous photocatalytic disinfection system, known as photoClO_2, was evaluated against HuNoV surrogates and *C. difficile*

endospores on stainless steel and nylon carpet. The process was optimized by utilizing 1% $NaClO_2$ and 10 ppm Eosin Y, which yielded a production rate of 60.64 ppm min^{-1} of ClO_2 within a 4.5×4.5 cm^2 area. Subsequently, the efficacy of the system was evaluated against FCV, TuV, and *C. difficile* endospores on both stainless steel and nylon carpet with two distinct backings under optimal lighting conditions. PhotoClO_2 was efficacious in reducing>5 log_{10} PFU of FCV in 45 min of contact time and >3 log_{10} $TCID_{50}$ of TuV in 60 min, but only 1.3 log_{10} CFU of *C. difficile* endospores in 120 min. On carpet Highlight®, photoClO_2 achieved a 2.9 log_{10} PFU reduction of FCV and 2.5 log_{10} $TCID_{50}$ reduction of TuV in 60 min, respectively, showcasing higher efficacy than carpet Color Accent® (a 1.3 log_{10} PFU reduction of FCV and 1.1 log_{10} $TCID_{50}$ reduction of TuV, respectively). Under indoor lighting conditions, photoClO_2 further exhibited its efficacy by inactivating 4.3 log_{10} PFU of FCV and 1.4 log_{10} $TCID_{50}$ of TuV on stainless steel after 120 min. While photoClO_2 proved highly effective against HuNoV surrogates, its efficacy against *C. difficile* endospores was limited regardless of surface material.

This dissertation provides some insights into surface disinfection strategies, with a particular focus on soft porous carpet commonly found in LTCFs and various public areas. As the alternatives to bleach, H_2O_2-based disinfectants exhibited efficacy against HuNoV and *C. difficile* endospores. The research outcomes emphasize the critical consideration of the active ingredient and product formulation when selecting disinfectants to effectively inactivate pathogens. However, the repeated use of chemical disinfectants may adversely impact carpet properties such as fiber strength and backing integrity. In comparison to chemical disinfectants, steam vapor performed well with short

contact time and can be used as a more suitable option for spot treatment or routine disinfection of HuNoV, SARS-CoV-2 and *C. difficile* endospores for carpet. It should be noted that the efficacy of these disinfection methods can be significantly affected by the carpet backing. This suggests the significance of considering carpet construction alongside materials when selecting disinfection approaches. While photoClO_2 has exhibited efficacy against HuNoV surrogates, it's necessary to emphasize that this process involves a slow yet long-lasting reaction. This specific characteristic could prove desirable in preventing the spread of pathogens between disinfection cycles. While this dissertation presented the efficacy of three types of disinfectants (chemical disinfectants, steam vapor, and photoClO_2) against HuNoV and SARS-CoV-2 surrogates on carpet surfaces, the efficacy of these disinfectants should be validated using pathogenic HuNoV and SARS-CoV-2 to ensure their practical applicability.

DEDICATION

I would like to dedicate this work to my mother, Xia Lei, my wife, Ye (Nina) Li, and my family for their profound sacrifices and the time they devoted to providing constant support and encouragement throughout this journey. My heartfelt gratitude extends to my mom for her unconditional support and selfless love, which has been a guiding light in my work and life. Without her, completing academic research would have been an insurmountable challenge. To my wife, Nina, I am incredibly fortunate to have you accompanied, embracing my imperfections even when I had nothing to share. This work holds a special place for our son, Albert Huang. I sincerely hope that our collective efforts contribute to creating a better world for him.

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CHAPTER ONE

LITERATURE REVIEW: PERSISTENCE AND DISINFFECTION OF VIRUSES AND *CLOSTRIDIOIDES DIFFICILE*

Introduction

Healthcare-associated infections (HAIs) can lead to high rates of morbidity and mortality and increased healthcare costs. Therefore, reducing the number of HAIs has been a top priority of public health agencies in the United States (1, 2). Long-term care facilities (LTCFs), where nearly 2.5 million older people live, are a common setting for HAIs (3), such as human norovirus (HuNoV) infections, *Clostridioides difficile* infections (CDIs), and more recently coronavirus disease 2019 (COVID-19). According to National Outbreaks Reporting System, 75% of 7,094 HuNoV infections between 2009-2017 were attributed to LTCFs (4). *C. difficile,* another common etiological agent, is a spore-former and can survive longer in harsh environments. *C. difficile* is also hard to be inactivated, which might cause recurrent CDIs and increase the mortality. *C. difficile* is of equal concern given estimated 59,900 LTCF-onset cases in 2017, and the fatality rate (24.7%) for LTCF residents was much higher than those from other common causes of acute gastroenteritis (5). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has raised new concerns of HAIs since the start of COVID-19 pandemic in late 2019. SARS-CoV-2 spread in >10,000 LTCFs in U.S. and caused over 186,000 deaths by December of 2020 (6).

Inanimate surfaces are important vehicles for the transmission of bloodborne, airborne, waterborne and foodborne pathogens, in addition to direct person to person

transmission (7). Indirect contact transmission can be prolonged, as some viruses and bacterial endospores persist in the environment for days or weeks (8). Thus, proper environmental cleaning and disinfecting is critical for effectively preventing the transmission of pathogens in both communities and healthcare settings. The effectiveness of several classes of chemical disinfectants against various human pathogens is well-understood in the laboratory (9, 10). Nevertheless, due to limited knowledge about disinfecting methods applicable on various surfaces found in public spaces, eliminating viruses and bacterial endospores in the real-world remains challenging.

The diverse characteristics of surfaces in public spaces, including healthcare facilities, restaurants, cruise ships, and transportation systems and so on, present significant challenge for effective surface disinfection (11-13). Surfaces present in these spaces are often a combination of both non-porous surfaces, such as stainless steel bench, glass and plastic containers, as well as porous surfaces including bricks, wooden furniture, fabrics, and rugs (14). In contrast to non-porous surfaces, porous surfaces have a greater tendency to retain pathogens due to their ability to absorb fluids and small particles within their porous structure and shield the pathogens from the action of disinfectants (12). However, the current understanding regarding the persistence of viruses and *C. difficile* endospore on porous surfaces is limited, as there is only one systematic review that has analyzed the available data specifically for enteric viruses on soft porous surfaces (15).

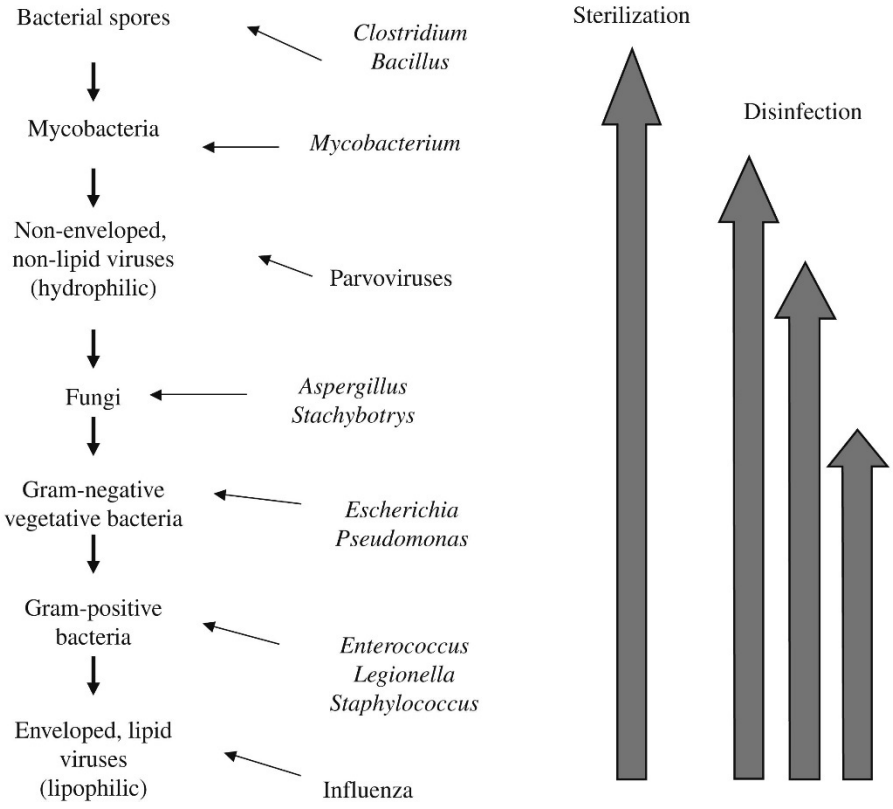
Within healthcare settings, a milestone for surface disinfection has been established, namely as the Spaulding Classification Scheme. The scheme designed in

1957 has provided a rational framework for determining necessary sterilization and disinfection protocols, initially for medical devices and utensils, and subsequently extended to environment surfaces in healthcare settings (16). The scheme devises to categorize surfaces into 3 major classifications, “critical”, “semi-critical”, and “non-critical” based on the risks associated with use of the object (Table 1.1). Critical surfaces are deemed high-risk when contaminated with any microorganisms (16), for example, items or surfaces which will contact with normally sterile tissue, or the vascular system. Thus, this category of surfaces must be sterile, which all microorganisms are destroyed, due the exceptionally high risk of disease transmission (17). Semi-critical surfaces pose a relatively lower risk possibly, as they may contact with patients’ mucous membranes or nonintact skins (16). High-level disinfection is recommended for these surfaces, allowing for the survival of only small numbers of microorganisms after disinfection. Non-critical surfaces, on the other hand, have limited chances of contacting unprotected patients' tissues (17). These surfaces require the inactivation of only enveloped viruses, vegetative bacteria, and fungi. Interestingly, despite their potential to contaminate sterile devices and patient’s body, most environmental surfaces fall within the non-critical category.

Beyond classifying the risk of surface contamination to patients' health, the Spaulding Classification Scheme takes into account the sensitivity by various microorganisms to disinfection (Figure 1.1). In this context, this dissertation focuses on the formidable microorganisms, with *C. difficile* endospores being the most challenging, followed by HuNoV and SARS-CoV-2. Notably, disinfectants with weaker formulations, though proven effective against bacteria, may not be equally effective against HuNoV

and *C. difficile* endospores. This indicates the need for specialized disinfection strategies tailored to specific microorganisms, as further discussed in the following sections.

Figure 1.1. Spaulding classification of disinfection currently used in healthcare facilities with the declined resistance (left), with examples of micro-organism types that are typical of each grouping and the equivalent levels of sterilization/disinfection (right). Within disinfection, from the left to right arrows indicate high-, medium-, and low-level disinfection.



Source: Figure from G. McDonnell, P. Burke. Disinfection: is it time to reconsider Spaulding?, Journal of Hospital Infection, 2011 78(3):163-170. (16)

To help decide disinfectant to be used on inanimate surfaces during infection disease outbreaks, the United States Centers for Diseases Control and Prevention (CDC) recommends 5,000 ppm bleach, or the use of the United States Environmental Protection Agency's (EPA) registered disinfectants for disinfection of specific microorganisms on hard non-porous surfaces (18). Of these, the most common active ingredients include chlorine, quaternary ammonium compounds (QACs), alcohols, organic acids, and peroxides, with each having limitations (10). For example, chlorine-based disinfectants, including sodium hypochlorite and chlorine dioxide, are highly efficacious against bacteria and viruses, but they can damage surfaces when used at high concentrations or over a prolonged period due to their strong oxidizing properties (19, 20). QACs and alcohols are widely used and less likely to cause damage to surfaces, but have weak activity against non-enveloped viruses (e.g., HuNoV) and bacterial spores (10). Organic acids can easily damage surface material such as metals and marbles, and they are generally more expensive than other products (10). Peroxides, including hydrogen peroxide (H_2O_2) and peracetic acids, can denature viral proteins, but efficacy data from published studies are limited (10). The EPA regulates chemical sanitizers and disinfectants applied on soft, porous surfaces, imposing a standard efficacy requirement of achieving a ≥3-log and ≥6-$\log_{10}$ CFU reduction of the target bacteria, respectively. For viruses, there is no mandatory requirements of disinfection efficacy. However, it should be noted that the claim of efficacy for disinfectants and sanitizers on soft, porous surfaces typically does not encompass the inactivation of viruses and *C. difficile* endospores.

Some human viruses pose significant technical challenges to be propagated in the lab (21). Additionally, the utilization of specialized protective systems (e.g., biosafety level 3 or 4 laboratories) is required to protect the researchers from infections of some highly contagious viruses, such as highly pathogenic avian influenza A viruses and Ebola virus (22, 23). It should be noted that *in vitro* culturing for certain viruses, availability of clinical samples or protection systems are not often accessible in all the laboratory settings. Consequently, only a limited number of research has been conducted to reliably determine the persistence and reduction of viable viral pathogens resulting from disinfection (9, 24). As a solution, surrogate viruses that are easy to culture and safe to human handling are often employed to study the persistence and disinfection instead of pathogenic viruses due to their structural similarities (25). It is acknowledged that surrogates are unable to perfectly mimic the exact characteristics of original pathogenic viruses. However, doing a comprehensive cross-examination of various animal viruses and their surrogates can potentially contribute to a deeper understanding of their response to disinfectants when exposed.

In brief, the primary objective of the review is to provide a comprehensive summary of the existing research progress and limitations associated with the persistence and disinfection of viruses and their surrogates, as well as *C. difficile* endospore on porous surfaces. To gather information, a systematic review was conducted to search for persistence and disinfection of viruses and *C. difficile* endospores. However, there is only a few persistence and disinfection studies related to *C. difficile* endospores on porous

surfaces. Thus, a comprehensive review was conducted to summarize the persistence and disinfection of *C. difficile* endospores on both porous and non-porous surfaces.

Table 1.1. The Spaulding Classification for disinfection.

Classification	Disinfection level	Virus [a]		Bacteria			Fungi
		Enveloped	Non-enveloped	Vegetative	Mycobacteria	Spore	
Critical	Sterilization	+	+	+	+	+	+
Semi-critical	High-level disinfection	+	+	+	+	-	+
Non-critical	Low-level disinfection	+	-	+	-	-	+

[a] + presents inactivation of a certain type of microorganisms, - presents no recommendation.

Persistence of viruses

The presence of a viral envelope has a significant impact on the persistence of viruses. However, other factors, including characteristics of surfaces, temperature, relative humidity (RH), transmission medium, deposition, and strain subtype can affect virus persistence (Table 1.2-3). Generally, non-enveloped viruses tend to survive longer on porous surfaces than enveloped viruses. Specifically, research indicated that non-enveloped viruses could remain infectious for periods ranging from one day to up to 15 days at room temperature, while most enveloped viruses typically last less than a day or two. However, enveloped influenza A virus H5N1 has shown remarkable resilience and can remain infectious for up to 17 to 44.7 days on feathers at room temperature (26).

Inanimate surface characteristics, pivotal in governing virus persistence, are typically categorized into two fundamental aspects: the surface materials and the structural construction. While the majority of studies investigated the effect of surface materials on virus persistence, the effect of surface construction, which refers to the arrangement and composition of components forming the surface, remained unclear. Furthermore, surfaces can be also categorized based on their launderability. However, this categorization methodology is tied to disinfection procedures and maybe not correlated to the persistence of viruses. Nevertheless, for the purpose of comparison with disinfection studies, we have presented data with surfaces identified as either launderable or non-launderable, as persistence data serve as a fundamental guide in the development of effective disinfection strategies.

Among studies of virus persistence to simulate real-world application, the transmission medium was typically solutions containing organic matters, fecal materials, or artificial saliva. Meanwhile, the process of deposition, which refers to the inoculation of viruses onto surfaces and can be accomplished through various approaches such as spraying, spiking, or the controlled release of virus-laden dust particles, might also affect the persistence of virus on these surfaces.

The effect of virus envelope

Non-enveloped viruses, specifically FCV, MNV, and PV were found to survive longer on porous surfaces, such as carpet, cotton, and wood, compared to non-porous surfaces of stainless steel (27, 28). However, MS2 was found to be less persistent on polyester tablecloths than on plastics (29). Among porous surfaces, vaccinia virus persisted longer on cotton than wool (30). Interestingly, the surface construction (looped or cut carpet) did not significantly affect the persistence of MS2 (31), while PV was more persistent on wool blanket than on wool garments (32).

Enveloped viruses including RSV, SARS-CoV, and SARS-CoV-2 were less persistent on cotton cloth and wood, compared to non-porous surfaces (33-35). In contrast, influenza A virus and porcine epidemic diarrhea virus were more persistent on feathers, wood, and cloth than on stainless steel (36, 37). Among porous surfaces, avian metapneumovirus, cytomegalovirus, equine herpesvirus, and SARS-CoV were more persistent on cotton cloth than on wood, while HCoV OC43, influenza A virus, and SARS-CoV-2 were just the opposite (35, 37-40). Moreover, avian metapneumovirus,

Ebola virus, influenza A virus, and SARS-CoV were reportedly more persistent on more hydrophobic materials such as feathers, polypropylene gown, and polyester fabrics (37, 41, 42). Furthermore, influenza A virus H9N2 was found to be more persistent than influenza A virus H6N2 on pine wood (43).

The enveloped viruses generally tend to be less stable than non-enveloped viruses on porous surfaces, as evidenced by shorter survival times. This phenomenon could be attributed to the inherent susceptibility of the envelope, which is composed of a monolayer of phospholipids (44). The viability of the virus can be compromised due to the impact of dehydration, surfactants, heat, and so on, on the phospholipid envelope (56-58). However, one exception to this trend was observed with the influenza A virus H5N1, which showed an exceptionally long survivability (17-44.7 d) on feathers at 25°C (26). The increased survivability of the viruses was attributed to the presence of preen oil on feathers, which concentrated the viruses and provided protective effects. However, the study failed to specify the effect of two critical factors: relative humidity (RH) and the transmission medium of the virus. Additionally, the study lacked a comprehensive description of the potential protective mechanism provided by the feathers, hence impeding the ability to compare this finding with other studies on virus persistence.

The effect of surface characteristics

Surface materials play a crucial role in the persistence and disinfection of viruses on porous surfaces. However, previous studies have yielded inconsistent results, mostly due to the inherent variability in surface materials and construction utilized across

different studies. Zuo et al. (42) investigated the effect of surface hydrophobicity on the persistence of influenza A virus H9N9 and concluded that hydrophobicity of the surface significantly influenced virus persistence more than the specific materials used. One possible explanation is that the increased hydrophobicity of surfaces promotes virus aggregation, which provides protection to the enclosed viruses against environmental stressors (45). Furthermore, the persistence of viruses is significantly affected by the construction of surfaces. Phi 6 survived longer on looped carpet than on cut carpet (31). Additionally, PV was more persistent on wool blanket than on wool garments (32). These studies suggested that the construction of soft porous could provide protection for viruses through diminished exposure to desiccation. This protection might be attributed to mechanisms such as a decreased surface area or the potential for viruses being absorbed into the porous texture of soft materials (31, 46). However, the impact of construction of carpet fiber on the survival of MS2 was negligible, suggesting that MS2 could be less susceptible to desiccation on porous surfaces (47).

The effect of temperature and relative humidity

Apart from surface characteristics, temperature and relative humidity (RH) were also explored as major impact factors in above virus persistence studies. The effect of temperature (4°C vs. room temperature) on persistence was studied for 11 viruses, and all viruses survived longer at lower temperature than higher temperature regardless of the presence of an envelope. At lower temperatures, the chemical and biological activities are decreased to maintain the structural integrity of viruses, hence protecting the viability of

viruses (15). Though 4°C or lower temperatures are typically used for preservation of most microorganisms, 20-25°C is more commonly found in indoor environments and in public spaces due to indoor environmental standards and regulations (48). Thus, studying the persistence of viruses at ambient temperatures is important to understand the transmission dynamics and develop preventive strategies.

There is evidence to suggest a correlation between temperature and virus persistence, which may explain the seasonal trends in outbreaks associated with airborne, waterborne, and foodborne viruses. However, RH also plays a key role in the occurrence of viral outbreaks, with effects on persistence varied among different viruses (49, 50). Higher RH generally led to a reduction in both non-enveloped and enveloped virus persistence, but with some exceptions (Table 3-4). Specifically, poliovirus survived longer at 35% RH compared to 78% RH on wool, but the reverse was observed on cellulose filter membranes (32, 51). Additionally, the persistence of some viruses, including adenovirus, hepatitis A virus, poliovirus, and rotavirus, was not significantly impacted by RH on cotton (32, 52). The persistence tendency of most viruses at high RH is probably due to the water activity. The increased water activity augments chemical reactions, such as the Maillard reaction and oxidation, which contribute to the inactivation of viruses exposed to air (53). This augmentation occurs from the increasing rate of diffusion as the reactants and products undergo dilution (53). Additionally, viral envelopes, composed primarily of phospholipids, are more susceptible to oxidation than spike proteins (53). Consequently, the influence of RH is greater for enveloped viruses

than some non-enveloped viruses, such as poliovirus, rotavirus, hepatitis A virus, and adenovirus.

The effect of transmission medium, deposition, strain subtype and pH

Only a few viruses have been studied in relation to factors such as transmission medium, deposition, strain subtype, and pH. The effects of transmission medium were evaluated on the persistence of five non-enveloped viruses, while Phi6 was the only enveloped virus studied in relation to transmission medium (Table 3-4). Savage et al. (54) revealed the different effects of transmission medium between two subtypes of avian reovirus. Specifically, fecal matter did not affect the survival of avian reovirus R2 on cotton, whereas it provided protection for avian reovirus S1133. It was observed that the presence of fecal material reduced the survival of adenovirus and poliovirus (52). But, specific soil loads, such as tripart soil load and artificial saliva, decreased the decay rates of Phi6 on wood from 1.98 to 0.08 and 1.30 log plaque forming unit (PFU)/h, respectively (55).

Consistent with a previous review, the effect of organic matter on the persistence of viruses varied among different surfaces (15). Generally, the protective effect conferred by the organic matter may act as antioxidants or enhance viscosity, hence impeding direct interaction between viruses and the atmospheric oxygen (53, 56, 57). However, organic matters such as fecal constituents may compete with virus for adsorption sites, resulting in shorter persistence period (52). Moreover, recovery efficiency from porous surface, which was merely reported in persistence studies, could be also reduced by the presence

of organic matters (58). This phenomenon could underestimate the persistence of viruses on porous surfaces.

Additionally, deposition had an impact on the persistence of influenza A virus, which showed longer survival when the virus was spiked onto surfaces compared to being dispersed by aerosol (42). However, under low RH, vaccinia virus exhibited longer survival through virus-containing dust contact, whereas under high RH, survival was longer in droplets (30). The deposition of virus particles is essential in determining the persistence of viruses on porous surfaces. There is a higher rate of survival when viruses are inoculated in the form of liquid droplets compared to aerosols. This is primarily attributed to the reduced surface area of liquid droplets, making them more resistant to evaporation compared to aerosols (59). Additionally, large areas of the air-water interface, viruses in aerosols tend to gather due to their hydrophobic nature, lead to an increased susceptibility to oxidation-induced damage (60).

The effect of pH was only investigated by one study, which reported increased sensitivity of FCV to lower pH levels (<3) on cotton sheet and carpet (unknown material) (61).

Table 1.2. Survival of non-enveloped viruses on porous surfaces.

Virus	Subtype studied	Surface type [a]		Material	Factors studied [b]				Key findings	Ref
		L	NL		T	RH	SC	Others		
Adenovirus	n.a. [c]	X		Cotton cloth	X	X		TM	● Fecal materials reduce persistence on cotton ● RH not affect ● Survive longer at low T ● Median reduction at 3.2-3.3 logs after dry for 3 to 5 h.	(52)
Avian reovirus	R2, S1133	X	X	Wood, cotton			X	TM Subtype	● Fecal materials not affect R2 on cotton ● R2 survived longer on wood with fecal materials ● S1133 survived longer with fecal materials on cotton and wood ● R2 survived for 1 d on cotton and 0.5-2 d on wood ● S1133 survived for 4 d on cotton and 1 d on wood	(54)
Bacteriophage MS2	n.a.	X	X	Polyester tablecloth, polyethylene terephthalate carpet			X		● Survive shorter on tablecloth (<14 d) than plastics (<23 d) ● Construction of carpet (cut or looped) not affect ● Decay rate on carpet=-(0.09-0.20) h^{-1}	(29, 31)

Virus	Subtype studied	Surface type [a]		Material	Factors studied [b]				Key findings	Ref
		L	NL		T	RH	SC	Others		
Echovirus	n.a.		X	Cellulose membrane	X	X			● Persistent at high RH (>80%) than at low RH (20%) ● Persistent at low T ● Survive for to 2 - >7 d	(51)
Feline calicivirus	n.a.		X	Unknown carpet, cotton, wool/nylon carpet		X	X	pH	● Sensitive to low pH (<3) ● Survive longer on carpet and cotton fabrics than Formica and vinyl ● Persistent at low RH (30%) than at high RH (70%) ● Survive for 1-15 d	(27, 61)
Hepatitis A virus	n.a.	X		Cotton cloth	X	X		TM	● Fecal materials not affect ● RH not affect ● Persistent at low T ● Median reduction at 0.8-1.6 logs after dry for 3 to 5 h.	(52)
Murine norovirus	n.a.		X	Wool, nylon carpet, wood		X	X		● Persistent at low RH (30%) than high RH (70%) ● Persistent on wood than stainless steel ● Survived for 3-15 d	(27, 62)

Virus	Subtype studied	Surface type [a]		Material	Factors studied [b]				Key findings	Ref
		L	NL		T	RH	SC	Others		
Poliovirus	n.a.	X	X	Cotton cloth, wool, cellulose membrane	X	X	X	TM	● Persistent at low RH (35%) on wool than high RH (78%) ● More persistence on wool blanket than on wool garments ● Fecal materials reduce persistence on cotton ● RH not affect on cotton ● Persistent at high RH (>80%) on cellulose membrane compared to low RH (20%) ● Persistent at low temperature ● More persistent on cotton than plastics and stainless steel ● Median reduction at 2.7-3.5 logs after dry for 3 to 5 h. ● Survive for to 2 - >7 d	(28, 32, 51, 52)
Rotavirus	n.a.	X		Cotton/polyester cloth	X	X	X	TM	● Persistent at low T on cotton ● Fecal materials not affect ● RH not affect ● Median reduction at 0.6-1.0 logs after dry for 3 to 5 h on cotton polyester cloth ● RNA detectable after 24 h on cotton cloth	(52, 63, 64)

Virus	Subtype studied	Surface type [a]		Material	Factors studied [b]				Key findings	Ref
		L	NL		T	RH	SC	Others		
Vaccinia virus	n.a.	X	X	Cotton, wool, industrial carpet	X	X	X	Deposition	● More persistent on cotton than wool ● Persistent at low RH (35%) than at high RH (78%) ● At low RH (1-10%), more persistent in virus-containing dust than droplets ● At high RH (89-100%), less persistent in virus-containing dust than droplets ● Persistent at low T (6-7C) and RH (1-10%)	(30, 38, 65)

[a] Surface type was classified into launderable (L) and non-launderable (NL).

[b] The effect factors studied were identified as temperature (T), relative humidity (RH), surface characteristics (SC) and others including transmission medium (TM).

[c] "n.a." indicated data not available or not compared.

Table 1.3. Survival of enveloped viruses on porous surfaces.

Virus	Subtype studied	Surface type [a]		Materials	Factors studied [b]				Key findings	Ref
		L	NL		T	RH	SC	Others		
Avian metapneumovirus	APV/MN-2a	X	X	Wood, cotton and polyester fabric, feather			X		● More persistent on feathers (6 d) than other porous surfaces (≤24 h)	(37)
Bacteriophage Phi6	n.a. [c]	X	X	Polyester fabric with and without zinc pyrithione, wood, polyethylene terephthalate carpet	X	X		TM	● Zinc pyrithione fabric coatings not affect ● Persistent at low T and RH ● Persistence: tripart soil load>artificial>PBS ● Persistence: looped>cut carpet	(31, 55, 66)
Cytomegalovirus	n.a.	X	X	Cotton blanket, sanded pine plywood and cotton cloth			X		● At least 1 hour on a cotton blanket at 25-27°C. ● More persistent on cotton cloth than pine wood	(39, 67)
Ebola virus	Makona variant	X	X	Cotton gown			X		● More persistent on respiratory mask than cotton gown	(41)
Equine herpesvirus type-1	n.a.	X	X	Pinewood shavings, polyester-cotton fabric	X		X		● Persistent at low T ● More persistent: polystyrene-cotton fabric than wood shavings	(40)

Virus	Subtype studied	Surface type [a]		Materials	Factors studied [b]				Key findings	Ref
		L	NL		T	RH	SC	Others		
Human coronavirus OC43	n.a.	X		Polyester, wool and cotton			X		● Persistence: Wool>polyester>cotton	(68)
Influenza A virus	H1N1 H6N2 H9N2 H5N1 H9N9 H13N7	X	X	Silver containing fabric, soft toy, wood, cotton cloth, microfiber, pinewood, duck feather (preen oil removed), polypropylene, polyester, polyamide, and polyester fabric	X	X	X	Deposition Subtype	● Persistence: duck feather>wood>cotton cloth>stainless-steel>pine wood ● Persistent on hydrophobic materials (polypropylene, polyester) than on hydrophilic materials (polyamide) ● Survived longer on microfiber than on cotton ● Persistent at low T and RH ● Persistence: liquid inoculum than aerosol ● Survival time on porous surfaces at room temperature: H1N1(<24 h), H6N2(1-21 d), H9N2(<5d), H5N1(17-44.7 d), H9N9(<24 h), and H13N7(1-6 d)	(26, 37, 42, 43, 69-72)

Virus	Subtype studied	Surface type [a]		Materials	Factors studied [b]				Key findings	Ref
		L	NL		T	RH	SC	Others		
Porcine epidemic diarrhea virus	n.a.	X		Unknown cloth	X		X		● More persistent on cloth than metal and nitrile gloves ● More persistent at low T	(36)
Respiratory syncytial virus	n.a.	X		Cloth gown (cotton/ polyester)			X		● Less persistent on cotton/polyester cloth than countertop	(33)
SARS-CoV	n.a.	X	X	Wood board, cloth, polypropylene gown and cotton gown			X		● Survive longer on cloth than on wood board, but shorter than on glass. ● Survive longer on polypropylene gown (2 d) than on cotton gown (24 h)	(34, 73)
SARS-CoV-2	n.a.	X	X	Cotton cloth, treated wood, gym pit foam, nylon and PET carpet	X		X		● Persistence: gym pit foam<cotton cloth<wood<non-porous ● Survive longer at low T ● Survive shorter on nylon than PET carpet, up to 4-8 h	(35, 74-77)

[a] Surface type was classified into launderable (L) and non-launderable (NL).

[b] The effect factors studied were identified as temperature (T), relative humidity (RH), surface characteristics (SC) and others including transmission medium (TM).

[c] "n.a." indicated data not available or not compared.

Disinfection of viruses on porous surfaces

All non-enveloped viruses for which persistence has been reported on porous surfaces have also been studied for disinfection, except for avian reovirus and vaccinia virus (Table 1.4). Specifically, adenovirus, MS2, hepatitis A virus, murine norovirus and poliovirus were investigated for disinfection on cotton fabrics during laundry, while MS2, coxsackievirus, echovirus, FCV, foot-and-mouth disease virus and poliovirus were studied on non-launderable surfaces including porous unglazed red clay, carpet, wood and cellulose membrane. Among enveloped viruses, only Phi6, Ebola virus and SARS-CoV-2 have been investigated regarding their persistence and disinfection on porous surfaces (Table 1.5). In addition, African swine fever virus and murine hepatitis virus have primarily been studied in terms of disinfection on concrete and bus seat fabric, respectively, but with limited data on their persistence.

While disinfectants can be categorized as chemical agents and physical treatments, EPA primarily regulates chemical disinfectants, imposing stringent requirements for standardized efficacy testing (78). Additionally, EPA provides comprehensive guidelines for standardized testing methods tailored to specific disinfection procedures and the surfaces they target (Table 1.6). According to the guidelines, disinfection procedures for porous surfaces were classified into two major categories: laundry and non-launderable surface disinfectants. Consequently, this review is presented with a clear distinction between disinfectants suitable for launderable surfaces and those designed for non-launderable surfaces.

Disinfection on launderable surfaces

EPA stipulated that disinfectants intended for laundry use must successfully undergo a standard suspension test, i.e., AOAC use-dilution methods (Table 1.6). This is due to the ability of water to remove viruses from fabric pores and hold them in suspension during the entire laundry process. Consequently, a significant level of virus inactivation occurs through contact with disinfectants in the suspension rather than on the fabrics (79, 80).

In laundry of clothes, hot water (54-60°C) was found to inactivate more polioviruses from cotton, wool, and nylon clothes than warm (38-43°C) and cold (21-27°C) water (81). However, the clothes drying cycle for 28 minutes after laundry was not effective against adenovirus, hepatitis A virus and rotavirus (80). While high water temperatures can contribute to virus inactivation during the laundry process, the addition of bleach can significantly enhance the inactivation of viruses (82). For example, the study on disinfection efficacy of these three viruses on cotton clothes in laundry showed that the addition of sodium hypochlorite (NaClO) in the wash cycle reduced the viruses by >4 logs after the final rinse (80). NaClO (0.07%) was also effective against SARS-CoV-2 during laundry, while 70% alcohol and Lysoform® (unknown product) reduced less SARS-CoV-2 (83). However, it is important to note that sodium hypochlorite is a strong oxidizer that has the potential to damage fabrics and bleach clothing (20). Therefore, there is a need for the development of alternative disinfectants to bleach to achieve effective virus inactivation and mitigate clothing damage in laundry process.

Chemical disinfection on non-launderable surfaces

According to the EPA, there is a lack of a standardized testing method for non-launderable soft porous surfaces (78). The lack of a standard testing method can be linked to challenges in recovering viruses from non-launderable porous surfaces (27, 84). Moreover, the efficacy of disinfectants against viruses can be significantly influenced by the characteristics of porous surfaces (85, 86). This further complicates the establishment of a universal standard testing method that accurately measures the efficacy of disinfectants on specific types of porous surfaces.

Chemical disinfectants are essential tools for effectively disinfecting non-launderable surfaces. The EPA oversees the regulation of disinfectants in the US, which encompasses a variety of active ingredients and formulations specifically designed for non-porous surfaces. However, only a limited subset of the available products has been tested on porous surfaces (85, 86).

On non-launderable surfaces, the effectiveness of NaClO or chlorine-based disinfectants was extensively reported against both non-enveloped and enveloped viruses. For example, 1.4-1.5% NaClO was able to effectively reduce >3 logs of Ebola virus in 10 min when applied to pilot seat-belt strapping (87). A NaClO solution at 0.5% or 5,000 ppm, a commonly used concentration, effectively reduced 3.92 logs of MS2 on wood and >3 logs of FCV and MNV on cotton fabrics in 5 min. Furthermore, the chlorine solution inactivated >4 logs of SARS-CoV-2 in 0.5 min on wood (88-90). Similarly, a solution containing 0.5% NaClO resulted in a reduction of Phi6 by 2.98 and 6.83 logs on wood and concrete surfaces, respectively, within 1 min at a temperature of 25°C and RH

of 23% (91). At increased RH (85%), the reduction of Phi6 was similar on wood, but decreased to 4.32 logs on concrete surfaces within 1 min (91). A lower concentration of NaClO solution at 1,076 ppm was also effective against MS2 on the polyethylene terephthalate carpet (31). At 500 ppm, NaClO was effective against Echovirus 25 on cellulose membrane, but not effective against Echovirus 6 (51). For murine hepatitis virus, wiping with detergent or chlorine-based disinfectants on seat fabric surface was found to be ineffective (92). Other than NaClO, PurTabs®, which can also produce hypochlorous acid as the active ingredient, resulted in a reduction of over 3 logs of Phi6 within 1 min when applied to polyethylene terephthalate carpet (31).

In addition to chlorine-based disinfectants, glutaraldehyde has been extensively studied and proven effective against FCV on carpet made from olefin, polyester, and nylon, as well as fabrics made from cotton, polyester, and cotton mix (86). However, it demonstrated limited efficacy on blended carpet made from unspecified materials (86). Nonetheless, glutaraldehyde with a 60-min contact time did not exhibit strong efficacy against coxsackievirus, echoviruses, and poliovirus on cellulose membranes (51).

