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Disinfectant Efficacy Against SARS-CoV-2 Surrogates, Bovine Coronavirus and Human Coronavirus OC43, on Hard and Soft Non-porous Surfaces

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DISINFECTANT EFFICACY AGAINST SARS-COV-2 SURROGATES,
BOVINE CORONAVIRUS AND HUMAN CORONAVIRUS OC43,
ON HARD AND SOFT NON-POROUS SURFACES

A Thesis
Presented to
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Master of Science.
Microbiology

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by
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ABSTRACT

The novel human coronavirus (HCoV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged from Wuhan, China in the latter part of 2019. The rapid spread of SARS-CoV-2 amid the most recent COVID-19 pandemic forced countless foodservice establishments (FSEs) across the United States to close, taking a tremendous toll on the foodservice industry. Although primarily transmitted person-to-person, previous studies indicate that the duration of SARS-CoV-2 survival on different environmental surfaces provides adequate time for secondary transmission to occur. To prevent the further spread of SARS-CoV-2, effective disinfection of surfaces in FSEs is necessary. While disinfectants approved for use against SARS-CoV-2 are found on the Environmental Protection Agency's (EPA's) List N, there is limited data available regarding the efficacy of these products against two SARS-CoV-2 surrogates, bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV OC43) on surfaces other than glass and stainless steel. Therefore, our aims for this study were to (I) optimize a neutralization method for chlorine- and quaternary ammonium compound (QAC)-based disinfectants during efficacy testing, (II) determine the efficacy of two separate batches of three ready-to-use (RTU) spray disinfectants of different active ingredients (chlorine, hydrogen peroxide, and QAC + alcohol) against SARS-CoV-2 surrogates, BCoV and HCoV OC43, in suspension and (III) on surfaces commonly found in the 'front-of-the-house' in FSEs (polyethylene terephthalate [PET] plastic and vinyl upholstery fabric). Cytotoxicity to the host cell line during efficacy testing was successfully eliminated via infection media paired with centrifugation in Pierce^{TM} detergent removal columns for the

QAC-based disinfectant, and a 5% fetal bovine serum (FBS) + 1% sodium thiosulfate ($Na_2S_2O_3$) solution for the chorine-based disinfectant. In suspension testing, all three RTU disinfectants tested achieved a ≥ 3.0 $\log_{10}$ reduction of BCoV and HCoV OC43 within 2 min. On PET carriers, all three RTU disinfectants reduced BCoV by ≥ 3.0 $\log_{10}$ $TCID_{50}$/mL, whereas Oxivir Tb was the only disinfectant efficacious against BCoV on vinyl carriers. None of the three RTU disinfectants tested achieved a ≥ 3.0 $\log_{10}$ reduction of HCoV OC43 on PET or vinyl. However, after a 2 min contact time, all three disinfectants reduced the infectivity of BCoV and HCoV OC43 below the limit of detection (LOD) on both PET and vinyl surfaces. Those cases with <3.0 $\log_{10}$ reduction of virus titer were due to the decreased dynamic range on the carrier prior to disinfection. Further analysis of carrier test results revealed that the mean reduction in titer of BCoV and HCoV OC43 after drying was greater on vinyl carriers. In addition, greater amounts of reduction of BCoV and HCoV OC43 were observed on both PET and vinyl carriers as the relative humidity (RH) level increased. At low RH (20-29%) conditions, the reduction in the titer of HCoV OC43 was significantly greater than that of BCoV on both PET and vinyl carriers. Our data showed that both SARS-CoV-2 surrogates are very sensitive to the tested disinfectants, and further analysis of the quantitative carrier test data indicates that additional factors (i.e., surface type, RH, and surrogate) must be carefully considered when performing disinfectant efficacy testing on carriers. Collectively, these data highlight the importance of verifying disinfectant suspension test data by performing disinfectant carrier tests which expose the virus and disinfectant to conditions (i.e.,

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surface type, relative humidity, surrogate) similar to those encountered during 'real-

world' application.

DEDICATION

I would like to dedicate this work to my parents, William and Lisa Kimbrell, my brother, William Kimbrell Jr., my grandparents, Paul and Linda Lowrey, Alex Samenko, family, and friends. I believe this thesis would not have been possible without all of your love and support. I am forever thankful for all that each of you has/have done for me as I have pursued my dreams.

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CHAPTER ONE:

LITERATURE REVIEW

INTRODUCTION

In late 2019, a novel pathogenic coronavirus (CoV) known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, and by January of 2020 the first case was reported in the United States (1). By the end of January, the World Health Organization (WHO) announced that the outbreak of coronavirus disease 2019 (COVID-19) was a pandemic (2). To date, the WHO has confirmed over 760,360,900 cases of COVID-19 globally (https://covid19.who.int). With an incubation time of 4-5 days (3) and the ability to be transmitted via aerosols or respiratory droplets, SARS-CoV-2 was able to spread rapidly throughout the world (4).

Due to the highly contagious nature and rapid spread of SARS-CoV-2 amid the most recent pandemic, a wide-range of industries within the United States were impacted. According to the U.S. Department of Labor and Statistics, the industries that had the largest percentage of establishments that experienced decreases in demand included air transportation (76%), accommodation and food services (71%), and mining, quarrying, and oil and gas extraction (70%) (https://www.bls.gov/brs/2020-results.htm). In addition, a number of establishments within various industries underwent government-mandated closures during the pandemic, including arts, entertainment, and recreation (48%), educational services (40%), and accommodation and food services (36%) (https://www.bls.gov/brs/2020-results.htm). In the earlier days of the COVID-19

pandemic, the unemployment rate in the United States rose abruptly. According to the

U.S. Bureau of Labor and Statistics, the national unemployment rate in 2020 increased

from 3.5 to 4.4% from February to March, and then peaked at 14.8% in April 2020 (5).

CHARACTERISTICS OF CORONAVIRUSES

Coronaviruses are spherical enveloped viruses that belong to the family

Coronaviridae and are further classified into one of four genera: *alpha-, beta-, gamma-,*

or *deltacoronavirus* according to the International Committee on Taxonomy of Viruses

(6). Since the discovery of the first human coronaviruses (HCoVs), i.e., HCoV 229E and

HCoV OC43 in the 1960s, a total of seven HCoVs have been found to infect humans thus

far (7). While four of them, HCoV-229E, HCoV OC43, HCoV-NL63, and HCoV-HKU1,

cause a self-limited infection of the upper respiratory tract (4, 8), both severe acute

respiratory syndrome coronavirus 1 (SARS-CoV-1) and Middle East respiratory

syndrome-coronavirus (MERS-CoV) infect the lower respiratory tract and can cause

severe respiratory syndrome (4, 9). In 2019, a novel CoV emerged in Wuhan, China and

caused a large outbreak of pneumonia (10, 2). This novel HCoV has since been identified

as SARS-CoV-2, a single-stranded RNA virus that belongs to the genus *Betacoronavirus*

(11, 2). The majority of individuals infected with SARS-CoV-2 develop mild to moderate

symptoms (e.g., cough, headache, fever, loss of taste or smell, sore throat, diarrhea, or

mild pneumonia), but some develop more severe symptoms, such as dyspnea, hypoxia,

respiratory failure, shock, or multiorgan dysfunction (3).

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SARS-COV-2 SURROGATE VIRUSES

SARS-CoV-2 is classified as a biosafety level-3 pathogen because it is easily transmitted via respiratory droplets or aerosols (https://www.cdc.gov/training/quicklearns/biosafety/). Due to the increased safety risk when working with SARS-CoV-2, surrogate viruses with lower health risks are used to allow persistence and disinfectant efficacy studies for SARS-CoV-2 to be conducted in biosafety level-2 laboratories (12, 4). Surrogates can be used for testing provided that they have similar biological and physiochemical properties that emulate those of the viruses they were chosen to represent (13, 14, 15). Bacteriophage phi 6 is easy to handle and be enumerated and has been used in previous persistence studies as a surrogate for SARS-CoV-2 (16, 17, 18); however, it may not be the most appropriate surrogate for CoVs because some CoVs were found to persist longer than phi 6 (19). Currently, the Environmental Protection Agency (EPA)-approved regulatory surrogates for registering a disinfectant as efficacious against SARS-CoV-2 include HCoV 229E, or other HCoVs (20). Although HCoV 229E and SARS-CoV-2 both belong to the family *Coronaviridae*, HCoV 229E is in the genus *Alphacoronavirus* while SARS-CoV-2 is in the genus *Betacoronavirus* so the two viruses could potentially have different responses to environmental stresses (12). Additionally, the HCoV 229E induced cytopathic effect (CPE) to the host cell line is difficult to assess when disinfectant efficacy testing is performed. Bovine coronavirus (BCoV), which causes gastrointestinal infections in cattle (21, 22), and HCoV OC43, responsible for upper and mild respiratory infections in humans (21), are both in the genus *Betacoronavirus*. However, there is limited published

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data regarding the disinfection of these viruses on surfaces (12, 4). When the survival of HCoV 229E and HCoV OC43 was previously evaluated in suspensions of either phosphate buffered saline (PBS) and culture medium, it was reported that HCoV OC43 displayed higher survival rates than HCoV 229E (21). HCoV OC43 also showed more significant CPE than HCoV 229E when their growth was compared on two different susceptible cell lines (23). In addition, previous research indicates that the environmental stability of HCoV OC43 is similar to that of SARS-CoV-2 (24, 25). A more accurate estimate of disinfectant efficacy against SARS-CoV-2 may be provided if efficacy testing is conducted using BCoV and HCoV OC43 as surrogates, as compared with HCoV 229E.

SURVIVAL OF SARS-COV-2 ON SURFACES

As the foodservice industry works to maintain operations during the COVID-19 pandemic, it is necessary to consider the ability of SARS-CoV-2 to survive on surfaces found within FSEs. While SARS-CoV-2 is primarily transmitted via respiratory droplets and aerosols (26), contaminated objects or surfaces (fomites) could potentially serve as another source of transmission (27, 28, 12, 29). Surfaces can be contaminated by infected individuals who shed SARS-CoV-2 via respiratory secretions, saliva droplets, and/or stool (30). A study that was conducted within twenty supermarkets in Italy reported SARS-CoV-2 contamination on several frequently touched surfaces, including shopping trolley handles, scales, refrigeration system handles, and keyboards (31). Other studies have reported the presence of SARS-CoV-2 on high-touch surfaces in public settings such as chairs, tables, keyboards, entry door handles, trash can handles, and ATMs (32,

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33, 34). Furthermore, a persistence study conducted using artificial inoculation reported that infectious SARS-CoV-2 can persist on a variety of surfaces (e.g., tissues, paper, cloth, glass, banknotes, stainless steel, plastic, surgical mask) at ambient conditions for times ranging from 30 min to 7 days (35). Although there are currently no reports of SARS-CoV-2 infection in humans via fomites, hamster models have provided evidence that fomite transmission may be possible (36, 37, 38, 39). The data from the aforementioned survival studies indicate that the duration of SARS-CoV-2 survival on different environmental surfaces provides adequate time for secondary transmission to occur.

Certain environmental factors (e.g., temperature, relative humidity [RH], soil load, surface type) can impact the survival of SARS-CoV-2 on surfaces. Previous studies reported that SARS-CoV-2 can survive longer on environmental surfaces at 4°C than at room temperature (40, 41). When SARS-CoV-2 was exposed to ambient indoor temperature and a range of relative humidity (RH) values, the results indicated that the virus was the most stable at a lower RH (20%) (42). Aside from differences in virus survival at varying temperatures and RHs, the persistence of SARS-CoV-2 has been reported to vary when evaluated on porous and non-porous surfaces (43). Riddell et al. (44) reported the presence of infectious SARS-CoV-2 up to 28 days on non-porous (e.g., glass, polymer notes, stainless steel, vinyl, and paper notes), but on porous surfaces (e.g., cotton cloth) no infectious virus was detected after 14 days (44). Studies have also indicated that the presence of a soil or organic load has a protective effect on the virus as it dries on surfaces (45, 46, 35, 44).

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SURFACE DISINFECTION IN THE FOODSERVICE INDUSTRY

As the second-largest private-sector employer in the United States and

contributing approximately $1 trillion of the $20.5 trillion U.S. GDP, the foodservice

industry is a critical component in the nation's food system and economy

(https://www.ers.usda.gov/topics/food-markets-prices/food-service-industry/market-

segments/, https://fortune.com/2020/04/20/coroanvirus-restaurants-food-service-industry-

takeout-delivery-covid-19/). Due to the highly contagious nature and rapid spread of

SARS-CoV-2 amid the most recent pandemic, many foodservice establishments (FSEs)

in the United States were forced to shut down. According to the U.S. Bureau of Labor

Statistics, the unemployment rate for food services and drinking places in 2020 increased

from 8.5 to 35.4% from March to April; much higher than the national averages of

unemployment rates from 4.4 to 14.7% in the same period (https://www.bls.

gov/iag/tgs/iag722.htm#workforce; https://data.bls.gov/timeseries/LNS14000000).

Additionally, sales for food service and drinking places were down 20.9% in the first

eight months of 2020 when compared to 2019 as indicated by the advance monthly sales

for retail and food services released by the Bureau of Census on September 16th, 2020

(47).

To prevent the further spread of SARS-CoV-2 in FSEs, implementation of

effective environmental cleaning is necessary. Currently, limited published data exist

regarding the disinfection of surfaces found in the 'front-of-the-house' in FSEs (i.e., any

place within a FSE that customers are allowed). By definition, disinfectants "destroy or

irreversibly inactivate bacteria, fungi and viruses, but not necessarily bacterial spores, in

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the inanimate environment", while sanitizers "reduce the bacteria population in the inanimate environment by significant numbers but do not destroy or eliminate all bacteria" (48, 49). Current regulations focus on sanitizing food-contact surfaces, which are typically found in the 'back-of-the-house' (i.e., food preparation and dishwashing areas). The U.S. Food and Drug Administration (FDA) Food Code does not require the sanitization of nonfood-contact surfaces and recommends only cleaning these surfaces as needed (49). At this time, the U.S. FDA Food Code does not define or address the disinfection of surfaces within FSEs (49, 50). In response to the COVID-19 pandemic, major United States government agencies (i.e., the Centers for Disease Control and Prevention [CDC], the EPA, and the FDA) compiled a series of recommendations that somewhat addresses the gap in the Food Code by promoting the frequent cleaning and disinfection of 'high-touch' surfaces (49, 51, 52, 50).

Types of Surfaces in FSEs

In FSEs, high-touch surfaces are found in the front-of-the-house and are oftentimes nonfood-contact surfaces (e.g., door handles, dining room tables/chairs, touchscreen ordering devices, etc.), many of which are hard, non-porous surfaces. As previously discussed, surfaces can be contaminated with SARS-CoV-2 via respiratory secretions or saliva droplets shed by an infected individual (30). Presently, the infectious dose of SARS-CoV-2 is not yet known; however, previous studies report that the virus has the ability to survive on hard, non-porous surfaces, such as plastic and stainless steel, for periods up to 28 days as reported by Riddell et. al (44). The role of contaminated

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surfaces has been found to be significant in the spread of other diseases, such as gastroenteritis, due to human norovirus (53, 54), but there is currently limited data regarding the transmission of SARS-CoV-2 via fomites. The ability of SARS-CoV-2 to survive on hard, non-porous surfaces for extended periods of time, in conjunction with frequent contact with potentially contaminated surfaces in the front-of-the-house in FSEs, provides an opportunity for secondary transmission to occur. Fortunately, previous studies have demonstrated that the use of disinfectants on contaminated hard, non-porous surfaces can effectively reduce the viral load present on the surface (12).

