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Quantitative Efficacy of Common Virucidal Disinfectants against Viral Surrogates on Porous and Nonporous Surfaces

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The objectives of this effort were threefold: (a) compare Ebola virus (EBOV) with a potential surrogate, the enveloped vaccinia virus (VACV), for susceptibility to disinfectants; (b) generate efficacy data for five U.S. Environmental Protection Agency-registered virucidal chemicals and one experimental disinfectant, 5% vinegar, against VACV; and (c) compare the efficacy of three chemicals against Phi 6 (a bacteriophage surrogate for EBOV) and VACV. The Organisation for Economic Co-operation and Development (OECD) method was adopted for efficacy evaluations. Of the three test viruses, VACV was found be the most persistent virus. Of six disinfectants, Peridox disinfectant (Contec; Spartanburg, SC) was most effective against all three viruses. Bioxy-S sanitizer (Atomes, Inc.; Quebec, Canada) was effective against all three viruses. The other four disinfectants were not very effective against VACV. Peridox disinfectant, 0.2% peracetic acid (PAA), and 0.5% bleach were effective against Phi 6 in the absence of blood. In the presence of blood (dried and wet), both Peridox disinfectant and PAA were equally effective against Phi 6. VACV is recommended as a potential surrogate for EBOV. More importantly, this study highlights that the DoD must generate its own database on efficacy of disinfectants for military surfaces to select effective chemicals in the event of a pandemic resulting from an infectious virus.

15. SUBJECT TERMS

Phi 6 surrogate	Disinfection	Vaccinia virus (VACV)
Ebola virus (EBOV)	Virucidal	Feline calicivirus
Organisation for Economic Co-operation and Development (OECD)	Viral persistence	Bioxy-S sanitizer

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PREFACE

The work described in this report was authorized under project no. CB10467 (HDTRA 1723635). The work was started in August 2015 and completed in December 2019. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD) was known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

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EXECUTIVE SUMMARY

This study on possible viral surrogates and disinfectant efficacy was executed by the U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD) BioDefense Branch. Participants in the study included Dr. Lalena Wallace, who was matrixed from Defense Threat Reduction Agency (DTRA; Aberdeen Proving Ground, MD); Ms. Savannah Hurst (U.S. Environmental Protection Agency; Fort Meade, MD); retired Lt. Col. Laura Burton (Oak Ridge Institute for Science and Education; Riverside, MD); and Dr. Glenn Lawson and Dr. Charles Bass (Hazard Mitigation Division, DTRA). The study was completed in three phases, beginning in August 2015, and was completed in December 2019. The objectives of this effort were threefold:

a. compare Ebola virus (EBOV) with a potential surrogate, the enveloped vaccinia virus (VACV), in terms of persistence and susceptibility to several disinfectants.
b. generate quantitative efficacy data for five U.S. Environmental Protection Agency (EPA)-registered virucidal chemicals (List L) and one experimental disinfectant, 5% vinegar, against VACV on nonporous surfaces in the presence or absence of blood and against a nonenveloped viral surrogate, feline calicivirus (FCV), on porous and nonporous surfaces.
c. compare the efficacy of three commercial off-the-shelf virucidal chemicals against Phi 6 (a bacteriophage recently proposed as an EBOV surrogate) and VACV in the absence and presence of blood.

The Organisation for Economic Co-operation and Development (OECD) test method was adopted for persistence and efficacy evaluations. Of the three test viruses, VACV was found to be the most persistent virus under three holding temperatures: 4, 22, and 37 °C. FCV was found to be persistent over 5 to 6 days, and EBOV was found to persist for less than 24 h. Of six EPA-listed disinfectants, Peridox ready-to-use (RTU) disinfectant (Contec; Spartanburg, SC) was found to be the most effective against all three animal viruses. Although vinegar (Heinz; Pittsburgh, PA) was effective against VACV and EBOV, it was found to be only partially effective against FCV. PureGreen 24 disinfectant (Pure Green LLC; Centre Island, NY) was found to be effective against FCV. The other disinfectants (Lysol cleaner [Reckitt Benckiser; Parsippany, NJ], Micro-Chem detergent disinfectant [National Chemical Laboratories; Philadelphia, PA], Zep Aviation RTU disinfectant [Zep, Inc.; Emerson, GA], and Spic and Span cleaner [Proctor & Gamble; Cincinnati, OH]) were found to be poorly effective against VACV. Micro-Chem detergent disinfectant and Spic and Span cleaner were both moderately effective against FCV. Bioxy-S sanitizer (Atomes, Inc.; Quebec, Canada) was found to be effective against all of the animal viruses.

Peridox disinfectant, 0.2% peracetic acid (PAA), and 0.5% bleach were found to be effective against the Phi 6 bacteriophage in the absence of blood. In the presence of blood (dried and wet), Peridox disinfectant and PAA were equally effective against Phi 6. However, a slight drop in bleach efficacy was observed against Phi 6 in the presence of blood. Peridox disinfectant was equally effective against VACV in the absence or presence of wet and dried blood. A slight drop in the efficacy of 0.5% bleach was observed against VACV in the presence

of dried blood. PAA efficacy was moderately affected by wet blood and severely affected in the presence of dried blood.

