U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER

ABERDEEN PROVING GROUND, MD 21010-5424

DEVCOM CBC-TR-1792

Sub-Scale Testing of Decontamination Technologies against SARS-CoV-2 BSL-2 Surrogate, HuCoV229E, and BSL-1 Surrogate, Phi6

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August 2022

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
XX-08-2022	Final	Mar 2020–Dec 2021

4. TITLE AND SUBTITLE
Sub-Scale Testing of Decontamination Technologies against SARS-CoV-2 BSL-2 Surrogate, HuCoV229E, and BSL-1 Surrogate, Phi6

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER
CB10497

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, DEVCOM CBC, ATTN: FCDD-CBR-BD, APG, MD 21010-5424
Excet Incorporated, 8001 Braddock Road, Suite 303, Springfield, VA 22151-2110

8. PERFORMING ORGANIZATION REPORT NUMBER
DEVCOM CBC-TR-1792

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Threat Reduction Agency, 8725 John J. Kingman Road, MSC 6201, Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)
DTRA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT: (Limit 200 words)
This report summarizes the efficacy results of virucidal chemicals against bacteriophage, Phi6 (biosafety level [BSL]-1), and human coronavirus (HuCoV229E; BSL-2) on three surface types: keyboard plastic, aluminum, and nylon webbing. The study was completed at two levels: (1) laboratory-scale using small coupons and (2) sub-scale using the control panel, seat cushion, and seat belt from a C-117 aircraft. A standardized Organization for Economic Co-operation and Development (Paris, France) method was used during the laboratory-scale phase. Virus was retrieved for the sub-scale phase by surface sampling. Before the experimental phase of the study, a literature review was conducted on possible surrogates of SARS-CoV-2 (the causative virus for Coronavirus disease-2019), disinfection approaches, and virucidal chemicals. Three products (Calla 1452, Lysol, and bleach) from the Environmental Protection Agency (Washington, DC) N-list and three experimental chemicals (DiChlor, OxiClean, and Bioxy) were selected for testing. The results showed high variability in virus recovery and inactivation. These experiments were conducted in a closed environment. An electrostatic sprayer was used to deliver the test chemicals. Partial inactivation by three chemicals (DiChlor, OxiClean, and Calla 1452) was observed. The search for novel technologies to combat emerging viral and bacterial threats will better prepare our Warfighters and first responders.

15. SUBJECT TERMS

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	Virucidal chemicals	Nylon webbing
Coronavirus disease-2019 (COVID-19)	Phi6	Keyboard plastic
Human coronavirus-229E (HuCoV229E)	MRC-5 cells	Aluminum

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Renu B. Rastogi
U	U	U	UU	36	**19b. TELEPHONE NUMBER** *(include area code)* (410) 436-7545

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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PREFACE

The work described in this report was authorized under project number CB10497. The work was started in March 2020 and completed in December 2021.

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Acknowledgments

The authors acknowledge the following individuals for their hard work and assistance with the execution of this technical program:

- Dr. Glenn Lawson and Dr. Jim Noah (Defense Threat Reduction Agency [DTRA]; Fort Belvoir, VA) for programmatic support,
- Orshuntis Cross (Oak Ridge Institute for Science and Education; Oak Ridge, TN) for his professional laboratory preparation work, and
- Dr. Lalena Wallace (DTRA) for her contribution to this program between May and July 2020.

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EXECUTIVE SUMMARY

This study was launched in March 2020 at the height of the worldwide Coronavirus disease-2019 (COVID-19) pandemic and was completed in December 2021. The focus of this study was two-fold: (a) to determine which surrogate could be used to demonstrate the effectiveness of virucidal chemicals under laboratory conditions and (b) to assess how DoD assets could be effectively and rapidly cleaned and decontaminated.

This report summarizes the efficacy results of three products selected from the U.S. Environmental Protection Agency (EPA; Washington, DC) N-List and three experimental virucidal chemicals against Phi6 (a biosafety level [BSL]-1 bacteriophage surrogate for severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) and human coronavirus-229E (HuCoV229E; surrogate for SARS-CoV-2) on three military-relevant surfaces: keyboard plastic, aluminum, and nylon webbing. The study was completed at the laboratory-scale level using small coupons and at the sub-scale level using the control panel, seat cushion, and seat belt from a C-117 aircraft. A standardized method developed at the Organization for Economic Co-operation and Development (Paris, France) and adapted by EPA was used for the laboratory-scale testing. A surface-sampling approach was used for the sub-scale testing to retrieve the virus from the aircraft components. Before commencing the experimental phase of the study, a literature review was conducted on possible surrogates of SARS-CoV-2 (the causative virus for COVID-19), disinfection approaches, virucidal chemicals, and the persistence of coronaviruses on inanimate surfaces. Three products from EPA's N-list (Calla 1452 [Zip-Chem Products; Morgan Hill, CA], Lysol [Reckitt Benckiser; Slough, United Kingdom], and bleach [Procter & Gamble Company; Cincinnati, OH]) and three experimental chemicals (dichloro-*S*-triazinetrione [Quick Dissolving Shock DiChlor; Nava Water Products; Charleston, WV], OxiClean [Church & Dwight Co., Inc.; Ewing Township, NJ], and Bioxy [Atomes, Inc.; Quebec, Canada]) were selected for testing in this study.

At the laboratory-scale level, the results showed effective inactivation of both Phi6 and HuCoV229E by Lysol, bleach, and Calla 1452 on all three test surfaces in the absence of added bioburden. Virus inactivation was the most challenging on nylon webbing. In the presence of 10% synthetic sputum (SS; bioburden; ClaremontBio; Upland, CA), virus inactivation was reduced by 0.5–1 log, and once more, nylon webbing proved to be the most challenging surface. Similar results were observed for the experimental chemicals (i.e., OxiClean, DiChlor, and Bioxy).

The results showed high variability in virus recovery and virus inactivation at the sub-scale level. These experiments were conducted in a closed environment (test chamber) using an electrostatic sprayer (Electrostatic Spraying Systems, Inc.; Watkinsville, GA) to administer the test chemicals. Partial inactivation by DiChlor, OxiClean, and Calla 1452 was observed.

In conclusion, although a direct comparative run was not performed, HuCoV229E was determined to be a suitable (relative to Phi6) surrogate for SARS-CoV-2. Inclusion of 10% SS as bioburden resulted in lower virus inactivation. Nylon webbing was observed to be the most challenging test surface. Method development and improvement for sub-scale testing are highly

recommended in the absence of a standard method because of variability in virus recovery and inactivation. The HuCoV229E virus preparation used in this study was crude and, as expected, contained cell debris as an organic burden. It is highly recommended that novel disinfection technologies be researched and assessed to combat emerging novel viral and bacterial threats effectively and better prepare our Warfighters and first responders.

CONTENTS

FIGURES

TABLE

SUB-SCALE TESTING OF DECONTAMINATION TECHNOLOGIES AGAINST SARS-COV-2 BSL-2 SURROGATE, HUCOV229E, AND BSL-1 SURROGATE, PHI6

1. INTRODUCTION

In December of 2019, a novel coronavirus, now recognized as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was detected in Wuhan City, Hubei Province, China. In under three months, an epidemic swept through Asia, then Europe, and by March of 2020, SARS-CoV-2 was declared a global pandemic. As recorded (AJMC Staff, 2021), over 46 million individuals have been infected in the United States, resulting in 759,000 deaths; the number of confirmed cases worldwide has exceeded 255 million. The virus is responsible for 5.1 million deaths globally.

SARS-CoV-2 belongs to a large family of coronaviruses (CoVs), which are common in many different species of animals, including camels, cattle, cats, and bats. Until 2020, it was rarely found to infect humans. The virus is a betacoronavirus, like Middle East respiratory syndrome and severe acute respiratory syndrome (SARS), both of which originated in bats. Initially, the respiratory illness outbreak linked to the large seafood and live animal market in Wuhan indicated that the virus spread from animal-to-person. However, as new cases continued to emerge, it became evident that this virus could also spread person-to-person. In fact, the unprecedented level of viral transmission and contagion was linked to the large number of asymptomatic human carriers who unknowingly spread the virus before the onset of symptoms.

Current studies assume that persistent viruses and bacteria on inanimate surfaces in the environment are the source of outbreaks of "new and emerging diseases" and nosocomial infections (Kramer et al., 2006; Casanova et al., 2010; Otter et al., 2016; Weber et al., 2019; Marquès and Domingo, 2021). Most viruses from the respiratory tract can persist for two to three days on surfaces. Environmental factors, such as ambient temperatures and relative humidity (RH), appear to severely affect the survival of two potential surrogates of SARS-CoV-2, murine hepatitis virus (MHV) and transmissible gastroenteritis virus (TGEV). In general, these two surrogate viruses persisted the longest at a low temperature of 4° C compared with the ambient temperature of 40° C (Casanova et al., 2010). Increased persistence was observed at a low RH of 20% rather than a high RH of 80%.