A number of porous surfaces (e.g., wood, upholstery, carpeting) can also be found in the front-of-the-house in FSEs. These surfaces can potentially be contaminated by soiled hands, infectious virus particles aerosolized by coughing and sneezing, or by airborne virus that settles following the disturbance of a contaminated surface (i.e., walking on contaminated carpet) (55, 56, 57). Previous studies reported that SARS-CoV-2 can survive on soft, porous surfaces for periods ranging from 2 to 3 days (58, 44). However, when evaluating the survival of viruses on porous surfaces it can be hard to determine whether any inactivation is due to an actual loss of infectivity or from insufficient recovery of the virus adsorbed in the porous material (13). Porous surfaces are not only challenging to clean but are also not required to be disinfected in FSEs. Currently, only 12 of the 660 products on the EPA's List N are approved for use on porous surfaces, with the majority labeled for laundry presoak (20).

Disinfectants used in FSEs

Chemical disinfectants are necessary to carry out effective environmental cleaning procedures within FSEs. A number of EPA-approved disinfectant products with various active ingredients are currently available to consumers in the United States. Compounds, such as alcohol, chlorine, peroxides, and quaternary ammonium compounds (QACs), are all frequently used as active ingredients (59); however, advantages and disadvantages exist for each category. Alcohol-based disinfectants are believed to act by targeting the viral envelopes to denature proteins (60, 61). Previous studies report that alcohols (ethanol and isopropanol) at concentrations ranging from 62 to 80% can effectively inactivate HCoV on hard surfaces (62, 12); however, reduced efficacy has been noted when testing was conducted with a heavy organic burden (59). Chlorine-based disinfectants are commonly used because of their broad-spectrum antimicrobial efficacy and their relatively low cost, but because they act as strong oxidizers, they can damage surfaces after repeated long-term use and when used at higher concentrations (59, 63). Peroxides demonstrate virucidal activity by oxidizing viral lipids, proteins and nucleic acids; however, there is limited published data regarding their efficacy against viruses on surfaces other than stainless steel and glass (64, 60). QACs are cationic detergents that work by disrupting a microorganism's lipid membrane and are less likely to damage surfaces, but they tend to be less effective against gram-negative bacteria and nonenveloped viruses (60, 59, 63). In the foodservice industry, the aforementioned active ingredients can be used as disinfectants in a dilutable or ready-to-use (RTU) form and are applied to surfaces via spraying or wiping.

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To help navigate which disinfectants to use during the most recent COVID-19 pandemic, the EPA compiled a list of disinfectant products approved for use against SARS-CoV-2 (List N, n = 660) (20). To appear on List N, a product must be able to achieve at least a 3-log reduction of HCoV 229E or other HCoVs within 10 min or be able to kill a more resistant pathogen than SARS-CoV-2, such as human norovirus (HuNoV) (20, 65). Although the disinfectants on List N are required to be tested against an EPA-approved, hard, non-porous surface (65), there is limited published data available to verify the efficacy of these products on surfaces other than glass and stainless steel. Some published studies have been conducted using products from List N, but most of these tests were only conducted in suspension and not on carriers (12, 66). As previously discussed, environmental temperature and relative humidity can impact the survival time of a virus on surfaces. In addition, the amount of soil load present in the virus inoculum has been shown to provide a protective effect to the virus during and after being dried on a surface (45, 46, 35, 44). Validation of the efficacy of products from EPA's List N on different surface materials and in the presence of a soil load is necessary to provide a more accurate estimation of a disinfectant's efficacy under field conditions.

DISINFECTANT TESTING METHODS

Disinfectant Suspension Testing Method

The antimicrobial efficacy of liquid disinfectants registered with the EPA decades ago was primarily evaluated using a suspension test method (67, 68). Disinfectant suspension testing requires the virus suspension to be exposed to the disinfectant in a test

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tube for a designated contact time, and a neutralization substance is added to stop the antimicrobial activity of the disinfectant after the contact time. While other standard methods do exist, the American Society of Testing and Materials (ASTM) E1052-20 (69) is commonly used for testing the virucidal efficacy of a disinfectant in suspension. Suspension tests are relatively simple to perform and can provide informative data on the efficacy of a disinfectant against certain viruses. However, since the virus is not required to be tested in the presence of a soil load (69) and is not exposed to other environmental factors (e.g., drying and relative humidity), suspension tests may overestimate the efficacy of a disinfectant when it is used in a field setting (70). Currently, the efficacy of a disinfectant must be evaluated on hard, non-porous surface carriers in order to be registered with the EPA (65).

Disinfectant Carrier Testing Method

Disinfectant carrier testing requires that the virus is first dried on the carrier, and then exposed to a disinfectant for the product's designated contact time. Unlike in suspension testing, during carrier tests the disinfectant must be able to successfully penetrate the dried inoculum in order to access and inactivate the virus on the carrier (71). For a disinfectant to be registered with the EPA, efficacy testing must be conducted on glass carriers (72, 73). Since the results from efficacy tests on glass may not accurately reflect a product's efficacy when used on other types of surfaces, there is an additional standard carrier testing method. The ASTM E2197-17 (74) uses brushed stainless steel disc carriers to evaluate the virucidal efficacy of disinfectants. The smaller carrier size (1

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cm in diameter) used in the ASTM E2197-17 method (74), compared to that of the 100 x15 mm glass Petri dishes used in the EPA method (72, 73), is advantageous when conducting disinfectant efficacy testing because less testing space and surface materials are required.

CYTOTOXICITY AND DISINFECTANT NEUTRALIZATION

During efficacy testing, effective neutralization of the disinfectant after the specified contact time with the virus is essential to eliminate potential toxicity to the host cell line. Improper neutralization of the disinfectant can prevent accurate estimation of its efficacy because it can be difficult to differentiate between cell death caused by cytotoxicity of the disinfectant, and the cytopathic effect (CPE) caused by viral infectivity (75). To achieve neutralization, a number of methods can be used: diluting the biocide to a level at which it has no inhibitory effect in the medium used for recovery, chemically neutralizing the biocide by using a non-toxic neutralizing agent or using membrane or gel filtration to physically remove the biocide (76, 77, 78).

Currently, a universal neutralizer for all classes of disinfectants does not exist. Previous studies have reported that Dulbecco's Modified Eagle Medium (DMEM) + 2% fetal bovine serum (FBS) can effectively neutralize alcohol-based disinfectants during disinfectant efficacy testing (62, 79), while another study reported that a 10% FBS solution combined with a ten-fold dilution can also completely eliminate cytotoxicity (80). The use of 0.1 and 1% sodium thiosulfate ($Na_2S_2O_3$) has been reported to remove cytotoxicity caused by chlorine-based disinfectants in previous disinfectant studies (79,

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81). Peroxide-based disinfectants have shown to be inactivated by neutralizing solutions composed of 10% FBS (82), 10% FBS + tryptic soy broth (TSB; 83), or 1,300 U/mL catalase (84). A number of neutralization methods have been previously reported to eliminate the cytotoxicity of QAC-based disinfectants such as Sephadex LH-20 gel filtration (85), tryptose phosphate broth (TPB; 70), 3% beef extract-0.05M glycine (86), and 4% Lecithin-28% Tween 80 (81). While the aforementioned neutralizers **(Table 1)** were reported to effectively neutralize their respective disinfectants, the cell lines used to conduct cytotoxicity and neutralization testing varied between studies. Since all cell culture lines are not equally robust, the effectiveness of a single neutralization method may not be successful when used on other cell lines. Therefore, effective optimization of disinfectant neutralization is a critical first step for efficacy testing.

CONCLUSION

In summary, studies have demonstrated that the novel pathogenic HCoV, SARS-CoV-2, can survive on surfaces long enough for secondary transmission to potentially occur. Currently, there is limited published data on the efficacy of disinfectants against SARS-CoV-2 on surfaces that are frequently found in FSEs. To conduct disinfectant efficacy testing, the appropriate surrogates, which can be handled in a BSL-2 laboratory, need to be determined. Furthermore, the regulations currently in place focus on sanitizing food-contact surfaces, which are typically found in the back-of-the-house, and surface disinfection is not defined or required in the FDA Food Code. This study aims to I) optimize a neutralization method for chlorine- and QAC-based disinfectants to eliminate

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cytotoxicity during efficacy testing, II) determine the efficacy of three RTU-spray disinfectants against SARS-CoV-2 surrogates, BCoV and HCoV OC43, in suspension and III) on surfaces commonly found in FSEs. The findings from this study can be used to inform the disinfection of surfaces found in the front-of-the-house in FSEs, which are typically nonfood-contact surfaces. Implementation of effective environmental cleaning is necessary, in conjunction with other infection control measures, to prevent the further spread of SARS-CoV-2.

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Table 1.1 Common disinfectant neutralization methods.

Disinfectant Active Ingredient	Neutralizer	Virus	Cell Line	Reference
62, 70, 75, 80, & 95% Ethanol	DMEM + 2% FBS	HCoV 229E	Huh7	62
20 & 75% Ethanol	DMEM + 2% FBS	SARS-CoV-2	Vero-E6	79
50, 70, & 90% Ethanol	10% FBS	FCV; MNV	CRFK; RAW 264.7	80
70, 75, 80, & 95% Isopropanol	DMEM + 2% FBS	HCoV 229E	Huh7	62
50, 70, & 90% Isopropanol	10% FBS + 10^{-1} dilution	FCV; MNV	CRFK; RAW 264.7	80
1,000 mg/L available chlorine	0.1% $Na_2S_2O_3$	SARS-CoV-2	Vero-E6	79
500 mg/L available chlorine	0.05% $Na_2S_2O_3$	SARS-CoV-2	Vero-E6	79
3% sodium hypochlorite	1% Na_2S_2O	MNV	RAW 264.7	81
4.25% accelerated hydrogen peroxide (AHP)	10% FBS	FCV; MNV	CRFK; RAW 264.7	82
7% AHP	10% FBS + 10% TSB	Reovirus; Sindbis virus	L929; BHK 21	83
0.5, 0.88, & 1.4% hydrogen peroxide (H_2O_2)	Catalase (1,300 U/mL)	FCV; TuV	CRFK; LLC-MK2	84
0.05% benzalkonium chloride	Sephadex LH-20 gel filtration	CCV, CPV, DBT; MHV, KRV	CRFK; Rat embryo cells	85
0.04% n-Alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	TPB	HCoV-229E	L-132	70
10% NaHCO3 + 10% n-Alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	3% beef extract-0.05 M glycine	FCV	CRFK	86
0.08% n-Alkyl dimethyl benzyl ammonium chloride (1% C_8, 1% C_{10}, 67% C_{12}, 25% C_{14}, 7% C_{16}, 1% C_{18}); 0.02% n-Alkyl dimethyl benzyl ammonium chloride (40% C_{12}, 50% C_{14}, 10% C_{16})	4% Lecithin + 28% Tween 80	MNV	RAW 264.7	81

CHAPTER TWO:

EFFICACY OF READY-TO-USE SPRAY DISINFECTANTS AGAINST SARS-COV-2 SURROGATES, BOVINE CORONAVIRUS AND HUMAN CORONAVIRUS OC43, IN SUSPENSION

ABSTRACT

Since the emergence of the novel pathogenic coronavirus (CoV) severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in late 2019, the World Health Organization (WHO) has confirmed over 760,360,900 cases of coronavirus disease 2019 (COVID-19) globally (https://covid19.who.int/). In the United States, a multitude of foodservice establishments (FSEs) were forced to close their doors due to the rapid spread of SARS-CoV-2. Effective environmental cleaning procedures in FSEs are necessary to prevent and control the further spread of SARS-CoV-2. However, data regarding the efficacy of disinfectants from the Environmental Protection Agency's (EPA's) List N against two SARS-CoV-2 surrogate viruses, bovine coronavirus (BCoV) and human coronavirus (HCoV) OC43, is limited. The aim of this study was to optimize experimental procedures to evaluate three ready-to-use (RTU) spray disinfectants of different active ingredients (chlorine, hydrogen peroxide, and quaternary ammonium compound [QAC] + alcohol) from EPA's List N against two SARS-CoV-2 surrogates, BCoV and HCoV OC43, in suspension. First, we optimized a neutralization method for each tested disinfectant. Then, we determined the efficacy of three RTU disinfectants against BCoV and HCoV OC43 in suspension with a soil load of 5% fetal bovine serum

(FBS). Infection media paired with centrifugation in Pierce^{TM} detergent removal columns was used to successfully neutralize the QAC-based disinfectant, whereas a 5% FBS + 1% sodium thiosulfate ($Na_2S_2O_3$) solution and 1,300 U/mL catalase, followed by centrifugation in Amicon® Ultra-4 centrifugal units, was used to neutralize the chlorine-based disinfectant and hydrogen peroxide-based disinfectant, respectively. For virus titration, the sample inoculum was removed from the 96-well plate after the 1 h incubation period and replaced with fresh infection media to further eliminate residual cytotoxicity from the disinfectant and neutralizer mixture. All three RTU disinfectants reduced the inoculated virus titers below the limit of detection (LOD) and were able to achieve a >3.0 $\log_{10}$ reduction against BCoV and HCoV OC43 in suspension with a 2 min contact time. Clorox Clean-Up Cleaner + Bleach reduced BCoV and HCoV OC43 by >4.7 and >4.4 $\log_{10}$ TCID$_{50}$/mL, respectively. Oxivir Tb achieved a >5.0 and >4.6 $\log_{10}$ reduction of BCoV and HCoV OC43, respectively. Cavicide 1 reduced BCoV and HCoV OC43 by >4.7 and >4.6 $\log_{10}$ TCID$_{50}$/mL, respectively. In conclusion, all three RTU disinfectants were found to be efficacious against BCoV and HCoV OC43 in suspension. In addition, we were able to optimize methods to effectively neutralize the chlorine- and QAC-based disinfectant used in this study.

INTRODUCTION

The novel pathogenic coronavirus (CoV) known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China in late 2019 (1). The highly contagious nature and rapid spread of SARS-CoV-2 amid the most recent

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pandemic forced many foodservice establishments (FSEs) in the United States to shut

down. According to the U.S. Bureau of Labor Statistics, the unemployment rate for food

services and drinking places increased from 8.5 to 35.4% from March to April in 2020

(https://www.bls.gov/iag/tgs/iag722.htm#workforce). Although SARS-CoV-2 is

primarily transmitted via direct contact with aerosols and respiratory droplets (2),

contaminated objects or surfaces (fomites) could potentially serve as another route of

transmission (3, 4, 5, 6). Surfaces can be contaminated with SARS-CoV-2 by infected

individuals who shed the virus via respiratory secretions, saliva droplets, and/or stool (7).