Taken together, the ease of production, quantification, and robust persistence of VACV lead us to recommend its use as a potential surrogate for EBOV and other enveloped viruses. We recognize that there is no relationship between the two viruses with respect to structure, shape, and genome apart from both being enveloped viruses. More importantly, this study also highlights that the DoD must generate its own database on the efficacy of EPA-listed disinfectants for military-relevant surfaces. Such a database would enable the selection of effective virucidal chemicals in the event of an endemic or a pandemic resulting from an infectious virus.

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QUANTITATIVE EFFICACY OF COMMON VIRUCIDAL DISINFECTANTS AGAINST VIRAL SURROGATES ON POROUS AND NONPOROUS SURFACES

1. INTRODUCTION

Emerging infectious diseases (EIDs) represent an ongoing threat to the health and livelihood of people everywhere, including Americans. Over the last few decades, EIDs including human immunodeficiency virus (HIV), severe acute respiratory syndrome (SARS), H1N1, and Ebola virus (EBOV), have taken the global community by surprise and drawn new attention to EIDs. EBOV was first detected in the Democratic Republic of the Congo in 1976 (Bausch et al., 2007; Julian et al., 2011; Cook et al., 2015; Fischer et al., 2015). In the absence of an effective drug and vaccine for the dreadful and deadly outbreak caused by Ebola virus disease (EVD; formerly known as Ebola hemorrhagic fever or EHF), there is growing concern for the public health burden it imposes on sub-Saharan Africa. EVD is infectious, resulting in fever and internal bleeding, and it remains highly contagious through infected bodily fluids, such as sweat, blood, secretions, saliva, tears, breast milk, stool, nasal discharge, and semen. The survival rate varies from 35 to 50%.

On 23 March 2014, the World Health Organization (WHO) issued its first communiqué on a new outbreak of EVD, which began in December of 2013 in the Republic of Guinea. By June 2016, West Africa was announced to be Ebola free, and in two and half years, EVD had claimed more than 11,325 lives out of 28,600 confirmed cases. EVD cases were reported in several locations: Liberia, Mali, and Sierra Leone. In support of the U.S. Agency for International Development, the DoD has committed 4,000 men and women in uniform to Monrovia, Liberia, as part of Operation United Assistance.

EVD is caused by a negative-sense, single-strand, ribonucleic acid (ssRNA)-enveloped virus belonging to the genus *Ebolavirus* in the family of Filoviridae. Filovirus particles are 80 nm in diameter and form twisted filaments of up to 1.1 μm in length. Marburg virus belongs to the same family and causes a similar disease to EVD. Because of the case fatalities, the members of Filoviridae have been classified as Category A potential bioterrorism agents by the Centers for Disease Control and Prevention. The discovery of the EBOV sequence in fruit bats near the locations of human outbreaks implied that EVD is a zoonosis, transmitted from a reservoir in bats.

The objectives of this effort funded over a three-year period were threefold:

a. compare EBOV with a potential surrogate, the enveloped vaccinia virus (VACV), in terms of persistence and susceptibility to several disinfectants;
b. generate quantitative efficacy data for five U.S. Environmental Protection Agency (EPA)-registered virucidal chemicals and one experimental disinfectant against VACV on nonporous surfaces in the presence or absence of blood and against a nonenveloped viral surrogate, feline calicivirus (FCV), on porous and nonporous surfaces;

c. compare the efficacy of three commercial off-the-shelf (COTS) virucidal chemicals against Phi 6 (a bacteriophage recently proposed as an EBOV surrogate) and VACV in the absence and presence of blood.

2. STUDY DESIGN

Viral genomes are packaged in a protein coat called a capsid. For some viruses, the capsid is surrounded by a lipid bilayer containing viral proteins. The combined lipid and protein structure is called the virus envelope. Human pathogenic enveloped viruses include those with RNA (e.g., EBOV) and those with DNA as genetic material (e.g., herpesviruses and poxviruses). In general, enveloped viruses are more labile than nonenveloped viruses. EBOV is a biosafety level 4 (BSL-4) pathogen, and only a handful of laboratories can work with the infectious agent. This highlights the need for a suitable surrogate.

In a recent study (Gallandat and Lantagne, 2017), the biosafety level 1 (BSL-1) bacteriophage Phi 6 was proposed as a surrogate for EBOV. Phi 6 belongs to the virus family Cystoviridae and infects *Pseudomonas syringae*. It is an enveloped, double-stranded RNA (dsRNA) bacteriophage with a 13 kilobase pair (kbp) genome. It was proposed as a surrogate because it has a low biosafety level classification and it performs similarly to EBOV against chlorine-based disinfectants. One caveat of the comparison study done by Gallandat and Lantagne is the relatively low challenge level (3–4 log) used within the study. Throughout our study, a 5–6 log minimum challenge level was used.