The direct correlation between contaminated surfaces and the transmission of respiratory viruses and other emerging disease pathogens is driving researchers to explore alternate methods for effective decontamination. Selecting the appropriate disinfection approach and technology is critical for controlling infection and minimizing spread of disease in medical treatment facilities and hospitals (Kim et al., 2016; Weber et al., 2016). These efforts are significantly more complicated with military relevant surfaces such as aircraft.

Surrogates for use in place of the SARS-CoV-2 virus include less-pathogenic members of other human coronaviruses (HuCoVs) such as HuCoV-NL63, HuCoV-OC43, and HuCoV-229E and non-pathogenic animal CoVs such as canine CoV, TGEV, and MHV. Taxonomically, the less-pathogenic HuCoV229E is closely related to SARS-CoV-2. In addition,

bacteriophage Phi6 (a double-stranded ribonucleic acid [RNA]-enveloped virus-infecting *Pseudomonas syringae*) has been suggested as a viral surrogate for an enveloped RNA virus. Most studies focused on its persistence on personal protective equipment and hard surfaces and in water. In the absence of direct side-by-side comparative studies for relative sensitivity or resistance of surrogate viruses with novel CoV, the appropriateness of animal CoV or bacteriophage as surrogates is at best speculative.

2. OBJECTIVES

The objectives for this study were designed to determine the following:

1. whether Phi6 is an appropriate surrogate for SARS-CoV-2 for efficacy of antiviral chemicals;
2. the efficacy of common disinfectants and household chemicals against biosafety level (BSL)-2 HuCoV surrogate, HuCoV229E; and
3. the effect of interfering material, that is, synthetic sputum (SS; ClaremontBio; Upland, CA) on the efficacy of disinfectants.

3. MATERIALS AND METHODS

Bacteriophage cultivation and bacterial cultures were performed in a laboratory at the U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD). Mammalian cell cultivation and human coronavirus work were also performed at the same location. The work was performed under ambient conditions with a temperature range of 20–25 °C and a RH range of 20–40%. No attempt was made to control either of the two parameters.

3.1 Viral Surrogates

Two surrogates for SARS-CoV-2 were included in this study: the first, HuCoV229E, is a BSL-2 surrogate, and the second, Phi6, is a BSL-1 surrogate. Both test viral surrogates were used in phase 1 of testing, but only HuCoV229E was used in phase 2.

3.2 Test Surfaces

The surface materials selected for this study were aluminum, keyboard plastic, and nylon webbing. The material selection was finalized after consultation with the Defense Threat Reduction Agency (DTRA; Fort Belvoir, VA) program manager.

3.3 Disinfection Technologies

The three Environmental Protection Agency (EPA; Washington, DC)-registered disinfectants selected for this program were Calla 1452 (Zip-Chem Products; Morgan Hill, CA), Lysol (Reckitt Benckiser; Slough, United Kingdom), and 1000 ppm Clorox bleach (Procter &

Gamble Company [P&G]; Cincinnati, OH). In addition, 1000 ppm of dichloro-*S*-triazinetrione (Quick Dissolving Shock DiChlor; Nava Water Products; Charleston, WV), 0.5% Bioxy (Atomes, Inc.; Quebec, Canada), and 10% OxiClean (Church & Dwight Co., Inc.; Ewing Township, NJ) were selected for evaluation.

3.4 HuCoV229E Preparation

MRC-5 cells were sub-cultured to 70–80% confluency. HuCoV229E virus was propagated with 2% fetal bovine serum (FBS; Gibco Life Technologies; Grand Island, NY) at 0.01 multiplicity of infection. The cells were allowed to grow for 48–72 h until cytopathic effects were visible. The infected cells were taken through three freeze-thaw cycles from –80 to 37 °C for 30 min for each cycle. After undergoing the freeze-thaw cycles, the cells were scraped from the surface, pooled together, mixed well, and centrifuged at 1K for 10 min to rid the samples of cell debris. The supernate was concentrated by passing it through Amicon Ultra-15 centrifugal filter units (MilliporeSigma; Boston, MA). The samples were concentrated 10- to 20-fold. The virus samples were aliquoted and stored at –80 °C.

3.5 Infection of MRC-5 Cells with HuCoV229E Virus

MRC-5 cells were cultured using 10% FBS in Eagle's minimum essential medium (EMEM) containing antibiotics. The cells were typically split 1:4 or 1:3 and grown up to passage 15 after which the cells began to show noticeable slowing of growth. The cells were seeded in a 24-well plate at 5×10^4 cells per plate for 24 h before infection. Viral dilutions were prepared in a final volume of 5 mL of 2% FBS/EMEM media. After the cell growth media over the cells was removed, 1 mL of the diluted virus was added to each well. From each dilution, four replicate wells were used for infection.

3.6 Phi6 Preparation, Transfection, and Phage Quantification

A broth culture of *P. syringae* from frozen strain stock or obtained from the American Type Culture Collection (Manassas, VA) was grown for 14–16 h at 25° C with shaking at 175 rpm. Soft agar and tryptic soy agar (TSA) plates were prepared before phage infection. Phi6 stock dilutions were prepared by making serial dilutions and aliquoted into 100 µL. A double agar overlay was used to process the test samples. The overlay was a mixture of 200 µL of overnight culture of bacterial cells, 50 µL of Phi6, and 4.25 mL of soft agar. It was poured quickly over the TSA plate surface, and the plate was swirled to ensure even distribution of the soft agar over the surface. Plates were incubated upside down at 25° C for 24 h. Small round plaques on a lawn of *P. syringae* were counted after two or three dilutions. To harvest Phi6, 1 mL of tryptic soy broth (TSB) was added to each transfected plate (zero, –1, –2, –3 dilution). The soft agar was scraped and transferred to a 50 mL sterile tube. The pooled solution was vortexed vigorously to mix and release the bacteriophage from the agar before being centrifuged for 10 min at 4000 rpm. The supernate was transferred into 0.22 µm cellulose acetate filter tubes and centrifuged for 2 min at 4000 rpm. Centrifugation was repeated periodically to optimize filtrate collection. The filtrate was stored in a refrigerator (for long term storage, the phage can be stored at –80° C, but the titer may drop over a period of time in cold storage).

3.7 Surface Sampling of HuCoV229E from Test Articles in Sub-Scale Studies

An aliquot of 50 mL was spotted on pre-marked areas on control and test sets. The virus was allowed to dry for 60–75 min. Drying time was dependent on the ambient temperature and RH. After the articles were dry, they were transferred to the test chamber. First, the control set was sprayed with sterile water for 10 min. The virus was recovered from articles by sampling using pre-wetted sterile polyester wipes. The wipes were transferred to 10 mL of 2% FBS/EMEM media. The virus was recovered from the wipes by vortexing for 1 min. The test set was then transferred to the chamber and sprayed with one of the three disinfectants. Three passes were made over the test articles. The articles were allowed to be in contact with the disinfectant for 10 min after which the virus was recovered in the same manner as the control set. The virus was recovered from the wipes by vortexing for 1 min. The control and test samples were diluted and used to infect MRC-5 cells.

3.8 Log Density Estimation and Data Reduction

Phi6 plaques on control and test plates were counted, and the numbers were recorded in Excel (Microsoft; Redmond, WA) spreadsheets. Log density of phage was calculated by multiplying the dilution factor with the volume factor. Log reduction was estimated by subtracting log density of treated samples from that of control log density.

4. RESULTS

One of the early questions addressed was whether the BSL-1 surrogate, Phi6, could be used as a surrogate for disinfection efficacy testing. Phi6 was cultivated and used as a test organism in early experiments. HuCoV229E was the BSL-2 surrogate used as an alternative test virus. Results are summarized in Sections 4.1 and 4.2 for Phi6, in Sections 4.3–4.6 for HuCoV229E, and in Section 4.4 for the sub-scale study.

4.1. Recovery of Phi6 and Effect of Bioburden on its Stability

One of the first experiments performed was the recovery of the two viruses from each of the three surfaces. Figure 1 shows the recovery of Phi6 from aluminum, nylon webbing, and keyboard plastics. As seen in the figure, the recovery of phage from nylon webbing was the lowest, but >3.5 log phage was recovered from all three surfaces. We determined whether the inclusion of bioburden affects the stability of Phi6 for up to 24 h. Two organic burdens, that is, 10% TSB and 10% SS, were evaluated for their effect on the stability of Phi6. Results are presented in Figure 2. The results show a slight effect on TSB and SS on the stability of Phi6 from the aluminum surface, and in 24 h, only 1/2 log of phage was lost. After 24 h, >3 log virus was recovered from samples with or without organic burden.