Depending on the surface type (i.e., porous or non-porous), SARS-CoV-2 has been

reported to survive for periods ranging from 2 to 28 days (8, 9) highlighting the

importance of the correct use of disinfectants to carry out effective environmental

cleaning.

To help navigate which disinfectants to use during the most recent COVID-19

pandemic, the EPA compiled a list of disinfectant products approved for use against

SARS-CoV-2 (List N, n = 660) (10). The products on List N must be able to achieve at

least a 3-log reduction of HCoV within 10 min or be able to kill a more resistant

pathogen than SARS-CoV-2 such as human norovirus (HNoV) (10, 11). Currently, the

EPA-approved regulatory surrogates for registering a disinfectant as efficacious against

SARS-CoV-2 include HCoV 229E or other HCoVs (10). Although both HCoV 229E and

SARS-CoV-2 belong to the family *Coronaviridae*, HCoV 229E is in the genus

Alphacoronavirus while SARS-CoV-2 is in the genus *Betacoronavirus* so the two could

potentially have different responses to environmental stresses (i.e., chemical disinfection

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and desiccation) (5). Bovine coronavirus (BCoV) and human coronavirus (HCoV) OC43 are both in the genus *Betacoronavirus* (12). There is currently limited published data regarding the efficacy of products on List N against these *Betacoronaviruses*. A more accurate estimation of disinfectant efficacy against SARS-CoV-2 may be provided if efficacy testing is conducted using BCoV and HCoV OC43 as surrogates, instead of HCoV 229E, which produces ambiguous cytopathic effect (CPE) towards the host cell line used for titer determination.

When conducting disinfectant efficacy testing, effective neutralization of the disinfectant after the specified contact time with the virus is essential to eliminate potential cytotoxicity to the host cell line. Improper neutralization of the disinfectant can prevent accurate estimation of its efficacy because it can be difficult to differentiate between cell death caused by cytotoxicity of the disinfectant, and the CPE caused by viral infectivity (13). Currently, a standard neutralization method does not exist. While previous studies have reported a number of effective neutralization methods (**Table 1.1**)**,** the cell lines used to conduct cytotoxicity and neutralization testing varied between studies. Since all cell culture lines are not equally robust, it is critical to identify an effective neutralization method prior to conducting disinfectant efficacy testing against the cell line for a specific virus.

The aim of this study was to evaluate the efficacy of three ready-to-use (RTU) spray disinfectants of different active ingredients (chlorine, hydrogen peroxide, and

QAC + alcohol) from EPA's List N against two SARS-CoV-2 surrogates, BCoV and HCoV OC43, in suspension. Prior to conducting disinfectant efficacy testing, a method to effectively neutralize the chlorine- and QAC-based disinfectants was optimized.

MATERIALS AND METHODS

Cell Culture

Human rectal tumor (HRT-18G) cells (ATCC CRL-11663) were used to propagate BCoV strain Mebus (BEI Resources NR-445) and HCoV strain OC43 (ATCC VR-1558). Cells were cultured in T75 and T175 vented capped flasks (Corning, Corning, NY) with Dulbecco's Modified Eagle Medium, 1x (DMEM; Corning, Corning, NY) supplemented with 3% low-endotoxin heat-inactivated fetal bovine serum (FBS; Corning, Corning, NY), 100 U/L penicillin (Corning, Corning, NY), and 100 mg/L streptomycin (Corning, Corning, NY). The CO_2 incubator (VWR International, Radnor, PA) was set at 37°C and 5% CO_2. HRT-18G cells were subcultured at ~90% confluency (~5 d) in a 1:4 split ratio using 0.25% trypsin EDTA (Thermo Fisher Scientific, Waltham, MA). Cells that have been passaged >30 times were not used for the median tissue culture infectious dose ($TCID_{50}$) assay for virus titration.

Viral Stock Preparation

Ninety percent (90%) confluent monolayers of HRT-18G cells were infected with BCoV and HCoV OC43 at a multiplicity of infection (MOI) of ~0.05. Initially, the virus had a 1 h adsorption phase with 5 mL infection media in a T75 flask that contained a

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monolayer of HRT-18G cells at 33°C and 5% CO_2 (VWR International, Radnor, PA).

The infection media consisted of DMEM supplemented with 2% FBS, 100 U/L

penicillin, and 100 mg/L streptomycin. During viral adsorption phase, the flask was

manually rocked every 15 min to ensure even distribution. Then, the infection media was

poured off and 25 mL of fresh infection media was added to the flask. The flask was

placed back in the incubator and held for 5-7 days at 33°C until at least ≥80% cytopathic

effect (CPE) was observed using an inverted microscope (Olympus CK2; Olympus

Optical Co., LTD, Tokyo, Japan). HRT-18G cells with ~80% CPE due to viral infection

were subjected to freeze-thaw cycles using a -80°C freezer and a biological safety cabinet

(Class II, Type A2, Thermo Scientific) at room temperature for ~2 h and ~1 h,

respectively. After three freeze-thaw cycles, the cell/virus solution was centrifuged at

5,000 x g for 10 min at 4°C (Model 5804 R; Eppendorf, Germany) to remove cell debris.

BCoV and HCoV OC43 stocks at ca. 10^8 to 10^9 $TCID_{50}$/mL were aliquoted and stored at

-80°C. The titers of infectious BCoV and HCoV OC43 were quantified by $TCID_{50}$ assay

as described below.

$TCID_{50}$ Assay for BCoV and HCoV OC43

HRT-18G cells were seeded in 96-well plates (Corning, Corning, NY) at a density

of ~2.0 x 10^4 cells/well, and incubated at 37°C and 5% CO_2 with 0.2 mL of cell culture

media. HRT-18G plates were used between 90-100% confluency (~5 d). Ten-fold serial

dilutions of virus/test samples, in triplicate, were prepared in infection media. The cell

culture media was removed from each 96-well plate, and 0.1 mL of the undiluted or

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serially diluted sample was placed onto replicate wells (n = 8) of the appropriate 96-well plate and then rocked 10-15 times. The inoculated HRT-18G plates were incubated at 33°C and 5% CO_2 for 1 h and rocked 10-15 times every 15 min. Infection media (0.1 mL) was added to each well, followed by incubation at 33°C and 5% CO_2 for 7 d, and then scored for CPE. There was a positive control (previous viral stock) and a negative control (infection media) for each passage of cells used. Quantification of the titer of BCoV and HCoV OC43 was determined via the improved Kärber method (14). The limit of detection (LOD) for this $TCID_{50}$ assay was calculated as 4.2 $TCID_{50}$/mL (0.6 $\log_{10}$ $TCID_{50}$/mL) if undiluted samples were readable, and 4.2 x 10^1 $TCID_{50}$/mL (1.6 $\log_{10}$ $TCID_{50}$/mL) if the lowest readable dilution is 1:10.

Disinfectant Selection

The three disinfectants **(Table 2.1)** tested in this study were selected based on the following criteria: (1) included on EPA List N and/or List G (for HNoV), (2) RTU, (3) different active ingredients (chlorine, hydrogen peroxide, QAC + alcohol), (4) have a ≤ 2 min contact time, and (5) are readily available for consumer purchase. Two separate lots of each disinfectant were purchased and tested in this study.

Cytotoxicity and Neutralization Testing

Cytotoxicity testing for the neutralizers in **Table 2.2** and validation of neutralization was conducted according to the methods outlined in the American Society of Testing and Materials (ASTM) E1052-20, with some modifications (15). To test the

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neutralizer cytotoxicity, each neutralizer in **Table 2.2** underwent ten-fold serial dilutions in infection media. HRT-18G monolayers were observed under an inverted microscope for any cytotoxicity after a 1 h, 24 h, and 7 d contact time with undiluted, 10^{-1}, and 10^{-2} dilutions of each neutralizer. Next, each disinfectant product was combined with the appropriate neutralizer, and underwent ten-fold serial dilutions. The disinfectant/neutralizer mixture was observed for any cytotoxicity against the HRT-18G cell line after 1 h, 24 h, and 7 d. If cytotoxicity was observed, an additional 'washing step' with phosphate buffered saline (PBS) was used to help remove any residual disinfectant that would cause cytotoxicity or interfere with BCoV and HCoV OC43 infectivity. Mixtures of Clorox Clean-Up Cleaner + Bleach and Oxivir Tb and their respective neutralizers **(Table 2.1)** were centrifuged using Amicon® Ultra-4 30K MWCO centrifugal units (MilliporeSigma, Burlington, MA) at 4,000 x g for 20 min at 4°C. Following centrifugation, the samples were washed by adding 3 mL PBS, and the samples were then centrifuged again. This process was repeated two additional times to remove any residual disinfectant from the mixture. After final centrifugation, samples were adjusted to 1 mL using infection media, and underwent a ten-fold serial dilution in infection media for $TCID_{50}$ assay.

Mixtures of Cavicide 1 and its respective neutralizer **(Table 2.1)** were centrifuged using Pierce[TM] Detergent Removal Spin Columns (Thermo Fisher Scientific, Waltham, MA), previously prepared according to manufacturer's instructions, at 1,000 x g for 2 min at 4°C. After centrifugation, the detergent-free filtrate was collected and underwent a ten-fold serial dilution in infection media. After serial dilutions were made for each

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disinfectant/neutralizer mixture, either 20 or 100 µL of the undiluted, 10^{-1}, and 10^{-2} diluted samples of the disinfectant/neutralizer 'washed' mixture were collected and assayed in 96-well plates with HRT-18G cells. The HRT-18G monolayers were observed for cytotoxicity after 1 h, 24 h, and 7 d.

To test neutralization effectiveness, 10 µL of either diluted BCoV (ca. 10^3 to 10^4 TCID_{50}/mL) or HCoV OC43 (ca. 10^3 to 10^4 TCID_{50}/mL) stock was mixed with the 'washed' disinfectant/neutralizer solution, and a control was mixed with infection media. The mixtures then underwent ten-fold serial dilutions, and the undiluted to 10^{-6} dilutions were used for titration via TCID_{50} assay with HRT-18G cells, as previously described. Neutralization was considered sufficient if, when compared to controls, more than 80 $\pm$5% of virus infectivity was recovered in the samples of disinfectant/neutralizer mixture that were inoculated with virus (15).

Quantitative Suspension Test

Efficacy of the three RTU disinfectants was evaluated against BCoV and HCoV OC43 in suspension according to ASTM E1052-20 (15) with modifications (**Figure 2.1**). All suspension tests were conducted in the presence of a 5% FBS soil load. First, 10 µL of BCoV or HCoV OC43 was mixed with 90 µL of undiluted disinfectant in a 2.0 mL centrifuge tube in triplicate at ambient laboratory conditions (20-25°C; 20-40% RH) and held for a 2 min contact time. As a control, 10 µL of BCoV or HCoV OC43 was mixed in triplicate with 90 µL of infection media instead and held for a 2 min contact time. After the 2 min contact time, both treatment and control samples were neutralized with 900 µL

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of the respective neutralizer (**Table 2.1**) and 'washed' using either Amicon® Ultra-4

centrifugal units or Pierce^{TM} Detergent Removal Columns, as described above. After the

removal of residual disinfectant, samples were collected, underwent a ten-fold serial

dilution, and were used for titration via TCID$_{50}$ assay with HRT-18G cells, as previously

described. To reduce the LOD and increase the dynamic range for efficacy testing, 100

μL of inoculum was used for TCID$_{50}$ assay.

Calculations

Log reductions **(Equation 1)** were calculated as previously described (15).

$$Log\ reduction = log\left(\frac{geometric\ mean\ Virus\ Control}{geometric\ mean\ Treatment\ samples}\right) \text{(Equation 1)}$$

A dynamic range, the maximum range of infectivity able to be observed, was

calculated in each experiment for all three RTU disinfectants tested:

$$Disinfectant\ dynamic\ range = \ log_{10}\ (Virus\ control) - \ log_{10}\ (LOD) \text{ (Equation 2)}$$

Statistical Analysis

Three replicates of ten-fold serial dilutions of each RTU disinfectant in the

suspension test were tested in two independent experiments. Statistical analysis was

performed with JMP (JMP Pro 16.0.0, SAS Inc., Cary, NC) using one-way analysis of

variance (ANOVA) and Student's t-test to determine the relationship between surrogates,

disinfectants, and log reduction. All results were expressed as mean ± standard deviation. Statistical significance was defined as a *p*-value of <0.05.

RESULTS

Neutralization Optimization

Due to the lack of a universal neutralizer or published studies relevant to the three disinfectants tested in this study, a neutralization method for each product was optimized prior to conducting disinfectant efficacy testing. The neutralization method used for testing hydrogen peroxide-based disinfectants in a previous study (16), i.e., 1,300 U/mL catalase followed by washing via Amicon® Ultra-4 centrifugal unit and PBS, was able to eliminate cytotoxicity to the HRT-18G cell line for Oxivir Tb in this study. However, neither the sole use of a chemical neutralizer or addition of membrane or Sephadex gel filtration could completely remove the residual toxicity of Clorox Clean-Up Cleaner + Bleach and Cavicide 1.

Cytotoxicity of Clorox Clean-Up Cleaner + Bleach to the HRT-18G cell line could only be eliminated when combining both chemical neutralization and membrane filtration with subsequent removal of the inoculum after the 1 h incubation period, and the addition of fresh infection media during the $TCID_{50}$ assay (**Table 2.3**).

More than twenty-five different experiments were conducted to optimize the neutralization method for the QAC-based disinfectant used in this study (**Table 2.4**). Cytotoxicity to the HRT-18G cell line was not completely removed when only chemical

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neutralization was used. Of the membrane filtration matrices that were tested in combination with chemical neutralizers, the use of infection media paired with centrifugation in Pierce^{TM} detergent removal spin columns, and subsequent removal of the inoculum, as described for the Clorox product, was the only method that removed all cytotoxicity caused by Cavicide 1.

Cytotoxicity and Neutralization Effectiveness Testing

As shown in **Table 2.2**, neutralization methods I, II, and III were evaluated based on their ability to prevent cytotoxicity and neutralize their respective disinfectant. Clorox, Oxivir Tb, and Cavicide 1 showed no apparent cytotoxicity toward HRT-18G cells after 1 h incubation and at the undiluted, 1:10, and 1:100 dilutions when using neutralization method I, II, and III, respectively. No adverse effects on the HRT-18G cells were observed after exposure to each of the three chemical neutralizers listed in **Table 2.1**. When validating neutralization, neutralization methods I, II, and III yielded >80% recovery of both BCoV and HCoV OC43 when compared to the controls (**Table 2.2**).