This report summarizes an extensive series of experiments completed in three phases, as listed in Table 1. VACV was selected as a suitable candidate for an EBOV surrogate based on three criteria: (a) virus type (it is an animal virus), (b) extended persistence, and (c) comparable sensitivity to virucidal chemicals. VACV is a large, complex, enveloped virus that belongs to the family Poxviridae. The envelopes are derived from the host cell membrane and include viral proteins. VACV contains a 192 kbp, double-stranded DNA genome. The viral particles are large, 250–300 nm, and shaped like bricks. VACV is a biosafety level 2 (BSL-2) virus and is reported to be persistent on environmental surfaces (Wood et al., 2013). Finally, VACV is also known to be resistant to drying. The number of infectious viral particles can be determined by performing plaque assays. The second test virus selected for this study was FCV. This is a nonenveloped, positive-sense, ssRNA virus, encapsulated by viral VP1 protein. FCV is listed as a BSL-2 virus and has been recommended as a surrogate for norovirus. This virus exhibits cytopathic effects (CPE). FCV is cultivated in the Crandell–Rees feline kidney (CRFK) cell line. Prior to this study, not much was known about the persistence and disinfectant sensitivity of this virus.

The surface materials selected for phase 1 were steel; transport isolation system (TIS), which is an enclosure material developed in 2014 by the DoD to safely transport patients with highly contagious diseases aboard a C-17 aircraft; anti-skid material; and nylon webbing. In phase 1, five disinfectants were evaluated: Calla 1452 cleaner (Zip-Chem Products; Morgan Hill, CA), hydrogen peroxide (H_2O_2), vinegar, Bioxy-S sanitizer (Atomes, Inc.; Quebec, Canada), and bleach. In phase 2, six disinfectants (Peridox disinfectant [Contec; Spartanburg, SC], Micro-Chem detergent disinfectant [National Chemical Laboratories; Philadelphia, PA], Spic and Span

cleaner [Proctor & Gamble; Cincinnati, OH], Zep Aviation RTU disinfectant [Zep, Inc.; Emerson, GA], Lysol cleaner [Reckitt Benckiser; Parsippany, NJ], and vinegar) and six surfaces (fabric [1005 cotton, heavyweight, woven, utility fabric], chemical agent resistant coating [CARC]-painted steel, nylon webbing, TIS, steel, and anti-skid material) were included. In phase 3, VACV and Phi 6 were compared for their sensitivity to bleach, Peridox disinfectant, and peracetic acid (PAA) on only a steel surface. The effect of blood (wet and dry) on the efficacy of these three disinfectants was evaluated.

Table 1. Work Plan in Three Phases

Study Phase (Work Year)	Virus	Surface	Disinfectant	Test Condition	Work Performed
Phase 1 (2015)	VACV	Steel, TIS, anti-skid, nylon webbing	Calla 1452, H_2O_2, Bioxy-S, bleach, vinegar	Efficacy and persistence with fetal bovine serum (FBS)	DEVCOM-CBC BSL-2
	Ebola	Steel, TIS	Calla 1452, Bioxy-S, Vinegar		U.S. Army Medical Research Institute of Infectious Disease (USAMRIID) BSL-4
	FCV	Steel	None	Persistence with FBS	DEVCOM-CBC BSL-2
Phase 2 (2017 and 2018)	VACV	Steel	Peridox,* Micro-Chem,* Spic and Span,* Zep AV,* Lysol,* vinegar	Efficacy with and without dry blood	DEVCOM-CBC BSL-2
	FCV	Steel	Peridox,* Micro-Chem,* Spic and Span,* Zep AV,* PureGreen24,* vinegar	Efficacy without blood	DEVCOM-CBC BSL-2
	FCV	Fabric, CARC-painted steel, nylon, anti-skid	Peridox,* PureGreen24*		
Phase 3 (2019)	VACV and Phi 6	Steel	Peridox,* bleach, PAA	Efficacy with and without wet or dry blood	DEVCOM-CBC BSL-2

*From EPA List L: Registered antimicrobial products that meet the Centers for Disease Control and Prevention criteria for use against the Ebola Virus.
DEVCOM CBC; U.S. Army Combat Capabilities Development Command Chemical Biological Center.

2.1 Persistence of Viral Surrogates

Persistence of both VACV and FCV was evaluated on steel and TIS at three holding temperatures: 4, 22, and 37 °C. The Organisation for Economic Co-operation and Development (OECD) quantitative method (EPA-approved) was adopted for this study. A few persistence studies (Sagripanti et al., 2010; Wood et al., 2013) suggest long-term surface persistence of VACV and alphaviruses.