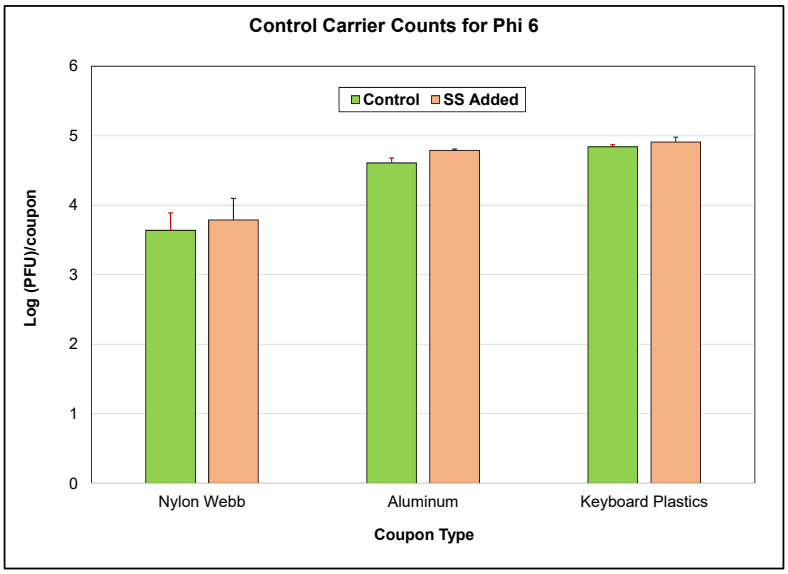


Figure 1. Recovery of Phi6 from test surfaces.

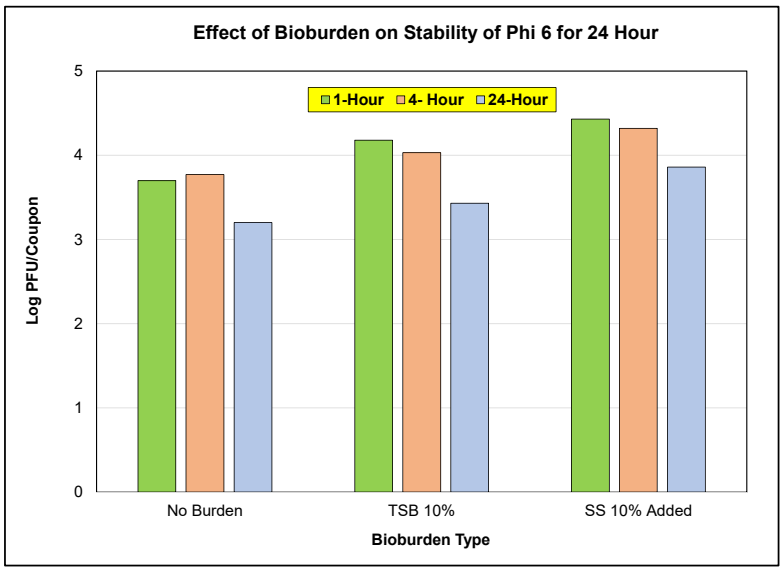


Figure 2. Effect of bioburden on stability of Phi6 for 24 h.

4.2. Efficacy of Four Disinfectants against Phi6

Efficacy of bleach, Bioxy, Lysol, and DiChlor against Phi6 was investigated in the presence and absence of SS (as a bioburden). The results are presented in Figures 3–6. The results clearly establish the following trends: (1) complete bacteriophage inactivation on aluminum and keyboard plastics in the absence or presence of SS by three of the tested chemicals, except in the case of Lysol when bioburden was added and (2) reduced efficacy in phage inactivation on nylon webbing, even in the absence of SS. In the presence of SS, the efficacy was lower by 1 log.

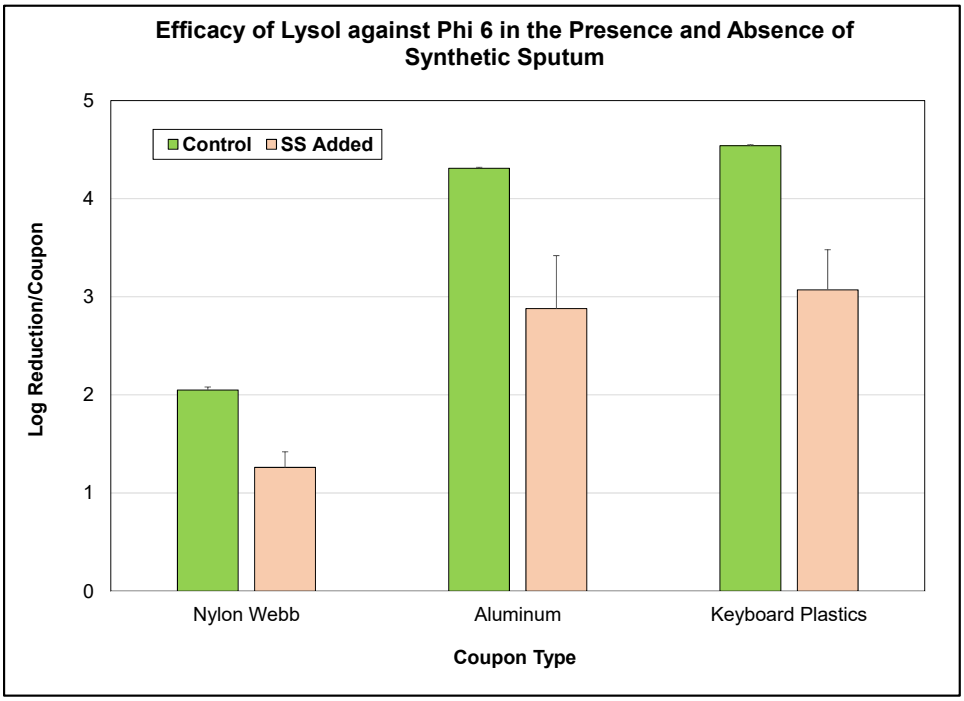


Figure 3. Efficacy of Lysol in the absence and presence of SS.

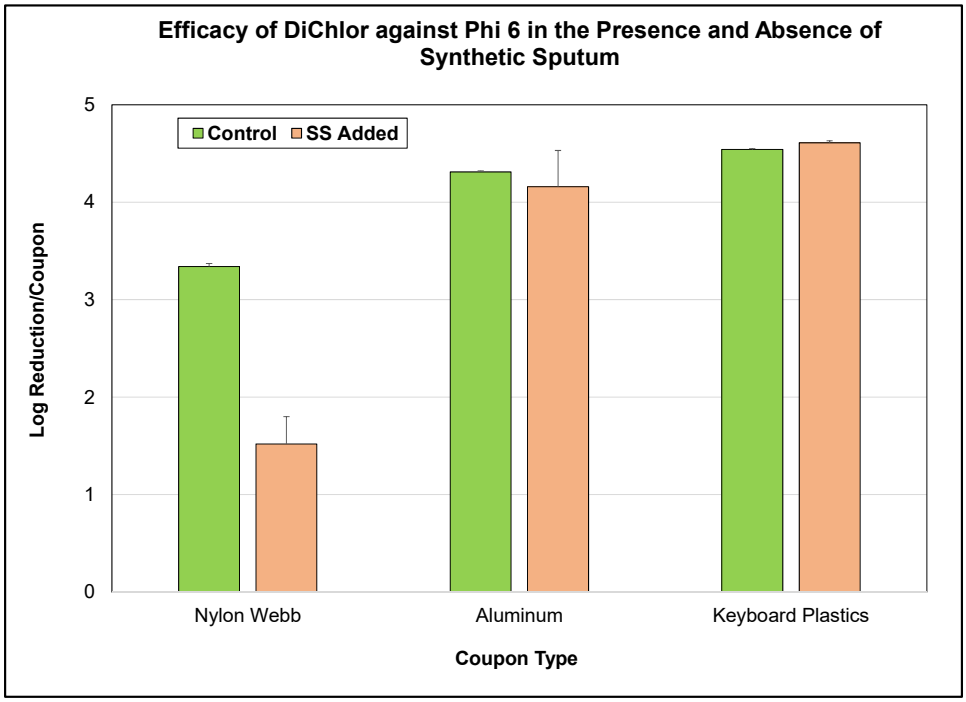


Figure 4. Efficacy of DiChlor in the absence and presence of SS.