Quantitative Suspension Test

The virucidal efficacy of three RTU disinfectants from the EPA's List N was evaluated against BCoV and HCoV OC43 in this study. All suspension tests were conducted in the presence of a soil load of 5% FBS. Disinfectants were considered efficacious if able to achieve a ≥ 3.0 log reduction in virus titer (11). **Table 2.5** shows the efficacy of two separate batches of Clorox, Oxivir Tb, and Cavicide 1 against BCoV and

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HCoV OC43 in suspension. The average initial inoculum levels for BCoV and HCoV OC43 were 7.7 ($\pm$0.2) and 7.7 ($\pm$0.4) $\log_{10}$ TCID$_{50}$/mL, respectively. After a 2 min contact time, all three disinfectants reduced the infectivity of BCoV and HCoV OC43 below the limit of detection (LOD). As a result, Clorox and Cavicide 1 achieved a >4.7 log reduction of BCoV, and Oxivir Tb achieved a >5.0 log reduction. When tested against HCoV OC43, Clorox was able to achieve a >4.4 log reduction, and Oxivir Tb and Cavicide 1 both achieved a >4.6 log reduction. Since the titers of recovered virus reached the LOD of the TCID$_{50}$ assay (0.6 $\log_{10}$ TCID$_{50}$/mL), the difference in disinfection efficacies of each product from two production lots or among disinfectant products cannot be compared.

DISCUSSION

The highly contagious nature and rapid spread of SARS-CoV-2 amid the most recent pandemic forced many FSEs in the United States to shut down. Implementation of effective environmental cleaning and disinfecting is necessary to prevent the further spread of SARS-CoV-2. The EPA's List N contains a number of disinfectant products approved for use against SARS-CoV-2 (10). However, there is currently limited published data regarding the efficacy of these products against two surrogate viruses, BCoV and HCoV OC43. In this study, we demonstrated the efficacy of three RTU disinfectants from List N against BCoV and HCoV OC43 in suspension. Prior to conducting efficacy testing, a neutralization method for the chlorine- and QAC-based disinfectants was optimized.

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Cytotoxicity and neutralization testing are critical for the successful evaluation of disinfectants intended for virucidal efficacy testing (17). Improper neutralization of the disinfectant can prevent accurate estimation of its efficacy because it can be difficult to differentiate between cell death caused by cytotoxicity of the disinfectant, and the cytopathic effect (CPE) caused by viral infectivity (13). Currently, a universal neutralizer does not exist. Enzymes known as catalases are well known for their ability to effectively degrade hydrogen peroxide in a solution (18); however, similar to results reported in a study by Huang et al. (16), we found that the use of 1,300 U/mL catalase as a neutralizer must be paired with membrane filtration to completely remove the residual cytotoxicity of Oxivir Tb to the HRT-18G cells.

Previous studies (**Table 1.1**) used 0.1 and 1% sodium thiosulfate ($Na_2S_2O_3$) alone to neutralize varying concentrations of sodium hypochlorite when conducting disinfectant efficacy testing because of its ability to convert sodium hypochlorite into sodium chloride and sodium sulfate (19); however, not all cell lines are equally robust. In addition to the active ingredient in a disinfectant product, other components may be harmful to the cell line so they may also require neutralization. For example, in a previous study conducted by Huang et al. (16) sodium hypochlorite at 1,000 ppm was neutralized with 0.1% $Na_2S_2O_3$ when CRFK and LLC-MK2 cell lines were tested. However, in the current study, the use of 0.1 and 1% $Na_2S_2O_3$ alone was not sufficient in terminating the residual toxicity of Clorox Clean-Up Cleaner + Bleach to the HRT-18G cells. Since the amount of chlorine present in a solution is reduced due to the reaction between chlorine and organic matter (19), previous studies reported using a 10% FBS solution as a neutralizer for

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sodium hypochlorite (20, 21, 22). Membrane filtration has also previously been reported as an effective method for removing cytotoxicity when conducting disinfectant efficacy testing (23, 24, 25, 26). When we combined the use of 1% $Na_2S_2O_3$ + 5% FBS and membrane filtration using Amicon® Ultra-4 centrifugal units, cytotoxicity was still observed. Due to the sensitivity of the HRT-18G cell line observed in this study, we were only able to eliminate the residual toxicity of Clorox by subsequently removing the sample inoculum from the 96-well plates after the 1 h incubation period, and then adding fresh infection media to each well during the $TCID_{50}$ assay.

QACs are classified as cationic surface-active agents and are one of the most commonly used disinfectants in the food industry (27). However, QACs can be challenging to neutralize during disinfectant efficacy studies because true neutralization is not achieved, but rather a sequestration of the active agent (28). QACs can form micelles due to their amphiphilic nature, so the critical concentration of micelle formation (CCM) is an important factor to consider when determining whether a neutralizing solution will be able to effectively neutralize the QAC during disinfectant efficacy testing (28). If the neutralizing solution is diluted below its CCM, the micelles break down which releases the QAC back into the solution where it is once again active. Previous studies (**Table 1.1**) have reported using a variety of different neutralizers to eliminate residual toxicity of QAC-based disinfectants, including Sephadex LH-20 gel filtration, 3% beef extract-0.05M glycine, and 4% Lecithin-28% Tween 80. As shown in **Table 2.4**, variations of these neutralizers tested with the HRT-18G cell line were not able to eliminate the residual toxicity of Cavicide 1. An additional study conducted with SARS-

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CoV-2 by Welch et al. (23) evaluated the effectiveness of five different filtration matrices (Sephadex LH-20, Sephacryl S400HR, Amicon Ultra 50kDa MWCO centrifugal filters, Pierce Detergent Removal Columns, and Bio-Bead SM2) at removing the cytotoxicity from multiple types of detergents (e.g., ionic, anionic, non-ionic, and zwitterionic). The authors also measured the percentage of virus recovery following one filtration step. Their results indicated that the Pierce detergent removal columns were able to achieve 100% virus recovery and remove 100% of the cytotoxicity from three of the four detergents tested (23). In our study, when using infection media as the neutralizer, followed by centrifugation in Pierce^{TM} detergent removal columns, and removal of the sample inoculum after the 1 h incubation period, we were able to eliminate all residual toxicity of Cavicide 1 to the HRT-18G cells without losing any virus during the filtration step (**Table 2.4**). Our results clearly confirmed the effectiveness of Pierce^{TM} Detergent Removal Columns as a tool for efficacy testing of disinfectants that contain detergent components.

When tested in suspension against BCoV and HCoV OC43, all three RTU spray disinfectants were able to reach the EPA standard for antiviral efficacy against SARS-CoV-2 (≥ 3 $\log_{10}$ reduction) within 2 min in the presence of a 5% FBS soil load (**Table 2.5**). For all three disinfectants, the amount of inactivation detected after disinfection was the maximum amount able to be detected by the $TCID_{50}$ assay. However, the dynamic range (**Equation 2**) for each disinfectant varied due to differences in the titers of the recovery control (RC) samples. Therefore, statistical differences between log reduction values for the products tested could not be determined.

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The log reductions of BCoV and HCoV OC43 (**Table 2.5**) observed in this study after treatment with Clorox Clean-Up Cleaner + Bleach are in line with previously published data regarding the efficacy of products containing sodium hypochlorite. Xiling et al. (29) found that 500 and 1,000 ppm of available chlorine inactivated SARS-CoV-2 in suspension in less than 5 and 0.5 min, respectively. Another study reported that when SARS-CoV-2 was exposed to ~75 ppm and ~150 ppm of household bleach in suspension, no infectious virus could be detected after a 5 min contact time (30). In addition, Sattar et al. (31) observed a >3.0 $\log_{10}$ reduction in virus titer after a 1 min contact time when 0.1 and 0.5% sodium hypochlorite were evaluated against HCoV 229E.

When BCoV and HCoV OC43 were treated with Oxivir Tb for 2 min, we observed a >5.0 and >4.6 $\log_{10}$ reduction in virus titer, respectively (**Table 2.5**). Our results are consistent with those of previous studies, which reported a >4.0 $\log_{10}$ reduction of infectious HCoV 229E in 1 min when treated with 0.5% accelerated hydrogen peroxide (32).

The log reductions observed in this study for BCoV and HCoV OC43 following treatment with Cavicide 1 (**Table 2.5**) are similar to those reported in previous studies. In a study conducted with murine hepatitis virus (MHV), a potential surrogate for SARS coronavirus, a formulation of 0.10% QAC + 79% ethanol was able to achieve a 3.0 $\log_{10}$ reduction after a 0.5 min contact time (33). An additional study that evaluated a disinfectant that containing 50% ethanol + 0.083% QAC against SARS-CoV-2 reported a $\geq$4.5 $\log_{10}$ reduction in virus titer after a 2 min contact time in suspension (34).

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Overall, BCoV and HCoV OC43 were found to be sensitive to the disinfectants tested in this study. These results are expected as coronaviruses are enveloped viruses, and the envelope can easily be damaged leading to a loss of infectivity. When considering the practical use of disinfectants in FSEs, further study is needed to determine the shortest contact time (e.g., 30 or 60 sec) for each product to achieve a >3 $\log_{10}$ reduction.

CONCLUSION

In summary, Clorox Clean-Up Cleaner + Bleach, Oxivir Tb, and Cavicide 1 were found to be efficacious against BCoV and HCoV OC43 in suspension with a 2 min contact time. In addition, we were able to optimize a method to effectively neutralize the chlorine- and QAC-based disinfectant used in this study. Considering the differences in robustness between cell lines and the varying compositions of disinfectant products, neutralization optimization is a critical step when conducting any efficacy study. Therefore, our approaches for disinfectant neutralization will be helpful for future studies when evaluating new disinfectant products. The findings from this study can be used to inform the disinfection of surfaces found in the front-of-the-house in FSEs, which are typically nonfood-contact surfaces. To prevent the further spread of SARS-CoV-2 in FSEs, implementation of effective environmental cleaning, in conjunction with other infection control measures, is necessary.

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Inoculate BCoV or HCoV OC43 in centrifuge tubes as Treatment (Tr) and Virus Control (VC) samples, and infection media as the Cytotoxicity Control/Neutralization Effectiveness Control (CC/NEC) sample.

Add 90 µL disinfectant to Tr and CC/NEC samples.

Add 90 µL infection media to VC samples.

Let all centrifuge tubes stand for 2 min contact time.

Add 900 µL of appropriate neutralizer to all centrifuge tubes.

Transfer samples and wash 3 times with PBS via centrifugation (Amicon Ultra-4 30K MWCO ultrafiltration units or Pierce^{TM} Detergent Removal Columns).

Dilute samples and assay samples by TCID$_{50}$ assay.
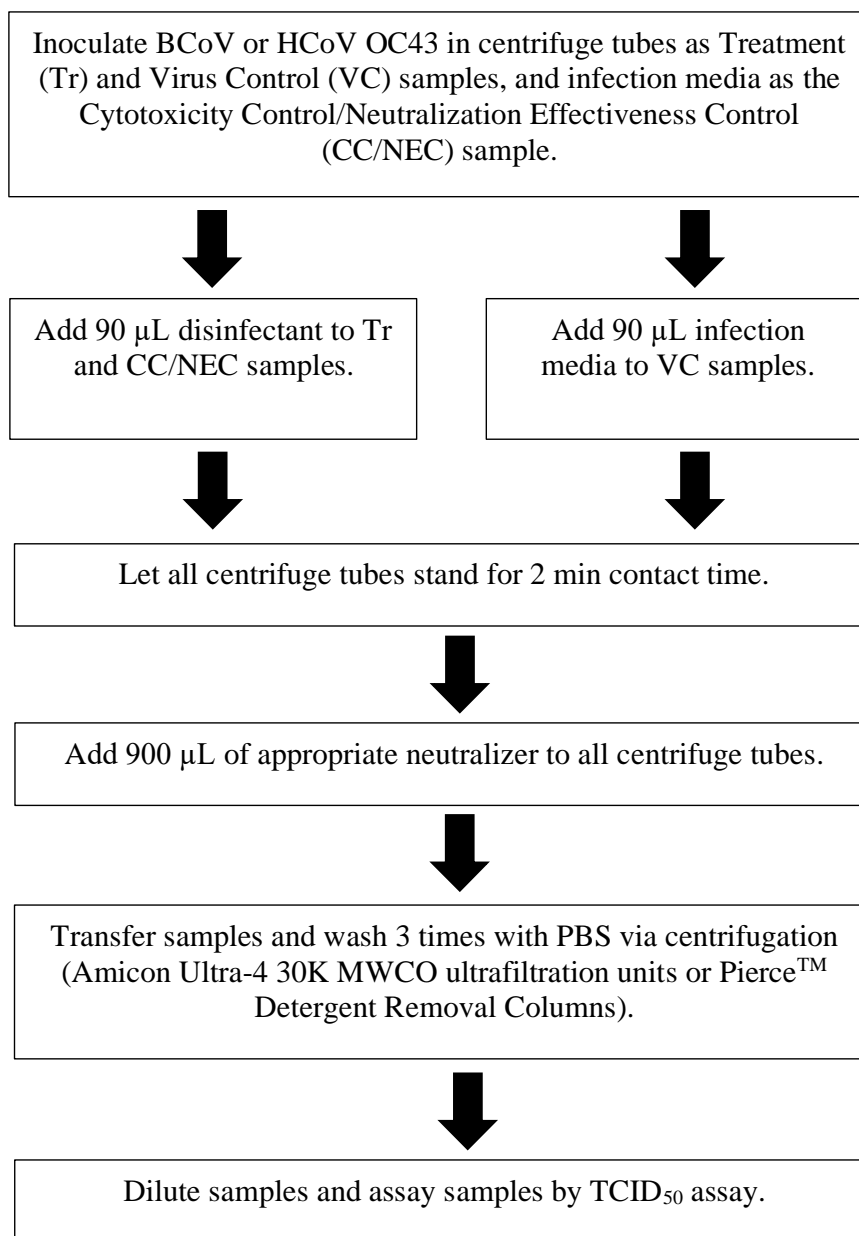
Figure 2.1: Workflow for disinfectant suspension test.

Table 2.1: Active Ingredients of Selected RTU Disinfectants and Neutralization Methods.

Ready-to-Use Disinfectant	Active Ingredient(s)	Neutralizer	Centrifugation
Clorox Clean-Up Cleaner + Bleach	1.84% sodium hypochlorite	5% (v/v) FBS + 1% (v/v) sodium thiosulfate	Amicon® Ultra-4 centrifugal unit
Oxivir Tb	0.5% hydrogen peroxide	1,300 U/mL catalase	Amicon® Ultra-4 centrifugal unit
Cavicide 1	0.76% didecyldimethylammonium chloride + 7.50% ethanol + 15.0% isopropanol	Infection media	Pierce™ Detergent Removal Spin Column

Table 2.2: Cytotoxicity test on HRT-18G cells and Neutralization Effectiveness Test for BCoV and HCoV OC43.

Disinfectant	Neutralization Method	Cytotoxicity Test			Neutralization Test Reduction ($\log_{10}$ TCID$_{50}$/mL)	
		10^0	10^{-1}	10^{-2}	BCoV	HCoV OC43
Clorox Clean-Up Cleaner + Bleach	[I] 5% FBS + 1% $Na_2S_2O_3$ & Amicon® Ultra-4 centrifugal unit + PBS[a]	-[d]	-	-	0.0 ± 0.1[e]	0.4 ± 0.2
Oxivir Tb	[II] 1,300 U/mL catalase & Amicon® Ultra-4 centrifugal unit + PBS[b]	-	-	-	0.0 ± 0.2	0.0 ± 0.1
Cavicide 1	[III] Infection media & Pierce™ Detergent Removal Spin Column[c]	-	-	-	0.3 ± 0.2	0.2 ± 0.2

[a][I] Method referred to here and after as Neutralization Method I. Samples were washed with phosphate buffered saline (PBS) three times. The inoculum was removed after 1 h incubation and then fresh infection media was added.
[b][II] Method referred to here and after as Neutralization Method II. Samples were washed with PBS two times.
[c][III] Method referred to here and after as Neutralization Method III. The inoculum was removed after 1 h incubation and then fresh infection media was added.
[d]The data are expressed as CT/-, CT presented as cytotoxicity caused by incomplete neutralization.
[e]The data is expressed as means $\pm$SD. All data were collected from three independent experiments.