Peroxide-based disinfectants have demonstrated promise against both non-enveloped and enveloped viruses. Specifically, disinfectants utilizing H_2O_2 as the active ingredient was effective against FCV on cotton fabric in 5 min and murine hepatitis virus on seat fabric in 30 s (90, 93). The effectiveness of ozone at concentrations of 20-25 ppm was shown against FCV on fabric, cotton, and carpet in office environments (90, 94).

Silver dihydrogen citrate was also effective against FCV with a 3.62 log- and 1.82 log-reduction on nylon and wool carpet in 60 min, respectively (85). Conversely,

chlorophenol/phenylphenol-based, QAC-based, and alcohol-based disinfectants were not effective against FCV on either fabric or carpet (86). Despite the limited effectiveness against non-enveloped viruses, two QAC-based disinfectants Ardrox 6092 and Desintex achieved >3- $\log_{10}$ $TCID_{50}$ reduction of efficacy against Ebola virus in 10 min on pilot seat-belt strapping, while another QAC-based disinfectant presented efficacy against murine hepatitis virus (87, 93). Interestingly, the effectiveness of disinfection may also be affected by method used to dispense the disinfectant, as one study reported use of an electrostatic sprayer decreased the disinfection efficacy against murine hepatitis virus compared to a trigger-pull sprayer (93). Other than the aforementioned chemical disinfectants, the disinfectant Virkon, which contains potassium peroxymonosulfate as an active ingredient, was found to be effective against African swine fever virus when used at concentrations greater than 2%, resulting in a reduction of over 2.2 logs on concrete surfaces after a 5 min contact time (84).

In summary, an evaluation was conducted on various active ingredients, including chlorine, quaternary ammonium chemicals (QACs), alcohols, glutaraldehyde, silver, and peroxides, to assess their effectiveness against some viruses on non-launderable porous surfaces, with each having limitations. For example, chlorine-based disinfectants are highly effective against Echovirus, FCV, MNV, PV, Phi6 and murine hepatitis virus, but they can damage surfaces when used at high concentrations (e.g., 5,500 ppm) or after prolonged use due to the strong oxidizing properties (19, 20). QACs and alcohols are less likely to cause surface damages, however they demonstrated weak activity against non-enveloped viruses (86, 95). The efficacy of glutaraldehyde and silver in virus disinfection

on specific surfaces such as cellulose membrane, nylon carpet, and olefin carpet has been demonstrated (51, 85, 86). However, exposure to glutaraldehyde can potentially pose health risks (96). Additionally, the use of silver dihydrogen citrate has been found to lead to the formation of a sticky film on carpet (85). Peroxides, including peracetic acid and hydrogen peroxide (H_2O_2), can denature viral capsid proteins, but only limited data on porous surfaces are available (90, 97).

Physical disinfection on non-launderable surfaces

Other than chemical disinfectants, the CDC also recommends implementation of steam cleaning to address carpet contamination subsequent to a human norovirus outbreak (98). However, the CDC has not published any specific protocols in compliance with this guidance. Some studies provided information about disinfection with steam vapor. For example, steam vapor was reportedly effective to reduce MS2 by 6 logs on unglazed red clay in 5 s (99). Steam vapor was also effective against FCV on wool and nylon carpet within 1.5 min, and Phi6 on polyethylene terephthalate carpet within 1 min (31, 85). These results are probably attributed to the rapid denaturation of viral proteins in the outer structures due to heat (31, 85, 99). However, the potential effect of various external factors, such as surface construction, steam temperature, heat distribution on surfaces, on the effectiveness of steam vapor has not been examined.

UV-C, another physical method, has been found in effectively eliminating bacteria on hard porous surfaces (100). However, there has been one reported instance where its application failed to disinfect MS2 on T-shirts (88). Specifically, UV radiation

at 100 mJ/cm^2 for 70 seconds reduced MS2 by 1.27-1.58 logs on the front sections and -0.07 to 1.36 logs on the side sections of a cotton T-shirt (88). Furthermore, UV-C treatment (396 mJ/cm^2) showed efficacy against SARS-CoV-2 only on fabrics of bus seats and clothing (101). This may be attributed to the inherent resistance of viruses to UV-C and the challenges associated with the limited penetration of UV-C into fabric materials (88).

Only one study explored the combination of physical and chemical disinfectants, and revealed that heat treatment combined with ozone (800 ppm) for a duration of 10 minutes was effective against SARS-CoV-2 on cotton fabric (102).

Table 1.4. Disinfection of non-enveloped viruses on porous surfaces.

Virus	Surface type [a] L	NL	Surfaces	Disinfectants	Key findings	Ref
Adenovirus	X		Cotton clothes	Drying cycle, NaClO	● Washing with detergent not effective ● NaClO (180 ppm) reduced >4 logs after the final rinse ● Drying for 28 min not effective	(80)
Bacteriophage MS2	X	X	Porous unglazed red clay coupon, cotton T-shirt, polyethylene terephthalate carpet, wood	Steam, UV-C, chlorine-based disinfectant	● Steam effective on unglazed clay and polyethylene terephthalate carpet, affected by cell culture medium ● UV-C (~100 mJ/cm^2) with weak efficacy on T-shirt (≤1.58 logs for 70 s) ● Chlorine (1,076 ppm) effective on polyethylene terephthalate carpet ● NaClO effective (5,000 ppm) on wood (3.92 logs after 5 min)	(31, 88, 89, 99)
Coxsackievirus		X	Cellulose membrane	Glutaraldehyde, chlorine	● Chlorine (500 ppm) with weak efficacy in 10 min ● Glutaraldehyde (500 ppm) not effective in 10 min	(51)
Echoviruses		X	Cellulose membrane	Glutaraldehyde, chlorine	● Chlorine (500 ppm) not effective in 10 min against Echovirus 6, but effective against Echovirus 25 ● Glutaraldehyde (500 ppm) not effective in 10 min	(51)

Virus	Surface type [a]		Surfaces	Disinfectants	Key findings	Ref
	L	NL				
Feline calicivirus		X	Nylon, wool, and olefin polyester carpet, cotton fabric, cotton, polyester and cotton blended cloth	Silver dihydrogen citrate and steam vapor, hydrogen peroxide- and chlorine-based disinfectants, glutaraldehyde-based, chlorophenol/ phenylphenol-based, QAC-based, alcohol-based disinfectants, ozone	● Steam effective (≥3.68 logs) in 90 s on wool and nylon carpet ● Silver dihydrogen citrate (30 ppm) not effective on the wool carpet, but effective on the nylon carpet ● Both H_2O_2- and chlorine-based disinfectants effective on cotton fabric. ● Glutaraldehyde (26,000 ppm) effective (>3 logs) in 10 min on olefin, polyester and nylon carpet, cotton, polyester and cotton blended fabrics, but not effective on blended carpet ● Chlorophenol/phenylphenol-based, QAC-based, alcohol-based disinfectants not effective. ● Ozone (20-25 ppm) effective on fabric, cotton, and carpet in an office	(85, 86, 90, 94)
Foot-and-mouth disease virus		X	Concrete	Virkon (potassium peroxymonosulfate)	● >Virkon (1%) effective in 10 min on concrete	(84)
Hepatitis A	X		Cotton clothes	Drying cycle in laundry, the addition of NaClO	● Washing with detergent not effective ● NaClO (180 ppm) reduced >4 logs after the final rinse ● Drying for 28 min not effective	(80)

Virus	Surface type [a]		Surfaces	Disinfectants	Key findings	Ref
	L	NL				
Murine norovirus	X		Cotton fabric	Hydrogen peroxide- and chlorine-based disinfectants	● H_2O_2-based disinfectants not effective ● Chlorine-based disinfectants effective	(90)
Poliovirus	X	X	Cotton, wool, and nylon clothes in laundry studies Cellulose membrane	Use of detergent, washing time, water temperature, detergent types Glutaraldehyde, NaClO	● Detergent effective ● Hot water (54-60°C) more effective than cold (21-27°C) and warm (38-43°C) water. ● NaClO (500 ppm) more effective than 0.05% glutaraldehyde on cellulose membrane	(51, 81, 82)
Rotavirus	X		Cotton clothes	Drying cycle in laundry, the addition of NaClO	● Washing with detergent not effective ● NaClO (180 ppm) reduced >4 logs after the final rinse ● Drying for 28 min not very effective	(80)

[a] Surface type was classified into launderable (L) and non-launderable (NL).

Table 1.5. Disinfection of enveloped viruses on porous surfaces.

Virus	Surface type [a] L	Surface type [a] NL	Surfaces	Disinfectants	Key findings	Ref
African swine fever virus		X	Concrete	Virkon (Potassium peroxymonosulfate)	● >2 and 5% Virkon reduced >2.2 logs	(84)
Bacteriophage Phi6		X	wood, concrete, polyethylene terephthalate carpet	NaClO (0.5%), steam, PurTabs® (sodium troclosene and hypochlorous acid, 1076 ppm free chlorine)	● At 25°C, 23% RH, 0.5% NaClO reduced 2.98 and 6.83 logs on wood, concrete in 1 min, respectively. ● At 25°C, 85% RH, 0.5% NaClO reduced 2.93 and 4.23 on wood and concrete in 1 min, respectively. ● Steam and PurTabs® reduced >3 logs in 1 min on polyethylene terephthalate carpet.	(31, 89, 91)
Ebola virus		X	Pilot seat-belt strapping	QAC-based disinfectants and sodium hypochlorite	● Ardrox 6092(QAC-based), Desintex(QAC-based) and 1.4-1.5% sodium hypochlorite reduced >3 logs in 10 min on pilot seat-belt strapping	(87)
Murine hepatitis virus		X	Bus seat fabric	Detergent, QAC-, oxide-, chlorine-based disinfectants	● Detergent nor chlorine-based disinfectants ineffective by wiping ● QAC- and oxide-based disinfectants effective ● Electrostatic sprayer decreased efficacy compared to trigger-pull sprayer	(92, 93)
SARS-CoV-2	X	X	Tricoline fabric, wood, fabrics from bus seat, car, hospital bed linen, hospital clothing, cotton fabric and strap flap	Detergent, formaldehyde-based disinfectant, sodium hypochlorite, 70% alcohol, UV-C, ozone, heat (40°C)	● Only sodium hypochlorite (0.07%) effective during the wash, not 70% alcohol and Lysoform® ● 0.5% NaClO effective on wood after 0.5 min ● UV-C (396 mJ/cm^2) effective only on fabrics from bus seat and clothing, but not on fabrics from car ● UV-C (460 mJ/cm^2) effective on cotton fabric, but not on strap flap ● Heat +ozone (800 ppm) for 10 min effective on cotton fabric	(83, 89, 101-103)

[a] Surface type was classified into launderable (L) and non-launderable (NL).

Table 1.6. Testing standards for soft porous surface disinfection and sanitization.

Disinfectants		Testing methods [a]	Evaluation of successful efficacy
Launderable surface			
Pre-soak treatments	limited and broad-spectrum disinfectant	AOAC Use-Dilution Methods (AOAC 955.14; 955.15; 964.02)	6 log-reduction of S*almonella enterica* (ATCC 10708) and *S. aureus* (ATCC 6538) ≤10 min
	healthcare disinfectants	AOAC Use-Dilution Methods (AOAC 955.14; 955.15; 964.02)	6 log-reduction of *Pseudomonas aeruginosa* (ATCC 15442) and *S. aureus* in ≤10 min
	Sanitizers	ASTM E1153	3 log-reduction of *S. aureus* and *Klebsiella pneumoniae* (ATCC 4352) in ≤5 min
Laundry additives	Disinfectants	ASTM E2274 or E2406	4 log-reduction of *S. aureus* and *K. pneumoniae* in a wash cycle, in addition of *P. aeruginosa* for healthcare facilities
	Sanitizers	ASTM E2274 or E2406	3 log-reduction of *S. aureus* and *K. pneumoniae* in a wash cycle
	Self-sanitizing additives	AATCC Test Method 100-2004 or ASTM E2149-01	3 log-reduction of *S. aureus* and *K. pneumoniae* in no more than 24 hr intervals on a case-by-case basis.

Disinfectants	Testing methods [a]	Evaluation of successful efficacy
Non-launderable surface		
Carpet Sanitizers	Not available [b]	3 log-reduction of *S. aureus* and *Enterobacter aerogenes* (ATCC 13048), in addition of *P. aeruginosa* for healthcare facilities
Mattress, pillows and upholstered furniture treatments	Not available	Efficacy is on a case-by-case basis. Tested microorganisms should be *Bacillus subtilis* (ATCC 19659) and *Clostridium sporogenes* (ATCC 3584) for claims of sterilization, *S. aureus* and *S. enterica* for claims of broad-spectrum disinfection, in addition of *P. aeruginosa* for healthcare facilities, *S. aureus* and *K. pneumoniae* (or *E. aerogenes*) for claims of sanitization
Surface sanitizers for fabric and textiles	ASTM E1153-03	3 log-reduction of *S. aureus* and *K. pneumoniae* (or *E. aerogenes*)

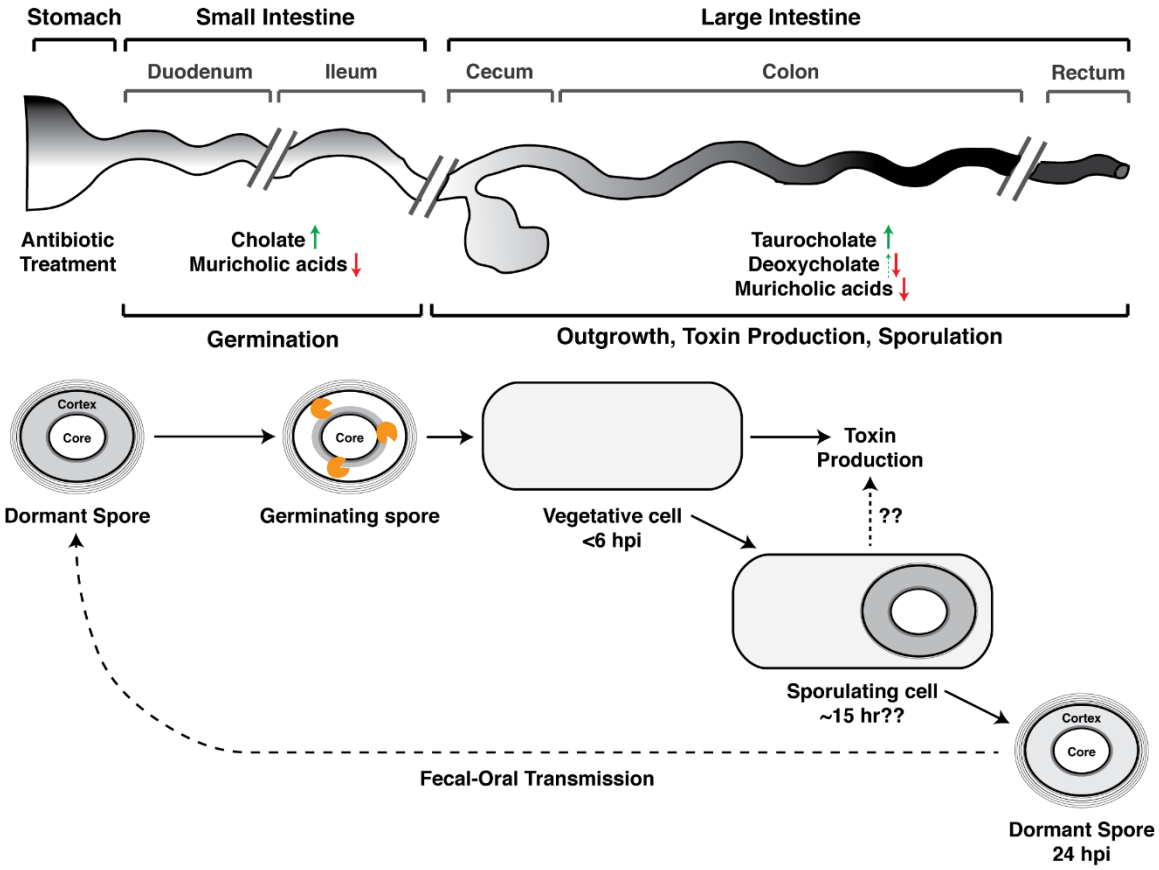
[a] All the testing methods should be conducted in the presence of 5% organic load.

[b] "Not available" indicates no standard testing method is recommended by EPA, but the testing method to achieve the claim needs to be discussed with EPA.

Persistence and disinfection of *C. difficile* endospore on non-porous and porous surfaces

The infection cycle of *C. difficile*, a gram-positive anaerobic bacterium, typically starts with the ingestion of dormant *C. difficile* endospores (Figure 1.2). Once these endospores travel through the stomach, germination is triggered by bile salts (e.g., sodium taurocholate) in the small intestine (104). While the cortex layer starts to degrade, a thicker layer of modified peptidoglycan persists, maintaining metabolic dormancy (105). The germinated spores then grow in the large intestine, where they may transform into toxin-producing vegetative cells. Despite the sporulation process of *C. difficile* remaining unclear, it's noteworthy that *C. difficile* forms spores before exiting the host, allowing its survival in ambient environments (106, 107). This is attributed to the presence of the cortex layer of *C. difficile* endospores, which provides the oxygen-tolerance (107).

Figure 1.2. Life cycle of *C. difficile* in host and environment.



Source: Figure from A. Shen. A gut odyssey: The impact of the microbiota on *Clostridium difficile* spore formation and germination. Plos Pathogen, 2015 11(10):e1005157. (105)

While *C. difficile* vegetative cells have a limited survival period of 15 min on dry surfaces and up to 6 h on wet surface aerobically, *C. difficile* endospores exhibited prolonged persistence (108). For example, Weaver et al. (109) reported that *C. difficile* endospores was only reduced for 1 log on stainless steel surface even after 7 days. Furthermore, *C. difficile* endospores can survive on hospital floors for an extended period

of up to 5 months (110). In contrast, *C. difficile* endospores can be inactivated rapidly on metal surface containing >70% copper achieving a >5- $\log_{10}$ CFU reduction within 24-48 h (109).

Temperature is the key factor for survival of *C. difficile* endospores as it can survive longer at 4°C than at room temperature on inanimate surfaces (111). *C. difficile* endospores is reported to survive longer in humid conditions (>80%) (110). Despite being found in healthy people (112), several ribotypes of *C. difficile* can cause antibiotic-associated diarrhea (113). Vegetative cells of different ribotypes have different stress resistance, but endospores are extremely resistant to chemicals (e.g., ethanol, butanol and chlorine) and high temperature (60-75°C) without significant difference among strains (114). However, the persistence of *C. difficile* has only been studied on non-porous surfaces, with no available data on porous surfaces. This gap in knowledge may be attributed to the fact that CDIs are predominantly healthcare-associated, where porous surfaces are not recommended in patients' area, in contrast of other settings including LTCFs, restaurants and cruise ships (18).

Currently, only a few disinfectants have been investigated for their efficacy in disinfecting *C. difficile* endospores on inanimate surfaces. While EPA mandates a stringent requirement of a 6 $\log_{10}$ CFU reduction of *C. difficile* endospores on stainless steel for the registration of disinfectants' claims, EPA maintains a list (EPA list K) of chemical disinfectants against *C. difficile* endospores, which the majority of registered disinfectants on the list contain chlorine as active ingredients (115). However, Dyer et al. (116) reported that *C. difficile* endospores were resistant to 1,000 ppm sodium

dichloroisocyanurate (NaDDC), a slow-release source of chlorine, with <6 $\log_{10}$ CFU reduction after a 10-min contact time. Moreover, chlorine tends to damage surfaces including stainless steel, plastics and fabrics as discussed above, the alternatives are needed for LTCFs. Hydrogen peroxide and peracetic acid were also reported to be effective against *C. difficile* endospores in hospital (117). However, ten commercial sporicidal wipes have been tested against *C. difficile* endospore on stainless steel, none of them effectively achieved >4 $\log_{10}$ CFU reduction in a 5 min of contact time (118). Considering different sensitivities of *C. difficile* vegetative cells and endospores to disinfection, the addition of germinants reportedly strengthened the efficacy of chemical disinfectants with weaker active ingredients (e.g., QAC-based), for example, from 0.3-0.6 logs to 2.0-3.2 logs of *C. difficile* spores after 24-h treatment. This innovative approach could potentially augment the efficacy of disinfectants that exhibit initially weak efficacy (119). However, the efficiency of sensitization varied among different *C. difficile* strains, as well as conditions including the presence of oxygen, metal ions and different amino acids (104, 107, 120). As a result, predicting the effect of sensitization on disinfection efficacy remains uncertainty based on current knowledge.

Ultraviolet irradiation has been extensively studied for disinfection of *C. difficile* endospores, none of the studies reported a >6 $\log_{10}$ CFU reduction on glass or stainless steel (121-123). Moreover, in those studies, high power (842 Wsec/cm^2) and particular exposure locations (direct exposure at 1.3 m from the instrument) of ultraviolet irradiation is needed for achieving >4 $\log_{10}$ CFU reduction against *C. difficile* endospores (122), which is impractical for disinfection in LTCFs. In addition to ultraviolet

irradiation, steam vapor, another physical disinfectant could reduce 2 $\log_{10}$ CFU of *C. difficile* endospores in 44 min in an isolation room with lowest cost, compared to other chemical disinfection methods (117). Therefore, only a few types of disinfectants, such as hydrogen peroxide and steam vapor, might be potential alternatives to chlorine-based disinfectants against *C. difficile* endospores on inanimate surfaces.

Photocatalysis disinfectants

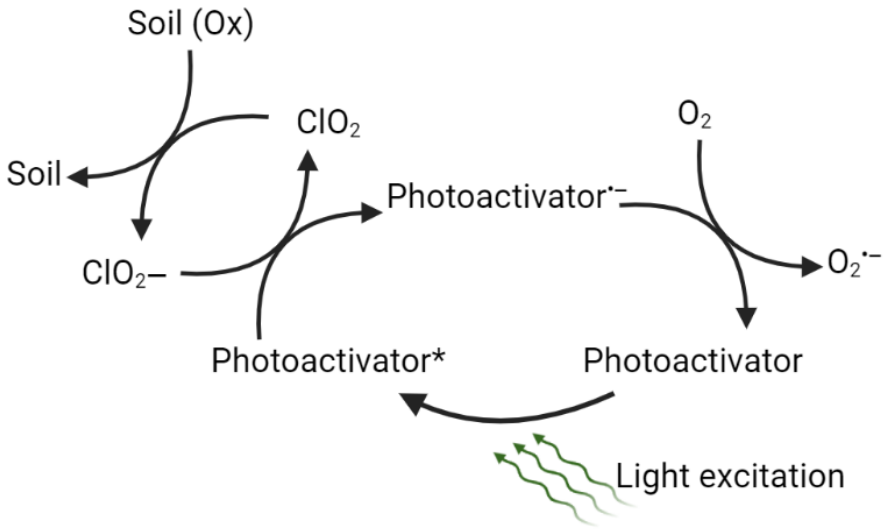
Photocatalysts, which harness light radiation to generate electrons or electron holes, have been extensively studied for microbial inactivation (124, 125). Typically, photo-induced electrons react with oxygen to form peroxide radicals, while electron holes can accept electrons from water molecules and produce hydroxide radicals (126). Both peroxide and hydroxide radicals possess antimicrobial activities due to their reactive properties. Some photocatalysts have been explored in disinfection studies, including titanium dioxide, graphitic carbon nitride, and nanocomposites (124). However, most of photocatalysts used in wastewater and surface disinfection against bacteria and viruses is often limited by their cost and solid state at room temperature (125). Due to the limitations of solid-state photocatalysts, aqueous photocatalysis disinfection systems have been recently patented and shown effectiveness against various microorganisms (127, 128).

Photocatalysis chlorine dioxide (photoClO_2), a novel technique patented in 2017 (129). This disinfectant is mainly composed of a photo-activator which can be excited by a radiation at 350-750 nm of wavelength, which can produce chlorine dioxide (ClO_2) to

inactivate microorganisms after excitation (129). In brief, the photoactivator is excited by light and converts chlorine ions to ClO_2 (Figure 1.3). Then ClO_2 can oxidize microorganisms, followed by the reduction of ClO_2 back to chlorite. Moreover, oxygen in the air plays a role by removing electrons from excited photoactivators and then restoring it back to a steady state. Compared to other photocatalysis disinfectants, this aqueous-based technology is a continuous disinfection method and may provide an efficient disinfection method on surfaces.

A recent study explored the use of photoClO_2 to treat biofilms of *Pseudomonas aeruginosa* (130). After 120 min, photoClO_2 effectively reduced 7 logs of viable *P. aeruginosa* cells within a biofilm characterized by a thickness ranging 40-260 μm. The imaging results further revealed a moderate 73% reduction in biovolume, underscoring the promising potential of photoClO_2 as an effective biofilm treatment. The demonstrated efficacy of photoClO_2 against thick biofilms suggests a potential of photoClO_2 for penetration into surfaces, which might be suitable for porous surfaces. Additionally, another previous study has demonstrated the effective inactivation of bacteriophage MS2, a non-enveloped virus, and *C. difficile* endospores on stainless steel surfaces using photoClO_2 (129). However, no research has investigated its application against HuNoV on porous or non-porous surfaces and *C. difficile* endospores on porous surfaces.

Figure 1.3. Mechanisms of photoClO_2. The system is a catalysis reaction, as neither ClO_2 nor photoactivator is consumed in the reaction.



Prevalence of carpet in healthcare settings

Most non-launderable surfaces (i.e., mattress, carpet and fabric sofa) in healthcare settings are classified as non-critical items that only enveloped viruses and bacteria were supposed to be eliminated according to current Spaulding classification (16). However, the role of flooring in pathogen transmission within healthcare settings is pivotal, as viruses and bacterial spores may reside on floors which are not subjected to high-level disinfection (16). For example, *C. difficile*, in addition to Methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus*, was identified in floor samples retrieved from both hospitals' rooms with CDI and non-CDI patients, exhibiting a positive rate ranging between 45-55% (131). Surprisingly, even after discharge cleaning in patients' rooms, 44% of floor samples still tested positive for *C. difficile (131)*. Furthermore, heavy bacterial colonization was observed on floors in hospitals, particularly with soilborne bacteria regardless of floor types (132, 133). On the other hand, HuNoV was detected on carpet in diverse settings such as hotels, performance halls, and hospitals during gastroenteritis outbreaks (134-137). These collective findings strongly imply a potential oversight in recognizing the role of carpet in the transmission of pathogens.

Pathogens can survive on carpet for a long period of time (15, 138), which allows pathogens having more chances to spread from contaminated carpet during the period of survival. Direct contact is one possible route transmitting pathogens from those reservoirs to patients. The efficiency of transferring pathogens from surface to human bodies raises the possibility of this hypothetical route (139). Thus, pathogens may infect workers in

cleaning or uninstalling contaminated carpet via direct contact (136). Other than direct contact, carpet can spread pathogens through dust, too. Specifically, dusts as vehicles can assist pathogens to spread through air (140, 141). Additionally, these pathogens can be resuspended from carpet into the air through walking, vacuuming, or other movements, thereby leading to further transmission over time and space (142, 143).

Although the use of carpeted flooring is not recommended in patients' areas within healthcare settings, it remains popular in LTCFs for its comfort, sound insulation, and fall prevention properties (144, 145). Moreover, an industry report indicates that carpet and other soft-surface flooring constitute a significant share, comprising 35% of the flooring market and reaching a value of $16.3 billion in the U.S. in 2023 (146). Overall, 42.3% of carpet was sold to commercial settings, with the remaining 57.7% designated for residential use (147). Leading the market are top manufacturers Shaw Inc. (31.1%), Mohawk Inc. (12.6%), and Engineered Floors LLC (5.7%), alongside some smaller companies (147). Consequently, the selection of a representative carpet for testing, to simulate disinfection procedures in LTCFs, should be made from these popular brands.

Considering that carpet is the primary consumers of synthetic fibers, the share of synthetic fiber manufacturing provides insights into the prevalent carpet fiber types (148). In 2023, nylon fibers constituted the majority at 41.5% of synthetic fibers primarily used in carpet manufacturing (148). In contrast, polyester (PET), although cost-effective, accounted for only 13.0% of the market due to its lower durability (148). Other fibers, such as polyolefin, thermoplastic resins, and plastics material, collectively comprised less

than 10.0% of the market share (148). In summary, nylon and PET carpet could be representative of application in public settings including LTCFs.

Conclusions

This review indicates that the persistence of viruses on porous surfaces is significantly affected by the presence of envelopes in virus structures, surface materials and construction, temperature, relative humidity, transmission medium, deposition, and subtype of viruses. In contrast, only temperature and relative humidity affected the persistence of *C. difficile* endospores on surfaces. These findings highlight the prevalence of these pathogens on surfaces and emphasize the need for disinfection measures to prevent transmission. Additionally, these findings also suggest that several environmental factors such as temperature, humidity, transmission medium (presence of soil load) and surface characteristics need to be controlled in the disinfection studies.

Floors, especially carpet, harbor a variety of microorganisms and contribute to the transmission of pathogens. However, floors are classified as non-critical surfaces, not requiring the high-level disinfection against viruses and bacterial spores per the Spaulding Classification Scheme. This reveals a significant knowledge gap in the disinfection of viral pathogens and *C. difficile* endospores on floors in LTCFs. Considering the popularity of carpet in LTCFs, it becomes crucial to address the disinfection of HuNoV, SARS-CoV-2, and *C. difficile* endospores on carpet other than other floor materials.

Regarding disinfection of virus and bacterial spore on non-launderable porous surfaces, it is noteworthy that there is a lack of standard testing methodologies employed to assess the effectiveness of disinfection. Despite this, chlorine-based disinfectants, particularly NaClO, have been extensively studied and demonstrated to be highly effective against both non-enveloped and enveloped viruses on both launderable and non-launderable surfaces. On the other hand, steam vapor was found to be the most effective physical disinfectant against non-enveloped and enveloped viruses, also exhibiting efficacy against *C. difficile* endospores. Furthermore, peroxide-based disinfectants and a novel disinfectant--photoClO2--show potential efficacy against non-enveloped viruses and *C. difficile* endospores on porous surfaces when used at higher concentrations or with extended contact time. However, it is important to emphasize that further research is necessary to validate these findings.

Consequently, the results derived from such assessments could inform effective disinfection strategies in LTCFs, playing a vital role in preventing HAIs. Therefore, the following are the objectives of this dissertation study:

1) Screening EPA-registered chemical disinfectants with one general and one novel HuNoV surrogate and *C. difficile* endospores on hard non-porous surfaces;

2) Assessing the efficacy of selected chemical disinfectants and steam vapor against HuNoV and *C. difficile* endospores on carpet;

3) Assessing the persistence of two SARS-CoV-2 surrogates on carpet and developing a disinfection strategy;

4) Assessing the efficacy of photoClO_2 against HuNoV surrogates and *C. difficile* endospores on stainless steel and carpet and

5) Assessing the carpet damage caused by repeated disinfection.

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CHAPTER TWO

EFFICACY OF EPA-REGISTERED DISINFECTANTS AGAINST TWO HUMAN NOROVIRUS SURROGATES AND *CLOSTRIDIOIDES DIFFICILE* ENDOSPORES

Abstract

Aims: Determining the efficacy of a panel of nine EPA-registered disinfectants against two human norovirus (HuNoV) surrogates [feline calicivirus (FCV) and Tulane virus (TuV)] and *Clostridioides difficile* endospores.

Methods and Results: Nine products on EPA's List G were selected using four criteria: 1) ready-to-use, 2) nonchlorine-based active ingredient, 3) commercially available, and 4) limited known health risks. Active ingredients of the products included hydrogen peroxide (H_2O_2), peracetic acid, quaternary ammonium compounds, or alcohols. The efficacy of the products against FCV, TuV and *C. difficile* spores was tested using the ASTM suspension test and on stainless steel coupons for 1, 5 and 10 min (FCV, TuV) and 10 min (*C. difficile* spores).

Nine EPA-registered products, five of which contained H_2O_2 as active ingredient, were tested against infectious FCV, TuV, and *C. difficile* endospores using two ASTM methods, a suspension and carrier test. Efficacy claims against FCV were confirmed for 8 of 9 products. The most efficacious product containing H_2O_2 as ingredient, achieved a >5.1 $\log_{10}$ PFU reduction of FCV and >3.1 $\log_{10}$ $TCID_{50}$ reduction of TuV after 5 min, and >6.0 $\log_{10}$ CFU reduction of *C. difficile* endospores after 10 min. Of the five products

containing H_2O_2, no strong correlation (R^2=0.25, p=0.03) was observed between disinfection efficacy and H_2O_2 concentration. Addition of 0.025% ferrous sulfate to 1%

Conclusions: Disinfectants containing H_2O_2 are the most efficacious disinfection products against FCV, TuV and *C. difficile* endospores. Product formulation, rather than the concentration of H_2O_2 in a product, impacts the efficacy of a disinfection product.

Importance: H_2O_2-based disinfectants are efficacious against surrogate viruses for HuNoV and *C. difficile* endospores.

Introduction

Healthcare-associated infections (HAIs) are linked to high morbidity, mortality, and increased healthcare costs (1-3). For many years, reduction in HAIs has been a top priority of public health agencies in the United States (2, 3). While progress has been made, a substantial burden from HAIs still persists. Many HAIs are transmitted through contact with contaminated surfaces, illustrating the importance of environmental disinfection as a strategy to prevent their spread (4, 5). Disinfectants are essential tools for effective environmental disinfection. As a result, hundreds of disinfectants with an array of active ingredients and formulations are commercially available. The most common active ingredients used are chlorine, quaternary ammonium chemicals (QACs), alcohols, and peroxides (6), with each having limitations. For example, chlorine-based disinfectants are highly efficacious against bacteria and viruses because they are strong oxidizers, but they can damage surfaces when used at high concentrations or after prolonged use (7, 8). QACs and alcohols are less likely to damage surfaces but show

weak efficacy against non-enveloped viruses [e.g., human norovirus (HuNoV)] and bacterial endospores (e.g., *Clostridioides difficile*) (6). Peroxides, including accelerated hydrogen peroxide (H_2O_2), can denature viral proteins, but efficacy data from published studies are limited (6).

To help users decide which disinfectant to use, the U.S. Environmental Protection Agency (EPA) maintains 15 individual lists of antimicrobials registered for use against specific pathogens (9). Two of these lists address two of the hardest-to-kill pathogens, HuNoV (List G) and *C. difficile* endospores (List K) (10, 11). To be registered as effective against HuNoV, products must achieve a 4-$\log_{10}$ plaque forming unit (PFU) reduction of feline calicivirus (FCV), a surrogate for HuNoV, within 10 min (12). To be registered as effective against *C. difficile* endospores, products must achieve at least a 6-$\log_{10}$ colony forming unit (CFU) reduction of *C. difficile* endospores within 10 min (13). HuNoV is difficult to culture *in vitro* which is why FCV is used to study it. Other surrogates, such as murine norovirus, sapovirus, and Tulane virus (TuV), could be used but none, including FCV, perfectly mimics HuNoV (14, 15). TuV is a promising surrogate as it is more resistant to disinfectant activity than other HuNoV surrogates, including FCV (15). Hence, testing product efficacy against both FCV, required by EPA, and TuV could yield better estimates of disinfectant efficacy.

The EPA requires glass carrier tests be used to conduct efficacy tests (10, 16). Results using glass coupons might not translate to other materials (e.g., brushed stainless-steel and plastics). As stainless-steel is widely used to construct surfaces in healthcare settings (17, 18), an alternative method, ASTM E2197-17 (19) uses brushed stainless-

steel coupons. At present, no published data is available comparing these two testing methods.

The aim of this study was to determine the efficacy of a panel of nine EPA-registered disinfectants against two HuNoV surrogate viruses (FCV and TuV) and *C. difficile* endospores, using two ASTM methods (E1052-11 & E2197-7). These findings can inform standard testing methods used to determine efficacy of disinfectants on hard non-porous surfaces.

Materials and Methods

Virus propagation and assays

Crandell-Rees Feline Kidney (CRFK) cells (ATCC CCL-94) were cultured in Eagle's modified essential medium (MEM; Gibco Life Technologies, NY, USA) containing 5% low-endotoxin heat-inactivated fetal bovine serum (FBS) (Seradigm, VWR International, Radnor, PA, USA), 100 U l^{-1} penicillin (HyClone, GE, MA, USA), and 100 mg l^{-1} streptomycin (HyClone). Ninety percent of confluent monolayers of CRFK cells were infected with FCV strain F9 (ATCC VR-782; American Type Culture Collection, VA, USA) at a multiplicity of infection (MOI) of 0.01 and held at 37°C for 2 days. FCV was then harvested from cell lysates by three freeze-thaw cycles followed by centrifugation for 10 min at 5,000 × g and 4°C. FCV stocks at ca. 10^8 plaque-forming unit (PFU) l^{-1} were aliquoted and stored at -80°C. Infectious FCV was quantified by standard plaque assay as previously described (20). To test for cell line permissiveness

and contamination, FCV and PBS served as a positive and negative control, respectively. CRFK cells were passaged less than 30 times.