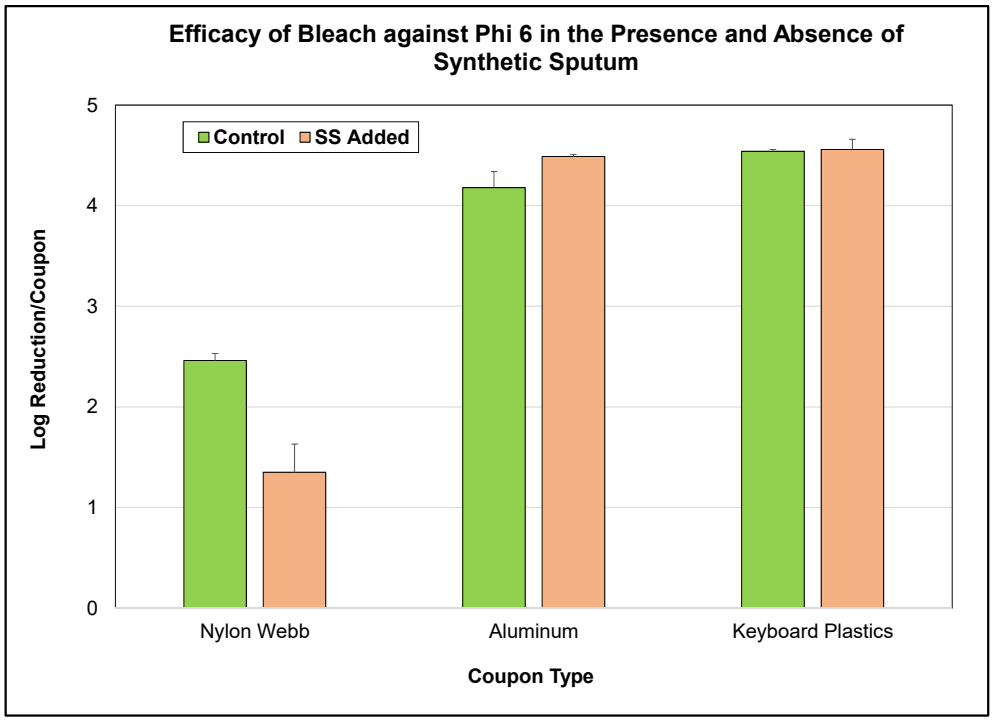


Figure 5. Efficacy of bleach in the absence and presence of SS.

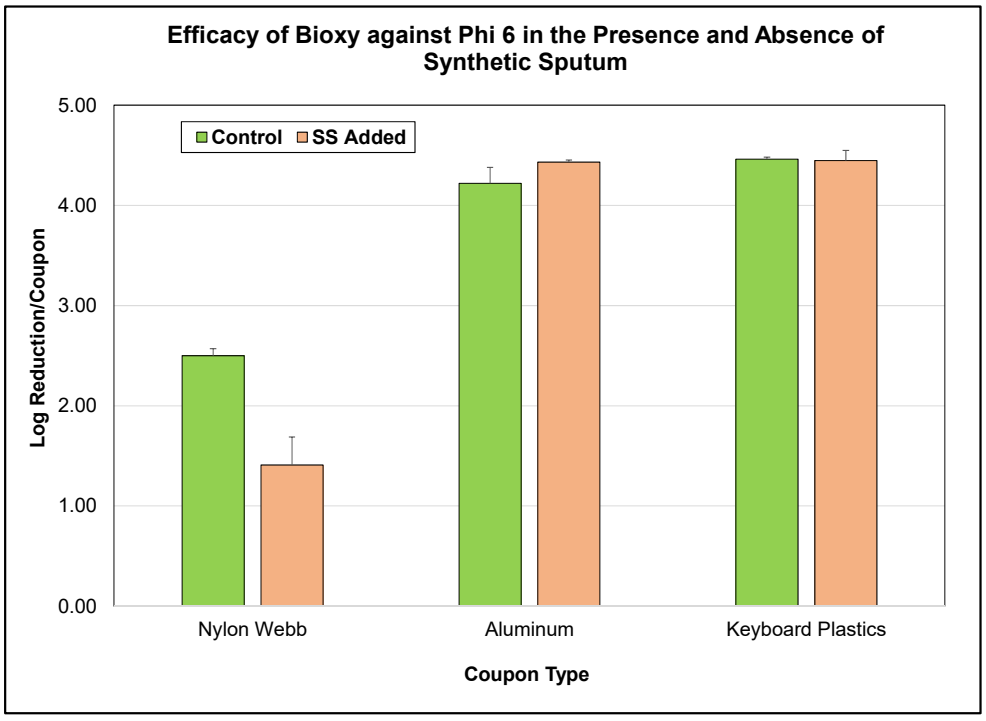


Figure 6. Efficacy of Bioxy in the absence and presence of SS.

4.3. Recovery of HuCoV229E from Three Surfaces

An earlier laboratory-scale experiment (conducted in-house) assessed the recovery of human coronavirus from the three test surfaces. The results summarized in Figure 7 show a recovery of >4 log from aluminum and keyboard plastics; for nylon webbing, the recovery was >3 log. The results are similar to those for Phi6. There was least viral recovery from nylon webbing and comparable recovery of virions from the other two surfaces.

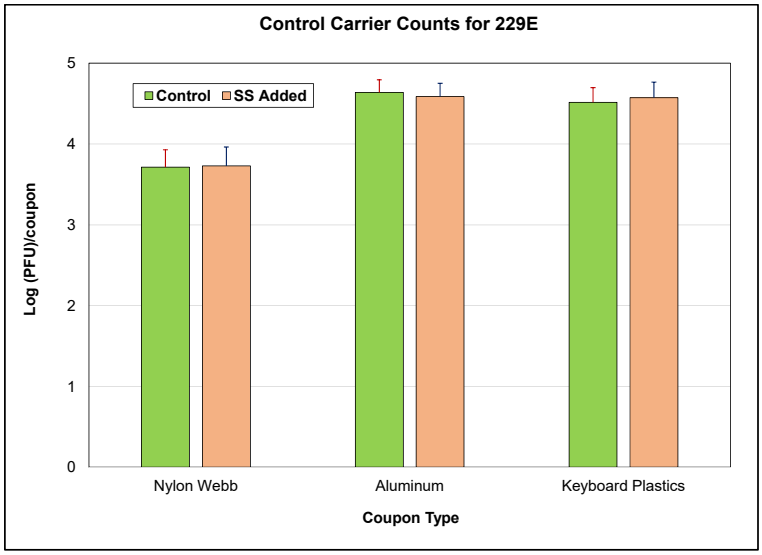


Figure 7. Recovery of HuCoV229E from test surfaces.

4.4. Efficacy of Five Disinfectants against HuCoV229E in the Presence and Absence of SS

Laboratory-scale efficacy runs were completed in accordance with the Organization for Economic Co-operation and Development (OECD; Paris, France) test method (OECD, 2020) using the BSL-2 virus surrogate, HuCoV229E, on three selected test surfaces. Runs were performed in the absence and presence of the selected bioburden, SS (10% final concentration with the virus). The five disinfectants used included Clorox bleach (1000 ppm), Lysol (as per manufacturer's recommendation, 2 oz/gal), Calla 1452 (as per manufacturer's recommendation, 4 oz/gal), and two experimental chemicals (Bioxy [1000 ppm] and DiChlor [1000 ppm]). All exposure times were for 10 min. The results show high efficacy of viral inactivation in the absence of bioburden and lower efficacy in the presence of bioburden. In addition, efficacy was higher on aluminum and keyboard plastics than on nylon webbing. Based on efficacy, chemicals can be ranked (from high to low) in the following order: DiChlor, bleach, Bioxy, Calla 1452, and Lysol. Interestingly, Calla 1452 was more effective on nylon webbing than on the other two test surfaces. We suggest that these tests be repeated to confirm the primary observations. Figures 8–12 show the efficacy test results.

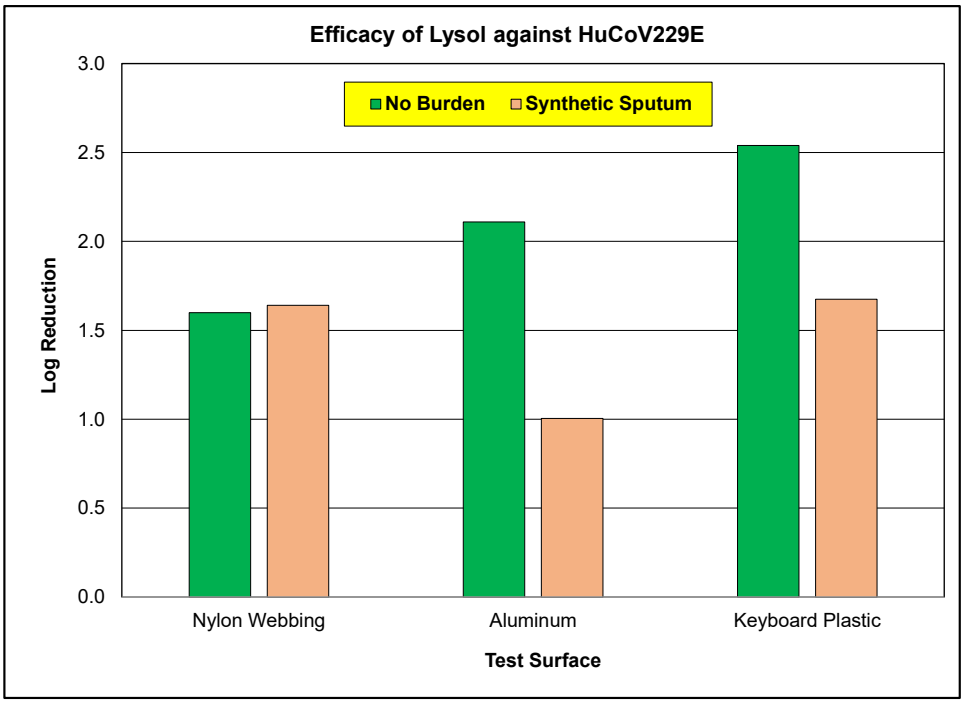


Figure 8. Efficacy of Lysol against HuCoV229E.