2.2 Disinfection Studies

A few disinfection studies (e.g., Lombardi et al., 2008; Smither et al., 2016) suggest that many virucidal chemicals are effective against RNA viruses. Five EPA-registered disinfectants were selected for this program: Peridox RTU disinfectant (registration no. 88089-4), Spic and Span cleaner (registration no. 6836-245-3575), Micro-Chem plus detergent disinfectant (registration no. 1839-95-2296), Zep Aviation RTU cleaner disinfectant (registration no. 6836-152-1270), and Lysol Brand Clean & Fresh multi-surface cleaner (registration no. 777-89). For FCV, PureGreen 24 disinfectant (registration no. 72977-3-84364) was used instead of Lysol cleaner because of the cytotoxic effects of Lysol cleaner on the CRFK cell line. All of these disinfectants were selected from EPA's List L and are indicated for use in hospital and healthcare facilities and institutional and residential sites. In addition, household vinegar (used previously at the U.S. Army Edgewood Chemical Biological Center [ECBC]; Aberdeen Proving Ground, MD, now DEVCOM CBC) was selected as an experimental virucidal disinfectant.

The BSL-2 work with VACV and FCV and the BSL-1 work with Phi 6 were conducted at ECBC. The objectives of this three-year effort were threefold:

a. to evaluate persistence of enveloped viral surrogate VACV over 10–12 weeks and determine the effect of temperature on environmental persistence;
b. to generate quantitative data on and evaluate efficacy of five EPA-registered virucidal chemicals against the enveloped viral surrogate, VACV, and the nonenveloped viral surrogate, FCV, and one experimental disinfectant on porous and nonporous surfaces in the presence and absence of serum protein or blood; and
c. to compare the efficacy of three COTS virucidal chemicals against Phi 6 and VACV in the absence and presence of blood.

3. MATERIALS AND METHODS

For this study, the BSL-1 and -2 work was conducted in three phases (as summarized in Table 1). Briefly, in phase 1, VACV and EBOV were compared for their persistence and sensitivity to five disinfectants (3% hydrogen peroxide, general-purpose Calla 1452 cleaner [Zep Chemical; Atlanta, GA], distilled white vinegar, Bioxy-S sanitizer [Atomes, Inc.; Quebec, Canada], and 5.66–6% laboratory-grade sodium hypochlorite [Thermo Fisher Scientific; Waltham, MA]). In phase 1, VACV was tested on four surfaces, and EBOV was tested on steel and TIS. In phase 2, VACV (enveloped) and FCV (nonenveloped) were compared. Six surfaces were included in this phase of testing: CARC-painted steel, fabric, steel, TIS, anti-skid material, and nylon webbing. Six EPA-registered virucidal chemicals were included in phase 2: Peridox RTU disinfectant, Spic and Span cleaner, Micro-Chem plus detergent disinfectant, Zep Aviation RTU disinfectant, and Lysol Brand Clean & Fresh multi-surface cleaner. PureGreen 24 disinfectant was used instead of Lysol cleaner for FCV because Lysol cleaner has cytotoxic effects on the CRFK cell line. In phase 3, with two viruses, Phi 6 (a bacteriophage of *P. syringae*) and VACV were evaluated on only one test surface, namely, steel.

Three disinfectants were selected for this phase: Peridox disinfectant, 0.5% bleach, and 0.2 and 0.5% PAA. Small coupons (1 cm circles) of the selected surfaces were used as carriers in all three phases. All of the listed disinfectant technologies are recommended for use as in hospital and healthcare facilities and institutional and residential sites (Table 1).

3.1 Vero Cell Maintenance

The African green monkey kidney cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin–streptomycin antibiotics and 10% FBS. The cells were grown in the presence of 5% CO_2 at 37 °C. The cells were maintained in T-150 flasks and split as needed after reaching nearly 95% confluence.

3.2 VACV Cultivation and Infection

A portion of virus suspension was removed from a –80 ºC freezer and added to a T-150 flask at 70% confluence. The flasks were incubated at 37 ºC with 5% CO_2 and were periodically observed for CPE. Once CPE were observed, the supernate was harvested by scraping the cells and pooling the supernate and lysed cells in a 50 mL sterile tube. The suspension was centrifuged at 2500 rpm for 5 min to pellet the cells. The supernate containing the viruses was then pipetted into 2 mL sterile tubes for long-term storage.

3.3 Coupon Inoculation and OECD Efficacy Testing

The 1 cm coupons were sterilized in glass Petri plates. The sterile coupons were inoculated with 10 µL of aliquot suspension. The titer was ~10^9/mL virus infectious particles containing 10% human whole blood for VACV or 10% FBS for FCV as the organic burden. The suspension was left to dry in a BSL-2 cabinet under sterile airflow for 45–60 min. The dried coupons were used for disinfection testing within 60 min after drying. The inoculated coupons were transferred to sterile plastic vials with the inoculated side facing up. An aliquot of control (DMEM containing 2% FBS) or test chemical was carefully added on top of the dried viral inoculum, ensuring nearly full coverage of the inoculum by the test solution. After 10 min, an aliquot of 10 mL of neutralizer (DMEM containing 2% FBS) was added and the solution was vigorously vortexed for 60 s. This vial was labeled as –1 (undiluted). Appropriate 10-fold dilutions were made using the same media. For controls, –3, –4, and –5 dilutions were used to infect the monolayers of Vero cells. For tests, –1 and –2 dilutions were used to infect the Vero cells.