LLC-MK2 cells (ATCC CCL-7) were cultured in Opti-MEM I reduced serum medium (Gibco Life Technologies) supplemented with 2% low-endotoxin heat-inactivated FBS, 100 U l^{-1} penicillin, and 100 mg l^{-1} streptomycin. Ninety percent confluent monolayers of LLC-MK2 cells were infected with TuV, kindly provided by Dr. Jason Jiang (Cincinnati Children's Hospital, OH, USA), at an MOI of 0.1 and held at 37°C for 2 days. TuV was harvested from cell lysates similar as for FCV. Infectious TuV titer was quantified by the median tissue culture infectious dose ($TCID_{50}$) assay as described with modifications (21). Twenty microliters of serially diluted viruses were added in each quantification well per column and 8 wells used for each dilution. TuV stocks at ca. 10^7 $TCID_{50}$ l^{-1} were aliquoted and stored at -80°C. LLC-MK2 cells were passaged fewer than 30 times.

Preparation and purification of C. difficile endospores

C. difficile (ATCC 43593) was cultured on modified brain heart infusion agar plates containing 5 g l^{-1} yeast extract, 1 g l^{-1} cysteine and 1 g l^{-1} sodium taurocholate (BHIA/YE/CYS/T) and anaerobically incubated at 37°C for 7 days. All plates were sealed with parafilm (Pechiney, IL, USA) and incubated in ambient conditions for an additional 7 days. The agar plate was flooded with 5 ml of 0.01 M PBS with 0.1% Tween-80 and the colony mass was scraped from the agar plates using sterile cotton swabs. The cell suspension was washed 5 times by ice-cold sterile deionized (DI) water

followed by centrifugation at 7,000 × g for 5 min at 4°C. Vegetative cells of *C. difficile* were removed by gradient centrifugation in 50% (w/v) sucrose solution (22). The endospore suspension was washed three times with sterile ice-cold water after purification. Concentration of endospores was enumerated on BHIA/YE/CYS/T plates and the purity of prepared endospores confirmed via endospore staining. The stock culture of *C. difficile* endospores at ca. 10^8 colony-forming unit (CFU) l^{-1} was stored at 4°C for routine tests and at -80°C for long-term storage.

Candidate disinfectants

Selection criteria included:1) ready-to-use (RTU), 2) nonchlorine-based, 3) commercially available and affordable for small businesses, and 4) limited known health risks (Figure 2.1A, Table A1). Nine products selected from List K (n=64) and List G (n=148) met our criteria and were used for this study. The active ingredients, claimed contact times, and pH of selected products are listed in Table 1. A sodium hypochlorite solution (1,000 ppm) was also evaluated as a positive control in the carrier test.

As contact times listed on product labels for products on List G (i.e., efficacious against FCV, a surrogate of HuNoV) ranged from 30 s to 10 min (Table 2.1), 1 min was used to determine efficacy against FCV in the suspension test and three contact times – 1, 5, and 10 min were used in the quantitative carrier test. No products provided claims against TuV, therefore, 10 min was used in suspension test and 1, 5, and 10 min were used for TuV in the carrier test in order to compare FCV and TuV results. Only product

D had a claimed contact time for *C. difficile* endospores, which was 10 min, so this contact time was used in both the suspension and the carrier test for all products.

Cytotoxicity and neutralization tests

Ingredient-specific neutralizers (Table 2.1) were evaluated for use with each product as previously described with modifications (20). An additional "wash step" was used to eliminate all residue cytotoxicity and antimicrobial activity. Briefly, mixtures of products and neutralizers were diluted by adding 3 ml PBS then it was concentrated via centrifugation using Amicon® Ultra-4 centrifugal 30K MWCO filters (Millipore Sigma, MA, USA) at 4,000 × g, 4°C, repeated 3 times to remove disinfectant residue in the mixture. Following the wash step, undiluted, 10^{-1} and 10^{-2} diluted solutions of product mixtures were assayed by plaque assay for CRFK cells and $TCID_{50}$ assay for LLC-MK2 cells, as described above. Cytotoxicity against these cell lines was observed under an inverted microscope (Olympus CK2) and recorded at days 2 and 5. To test neutralization effect, 10 µl of either diluted FCV (ca. 10^4 PFU l^{-1}) or TuV (ca. 10^7 $TCID_{50}$ l^{-1}) stock were mixed with product-neutralizer solution and assayed as described above. As for *C. difficile*, 10 µl of endospores (ca. 10^4 CFU l^{-1}) were directly added to the product-neutralizer solution.

Quantitative suspension test

Efficacy was first tested using ASTM standard E1052-20 (23) with several modifications (Figure 2.1B). Briefly, 10 µl of FCV, TuV, or *C. difficile* endospores were

each mixed separately with 90 µL of undiluted disinfectant in a 1.5 ml centrifuge tube at room temperature for a designated contact time. Contact times were 1 min for FCV, 10 min for TuV and 10 min for *C. difficile* endospores. PBS was used as a negative control. Mixtures were neutralized by adding 900 µl of neutralizer (Table 2.1) then washed using Amicon® Ultra-4 centrifugal 30K MWCO filters for both FCV and TuV, as described above. After removal of product residue, the retentate was collected and assayed with CRFK cells and LLC-MK2 cells for FCV and TuV, respectively (Figure 1B). Without using centrifugal filters, *C. difficile* endospores were collected directly by centrifugation after neutralization and enumerated as described above.

Quantitative carrier test

Efficacy of the nine products was tested using ASTM standard E2197-17 with modifications (19). A sodium hypochlorite solution (1,000 ppm) (Clorox, CA, USA) was used as a positive control. Briefly, each coupon of brushed stainless-steel (Muzeen & Blythe Ltd., MB, Canada) disk (1 cm in diameter) placed in a 24-well plate (Corning, NY, USA) was inoculated with 10 µL of one stock suspension of FCV, TuV, or *C. difficile* endospores and dried for 1.5 h inside a biological safety cabinet set at room temperature (20-25°C) with 30-50% relative humidity. Dried disks were then incubated with 90 µL of each disinfectant, whereas control disks only received 90 µL of appropriate neutralizers. After the designated contact time (1, 5, and 10 min for FCV and TuV, and 10 min for *C. difficile*), 900 µL of respective neutralizing broth (Table 2.1) were pipetted into each well to neutralize biocidal activity of disinfectant and to facilitate elution of

virus or endospores from coupons. Samples were then assayed as described above. As for *C. difficile* endospores, coupons were first sonicated for 15 s at 40 kHz in a sonication bath (FS110; Fisher Scientific International, PA, USA) after neutralization then pipetted up and down 10 times to remove endospores from carrier coupons. Endospore suspensions were collected and enumerated as described above. Neutralization verification and cytotoxicity elimination were conducted as described in the ASTM standard (19). "Efficacious" was defined as a 4-$\log_{10}$ PFU reduction of FCV and a 6-$\log_{10}$ CFU reduction of *C. difficile* endospores on hard non-porous surfaces (12, 13). As TuV is not recognized by EPA as a target agent, "Efficacious" was defined as a 3-$\log_{10}$ $TCID_{50}$ reduction of general viral surrogates was used (12).

Inactivation kinetics determination of four products against C. difficile endospores in suspension test

To determine if concentration of active ingredients was correlated with efficacy of H_2O_2-based disinfectants, D-values of four products (A, C, D, and E) that significantly inactivated *C. difficile* endospores were compared. The D-value, which indicates contact time needed to achieve a 1-$\log_{10}$ reduction of microorganism, was calculated from the inactivation kinetic curve using the following equation:

$$D = \frac{t}{\log_{10} \frac{N_0}{N_d}}$$

where D means D-value (min) at ambient conditions, N_0 indicates endospore population in the positive endospore control, and N_d indicates surviving endospore population after a

contact time of t (min). To accurately calculate D-values, microbial reduction for each of those products at five contact times were collected in suspension tests. When considering different inactivation rates, contact times for products A and D were 1, 2, 3, 4 and 5 min, while longer contact times of 5, 10, 15, 20 and 25 min were used to test products C and E.

Determination of synergistic effect of hydrogen peroxide and ferrous sulfate

Only H_2O_2-based products presented strong antimicrobial activity against FCV, TuV, and *C. difficile* endospores in either suspension or carrier tests. Therefore, the efficacy of H_2O_2 (Honeywell, NC, USA) against FCV, TuV, and *C. difficile* endospores was tested with the addition of $FeSO_4$ to H_2O_2-based products, known as the Fenton reaction. The Fenton reaction catalyzes H_2O_2 to produce more hydroxyl radicals to oxidize proteins in microbial structures. This was done to better understand the effect of H_2O_2-based formulations against both HuNoV surrogates and *C. difficile* endospores. Hydrogen peroxide solutions of 0.5, 1, 3 and 5% (w/v) were prepared by diluting the concentrated H_2O_2 solution (50%) in deionized (DI) water and the pH adjusted to 2.90 ± 0.05 with 1 M citric acid. To determine the impact of the Fenton reaction, 0.025% (w/v) $FeSO_4$ was added into 1% (w/v) H_2O_2 solution compared to 0.025% (w/v) $FeSO_4$ in DI water, which was used as a negative control.

Statistical analysis

Four replicates of 10-fold serial dilutions of each product were tested in two independent experiments. Microbial reductions were calculated by $\log_{10}$ (N_0/N_d), where N_d is the average microbial population from the treatment samples and N_0 is the average microbial population from each control sample. Statistical analysis was performed using a one-way multiple-comparison t-test to determine the relationship between contact time and microbial reduction. All results were expressed as mean ± standard deviation. Statistical significance was defined as a *p*-value of <0.01 to establish a more conservative estimate of efficacy. Statistical analyses were conducted using GraphPad Prism 6.01 (GraphPad Software, Inc., CA, USA).

Results

Quantitative suspension test

Cytotoxicity of each product was eliminated with an ingredient-specific neutralizer (Table 2.1) before efficacy testing began. Although 5% FBS initially did not neutralize product I, the wash step using centrifugal filters eliminated all remaining cytotoxicity (<1 $\log_{10}$ reduction of viruses and *C. difficile* endospores).

After a 1 min contact time, the four H_2O_2-based products (A-D) and one ethanol-based product (I) achieved a 5.1, 4.1, 5.0, >5.4, and 5.2 $\log_{10}$ PFU reduction of FCV, respectively, whereas the remaining four products (E-H) achieved a 2.7, 2.2, 0.3, and 1.9 $\log_{10}$ PFU reduction of FCV (Table A2). Six products (A-D, H, and I) achieved a 3.8, 3.4, 3.8, 3.9, 3.8 and 4.3 $\log_{10}$ $TCID_{50}$ reduction of TuV after 10 min contact time,

respectively, whereas products E, F and G achieved a 2.5, 0.2 and 1.8 $\log_{10}$ $TCID_{50}$ reduction, respectively. Only product D listed a 10-min contact time against *C. difficile* endospores on its label (Table 2.1), but products A and D both showed a >6.0 $\log_{10}$ CFU reduction of *C. difficile* endospores after 10 min. All other products were not efficacious against *C. difficile* endospores.

Quantitative carrier test

Seven of 9 products (i.e., A-D, F, H and I) were efficacious against FCV, all achieving a >5.1 $\log_{10}$ PFU reduction after 5 min (Figure 2.2). Although product E was not efficacious against FCV after 5 min, it was efficacious after 10 min which was in agreement with the label claim (Table A3). Sodium hypochlorite solution (1,000 ppm) and product D were efficacious against TuV (≥3 $\log_{10}$ $TCID_{50}$ reduction after 5 min) (Figure 2.2). As for *C. difficile* endospores, four products (A, C, D and E) showed sporicidal activity, but only product D was considered efficacious (⩾6 $\log_{10}$ CFU reduction of *C. difficile* endospores after 10 min) (Figure 2.2).

Inactivation kinetics against C. difficile endospores in suspension test

Because only four products (A, C, D and E) inactivated *C. difficile* endospores in the carrier test, inactivation kinetics of those four products were determined based on a suspension test to clearly illustrate relationships between concentration and efficacy. Products A and D achieved a >6.0 $\log_{10}$ CFU reduction of *C. difficile* endospores in 4 and 3 min, respectively (Figure 2.3), whereas products C and E failed to achieve 6.0 $\log_{10}$

CFU reduction by 25 min. When compared with D-values of 0.7 and 0.4 min for products A (0.5% H_2O_2) and D (3.13% H_2O_2), respectively, product E had a higher H_2O_2 concentration (5%) but a higher D-value (5.6 min) against *C. difficile* endospores. Although product C had a H_2O_2 concentration (1.4%) higher than product A, the D-value (6.2 min) for product C was greater than that of product A.

Synergistic effect of hydrogen peroxide and ferrous sulfate

Laboratory-prepared solutions of 3% H_2O_2 and 5% H_2O_2 achieved a 3.9- and 4.1-$\log_{10}$ PFU reduction of FCV after 1 min (Figure 2.4) in a suspension test, with both solutions also achieving a 1.6 and 1.8 $\log_{10}$ $TCID_{50}$ reduction of TuV after 10 min and a 1.5 and 2.1 $\log_{10}$ CFU reduction of *C. difficile* endospores after 10 min, respectively. At lower concentrations of H_2O_2 (≤1%), the efficacy was diminished for FCV (<2.1 $\log_{10}$ PFU after 1 min), TuV and *C. difficile* endospores (0.7 $\log_{10}$ $TCID_{50}$ and 0.3 $\log_{10}$ CFU after 10 min, respectively).

The efficacy of 5 commercial H_2O_2-based disinfectants (products A-E) were compared with laboratory-prepared H_2O_2 solutions. Except for product E, four H_2O_2-based products (A-D) showed higher antiviral activity (additional ≥1.5 $\log_{10}$ reduction of FCV or TuV) than pure H_2O_2 solutions with equivalent concentrations (Figure 2.4). Three of these 5 products (products A, C, and D) were more efficacious against *C. difficile* endospores than pure H_2O_2 solutions. Products A and D with lower concentrations of H_2O_2 achieved a >6.0 $\log_{10}$ CFU reduction of *C. difficile* endospores as compared with a 2.0-$\log_{10}$ CFU reduction by a 5% H_2O_2 solution. Product C containing

1.4% H_2O_2 achieved a 1.4 $\log_{10}$ CFU reduction of *C. difficile* endospores while 1% H_2O_2 solution only achieved a 0.3 $\log_{10}$ CFU reduction. Surprisingly, product E with 5% H_2O_2 had lower activity against FCV and *C. difficile* than 5% pure H_2O_2 solution. Overall, no strong correlation (R^2=0.25, p=0.03) between reduction and H_2O_2 concentration was observed.

Ferrous sulfate at 0.025% had a minimal effect on FCV, TuV, and *C. difficile* endospores with ≤ 0.2 $\log_{10}$ reduction after 1, 10, and 10 min, respectively (Figure 2.5). Addition of 0.025% ferrous sulfate to 1% H_2O_2 solution improved efficacy against FCV, TuV, and *C. difficile* resulting in additional 1.4, 0.4 and 0.9 $\log_{10}$ reduction, respectively.

Discussion

We determined the efficacy of a panel of nine EPA-registered disinfectants against two HuNoV surrogate viruses (FCV and TuV) and *C. difficile* endospores. First, we found that 8 of the 9 product claims could be verified via our testing methods, suggesting our methods were more conservative than those required by the EPA. Secondly, H_2O_2-based products presented strong disinfection efficacy against FCV, TuV and *C. difficile* endospores. Lastly, the production formulation, not just concentration of active ingredients, affects product efficacy.

Product claims for all nine disinfectants, except for product G, were verified against FCV (10). Product G was the only product not efficacious against FCV or TuV presumably due to differences in test conditions (inoculum volume and drying time) recommended by the EPA and ASTM testing methods (16, 19, 24). A likely explanation

for this observation is that the differences in inoculum volume and drying time between the two methods led to differences in virus susceptibility to tested disinfectants. Viruses are more susceptible to disinfectant activity in suspension than when dried on carriers (25). Moreover, our modified ASTM testing methods used smaller inoculum volume and longer drying time leading to more conservative estimates of disinfectant efficacy than what was reported on product claim labels. Neutralizing the disinfectants after the specified contact time to eliminate potential cytotoxicity is critical for an efficacy test when using a cell culture to measure reduction of viral infectivity. Cell death caused by potential cytotoxicity of disinfectants cannot be distinguished from the cytopathic effect caused by viral infectivity thus strong cytotoxicity can result in difficulties to estimate product efficacy (26). Our testing protocol which included a "wash step" was designed to minimize the cytotoxicity of the disinfectants. In addition, concentrating disinfectant-treated viruses by ultrafiltration has been shown to maintain infectivity of SARS-CoV-2, a more sensitive virus than HuNoV, suggesting our testing method was more conservative than the EPA carrier methods (27).

Chlorine-based disinfectants show efficacy against *C. difficile* endospores due to their oxidation activity whereas QACs and alcohols are ineffective against bacterial endospores and HuNoV (6, 15, 28). In our study, only product D with H_2O_2 as the main active ingredient, made a claim against both HuNoV and *C. difficile* endospores. The other 3 H_2O_2-based products (A, C and E), without any claim against TuV and *C. difficile* endospores, showed efficacy against FCV, TuV, and *C. difficile* endospores in a suspension test. Moreover, two products (A and D) containing H_2O_2 were efficacious

against FCV, TuV and *C. difficile* endospores, while disinfectants that contained other active ingredients (i.e., QACs and alcohols) were not efficacious against *C. difficile* endospores. Alcohol and QAC have a limited impact on the surface structure of bacterial endospores(29), whereas H_2O_2, which yields hydroxyl radicals, was reported to be toxic to some bacterial endospores and viral particles (30, 31).

No strong correlation was found between the concentration of H_2O_2-based disinfectants and reduction. Specifically, the D-values (>5 min) of two H_2O_2-based products (C and E) were greater than those of products A and D, which contained even lower concentrations of H_2O_2. These higher D-values may be explained by the interactions between active ingredients and inert ingredients (15), added to improve cleaning performance, aesthetics, formulation stability, and hard water tolerance (32). In addition to H_2O_2, accelerated hydrogen peroxide contains surfactants and other inert ingredients, which act synergistically to yield an efficacious disinfectant (33-36). For example, H_2O_2 is commonly stabilized by organic ligands (e.g., citric acid and malonic acid) to prevent self-degradation (33). Adding ferrous ions to a H_2O_2 solution, known as Fenton reaction, enhances H_2O_2 reactivity (37-41). Production of hydroxyl radicals and hydroperoxyl radicals during the Fenton reaction is believed to cause cytotoxicity leading to DNA damage and protein denaturation (39, 40). In our study, the inclusion of ferrous ions increased the efficacy of 1% stabilized H_2O_2 solution and resulted in an additional reduction of FCV, TuV, and *C. difficile* endospores.

In agreement with previously reported data (15), TuV was more resistant to disinfectants than FCV presumably due to differences in their viral capsid structures (42).

Preserving amino acid residue G329 of the S-P1 hinge region of FCV is critical to maintain its infectivity (43). However, TuV has an isoleucine residue instead of glycine at this position, which is less impacted by oxidation (44, 45). In addition, the structure of TuV virion is more similar to HuNoV than other genera in the family of *Caliciviridae* (44). Furthermore, like HuNoV, TuV utilizes histo-blood group antigens as binding ligands to infect cells (46).

Conclusion

The differences in the efficacy claims by the manufacturer and our data likely can be explained by the use of different testing methods. Though we conservatively estimated the efficacy of EPA-registered disinfectants on stainless-steel carriers, efficacy needs to be validated on other surfaces due to the effect of different surface characteristics (e.g., roughness and water absorbance). Although TuV was confirmed as a more conservative surrogate for HuNoV than FCV, ultimately our findings need to be validated using the recently reported human intestinal enteroid system for HuNoV (47).

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conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Table 2.1. Active ingredients of selected disinfecting products and appropriate neutralizers.

Product	Active ingredient	Label contact time (min)[a]		pH	Neutralizer (concentration)	Cytotoxicity/CPE after neutralization
		FCV	*C. difficile*			
A	0.5% hydrogen peroxide	1	NA	2.64	catalase (1300 U ml^{-1})	-[b]
B	0.88% hydrogen peroxide	10	NA	2.85	catalase (1300 U ml^{-1})	-
C	1.4% hydrogen peroxide	1	NA	2.38	catalase (1300 U ml^{-1})	-
D	3.13% hydrogen peroxide/0.099% octanoic acid/0.05% peracetic acid	4	10	2.95	catalase (1300 U ml^{-1})	-
E	5% hydrogen peroxide/0.005% silver	10	NA	3.07	catalase (1300 U ml^{-1})	-
F	4.85% citric acid/0.003% silver	10	NA	1.79	FBS (5%)	-
G	0.2% chlorine dioxide/0.125% alkyl dimethyl benzyl ammonium chloride/0.125% alkyl dimethyl ethylbenzyl ammonium chloride	5	NA	8.68	FBS (5%) +sodium thiosulfate (0.1%)	-

H	15% isopropanol/7.5% ethanol/0.76% didecyldimethylammonium chloride	1	NA	12.17	FBS (5%) +sodium thiosulfate (0.1%)	-
I	29.4% ethanol	0.5	NA	13.07	FBS (5%)	+[c]

[a] Recommended contact time against FCV listed on product labels; NA represents "not available".

[b] No cytotoxicity/CPE to both cell lines after neutralization, i.e., <1 $\log_{10}$ reduction of viruses or spores in neutralization effectiveness treatments.

[c] Cytotoxicity of samples was finally neutralized by washing with centrifugal filters.

Figure 2.1. Selection criteria of disinfectants (A), and workflow for suspension test and carrier test (B).

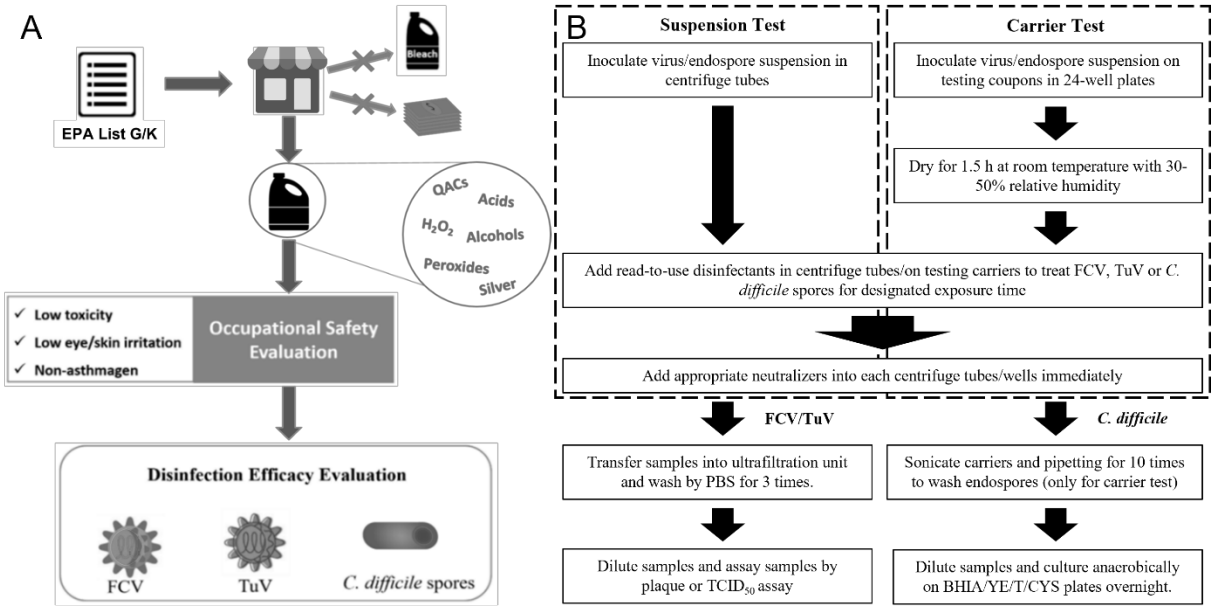


Figure 2.2. Efficacy of nine EPA-registered disinfectants and bleach (1,000 ppm) against FCV, TuV, and *C. difficile* spores on stainless-steel carriers. Contact time for FCV, TuV, and *C. difficile* spores was 5, 5 and 10 min, respectively. Error bars represent standard deviations from replicates in two independent experiments, and stars represent reaching limits of detection.

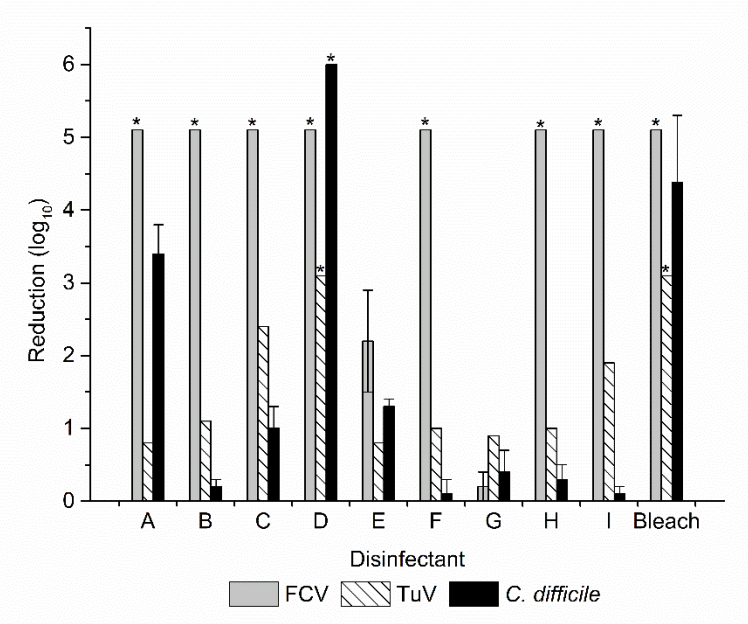


Figure 2.3. Inactivation curves of products A (■), C (□), D (●) and E (○) against *C. difficile* spores. Error bars represent standard deviations from replicates in two independent experiments. Contact times were only for accurate calculation of D-values, not for comparison of disinfectant efficacies.

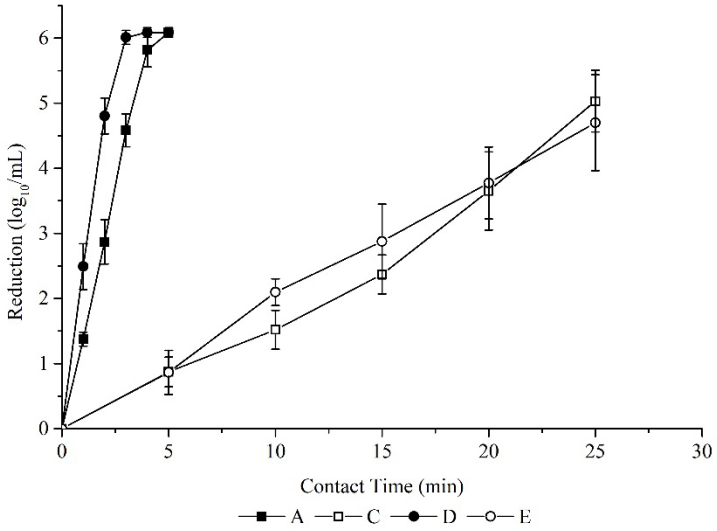


Figure 2.4. Efficacy of H_2O_2 against FCV, TuV and *C. difficile* spores at contact times of 1, 10 and 10 min, respectively. Solid squares indicate efficacy of laboratory prepared H_2O_2 solutions at various concentrations (0.5, 1, 3 and 5%), and open symbols indicate inactivation efficacy of 5 commercial H_2O_2-based disinfectants (A, B, C, D and E). Error bars represent standard deviation from replicates in two independent experiments, and stars represent reaching limits of detection.

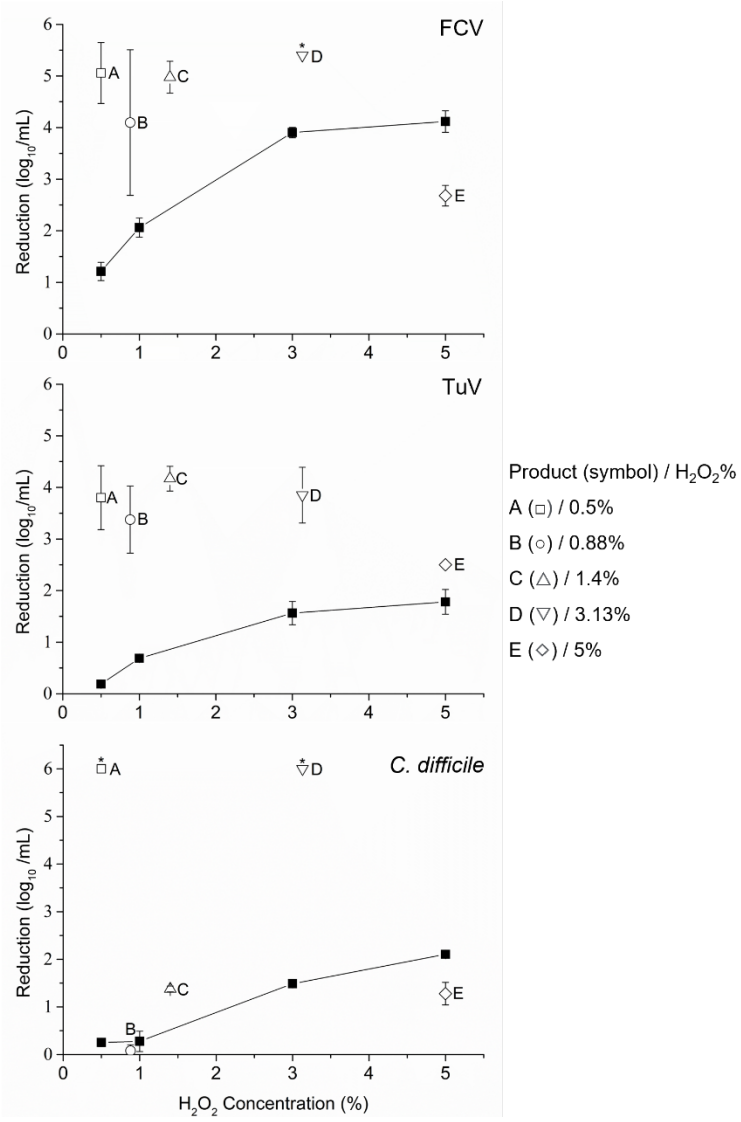
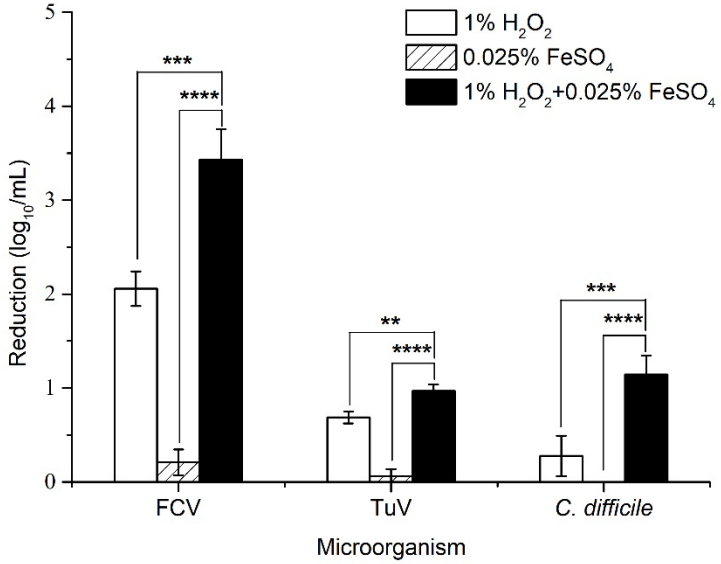


Figure 2.5. Efficacy of H_2O_2 against FCV, TuV and *C. difficile* spores as affected by ferrous sulfate. White bars indicate efficacy of 1% H_2O_2, bars with slash pattern indicate efficacy of 0.025% $FeSO_4$, and black bars indicate efficacy of 1% H_2O_2 +0.025% $FeSO_4$. The contact times for FCV, TuV and *C. difficile* spores were 1, 10 and 10 min, respectively. Error bars represent standard deviations from replicates in two independent experiments. The *p*-value among treatments for each microorganism was ≤0.01 (**), ≤0.001 (***) and ≤0.0001 (****), respectively.



CHAPTER THREE

EFFICACY OF EPA-REGISTERED DISINFECTANTS AND STEAM VAPOR AGAINST TWO HUMAN NOROVIRUS SURROGATES ON NYLON CARPET WITH TWO DIFFERENT BACKINGS

Abstract

Aims: Determining the efficacy of three chemical disinfectants--two hydrogen peroxide-based (H_2O_2) products (products A and B) and one chlorine-based product (product C), and steam vapor against two HuNoV surrogates, feline calicivirus (FCV) and Tulane virus (TuV), on nylon carpet with two types of backings (water-permeable and waterproof).

Methods and Results: A mixture of FCV and TuV was inoculated on carpet coupons (5×5 cm^2) and then dried at room temperature with a relative humidity at 30-50%. After treated virus inocula with either chemical disinfectants or steam vapor for different contact times, the treatment was neutralized and the inocula were assayed for calculation of titer reduction. The effect of repeated disinfection on carpet properties (color and tensile strength) was also evaluated.

The results showed that for carpet with the WPerB (Color Accent®) and 30 min contact time, products A, B, and C showed a 0.8, 3.1, and 0.9 log_{10} PFU reduction of FCV and 0.3, 2.5, and 0.4 log_{10} $TCID_{50}$ reduction of TuV, respectively. For carpet with a WProB (Highlight®), only product B reduced 5.0 log_{10} PFU of FCV and >3.0 log_{10} $TCID_{50}$ of TuV, while products A and C reduced 2.4 and 1.6 log_{10} PFU of FCV and 1.2 and 1.2 log_{10} $TCID_{50}$ of TuV, respectively. Steam vapor showed a ≥5.2 log_{10} PFU

reduction of FCV and >3.2 $\log_{10}$ $TCID_{50}$ reduction of TuV in 15s on carpet with both types of backing. Two H_2O_2-based disinfectants changed the tensile strength of carpet backings, while only product B caused cracks on nylon carpet fibers.

Conclusions: Steam vapor was effective against HuNoV on both tested carpet, but only H_2O_2-based product B showed efficacy after a 30-minute exposure on waterproof carpet. Furthermore, the process of repeated disinfection had discernible effects on both the carpet fiber and backing.

Importance: The results of this study can provide valid information for the development of effective strategies for the disinfection of carpet contaminated with HuNoV.

Introduction

Human norovirus (HuNoV) is a highly contagious virus causing acute gastroenteritis outbreaks and is responsible for more than 50% of all foodborne infections with known etiologic agent (1). In USA, 75% of HuNoV outbreaks occurred in long-term care facilities (LTCFs), as compared with 4% of the outbreaks happened in hospitals or healthcare facilities, and approximately 21% of outbreaks are linked to other settings (2). While HuNoV can be directly transmitted via person-to-person, the indirect transmission of HuNoV is predominantly through fomites, which are objects or materials that can carry the virus and facilitate its spread (3). Unlike hospitals where surfaces are primarily non-porous and easy to disinfect, other settings such as LTCFs, private homes, restaurants, and cruise ships have a wide range of surface types, from non-porous to

porous surfaces, making it difficult to eradicate the virus (4). Although people have taken special precautions to disinfect high-contact surfaces during outbreaks, epidemiological investigations have shown other possible transmission routes, such as floorings (4, 5).

The carpet floorings are potential fomite for the transmission of HuNoV, although there is limited knowledge on how to prevent the spread of the virus through carpet (4, 6). When people infected with HuNoV shed the virus in their stool or vomit, it can land on carpet surfaces and persist for an extended period of time (7-9). For example, a HuNoV outbreak at a hotel lasted for over 7 days and was reportedly associated with the contaminated carpet, suggesting that carpet can serve as a reservoir for HuNoV and perpetuate its spread (10). This is because the complex structure of carpet, including fibers and backing materials, can create an environment that is conducive for the survival of HuNoV (11). Moreover, the act of walking on carpeted surfaces can lead to the resuspension of contaminated dust particles, which can further spread to the high-contact surfaces (7, 11, 12).