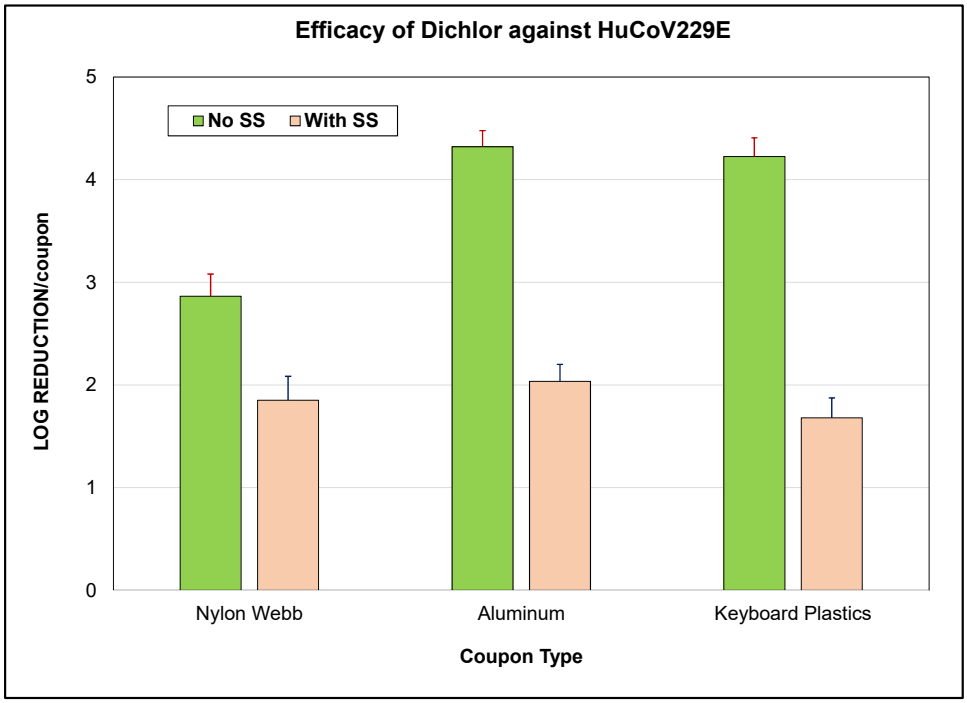


Figure 9. Efficacy of DiChlor against HuCoV229E.

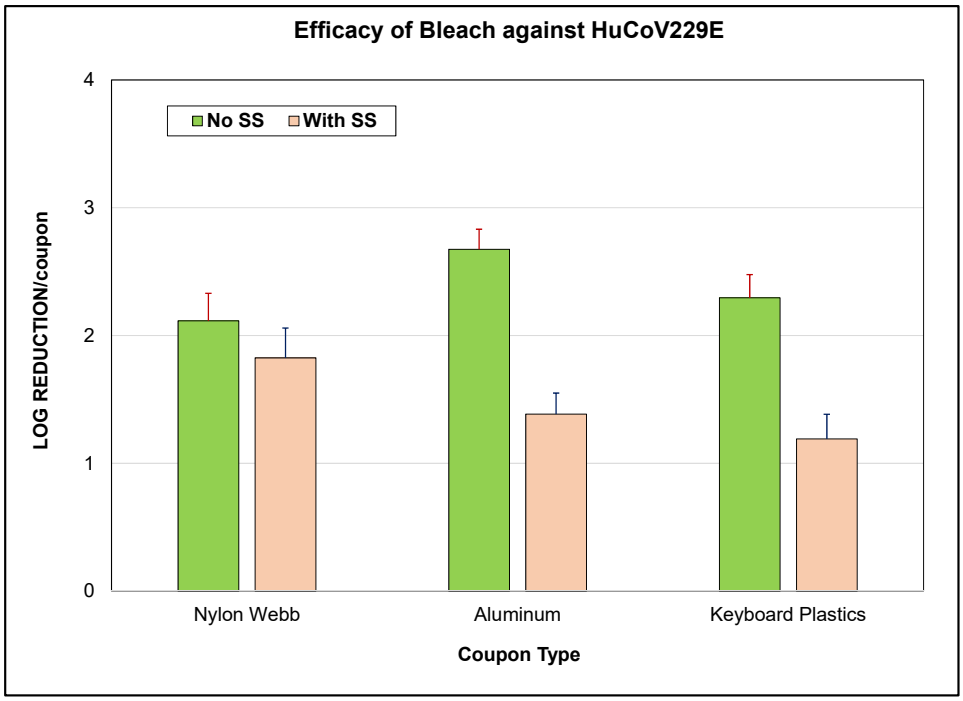


Figure 10. Efficacy of bleach against HuCoV229E.

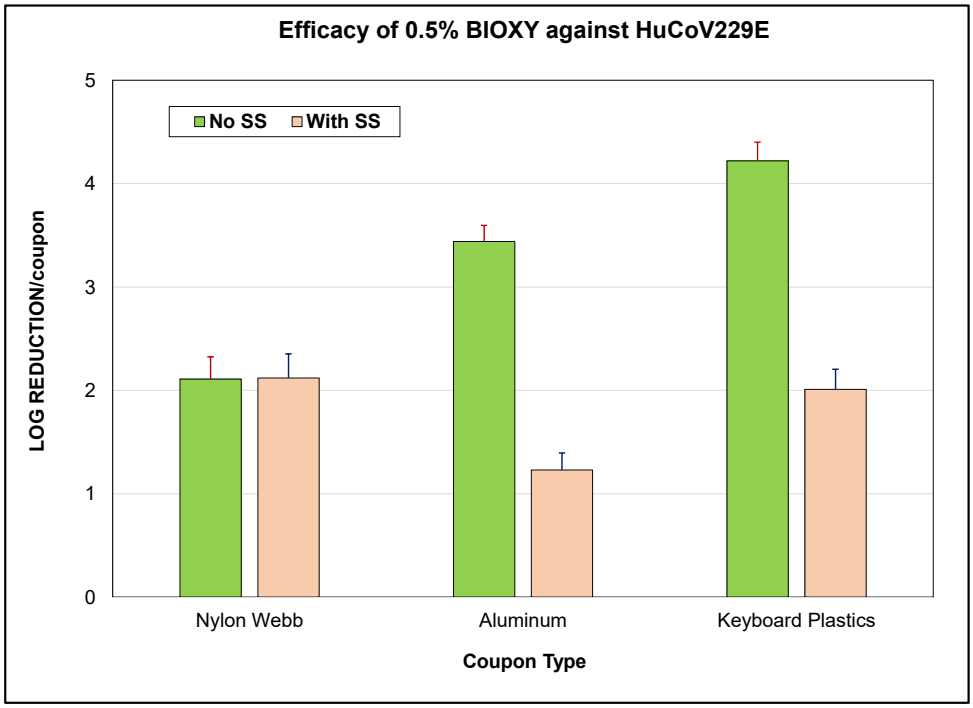


Figure 11. Efficacy of Bioxy against HuCoV229E.

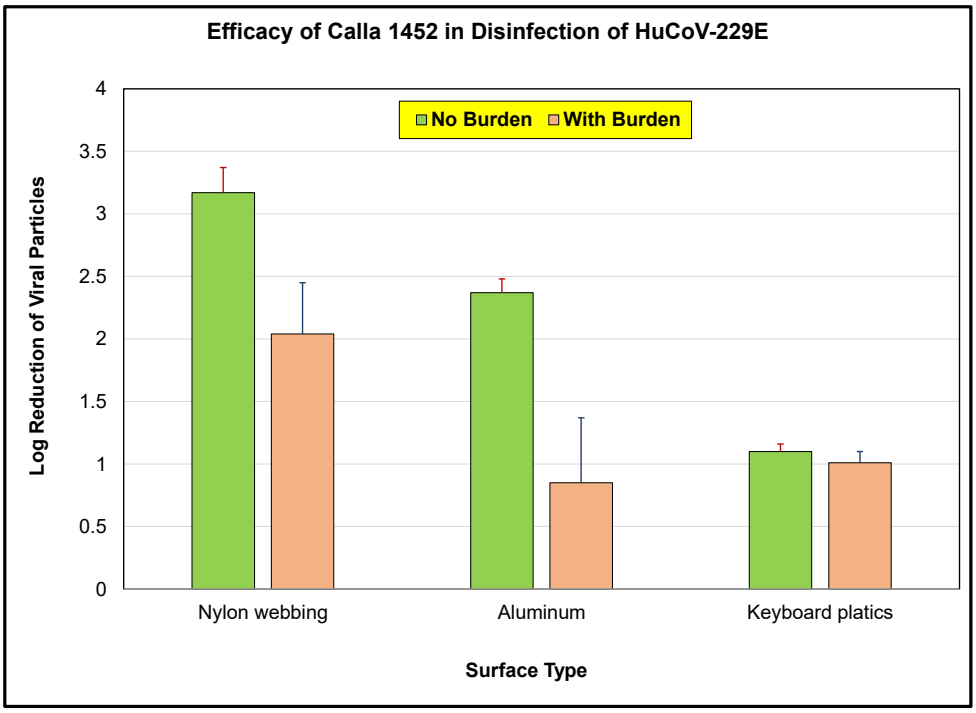


Figure 12. Efficacy of Calla 1452 against HuCoV229E.