Table 2.3: Cytotoxicity of Chlorine Neutralization Method with Clorox Clean-Up Cleaner + Bleach on HRT-18G cells.

Neutralization Method		TCID$_{50}$ Assay[a]	Cytotoxicity Test[b]		
Neutralizer	Washing Step	Media Replacement	10^0	10^{-1}	10^{-2}
5% FBS + 0.1% Na$_2$S$_2$O$_3$	-	-	CT[c]	-	-
5% FBS + 0.1% Na$_2$S$_2$O$_3$	Amicon® Ultra-4 centrifugal unit + PBS[d]	-	CT	-	-
5% FBS + 1% Na$_2$S$_2$O$_3$	-	-	CT	CT	-
5% FBS + 1% Na$_2$S$_2$O$_3$	PD-10 Desalting Column containing 8.3 mL Sephadex G-25 Resin	-	CT	-	-
5% FBS + 1% Na$_2$S$_2$O$_3$	Amicon® Ultra-4 centrifugal unit + PBS[d]	-	CT	-	-
5% FBS + 1% Na$_2$S$_2$O$_3$	Amicon® Ultra-4 centrifugal unit + PBS[d]	Removed inoculum after 1 h incubation period, then added fresh infection media	-	-	-

[a]For TCID$_{50}$ assay, 100 µL samples were used.
[b]All experiments were performed in duplicate.
[c]The data are expressed as CT/-, CT presented as cytotoxicity caused by incomplete neutralization.
[d]Samples were washed with phosphate buffered saline (PBS) three times.

Table 2.4: Cytotoxicity of QAC Neutralization Methods with Cavicide 1 on HRT-18G cells.

Neutralization Method			TCID$_{50}$ Assay		Cytotoxicity Test		
Neutralizer	Washing Step		Media Replacement	Sample Amount (µL)	10^0	10^{-1}	10^{-2}
DE Broth	-		-	20	CT[a,b]	CT	-
DE Broth	AcroPrep™ 24-well 30K Omega™ Filter Plate		-	20	CT[b]	ND[c]	ND
5% FBS + 0.1% $Na_2S_2O_3$	AcroPrep™ 24-well 30K Omega™ Filter Plate		-	20	CT[b]	ND	ND
5% FBS + 0.1% $Na_2S_2O_3$	Amicon® Ultra-4 centrifugal unit + PBS[d]		-	20	CT[e]	ND	ND
5% FBS + 0.1% $Na_2S_2O_3$	Amicon® Ultra-4 centrifugal unit + HBSS		-	20	CT[b]	ND	ND
10% DE Broth	-		-	20	CT[e]	CT	-
Tween80 (5.0 g/L) + Lecithin (0.7 g/L)	-		-	20	CT[e]	CT	-
Tween80 (5.0 g/L) + Lecithin (1.05 g/L)	-		-	20	CT[e]	CT	-
Tween80 (5.0 g/L) + Lecithin (1.4 g/L)	-		-	20	CT[e]	CT	-
10% DE Broth	Pierce Protein Concentrator PES, 100K MWCO		-	20	CT[b]	ND	ND
Tween80 (5.0 g/L) + Lecithin (1.05 g/L)	Pierce Protein Concentrator PES, 100K MWCO		-	20	CT[b]	ND	ND
10% DE Broth	PD-10 Desalting Column containing 8.3 mL Sephadex G-25 Resin		-	20	CT[b]	CT	-

100% FBS	-	-	20	CT [b]	CT	-
100% FBS	-	-	100	CT [b]	CT	CT
100% FBS	Pierce Protein Concentrator PES, 100K MWCO	-	20	CT [b]	CT	-
100% FBS	PD-10 Desalting Column containing 8.3 mL Sephadex G-25 Resin	-	20	CT [b]	-	-
100% FBS	PD-10 Desalting Column containing 8.3 mL Sephadex G-25 Resin	-	100	CT [b]	-	-
5% FCS + 3% Glycine	-	-	20	CT [b]	CT	CT
5% FCS + 3% Glycine	PD-10 Desalting Column containing 8.3 mL Sephadex G-25 Resin	-	20	CT [b]	-	-
5% FCS + 3% Glycine	Amicon® Ultra-4 centrifugal unit + PBS	-	20	CT [b]	-	-
Infection media	Amicon® Ultra-4 centrifugal unit + PBS[f]	-	20	CT [b]	-	-
Infection media	Amicon® Ultra-4 centrifugal unit + PBS[f]	-	100	CT [b]	-	-
Infection media	PD-10 Desalting Column containing 8.3 mL Sephadex G-25 Resin	-	20	CT [b]	-	-
1 mL 5% FCS + 3% Glycine[g]	-	-	20	CT [b]	-	-
Infection media	Pierce™ Detergent Removal Column	Removed inoculum after 1 h incubation period, then added fresh infection media	100	- [b]	-	-

Table 2.5: Virucidal efficacy of two separate lots of three RTU disinfectants from EPA's List N against BCoV and HCoV OC43 in suspension.

Disinfectant[a]	Recovery Control Titer ($\log_{10}$ TCID$_{50}$/mL)		Reduction ($\log_{10}$ TCID$_{50}$/mL)	
	BCoV[b]	HCoV OC43[c]	BCoV	HCoV OC43
Clorox Clean-Up Cleaner + Bleach	5.4 ($\pm$0.3)[c]	5.0 ($\pm$0.1)	>4.7 ($\pm$0.3)[d]*	>4.4 ($\pm$0.1)*
Oxivir Tb	5.6 ($\pm$0.2)	5.3 ($\pm$0.1)	>5.0 ($\pm$0.2)*	>4.6 ($\pm$0.1)*
Cavicide 1	5.4 ($\pm$0.2)	5.2 ($\pm$0.2)	>4.7 ($\pm$0.2)*	>4.6 ($\pm$0.2)*

[a]A 2 min contact time was used for all disinfectants.
[b]Average inoculum titer for BCoV was 7.7 ($\pm$0.2) $\log_{10}$ TCID$_{50}$/mL.
[c]Average inoculum titer for HCoV OC43 was 7.7 ($\pm$0.4) $\log_{10}$ TCID$_{50}$/mL.
[d]The data is expressed as means $\pm$SD. All data was collected from three replicates in two batches of disinfectants in two independent experiments.
[*]The detection limit (0.6 $\log_{10}$ TCID$_{50}$/mL) of the TCID$_{50}$ assay was reached.

CHAPTER THREE:

EFFICACY OF READY-TO-USE SPRAY DISINFECTANTS AGAINST
SARS-COV-2 SURROGATES, BOVINE CORONAVIRUS AND HUMAN
CORONAVIRUS OC43, ON SURFACES COMMONLY FOUND IN THE
'FRONT-OF-THE-HOUSE' IN FOOD SERVICE ESTABLISHMENTS

ABSTRACT

The rapid spread of the novel pathogenic coronavirus (CoV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), after its emergence in the latter part of 2019 forced many foodservice establishments (FSEs) in the United States to shut down. In order to control and prevent the further spread of SARS-CoV-2, effective environmental cleaning procedures in FSEs are necessary. However, data regarding the efficacy of disinfectants from the Environmental Protection Agency's (EPA's) List N against two SARS-CoV-2 surrogate viruses, bovine coronavirus (BCoV) and human coronavirus (HCoV) OC43, is limited. The aim of this study was to evaluate the efficacy of three ready-to-use (RTU) spray disinfectants of different active ingredients (chlorine, hydrogen peroxide, and quaternary ammonium compound [QAC] + alcohol) from EPA's List N against two SARS-CoV-2 surrogates, BCoV and HCoV OC43, on two different non-porous surfaces (hard polyethylene terephthalate [PET] plastic and soft vinyl upholstery fabric) commonly found in the 'front-of-the-house' in FSEs in the presence of a soil load of 5% fetal bovine serum (FBS). The average initial inoculum levels for BCoV and HCoV OC43 were 7.0 ($\pm$0.6) and 6.5 ($\pm$0.2) $TCID_{50}$/carrier, respectively. After a 2

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min contact time, all three disinfectants reduced the infectivity of BCoV and HCoV OC43 below the limit of detection (LOD) on both PET and vinyl surfaces. In some cases, a ≥ 3.0 $\log_{10}$ reduction of virus titer could not be observed due to the decreased dynamic range on the carrier prior to disinfection. An additional analysis of the quantitative carrier test data was conducted to determine the impact of surface type, relative humidity (RH), and surrogate on the reduction of BCoV and HCoV OC43 after drying on carriers during efficacy testing. Overall, the results showed the mean reduction in titer of BCoV and HCoV OC43 after drying was greater on vinyl carriers. In addition, greater amounts of reduction of BCoV and HCoV OC43 were observed on both PET and vinyl carriers as the RH level increased. At low RH (20-29%) conditions, the reduction in the titer of HCoV OC43 was significantly ($p < 0.05$) greater than that of BCoV on both PET and vinyl carriers. In conclusion, both SARS-CoV-2 surrogates are very sensitive to the tested disinfectants, but BCoV is a more robust surrogate when compared to HCoV OC43. Further analysis of the quantitative carrier test data indicates that additional factors (i.e., surface type, RH, and surrogate) must be carefully considered when performing disinfectant efficacy testing on carriers.

INTRODUCTION

The first case of the novel pathogenic coronavirus (CoV) known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was reported in the United States in January of 2020 (1). Less than a month later, the World Health Organization (WHO) declared the outbreak of COVID-19 a pandemic (2). A multitude of foodservice

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establishments (FSEs) across the United States were forced to close their doors due to the spread of SARS-CoV-2, which resulted in a 26.9% industry increase in the unemployment rate from March 2020 to April 2020 (https://www.bls. gov/iag/tgs/iag722.htm#workforce). The highly contagious nature and rapid spread of SARS-CoV-2 has drawn an unprecedented amount of attention to infection control procedures in the public sector. Despite primary transmission from person to person with aerosols and respiratory droplets, the potential for transmission via contaminated objects or surfaces (fomites) should also be considered (3, 4, 5, 6, 7). Sources of surface contamination may include respiratory secretions, saliva droplets, and/or stool of an individual infected with SARS-CoV-2 (8).

Within FSEs, surfaces are categorized as being either food contact or non-food contact. In the 'back-of-the-house' in FSEs, (i.e., where food preparation and storage occur), food contact surfaces are those that have direct contact with food during the preparation process (i.e., pots, pans, cutting boards, utensils, food preparation surfaces, cooking surfaces), and they are commonly composed of stainless steel or plastic. Non-food contact surfaces are those that do not typically come in contact with food, and in the 'front-of-the-house' in FSEs (i.e., any place within a FSE customers are allowed) these surfaces are often constructed with materials, such as stainless steel, plastic, vinyl fabric, and wood. Notably, SARS-CoV-2 has been found to survive on plastic and stainless steel surfaces for 2-3 days when experiments were performed under laboratory conditions (5). In addition, a variety of high-touch surfaces in public settings (e.g., refrigeration system handles, tables, chairs, trash can and entry door handles) have been reported to be

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contaminated with SARS-CoV-2 by previous studies (9, 10, 11). Currently, the U.S. Food and Drug Administration (FDA) Food Code does not define or address the disinfection of surfaces within FSEs (12, 13). The ability of SARS-CoV-2 to survive on environmental surfaces for prolonged periods of time, and the lack of regulation regarding surface disinfection in the front-of-the-house in FSEs highlight the importance of effective environmental cleaning procedures in FSEs to prevent the further spread of SARS-CoV-2.

The aim of this study was to evaluate the efficacy of three RTU spray disinfectants of different active ingredients (chlorine, hydrogen peroxide, and QAC + alcohol) from EPA's List N against two SARS-CoV-2 surrogates, BCoV and HCoV OC43, on two types of non-porous surfaces (PET and vinyl upholstery fabric) that are frequently found in the front-of-the-house in FSEs. In addition, the data obtained during disinfectant carrier testing was subsequently analyzed to determine if the surface carrier type or the relative humidity (RH) during testing had any impact on the survival of BCoV and HCoV OC43 on the tested surfaces during the drying step.

MATERIALS AND METHODS

Cell Culture

Human rectal tumor (HRT-18G) cells (ATCC CRL-11663) were used to propagate BCoV strain Mebus (BEI Resources NR-445) and HCoV strain OC43 (ATCC VR-1558). Cells were cultured in T75 and T175 vented capped flasks (Corning, Corning, NY) with Dulbecco's Modified Eagle Medium, 1x (DMEM; Corning, Corning, NY)

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supplemented with 3% low-endotoxin heat-inactivated fetal bovine serum (FBS; Corning, Corning, NY), 100 U/L penicillin (Corning, Corning, NY), and 100 mg/L streptomycin (Corning, Corning, NY). The CO_2 incubator (VWR International, Radnor, PA) was set at 37°C and 5% CO_2. HRT-18G cells were subcultured at ~90% confluency (~5 d) in a 1:4 split ratio using 0.25% trypsin EDTA (Thermo Fisher Scientific, Waltham, MA). Cells that have been passaged >30 times were not used for the median tissue culture infectious dose ($TCID_{50}$) assay for virus titration.

Viral Stock Preparation

Ninety percent (90%) of confluent monolayers of HRT-18G cells were infected with BCoV and HCoV OC43 at a multiplicity of infection (MOI) of ~0.05. Initially, the virus had a 1 h adsorption phase with 5 mL infection media in a T75 flask that contained a monolayer of HRT-18G cells at 33°C and 5% CO_2 (VWR International, Radnor, PA). The infection media consisted of DMEM supplemented with 2% FBS, 100 U/L penicillin, and 100 mg/L streptomycin. During viral adsorption phase, the flask was manually rocked every 15 min to ensure even distribution. Then, the infection media was poured off and 25 mL of fresh infection media was added to the flask. The flask was placed back in the incubator and held for 5-7 days at 33°C until at least ~80% cytopathic effect (CPE) was observed using an inverted microscope (Olympus CK2; Olympus Optical Co., LTD, Tokyo, Japan). HRT-18G cells with ~80% CPE due to viral infection were subjected to three freeze-thaw cycles using a -80°C freezer and a biological safety cabinet (Class II, Type A2, Thermo Scientific) at room temperature for ~2 h and ~1 h,

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respectively. After the freeze-thaw cycles, the cell/virus solution was centrifuged at 5,000 x g for 10 min at 4°C (Model 5804 R; Eppendorf, Germany) to remove cell debris. BCoV and HCoV OC43 stocks at ca. 10^8 to 10^9 $TCID_{50}$/mL were aliquoted and stored at -80°C. The titers of infectious BCoV and HCoV OC43 were quantified by $TCID_{50}$ assay as described below.