Given the potential for carpet to act as fomites for spreading HuNoV, it is important to understand the efficacy of disinfection methods for these surfaces. There is a very limited number of USA Environmental Protection Agency (EPA)-registered products for use on non-launderable textiles against bacteria, but none of them is approved for use against human HuNoV on carpet (13). Moreover, the complex structure of carpet can pose additional challenges for effective disinfection, as the construction of fibers and backing can trap and protect the virus from the actions of disinfectant (11). Due to the lack of standardized method for testing, the efficacy of disinfection methods

for HuNoV on carpet is still largely unknown, except for one study investigating the efficacy of silver dihydrogen citrate and steam vapor on carpet (14). Buckley et al. (14) reported that silver dihydrogen citrate achieved 1.82- and 3.62- $\log_{10}$ PFU reductions of feline calicivirus (FCV), a HuNoV surrogate, on wool and nylon carpet, respectively. Moreover, they also found that steam vapor required a 90-s exposure time to achieve acceptable efficacy (14). Besides silver dihydrogen citrate and steam vapor, both hydrogen peroxide- and chlorine-based disinfectants have been reported to be effective against HuNoV surrogates on non-porous surfaces (15-17). Therefore, it is crucial to explore other disinfectants for carpet that have proven efficacy against HuNoV on non-porous surfaces.

It is important to note that the efficacy of disinfectants for carpet disinfection can vary depending on the type of carpet (14). Carpet is typically constructed of fibers and backings, which can influence the efficacy of disinfectants on surfaces (4). While some studies have focused on the effect of fibers on disinfection efficacy, none have reported the impact of carpet backings on the effectiveness of disinfectants against HuNoV or other pathogens (11, 14, 18, 19). In order to select the disinfectants that are effective against HuNoV on the carpet commonly found in public space, the impact of fibers and backings on disinfection efficacy should be investigated.

The use of disinfectants on carpet can sometimes result in damage to the carpet fibers and backing materials following repeated uses (20-23). This is because some disinfectants contain chemicals that can cause deterioration of the carpet fibers and backing materials, such as fading and lack of shock absorption. Furthermore, exposure to

chemicals for a long time can also cause carpet delamination, where the adhesive holding the carpet fibers in place becomes weakened, leading to the separation of the fibers from the backing material (4, 24). To minimize the risk of damage to carpet during disinfection, it is important to evaluate the effect of repeated disinfection on carpet fibers and backings in addition to the efficacy tests against target microorganisms.

In this study, we evaluated the efficacy of three EPA-registered disinfectants and steam vapor against two HuNoV surrogates, FCV and Tulane virus (TuV), on nylon carpet with two types of backings. Our hypothesis was that the type of carpet backing might affect distribution of virus and disinfectants, and the efficacy of disinfectants against HuNoV surrogates. Meanwhile, we also evaluated the effect of repeated disinfection by chemical products on properties of both carpet fibers and backings.

Materials and Methods

Virus propagation and assays

Crandell-Rees Feline Kidney (CRFK) cells (ATCC CCL-94) were cultured in Eagle's modified essential medium (MEM; Gibco Life Technologies, NY, USA) containing 5% low-endotoxin heat-inactivated fetal bovine serum (FBS) (Seradigm, VWR International, Radnor, PA, USA), 100 U l^{-1} penicillin (HyClone, GE, MA, USA), and 100 mg l^{-1} streptomycin (HyClone). Ninety percent of confluent monolayers of CRFK cells were infected with FCV strain F9 (ATCC VR-782; American Type Culture Collection, VA, USA) at a multiplicity of infection (MOI) of 0.01 and held at 37°C for 2 days. FCV was then harvested from cell lysates by three freeze-thaw cycles followed by

centrifugation for 10 min at 5,000 × g and 4°C. FCV stocks at ca. 10^8 plaque-forming unit (PFU) l^{-1} were aliquoted and stored at -80°C. Infectious FCV was quantified by standard plaque assay as previously described (14). To test cell line permissiveness and contamination, FCV and PBS served as a positive and negative control, respectively. CRFK cells were passaged less than 30 times.

LLC-MK2 cells (ATCC CCL-7) were cultured in Opti-MEM I reduced serum medium (Gibco Life Technologies) supplemented with 2% low-endotoxin heat-inactivated FBS, 100 U l^{-1} penicillin, and 100 mg l^{-1} streptomycin. Ninety percent confluent monolayers of LLC-MK2 cells were infected with TuV, kindly provided by Dr. Jason Jiang (Cincinnati Children's Hospital, OH, USA), at an MOI of 0.1 and held at 37°C for 2 days. TuV was harvested from cell lysates similar to FCV. Infectious TuV titer was quantified by the median tissue culture infectious dose ($TCID_{50}$) assay as described with modifications (25). Twenty microliters of serially diluted viruses were added in each quantification well per column and 8 wells used for each dilution. TuV stocks at ca. 10^7 $TCID_{50}$ l^{-1} were aliquoted and stored at -80°C. LLC-MK2 cells were passaged fewer than 30 times.

RNA extraction and qRT-PCR

Viral RNA extraction was performed as previously described (14). Briefly, the RNAs of FCV and TuV were extracted from 0.15 ml of samples using an ENZA viral RNA kit (Omega Bio-Tek, GA, USA) per the manufacturer's guidance. After extraction, RNAs were stored at -80°C before further analysis. Quantitative reverse transcription

PCR (qRT-PCR) was performed for FCV and TuV separately to determine the loss of viral genome copies using Platinum SYBR Green PCR kit (Invitrogen, CA, USA). The forward and reverse primer sequences for FCV qRT-PCR analysis were GCCATTCAGCATGTGGTAGTAACC and GCACATCATATGCGGCTCTG, respectively, and for TuV analysis were TTCACCCGACCAACCCTG and ACGCCCCAACGCACCTA, respectively (14, 16). The standard curves were prepared individually for FCV and TuV by 7-step 10-fold dilutions of virus stocks.

Selection of disinfectants and preparation of carpet coupons

Two H_2O_2-based (products A and B) and one chlorine-based (product C) disinfectants were selected from a previous study based on the disinfection efficacy against HuNoV surrogates on stainless steel (Table A4) (15). As few studies reported the efficacy of steam cleaners against viruses, a professional steam cleaner (Ladybug 2300, Advap, WA, USA) was also evaluated in this study.

The nylon loop pile carpet Color Accent® (Shaw Inc., GA, USA) was chosen according to the Carpet and Rug Institute Test Method 112 (26). To compare the effect of carpet backings, another nylon loop pile carpet, Highlight® (Shaw Inc., GA, USA), was selected to have almost the exact specifications of Color Accent® other than the backing (Table 3.1). Color Accent® had a water-permeable backing (WPerB), whereas Highlight® had a waterproof backing (WProB). Although both carpet had anti-soil coatings, the carpet were confirmed as free of viricidal activities against FCV and TuV. The carpet was cut into 5×5 cm^2 coupons (in Interface Inc., GA, USA) and dusted by hand to

remove loose fibers. Before the tests, the carpet were autoclaved on a 20-min dry cycle and cooled at room temperature overnight.

Determination of H_2O_2 stability on carpet

Preliminary results showed the reduced efficacy of tested chemical disinfectants against FCV and TuV on carpet within 10 min of exposure. Therefore, a longer contact time was explored by determining the loss of active ingredients over an extended exposure period. To specifically assess the efficacy on carpet, the concentration of hydrogen peroxide (H_2O_2) was monitored for two selected H_2O_2-based disinfectants (referred to as products A and B) to determine the optimal contact time required for efficacy testing on carpet. The diluted 1% H_2O_2 solutions at pH 3.0 and 5.5 were also tested. Each carpet coupon received 6 mL of antimicrobials and scrubbed clockwise and counterclockwise for 30 s each with an antimicrobial-saturated (approximately 1 ml) surgical scrub brush (BD E-Z Scrub, BD, NJ, USA) as described previously (14). After 1-, 5-, 10-, 15-, 30-, and 60-min exposure times, carpet coupons were immediately transferred into a flask with 100 mL water to elute H_2O_2. The concentration of H_2O_2 was measured by a Hydrogen Peroxide Test Kit (HYP-1, Hach, IL, USA) within 10 min after sampling per the user's manual.

Quantitative carpet test

As no standard testing method was available for carpet, the efficacy of chemical disinfectants and the steam vapor was evaluated as previously described with

modifications (14). In brief, all carpet coupons were inoculated with a mixture of FCV and TuV at ca. 7 $\log_{10}$ PFU or $TCID_{50}$ per coupon, respectively, then dried within 1 h at room temperature and a humidity level between 30 and 50%. Next, 6 ml of one of three chemical disinfectants were sprayed onto coupons and held for 30 min or a steam cleaner head wrapped with sterile terry cloth was put over coupons for 15s, 30s, and 60s. There were two sets of controls, unscrubbed and scrubbed. The unscrubbed controls were enumerated immediately after drying, whereas the scrubbed control samples were either scrubbed with a phosphate-buffered saline saturated surgical scrubber (BD E-Z Scrub, BD, NJ, USA) or using a cool steam cleaner head. After defined contact times, the treatment and control samples were neutralized by 100 ml of appropriate neutralizer plus 0.02% Tween-80. Then, inocula on coupons were recovered by ultrasonicating for 1 min at 40 kHz and stomaching at 200 rpm in a stomacher (Model 400, Seward, NY, USA) for 3 min, and titrated using a plaque assay for FCV and $TCID_{50}$ assay for TuV. The viral genome reduction following treatment (i.e., three chemical disinfectants or steam vapor) was measured using the RT-qPCR method described above.

Effects of repeated disinfection on carpet color and tensile strength

To simulate repeated disinfection, carpet coupons (5×12.5 cm^2) with two types of backings were immersed in 500 ml of tested disinfectants at room temperature for 15 h or treated with steam for 450 s, equals to 30 cycles of disinfection treatments. Carpet immersed in water were used as cleaning controls. After treatment, the carpet coupons

were rinsed with DI water until no residue was observed and dried completely at room temperature for at least 4 h before the determination of color and tensile strength.

A colorimeter (CR-400, Konica Minolta, NJ, USA) was used to determine the color of carpet coupons using CIE LAB color space evaluation according to ASTM E2828-20 with modifications (27). The colors were read in three positions on each of the cleaning controls or the treated carpet coupons. ΔE values, which measured the total color and brightness difference between untreated and the cleaning controls or treated carpet coupons, were calculated as the following formula:

$$\Delta E=\sqrt{(L_1-L_0)^2+(a_1-a_0)^2+(b_1-b_0)^2}$$

Where L_0, a_0, and b_0 were color parameters of untreated carpet, and L_1, a_1, and b_1 were color parameters of either cleaning controls or treated carpet. Additionally, fiber damage was observed under a confocal microscope (LEXT OLS4100, Olympus, PA, USA) as reported previously (28). Briefly, the fibers of each untread, treated, and cleaning control carpet coupons were observed under a 40× lens. Images of untreated, cleaning control, and treated coupons were captured without any modifications.

To evaluate the backing damage due to repeated disinfection, the tensile strength of treated carpet coupons was determined using the ASTM D5034-21 method (29). Briefly, the test involved clamping a carpet coupon (2.5×12.5 cm^2), either untreated or repeatedly disinfected, in an Instron Universal Testing Machine (Model 4201, Instron Corp, MA, USA). The clamps, initially positioned 5.1 cm apart, were moved at a speed of 30.5 cm min^{-1} to stretch the carpet coupon until it broke. The breaking force (MPa) recorded by the machine was used to represent the tensile strength.

Statistical analysis

Microbial reductions were calculated by $\log_{10}$ (N_0/N_d), where N_d is the average microbial population from the treatment samples and N_0 is the average microbial population from each control sample. Statistical analysis was performed using a one-way multiple-comparison t-test to determine the relationship between contact time and microbial reduction. All results were expressed as mean ± standard deviation. Statistical significance was defined as a *p*-value of <0.05 to establish a conservative estimate. Statistical analyses were conducted using GraphPad Prism 6.01 (GraphPad Software, Inc., CA, USA).

Results

Stability of H_2O_2 on carpet

As the hydrogen peroxide is generally stabilized by acids at low pH in commercial products (15), two 1% hydrogen peroxide solutions at pH 5.5 (non-stabilized solution) and 3.0 (stabilized solution) were evaluated. After applied to the carpet, the concentration of 1% H_2O_2 solution at pH 5.5 dropped from 1% to 0.69% after 1 h of incubation, while the 1% H_2O_2 solution at pH 3.0 was reduced to 0.76%. There was no significant difference (p>0.05) in concentration loss between H_2O_2 solutions at those two pHs (Figure 3.1). Slightly different from products' labels (Table A4) still within the range of chemical manufacturing standards that are permissible by law (30), the initial concentration of H_2O_2 in products A was measured as 0.6%. However, the initial

concentration of H_2O_2 in products B (containing 0.05% peracetic acid as well) was measured as 3.9% higher than the claims on the label, probably due to the presence of peracetic acid as it is indistinguishable from H_2O_2 when measured by the Hydrogen Peroxide Test Kit (31). The concentration of H_2O_2 in product A was relatively stable, decreasing from 0.6% to 0.4% in 30 min and further to 0.3% in 60 min. In contrast, H_2O_2 in product B rapidly reduced to 1.9% during 15 min of incubation and then slowly declined to 1.1% after incubating for 60 min.

Efficacy of chemical disinfectants on carpet coupons

Products A (H_2O_2-based) reduced 0.8 $\log_{10}$ PFU of FCV and 0.3 $\log_{10}$ $TCID_{50}$ of TuV on carpet Color Accent®, respectively, while it reduced 2.4 $\log_{10}$ PFU of FCV and 1.2 $\log_{10}$ $TCID_{50}$ of TuV on carpet Highlight® (Figure 3.2). Product B (H_2O_2-based) reduced 3.1 $\log_{10}$ PFU of FCV and 2.5 $\log_{10}$ $TCID_{50}$ of TuV on carpet Color Accent®, but efficaciously reduced 5.0 $\log_{10}$ PFU of FCV and >3.0 $\log_{10}$ $TCID_{50}$ of TuV on carpet Highlight®. In contrast, product C (chlorine-based) reduced 0.9 and 1.6 $\log_{10}$ PFU of FCV and 0.4 and 1.2 $\log_{10}$ $TCID_{50}$ of TuV on carpet Color Accent® and Highlight®, respectively.

Moreover, on Color Accent®, products A, B, and C reduced 0.4, 1.7, and 0.5 $\log_{10}$ genome copy (gc) of FCV, respectively, and 0.5, 0.3, and 0.3 $\log_{10}$ gc of TuV, respectively (Figure 3.3). On Highlight®, products A and B reduced 2.0 and 1.8 $\log_{10}$ gc of FCV and 0.5 and 0.3 $\log_{10}$ gc of TuV, respectively. However, product C did not

significantly impact either FCV or TuV genomes with a 0.2 $\log_{10}$ gc reduction on Highlight®.

Efficacy of steam vapor on carpet

Following treatment by the steam vapor, no viable FCV and TuV was detected on any of carpet coupons, except for one treatment whereas 1.9 $\log_{10}$ PFU of FCV was detected after 15 s steaming on Color Accent® (Figure 3.4). Therefore, the steam vapor reduced 5.2, >5.3 and >5.3 $\log_{10}$ PFU of FCV and >3.2 $\log_{10}$ $TCID_{50}$ of TuV on Color Accent® with exposure times of 15, 30 and 60 s, respectively. On Highlight®, steam vapor reduced >5.3 $\log_{10}$ PFU of FCV and >3.2 $\log_{10}$ $TCID_{50}$ of TuV in all exposure times.

Meanwhile, steam vapor reduced 0.0, 0.3 and 1.6 $\log_{10}$ gc of FCV and 0.4, 0.6, and 1.8 $\log_{10}$ gc of TuV on Color Accent ® with exposure times of 15, 30, and 60 s, respectively (Figure 3.5). On Highlight®, steam vapor reduced 0.0, 0.5 and 0.9 $\log_{10}$ gc of FCV and 0.6, 1.1, and 2.0 $\log_{10}$ gc of TuV with exposure times of 15, 30, and 60s, respectively.

Effect of repeated disinfection on carpet properties

After repeated disinfection for 30 cycles, there was no noticeable color change by the naked eye. The average of ΔE of carpet Color Accent® treated with water, steam vapor, products A, B, and C were 1.34, 1.58, 1.21, 1.08, and 0.74, respectively, while ΔE of carpet Highlight® were 0.54, 0.98, 0.77, 0.74, and 0.54, respectively (Table A5).

However, there was no significant (p>0.05) difference in ΔE among cleaning control and all disinfection treatments on either Color Accent® or Highlight®. For the fiber damage, there was no significant difference between the controls and treatments under a 40× magnification with a confocal microscope, except for product B (Figure 3.6). Product B caused significant cracks on both Color Accent® and Highlight® fibers.

The tensile strength of Color Accent® backing was decreased from 33.81 MPa to 26.59 and 27.36 MPa after treated with water and steam vapor, respectively, while the tensile strength of Highlight® backing was decreased from 41.24 to 35.64 and 37.62 MPa, respectively (Figure 3.7). Following treatment with disinfectants, the tensile strength of Color Accent® backing was reduced to 19.51, 19.45, and 26.14 MPa for products A, B and C, respectively, while the tensile strength of Highlight® backing was decreased to 33.43, 32.20, and 34.81 MPa, respectively. There were no significant differences among cleaning control, steam vapor and product C on the tensile strength of Color Accent® backing, but products A and B significantly decreased the tensile strength compared to them. In contrast, there was no significant difference between the cleaning control and all the disinfection treatments on Highlight® backing.

Discussion

We evaluated the efficacy of three EPA-registered chemical disinfectants and steam vapor against two human HuNoV surrogate viruses, FCV and TuV, on nylon carpet with two distinct backings. Our findings demonstrated that only one chemical disinfectant (product B) showed efficacy against both surrogates on one of the carpet

(Highlight®), while steam vapor was highly efficacious against FCV and TuV. The impact of carpet backing on disinfection efficacy was significant, with FCV and TuV showing greater sensitivity to disinfectants on carpet Highlight® as compared to Color Accent®. Last, we demonstrated the effect of repeated disinfection on carpet properties by simulating routine disinfection of carpet.

As most EPA-registered disinfectant products are recommended and used for disinfecting non-porous hard surfaces in healthcare settings, there is no disinfectant with efficacy claim against HuNoV on the carpet currently (32). Our study was to determine if some disinfectants approved for hard non-porous surfaces may be efficacious on carpet. However, only one out of three EPA-registered disinfectants tested in this study was efficacious against FCV on carpet Highlight® with a contact time of 30 min, which is much longer than the product's claimed contact times (10 min) for hard non-porous surfaces. The slow action of disinfectant on carpet may be due to the fact that viruses can form more aggregates in carpet fibers, and are shielded by hydrophobic nylon fibers from the disinfectants (33). In contrary, steam vapor for 15 s was found to be more efficacious against FCV and TuV on two tested carpet types in our study. However, Buckley et al. (14) reported that FCV was only reduced by 3.68 and 3.80 $\log_{10}$ PFU on nylon and wool carpet by steam vapor in 90 s, respectively. One possible explanation for the high efficacy of steam vapor in this study is that the thickness (2.92-3.20 mm) of Color Accent® and Highlight® is much thinner than the carpet tested in the previous study (6.35-10.6 mm), allowing steam to penetrate fibers quickly and thoroughly. Based on our

results, we can conclude that carpet with low piles of fibers can be steamed for 15 s, but thicker carpet may require longer exposure time.

A previous study reported that disinfectants require longer contact time on carpet (90 s) than on glass (<10 s) to achieve the same level of efficacy (14). Moreover, extension of contact time for chemical disinfectants could enhance the efficacy (34). But our results suggest that excessively long contact time is not only impractical but also ineffective for chemical disinfectants due to the instability of active ingredients (Figure 1). Active ingredients of chemical disinfectants, such as chlorine and peroxides, were not stable both in suspension and on surfaces during the prolonged contact time due to degradation (35). Thus, to better understand the performance of chemical disinfectants, we assessed the stability of H_2O_2-based disinfectants during prolonged contact with carpet surfaces and revealed that high concentration (3.13%) of H_2O_2 in product B were rapidly lost. In contrast, lower concentration (0.5%-1%) of H_2O_2 (product A and H_2O_2 controls) was lost slowly, highlighting the importance of stability of active ingredients in disinfectants. Based on the stability test results, we would recommend the contact time on carpet should be limited to 30 min.

Compared to infectivity loss, the impact of disinfectants on the genomes of FCV and TuV was relatively minimal. Overall, the reductions in genome copies were less than half of the infectivity loss caused by either disinfectant. This observation agreed with previous reports (14, 36), suggesting that viral genomes are well-protected by capsids. Moreover, this study revealed that TuV genomes displayed relatively more sensitive to steam vapor while exhibiting more resistance to the chemical disinfectants when

compared to FCV genomes. One possible explanation is the presence of specific amino acid sequences in TuV capsid proteins that confer resistance to oxidation (15). However, the capsid integrity of both FCV and TuV was affected by heat allowing the steam vapor to breach the capsid and affect viral genomes (37).

As nylon carpet Color Accent® and Highlight® are constructed with a WPerB and WProB, respectively, our study also revealed the role of carpet backings in differences of disinfection efficacy against FCV and TuV between those two carpet types. Specifically, capillary action facilitated disinfectant absorption and retention by carpet fibers (38). However, the carpet fibers tufted in clusters on the backing interface create areas where only receive limited disinfectant (38). On the other hand, the WPerB of Color Accent® facilitated the infiltration of the virus inoculum in the form of large droplets, which were driven through the primary backing layer by gravity, not the capillary action (39). Thus, despite thorough scrubbing, disinfectant has limited access to viruses in the WPerB of Color Accent®. In contrast, a significantly more portion of FCV and TuV virions which cannot penetrate the hydrophobic material of WProB, stay above the Highlight® backing, allowing more contact between disinfectant and viruses (38, 39). This disparity in the distribution of disinfectants and viruses on carpet with WPerB and WProB led to variations in disinfection efficacy. As a result, FCV and TuV were more sensitive to disinfectants on carpet Highlight® than on Color Accent®.

In order to better understand the impact of disinfectants on carpet properties, color analysis, microscopic examination and tensile strength were examined. In this study, our results showed that steam vapor, chemical disinfectant products A and C did not cause

any significant changes in the appearance of the fibers, confirming that nylon fibers are durable to withstand repeated disinfection (4). However, product B containing 3.13% H_2O_2 and 0.05% peracetic acid, was found to cause cracks in the nylon fibers after repeated disinfection for 30 times. This observation can be attributed to the strong oxidation activity of product B (40, 41). The active ingredients of all three chemical disinfectants tested in this study are strong oxidizers, which typically result in color loss of textiles (20, 21, 42). Surprisingly, no significant difference in color change impact was found among disinfectants and DI water in this study, likely due to development of dyeing system for nylon fibers (43, 44). Additionally, the dye was also resistant to the heat provided by the steam vapor in this study. Nevertheless, our results also showed that the impact of the disinfectant on Color Accent® (black color) was greater than that of Highlight® (navy blue color), suggesting that the different dyes used in the carpet might have played a role in the color change. However, the specific components of the dyes remain unknown, and further research is required to understand the mechanism behind this difference.

In addition to assessing the impact of repeated disinfection on carpet fibers, our study also investigated the effect of repeated disinfection on the mechanical properties of carpet backings. Consistent with previous research (45), the tensile strength of Color Accent® backings was influenced by repeated water-wash and steam vapor in this study, whereas the Highlight® backing due to its hydrophobicity was mildly impacted. Interestingly, products A and B significantly decreased the tensile strength of the Color Accent®'s backing compared to other treatments. This can be explained by the presence

of hydroxyl radicals in these two disinfectants, which accelerated oxidation of latex used in Color Accent®'s backing, ultimately leading to changes in its mechanical properties (46, 47). In contrast, Highlight®'s backing exhibited higher strength due to its multilayers of the secondary backing. Furthermore, Highlight® also had greater resistance to prolonged exposure to disinfectants than Color Accent®. The observation suggests potential improvements in the anti-oxidation property of polymers used in Highlight®'s backing (4). However, the specific materials utilized in manufacturing of Highlight®'s backing remained undisclosed, which prevented a direct comparison to Color Accent®. In contrast to previous reports, our results suggested that appearance change analysis alone might not be sufficient to evaluate the overall impact of disinfectants on carpet (4, 14, 48). Therefore, a comprehensive evaluation to include both fibers and backings is essential for evaluating the long-term impact of disinfectants on carpet.

Although our study compared efficacies of three chemical disinfectants and steam vapor against FCV and TuV on carpet with two different backings, effect of other characteristics of carpet was not investigated. For example, fiber material (i.e., wool, polyester, and nylon) and construction of fiber (i.e., looped and pile cut) have been reported to affect efficacy of disinfectants (14). Furthermore, these fiber characteristics may also interact with carpet backings in complex ways (4). Therefore, our research findings may not be extrapolated to other types of carpet to provide general disinfection recommendations for carpet due to these variations.

Conclusion

In conclusion, our study has shown that steam vapor is an effective disinfection tool against HuNoV on carpet with the minimal impact on the properties of carpet fibers and backings. Despite some H_2O_2-based disinfectants, as alternatives of chlorine-based disinfectants, were also effective to be used during HuNoV outbreaks, it is important to be aware of the potential damage to carpet by repeated disinfectant application. Therefore, strong disinfectants are recommended for spot treatment, rather than for routine use. Additionally, the effect of carpet backing on disinfection efficacy should be considered while selecting appropriate disinfectants. The results of this study can inform the development of effective strategies for the disinfection of carpet contaminated with HuNoV.

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Table 3.1. Characteristics of selected carpet.

Specifications	Color Accent®	Highlight®
Fiber (Construction method)	Nylon 6 (loop)	Nylon 6 (loop)
Fiber finishing	Solution-dyed, soil-resistant coating	Solution-dyed, soil-resistant coating
Average density	0.348 g/cm^3	0.317 g/cm^3
Finished pile thickness	2.92 mm	3.20 mm
Primary Backing	Synthetic	Synthetic
Secondary Backing	Stalok®	Ecoworx® Performance Broadloom
Backing to moisture	Water-permeable	Waterproof

Figure 3.1. Changes of H_2O_2 concentrations in products A (○), B (□), 1% H_2O_2 solution (pH 5.5, △) and 1% H_2O_2 solution (pH 3.0, ◊) after applied to carpet. Error bars represent standard deviations from triplicates.

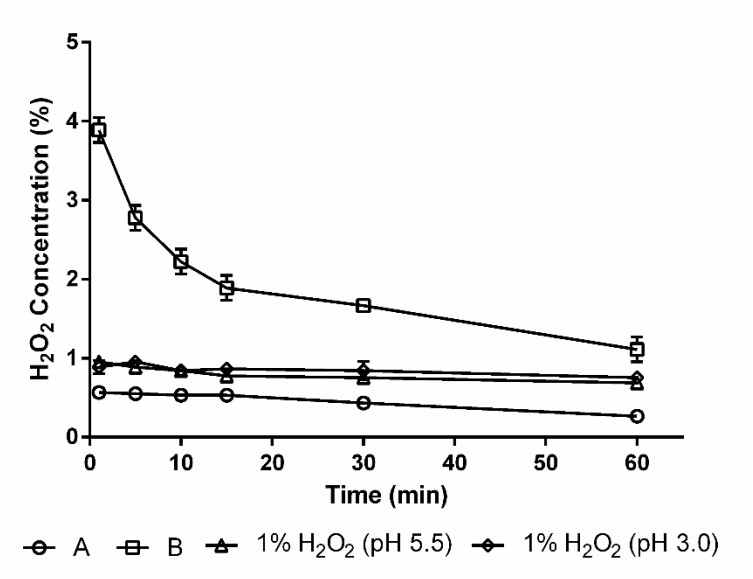


Figure 3.2. Efficacy of three chemical disinfectants against FCV and TuV on carpet with WPerB (Color Accent®) and WProB (Highlight®). The contact time was 30 min. The error bars were standard deviations (SDs) from ten replicates. Stars indicate reaching the detection limit (3.0 $\log_{10}$ $TCID_{50}$ reduction of TuV).

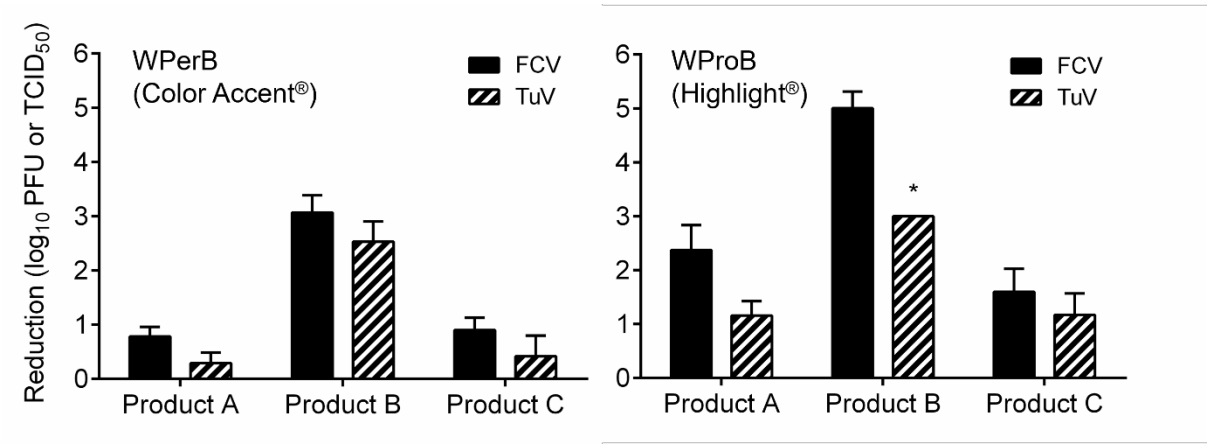


Figure 3.3. Reduction of FCV and TuV genome copies on carpet Color Accent® and Highlight® treated with chemical disinfectants. The contact time was 30 min. The error bars were standard deviations (SDs) from ten replicates.

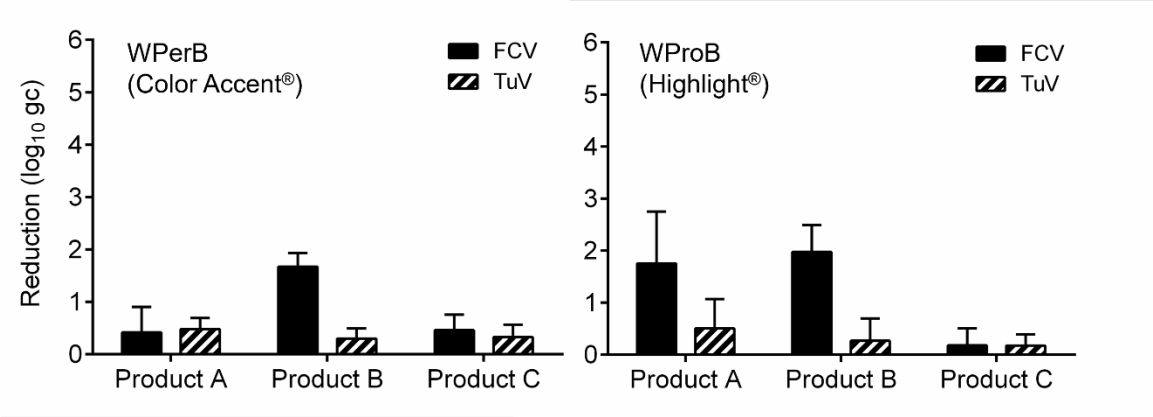


Figure 3.4. Efficacy of steam vapor against FCV and TuV on carpet Color Accent® and Highlight®. The error bars were standard deviations (SDs) from ten replicates. Stars indicate reaching the detection limit (5.3 $\log_{10}$ PFU reduction for FCV and 3.2 $\log_{10}$ $TCID_{50}$ reduction for TuV).

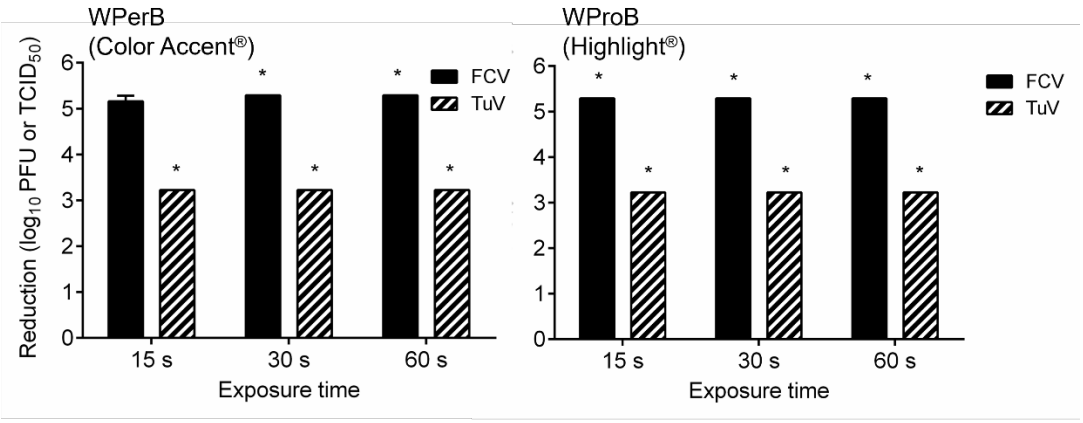


Figure 3.5. Reduction of FCV and TuV genome copies on carpet Color Accent® and Highlight® treated by steam vapor. The error bars were standard deviations (SDs) from ten replicates.

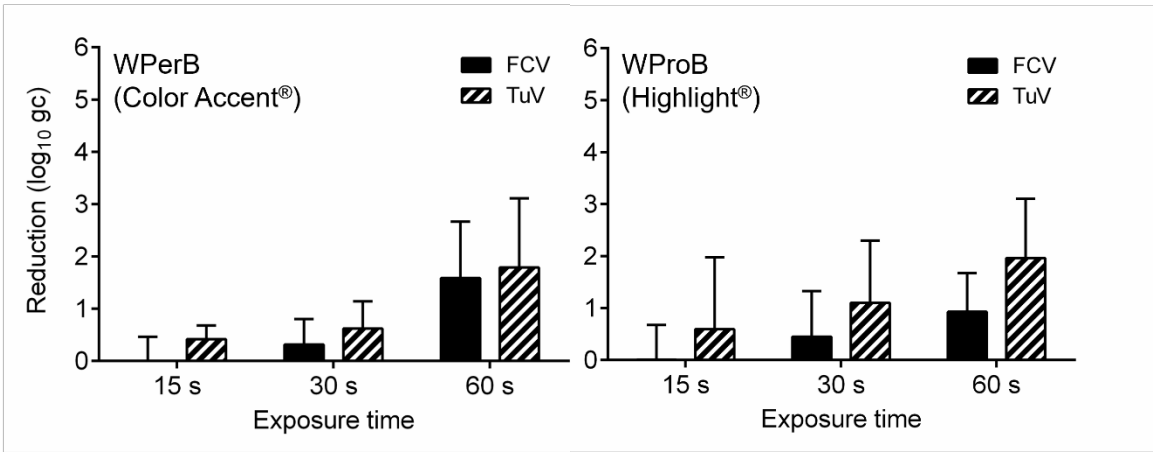


Figure 3.6. Carpet fibers of Color Accent® and Highlight® under a 40× magnification with the confocal microscope: (1) untreated carpet, (2) water-treated carpet, (3) steam-treated carpet, (4) product A-treated carpet, (5) product B-treated carpet, (6) product C-treated carpet. The yellow rectangles indicate cracks on the fibers and the green rectangles indicate random residues on fibers due to manufacturing. The scale bars (yellow lines) indicate 50 μm.