The efficacy test results data are presented in the Table. The green shaded-cells represent a >3 log reduction, and the yellow-shaded cells depict a 1–2 log reduction. In addition, the media had to be changed in the case of quaternary ammonium-based disinfectants like Lysol and Calla 1452, as these chemicals were found to be cytotoxic.

Table. Summary of Disinfectant Efficacy and Cytotoxic Circumvention

Non Porous Materials- Aluminum and Keyboard Plastics					
Virucidal Chemical	**Description**	**+SS**	**-SS**	**Cytotoxicity**	**Media Change**
1	Calla 1452 Neutral Disinfectant Cleaner				Yes
2	Lysol Clean & Fresh Multi-surface Cleaner				Yes
3	Clorox 8.25%				No
4	Dichlor Quick Dissolve Shock				No
5	Bioxy				No
UVC					No

Porous Materials- Nylon Webbing					
Virucidal Chemical	**Description**	**+SS**	**-SS**	**Cytotoxicity**	**Media Change**
1	Calla 1452 Neutral Disinfectant Cleaner				Yes
2	Lysol Clean & Fresh Multi-surface Cleaner				Yes
3	Clorox 8.25%				No
4	Dichlor Quick Dissolve Shock				No
5	Bioxy				No
UVC					No

In the final segment of the laboratory-scale study, the effects of an all-purpose cleaner (OxiClean) and wetting surfactant (Dawn dishwashing liquid; P&G), were investigated. Figure 13 summarizes the efficacy of OxiClean on the three test surfaces. The results show >3 log reduction of the virus on all three surfaces in the absence of bioburden. However, in the presence of bioburden, the efficacy dropped by 1 to 1.5 log on aluminum and nylon webbing. The effect of wetting agent, Dawn soap (0.5%), on the efficacy of Calla, DiChlor, and OxiClean in the presence of bioburden is summarized in Figure 14. The results show marginal improvement (0.3–0.6 log) in the efficacy of the three test chemicals.

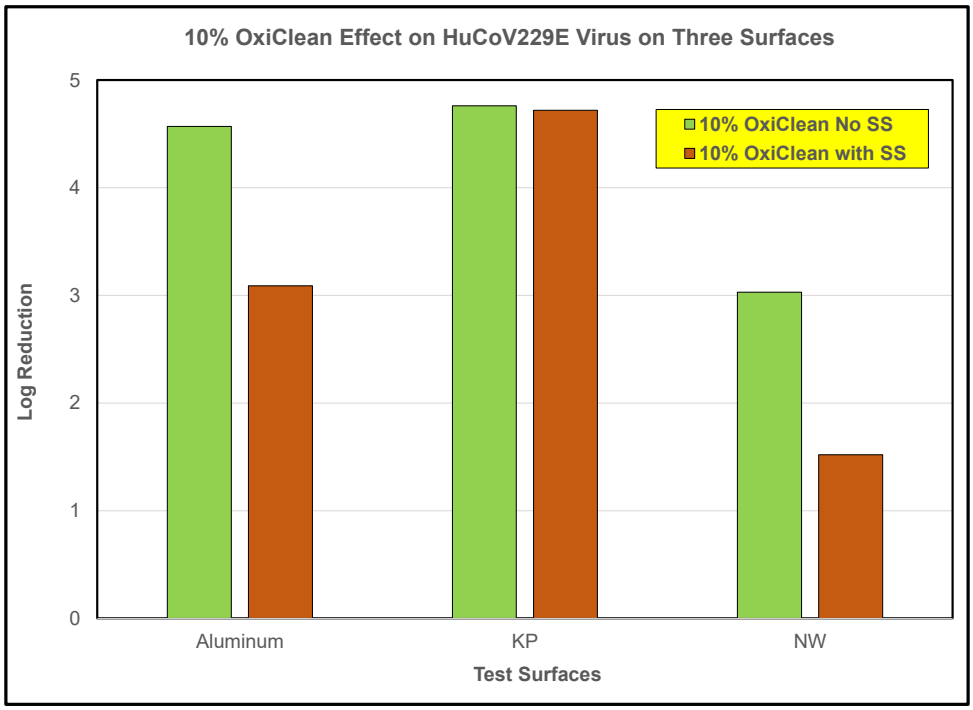


Figure 13. Efficacy of OxiClean against HuCoV229E.

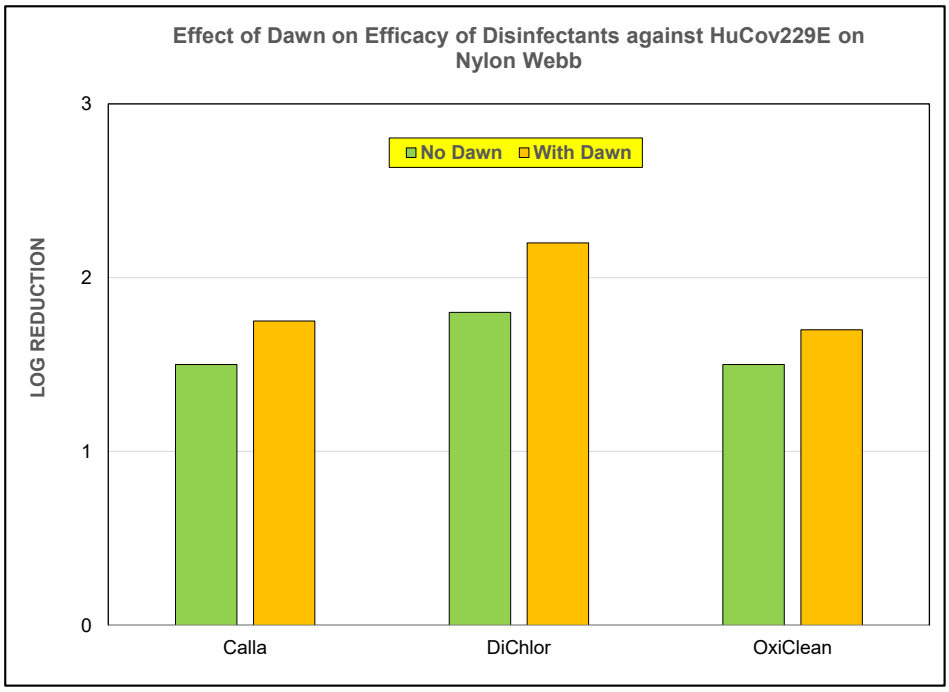
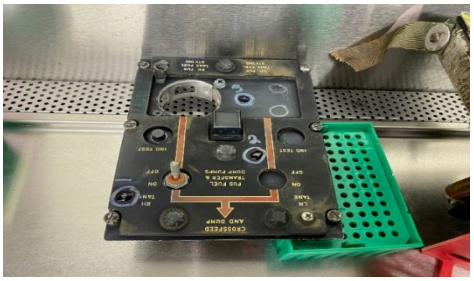


Figure 14. Effect of Dawn soap on efficacy of three disinfectants against HuCoV229E.

Sub-Scale Study

One objective of this study was to investigate the effectiveness of antiviral chemicals at a sub-scale level. For this task, actual objects retrieved from an aircraft were used in a chamber. Three pieces of an aircraft (a seat belt, seat cushion, and control panel parts) were selected and are shown in Figure 15. Figure 16 shows the electrostatic sprayer (Electrostatic Spraying Systems [ESS], Inc.; Watkinsville, GA) used to apply antiviral chemicals for testing. Only three disinfectants (DiChlor, OxiClean, and Calla 1452) were selected as the test chemicals. Based on the comparative efficacy results between Phi6 and HuCoV229E, the latter was selected as the test virus. Because HuCoV229E is a BSL-2 virus, the tests were conducted within a chamber in the Aerosol Sciences Branch, DEVCOM CBC. On the day of the test, an aliquot of 50 µL was inoculated on each of the three test objects within the BSL-2 cabinet and allowed to dry for 60–90 min. After the inoculum was dry, the test objects were transferred to the BSL-2 chamber. The control set was sprayed first with sterile water, then the test set was sprayed with either 1000 ppm OxiClean, 1000 ppm DiChlor, or Calla 1452 (in accordance with the manufacturers' recommendations). Three passes of spray were made within 1–2 min. After a 10 min contact period, pre-wetted polyester wipes (wetted with 2% FBS/EMEM media) were used to retrieve the virus from the surface, and the virus was extracted in 10 mL of 2% FBS/EMEM media. Infection of MRC-5 cells was performed after the control and test samples were diluted.