3.4 Plaque Assays

A 12-well plate was seeded with 1 mL of a suspension of 4×10^5 Vero cells in DMEM containing 10% FBS. After incubation at 37 ºC with 5% CO_2, the media was carefully aspirated and replaced with 0.25 mL of viral dilutions or controls. For each dilution, three replicate wells were used. During this step of viral adsorption, the plates were rocked every 15 min for 60 min. After viral adsorption, the supernate was aspirated and replaced with 1 mL of DMEM containing 10% FBS to allow cell growth. The plates were incubated for 48 h, after which the plaques (cleared zones) were counted (Figure 1). Total viral titer was estimated by multiplying the plaques by a volume factor (4) and a dilution factor. Log reduction was estimated by subtracting the log viral titer per sample (test) from the control sample per sample.

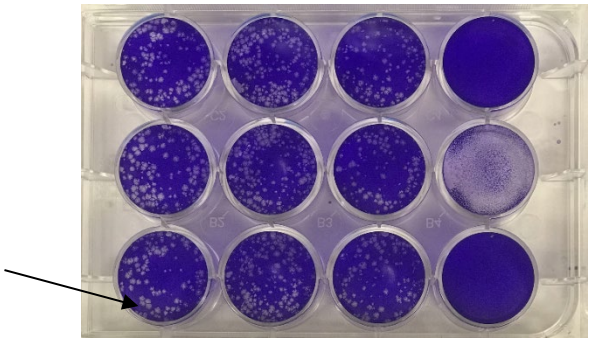

Figure 1. Plaque formation in Vero cell line (arrow shows the plaque location).

3.5 FCV Cultivation and Infection

The CRFK (American Type Culture Collection [ATCC] no. CCL-94; Manassas, VA) cell line was cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS. The cells in a T-150 flask attained confluence within 2 to 3 days (Figure 2). The cells were grown at 37 ºC in the presence of 5% CO_2. Virus infection occurred in the presence of 2% FBS protein. The CPE manifest even if one viral particle is infective (Figure 3), and the CPE were monitored for 5–7 days. Presence or absence of CPE was recorded and used in calculations to estimate the log reduction based on minimum probable number (MPN). The MPN per carrier was calculated using the MPN calculator spreadsheet (Briones and Reichardt, 1999). The log density per carrier was calculated from the $\log_{10}$ of the number of plaque-forming units (PFU) per carrier.

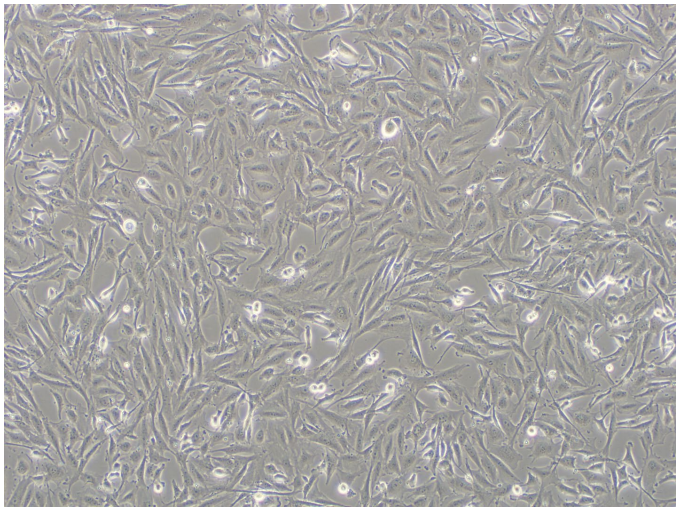

Figure 2. Healthy CRFK monolayer cells at 95% confluence.

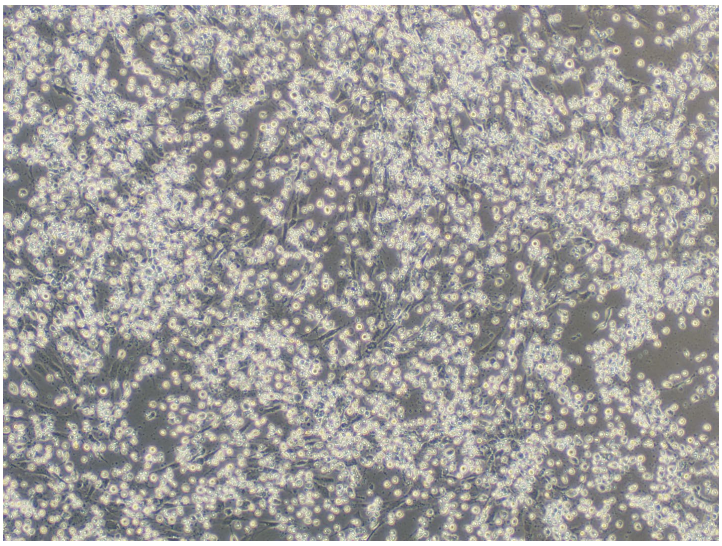

Figure 3. FCV in CRFK cell line (CPE).