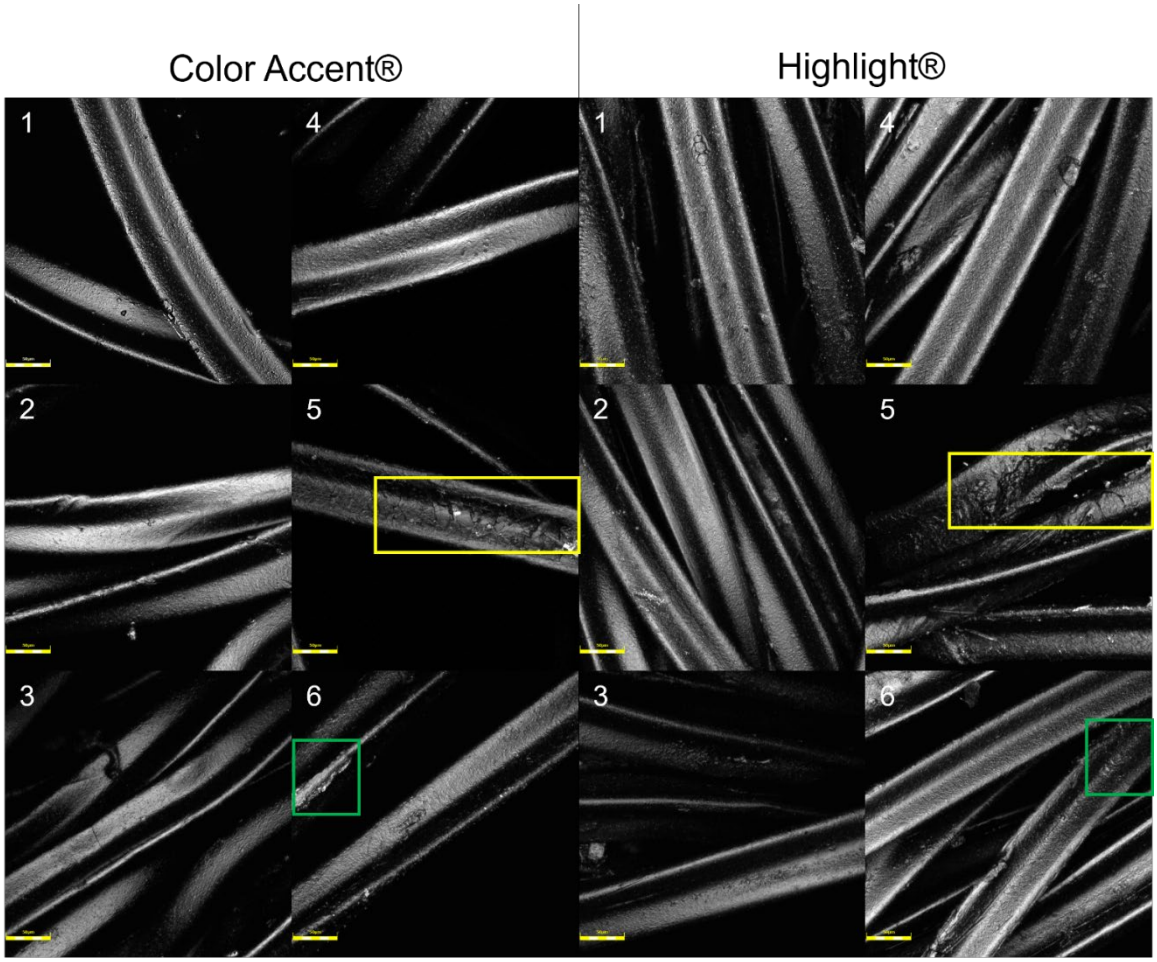
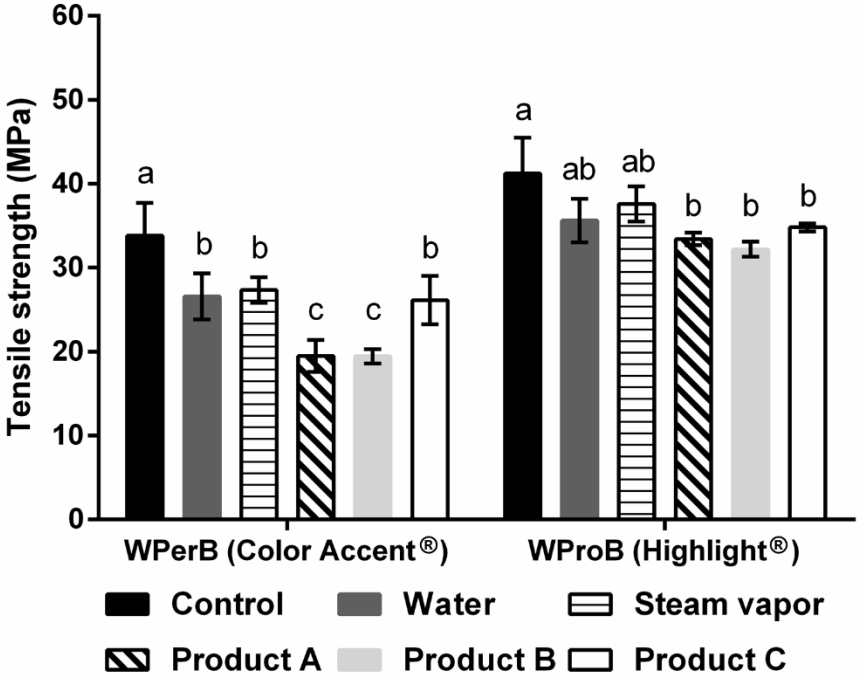


Figure 3.7. Tensile strengths of Color Accent® and Highlight® backing affected by various treatments. Error bars were standard deviation from six replicates. Different letters (i.e., a and b) on each column of each carpet type indicate significant difference among treatments.



CHAPTER FOUR

PERSISTENCE OF AND STEAM VAPOR EFFICACY AGAINST TWO SARS-COV-2 SURROGATES ON TWO TYPES OF CARPET

Abstract

Aims: Evaluating the persistence of and steam vapor efficacy against two surrogates of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) on carpet.

Methods and Results: First, the persistence of two SARS-CoV-2 surrogates [bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV OC43)] on polyethylene terephthalate (PET) and nylon carpet was studied using infectivity and RT-qPCR assays. Then the efficacy of steam vapor was evaluated against both surrogates on nylon carpet. Immediately after inoculation of carpet coupons, 3.87% and 24.37% of HCoV OC43 were recovered from PET and nylon carpet, respectively, compared to 32.50% and 34.86% of BCoV recovered from PET and nylon carpet, respectively. After incubation at room temperature for 1 h, BCoV showed a 3.6 $\log_{10}$ $TCID_{50}$ reduction and HCoV OC43 a >2.8 $\log_{10}$ $TCID_{50}$ reduction on PET carpet and a 0.6 $\log_{10}$ $TCID_{50}$ reduction of BCoV and 1.8 $\log_{10}$ $TCID_{50}$ reduction of HCoV OC43 on nylon carpet. Based on first-order decay kinetics, the total gRNA of BCoV and HCoV OC43 were stable with k values of 1.19 and 0.67 h^{-1} on PET carpet and 0.86 and 0.27 h^{-1} on nylon carpet, respectively. A 15-s steam vapor treatment achieved a >3.0 $\log_{10}$ $TCID_{50}$ reduction of BCoV and >3.2 $\log_{10}$ $TCID_{50}$ reduction of HCoV OC43 on nylon carpet.

Conclusion: More infectious BCoV was recovered than HCoV OC43 from both PET and nylon carpet. BCoV was more resistant to desiccation on both carpet types than HCoV OC43, but both lost infectivity quicker on PET carpet than on nylon carpet. Steam vapor inactivated both SARS-CoV-2 surrogates on nylon carpet.

Importance: Our study demonstrated that SARS-CoV-2 can survive on carpet supporting the need to periodically disinfect carpet. A 15-s steam vapor treatment inactivated two surrogates of SARS-CoV-2 on the low pile nylon carpet, demonstrating it is an efficacious disinfection method.

Introduction

The emergence of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in late 2019 has resulted in over 700 million cases of illness and nearly 7 million deaths worldwide (1). The high transmissibility and severity of COVID-19 infections catalyzed the rapid development of multiple vaccines to stem its spread (2). However, due to the ongoing emergence of various SARS-CoV-2 mutants, the sustained effectiveness of the available vaccines is limited (3). Because the complete eradication of SARS-CoV-2 is near impossible, the need to supplement vaccination efforts with other infection control strategies, such as surface disinfection, is essential.

SARS-CoV-2 is primarily transmitted person-to-person through contact with aerosols and droplets with indirect transmission possible (4). Multiple laboratory studies confirmed its persistence on fomites (e.g., furniture, remote controls, counters) but persistence varies widely depending on surface material (4-7). For example, stainless

steel coupons, a non-porous material, inoculated with SARS-CoV-2 showed a 1 $\log_{10}$ $TCID_{50}$ reduction between 1 and 1.74 days, but cotton, a porous material, showed more wide ranging results, <1- to >4- $\log_{10}$ $TCID_{50}$ reduction in 1 day (5, 8). These variations suggest the need to continue to determine persistence of SARS-CoV-2 on a wider range of materials.

Hard flooring has been reported to be a reservoir for viral particles, especially in dust (9). These particles can be re-suspended in the air through mechanical agitation (e.g., walking and vacuuming) (9, 10). Less is known about porous flooring materials, such as carpet. Carpet is unique due to its composition and structure, which presents unique challenges when assessing recovery and persistence of viruses. Carpet is widely used in public spaces, as it provides comfort, insulating sound and preventing falling so is often impractical to replace with non-porous materials (11, 12). Traditionally, carpet is cleaned by daily vacuuming (13, 14). However, the process of vacuuming might unintentionally resuspend viral particles, dispersing them into the surrounding environment (10, 15). To disinfect carpet after a bodily fluid event, the U.S. Centers for Disease Control and Prevention (CDC) recommends steam cleaning (15, 16). Steam vapor is reportedly effective against FCV with a >3 $\log_{10}$ plaque-forming-unit reduction on wool and nylon carpet within 1.5 min, and Phi6 on polyethylene terephthalate carpet within 1 min (10, 17). While steam vapor has demonstrated efficacy against some viruses on carpet, its efficacy against coronaviruses, particularly SARS-CoV-2, has yet to be confirmed.

Because SARS-CoV-2 is a highly communicable pathogen, surrogates are typically used to mitigate health risks of those handling samples (18, 19). While

bacteriophage Phi6 is most commonly used as a model to study enveloped viruses, bovine coronavirus (BCoV), human coronavirus (HCoV) 229E, and HCoV OC43 are primarily used to study SARS-CoV and SARS-CoV-2, due to structural similarities. HCoV 229E is classified within the genus of *Alphacoronavirus*, whereas BCoV, HCoV OC43, and SARS-CoV-2 belong to the *Betacoronavirus* genus. Additionally, HCoV 229E has shown greater heat-sensitivity compared to HCoV OC43 (20). Consequently, using both HCoV OC43 and BCoV as surrogates to investigate persistence and disinfection of SARS-CoV-2 could potentially yield more comparable estimates of disinfection efficacy.

The recovery and detection of viruses from porous materials are also formidable obstacles. SARS-CoV-2 has exhibited sensitivity to recovery methodology [e.g., detergents used for recovery (21)], supporting the need to identify a better recovery method. Some methods exclusively rely on the degradation of viral RNA as the sole metric for assessing virus survival. While this approach provides valuable insights into both persistence of the viral genome and structural integrity, it does not provide an assessment of SARS-CoV-2 infectivity.

We aimed to fill these knowledge gaps by first evaluating the persistence of two SARS-CoV-2 surrogates, BCoV and HCoV OC43, on two types of carpet -- polyethylene terephthalate (PET) and nylon, then, test the disinfection efficacy of steam vapor against these two surrogates on nylon carpet. Our findings can be used to inform disinfection strategies on complex surfaces, such as carpet.

Materials and Methods

Virus propagation and assays

Human rectal tumor (HRT-18G) cells (ATCC CRL-11663) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g l^{-1} glucose, 3% low-endotoxin heat-inactivated fetal bovine serum (FBS), 100 U l^{-1} penicillin, and 100 mg l^{-1} streptomycin. Ninety percent (90%) confluent monolayers of HRT-18G cells were infected with bovine coronavirus (BCoV) strain Mebus (BEI, NR-445) or HCoV OC43 (ATCC, VR-1558) at a multiplicity of infection (MOI) of 0.01 then incubated at 37°C for five days. BCoV and HCoV OC43 were then harvested from cell lysates by three freeze-thaw cycles followed by centrifugation for 10 min at 5,000 × g and 4°C. BCoV and HCoV OC43 stocks at ca. 10^8 50% tissue culture infectious dose ($TCID_{50}$) ml^{-1} were aliquoted and stored at -80°C. HRT-18G cells were passaged less than 30 times.

Infectious BCoV and HCoV OC43 were quantified by $TCID_{50}$ assay as previously described, with modifications (22). Briefly, monolayers of HRT-18G cells at 90% confluency were infected with 100 µl of viral samples at 33°C for 1 h in a humidified 5% CO_2 incubator, followed by the addition of 100 µl of infection medium, which is DMEM containing 4.5 g l^{-1} glucose, 2% low-endotoxin FBS, 100 U l^{-1} penicillin, and 100 mg l^{-1} streptomycin. After incubating at 33°C in a humidified 5% CO_2 incubator for seven days, the virus titer was determined by the improved Kärber method (23). To test cell line susceptibility to infection and viability, BCoV or HCoV OC43 stock and phosphate buffer saline (PBS) were used as positive and negative controls.

Carpet coupon preparation and selection of steam cleaner

Two common commercial carpet materials, PET carpet (Profusion 20®, Shaw Inc., GA, USA) and nylon carpet (Color Accent®, Shaw Inc., GA, USA), were tested. Both PET and nylon carpet had no antimicrobial coating and were low pile carpet with the thickness of the fiber pile at 3.58 and 2.92 mm, respectively. Carpet samples were cut into 5×5 cm^2 coupons with a mechanical cutting die (model 1500, Freeman Schwabe, OH, USA) (kindly provided by Dr. Daniel Price, Interface Inc., GA, US) then dusted by gloved hand to remove loose fibers. To remove additional residue, coupons were scoured using a boiling solution of 5 g l^{-1} Tergitol N-101 (Spectrum Chemical Inc., New Brunswick, NJ) and 5 g l^{-1} of Na_2CO_3 (Fisher Scientific, MA, USA), then rinsed with cold tap water until visibly clean.

A household steam cleaner (IVASTEAMR20, Ivation, NJ, USA) that can generate 170°C, 29-65 psi steam in its boilerwas used with a small round head (4 cm in diameter) to test the efficacy of steam vapor.To prevent cross-contamination during the steam vapor treatment, the head of a steam cleaner was wrapped with a sterile terry cloth that was folded into 4 layers.

Persistence of coronaviruses on carpet

BCoV and HCoV OC43 were prepared at ca. 10^8 $TCID_{50}$ ml^{-1} with 5% FBS as soil load. Each pre-cut carpet coupon was inoculated with 100 µl suspension of either BCoV or HCoV OC43 then kept for 2 h under a biosafety cabinet (Model 1300 A2, Thermo Fisher, MD, USA) at room temperature with a relative humidity at 30-50%.

After 0, 10, 20, 30, 60, 90 and 120 min, three coupons were immediately transferred into a flask with 100 mL of PBS plus 0.02% Tween-80. All flasks were ultrasonicated for 1 min at 40 kHz (Model FS110, Fisher, PA, USA) then vigorously shaken by hand for 30 s. Samples were recovered and concentrations of each surrogate in each sample were assayed as described above.

PCR and RNase-treated PCR

Viral genome RNA (gRNA) extraction was performed as previously described (17). Briefly, BCoV and HCoV OC43 gRNA was extracted from 0.15 ml of carpet samples using an ENZA viral RNA kit (Omega Bio-Tek, GA, USA) per manufacturer instructions. After extraction, gRNAs were stored at -80°C before further analysis.

Quantitative reverse transcription PCR (qRT-PCR) was performed for BCoV and HCoV OC43 separately to determine the loss of viral genome copies using Platinum SYBR Green PCR kit (Invitrogen, CA, USA). For qRT-PCR analysis, the forward and reverse primer sequences were CTGGAAGTTGGTGGAGTT and ATTATCGGCCTAACATACATC for BCoV, respectively, and CGATGAGGCTATTCCGACTAGGT and CCTTCCTGAGCCTTCAATATAGTAACC for HCoV OC43, respectively. The standard curves were individually prepared for BCoV and HCoV OC43 by 7-step, 10-fold dilutions of virus stocks.

To assess the structural integrity of the viral capsid, the exposed gRNA due to capsid cleavage was removed by RNase I pretreatment of samples prior to RNA extraction (24). Briefly, 0.1 U μl^{-1} RNase I (Thermo Fisher, MD, USA) was mixed with

250 µl of samples and incubated at 37°C for 15 min. RNA extraction was performed immediately after the RNase-I pretreatment as described above.

Determination of disinfection efficacy of steam vapor against coronaviruses on nylon carpet

As both surrogates did not survive long (>3 $\log_{10}$ $TCID_{50}$ reduction after 1-h desiccation) on the PET carpet, disinfection efficacy of steam vapor using the steam cleaner was determined only on nylon carpet following a previous study with minor modifications (17). Briefly, each pre-cut coupon was inoculated with a 100 µl suspension of either BCoV or HCoV OC43 then dried for 1 h at room temperature at a relative humidity at 30-50%. To evaluate steam vapor efficacy, the steam cleaner was preheated, then the wrapped head and hose were saturated with steam for 10 s per the manufacturer instructions. Coupons were scrubbed vertically for 15 s with steam. All coupons were transferred into a flask with 100 ml of PBS plus 0.02% Tween-80 to elute virus from carpet coupons. To evaluate the effect of scrubbing on virus inoculum, three scrubbed coupons were scrubbed using the wrapped head of steam cleaner without heat, while three unscrubbed coupons were immediately transferred to elution buffer after drying to evaluate desiccation effect. All flasks were ultrasonicated for 1 min at 40 kHz then vigorously shaken by hand for 30 s to recover virus inoculum from carpet coupons. Titers of BCoV and HCoV OC43 in samples were assayed by $TCID_{50}$ assay as described above. The temperature of steam vapor was measured using type T thermocouples (HotMux, DCC Corporation, NJ, USA).

Carpet absorption capacity

To measure the hydrophobicity of carpet fibers, the water absorption capacity of carpet fibers was tested as described previously (25). Briefly, the carpet fibers were cut from the coupons using a disposable scalpel (Sklar, PA, USA) then 0.1 g of fibers were packed in a 2-ml microcentrifuge tube (Fisher Scientific, CA, USA). PET and nylon fibers were thoroughly mixed with (0.1%) safranin solution in increments of 0.05 ml and removed afterwards. The weight of residual liquid was obtained by subtracting the weight of the empty microcentrifuge tube from the weight of the microcentrifuge tube after treatment.

Statistical analysis

Six replicates were tested in two independent tests to determine persistence of surrogates, whereas 10 replicates were used for evaluating disinfection efficacy of steam vapor against surrogates. Microbial reductions were calculated using $\log_{10}$ (N_0/N_d), where N_0 is the average surrogate titers from the samples at 0 min or the control samples, and N_d is the average surrogate titers from the samples at different sampling times or the steam-treated samples.

For RNA determination, the first-order decay rate constants (k) were calculated following equation (1), where N_0 is the average amount of coronavirus RNA at 0 h, and N_t the average amount of coronavirus RNA at time (t). k values were calculated by

plotting ln (N_t/N_0) versus time (t) and calculating the slope, its standard error, with β_0 as the intercept.

$$\ln\left(\frac{N_t}{N_0}\right) = \beta_0 - kt \quad (1)$$

Statistical analysis was performed using a one-way multiple-comparison ANOVA to determine the relationship between steam vapor and microbial reduction. All results were expressed as mean ± standard deviation. Statistical significance was defined as a *p*-value of <0.05. Statistical analyses were conducted using GraphPad Prism 6.01 (GraphPad Software, Inc., CA, USA).

Results

Persistence of infectious coronaviruses on carpet

The immediate recovery rate of BCoV from PET and nylon carpet was 32.50% and 34.86%, respectively, which was significantly higher than the recovery rate of HCoV OC43 from nylon carpet, which was 3.87% and 24.37% of HCoV OC43, respectively (Table A6). After 0, 10, 20, 30, 60, and 90 min of inoculation, the titers of BCoV on PET carpet were 6.1, 5.8, 5.6, 5.1, 2.7, and <2.6 $\log_{10}$ $TCID_{50}$, respectively, and the titers of HCoV OC 43 were 5.4, 4.8, 4.4, 4.1, <2.6, and <2.6 $\log_{10}$ $TCID_{50}$, respectively (Figure 4.1A). In contrast, 6.8, 6.7, 6.5, 6.2, 6.1, 6.0 and 5.3 $\log_{10}$ $TCID_{50}$ of BCoV were detected on nylon carpet after 0, 10, 20, 30, 60, 90, and 120 min, respectively (Figure 4.1B), and 6.7, 6.5, 6.6, 6.4, 5.7, 5.5 and 4.9 $\log_{10}$ $TCID_{50}$ of HCoV OC43, respectively.

Persistence of coronavirus total genome on carpet

On PET carpet, gRNAs of BCoV were decreased by 0.23, 0.69, 0.90. 0.97, and 1.08 $\log_{10}$ genome copy (gc), respectively, and a 0.22, 0.35, 0.50, 0.57, and 0.61 $\log_{10}$ gc reduction of HCoV OC43 after 10, 20, 30, 60, and 90 min , respectively (Figure 4.2A). On nylon carpet, gRNAs of BCoV showed a 0.11, 0.13, 0.14, 0.47, 0.52, and 0.87 $\log_{10}$ gc reduction after 10, 20, 30, 60, 90, and 120 min, respectively, while gRNA of HCoV OC43 had a 0.20, 0.21, 0.16, 0.06, 0.28, and 0.44 $\log_{10}$ gc reduction, respectively (Figure 4.2B). Based on first-order decay kinetics, the *k* values of BCoV and HCoV OC43 total gRNA on PET carpet were 1.19 and 0.67 h^{-1}, respectively, and on nylon carpet were 0.86 and 0.27 h^{-1}, respectively (Table 4.1).

Persistence of coronavirus unexposed genome on carpet

Compared to the total gRNA, unexposed gRNA wrapped inside viral capsids, which represent for intact capsid, was decreased more slowly on PET carpet by a 0.03, 0.18, 0.10, 0.14, and 0.33 $\log_{10}$ gc reduction of BCoV, and a 0.38, 0.29, 0.36, 0.52, 0.40 $\log_{10}$ gc reduction of HCoV OC43 after 10, 20, 30, 60, and 90 min, respectively (Figure 4.2C). On nylon carpet, gRNA of BCoV was decreased by 0.07, 0.33, 0.19, 0.23, 0.43, and 0.73 $\log_{10}$ gc reduction after 10, 20, 30, 60, 90, and 120 min, respectively, whereas gRNA of HCoV OC43 had a 0.38, 0.29, 0.36, 0.52, 0.40, and 0.63 $\log_{10}$ gc reduction, respectively (Figure 4.2D). As the exposed gRNA was removed by RNase I, the *k* values of unexposed gRNA from BCoV and HCoV OC43 were 0.61 and 0.28 h^{-1} on PET carpet, respectively, and 0.84 and 0.43 h^{-1}on nylon carpet, respectively (Table 4.1).

Efficacy of steam vapor against coronaviruses on nylon carpet

Following a 1-hour drying period on nylon carpet, desiccation resulted in a 0.4 $\log_{10}$ $TCID_{50}$ reduction for BCoV and 0.6 $\log_{10}$ $TCID_{50}$ reduction for HCoV OC43 (Table 4.2). The temperature of steam vapor from the steam cleaner head reached 99.44±0.98°C. Subsequent treatment with steam vapor inactivated both BCoV and HCoV OC43 across all carpet coupons in 15 s, indicating virucidal efficacy of this approach. Specifically, steam vapor achieved a >3.0 $\log_{10}$ $TCID_{50}$ reduction of BCoV and >3.2 $\log_{10}$ $TCID_{50}$ of HCoV OC43 on nylon carpet. Mechanical scrubbing alone resulted in a 0.2 $\log_{10}$ $TCID_{50}$ reduction for both BCoV and HCoV OC43.

Carpet absorption capacity

PET fiber (0.1g) absorbed up to 0.65-0.70 ml of safranin solution (Table 4.3). In contrast, 0.1 g of nylon fibers reached saturation, retaining only 0.50-0.55 ml of safranin solution. When 0.55 ml of safranin solution was added, nylon fibers had a greater amount of residual liquid than did PET fibers. PET fibers tested exhibited a higher degree of hydrophilicity compared to nylon fibers.

Discussion

The persistence of two SARS-CoV-2 surrogates on PET and nylon carpet and the efficacy of steam disinfection of both surrogates on nylon carpet was studied. In our study, more viable BCoV was recovered than HCoV OC43 from both carpet types, while

BCoV was more resistant to desiccation on surfaces with a slower loss in infectivity. The hydrophobic PET carpet had significant loss of infectivity for both surrogates and possible viral capsid damage, highlighting that infection assays are more accurate in assessing coronavirus infectivity loss than RT-qPCR assays. Our persistence results suggest the use of disinfection protocols may be unnecessary after the contamination of PET carpet by coronaviruses due to the rapid loss of infectivity. Conversely, both viruses declined slowly on nylon carpet. Lastly, steam vapor was efficacious enough to eliminate both surrogates within 15 s, indicating the potential of steam vapor as a rapid and effective disinfectant against SARS-CoV-2 on porous surfaces like nylon carpet.

Coronaviruses including SARS-CoV-2 are hard to recover from environmental surfaces, with the recovered titer affected by the surface material and recovery methods (21). For example, Riddell and colleagues (5) recovered viable viruses by repeated pipetting with an approximate 3-log loss from cotton cloth. In addition, studies also revealed the adverse effect of recovery media, composition of surfactants and elution methods on the recovery rate of coronaviruses (26). To our knowledge, no study has reported the recovery of coronaviruses from carpet. This is possibly attributed to the fact that surfactants and mechanical agitation used for recovery could chemically and physically affect the phospholipid layer and spike proteins on viral envelopes (21). The envelope structure of coronaviruses plays a critical role in its attaching and entering host cells, hence, any damage to the envelope structure including the phospholipid layer and spike proteins might lead to loss of infectivity (21, 27). In our study, BCoV and HCoV OC43 were successfully recovered, with less than a 1-$\log_{10}$ $TCID_{50}$ reduction from PET

and nylon carpet using the method reported previously for norovirus (17). In addition, more HCoV OC43 was lost in recovery than BCoV, suggesting BCoV was more resistant to the recovery method.

SARS-CoV-2 has been shown to survive 1-7 days at 20-30°C on cotton cloth, whereas HCoV OC43 survived for 2 days at room temperature (5, 28). In contrast, HCoV OC43 was less persistent on PET fabrics -- >3 $\log_{10}$ $TCID_{50}$ reduction after 1 h (29) at 35°C. A similar result was observed in our study. Infectious BCoV and HCoV OC43 were rapidly inactivated on PET carpet, reaching the detection limit within one hour. In comparison, both surrogates survived on nylon carpet with ≤2 $\log_{10}$ $TCID_{50}$ reduction of viable viruses after two hours. Different levels of persistence of these two viable surrogates were also observed on plastic and vinyl surfaces in a previous study (30). The correlation between persistence of coronaviruses and surface material and construction was explained by the fact that coronaviruses were rapidly inactivated on carpet fabric with a faster water absorption (29), which our absorption data also supported. Hydrophilic synthetic fibers have been a source of viruses due to larger area of exposure surfaces to desiccation, whereas the surface hydrophobicity promotes virus aggregation and concentration to protect viruses within the aggregates (6, 31). Additionally, the rate of decline for unexposed gRNAs was significantly slower than that of total gRNAs for both BCoV and HCoV OC43 on PET carpet. This difference was not observed on nylon carpet. These results also confirmed that nylon fibers are more hydrophobic than PET fibers (Table 3), subsequently promote a high level of desiccation on PET carpet as

discussed above (6). As a result, surrogate capsids are more likely to be damaged on PET carpet than on nylon carpet.

Infectivity assays are not always used in studies regarding non-porous and porous environmental surfaces, partially due to the challenges associated with the recovery of infectious viruses from surfaces (21, 32, 33). In our study, the reduction of surrogate gRNA on PET carpet occurred at a slower rate (<1 $\log_{10}$ gc h^{-1}) than did the decline in viral infectivity (>2.8 $\log_{10}$ $TCID_{50}$ h^{-1}) at room temperature. SARS-CoV-2 gRNAs are encased within viral capsids, protecting the structure (34). Environmental factors can facilitate the disruption of viral envelopes and capsids before acting upon the gRNAs. As such, relying solely on gRNA detection may not accurately reflect the persistence of SARS-CoV-2 and its disinfection efficacy.

Interestingly, we found that unlike infectivity, total gRNAs of HCoV OC43 degraded slower than BCoV on both PET and nylon carpet (Figure 2A-B). This is likely due to the capsid protein of HCoV OC43 being more resistant to desiccation. As most coronaviruses only share 43% identity on the structural protein-coding region (35), it is not surprising that the HCoV OC43 capsid is more resistant than BCoV to desiccation but still within the same order of magnitude. Apart from structural proteins, HCoV OC43 shared similar spike proteins with BCoV, particularly both viruses having a deletion within the S1 subunit of the spike protein (36). While oxidation-sensitive amino acids (i.e., tyrosine, tryptophan and histidine) are abundant in the receptor binding domain of the spike protein of HCoV OC43(37), the spike proteins of HCoV OC43 are more

sensitive than BCoV to oxidation (37), resulting in the significant loss of infectivity for HCoV OC43 when drying on PET carpet.

Heat is associated with persistence and disinfection efficacy of coronaviruses. More than 3 $\log_{10}$ $TCID_{50}$ of Middle East respiratory syndrome coronavirus (MERS-CoV), SARS-CoV, and SARS-CoV-2 were reduced in cell culture medium when exposure to temperatures ≥60°C was as short as 15 minutes (38-40). However, such heat inactivation of coronaviruses has been investigated in suspension only. Because HCoV OC43 and BCoV were reduced below the detection limit during a 1-hour desiccation on PET carpet, we investigated the efficacy of steam vapor against both coronavirus surrogates only on nylon carpet. Steam vapor was efficacious against both surrogates on nylon carpet, achieving a >3-$\log_{10}$ $TCID_{50}$ reduction within 15 s. This robust virucidal activity can be attributed to the potential of steam vapor to reach temperatures as high as 99.44±0.98°C on carpet. However, it's important to acknowledge that the efficacy of disinfectants including steam vapor could be influenced by other factors (41). Specifically, fiber construction, including characteristics like looped or pile cut, materials employed, and fiber length, all could affect the performance of steam vapor (17). Due to the scope of this study, we were unable to comprehensively evaluate these factors, leaving room for further investigation in subsequent research.

Conclusion

In summary, this study examined the persistence of two SARS-CoV-2 surrogates, BCoV and HCoV OC43 on PET and nylon carpet. Our results revealed that more viable

BCoV was recovered than HCoV OC43 from both carpets. Moreover, viable surrogates were rapidly inactivated on PET carpet, but relatively stable on nylon carpet. The infectivity assay was validated to be a better approach than the qPCR method to accurately determine persistence of both surrogates and disinfection efficacy against them. Furthermore, we confirmed that steam vapor is an effective disinfectant against both surrogates on nylon carpet. However, the results might not be well-translated to other types of carpet due to different characteristics of carpet material. Additionally, our results need to be confirmed with SARS-CoV-2, as only surrogates were tested.

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Table 4.1. First-order decay rate constants *k* for total gRNAs and unexposed gRNAs of BCoV and HCoV OC43 on different carpet materials.

	Decay rate constants k (h^{-1}) [a]			
	Total gRNA		Unexposed gRNA	
Virus	PET carpet	Nylon carpet	PET carpet	Nylon carpet
BCoV	$1.19\pm0.62^{A/A}$	$0.86\pm0.29^{A/A}$	$0.61\pm0.20^{A/A}$	$0.84\pm0.21^{A/A}$
HCoV OC43	$0.67\pm0.30^{A/A}$	$0.27\pm0.14^{A/A}$	$0.28\pm0.33^{A/A}$	$0.43\pm0.20^{B/A}$

[a] Data are expressed as mean ± standard deviation (SD), calculated based on triplicates at each of the five sampling time intervals. Values with different letters within/across carpet type (e.g., A/A, B/A) for the total gRNA or unexposed gRNA indicate significant difference in Tukey test grouping.

Table 4.2. Efficacy of steam vapor against two coronavirus surrogates on nylon carpet.

	Reduction (log_{10} $TCID_{50}$) [a]	
Process	BCoV	HCoV OC43
Desiccation	0.4 ± 0.3^{A}	0.6 ± 0.4^{A}
Scrub	0.2 ± 0.2^{A}	0.2 ± 0.1^{A}
Steam vapor	$>3.0^{B}$	$>3.2^{B}$

[a] Data are expressed as mean ± standard deviation (SD) from triplicates (desiccation and scrub) and five replicates (steam vapor) at each of 2 independent experiments. Values with different letters within the same column (e.g., A, B) indicate a significant difference in Tukey test grouping.

Table 4.3. Absorptive capacity of carpet fibers.

Sample[a]	Vol added (ml)	Residual wt (μg)[b]
PET	0.500	6.2 ± 1.3^{A}
	0.550	7.2 ± 1.8^{AB}
	0.600	8.5 ± 2.3^{AB}
	0.650	12.5 ± 5.2^{BC}
	0.700	15.3 ± 5.2^{C}
Nylon	0.400	5.7 ± 1.0^{A}
	0.450	8.8 ± 1.8^{A}
	0.500	9.7 ± 2.5^{AB}
	0.550	16.3 ± 6.0^{B}
	0.600	23.5 ± 6.9^{C}

[a] Carpet fiber samples were 0.1 g each.

[b] Data are expressed as mean ± standard deviation (SD) from triplicates at each of 2 independent experiments. Values of residual weight with different letters for the same fiber (e.g., A, B) indicate significant difference in Tukey's test grouping.

Figure 4.1. Infectious coronaviruses on PET (A) and nylon (B) carpet. The data are expressed as mean ± standard deviation (SD) from six replicates in two trials. Dotted lines indicate the detection limit at 2.6 $\log_{10}$ $TCID_{50}$.