A. Seat cushion and seat belt



B. Control panel part

C. Virucidal agent sprayed using an ESS sprayer

Figure 15. (A, B) Sub-scale test articles used in this study and (C) scientist using the ESS sprayer.

Figure 16. ESS sprayer.

4.5 Recovery of HuCoV229E from Test Articles

HuCoV229E recovery from the test articles is presented in Figure 17. As seen in the figure, recovery was highly variable from all three articles: seat cushion (2.9–3.8 log), seat belt (2–3.2 log), and control panel (3.2–4.6 log). The recovery was lowest from the seat belt. Variability in recovery could largely be due to inefficient sampling of virus from the test articles.

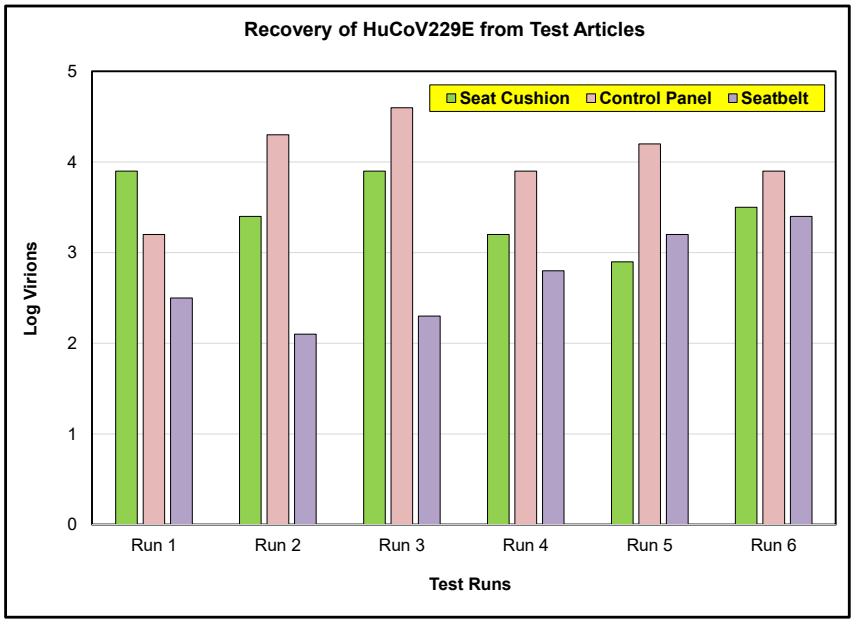


Figure 17. Recovery of HuCoV229E from test articles by surface sampling.

4.6 Efficacy of Disinfectants against HuCoV229E Inoculated on Test Articles

The efficacy test results are summarized in Figure 18. High variability in log reduction is evident (0.2–1.0 log). Efficacy was lowest for the seat belt (2–2.5 log), whereas the efficacy for the seat belt and control panel ranged between 2.2 and 3 log. All three test chemicals (Calla 1452, DiChlor, and OxiClean) appear comparably efficacious.

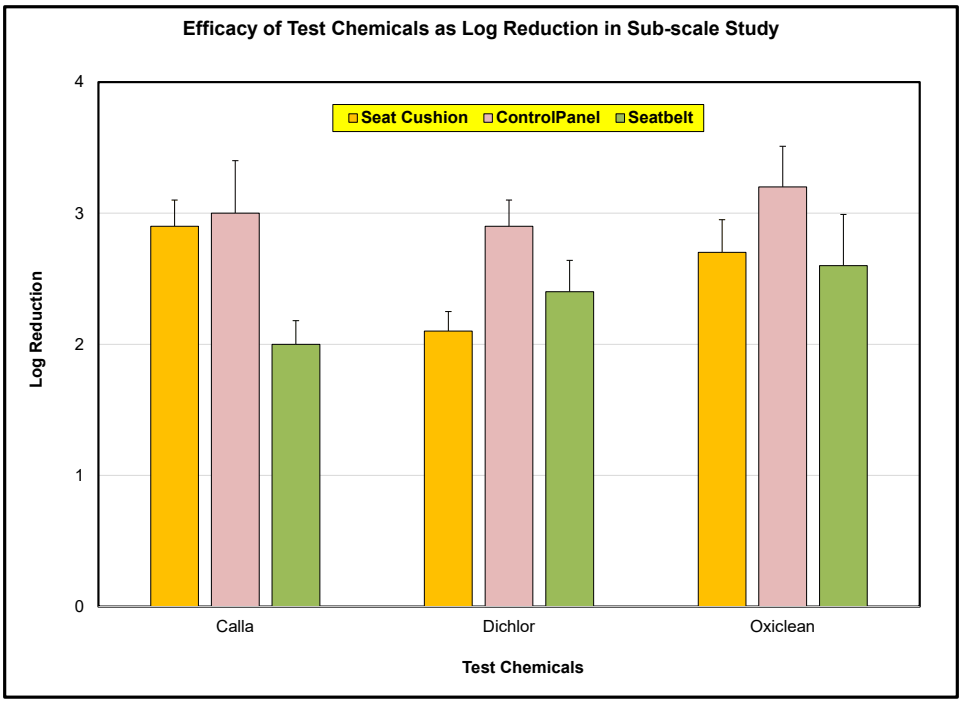


Figure 18. Efficacy of three disinfectants against HuCoV229E.

5. DISCUSSION AND CONCLUSIONS

In early 2020, the COVID-19 pandemic spread exponentially, and there was national attention on the implementation of technologies for rapid cleanup of surfaces to control the spread of infection. To meet the demand, the DTRA Hazard Mitigation Division requested that a team from DEVCOM CBC conduct a literature survey on (1) BSL-1 and -2 surrogate selections for the COVID-19 virus, SARS-CoV-2; (2) disinfection options for rapid disinfection of the SARS-CoV-2 virus; and (3) selection of non-chlorinated technologies that could be deployed for cleanup of military assets, especially aircraft and transport vehicles. The literature survey was completed by April 2020.

Based on the literature survey, two surrogates were identified. First, Phi6, a BSL-1 bacteriophage for *P. syringae*, was suggested as a surrogate for RNA viruses in general (Ye et al., 2016; Aquino de Carvalho et al., 2017; Cadnum et al., 2020). Second, HuCoV229E, a BSL-2 surrogate that has been used in other studies, was recommended as a substitute for SARS-CoV-2 (Gundy et al., 2009; Warnes et al., 2015). This study was completed using both test organisms. Initially, the Phi6 bacteriophage was included as a test organism, and its sensitivity to Lysol, bleach, DiChlor, and Bioxy was investigated in comparison with that of HuCoV229E. Based on the results summarized in this report, it was concluded that the HuCoV229E virus was more resistant to test chemicals. The remainder of the study, therefore, was focused on human coronavirus.

Throughout the pandemic, the EPA's N-list was used as a guide for selecting effective antiviral chemicals for surface disinfection in household and hospital settings. In the spring of 2020, due to sustained high demand for conventional disinfectants, many of these

chemicals were in short supply or unavailable. From the DoD perspective, there was an acute need to use specific chemicals in an aircraft. A number of common classes of disinfectants, specifically those that are chlorinated or peroxide-based, are not acceptable for use within an aircraft due to high reactivity or flammability. However, Calla 1452 is an approved antiviral chemical for use within an aircraft. DiChlor, OxiClean, and Bioxy were also screened in this study to assess their applicability as potential virucidal agents.

DiChlor, which is chemically known as dichloroisocyanuric acid, is marketed as a tablet or in granular form. It is typically used to shock pool water (1 lb/10,000 gal of pool water). When dissolved in water, it turns into hydrochloric acid and hypochlorite ions (free chlorine), and the pH is near neutral (6.5). Free chlorine is the key disinfecting molecule. OxiClean is a combination of sodium percarbonate, sodium carbonate, surfactants, and polymer and in solution, it generates hydrogen peroxide and washing soda. It is generally used as an all-purpose cleaner and stain remover on surfaces and fabric. In an aqueous solution, it forms oxygen and hydrogen peroxide. Bioxy is a powder that generates hydrogen peroxide and peracetic acid at neutral pH. Bioxy is a hard surface disinfectant used in the food and beverage industry and agricultural community.