3.6 Phi 6 Cultivation and Decontamination Assay

Sterile Luria–Bertani (LB) agar plates (20 g of LB broth powder and 15 g of agar per liter of water), LB broth (20 g of LB broth powder per liter of water), and soft LB agar (20 g of LB broth powder and 6 g of agar per liter of water) were prepared. *P. syringae* culture was streaked on the LB agar plates, and the plates were incubated at 25 °C for 2–3 days. One colony of *P. syringae* was inoculated from the LB agar plate into 10 mL of LB broth and was grown overnight at 25 °C with 175 rpm shaking. Molten soft agar (4.75 mL) was aliquoted in a 10 mL sterile plastic tube and equilibrated to 50 °C. Phi 6 phage 10-fold dilutions were prepared in a 0.1 mL final volume of LB broth, ranging from –1 through –9. Two hundred milliliters of overnight-grown *P. syringae* broth culture was added into soft agar equilibrated to 50 °C, and to this, 50 µL of diluted phage was added (control or test sample). The suspension was mixed rapidly by inversion (avoiding air bubbles) and gently poured over the entire top surface of the LB agar plates over (using swirling to ensure even coverage). The soft agar was allowed to solidify over the plates, then the plates were incubated overnight at 25 °C. The number of plaques (cleared zone) were counted. The numbers of total plaques were calculated to determine the phage count per coupon by multiplying the number of plaques × 2 (volume factor, 50 out of 100 µL) × 1/dilution × 10 (for 1 mL volume) × 10 (for total extraction volume).

For disinfection assays, each coupon was inoculated with 10 µL of Phi 6 phage and dried for 60 min in open air inside a BSL-2 cabinet. Once dried, the inoculated coupons were placed inside a sterile 20 mL vial. Then 50 µL of test disinfectant (test samples) or LB broth (control sample) was added over the entire inoculated surface. After 10 min of contact time, 9.95 mL of LB broth was added. The vial was vortexed to dislodge the phage from the coupon surface. The appropriate dilutions were prepared: –3 through –5 for control samples, and –3 through –1 (undiluted) for test samples. The total number of phages was calculated in both control and test samples. Log reduction was estimated by subtracting average log (test sample) from average log (control sample).

3.7 Coupons for Persistence and Disinfection

All coupons were sterilized via autoclave within sample vials before use on an assay. Virus was added (10 µL per coupon; approximately 3.4×10^6 PFU) and dried within a biosafety cabinet at ambient temperature (19–22 °C) until visibly dry, approximately 60 min. The inoculated coupons were placed in a glass or plastic vial, and 10 or 20 mL of LB (Phi 6) or DMEM/EMEM (animal viruses) was added to the vial. (Volume added was determined prior to experimentation based on cytotoxicity results.) The coupons were vortexed for 60 s before dilution and viral adsorption. The dilutions were made in media containing 2% FBS protein. In some instances, viral media was also replaced 1 h post infection to combat cytotoxicity. The viral laboratory work setup is detailed below.

3.8 Cytotoxicity Test for CRFK

Due to the nature of the MPN assay, in which cytopathic effects are observed over a period of time, chemicals had to be evaluated before use to ensure that they were not cytotoxic to the host cell line. Cytotoxicity was evaluated by adding the neutralized disinfectant (50 µL of disinfectant and 10 or 20 mL of EMEM) directly to the cells in the absence of virus and observing the cells as normal. If no CPE were present, the chemical was determined not to be cytotoxic. If CPE were observed, lower concentrations were evaluated in the same fashion to see whether they could be acceptable for use. Lysol cleaner was the only selected chemical that could not be tested against FCV due to cytotoxicity to the CRFK cell line. It was replaced with PureGreen 24 disinfectant.

3.9 EBOV Cultivation and Infection

All EBOV work was performed by Dr. Jay Goff and Dr. Sara Ruiz (Virology Division, United States Army Medical Research Institute for Infectious Diseases; Fort Detrick, MD) in a BSL-4 laboratory. The Ebola–Zaire virus was expanded by inoculating T-150 flasks seeded with Vero E6 cells. The flasks were incubated at 37 °C and checked daily for CPE. Virus was harvested when flasks reached 90–100% CPE. Virus was frozen into single-use stocks at an approximate titer of 1.7×10^7 PFU/mL. For each assay, the appropriate number of vials were thawed and combined prior to usage.

3.10 Coupons for EBOV

Steel and TIS were provided by ECBC and sterilized via autoclave within sample vials prior to use on an assay. Virus was added at 20 µL per coupon (approximately 3.4×10^5 PFU) and dried within a biosafety cabinet at ambient temperature (19.5 °C) until visibly dry, approximately 2 h.