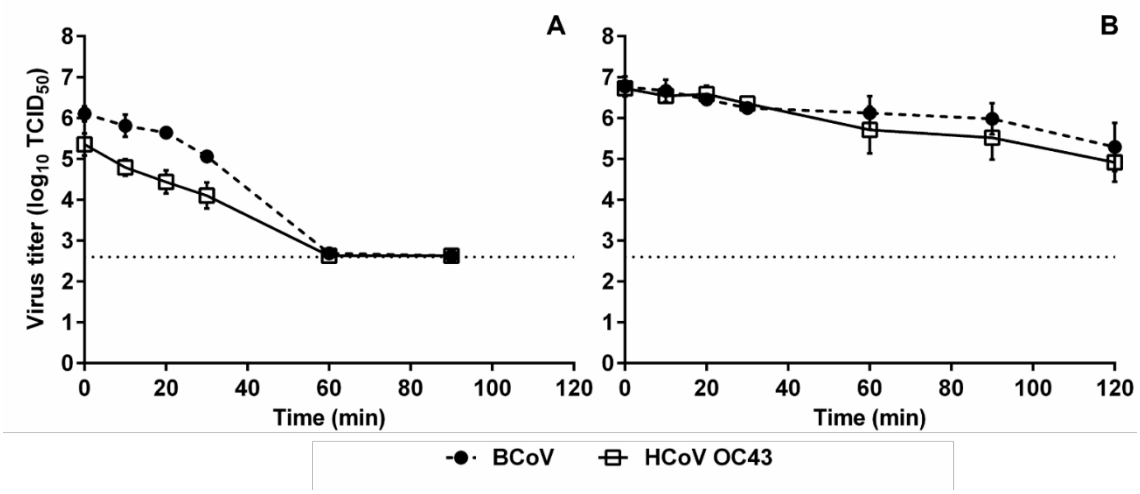
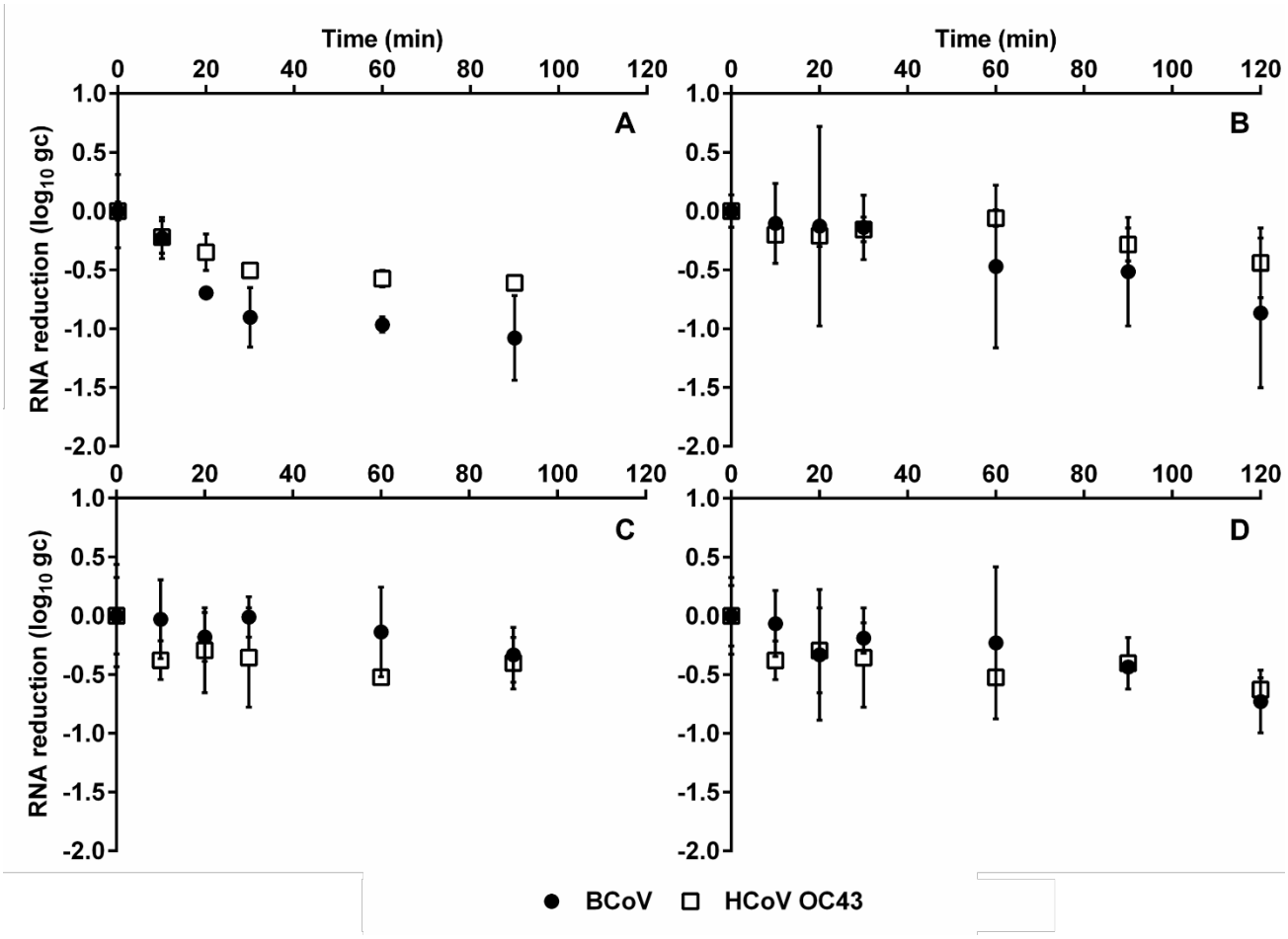


Figure 4.2. RNA copy reduction of BCoV and HCoV OC43 on PET (A) and nylon (B) carpet, and RNase I-treated BCoV and HCoV OC43 on PET (C) and nylon (D) carpet. The data are expressed as mean ± standard deviation (SD) from six replicates in two trials.



EFFICACY OF THREE EPA-REGISTERED CHEMICAL DISINFECTANTS AND STEAM VAPOR AGAINST *CLOSTRIDIOIDES DIFFICILE* ENDOSPORES ON NYLON CARPET WITH TWO DIFFERENT BACKING SYSTEMS

Abstract

Aims: Determining the efficacy of three chemical disinfectants and steam vapor against *Clostridioides difficile* endospores on nylon carpet with two different backing systems.

Methods and Results: We first optimized the recovery method for *C. difficil*e endospores from carpet, using different concentrations of Tween-80 and for different stomaching times. Then the efficacy of three EPA-registered disinfectants (two H_2O_2-based and one chlorine-based) and steam vapor was determined against *C. difficile* endospores on nylon carpet with two types of backing (water-permeable and waterproof). The addition of 0.2% Tween-80 followed by stomaching for 3 min and sonication improved the recovery rate of *C. difficile* endospores to >60%. Among three chemical disinfectants, product B was the most efficacious with 5.8 and 4.9 $\log_{10}$ CFU reduction of *C. difficile* endospores in 30 min on carpet with water-permeable backing and waterproof backing, respectively. Steam vapor for 120 s showed a 4.9 and >6.0 $\log_{10}$ CFU reduction of *C. difficile* endospores on carpet with water-permeable and waterproof backings, respectively. On carpet with water-permeable backing, 120-s steam vapor treatment followed by product A reduced 6.1 $\log_{10}$ CFU of sensitized *C. difficile* endospores.

Conclusions: This study established a high-recovery-efficiency protocol for *C. difficile* endospore to assess the efficacy of carpet disinfection. Notably, the carpet

backing played a role in influencing the efficacy of chemical disinfectants and steam vapor against *C. difficile*. Steam vapor was efficacious against *C. difficile* on carpet with waterproof backing, whereas only product B showed some efficacy. Moreover, combining steam with chemical disinfectants proved to enhance the potency of weaker disinfectants against sensitized *C. difficile* endospores.

Importance: The findings provide validated data to inform the development of disinfection guidelines for carpet to prevent healthcare-associated infections caused by *C. difficile*.

Introduction

Clostridioides difficile, a gram-positive spore-forming anaerobic bacterium, is the leading cause of healthcare-associated infections (HAIs) (1). Due to its antibiotic resistance, patients with *C. difficil*e infections (CDIs) progressively increased the mortality rate to 40.9% compared to 7.4% among patients without CDIs in healthcare settings (2). Healthcare-associated CDIs are generally transmitted through contacting the contaminated surfaces (3). Despite the common understanding that high-contact surfaces play a significant role in pathogen transmission, floors are often overlooked and receive limited attention, partially due to the lack of guidance for the disinfection of floors (3, 4). However, some reports suggested floors, especially carpeted floors, as possible reservoirs of *C. difficile* endospores in healthcare settings such as hospitals and long-term care facilities (LTCFs) (5, 6). Moreover, recent epidemiological evidence indicated that pathogens could move from the floor to hand-contact surfaces in healthcare facilities via

air movement (7, 8). Therefore, adequate cleaning and disinfection of floors is needed in healthcare settings to prevent recurrent CDIs among patients.

Floors used in hospitals and LTCFs are constructed with either non-porous (e.g., vinyl tile, rubber) or porous (e.g., nylon carpet) floorings (9). As previously reported, porous surfaces, such as carpet, are more challenging to clean and disinfect than non-porous surfaces due to their complex three-dimensional structures and the ability to absorb dust, microbes, and moisture, allowing pathogens to persist in the fibers of carpet (10). Although vacuum cleaning can remove dust and moisture from carpet, mechanical agitation may facilitate pathogens spreading by resuspending pathogens attached to the loosened carpet fibers and dust to the air (11, 12). Additionally, bacterial spores, due to their small size and lightweight nature, are more likely to be resuspended into the air from carpet compared to polyvinyl chloride flooring (11). This is likely due to the greater propulsion of lightweight spores that occurs upon stretch after deflection of relatively loose fibers in carpet (11). This increased potential for airborne transmission of bacterial spores from carpet highlights the importance of developing effective cleaning and disinfection protocols for carpet. Since carpet are non-launderable porous surfaces that cannot be removed, cleaned and disinfected by laundry with chlorine-based disinfectants (13), effective on-site cleaning and disinfection methods are needed for carpet floorings.

The USA Environmental Protection Agency (EPA) maintains lists of disinfectants designed for use on inanimate surfaces, such as hard non-porous surface and soft porous surfaces, but a few commercial disinfectants were specifically formulated for non-launderable soft porous surfaces (13, 14). According to the EPA's list of registered

disinfectants, the two major types that have been proven efficacious against *C. difficile* endospores are chlorine-based and hydrogen peroxide-based disinfectants (15). However, the use of chlorine-based products at high concentrations or over extended periods can result in damage to surfaces (16). Hydrogen peroxide-based disinfectant sprays reported effectively against human norovirus and *C. difficile* endospores on non-porous and porous surfaces could be a potential alternative to chlorine-based disinfectants in consideration of occupational safety and exposure hazards (17). Moreover, those H_2O_2-based disinfectants don't visibly damage fabrics, whereas chlorine-based disinfectants can bleach fabrics (18, 19). In addition to chemical disinfectants, physical methods, such as steam vapor, can be sporicidal. While EPA does not regulate the efficacy of steam-vapor generation devices, our previous study has shown that steam vapor could efficaciously inactivate human norovirus surrogates on carpet without visible damage (20). However, to date, there has been no research investigating the application of steam vapor against *C. difficile* endospores on non-launderable soft surfaces.

The type of fiber used was commonly a focus of research in microbial persistence and disinfection studies on fabrics. While various fibers may have distinct effects on the persistence of pathogens and disinfection efficacy, nylon carpet are the most popular in LTCFs due to their durability, cost-effectiveness, and functionality (21, 22). For example, looped and cut fibers affected the persistence of bacteriophages Phi6 and MS2 on carpet differently (23). The backing of a carpet is another essential component in its construction, as it provides stability and support to the carpet fibers (24). In commercial carpet, the backing is typically waterproof and made of materials such as polyvinyl

chloride or polyurethane, whereas residential carpet commonly use latex backing which is water-permeable (24). However, the effect of carpet backing type on microorganisms and chemicals is not yet understood (24). To our best knowledge, the disinfection efficacy of WPerB and WProB of carpet has never been compared (25).

As the endospores are notoriously resistant to environmental stress, a few studies have explored the strategies for sensitizing spores prior to disinfection steps (26-28). These approaches aim to enhance the susceptibility of endospores to subsequent disinfection procedures (28). Notably, the sensitization of *C. difficile* endospores has demonstrated the effect of rendering them more vulnerable, even to those disinfectants with relatively weaker efficacy (27).

In this study, we improved the recovery method of *C. difficile* endospores from carpet, and conducted disinfection efficacy studies on carpet surfaces with two types of carpet backing systems using three EPA-registered disinfectants and a commercial steam cleaner to treat nylon carpet. Additionally, we explored the possibility of combining steam vapor and weak disinfectant to effectively inactivate *C. difficile* endospores.

Materials and methods

Preparation and purification of C. difficile endospores

C. difficile ATCC 43593 (toxic A-, toxic B-) was employed in most tests in this study, while two other strains *C. difficile* ATCC BAA2155 (toxic A+, toxic B+) and *C. difficile* Isolate #96 (toxic A+, toxic B+) (isolated previously in our lab from food waste) were only tested in sensitization of *C. difficile* endospores (29). All three strains of *C.*

difficile were cultured on modified brain heart infusion agar plates containing 5 g l^{-1} yeast extract, 1 g l^{-1} cysteine, and 1 g l^{-1} sodium taurocholate (BHIA/YE/CYS/T) and incubated inside an anaerobic chamber (BactronEZ, Sheldon Manufacturing, OR, USA) at 37°C for 7 days as previously described (17). Then, all plates were sealed with parafilm (Pechiney, IL, USA) and incubated at ambient conditions for another 7 days. Each agar plate was then flooded with 5 ml of 0.01 M phosphate buffered saline (PBS) with 0.1% Tween-80, and the colony mass was scraped from the agar plates using sterile cotton swabs. The cell suspension was washed 5 times with ice-cold sterile deionized (DI) water, followed by centrifugation at 7,000 × g for 5 min at 4°C. Vegetative cells of *C. difficile* were removed by gradient centrifugation in 50% (w/v) sucrose solution (30), and the endospore suspension was washed 3 times with sterile ice-cold water. The concentration of endospores was enumerated on BHIA/YE/CYS/T plates, and the purity of prepared endospores was confirmed under a microscope after endospore staining. The stock culture of *C. difficile* endospores at ca. 10^8 colony-forming unit (CFU) ml^{-1} was stored at 4°C for routine tests and at -80°C for long-term storage. Before the recovery optimization and efficacy test, *C. difficile* endospores (ATCC 43593) were prepared with the soil load by mixing 340 µl of endospore suspension with 25 µl of a 5% bovine serum albumin (BSA), 35 µl of 5% yeast extract and 100 µl of 0.4% bovine mucin according to ASTM E2197-17 (31).

Selection of disinfectants and preparation of carpet coupons

One chlorine-based and two H_2O_2-based disinfectants (Table 5.1) were selected based on the effectiveness against *C. difficile* endospores from a previous study (17), although all three products don't have disinfection claim against *C. difficile* endospores on carpet. To test the efficacy of steam cleaners, a household steam cleaner (IVASTEAMR20, Ivation, NJ, USA) was chosen for this study. Specifically, the steam cleaner had a 1.8-liter water tank and can reach a maximum temperature of 170°C and a maximum pressure of 500 kPa. The nylon loop pile carpet Color Accent® (Shaw Inc., GA, USA) was selected according to the Carpet and Rug Institute Test Method 112 (32). To compare the effect of carpet backings, another nylon loop pile carpet, Highlight® (Shaw Inc., GA, USA), was chosen to have almost the exact specifications of Color Accent® except for the backing (Table 5.2). Color Accent® has a water-permeable backing (WPerB), whereas Highlight® has a waterproof backing (WProB). Although both carpet contained anti-soil coatings, the carpet were confirmed as free of sporicidal activities. The carpet were cut into 5×5 cm^2 coupons with a mechanical cutting die (Model 1500, Freeman Schwabe, OH, USA) (kindly provided by Dr. Daniel Price, Interface Inc., GA, USA) then dusted by hand to remove loose fibers. Before the tests, the carpet coupons wrapped in aluminum foil were autoclaved on a 30-min dry cycle and cooled at room temperature overnight.

Recovery optimization of C. difficile endospores from nylon carpet

As the addition of Tween-80, a common surfactant, can improve the recovery rate of *C. difficile* endospores from carpet (25), the concentration of Tween-80 in the neutralizer was optimized. Each of the 6 pre-cut coupons received 100 µl *C. difficile* spore suspension (~10^8 CFU ml^{-1}) with or without soil load and then dried for 1 h under ambient conditions inside a biological hood; relative humidity was maintained at 40±10% in the lab and confirmed by a humidity meter (VWR, PA, USA). After drying, three coupons were immediately transferred into a flask with 100 ml of PBS plus 0.02%, 0.1%, and 0.2% Tween-80, respectively. All flasks were ultrasonicated for 1 min at 40 kHz (FS110, Fisher Scientific, PA, USA) and then vigorously shaken at 250 rpm in a shaking incubator (New Brunswick Scientific C25, NJ, USA) for 15 min, followed by *C. difficile* endospore enumeration on BHIA/YECYS/T plates.

Our preliminary study showed that the combination of sonication and shaking could not effectively recover *C. difficile* endospores from the carpet. Therefore, a stomacher (Seward, NY, USA) was employed to improve the recovery rate of *C. difficile* endospores by optimizing the stomaching time. Each of the 6 pre-cut coupons received 100 µl *C. difficile* spore suspension and dried as described above, and the dried coupons were immediately transferred into a flask with 100 ml of PBS plus 0.2% Tween-80. All flasks were ultrasonicated for 1 min at 40 kHz and then vigorously stomached at 200 rpm in a stomacher for 1-, 2-, and 3-min. *C. difficile* endospore population from the inoculum suspension and all carpet coupons were enumerated on BHIA/YE/CYS/T plates.

Sensitization of C. difficile endospores attached to the stainless steel

A previous study reported that quaternary ammonium compounds inactivated more sensitized *C. difficile* endospores than dormant *C. difficile* endospores (27). Sensitization of *C. difficile* endospores were explored in this study. Specifically, a germinant solution with 0.5% sodium taurocholate, 50 mM glycine and 60 mM calcium chloride were mixed with ca. 10^6 CFU ml^{-1} *C. difficile* endospores. Then 10 µl of above mixture was applied to a stainless steel disc (Ø=1 cm) and incubated at room temperature aerobically and anaerobically. After 0, 60, and 120 min, the inoculum was recovered in 1 ml of PBS with 0.2% Tween-80 by sonication for 30 s and repeated pipetting from the stainless steel disc. Meanwhile, the difference among *C. difficile* strains (ATCC 43593, BAA 2155, and Isolate #96) were also compared by incubating aerobically. The sensitized *C. difficile* endospores were enumerated as vegetative cells on BHIA/YE plates without germinant.

Efficacy test on carpet

As no standard efficacy testing method was available for carpet, the efficacy of chemical disinfectants and the steam vapor against *C. difficile* endospores on carpet was evaluated as previously described with some modifications (20). In brief, each pre-cut coupon received 100 µl of *C. difficile* endospore suspension, and then dried for 1 h under ambient conditions with relative humidity being monitored and controlled at 40±10%. After drying, unscrubbed coupons (n=3) were immediately transferred into a flask with 100 ml of PBS plus 0.2% Tween-80. Each of treatment coupons (n=5) received about 6

ml of disinfectants which was sprayed for ca. 8 s on each carpet coupon using Preval sprayers (Nakoma, IL, USA). Then coupons were scrubbed clockwise and counterclockwise for 30 s each with a disinfectant-saturated (approximately 1 ml) surgical scrub brush (E-Z Scrub, Becton Dickinson, NJ, USA) and left at ambient conditions for a contact time of 30 min. For scrubbed control, the disinfectant was replaced by PBS. After 30 min contact time, scrubbed control coupons and testing coupons were transferred into a flask with 100 mL of appropriate neutralizers plus 0.2% Tween-80.

As for the steam vapor treatment, the head of the steam cleaner was wrapped with sterile terry cloth and folded into 4 layers The head was saturated by the steam for 10 s before each application, and then coupons were scrubbed vertically for a contact time of 30, 60, and 120 s with steam, respectively. For the scrubbed controls, coupons (n=3) were scrubbed by the wrapped head of steam cleaner without heat for 120 s. Scrubbed control coupons (n=3) and treatment coupons (n=5) were immediately transferred into a flask with 100 ml of PBS plus 0.2% Tween-80. All flasks were ultrasonicated for 1 min at 40 kHz and then vigorously stomached at 200 rpm for 3 min. *C. difficile* endospore population from the inoculum suspension and all carpet coupons were enumerated on BHIA/YECYS/T plates.

To increase the disinfectant efficacy, thus, a combination approaches i.e., applying product A for 30 min then followed by treatment with steam for 60 s and the reverse application order was explored on Color Accent® as described above. Three replicates of treatment coupons were used for each trial.

Measurements of carpet treated by the steam cleaner

During steam vapor treatment of carpet, there was condensed water under the carpet. The condensed water was measured by weighing carpet coupons before and after 120 s-steaming. Meanwhile, temperatures above and below the carpet backing were immediately measured in triplicates by type T thermocouples (HotMux, DCC Corporation, NJ, USA) and recorded every 3 s. All above experiments were repeated once.

Statistical analysis

Ten replicates of each product were tested in two independent efficacy tests on carpet. Reductions were calculated by $\log_{10}(N_0/N_d)$, where N_d is the average *C. difficile* endospore population from the treatment samples and N_0 is the average *C. difficile* endospore population from the scrubbed control samples. Statistical analysis was performed using a one-way multiple-comparison ANOVA to determine the relationship between disinfectants and reduction. All results were expressed as mean ± standard deviation. Statistical significance was defined as a p-value of <0.05 to establish a more conservative estimate of efficacy. Statistical analyses were conducted using GraphPad Prism 6.01 (GraphPad Software, Inc., CA, USA).

Results

Recovery optimization of C. difficile endospores from nylon carpet

Both Tween-80 concentration and stomaching time were optimized for recovering *C. difficile* endospores from nylon carpet at the presence and absence of soil load. After ultrasonication, the recovery rates of *C. difficile* spores with 0.02%, 0.1% and 0.2% Tween-80 were 11.3%, 15.7%, and 20.8% without soil load and 10.9%, 14.7%, and 18.9% with soil load, respectively (Table 5.3). Since the recovery rates for 0.2% Tween-80 were significantly higher than other concentrations at the presence or absence of soil load, 0.2% was used for further studies. By incorporating the stomaching step, the recovery rates with sonication followed by stomaching for 1, 2, and 3 min at 200 rpm were increased to 59.8%, 72.1%, and 89.4% in the absence of soil load, respectively, and 45.3%, 64.2%, and 66.5% in the presence of soil load, respectively (Table 5.4). A longer stomaching time (3 min) significantly improved recovery efficiency compared with 1 min.

Sensitization of C. difficile endospores attached to the surfaces

For freshly prepared spores, *C. difficile* ATCC 43593, ATCC BAA2155 and Isolate #96 contained 2.9%, 2.6% and 2.1% vegetative cells, respectively.

Upon the attachment to stainless steel coupon for 60 min at room temperature under aerobic conditions, 48.8% of *C. difficile* endospores were sensitized (Figure 5.1A). However, the sensitization was reversible, as only 13.5% of *C. difficile* endospores remained sensitized after an extended 120-min period. Under anaerobic conditions, a

higher rate of spore sensitization was observed after 60 min, with 59.9% of *C. difficile* endospores sensitized. Additionally, 55.9% of *C. difficile* endospores maintained their sensitized status after the prolonged 120-min period.

The sensitization efficiency varied among strains when incubated aerobically (Figure 5.1B). After incubating on stainless steel coupon for 60 min, 48.8%, 49.9% and 52.3% of *C. difficile* ATCC 43593, ATCC BAA2155 and Isolate #96 were sensitized endospores, respectively. But only 13.5%, 9.2% and 14.1% of *C. difficile* ATCC 43593, ATCC BAA2155 and Isolate #96 remained sensitized after 120 min, respectively.

Efficacy of chemical disinfectants and steam vapor against C. difficile endospores on carpet

Hydrogen peroxide-based products A and B inactivated 0.9 and 5.8 $\log_{10}$ CFU of *C. difficile* endospores on Color Accent® with 30-min contact time, respectively, as compared to 0.7 and 4.9 $\log_{10}$ CFU reduction of *C. difficile* endospores on Highlight® carpet, respectively (Figure 5.2). Chlorine-based product C inactivated more *C. difficile* endospores on Color Accent® with a 1.2 $\log_{10}$ CFU reduction, compared to 0.4 $\log_{10}$ CFU on Highlight®.

The steam vapor reduced 3.3, 3.7, and 4.9 $\log_{10}$ CFU of *C. difficile* endospores on Color Accent® after a contact time of 30, 60, and 120 s, respectively (Figure 5.3). In contrast, the steam reduced 2.8, 3.8, and >6.0 $\log_{10}$ CFU of *C. difficile* endospores after 30, 60, and 120 s on Highlight® carpet, respectively.

As shown in Figures 2 and 3, either disinfectants or steam vapor alone couldn't achieve a 6 $\log_{10}$ CFU reduction of *C. difficile* endospores on Color Accent®. To increase disinfection efficacy of product A on Color Accent®, a combination of product A with steam vapor was tested against the sensitized *C. difficile* endospores. A 60-s steam treatment followed by a 30-min treatment using product A was effective in reducing 5.8 $\log_{10}$ CFU of sensitized *C. difficile* endospores on Color Accent® (Figure 5.4). Conversely, the efficacy of the combination using the reverse order was weakened to a 4.1 $\log_{10}$ CFU reduction of sensitized *C. difficile* endospores. Furthermore, when extended the exposure time of steam vapor to 120 s, above combination treatments improved the disinfection efficacy of sensitized *C. difficile* endospores, as steam vapor followed by product A treatment and the reverse order with a 6.1 and 4.4 $\log_{10}$ CFU reduction, respectively.

Effect of steam vapor on carpet

After 120 s steaming, 0.57 and 0.69 g of water were condensed on Color Accent® and Highlight®, respectively (Figure 5.5A). The condensed water was more obvious under Color Accent® than under Highlight®. The temperature increased rapidly, and reached and maintained at 99.26 and 99.36°C above the backing of Color Accent® and Highlight® between 3-120 s, respectively (Figure 5.5B). Below the backing of the Color Accent® and Highlight®, the maximum temperature reached 81.83 and 70.86°C, respectively. Clearly, the temperature below the backing of Color Accent® was higher than Highlight® during the 120 s exposure time, suggesting the passing of steam through

the backing of Color Accent®. When the carpet was treated with steam vapor before product A treatment, the maximum temperature reached 77.95°C below the backing of Color Accent® during the 60 s contact time (Figure 5.5C). However, when the order of treatment was reversed, with product A applied before steam vapor, the maximum temperature achieved was slightly cooler at 74.96°C.

Discussion

Efficacy testing of disinfectants on carpet can be challenging due to complex structures of the carpet and low recovery of tested microorganism. In this study, first we optimized the experimental protocol to recover *C. difficile* endospores from soft porous surfaces, then we evaluated the efficacy of three EPA-registered disinfectants against *C. difficile* endospores on carpet with two types of backing. Only one chemical product (product B) was efficacious against *C. difficile* endospores on carpet in 30 min. A commercial steam cleaner as an alternative to chemical disinfectants has shown the potential for effective disinfection of carpet. To improve the disinfection efficacy, either the combination of chemical and physical approaches or sensitization of *C. difficile* endospores were explored.

The efficacy of several disinfectants (i.e., H_2O_2 vapor, gaseous plasma, and UV-C radiation) has been evaluated against *C. difficile* endospores on a hard surface, but it is challenging to study the efficacy on porous surfaces due to the lack of appropriate recovery method (33-36). Sadowsky et al. (25) reported that the addition of a surfactant, such as Tween-80, in the buffer can significantly enhance the recovery of *C. difficile*

endospores from roughly 10% to 39.8% from olefin carpet. Similarly in this study, adding Tween-80 increased the recovery rate from 8% to 22% from nylon carpet. On the basis of another study (37), we improved a sample preparation method using sonication and a stomacher to vigorously detach *C. difficile* endospores from the carpet samples. Our optimized recovery method (0.2% Tween-80, sonication and stomaching) with over 60% recovery rate was more effective than the previous studies which had <40% of recovery rate (25, 38).

Furthermore, our results revealed that the presence of soil load decreased the recovery rate of *C. difficile* endospores from nylon carpet. This finding implies that a considerable amount of *C. difficile* endospores may persist on carpet following inadequate cleaning procedure, rendering inactivation of *C. difficile* endospores to an undetectable level more challenging during disinfection. Additionally, the presence of soil load also significantly weakens the efficacy of surface disinfectants(39). This highlights the necessity of a thorough cleaning prior to disinfection procedure to ensure the efficacy of disinfectants.

Carpet fibers are generally bound to a primary backing (Figure 5.6A), and then attached to another functional secondary backing to meet different application scenarios. Healthcare settings (e.g., clinics and nursing homes) usually select nylon carpet for a comfortable environment with a WProB for easy cleaning and preventing floor damage (40). Though some studies revealed that the role of fibers in attracting dust and affecting the efficacy of disinfectants (20, 41, 42), none has reported the effects of carpet backing system on the disinfection efficacy.

C. difficile endospores were found to be more sensitive to chemical disinfectants on carpet with WPerB (Color Accent®) compared to WProB (Highlight®) . This difference in sensitivity might be due to some differences in the distribution of microorganisms and disinfectants on the carpet. The fibers in carpet backings tended to bind in clusters, which did not fully cover the entire backing (Figure 5.6B). According to Carr et al. (43), the high content of water (50% dry weight of carpet) was retained in carpet fibers with backing received less liquid due to capillary action. As a result, some of the primary backings might not receive chemical disinfectants during the application of disinfectants. On the other hand, a study revealed that blood drops penetrated fibers and even into the primary backing, which could suggest microorganism inoculum could also penetrate through fibers to backings due to the size of droplets other than the capillary action (43, 44). Moreover, the hydrophobic *C. difficile* endospores can attach and be absorbed by the primary layer of a more hydrophobic and WProB used in Highlight® carpet (Figure 5.6C). Thus, *C. difficile* endospores resided in the backing layer of Highlight® carpet were barely accessible by disinfectants, and then shielded from the action of disinfectants.

Steam vapor effectively acted as a biocide due to its high temperature and penetration, which kills microorganisms (45). In addition, the heat transfer to suspensions or surfaces led to indirect inactivation of the microbes (46). For example, steam vapor has been reported as efficacious against norovirus, molds and antibiotic-resistance bacteria on carpet and other hard surfaces (20, 46-48). Moreover, some studies reported that *C. difficile* endospores can be inactivated by steam vapor on stainless steel carriers or

temperature at ≥75°C in suspension (49, 50). But this study is the first one reporting the efficacy of steam vapor against *C. difficile* endospores on carpet. Different efficacies were observed on two types of carpet because of the interactions between steam and carpet backings. When the steam first contacted a dried carpet coupon, it quickly penetrated carpet fibers and heated the backing, while fraction of the steam condensed on carpet backings and fibers. Based on the temperature monitoring results and condensed water measurements (Figure 5.5), the steam quickly went through the backing of Color Accent®, while the Highlight® carpet's WProB could block the steam flow through the backing and retain more heat and condensed water within the carpet. As a result, *C. difficile* endospores are more inactivated on carpet with WProB (Highlight®) than WPerB (Color Accent®) (Figure 5.6C).

We also observed a mixed effect in the efficacy of steam vapor against *C. difficile* endospores when used in conjunction with the application of product A on Color Accent®, compared to using either method alone. This effect likely stems from the distinct distribution patterns of steam vapor and aqueous disinfectants on carpet (Figure 5.6). Applying steam vapor could reach most of the *C. difficile* endospores on the carpet in a short time. In contrast, product A was able to stay in carpet fibers and most of backings for ensuring a prolonged contact time. However, the reversed application order led to a reduction in the maximum temperature attainable on the carpet. This decrease in temperature was caused by the presence of product A, an aqueous disinfectant, on the carpet, subsequently weakened the efficacy of the steam vapor treatment. Therefore,

steam vapor should be used prior to chemical disinfectants to achieve higher levels of efficacy.

As vegetative *C. difficile* cells are more sensitive to disinfectants than endospores, Nerandzic et al. reported sensitizing *C. difficile* endospores to increase the efficacy of weak disinfectants (27). In agreement with the previous study, sensitization of *C. difficile* endospores increased the efficacy on Color Accent® according to our results, in addition to the combination of physical and chemical disinfectants (27). Moreover, the sensitization efficiency was different between aerobic and anaerobic conditions and among *C. difficile* strains (51, 52). Our findings align with previous research (52), which demonstrated a greater sensitization of *C. difficile* endospores on stainless steel surfaces under anaerobic conditions. However, it is important to note that creating anaerobic conditions for surface disinfection in real-world environments is not practical. Additionally, our studies confirmed that the efficiency of the germinants in sensitizing *C. difficile* endospores varies among different *C. difficile* strains (51). Moreover, some studies have correlated the heightened sensitivity of *C. difficile* endospores and the elevated recurrence rate of infections (53, 54), suggesting that the combination of germinants and disinfectants could potentially serve as a preventative measure against certain recurrent CDIs.

Despite our investigation into potential routine disinfection methods for disinfecting *C. difficile* endospores on carpet, limitations were encountered. Firstly, EPA-registered disinfectants are intended for use on inanimate surfaces, such as hard non-porous surface, and soft porous surfaces (55, 56). However, there are limited commercial

products for certain types of soft surfaces, such as upholstery and carpet (13). Thus, our results can only demonstrate potential disinfection strategies. Secondly, variations in sensitivity to disinfection were noted among different ribotypes of *C. difficile*, in addition to differences in virulence (25), thus, other ribotypes of *C. difficile* endospores need to be examined. Thirdly, even though we proposed a testing condition to simulate the real-world application, our data collected from lab conditions may differ from applications in real-world such as LTCFs, which have more complicated environments (i.e., carpet specification, airflow, and disinfection frequency).

Conclusions

In conclusion, we have developed a modified protocol with high recovery efficiency for evaluating the effectiveness of carpet disinfection. This protocol can be utilized to accurately determine the efficacy of disinfectants against *C. difficile* endospores on carpet. Steam vapor was found to be more effective than EPA-registered disinfectants against *C. difficile* endospores on carpet with a shorter contact time and without leaving any chemical residues. As such, steam vapor may be used for spot treatment during outbreaks or routine practice in conjunction with proper cleaning. It is important to note that the efficacy of disinfectant agents is significantly impacted by the type of carpet backing, highlighting the need for guidance in selecting carpet in healthcare facilities for safety purposes. Additionally, a combination of steam and weak disinfectant (product A) against sensitized *C. difficile* endospores, with steam vapor applied first, may be a promising approach for disinfection of *C. difficile* endospores on

carpet with WPerBs, like Color Accent®, when the selection of disinfectants in public settings is limited.

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Table 5.1. Selection of EPA-registered disinfectants.

Product	Active ingredient	Working concentration	Working pH	Neutralizer (concentration)
A	0.5% hydrogen peroxide	Ready-to-use	2.64	catalase (1300 U/ml)
B	3.13% hydrogen peroxide/0.099% octanoic acid/0.05% peracetic acid	Ready-to-use	2.95	catalase (1300 U/ml)
C	6% sodium hypochlorite	Diluted to 1,000 ppm	10.01	FBS (5%) + sodium thiosulfate (0.1%)

Table 5.2. Characteristics of tested carpet.

Specifications	Color Accent®	Highlight®
Fiber (Construction method)	Nylon 6 (loop)	Nylon 6 (loop)
Fiber finishing	Solution-dyed, soil-resistant coating	Solution-dyed, soil-resistant coating
Average density	0.348 g/cm^3	0.317 g/cm^3
Finished pile thickness	2.92 mm	3.20 mm
Primary Backing	Synthetic	Synthetic
Secondary Backing	Stalok®	Ecoworx® Performance Broadloom
Backing to moisture	Water-permeable	Waterproof
Backing thickness	3.96 mm	3.56 mm

Table 5.3. Effect of Tween-80 concentration on *C. difficile* endospore recovery rate.

	Recovery rate (%) [a]	
Concentration of Tween-80	Without soil load	With soil load
0.02%	11.3±1.8 [A]	10.9±1.1 [A]
0.1%	15.7±4.1 [AB]	14.7±2.0 [AB]
0.2%	20.8±1.4 [B]	18.9±2.8 [B]

[a] The data are expressed as means ± standard deviations (SDs) from duplicates in each of 2 independent experiments. The recovery rate was calculated based on plate count data (CFU). Values with different letters within the same column indicate a significant difference ($p<0.05$).

Table 5.4. Effect of stomaching time on *C. difficile* endospore recovery rate.

	Recovery rate (%) [a]	
Stomaching time	Without soil load	With soil load
1 min	59.8 ± 3.3^{AB}	45.3 ± 7.3^{A}
2 min	72.1 ± 6.4^{BC}	64.2 ± 5.9^{AB}
3 min	89.4 ± 13.0^{C}	66.5 ± 7.4^{BC}

[a] The data are expressed as means ± standard deviations (SDs) from duplicates in each of 2 independent experiments. The recovery rate was calculated based on plate count data (CFU). Values with different letters within the same column indicate a significant difference ($p<0.05$).

Figure 5.1. Sensitization of *C. difficile* endospores (ATCC 43593) under aerobic and anaerobic conditions (A), and efficiency among strains under aerobic condition (B). Error bars represent standard deviations from three replicates in each of two independent experiments. Different letters (i.e., a and b) indicate a significance within the same group (time).