Overall, the results show that in the absence of 10% SS, Calla 1452, Lysol, bleach, DiChlor, and OxiClean are effective virucidal chemicals on aluminum and keyboard plastic surfaces. The disinfection efficacy on nylon webbing appears to be more challenging, as log reduction showed <3 log. SS contains amino acids, mucin, saliva, and mucous membranes. The inclusion of this bioburden was justified because SARS-CoV-2 is a respiratory virus and likely to be complexed with such materials. A number of other studies have included 5 or 10% FBS or three-part organic soil load (EPA's OECD protocol). All contact times in this study were 10 min; however, future studies with longer contact times may be necessary to achieve complete virus inactivation. The results from the laboratory-scale testing summarized in this report are instructive in two respects. First, Lysol, bleach, and Calla 1452 are effective disinfectants against the BSL-2 surrogate, HuCoV229E. Second, OxiClean, DiChlor, and Bioxy are equally effective against this surrogate. Nylon webbing is consistently the most difficult material to disinfect.

The second phase of this study demonstrated disinfection practices at a sub-scale level. Three test articles (a seat cushion, control panel, and seat restrainer) were selected and retrieved from a decommissioned C-117 aircraft. One of the first challenges was the lack of a standardized test protocol for evaluation at this level. An ESS sprayer was used to apply the disinfectant. It was difficult to assess a uniform application of test chemical. In a laboratory-scale study, virus can typically be retrieved by extraction. However, extraction from test articles was not possible in this study. Surface sampling using pre-wetted polyester wipes was used to retrieve virus from the test articles. In the initial experiments, >3–4 log virus was recovered. Two challenges were immediately recognized: (1) high variability of virus recovery from control set and (2) high variability in log reduction values. Because of these two observations, the results from our study phase must be taken as preliminary. Overall, the results show a trend (i.e., ~3 log reduction from the control panel and <3 log reduction from the other two test articles). Here again, the seat belt surface results showed the least efficacy.

In conclusion, the present study clearly established the following:

1. suitability of the OECD test method for quantitative efficacy evaluations of virucidal chemicals on DoD-relevant surfaces;
2. high virucidal efficacy of three N-listed disinfectants (Lysol, Calla 1452, and bleach) against all three surface types;
3. partial loss of efficacy in the presence of 10% SS on surfaces;
4. high virucidal efficacy for three experimental disinfectants or cleaners (DiChlor, OxiClean, and Bioxy);
5. deleterious effect of bioburden inclusion for experimental disinfectants;
6. at the sub-scale level, suitability of ESS sprayer in a closed environment for spraying test chemicals;
7. high variability in virus recovery and log reduction values at sub-scale level;
8. effectiveness of Calla 1452 and OxiClean on sensitive military assets, including aircraft interiors; and
9. effectiveness of DiChlor on non-sensitive assets.

We strongly recommend the following future research efforts:

1. disinfection studies in the presence of bioburden types other than 10% SS to harmonize and standardize the OECD test method, allowing comparison of test results from different laboratories;
2. evaluation of other liquid-spraying devices, especially in field conditions, to assess the effectiveness of disinfectant application in an operational environment;
3. development of a test method for sub-scale level testing with reduced variability;
4. direct comparative studies between a BSL-2 surrogate and SARS-CoV-2, including key variants (highly recommended), to enhance confidence in test results and disinfectant applicability in field conditions;
5. continued search for benign and effective novel decontamination materials on sensitive and porous surfaces; and
6. comparative studies among variant strains of the COVID-19 virus to determine whether more pathogenic strains show altered disinfectant sensitivity.

In closing, several experts believed that the COVID-19 pandemic would be over within months after vaccine development. Contrary to such opinions, it appears that COVID-19 (as of 5 January 2022) is still spreading in high numbers, even two years after first detection in humans. The fundamental reasons for this are (1) the virus is acquiring multiple mutations, resulting in variant strains with high contagious characteristics, and (2) a significant number of unvaccinated people are providing conducive hosts for the emergence of highly contagious variants with varying lethality. The emergence of novel threats, including viruses and antibiotic-

resistant bacterial strains, necessitates a continued search for new and novel technologies to clean contaminated surfaces to limit the spread of infectious biologicals. In this endeavor, efforts directed and funded by the DTRA Hazard Mitigation Division are significant in preparing Warfighters, first responders, and this nation to face and combat such novel emerging biological threats.

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LITERATURE CITED

American Journal of Managed Care (AJMC) Staff. A Timeline of Covid-19 Developments in 2020, 2021. https://www.ajmc.com/view/a-timeline-of-covid19-developments-in-2020 (accessed December 20, 2021).

Aquino de Carvalho, N.; Stachler, E.N.; Cimabue, N.; Bibby, K. Evaluation of Phi6 Persistence and Suitability as an Enveloped Virus Surrogate. *Environ. Sci. Technol*. **2017,** *51* (15), 8692–8700.

Cadnum, J.L.; Li, D.F.; Redmond, S.N.; John, A.R.; Pearlmutter, B.; Donskey, C.J. Effectiveness of Ultraviolet-C Light and a High-level Disinfection Cabinet for Decontamination of N95 Respirators. *Pathog. Immun*. **2020,** *5,* 52–67.

Casanova, L.; Rutala, W.A.; Weber, D.J.; Sobsey, M.J. Coronavirus Survival on Healthcare Personal Protective Equipment. *Infect. Control Hosp*. *Epidemiol*. **2010,** *31* (5), 560–561.

Gundy, P.M.; Gerba, C.P.; Pepper, I.L. Survival of Coronaviruses in Water and Wastewater. *Food Environ. Virol*. **2009,** *1*, 10–14.

Kampf, G. Potential Role of Inanimate Surfaces for the Spread of Coronaviruses and their Inactivation with Disinfectant Agents. *Infect. Prev. Pract*. **2020,** *2*(2), 100044.

Kim, S.H.; Chang, S.Y.; Sung, M.; Park, J.H.; Kim, H.B.; Lee H.; Choi, J.P.; Choi, W.S.; Min, J.Y. Extensive Viable Middle East Respiratory Syndrome (MERS) Coronavirus Contamination in Air and Surrounding Environment in MERS Isolation Wards. *Clin. Infect. Dis.* **2016**, *63* (3), 363–369.

Kramer, A.; Schwebke, I.; Kampf, G. How Long Do Nosocomial Pathogens Persist on Inanimate Surfaces? A Systematic Review. *BMC Infect. Dis.* **2006,** *6,* 1–8.

Marquès, M.; Domingo, J.L. Contamination of Inert Surfaces by SARS-CoV-2: Persistence, Stability and Infectivity. A Review. *Environ. Res.* **2021,** *193,* 110559.

Organization for Economic Co-operation and Development (OECD). Quantitative method for evaluating virucidal activity of biocides on hard surfaces, Section C, 2013, www.oecd.org. (accessed February 15, 2020).

Otter, J.A.; Donskey, C.; Yezil, S.; Douthwaite, S.; Goldenberg, S.D.; Weber, D.J. Transmission of SARS and MERS Coronaviruses and Influenza Virus in Healthcare Settings: The Possible Role of Dry Surface Contamination. *J. Hosp. Infect.* **2016,** *92* (3), 235–250.

Warnes, S.L.; Little, Z.R.; Keevil, C.W. Human Coronavirus 229E Remains Infectious on Common Touch Surface Materials. *mBio.* **2015,** *6* (6), e01697–15.

Weber, D.J., Rutala, W.A., Fischer, W.A., Kanamori, H., Sickbert-Bennett, E.E. Emerging Infectious Diseases: Focus on Infection Control Issues for Novel Coronaviruses (Severe Acute Respiratory Syndrome-CoV and Middle East Respiratory Syndrome-CoV), Hemorrhagic Fever Viruses (Lassa and Ebola), and Highly Pathogenic Avian Influenza Viruses, A (H5N1) and A (H7N9). *Am. J. Infect. Control.* **2016,** *44*, e91–100.

Ye, Y.; Chang, P.H.; Hartert, J.; Wigginton, K.R. Reactivity of Enveloped Virus Genome, Proteins, and Lipids with Free Chlorine and UV254. *Environ. Sci. Technol.* **2018,** *52*, 7698–7708.

ACRONYMS AND ABBREVIATIONS

BSL	biosafety level
DEVCOM CBC	U.S. Army Combat Capabilities Development Command Chemical Biological Center
DTRA	Defense Threat Reduction Agency
CoV	coronavirus
COVID-19	coronavirus disease-2019
EMEM	Eagle's minimum essential medium
EPA	Environmental Protection Agency
ESS	Electrostatic Spraying Systems
FBS	fetal bovine serum
HuCoV	human coronavirus
MERS	Middle East respiratory syndrome
MHV	murine hepatitis virus
OECD	Organization for Economic Co-operation and Development
P&G	Procter & Gamble Company
RH	relative humidity
RNA	ribonucleic acid
SARS	severe acute respiratory syndrome
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SS	synthetic sputum
TGEV	transmissible gastroenteritis virus
TSA	tryptic soy agar
TSB	tryptic soy broth

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