3.11 EBOV Cultivation and Infection

Once the virus was visibly dry on the coupon (T0), the sample vials were stored loosely capped at 22.7 °C and 53% humidity, with a 12 h light/dark cycle, until the designated time point. Viral recovery was attempted at 0, 2, 18, and 24 h and 7 and 14 days. At that time, 10 mL of media (1× DMEM) with 10% FBS) was added to the sample vial and vortexed for 60 s. The recovered viral suspension was then serially diluted 10-fold and plated on a Vero E6 cell monolayer that was at 95–98% confluence. The remaining volume was then plated on T-150 flasks with a Vero E6 cell monolayer and was monitored up to seven days for cytopathic effect.

Dried virus on both coupon materials had 50 µL of either vinegar, 1% Bioxy-S sanitizer, or Calla 1452 cleaner added directly over the dried viral inoculum. Following a contact time of 30 min at ambient temperature, 9.95 mL of cell media was added to the sample vial and vortexed for 60 s. The recovered viral suspension was then serially diluted 10-fold and plated on a Vero E6 cell monolayer that was at 95–98% confluence. The neutralized disinfectant (50 µL of either vinegar, Bioxy-S sanitizer, or Calla 1452 cleaner with 9.95 mL of cell media) was added to separate wells to ensure there were no cytotoxic effects.

3.12 Neutral Red Plaque Assay

Inoculated plates were rocked every 15 min and incubated at 37 °C with 5% CO_2 for 1 h. Following this, 0.5% agarose was added to each well, and the plates were incubated at 37 °C with 5% CO_2 for 7 days. A neutral red agarose overlay (4%) was added to each well, and the plates were incubated overnight. The plaques were then enumerated and recorded.

4. RESULTS

4.1 Persistence of VACV on Test Surfaces

The effect of ambient temperature on VACV persistence was investigated. In addition to room temperature, low (4 °C) and high (37 °C) temperatures were selected for VACV persistence on steel. The results are summarized in Figure 4 and show high recovery (~6.5 log) from steel. VACV appears to be very persistent on steel and TIS (results on TIS not shown). At 37 °C, VACV appears to lose its infectivity; however, at low and room temperature, the virus appears to be persistent for at least three months and possibly longer.

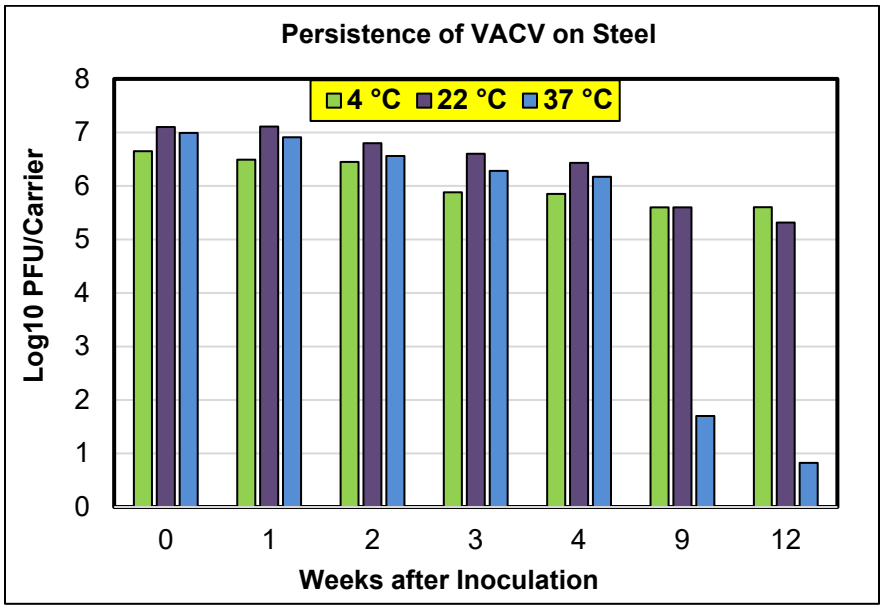

Figure 4. Effect of temperature on VACV persistence on steel.

4.2 Persistence of FCV on Test Surfaces

The persistence of FCV on a steel surface was investigated over one week, at ambient (22 °C), low (4 °C), and high (37 °C) temperatures. The results summarized in Figure 5 show that FCV lost its infectivity rapidly over a seven day period, as no virus was detectable after six days. Although the effects of low and high temperature were marginal, detectable levels (1.5–2 log) of FCV were observed under ambient temperature at day six, and barely detectable levels were observed at the other temperatures.