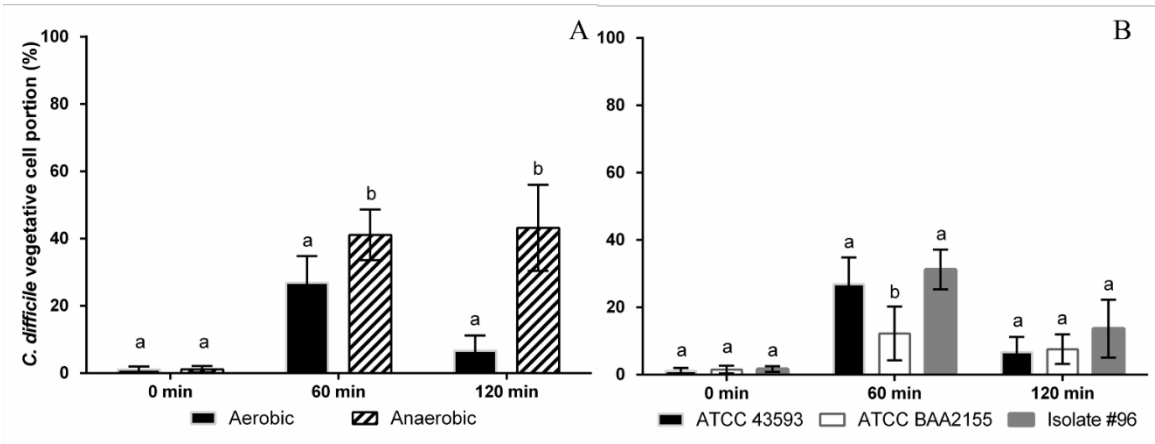


Figure 5.2. Efficacy of disinfectants against *C. difficile* endospores on carpet with different backings. Products A (0.5% H_2O_2), B (3.13% H_2O_2) and C (1,000 ppm sodium hypochlorite) were tested for a contact time of 30 min. White bars indicate the efficacy of disinfectants on Color Accent® carpet with WPerB; black bars indicate the efficacy of disinfectants on Highlight® carpet with WProB. Error bars represent standard deviations from ten replicates in two independent experiments. The *p*-value among treatments on each carpet was ≤0.01 (**), ≤0.001 (***), and ≤0.0001 (****), respectively.

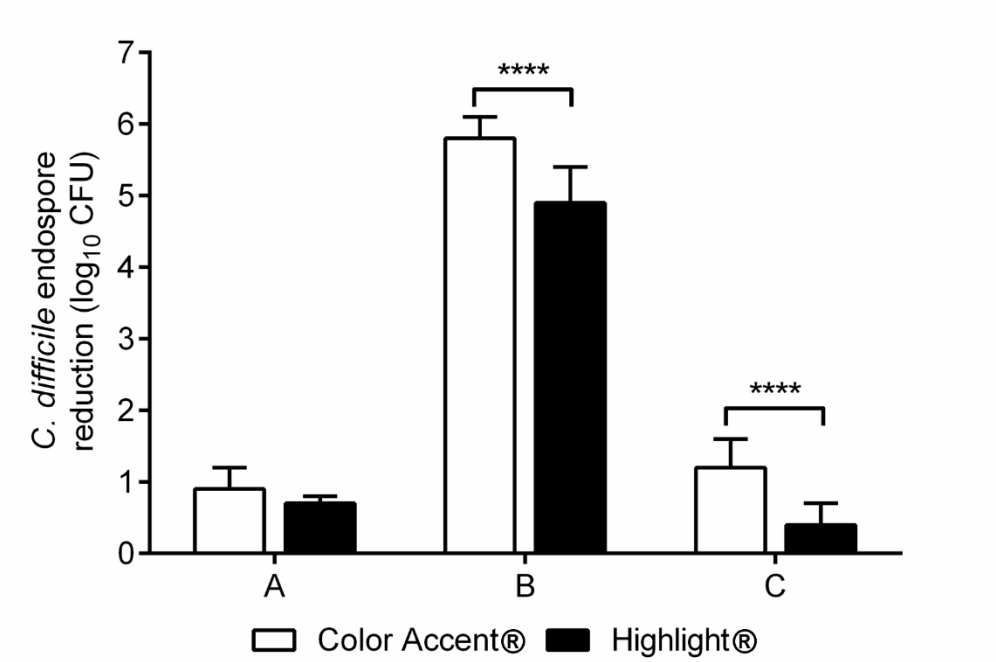


Figure 5.3. Efficacy of steam vapor against *C. difficile* endospore on different carpet. The dashed line with open squares represents efficacy on Color Accent® carpet with WPerB, and the solid line with solid squares represents efficacy on Highlight® carpet with WProB. Error bars represent standard deviations from replicates in two independent experiments. The dot line indicates the reach of detection limit which equals to a 6.0 $\log_{10}$ CFU reduction. The *p*-value among treatments for each microorganism was ≤0.05 (*), ≤0.01 (**), ≤0.001 (***), and ≤0.0001 (****), respectively.

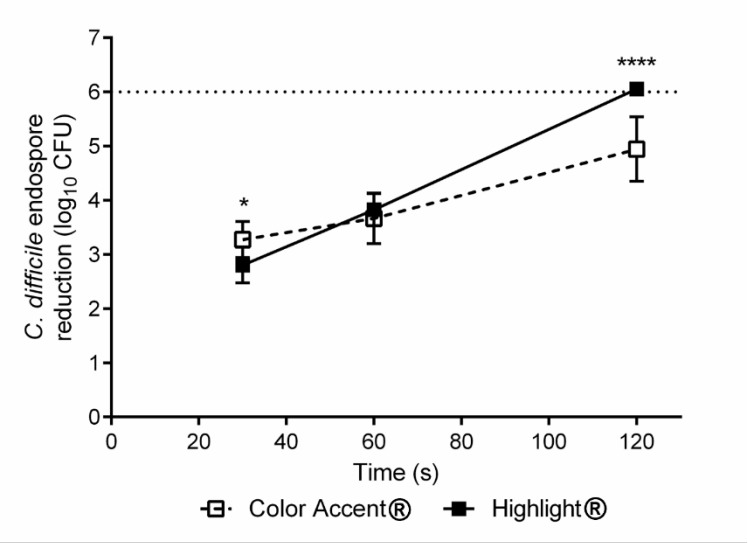


Figure 5.4. Efficacy of different application order of steam vapor and product A against the sensitized *C. difficile* endospore on Color Accent®. The exposure time for steam vapor was 60 s (black bars) and 120 s (white bars). Different letters (i.e., a and b) indicate a significance among different treatments in the Tukey's grouping test.

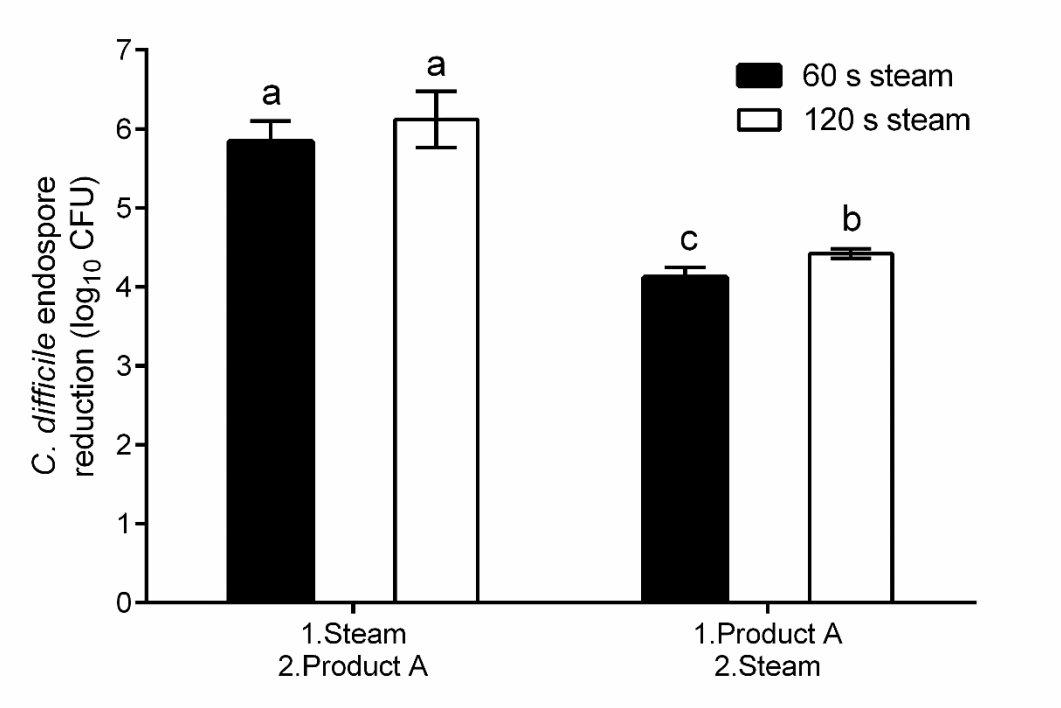


Figure 5.5. The condensed water weight of two carpet following steam treatment for 120 s (A), temperature of carpet above and below the backings of two carpet over 120 second steaming (B), and temperature of Color Accent® above and below the backings over 60 second steaming in combination of product A treatment (C). Each line indicates a mean temperature from six replicates.

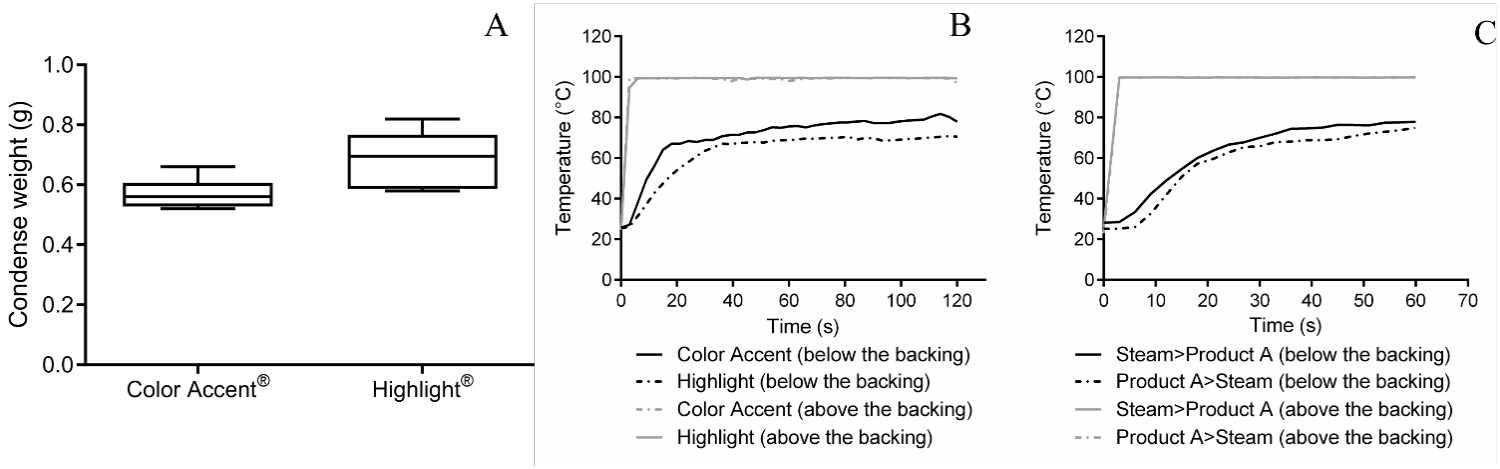
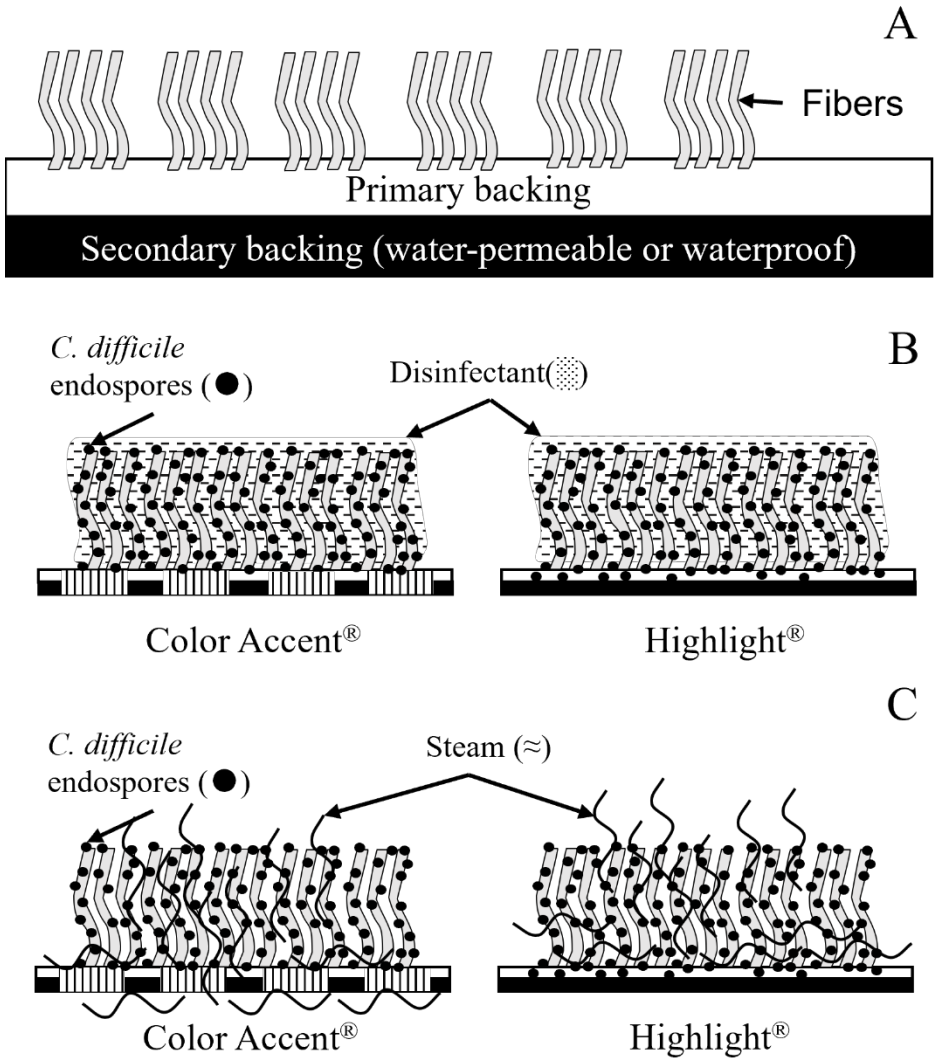


Figure 5.6. A general structure of carpet with backings. Color Accent® and Highlight® carpet are constructed of both primary and secondary backings (A). Distribution of disinfectants and *C. difficile* endospores on two tested carpet (B). Distribution of steam and *C. difficile* endospores on two tested carpet (C). Color Accent® carpet has a WPerB (double layer with stripes), while Highlight® carpet has a WProB (double layer).



CHAPTER SIX

EVALUATION OF PHOTOCLO$_2$ AGAINST TWO HUMAN NOROVIRUS SURROGATES AND *CLOSTRIDIOIDES DIFFICILE* ENDOSPORES ON SURFACES

Abstract

Aims: Evaluating the efficacy of an aqueous photocatalysis disinfection system, known as photoClO$_2$, against two human norovirus surrogates, feline calivirus (FCV) and Tulane virus (TuV), and *Clostridioides difficile* endospores on stainless steel and nylon carpet with two different backings.

Methods and Results: First, photoClO$_2$ was optimized with 1% NaClO$_2$ and 10 ppm Eosin Y to produce 60.64 ppm ClO$_2$ min^{-1} in a 4.5×4.5 cm^2 area. Then the system was tested against FCV, TuV and *C. difficile* endospores on stainless steel and nylon carpet with two different backings. PhotoClO$_2$ reduced >5 log$_{10}$ PFU of FCV in 45 min, >3 log$_{10}$ TCID$_{50}$ of TuV in 60 min, and 1.3 log$_{10}$ CFU of *C. difficile* endospores on stainless steel in 120 min. In addition, under an indoor lighting condition, photoClO$_2$ inactivated 4.3 log$_{10}$ PFU of FCV and 1.4 log$_{10}$ TCID$_{50}$ of TuV on stainless steel after 120 min. Besides, photoClO$_2$ achieved a 2.9 log$_{10}$ PFU reduction of FCV and 2.5 log$_{10}$ TCID$_{50}$ reduction of TuV on the nylon carpet with water-permeable backing in 60 min, respectively, a higher efficacy than on the carpet with water-permeable backing (a 1.3 log$_{10}$ PFU and 1.1 log$_{10}$ TCID$_{50}$ reduction, respectively).

Conclusion: The ClO$_2$ production rate of photoClO$_2$ can be influenced by light distribution, while its efficacy is affected by light intensity, surface characteristics and

targeting microorganisms. Our results showed exceptional efficacy of photoClO_2 in inactivating both norovirus surrogates on stainless steel surfaces and nylon carpet, particularly under optimal lighting conditions, but with a slight reduction in efficacy within indoor environments. Limited efficacy against *C. difficile* endospores was observed.

Importance: PhotoClO_2 is effective against norovirus on surfaces under both the optimal lighting conditions and indoor environments.

Introduction

Photocatalysts, which harness light radiation to generate electrons or electron holes, have been extensively studied for microbial inactivation (1, 2). Typically, light-induced electrons react with oxygen to form peroxyl radicals, while electron holes can accept electrons from water molecules and produce hydroxyl radicals (3). Both peroxyl and hydroxyl radicals possess antimicrobial activities due to their reactive properties. Some photocatalysts have been explored in disinfection studies, including titanium dioxide, graphitic carbon nitride, and nanocomposites (1). However, the use of most photocatalysts used in wastewater and surface disinfection against bacteria and viruses is often limited by their cost and poor solubility that cannot be easily spread over surfaces (2). Due to the limitations of solid-state photocatalysts, aqueous photocatalysis disinfection systems have been recently patented and shown effectiveness against various microorganisms (4-6).

Human norovirus (HuNoV), a non-enveloped virus responsible for more than 50% of known foodborne diseases, is easily transmitted through fomites (7). Consequently, environmental surface disinfection is critical to prevent the transmission of HuNoV. However, unlike enveloped viruses or bacterial pathogens, non-enveloped viruses have a stable capsid which is highly resistant to environmental stresses including oxidation, heat and extreme pHs (8, 9). This leads to challenges for environmental disinfection of HuNoV in healthcare settings, food service establishments, and other public spaces (7, 10, 11). Furthermore, disinfecting the spore-forming bacterium *Clostridioides difficile*, a major cause of healthcare-associated infections, presents an even greater challenge due to the resistance nature of bacterial endospores (12). Studies have reported that both HuNoV and *C. difficile* endospores exhibit high resistance to different classes of disinfectants (13).

Chlorine-based disinfectants, the most commonly used disinfectants in the world, are reportedly effective against HuNoV and *C. difficile* endospores, but they come with disadvantages (14). Specifically, high concentration of chlorine-based disinfectant can pose occupational hazards and cause surface damage due to their strong toxicity and oxidative activity (15). Moreover, active chlorine can be neutralized by organic matters and easily volatilize from aqueous solutions, leading to a decline in concentration and loss of disinfection efficacy (16). Chlorine dioxide (ClO_2), commonly used as an alternative to chlorine, is more effective under alkaline pH conditions and produces fewer harmful by-products when reacting with organic matters (17, 18). A novel subtype of photocatalytic chlorine-based disinfection system, referred to as photoClO_2, utilizes a

photoactivator and sodium chlorite to generate ClO_2 continuously at low concentrations, which is subsequently reduced back to chlorite after disinfection (4). A previous study has demonstrated the effective inactivation of bacteriophage MS2, a non-enveloped virus, and *C. difficile* endospores on stainless steel surfaces using photoClO_2 (19). Additionally, a recent study also reported the efficacy of photoClO_2 against thick *Pseudomonas aeruginosa* biofilm on glass with a 7 $\log_{10}$ CFU reduction, suggesting the potential use of this novel disinfectants against various microorganisms (6). However, no research has investigated its application against HuNoV on porous or non-porous surfaces and *C. difficile* endospores on porous surfaces.

In a previous study, the measurement of ClO_2 production rate was performed in cuvettes, where the liquid depth inside the cuvettes is much greater than liquid depth on environmental surfaces (19). Additionally, the ClO_2 production rate may vary near and away from the light source due to differences in light absorption (20, 21). Therefore, the ClO_2 production rate measured by cuvette method could not be accurately reflect and predict the application of photoClO_2 on surfaces, limiting its practical use.

In this study, we first optimized the measurement of ClO_2 production rate generated by photoClO_2 system. Then, the disinfection efficacy of the optimized photoClO_2 system was evaluated against two HuNoV surrogates, feline calicivirus (FCV) and Tulane virus (TuV), and *C. difficile* endospores on stainless steel and nylon carpet with two different backings. We also explored the potential use of photoClO_2 for surface decontamination in an indoor environment.

Materials and methods

Virus propagation, concentration and assays

Crandell-Rees Feline Kidney (CRFK) cells (ATCC CCL-94) were cultured in Eagle's modified essential medium (MEM; Gibco Life Technologies, NY, USA) containing 5% low-endotoxin heat-inactivated fetal bovine serum (FBS) (Seradigm, VWR International, Radnor, PA, USA), 100 U l^{-1} penicillin (HyClone, GE, MA, USA), and 100 mg l^{-1} streptomycin (HyClone). Ninety percent of confluent monolayers of CRFK cells were infected with FCV strain F9 (ATCC VR-782; American Type Culture Collection, VA, USA) at a multiplicity of infection (MOI) of 0.01 and incubated at 37°C for 2 days. FCV was then harvested from cell lysates by three freeze-thaw cycles followed by centrifugation for 10 min at 5,000 × g at 4°C. FCV stocks at ca. 10^8 plaque-forming unit (PFU) l^{-1} were aliquoted and stored at -80°C. Infectious FCV was quantified by standard plaque assay as previously described (22). To test for cell line permissiveness and contamination, FCV and PBS served as a positive and negative control, respectively. CRFK cells were passaged less than 30 times.

LLC-MK2 cells (ATCC CCL-7) were cultured in Opti-MEM I reduced serum medium (Gibco Life Technologies) supplemented with 2% low-endotoxin heat-inactivated FBS, 100 U l^{-1} penicillin, and 100 mg l^{-1} streptomycin. Ninety percent confluent monolayers of LLC-MK2 cells were infected with TuV, kindly provided by Dr. Jason Jiang (Cincinnati Children's Hospital, OH, USA), at an MOI of 0.1 and incubated at 37°C for 2 days. TuV was harvested from cell lysates similar as for FCV. Infectious TuV titer was quantified by the median tissue culture infectious dose ($TCID_{50}$) assay with

some modifications (23). Twenty microliters of serially diluted viruses were added in each quantification well per column and 8 wells used for each dilution. TuV stocks at ca. 10^7 $TCID_{50}$ l^{-1} were aliquoted and stored at -80°C. LLC-MK2 cells were passaged fewer than 30 times.

To concentrate viruses with stock concentrations below the desired inoculum level, viruses were precipitated by incubating with 8% polyethylene glycol 8000, 0.3 M NaCl and 1% bovine serum albumin at 4 °C overnight as described previously (15). Following centrifugation at 10,000 ×*g* for 30 min at 4°C to pellet the viruses, the pellet was then resuspended in 1 ml of PBS and centrifuged at 18,000 ×*g* for 1 min at 4°C. The supernatant containing the concentrated virus particles was carefully collected for subsequent tests. For the following tests, a mixture of FCV and TuV was prepared by mixing the stocks to achieve 8 $\log_{10}$ PFU ml^{-1} or 8 $\log_{10}$ $TCID_{50}$ ml^{-1} and 5% FBS was used as the soil load.

Preparation and purification of C. difficile endospores

C. difficile (ATCC 43593) was cultured on modified brain heart infusion agar plates containing 5 g l^{-1} yeast extract, 1 g l^{-1} cysteine, and 1 g l^{-1} sodium taurocholate (BHIA/YE/CYS/T) and anaerobically incubated at 37°C for 7 days and at room temperature for another 7 days as previously described (15). The *C. difficile* cell mass was scrapped from the plates with 5 ml of PBS supplementary with 0.1% Tween-80, then washed 5 times with ice-cold sterile deionized water, followed by centrifugation at 7,000 × g for 5 min at 4°C. Vegetative cells of *C. difficile* were removed by gradient

centrifugation in 50% (w/v) sucrose solution (24). The endospore suspension was washed three times with sterile ice-cold water after purification. The concentration of endospores was enumerated on BHIA/YE/CYS/T plates, and the purity of prepared endospores was confirmed via endospore staining (15). The stock culture of *C. difficile* endospores at ca. 10^8 colony-forming unit (CFU) l^{-1} was stored at 4°C for routine tests and at -80°C for long-term storage. Before the test, *C. difficile* endospores were mixed with 5% FBS as the soil load.

Preparation of photoClO_2 solution

Stock solutions of $NaClO_2$ (10%), eosin Y (200 ppm) and indigo carmine (200 ppm) were prepared separately in 18 MΩ deionized water as previously described (19). All experiments regarding photoClO_2 system were conducted in a dark room equipped with red lights (CrazyFire USA, Shenzhen H-Zone Industrial Technology Co., China). On the day of experiment, stock solutions were freshly mixed to yield working solutions with 1%, 2% or 4% $NaClO_2$ and 10 ppm eosin Y (EY). The indigo carmine (IC) at 100 ppm was used as an indicator to measure the ClO_2 production rate.

Carrier preparation

Type 304 stainless steel disks (1 cm in diameter) were produced by Muzeen & Blythe Ltd (MB, Canada). Nylon carpet Color Accent® (Shaw Inc., GA, USA) with a WPerB and Highlight® (Shaw Inc., GA, USA) with a WProB were cut into 5×5 cm^2 coupons with a mechanical cutting die (model 1500, Freeman Schwabe, OH, USA)

(kindly provided by Dr. Daniel Price, Interface Inc., GA, USA) then dusted by hand to remove loose fibers. Before the tests, the stainless steel disks and carpet coupons wrapped in aluminum foil were autoclaved on a 30-min dry cycle and cooled at room temperature overnight.

Light source and production rate of ClO_2 affected by the distribution of light intensity

Two light sources for photoClO_2 system were tested to mimic applications under various circumstances. The green flashlight (CrazyFire USA, Shenzhen H-Zone Industrial Technology Co., China) placed 4.7 cm away from tested surfaces (stainless steel and carpet coupon) could emit a range of light including 516 nm (Figure 1A). The light intensity at this distance was 9,960 lux measured by a light meter (LX-1330B, Thousandshores Inc., China). A fluorescence light tube equipped in the biosafety cabinet (Model 1300, Thermo Fisher, CA, USA) was used to simulate indoor lighting condition, which has an average light intensity at 683 lux.

Considering that the ClO_2 production rate of photoClO_2 is correlated to the intensity of light exposure, and recognizing the variability in light intensity across different surfaces, it becomes imperative to adjust the ClO_2 production rate of photoClO_2 in accordance with the distribution of light intensity. One hundred microliters of the working solution were added into each well of 96-well plates in an area of 5 by 5 wells. Blanks for the microplate reader were prepared to contain either 1%, 2% or 4% $NaClO_2$ and 10 ppm EY. After 60 s exposure to the green light, 100 µl of 10% glycine were added to terminate the reaction, and the concentration of IC was measured by a microplate

reader (μQuant, Bio-Tek, VT, USA) at 610 nm. The standard curves for IC concentration were prepared for each 96-well plate. ClO_2 production rates were calculated according to the previous study by the following equation (19):

$$[ClO_2] = (\mathrm{A}/\varepsilon \mathrm{l}) \times 4 \times mass_{ClO2}$$

Where $mass_{ClO2}$ is the molar mass of ClO_2, that is 67.45 g mol^{-1}, A is the absorbance of samples read at 610 nm wavelength, ε is the molar absorptivity of IC at 19,500 l $(mol \cdot cm)^{-1}$, and l is the light path length of the cuvette (1 cm) or 96 well plate (0.3 cm).

Optimization of neutralizers to terminate ClO_2 production

In order to measure ClO_2 production rate, the neutralizers, including glycine, sodium sulfite (Na_2SO_3), sodium thiosulfate ($Na_2S_2O_3$) and FBS, for terminating the photoClO_2 reaction were optimized. An area of 5 by 5 wells (4.5 x 4.5 cm^2) in a 96-well plate received similar radiation strengths from the flashlight were used for the optimization, and the ClO_2 production rate was adjusted based on the light distribution factor. To optimize the single composition of neutralizer, 100 μl of 10% of glycine, Na_2SO_3, or $Na_2S_2O_3$ were mixed with 100 μl of the solution containing 2% $NaClO_2$, 10 ppm EY, and 100 ppm IC as the testing mixtures. The mixtures were added into each well of 96-well plates, and exposed to the flashlight for 120 s to measure the continuous production rate ClO_2 after neutralization, in order to evaluate whether the reaction had been completely terminated. Additionally, two volumes (100 μl and 150 μl) and three concentrations (1%, 5%, and 10%) of glycine solutions were also optimized in a

preliminary test. After getting the optimized glycine solution, 5% FBS or 10% Na_2SO_3 was added to the glycine solution to improve the neutralization efficiency of the glycine solution. ClO_2 production rates after neutralization were calculated as described above and used to evaluate the effectiveness of neutralization.

In a previous study, the solution of 0.1% $Na_2S_2O_3$ and 5% FBS successfully neutralized chlorine-based disinfectants without toxicity against either cell lines, viruses, and *C. difficile* endospores (15). Thus, this neutralizer was solely evaluated for cell lines, viruses, and *C. difficile* endospores during efficacy tests, as these cells and viruses were identified as non-sensitive to the neutralized ClO_2 in contrast to the indicator IC used in the measurement described above.

Optimization of $NaClO_2$ concentration for photoClO_2 production

The previous study measured photoClO_2 production in cuvettes successfully (19). However, measuring ClO_2 production rate in cuvettes could not accurately reflect the application of photoClO_2 on surfaces. Thus, $NaClO_2$ concentration was optimized for the photoClO_2 system using the setup in a 96-well plate. One hundred microliters of the working solution (containing 1%, 2% or 4% $NaClO_2$, 10 ppm EY) of photoClO_2 system supplemented with 100 ppm IC were added into each well of 96-well plates in an area of 5 by 5 wells (4.5×4.5 cm^2). As a comparison, 2 ml of working solution (containing 1, 2 or 4% $NaClO_2$, 10 ppm EY) of photoClO_2 system supplemented with 40 ppm IC were added to each cuvette. IC concentration was reduced for measurement in cuvettes due to the different measurement range of devices.

Blanks for the microplate reader (μQuant, Bio-Tek, VT, USA) and spectrophotometer (SpectraMax QuickDrop, Molecular Devices, CA, USA) were prepared to contain either 1%, 2%, or 4% $NaClO_2$ and 10 ppm EY. One hundred microliters of 10% glycine were added to terminate the reaction in 96-well plates every 10 s over 50 s, whereas IC concentration in cuvettes was immediately read every 30 s over 150 s. ClO_2 production rates were calculated as described above.

Quantitative stainless steel carrier test for FCV/TuV mixture and C. difficile endospores

The disinfection experiments utilized 1% $NaClO_2$, as it was determined to be the optimal concentration for the photoClO_2 system. A previous study reported photoClO_2 was efficacious against MS2 on stainless in 60 min, thus 60 min of exposure time was tested for FCV and TuV (19). Due to the resistance nature of bacterial endospores, 120 min was tested for *C. difficile* endospores on stainless steel. Ten microliters of prepared FCV/TuV mixture or *C. difficile* endospore suspension were inoculated on each stainless-steel disk and placed in one 60 mm petri dish, then all carriers were dried for 1.5 h at room temperature with a relative humidity at 30-50%. After drying, each petri dish was put on a customized stage with coupons in the center (Figure 6.1). A ninety microliters of freshly prepared working solution were added to each of the disks in the dark room and then exposed to a green light for 15, 30, 45, and 60 min for FCV/TuV, and 30, 60, 90, and 120 min for *C. difficile* endospores, or to a fluorescent lamp inside a biosafety cabinet as indoor lighting conditions for 30, 60, 90, and 120 min for FCV/TuV. After the exposure, all disks were transferred into individual wells of a 24-well plate containing

900 µl of 5% FBS with 0.1% sodium thiosulfate for neutralization. Controls were kept in the dark all the time. Disks were ultrasonicated at 40 kHz for 15 s and pipetted 10 times to elute the inoculum. Samples were 10-fold serially diluted, and the concentration of FCV, TuV, and *C. difficile* endospores were assayed as described above.

Quantitative nylon carpet carrier test for FCV/TuV mixture and C. difficile endospores

Each of the pre-cut coupons (5×5 cm^2) received 100 µl of either FCV/TuV mixture or *C. difficile* endospore suspension, then dried for 1 h at room temperature under a biosafety cabinet (Model 1300, Thermo Fisher, CA, USA) with relative humidity at 30-50%. Freshly prepared photoClO_2 working solution was sprayed for ca. 8 s on each carpet coupon (approximately 6 ml). The coupons were scrubbed clockwise and counterclockwise for 30 s each with a working solution-saturated (approximately 1 ml) surgical scrub brush (BD E-Z Scrub, BD, NJ, USA). Then carpet coupons were exposed to the flashlight for 120 min. The scrubbed controls were kept in the dark along with the treatments. The unscrubbed controls were transferred into a flask with the neutralizers, 5% FBS plus 0.02% Tween-80 for FCV/TuV or 0.2% Tween-80 for *C. difficile* endospores, immediately after drying, while scrubbed control coupons and tested coupons were transferred into flasks with the neutralizers described above to neutralize photoClO_2. All flasks were ultrasonicated for 1 min at 40 kHz and then vigorously homogenized at 200 rpm in a stomacher for 3 min. Samples were 10-fold serially diluted, and the concentration of FCV, TuV, and *C. difficile* endospores were assayed as described above.

Statistical analysis

Four replicates were tested in two independent experiments for stainless steel carriers, while six replicates were tested for carpet. Reductions were calculated by $\log_{10}(N_0/N_d)$, where N_d is the average FCV, TuV or *C. difficile* endospore population from the treatment samples and N_0 is the average FCV, TuV or *C. difficile* endospore population from each control sample. Statistical analysis was performed using a one-way multiple-comparison ANOVA to determine the relationship between disinfectants and reduction. All results were expressed as mean ± standard deviation. Statistical significance was defined as a *p*-value of <0.05. Statistical analyses were conducted using GraphPad Prism 6.01 (GraphPad Software, Inc., CA, USA).

Results

Production rate of ClO_2 affected by the distribution of light intensity

Based on current experiment setup (Figure 1), 3×3 wells (4.5×4.5 cm^2) of a 96-well plate were exposed to the green light. As the distance from light source to each well is not equal, the highest production rate of ClO_2 was observed in the central area of the plate, reaching 61.21 ppm min^{-1}, while the lowest rate was recorded in the peripheral wells of the testing area at 41.64 ppm min^{-1}. As a result, the solutions in the peripheral areas produced significantly lower ClO_2 compared to the central area. The correlation between ClO_2 production rate and distance to the light source fits a linear regression with

an R^2 at 0.9960 (Figure 1C). Thus, the production rate of ClO_2 in each well can be standardized by the following equation:

$$P = P_0 - 2.625 \times (D - 47)$$

Whereas P_0 represents the production rate of ClO_2 in position 1 and D represents the distance to the light source.

Optimization of neutralizers for measuring ClO_2 product rate and disinfection efficacy

In order to measure the ClO_2 production rate, the chemical reaction of photo ClO_2 system should be terminated completely at each time point. Several neutralizers for the ClO_2 production rate were tested individually and in combination. Neutralization of the ClO_2 production solution with 10% glycine solely resulted in the lowest ClO_2 production of 5.20 ppm min^{-1}, while neutralization with Na_2SO_3 and $Na_2S_2O_3$ still produced 10.02 and 19.78 ppm min^{-1} of ClO_2 after neutralization, respectively (Figure 6.2A). The addition of the 5% FBS to 10% glycine significantly improved the efficiency of the neutralizer, by reducing the ClO_2 production rate from 5.20 ppm min^{-1} to 1.12 ppm min^{-1} (Figure 6.2B). In contrast, adding 10% $Na_2S_2O_3$ to glycine increased the ClO_2 production rate to 25.49 ppm min^{-1} after neutralization. Additionally, large volume (150 µl) of glycine at higher concentration (10%) had higher neutralization efficiency compared to lower volume and concentrations (Figure A1).

FBS (5%) plus 0.1% $Na_2S_2O_3$, which were used for neutralizing chlorine-based disinfectants in a previous study (15), could eliminate the effect of photoClO_2 on host cell

lines, tested viruses and *C. difficile* endospores (data not shown), thus this neutralizer was used for the efficacy test.

Optimization of ClO_2 production

In our previous study, cuvettes were employed to measure the rate of ClO_2 production (19). However, cuvettes might not accurately mimic the application of photoClO_2 on surfaces. To address this limitation, we compared the ClO_2 production rate measured in cuvettes with 96-well plates, which have a shallow liquid depth to provide a more actual measurement of ClO_2 production rate generated by photoClO_2 on surfaces. Then we used the data collected from 96-well plates to optimize the $NaClO_2$ concentration for photoClO_2 production.