Viral Concentration

BCoV and HCoV OC43 stocks, previously propagated and stored at -80°C, were thawed at room temperature inside a biological safety cabinet for ~1 h. In a separate centrifuge tube, polyethylene glycol (PEG) 8,000 MW (Alfa Aesar, Haverhill, MA) and sodium chloride (NaCl) were added to the virus stock to obtain final concentrations of 8% (w/v) and 0.3 M, respectively. The PEG and NaCl were dissolved by hand shaking, and then mixed with the virus stock. The resulting solution was incubated overnight at 4°C, followed by centrifugation at 10,000 x g for 30 min at 4°C (Model 5804 R; Eppendorf, Germany). The supernatant was removed, and the resulting pellet was resuspended in 0.4 mL of 5% FBS. The virus containing suspension was centrifuged at 18,000 x g for 1 min at 4°C (Model 5417 R; Eppendorf, Germany) to remove any denatured proteins in the PEG precipitation which could potentially affect the viability of the virus, and the supernatant was collected and stored at -80°C (14).

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TCID$_{50}$ Assay for BCoV and HCoV OC43

HRT-18G cells were seeded in 96-well plates (Corning, Corning, NY) at a density of ~2.0 x 10^4 cells/well and incubated at 37°C and 5% CO_2 with 0.2 mL of cell culture media. HRT-18G plates were used between 90-100% confluency (~5 d). Ten-fold serial dilutions of virus/test samples, in triplicate, were prepared in infection media. The cell culture media was removed from each 96-well plate, and 0.1 mL of the undiluted or serially diluted sample was placed onto replicate wells (n=8) of the appropriate 96-well plate. Each plate was rocked 10-15 times. The HRT-18G plates were incubated at 33°C and 5% CO_2 for 1 h and rocked 10-15 times every 15 min. After 1 h incubation, plates were removed from the incubator. Infection media (0.1 mL) was added to each well, followed by incubation at 33°C and 5% CO_2 for 7 d, and then scored for CPE. There was a positive control (previous viral stock) and a negative control (infection media) for each passage of cells used. Quantification of the titer of BCoV and HCoV OC43 was determined via the improved Kärber method (15). The limit of detection (LOD) for this TCID$_{50}$ assay was calculated as 4.2 TCID$_{50}$/mL (0.6 log$_{10}$ TCID$_{50}$/mL) if undiluted samples were readable, and 4.2 x 10^1 TCID$_{50}$/mL (1.6 log$_{10}$ TCID$_{50}$/mL) if the lowest readable dilution is 1:10.

Disinfectant and Surface Selection

The three disinfectants **(Table 2.1)** tested in this study were selected based on the following criteria: (1) included on EPA List N and/or List G (for HNoV), (2) RTU, (3) different active ingredients (chlorine, hydrogen peroxide, QAC + alcohol), (4) have a ≤ 2

min contact time, and (5) are readily available for consumer purchase. Two separate lots of each disinfectant were tested in this study.

Each of the three selected RTU disinfectants was tested on one hard nonporous surface, PET plastic (ePlastics, San Diego, CA), and one soft nonporous surface, vinyl upholstery fabric (Mayer Fabrics, Indianapolis, IN). These surfaces were selected based on the following criteria: (1) frequently found in the 'front-of-the-house' in FSEs, (2) are high-touch surfaces, and (3) limited published data exist regarding disinfectant efficacy on these surfaces. Though the American Society of Testing and Materials (ASTM) E2197-17 (16) uses stainless steel carriers, stainless steel was not selected for testing to provide efficacy data on novel surfaces.

Preparation of Surface Carriers

PET plastic and vinyl upholstery fabric were manually cut into 1 cm diameter disks using a 1 cm diameter punch (16). Plastic and vinyl carriers were washed with LoSUDS Liquid Glassware Detergent (Bar Maid, Pompano Beach, FL) diluted with deionized (DI) water, thoroughly rinsed with DI water, and dried completely. Vinyl carriers were wrapped in aluminum foil and steam sterilized at 121°C, 15 psi for 20 min. After sterilization, sterile forceps were used to transfer vinyl carriers to a sterile 24-well plate (VWR International, Radnor, PA) to be used for testing. Plastic carriers were sprayed on both sides with 75% ethanol until saturation and held for 20 min or until air-dried. Prior to inoculation, plastic carriers were transferred to sterile aluminum foil inside a biological safety cabinet to expose one side of the plastic to ultraviolet (UV) light for at

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least 30 min. Then, sterile forceps were used to transfer each plastic carrier to a sterile 24-well plate to expose the other side to UV light for at least 30 min prior to testing. After each experiment, all carriers were discarded following steam sterilization.

Cytotoxicity and Neutralization Effectiveness Testing

Cytotoxicity elimination and neutralization verification for the three RTU spray disinfectants used in this experiment were conducted as previously described in Chapter 2 (**Table 2.2**).

Quantitative Carrier Test

Efficacy of the three RTU disinfectants was evaluated against BCoV and HCoV OC43 on both a hard and soft nonporous surface, PET plastic and vinyl upholstery fabric, respectively, according to ASTM E2197-17 (16) with some modifications (**Figure 3.1**). All carrier tests were conducted in the presence of a 5% FBS soil load. PET or vinyl surface carriers (1 cm diameter), sterilized as previously described, were placed in separate wells of a 24-well plate and inoculated, in triplicate, with 10 μL of BCoV or HCoV OC43, previously concentrated via PEG precipitation as described above. All carriers were dried for 1 to 1.5 h inside a biological safety cabinet at ambient laboratory conditions (20-25°C; 20-40% RH). The RH was monitored with a digital hydrothermometer (EU 620-0915; VWR International). Dried carriers were then incubated with 90 μL of undiluted disinfectant, in triplicate, for a 2 min contact time. As a control, triplicate carriers containing dried virus were incubated with 90 μL of infection

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media for a 2 min contact time. After the 2 min contact time, 900 μL of the respective

neutralizer (**Table 2.1**) was pipetted into each well and pipetted up and down 10 times to

neutralize virucidal activity of the disinfectant. To recover virus from the surface carriers,

the 24-well plate was sealed with parafilm (Bemis Company, Inc., Neenah, WI) then

placed in a sterile stomaching bag, submerged under water, and sonicated for 30 s at 40

kHz (FS110D; Fisher Scientific International, Inc., Hampton, NH). After sonication,

samples were pipetted up and down 10 times, collected from each well, and 'washed'

using either Amicon® Ultra-4 centrifugal units or Pierce^{TM} detergent removal columns,

as described in Chapter 2. After the removal of residual disinfectant, samples were

collected, underwent a ten-fold serial dilution using infection media, and were used for

titration via TCID$_{50}$ assay with HRT-18G cells, as described above. Cytotoxicity

elimination and neutralization effectiveness verification were conducted according to the

methods outlined in ASTM 2197-17 (16). Disinfectants were considered efficacious if a

≥3.0 log reduction in virus titer could be achieved (17).

Calculations

Log reductions **(Equation 1)** were calculated as previously described (16).

$$Log\ reduction = log \left(\frac{geometric\ mean\ Virus\ Control}{geometric\ mean\ Treatment\ samples} \right)$$ **(Equation 1)**

A dynamic range, the maximum range of infectivity able to be observed on each

carrier, was calculated in each experiment for all three RTU disinfectants tested:

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$Disinfectant\ dynamic\ range = \ log_{10}\ (Virus\ control) - \ log_{10}\ (LOD)$ **(Equation 2)**

Statistical Analysis

Three replicates of ten-fold serial dilutions of each RTU disinfectant were tested in two independent experiments for each surrogate and surface combination. Statistical analyses were performed in JMP pro v.14.1.0 (SAS Inc., Cary, NC) using one-way analysis of variance (ANOVA) and Student's t-test ($\alpha = 0.05$) to determine the relationship between surrogates, disinfectants, surfaces, and log reduction. Data sets were confirmed to be from a normal distribution using the Shapiro-Wilk w-test for goodness of fit ($\alpha = 0.05$). If rejected for normalcy, transformations were performed with box-cox transformations (18). Once data sets were normalized, an outlier analysis was performed, and outliers were excluded from the analysis on a basis following constraints of Tukey's 1.5 inter quartile range rule (18). A one-way ANOVA and Student's t-test were performed upon completion of the listed procedures. In addition to the comparisons discussed above, analysis of subsequent effects of surface type and RH were performed by grouping of individual treatment factors within the test. Statistical significance was defined as a *p*-value of <0.05.

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RESULTS

Cytotoxicity and Neutralization Effectiveness Testing

As previously described in Chapter 2, neutralization methods I, II, and III were evaluated based on their ability to prevent cytotoxicity and neutralize their respective disinfectant (**Table 2.2**). Clorox, Oxivir Tb, and Cavicide 1 at the 1:10 and 1:100 dilution showed no apparent cytotoxicity toward HRT-18G cells after 1 h incubation when using neutralization method I, II, and III, respectively. No adverse effects on the HRT-18G cells were observed after exposure to each of the three chemical neutralizers listed in **Table 2.1**. When validating neutralization effectiveness, neutralization methods I, II, and III yielded >80% recovery of both BCoV and HCoV OC43 when compared to the controls (**Table 2.2**).

Quantitative Carrier Test

In this study, the virucidal efficacy of three RTU disinfectants from the EPA's List N was evaluated against BCoV and HCoV OC43 on both a hard and soft nonporous surface, PET plastic and vinyl upholstery fabric, respectively. All carrier tests were conducted in the presence of a soil load of 5% FBS under ambient conditions. Disinfectants were considered efficacious if a ≥ 3.0 log reduction in virus titer was achieved (17). **Table 3.1** shows the efficacy of two separate lots of Clorox, Oxivir Tb, and Cavicide 1 against BCoV and HCoV OC43 on PET plastic carriers. The average initial inoculum levels for BCoV and HCoV OC43 were 7.0 ($\pm$0.6) and 6.5 ($\pm$0.2) $\log_{10}$

TCID$_{50}$/carrier, respectively. After a 2 min contact time, all three disinfectants reduced the infectivity of BCoV and HCoV OC43 below the LOD. As a result, Clorox, Oxivir Tb, and Cavicide 1 showed a >3.8, >3.8, and >4.1 log reduction of BCoV, respectively. When tested against HCoV OC43, Clorox was able to demonstrate a >2.3 log reduction, and Oxivir Tb and Cavicide 1 both had a >2.9 log reduction (**Table 3.1**).

Table 3.2 shows the efficacy of two separate lots of Clorox, Oxivir Tb, and Cavicide 1 against BCoV and HCoV OC43 on vinyl upholstery fabric carriers. The average initial inoculum levels for BCoV and HCoV OC43 were 7.0 ($\pm$0.6) and 6.5 ($\pm$0.2) log$_{10}$ TCID$_{50}$/carrier, respectively. After a 2 min contact time, all three disinfectants reduced the infectivity of BCoV and HCoV OC43 below the LOD. As a result, Clorox, Oxivir Tb and Cavicide 1 showed a >2.9, >4.2, and >2.8 log reduction of BCoV, respectively. When tested against HCoV OC43, Clorox, Oxivir Tb and Cavicide 1 were only able to demonstrate a >1.1, >2.2 and >1.2 log reduction, respectively (**Table 3.2**).

Since the titers of recovered virus after disinfectant exposure reached the LOD (0.6 log$_{10}$ TCID$_{50}$/mL) of the TCID$_{50}$ assay, the difference in disinfection efficacies of each product from two separate lots and among products cannot be compared statistically.

Impact of Carrier Type, Relative Humidity, and Surrogates on Virus Survival

In consideration of the low virus titers recovered from the carriers immediately following the drying period, the virus recovery data obtained from the quantitative carrier

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tests were further analyzed to determine if the type of carrier material, relative humidity (RH), and surrogate had any impact on the titers of BCoV and HCoV OC43 during the drying step on the carrier surfaces.

Carrier Type: The impact of carrier surface type on the mean reduction in titer of BCoV and HCoV OC43 after drying on PET and vinyl carriers was determined in **Table 3.3.** The mean reduction in titers of BCoV observed on PET and vinyl carriers (2.4 and 3.1 $\log_{10}$ TCID$_{50}$/mL, respectively) were lower than those of HCoV OC43 (3.1 and 4.4 $\log_{10}$ TCID$_{50}$/mL, respectively). In addition, the mean reduction in the titers of both surrogates on PET was lower than that observed on vinyl. However, due to a non-normal data set, a statistical comparison could not be made.

The impact of carrier surface type on the mean reduction in titer of BCoV and HCoV OC43 under three different RH conditions was determined in **Table 3.4** There was no significant difference ($p > 0.05$) in the reduction of BCoV on PET and vinyl carriers under low RH conditions (1.6 and 1.7 $\log_{10}$ TCID$_{50}$/mL, respectively) and medium RH conditions (3.2 and 3.7 $\log_{10}$ TCID$_{50}$/mL, respectively). The mean reduction in titer of BCoV on vinyl under high RH conditions was found to be 4.1 $\log_{10}$ TCID$_{50}$/mL. No significant difference ($p > 0.05$) in the reduction of HCoV OC43 was found on PET and vinyl carriers under low RH conditions (2.7 and 2.4 $\log_{10}$ TCID$_{50}$/mL, respectively) and medium RH conditions (5.3 and 4.6 $\log_{10}$ TCID$_{50}$/mL, respectively). The mean reduction in titer of HCoV OC43 on vinyl under high RH conditions was 5.6 $\log_{10}$ TCID$_{50}$/mL.

Relative Humidity: In **Table 3.5** the results showed that the mean reduction in the titer observed for BCoV on both surfaces at low, medium and high RH conditions

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was 1.6, 3.2, and 4.1 $\log_{10}$ TCID$_{50}$/mL, respectively, as compared with 2.6, 4.2, and 5.6 $\log_{10}$ TCID$_{50}$/mL for the titer of HCoV OC43, respectively. It appeared that virus reduction was higher at high RH than at lower RH. However, due to a non-normal data set, a statistical comparison could not be made.

Table 3.6 shows the impact of different RH conditions on the mean reduction in titer of BCoV and HCoV OC43 on PET and vinyl carriers. The reduction of BCoV on PET observed under low RH conditions, 1.6 $\log_{10}$ TCID$_{50}$/mL, was significantly ($p < 0.05$) lower than that observed under medium RH conditions, 2.8 $\log_{10}$ TCID$_{50}$/mL. Similar to the trend on PET carriers, the reduction of BCoV after drying on vinyl carriers under low RH conditions, 1.7 $\log_{10}$ TCID$_{50}$/mL, was significantly ($p < 0.05$) lower than that observed at medium and high RH. However, there was no significant difference ($p > 0.05$) in the reduction of BCoV under medium and high RH conditions, 3.7 and 4.1 $\log_{10}$ TCID$_{50}$/mL, respectively. No significant difference ($p > 0.05$) in the reduction of HCoV OC43 on PET was observed under low and medium RH conditions, 2.7 and 3.5 $\log_{10}$ TCID$_{50}$/mL, respectively. On vinyl carriers, the mean reduction of HCoV OC43 after drying was found to be 2.4, 4.6, and 5.6 $\log_{10}$ TCID$_{50}$/mL when under low, medium, and high RH conditions, respectively. However, a statistical comparison cannot be made on these values because of the non-normal data set.