The slopes of photoClO_2 production for 1%, 2%, and 4% $NaClO_2$ were -1.747, -1.833, and -1.474 in 96-well plates, respectively, and -0.2040, -0.1701, and -0.1397 in cuvettes, respectively (Figure 6.3). The production rate of photoClO_2 for 1%, 2%, and 4% $NaClO_2$ was 60.64, 63.62, and 51.16 ppm/min in 96-well plates, respectively, as compared to only 7.08, 5.90, and 4.85 ppm min^{-1} in cuvettes, respectively (Table 6.1). There was no significant difference in ClO_2 production rate between 1% and 2% $NaClO_2$ observed in 96-well plates, but 1% $NaClO_2$ generated highest ClO_2 production rate in cuvettes. Therefore, to be comparable with the previous study, photoClO_2 system with 1% $NaClO_2$ was tested against FCV, TuV and *C. difficile* endospores on stainless steel and carpet in the following experiments.

Efficacy of photoClO_2 against FCV, TuV, and C. difficile on stainless steel

On stainless steel carriers with 5% FBS as soil load, photoClO_2 system illuminated by a green flashlight for 15, 30, 45, and 60 min reduced 2.1, 4.2, >5.3, and >5.3 log_{10} PFU of FCV and 2.1, 2.4, 2.9, >3.0 log_{10} $TCID_{50}$ of TuV, respectively (Figure 6.4A). In addition, after exposure to the indoor lighting for 30, 60, 90, and 120 min, photoClO_2 inactivated 1.3, 2.4, 3.7, and 4.3 log_{10} PFU of FCV, respectively, and 0.6, 0.7, 1.2 and 1.4 log_{10} $TCID_{50}$ of TuV, respectively (Figure 6.4B).

PhotoClO_2 reduced 0.0, 0.0, 0.4 and 0.5 log_{10} CFU of *C. difficile* endospores in the presence of soil load on stainless steel after 30, 60, 90, and 120 min, respectively (Figure 5), while reduced 0.4, 0.4, 0.9, and 1.3 log_{10} CFU of *C. difficile* endospores without soil, respectively.

Efficacy of photoClO_2 against FCV, TuV and C. difficile on nylon carpet

The average inoculum on each carpet coupon was 6.9-7.3 log_{10} PFU of FCV, 6.7-7.1 log_{10} $TCID_{50}$ of TuV, and 7.3-7.7 log_{10} CFU of *C. difficile* endospores. After 60 min exposed to green flashlight, photoClO_2 reduced 2.9 log_{10} PFU of FCV and 2.5 log_{10} $TCID_{50}$ of TuV on Highlight®, and 1.3 log_{10} PFU of FCV and 1.1 log_{10} $TCID_{50}$ of TuV on Color Accent® (Figure 6.6). As for *C. difficile* endospore, photoClO_2 only inactivated 0.3 l log_{10} CFU of *C. difficile* endospore on Color Accent® and Highlight® after exposed to the green flashlight for 120 min.

Discussion

As photocatalysis disinfection is generally a complex system, several factors such as light intensity and wavelength, and interactions among photoactivators, surface disinfectants and microorganisms highly impacted the disinfection efficacy (25, 26). Taking into consideration of factors that are usually overlooked in photocatalysis studies, our study optimized a novel photocatalyzed ClO_2 production system for the efficacy test. Firstly, we explored the correlation between light intensity distribution across different surface areas and ClO_2 production rates. Secondly, we developed a novel and simple method for measuring ClO_2 production from the photoClO_2 system in a 96-well plate format. Thirdly, we determined the efficacy of the optimized photoClO_2 system against HuNoV surrogates, FCV and TuV, as well as *C. difficile* endospores on both stainless steel. To mimic real-world applications, we also evaluated the effect of indoor lighting of the photoClO_2 efficacy against FCV and TuV on stainless steel. Furthermore, the efficacy of photoClO_2 on nylon carpet with two different backings was evaluated to explore the potential use on soft porous surfaces.

A few studies have raised the concerns about uneven light distribution during photocatalysis disinfection even in a small area (27, 28). When light is applied in disinfection treatment, the different light distribution patterns affected disinfection efficacy in some of those cases (27). For example, Weaver et al. (29) reported significant variations in UV light emission across different surface areas within a biosafety chamber. Specifically, one area received 176 μW cm^{-2} while three adjacent areas received only 79, 85, and 142 μW cm^{-2}, respectively. Similarly, our study found significant differences in

ClO_2 production rates across a small surface area (4.5×4.5 cm^2) due to variations in light distribution. Therefore, it is important to take into account the factor of light distribution on surfaces when conducting photocatalysis studies.

Cuvettes-based assay allowing consistent light distribution and eliminating potential variations was used to measure the rate of ClO_2 production in a previous study (19). However, when comparing our experimental setup using 96-well plates with cuvettes, we observed higher photoClO_2 production rates in the plates. This discrepancy can be attributed to the difference in the depth of liquid that the light needs to penetrate: 3.0 mm in each well of a 96-well plate versus 48.0 mm in a cuvette. The depth of the liquid directly impacts light penetration and catalytic efficiency, as the absorption of light by the substrate or liquid can impede its penetration and influence the effectiveness of photocatalytic disinfection (30). Additionally, the reaction is significantly influenced by two diffusion steps. The first diffusion step involves the dispersion of ClO_2, which is more concentrated near the light source (31). The second diffusion step relates to the dispersion of the oxidized indicator IC. In the absence of agitation in the cuvettes, the production rate of ClO_2 was underestimated. It is important to note that evaluating the efficacy of photocatalytic disinfection in large volumes of liquid may not accurately mimic real-life applications, as surfaces typically retain a thin layer of disinfectant that can evaporate within a few minutes.

Choosing neutralizers was critical to terminate the photocatalysis reaction for accurately measuring ClO_2 production rate and neutralize toxic ClO_2 to host cells or testing microorganisms to evaluate disinfection efficacy. Although several studies have

suggested that glycine, sodium thiosulfate, and sodium sulfite can neutralize hypochlorite or ClO_2 (32-34), none of these neutralizers alone were able to completely terminate photocatalysis reaction during the measurement of ClO_2 production rate in our study. This limitation stems from the unique nature of the photoClO_2 system. In this system, the photoactivator is stimulated by light, enabling it to receive electrons and convert chlorite ions to ClO_2 (4). This process leads to the oxidation of the surface of microorganisms or the indicator IC, followed by the reduction of ClO_2 back to chlorite (4). Moreover, oxygen from the air can remove electrons from photoactivators that have already received them, restoring the photoactivators to a steady state (4). Consequently, the presence of ClO_2 is transient, and its reaction with the indicator IC, used in the measurement, is limited. Furthermore, IC is susceptible to both oxidation and reduction, and excessive amounts of neutralizers, which are typically reducing agents, can also cause the loss of color in IC (35). Therefore, a neutralizer that specifically reacts with chlorine dioxide but not IC was required for accurate measurement of the ClO_2 production rate in our study.

This is the first report on photoClO_2 being effective against both FCV and TuV on stainless steel when exposed to the optimal light source. Nonetheless, the contact time required for photoClO_2 to achieve a >3 $\log_{10}$ reduction was longer than the claims of most ready-to-use disinfectants, typically a 10-min contact time (15). That's probably due to two reasons. First, photoClO_2 maintained a relatively low concentration of ClO_2 compared to ready-to-use disinfectants, so it naturally has weaker efficacy (15, 36). Second, ClO_2, especially at such reduced concentrations, is susceptible to interference from organic matters (37). To simulate real-world conditions, our study included 5% FBS

as soil load, which contributed to the extended contact time requirement observed. Moreover, as a photocatalyzed disinfectant, photoClO_2 displayed sustained disinfection activity against viruses on surfaces. Unlike traditional chlorine-based disinfectants, the efficacy of photoClO_2 remained stable over time and did not exhibit the typical decay observed with these conventional disinfectants (38). This feature underscores its potential as a promising alternative for maintaining long-lasting and effective surface disinfection to prevent cross contamination in between disinfection cycles.

In agreement with the previous study, our results showed that photoClO_2 was not effective against *C. difficile* endospores, and the presence of soil further decreased its efficacy (19). The low concentration of ClO_2 (60.64 ppm min^{-1}) produced by the photoClO_2 was proved ineffective in fully damaging *C. difficile* endospore coat (15, 39). Furthermore, its oxidation activity was significantly hindered by the presence of proteins in the soil load, further limiting its efficacy (40). Therefore, *C. difficile* endospores disinfection needs stronger disinfectants or the combination of photoClO_2 with other approaches.

As for practical use of photoClO_2 system, we explored the disinfection under indoor light conditions. Our results demonstrated that photoClO_2 system, effective against FCV, can be non-specifically activated by light with a wide range of wavelengths. Despite its potential, as compared to the control conditions, the efficacy of photoClO_2 against both viruses were lower under indoor lighting conditions, likely due to the lower light intensity indoors. Specifically, our measurements revealed that the intensity of indoor lighting only reached 683 lux, while the green flashlight emitted a 9,960-lux light.

The green flashlight's ability to emit light at the optimal wavelength for the photoClO_2 system also played a crucial role in maximizing ClO_2 production (19), resulting in significantly stronger efficacy compared to the indoor lighting setup. As compared to FCV, photoClO_2 was not potent enough to inactivate TuV, which confirmed by previous studies that TuV is a more conservative surrogate than FCV to HuNoV (15, 41).

Further efficacy testing on carpet revealed the decreased efficacy of photoClO_2 against all tested microorganisms. This reduced efficacy can be attributed to the interference caused by the stacks of carpet fibers, impeding the effective penetration of light into the deeper layers. As discussed above, the decline in light intensity within the carpet's depths directly correlated with a reduction in ClO_2 production, consequently yielding reduced efficacy. Moreover, in similarity to the different efficacy of photoClO_2 between in suspension and stainless steel (19), the contact of photoClO_2 working solution with inoculated microorganisms limited by the complex three-dimensional structure of carpet also contributed to the decrease efficacy.

Studies on carpet disinfection generally evaluated the effect of fiber materials, but none of them investigated the effect of carpet backing (42). While carpet backings are expected to come into contact with only a fraction of the microorganisms in the structure of the carpet, they nonetheless have a notable influence on the distribution of chemicals across the carpet surface (42). To assess the effect of carpet backing on the efficacy of photoClO_2, we tested nylon carpet with two distinct backing types: one with water-permeable characteristics and another with waterproof properties. Our study revealed that efficacy of photoClO_2 against FCV and TuV was more effective on the nylon carpet with

WProB than the one with WPerB. One of the explanations may be due to the different distribution of the viruses on these two types of carpet. The carpet with a WProB is inherently more hydrophobic, which can prevent virus suspensions from penetrating into the carpet backing layer, whereas the WPerB can absorb some virus suspension (43). Additionally, our results indicate that photoClO_2 is more effective when it is present as a thin layer, as demonstrated by the comparison of ClO_2 production rates between 96-well plates and cuvettes. This suggests that the efficacy of photoClO_2 treatment may be optimized by ensuring that the solution is applied in a thin layer, which can maximize contact with the target surface and promote efficient ClO_2 production.

Conclusion

In this study, we developed a method by addressing the light distribution factor to more accurately measure ClO_2 product rate. Subsequently, this method was applied to validate optimal aqueous photoClO_2 system reported in a previous study. Moreover, our findings demonstrated the promising use of this disinfecting system against HuNoV on stainless steel both in controlled optimal conditions and within indoor environments. In regarding to carpet disinfection, photoClO_2 has some efficacy against both HuNoV surrogates on carpet, despite weaker than its performance on nonporous surfaces. The efficacy of this system is limited when targeting *C. difficile* endospores. Therefore, the utilization of photoClO_2 system necessitates careful consideration of factors such as light distribution, light intensity, surface characteristics and targeting microorganisms to achieve optimal disinfection efficacy. Despite being effective against HuNoV, the

inactivation of microorganisms by photoClO_2 is a slow but long-lasting reaction. This particular characteristics may be effective in preventing the spread of pathogens between disinfection cycles.

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Table 6.1. Production rate of ClO_2 under different concentrations of $NaClO_2$.

	ClO_2 production rate (ppm/min) [a]	
$NaClO_2$ concentration	96-well plates	Cuvettes
1%	60.64±2.43 [A]	7.08±0.37 [A]
2%	63.62±1.41 [A]	5.90±0.17 [B]
4%	51.16±1.55 [B]	4.85±0.16 [C]

[a]The data are expressed as means ± standard deviations (SDs) from triplicates. Values with different letters within the same column (e.g., AB) indicate a significant difference in Tukey's test grouping.

Figure 6.1. Testing set-up (A), the distance of different area to light source (B) and correlation of ClO_2 production rate and distance to the light source (C). All production rates of ClO_2 in each well were collected from triplicates. The ClO_2 production rate for each position equals P_0-2.625×(D-47), R^2=0.9960, whereas P_0 presents the production rate of ClO_2 in position 1 and D presents the distance to the light source. The blue square and gray circle in (A) indicate carpet and stainless steel coupons, respectively. The dotted line in (C) indicates the base production rate at position 1.

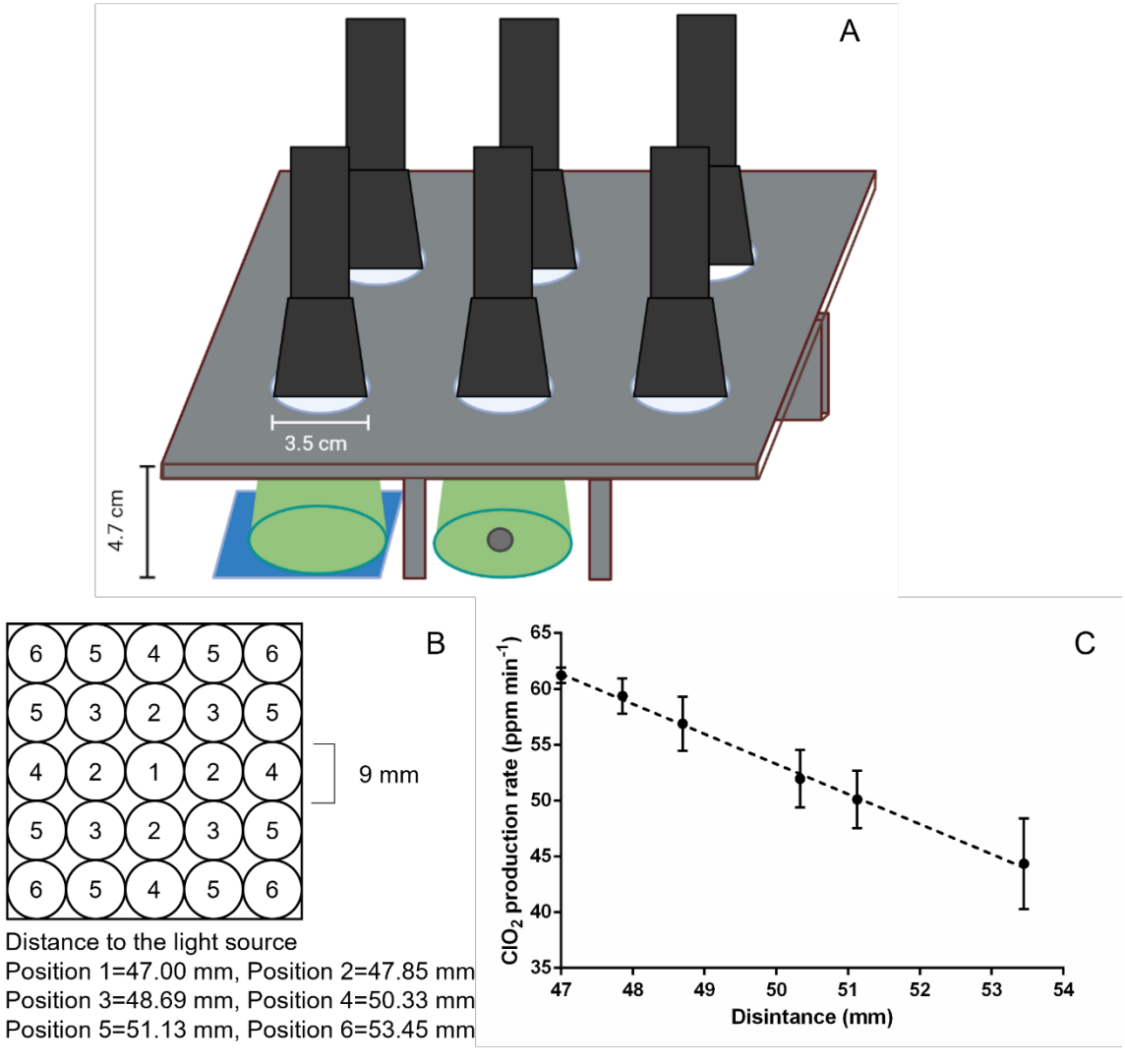


Figure 6.2. Neutralizer optimization for measuring ClO_2 production rate. A. Efficiency of 100 µl neutralizers at a concentration of 10% each; B. Efficiency of combination of 10% glycine, 5% FBS and 10% sodium sulfite. Error bars represent standard deviations from 6 replicates performed in 2 trials in 96-well plates. The *p*-value among treatments was ≥0.05(ns), <0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****), respectively.

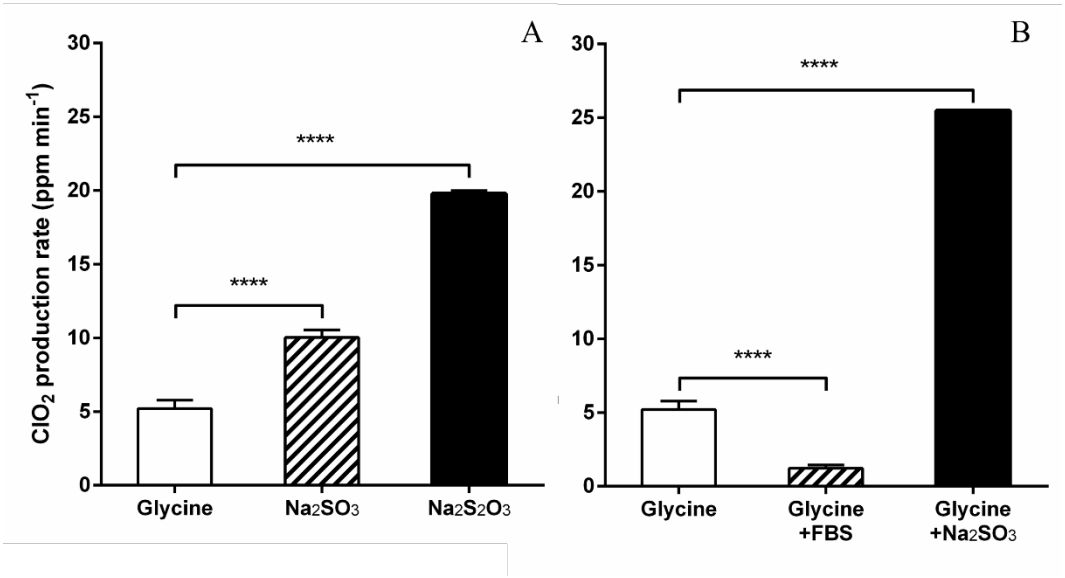


Figure 6.3. Estimated production of photoClO_2 in cuvettes (A) and 96-well plates (right) using 1% (solid line), 2% (dashed line) and 4% (dotted line) $NaClO_2$. This experiment is representative of 10 and 3 replicates for cuvettes and 96-well plates, respectively. Error bars are representative of standard deviations from 20 replicates for cuvettes and 6 replicates for 96-well plates performed in 2 trials.

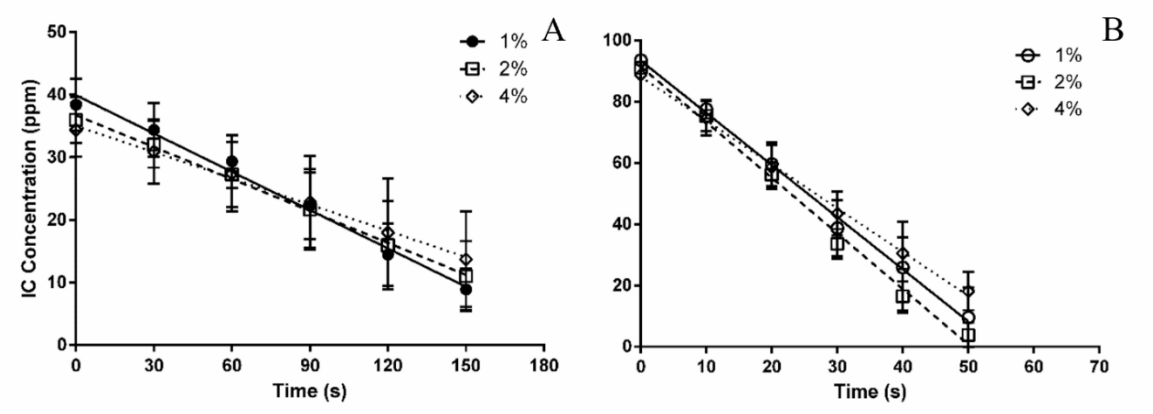


Figure 6.4. PhotoClO$_2$ efficacy against FCV and TuV under a green flashlight (A) and indoor lighting (B). Error bars are representative of standard deviations from 4 replicates performed in 2 trials. Dash and dotted lines represent the detection limit of FCV and TuV, respectively.

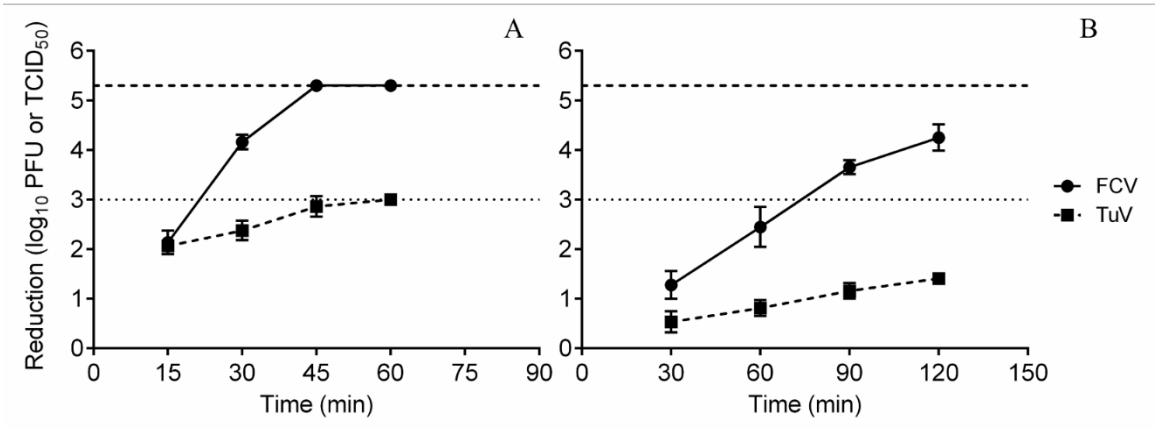


Figure 6.5. Efficacy of photoClO_2 against *C. difficile* endospores on stainless steel carriers. Error bars are representative of standard deviations from 4 replicates performed in 2 trials.

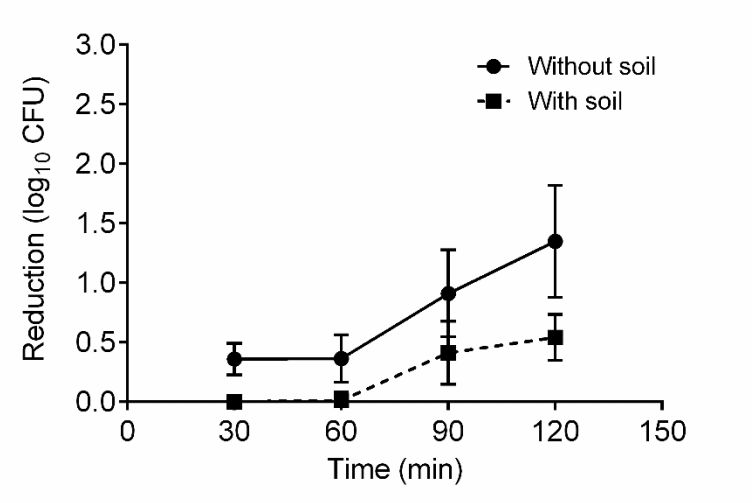
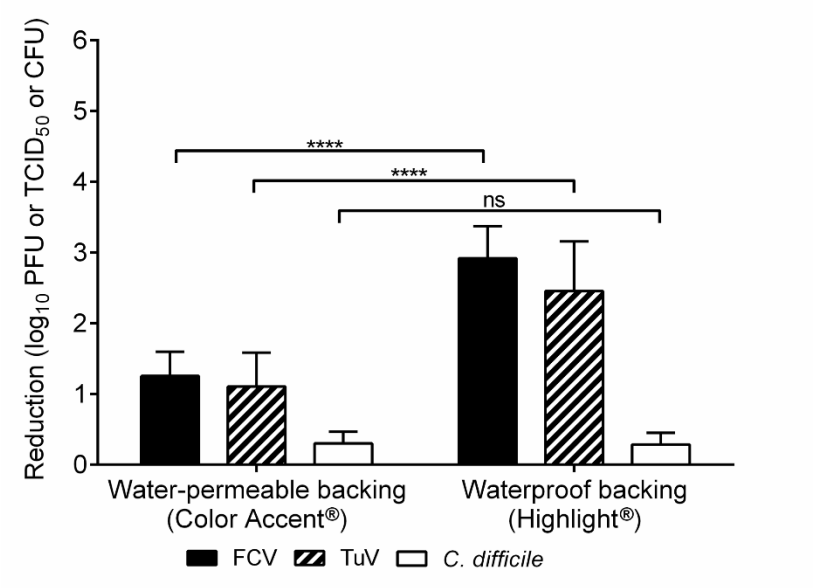


Figure 6.6. Efficacy of photoClO_2 against FCV, TuV, and *C. difficile* endospore on two types of carpet (Color Accent® and Highlight®). The contact time was 60 min for FCV and TuV, and 120 min for *C. difficile* endospores. Error bars are representative of standard deviations from 10 replicates. The *p*-value among treatments was ≥0.05(ns) and <0.0001 (****), respectively.



CONCLUSION

In summary, this dissertation evaluated the efficacy of three types of disinfectants (chemical disinfectants, steam vapor, and photoClO_2) against surrogates of HuNoV and SARS-CoV-2, and *C. difficile* endospores on stainless steel and carpets. Among chemical disinfectants, hydrogen peroxide (H_2O_2)-based disinfectants showed promising efficacy against both HuNoV surrogates and *C. difficile* spores on stainless steel and carpet, proving as effective alternatives to chlorine-based disinfectants. Our findings emphasized that the formula of disinfectants and active ingredient concentration are essential for the disinfection efficacy. Moreover, the efficacy of chemical disinfectants was also affected by carpet backing due to different distribution patterns of disinfectants and microorganisms. In considering potential carpet damage from repeated disinfection procedures, it is recommended to use strong chemical disinfectants as the spot treatment rather than routine preventive measures.

Importantly, our research findings validated that steam vapor disinfection for 30 s was effective against HuNoV and SARS-CoV-2 surrogates on nylon carpet with minimal impact on fiber and backing properties. Although steam vapor disinfection for 120 s was effective against *C. difficile* on carpet with waterproof backing, it's less effective on carpet with water-permeable backing. Therefore, the combination of steam vapor with a milder disinfectant becomes a viable approach, especially on carpet with water-permeable backing when individual disinfectants may fall short of achieving sufficient efficacy.

Furthermore, this study evaluated the efficacy of a novel photocatalysis disinfection technology, photoClO_2. The results revealed its efficacy against HuNoV surrogates on both hard non-porous and soft porous surfaces under optimal lighting conditions, albeit with limitations in inactivating *C. difficile* endospores. Additionally, the efficacy of photoClO_2 against HuNoV surrogates under indoor lighting conditions suggests the practical use of this technology for real-world application. Although photoClO_2 has been found to be effective against HuNoV surrogates, it is important to note that this process is a slow but long-lasting reaction. This particular characteristic may be desirable in preventing the spread of pathogens between disinfection cycles.

Given that this dissertation used surrogates to investigate the disinfection of HuNoV and SARS-CoV-2, it is imperative to validate current research findings, particularly on HuNoV using human intestinal enteroid systems, or SARS-CoV-2. Further research is also needed to validate the efficacy of these disinfectants under a wider range of temperature and relative humidity on various surfaces, in order to expand the applicability of these findings.

APPENDICES

Appendix A

Supplementary Data

Table A1. Occupational safety evaluation of active ingredients.

Product	Active ingredients	GHS hazard class[a,b]
A	0.5% hydrogen peroxide	skin corrosion 2, acute toxicity 4
B	0.88% hydrogen peroxide	skin corrosion 2, acute toxicity 4
C	1.4% hydrogen peroxide	skin corrosion 2, acute toxicity 4
D	3.13% hydrogen peroxide/0.099% octanoic acid/0.05% peracetic acid	eye irritation 1, skin corrosion 1, acute toxicity 3
E	5% hydrogen peroxide/0.005% silver	skin corrosion 2, acute toxicity 4
F	4.85% citric acid/0.003% silver	eye irritation 2
G	0.2% chlorine dioxide/0.125% alkyl dimethyl benzyl ammonium chloride/0.125% alkyl dimethyl ethylbenzyl ammonium chloride	eye irritation 1, skin corrosion 1, acute toxicity 1
H	15% isopropanol/7.5% ethanol/0.76% didecyldimethylammonium chloride	eye irritation 2, skin corrosion 1, acute toxicity 3
I	29.4% ethanol	eye irritation 2

[a] GHS hazard class indicates for the Globally Harmonized System of Classification and Labelling of Chemicals. Category 1 of eye irritation and skin corrosion refers to serious, irreversible eye damage and skin corrosion, respectively. Category 2 of eye irritation and

skin corrosion refers to irritation (2A) or mild irritation (2B). Category 3 and 4 of acute toxicant are based on median lethal doses of >500 mg/kg and >5,000 mg/kg, respectively.

[b] Data present most serious hazard class of active ingredients in one product.

Table A2. Efficacy of commercially available disinfectants against FCV, TuV and *C. difficile* spores in a suspension test [a].

	Reduction (logs)		
Product	FCV [b]	TuV	*C. difficile*
A	5.1 ± 0.6^{A}	3.8 ± 0.6^{AB}	$>6.0^{A}$
B	4.1 ± 1.4^{AB}	3.4 ± 0.7^{AB}	0.1 ± 0.1^{B}
C	5.0 ± 0.4^{A}	3.8 ± 0.8^{A}	1.4 ± 0.1^{C}
D	$>5.4^{A}$	3.9 ± 0.5^{A}	$>6.0^{A}$
E	2.7 ± 0.2^{B}	2.5 ± 0.0^{B}	1.3 ± 0.2^{C}
F	2.2 ± 0.2^{B}	0.2 ± 0.2^{C}	0.0 ± 0.1^{B}
G	0.3 ± 0.1^{C}	1.8 ± 0.8^{B}	0.4 ± 0.3^{B}
H	1.9 ± 1.3^{B}	3.8 ± 0.6^{AB}	0.4 ± 0.4^{B}
I	5.2 ± 0.8^{A}	4.3 ± 0.2^{A}	0.0 ± 0.1^{B}

[a] Contact time was 1 min for FCV, 10 min for TuV and 10 min for *C. difficile* spores.

[b] The data are expressed as means ± standard deviations (SDs) from duplicates of 2 independent experiments. Values with different letters within the same column (e.g., AB) indicate significant difference ($p<0.01$) in Tukey test grouping.

Table A3. Inactivation kinetics of FCV and TuV by selected disinfectants on stainless steel carriers [a].

	FCV reduction (log_{10} PFU)			TuV reduction (log_{10} $TCID_{50}$)		
Product	1 min [b]	5 min	10 min	1 min	5 min	10 min
A	5.0 ± 0.2^{A}	$>5.1^{A}$	$>5.14^{A}$	0.8	0.8	1.3
B	4.9 ± 0.2^{A}	$>5.1^{A}$	$>5.1^{A}$	0.3	1.1	1.3
C	4.7 ± 0.3^{A}	$>5.1^{A}$	$>5.1^{A}$	0.6	2.4	2.4
D	$>5.0^{A}$	$>5.1^{A}$	$>5.1^{A}$	2.5	> 3.1	> 3.1
E	1.8 ± 0.3^{B}	2.2 ± 0.7^{C}	$>5.1^{A}$	0.8	0.8	1.4
F	2.5 ± 0.3^{B}	$>5.1^{A}$	$>5.1^{A}$	0.4	1.0	1.8
G	0.4 ± 0.3^{C}	0.2 ± 0.2^{B}	0.3 ± 0.1^{B}	0.6	0.9	1.0
H	0.4 ± 0.3^{C}	$>5.1^{A}$	$>5.1^{A}$	1.0	1.0	1.6
I	$>5.0^{A}$	$>5.1^{A}$	$>5.1^{A}$	2.0	1.9	1.9
Bleach[c]	1.7 ± 0.2^{B}	$>5.1^{A}$	$>5.1^{A}$	> 3.1	> 3.1	> 3.1

[a] Contact times for FCV and TuV were 1, 5 and 10 min.

[b] The data are expressed as means ± standard deviations (SDs) from duplicates in 2 independent experiments. Values with different letters within the same column indicate significant difference ($p<0.01$) in Tukey test grouping.

[c] 1,000 ppm sodium hypochlorite

Table A4. Selection of EPA-registered chemical disinfectants.

Product	Active ingredient	Working concentration	Working pH	Neutralizer (concentration)
A	0.5% hydrogen peroxide	Ready-to-use	2.64	catalase (1300 U ml^{-1})
B	3.13% hydrogen peroxide/0.099% octanoic acid/0.05% peracetic acid	Ready-to-use	2.95	catalase (1300 U ml^{-1})
C	6% sodium hypochlorite	Diluted to 1,000 ppm	10.01	FBS (5%) + sodium thiosulfate (0.1%)

Table A5. Color change of Color Accent® and Highlight® after treatments.

	ΔE[1]	
Treatment	Color Accent®	Highlight®
Water	1.34±0.60[a]	0.54±0.39[a]
Steam vapor	1.58±0.46[a]	0.98±0.41[a]
Product A	1.21±0.48[a]	0.77±0.33[a]
Product B	1.08±0.85[a]	0.74±0.30[a]
Product C	0.74±0.52[a]	0.54±0.26[a]

[1] The data are expressed as means ± standard deviations (SDs) from six replicates. Values with different letters within the same column indicate a significant difference ($p<0.05$).

Table A6. Recovery rate of BCoV and HCoV OC43 from carpet.

	Recovery rate (%) [a]	
Virus	PET carpet	Nylon carpet
BCoV	32.50±14.32[A]	34.86±12.44[A]
HCoV OC43	3.87±2.02[B]	24.37±6.21[B]

[a] Data are expressed as mean ± standard deviation (SD) from triplicates at each of 2 independent experiments. Values with different letters for the same fiber (e.g., A, B) indicate significant difference in Tukey's test grouping.

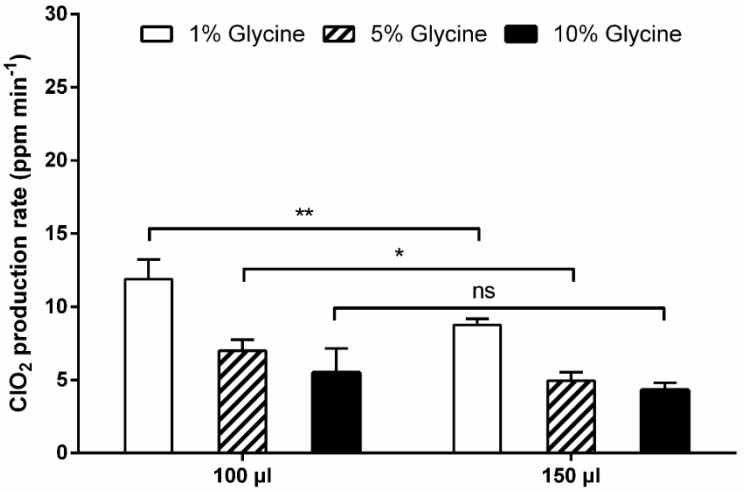


Figure A1. Efficiency of glycine at different concentrations and volumes; Error bars represent standard deviations from 6 replicates performed in 2 trials in 96-well plates. The *p*-value among treatments was ≥0.05(ns), <0.05 (*), and <0.01 (**), respectively.