Surrogate: The impact of the surrogate on the mean reduction in titer of BCoV and HCoV OC43 after drying on PET and vinyl carriers was determined in **Table 3.7**. The reduction in BCoV titer on vinyl was significantly ($p < 0.05$) lower than the reduction in HCoV OC43 titer, 3.1 and 4.4 $\log_{10}$ TCID$_{50}$/mL, respectively. However,

there was no significant difference ($p > 0.05$) in the reduction of BCoV and HCoV OC43 after drying on PET, 2.4 and 3.1 $\log_{10}$ TCID$_{50}$/mL, respectively. As indicated in **Table 3.8**, the reduction of BCoV titer under low RH conditions, 1.6 $\log_{10}$ TCID$_{50}$/mL, was significantly ($p < 0.05$) lower than the 2.6 $\log_{10}$ TCID$_{50}$/mL reduction that was observed for HCoV OC43. The reduction of BCoV titer was also significantly lower than that of HCoV OC43 after being dried under medium RH conditions, 3.2 and 4.2 $\log_{10}$ TCID$_{50}$/mL, respectively. When BCoV and HCoV OC43 were dried on surface carriers under high RH conditions, the mean reduction in titer observed for each virus was 4.1 and 5.6 $\log_{10}$ TCID$_{50}$/mL, respectively. However, due to a non-normal data set, a statistical comparison could not be made.

The impact of the surrogate on the survival of BCoV and HCoV OC43 after drying on PET and vinyl carriers under different RH conditions is shown in **Table 3.9**. The reduction of BCoV titer observed on PET under low RH conditions, 1.6 $\log_{10}$ TCID$_{50}$/mL, was significantly ($p < 0.05$) lower than that observed for HCoV OC43, 2.7 $\log_{10}$ TCID$_{50}$/mL. However, no significant difference ($p > 0.05$) in the reduction of BCoV and HCoV OC43 on PET was found under medium RH conditions, 2.8 and 3.5 $\log_{10}$ TCID$_{50}$/mL, respectively. The reduction of BCoV titer on vinyl was significantly ($p < 0.05$) lower than the reduction observed for HCoV OC43 under low RH conditions, 1.7 and 2.4 $\log_{10}$ TCID$_{50}$/mL, respectively. Under medium RH conditions, there was no significant difference ($p > 0.05$) in the reduction of BCoV, 3.7 $\log_{10}$ TCID$_{50}$/mL, and HCoV OC43, 4.6 $\log_{10}$ TCID$_{50}$/mL, titer. The mean reduction of BCoV (4.1 $\log_{10}$ TCID$_{50}$/mL) on vinyl carriers under high RH conditions was less than that of HCoV

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OC43 (5.6 $\log_{10}$ TCID$_{50}$/mL). However, due to a non-normal data set, a statistical comparison could not be made.

DISCUSSION

Due to the highly contagious nature and rapid spread of SARS-CoV-2 amid the most recent pandemic, many FSEs in the United States were forced to shut down at the start of the COVID-19 pandemic. To prevent further spread of SARS-CoV-2, implementation of effective environmental cleaning and disinfecting procedures is necessary. The EPA's List N is composed of a number of disinfectant products approved for use against SARS-CoV-2 (19). However, there is currently limited published data regarding the efficacy of these products against two additional surrogate viruses, BCoV and HCoV OC43, on surfaces other than glass and stainless steel. In this study, we demonstrated the efficacy of three RTU disinfectants from List N against BCoV and HCoV OC43 on both a hard and soft nonporous surface, PET plastic and vinyl upholstery fabric, respectively. In addition, the data obtained from the disinfectant carrier testing was subsequently analyzed to determine if the surface carrier type, RH during testing, or surrogate had any impact on the survival of BCoV and HCoV OC43 during the drying step on the surfaces.

Quantitative Carrier Test

When tested on PET surface carriers against BCoV, all three RTU spray disinfectants were able to reach the EPA standard for antiviral efficacy against SARS-

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CoV-2 ($\geq$3 $\log_{10}$ reduction) within 2 min in the presence of a 5% FBS soil load (**Table 3.1**). However, none of the three RTU spray disinfectants were able to reach a $\geq$3 $\log_{10}$ reduction against HCoV OC43 on PET. When tested on vinyl surface carriers, Oxivir Tb was the only RTU disinfectant product that achieved a >3.0 $\log_{10}$ reduction of BCoV. None of the three RTU disinfectant products were able to achieve a $\geq$3.0 $\log_{10}$ reduction of HCoV OC43 on vinyl carriers. For all three disinfectants, the amount of virus inactivation detected after disinfection was the maximum amount able to be detected by the $TCID_{50}$ assay. However, the dynamic range (**Equation 2**) for each disinfectant varied due to the differences in the titers of the recovery control (RC) samples. In other words, the amount of virus that was inactivated on the carriers during the drying period varied. As a result, in some cases a >3.0 $\log_{10}$ reduction could not be observed due to the decreased dynamic range on the carrier. Therefore, statistical differences between log reduction values for the disinfectant products tested could not be determined.

The log reduction of BCoV on PET (**Table 3.1**) observed in this study after treatment with Clorox Clean-Up Cleaner + Bleach aligns with previously published data regarding the efficacy of sodium hypochlorite products. A >3.0 $\log_{10}$ reduction in virus titer after a 1 min contact time was reported by Sattar et al. (20) when 0.10 and 0.50% sodium hypochlorite were evaluated against HCoV 229E on stainless steel carriers. Another study found that no infectious SARS-CoV-2 could be detected after exposure to ~75 ppm and ~150 ppm of household bleach for a 5 min contact time (21). In addition, Xiling et al. (22) reported that 500 and 1,000 ppm of available chlorine achieved a $\geq$4.75 $\log_{10}$ reduction of SARS-CoV-2 in less than 5 and 0.5 min, respectively. The latter two

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studies that evaluated chlorine-based disinfectants were conducted in suspension, which highlights the importance of confirming disinfectant efficacy via quantitative carrier testing. While the log reductions of BCoV on vinyl and HCoV OC43 on PET and vinyl in our study did not meet the EPA standard ≥ 3.0 $\log_{10}$ reduction, due to the decreased dynamic range caused by low virus titer on carriers after drying, further analysis of our quantitative carrier test data indicated additional factors (i.e., surface type, RH, and surrogate) may have impacted the results of this study. The potential impact of these additional factors will be discussed later in this section.

When BCoV was treated with Oxivir Tb for 2 min on PET and vinyl, we observed a >3.8 and >4.2 $\log_{10}$ reduction in virus titer, respectively (**Table 3.1 and 3.2**). These findings are consistent with those in a previous study conducted by Omidbakhsh et al. (23), which reported a >4.0 $\log_{10}$ reduction of infectious HCoV 229E in 1 min after treatment with 0.5% accelerated hydrogen peroxide. The inability to demonstrate a ≥ 3.0 $\log_{10}$ reduction of HCoV OC43 on PET and vinyl in this study could be due to the aforementioned additional factors, which will be further discussed later in this section.

The log reduction observed in this study for BCoV on PET following treatment with Cavicide 1 (**Table 3.1**) is similar to those reported in previous studies. A study conducted with murine hepatitis virus (MHV), a potential surrogate for SARS coronavirus, reported a 3.0 $\log_{10}$ reduction in virus titer after treatment with 0.10% QAC + 79% ethanol for 0.5 min (24). In addition, Ijaz et. al (25) reported a ≥ 4.5 $\log_{10}$ reduction of SARS-CoV-2 following treatment with a disinfectant that contained 50% ethanol + 0.083% QAC for 2 min.

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Although a ≥ 3.0 $\log_{10}$ reduction of both surrogates for all treatments was not observed during the quantitative carrier tests, we believe the primary reason was the low amount of inoculated virus recovered from the carriers after drying rather than the efficacy of the disinfectant. In Chapter 2, our suspension tests demonstrated a ≥ 4.4 $\log_{10}$ reduction of BCoV and HCoV OC43 for all three disinfectants. Even with the minimal LOD (0.6 $\log_{10}$ $TCID_{50}$/mL) our testing method allowed, the dynamic range of virus inactivation on the carriers was dependent upon the titer of the inoculated virus after the drying period (i.e., a minimum of 3.6 $\log_{10}$ $TCID_{50}$/carrier was needed to pass the efficacy test). Therefore, further analysis of the quantitative carrier test data suggest that the aforementioned additional factors potentially impacted our results which will be addressed below.

Impact of Carrier Type on Virus Survival

The quantitative carrier test data obtained in this study was further analyzed to determine the impact of the carrier surface on the reduction in titer of BCoV and HCoV OC43 after the drying period. The mean reduction in titer for both BCoV and HCoV OC43 was found to be greater on vinyl carriers than on PET carriers (**Table 3.3**). Additional analysis of the quantitative carrier test data was performed to determine the impact of the carrier surface on the survival of each surrogate after the drying period for three different RH levels. However, there was no significant difference in the reduction in titer of BCoV or HCoV OC43 on PET when compared to vinyl under low or medium RH conditions (**Table 3.4**). A comparison of the reduction of each surrogate as a result of

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surface type for high RH conditions could not be made because the mean reduction in

virus titer at high RH was only observed on vinyl carriers (**Table 3.4**).

During the quantitative carrier tests, Oxivir Tb was the only RTU disinfectant

product that was able to achieve a >3.0 $\log_{10}$ reduction of BCoV on vinyl carriers (**Table

3.2**). A ≥ 3.0 $\log_{10}$ reduction of HCoV OC43 on vinyl was not observed for any of the

three RTU products tested in this study, which could be the result of having used a

slightly higher inoculum titer of BCoV (7.0 $\log_{10}$ $TCID_{50}$/carrier) than HCoV OC43 (6.5

$\log_{10}$ $TCID_{50}$/carrier). In all cases, the LOD for the $TCID_{50}$ assay was reached. The higher

mean reduction in titer for both BCoV and HCoV OC43 after drying on the vinyl carriers

could potentially be due to insufficient recovery of the virus from the carriers. Although

the vinyl upholstery fabric was considered a nonporous surface in this study, it had a

textured surface and a brushed polyester knit backing. Since the entire carrier was

submerged in the disinfectant/neutralizer mixture during the recovery process, some of

the virus could have been absorbed by the porous backing which could explain why a

≥ 3.0 $\log_{10}$ reduction in virus titer could not be observed during efficacy testing even for

BCoV, except for Oxivir Tb.

While not evaluated in this study, certain properties (i.e., surface roughness and

hydrophobicity) of the surfaces used for testing could also potentially impact the titer of

virus recovered from carriers after drying. A study conducted by Dika et al. (26) reported

that an increased surface roughness will increase the overall available surface area for

virus adhesion. In addition, phages were found to adhere most significantly to

hydrophobic polypropylene surfaces versus hydrophilic glass (26). Further investigation

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is needed to determine whether specific characteristics of each tested surface impact the amount of virus that can be recovered from carriers after drying.

Impact of Relative Humidity on Virus Survival

To evaluate the impact of RH on the survival of BCoV and HCoV OC43 after drying, the quantitative carrier test data was further analyzed. Overall, the mean reduction in titer of both surrogates on all surfaces increased as the RH levels increased (**Table 3.5**). The impact of RH on virus survival was also evaluated for each surface type, which showed a more significant reduction of BCoV titer at medium and high RH levels on PET and vinyl carriers, respectively, than at low RH levels (**Table 3.6**). While there was no significant difference in the reduction of HCoV OC43 due to RH for PET, the mean reduction in titer was higher at high RH than low RH for vinyl.

The temperature and RH ranges (20-25°C and 20-40%, respectively) selected for the quantitative carrier tests in this study were based on FDA recommendations for ambient laboratory conditions, and were also intended to mimic indoor dining conditions (27). Prior to the start of the quantitative carrier tests, it was not our intent to control the RH beyond the range previously stated. Although the temperature in our laboratory setting fluctuated very little, the RH ranged from 20 to >40% depending on local weather conditions. As a result, further analysis of the data obtained from the carrier tests indicated that RH is indeed an important factor to consider.

The results of our analysis to determine the impact of RH on the survival of BCoV and HCoV OC43 after drying generally align with the data that is currently

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available, which shows that coronaviruses survive longer at lower temperatures and low RH conditions (28). Chan et al. (29) reported that at 33°C on a plastic surface at 80-90% RH and >95% RH, there was a 0.75 and 1 log loss in the titer of SARS-CoV-1 after 1 day, while a 2 and 3.5 log reduction in virus titer was seen at 38°C. In another study, MERS-CoV was deposited on plastic and stainless steel surfaces at 30°C at both 30% and 80% RH, and complete decay of the virus was observed after 2 and 1 days, respectively (30). However, a 40% RH paired with a lower temperature (20°C) extended the time required for complete decay of MERS-CoV on plastic and stainless steel surfaces to 3 days (30). A study conducted by Chan et al. (31) reported that at 20-25°C and 63% RH, SARS-CoV-2 could maintain viability on a glass surface for 3 to 5 days. When the temperature was decreased to 4°C and 63% RH, the survival of SARS-CoV-2 was prolonged to >14 days (31). However, the virus lost its infectivity within just 1 day when the temperature was increased to 37°C and 63% RH (31).

Overall, we believe that higher RH conditions observed in this study contributed to the increased reduction of BCoV and HCoV OC43 titer after drying on the carriers, which ultimately affected the ability of the three RTU disinfectant products tested to demonstrate the EPA standard ≥ 3 $\log_{10}$ reduction. At low RH conditions, the virus inoculum on the carrier dried much faster, and the absorbed virus particles are more stable compared to a free, unbound virion. A previous study in which the survival of human norovirus (HNoV) surrogates, feline calicivirus (FCV) and murine norovirus (MNV), was evaluated on carpet found that 30% RH provided a more hospitable environment for these non-enveloped viruses than 70% RH (32). Enveloped viruses are

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typically more sensitive to environmental conditions, which could explain why the mean reduction in titer for BCoV and HCoV OC43 in our study was greater at medium RH (30-39%) than at low RH (20-29%).

Impact of Surrogate on Virus Survival

The data obtained from the quantitative carrier tests in this study was further analyzed to determine the impact of the virus surrogate on the reduction in titer of BCoV and HCoV OC43 after being dried on surface carriers under different RH conditions. Under low and medium RH conditions, the reduction of HCoV OC43 was significantly ($p < 0.05$) greater than that of BCoV (**Table 3.8**). The quantitative carrier test data was also evaluated to determine the impact of the surrogate on the reduction in virus titer after drying on PET and vinyl carriers under different RH conditions. Overall, the results showed that the reduction of HCoV OC43 was more significant than BCoV on both PET and vinyl at low RH conditions (**Table 3.9**).

The reduction in HCoV OC43 titer observed in this study after the 1 h drying period aligns with the rapid loss of infectivity of HCoV OC43 after drying for 1 h on both porous and nonporous surfaces (e.g., aluminum, sterile latex surgical gloves and sterile sponges) reported by Sizun et al. (33). While there is limited published data regarding the survival of BCoV on surfaces, Todt et al. (34) noted similarities between BCoV and SARS-CoV-2 after desiccation on banknotes and coins. When performing our study, we observed that HCoV OC43 is more susceptible to environmental stresses, and lost viability faster than BCoV. Overall, the results of our comparison to determine the impact

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of each surrogate on the reduction in titer observed after drying indicated that BCoV was a more robust surrogate when compared to HCoV OC43.

CONCLUSION

In summary, our carrier tests revealed that all three types of disinfectants reduced the infectivity of BCoV and HCoV OC43 below the LOD on both PET and vinyl surfaces following a 2 min exposure time. In some cases, a ≥ 3.0 $\log_{10}$ reduction could not be observed due to the decreased dynamic range on the carrier that resulted from the low titer of the inoculated virus that was recovered from the carrier prior to disinfection. The after-drying recovery data showed that HCoV OC43 was more sensitive to drying than BCoV, and both surrogates were inactivated faster under higher RH conditions and on vinyl surface carriers. These results emphasized that the impact of some key factors (i.e., surface type, RH, and surrogate) on virus survival should be considered when performing efficacy testing and/or interpreting the efficacy data. Since SARS-CoV-2 is an enveloped virus, which is very susceptible to environmental stresses, disinfectant efficacy tests can be very challenging. The use of BCoV seems to be a more conservative surrogate for SARS-CoV-2 for disinfectant efficacy testing. Further study on increasing the dynamic range for the carrier test by increasing the virus titer prior to disinfection should be pursued. Collectively, our data highlight the importance of verifying disinfectant suspension test data by performing disinfectant carrier tests which expose the virus and disinfectant to conditions (i.e., surface type, RH, and surrogate) similar to those encountered during 'real-world' application. The findings from this study can be used to

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inform the disinfection of surfaces frequently found in the front-of-the-house in FSEs.

Implementation of effective environmental cleaning, in conjunction with other infection

control measures, is necessary to prevent the further spread of SARS-CoV-2.

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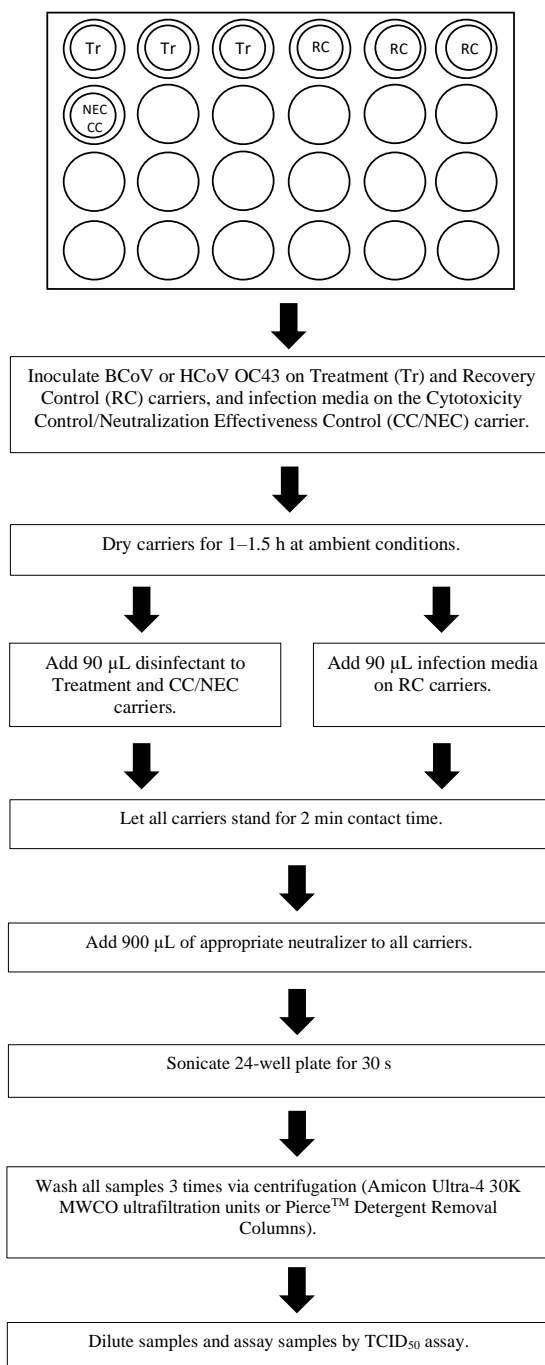
Figure 3.1: Workflow for disinfectant carrier test.

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Table 3.1: Virucidal efficacy of two separate lots of three RTU disinfectants from EPA's List N against BCoV and HCoV OC43 on PET plastic carriers.

Disinfectant	Contact Time (min)	Recovery Control Titer ($\log_{10}$ TCID$_{50}$/mL)		Reduction ($\log_{10}$ TCID$_{50}$/mL)	
		BCoV[a]	HCoV OC43[b]	BCoV	HCoV OC43
Clorox Clean-Up Cleaner + Bleach	2	4.4 ($\pm$1.1)[c]	2.9 ($\pm$1.9)	>3.8 ($\pm$1.1)[c]*	>2.3 ($\pm$1.9)*
Oxivir Tb	2	4.5 ($\pm$0.6)	3.6 ($\pm$0.3)	>3.8 ($\pm$0.6)*	>2.9 ($\pm$0.3)*
Cavicide 1	2	4.7 ($\pm$1.7)	3.6 ($\pm$3.0)	>4.1 ($\pm$1.7)*	>2.9 ($\pm$3.0)*

[a]Average inoculum titer for BCoV was 7.0 ($\pm$0.6) $\log_{10}$ TCID$_{50}$/carrier.
[b]Average inoculum titer for HCoV OC43 was 6.5 ($\pm$0.2) $\log_{10}$ TCID$_{50}$/carrier.
[c]The data is expressed as means $\pm$SD. All data was collected from three replicates in two independent experiments.
[*]The detection limit (0.6 $\log_{10}$ TCID$_{50}$/mL) of the TCID$_{50}$ assay was reached.

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Table 3.2: Virucidal efficacy of two separate lots of three RTU disinfectants from EPA's List N against BCoV and HCoV OC43 on vinyl upholstery fabric carriers.

Disinfectant	Contact Time (min)	Recovery Control Titer ($\log_{10}$ TCID$_{50}$/mL)		Reduction ($\log_{10}$ TCID$_{50}$/mL)	
		BCoV[a]	HCoV OC43[b]	BCoV	HCoV OC43
Clorox Clean-Up Cleaner + Bleach	2	3.6 ($\pm$0.2)[c]	1.8 ($\pm$1.0)	>2.9 ($\pm$0.2)[c]*	>1.1 ($\pm$1.0)*
Oxivir Tb	2	4.8 ($\pm$0.8)	2.8 ($\pm$2.0)	>4.2 ($\pm$0.8)*	>2.2 ($\pm$2.0)*
Cavicide 1	2	3.5 ($\pm$0.8)	1.8 ($\pm$1.6)	>2.8 ($\pm$0.8)*	>1.2 ($\pm$1.6)*

[a]Average inoculum titer for BCoV was 7.0 ($\pm$0.6) $\log_{10}$ TCID$_{50}$/carrier.
[b]Average inoculum titer for HCoV OC43 was 6.5 ($\pm$0.2) $\log_{10}$ TCID$_{50}$/carrier.
[c]The data is expressed as means $\pm$SD. All data was collected from three replicates in two independent experiments.
[*]The detection limit (0.6 $\log_{10}$ TCID$_{50}$/mL) of the TCID$_{50}$ assay was reached.

Table 3.3: Titer Reduction as a function of carrier surface for BCoV and HCoV OC43.

Surface	Reduction[a] ($\log_{10}$ TCID$_{50}$/mL)	
	BCoV	HCoV OC43
PET	2.4 ($\pm$0.3)	3.1 ($\pm$0.4)
Vinyl	3.1 ($\pm$0.3)	4.4 ($\pm$0.4)

[a]The data is expressed as means $\pm$SEs from three replicates in two independent experiments. Due to a non-normal data set, a statistical comparison could not be made.

Table 3.4: Titer Reduction as a function of carrier surface for BCoV and HCoV OC43 at three relative humidity levels.

Surface	Reduction[a] ($\log_{10}$ TCID$_{50}$/mL)					
	BCoV			HCoV OC43		
	Low RH (20-29%)	Medium RH (30-39%)	High RH (>40%)	Low RH (20-29%)	Medium RH (30-39%)	High RH (>40%)
PET	1.6 ($\pm$0.1) A	3.2 ($\pm$0.2) A	ND[b]	2.7 ($\pm$0.1) A	5.3 ($\pm$0.5) A	ND
Vinyl	1.7 ($\pm$0.1) A	3.7 ($\pm$0.2) A	4.1 ($\pm$0.6)	2.4 ($\pm$0.2) A	4.6 ($\pm$0.3) A	5.6 ($\pm$0.0)

[a]The data is expressed as means $\pm$SEs from three replicates in two independent experiments. Values with different uppercase letters in the same column for each surrogate are significantly different ($p < 0.05$).
[b]ND: Not determined.

Table 3.5: Titer Reduction as a function of <u>relative humidity</u> for BCoV and HCoV OC43.

Relative Humidity	Reduction[a] ($\log_{10}$ TCID$_{50}$/mL)	
	BCoV	HCoV OC43
Low RH (20-29%)	1.6 ($\pm$0.2)	2.6 ($\pm$0.5)
Medium RH (30-39%)	3.2 ($\pm$0.2)	4.2 ($\pm$0.4)
High RH (>40%)	4.1 ($\pm$0.5)	5.6 ($\pm$0.9)

[a]The data is expressed as means $\pm$SEs from three replicates in two independent experiments. Due to a non-normal data set, a statistical comparison could not be made.

Table 3.6: Titer Reduction as a function of <u>relative humidity</u> for BCoV and HCoV OC43 on PET and Vinyl carriers.

Relative Humidity	Reduction[a] ($\log_{10}$ TCID$_{50}$/mL)			
	BCoV		HCoV OC43	
	PET	Vinyl	PET	Vinyl[b]
Low RH (20-29%)	1.6 ($\pm$0.4) A	1.7 ($\pm$0.2) A	2.7 ($\pm$0.6) A	2.4 ($\pm$0.7)
Medium RH (30-39%)	2.8 ($\pm$0.3) B	3.7 ($\pm$0.2) B	3.5 ($\pm$0.6) A	4.6 ($\pm$0.3)
High RH (>40%)	ND[c]	4.1 ($\pm$0.3) B	ND	5.6 ($\pm$0.7)

[a]The data is expressed as means $\pm$SEs from three replicates in two independent experiments. Values with different uppercase letters in the same column for each surface are significantly different ($p < 0.05$).
[b]Due to a non-normal data set, a statistical comparison could not be made.
[c]ND: Not determined.

Table 3.7: Titer Reduction as a function of <u>surrogate</u> type for PET and Vinyl carriers.

Surrogate	Reduction[a] ($\log_{10}$ TCID$_{50}$/mL)	
	PET	Vinyl
BCoV	2.4 ($\pm$0.4) A	3.1 ($\pm$0.4) A
HCoV OC43	3.1 ($\pm$0.3) A	4.4 ($\pm$0.3) B

[a]Data is expressed as means $\pm$SEs from three replicates in two independent experiments. Values with different uppercase letters in the same column are significantly different ($p < 0.05$).

Table 3.8: Titer Reduction as a function of <u>surrogate</u> type for three relative humidity levels.

Surrogate	Reduction[a] ($\log_{10}$ TCID$_{50}$/mL)		
	Low RH (20-29%)	Medium RH (30-39%)	High RH[b] (>40%)
BCoV	1.6 ($\pm$0.1) A	3.2 ($\pm$0.4) A	4.1 ($\pm$0.4)
HCoV OC43	2.6 ($\pm$0.1) B	4.2 ($\pm$0.4) B	5.6 ($\pm$0.4)

[a]Data is expressed as means $\pm$SEs from three replicates in two independent experiments. Values with different uppercase letters in the same column are significantly different ($p < 0.05$).
[b]Due to a non-normal data set, a statistical comparison could not be made.

Table 3.9: Titer Reduction as a function of <u>surrogate</u> type for PET and Vinyl carriers at three relative humidity levels.

Surrogate	Reduction[a] ($\log_{10}$ TCID$_{50}$/mL)					
	PET			Vinyl		
	Low RH (20-29%)	Medium RH (30-39%)	High RH (>40%)	Low RH (20-29%)	Medium RH (30-39%)	High RH[c] (>40%)
BCoV	1.6 ($\pm$0.2) A	2.8 ($\pm$0.6) A	ND[b]	1.7 ($\pm$0.1) A	3.7 ($\pm$0.4) A	4.1 ($\pm$0.4)
HCoV OC43	2.7 ($\pm$0.1) B	3.5 ($\pm$0.7) A	ND	2.4 ($\pm$0.1) B	4.6 ($\pm$0.3) A	5.6 ($\pm$0.4)

[a]The data is expressed as means $\pm$SEs from three replicates in two independent experiments. Values with different uppercase letters in the same column for each surface are significantly different ($p < 0.05$).
[b]ND: Not determined.
[c]Due to a non-normal data set, a statistical comparison could not be made.

CONCLUSION

In summary, previous studies have demonstrated that the novel pathogenic HCoV, SARS-CoV-2, can survive on surfaces long enough for secondary transmission to potentially occur, highlighting the importance of proper surface disinfection in FSEs. Due to the differences in robustness between cell lines and the varying compositions of disinfectant products, neutralization optimization is a critical first step when conducting disinfectant efficacy testing. Prior to the start of disinfectant testing, we were able to optimize methods to effectively neutralize the chlorine- and QAC-based disinfectants used in this study. When tested in suspension, Clorox Clean-Up Cleaner + Bleach, Oxivir Tb, and Cavicide 1were found to be efficacious against BCoV and HCoV OC43 with a 2 min contact time. Our subsequent carrier tests revealed that all three disinfectants tested reduced the infectivity of BCoV and HCoV OC43 below the LOD on both PET and vinyl surfaces following a 2 min exposure time. However, a ≥ 3.0 $\log_{10}$ reduction could not be observed in some cases due to the decreased dynamic range on the carrier that resulted from the low titer of the inoculated virus that was recovered from the carriers prior to disinfection. Our further analysis of the carrier test data found that HCoV OC43 was more sensitive to drying than BCoV, and both surrogates were inactivated faster at higher RH conditions and on vinyl surface carriers. Overall, the results of this study highlight the importance of properly neutralizing the disinfectants and verifying disinfectant suspension test data by performing disinfectant carrier tests to expose the virus and disinfectant to conditions (i.e., surface type, RH, and surrogate) comparable to what would be encountered during 'real-world' application.

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Further investigation regarding increasing the dynamic range for the carrier test by increasing the virus titer prior to disinfection is needed. In addition, future studies could be conducted to determine the shortest possible contact time (e.g., 30 or 60 seconds) in which the three RTU disinfectants tested in this study can inactivate SARS-CoV-2 surrogates on PET and vinyl fabric surfaces.