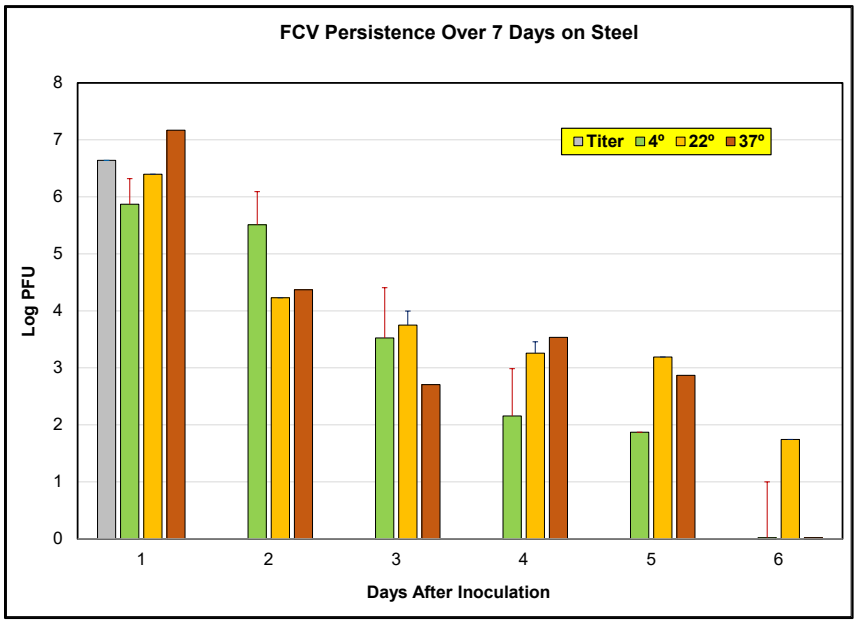

Figure 5. Effect of temperature on FCV persistence on steel.

4.3 Persistence of EBOV on Steel and TIS

This set of experiments was performed at USAMRIID by Drs. Jay Goff and Sara Ruiz. EBOV is not persistent, and within 24 h, less than detectable levels of virus were observed. Results summarized in Figure 6 show a rapid decline of infectivity of EBOV within 24 h after drying. EBOV appeared to persist a bit longer on steel than on TIS.

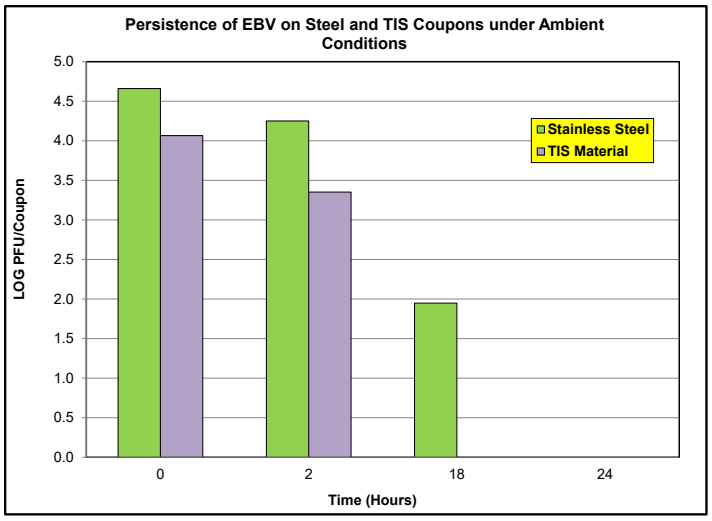

Figure 6. Persistence of EBOV on steel and TIS surfaces.

4.4 Recovery of Phi 6

Bacteriophage Phi 6 infects bacterial cells of *P. syringae*. The phage was recovered from a steel surface (after 60–75 min of drying), and the results are summarized in Figure 7. The results show a 7 log recovery of phage in the absence of blood. In the presence of wet and dried blood, a slightly higher (0.5–1 log) level of Phi 6 was recovered, indicating some protective effect of blood on infectivity of this bacteriophage. It is unclear why blood would have any protective effect on Phi 6, given this a bacteriophage.

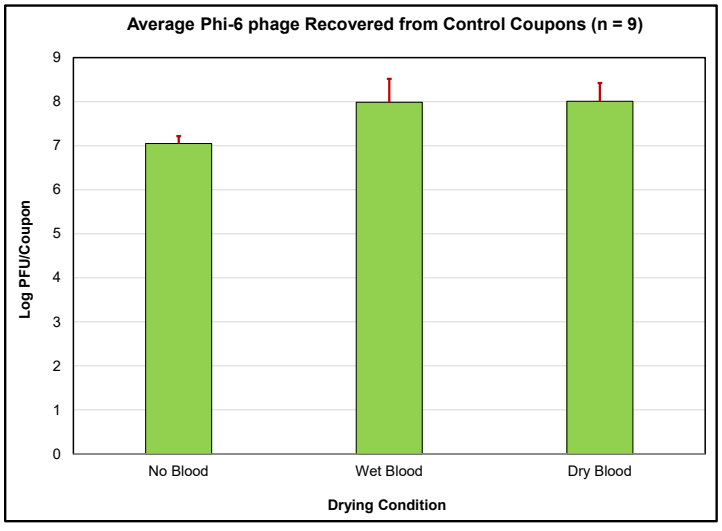

Figure 7. Recovery of Phi 6 phage from steel.

4.5 Efficacy of Disinfectants on EBOV

In phase 1, three out of four chemicals were investigated for their potential as effective virucidal approaches against EBOV. The contact time for all chemicals was 30 min. The results summarized in Figure 8 show that Calla 1452 cleaner, vinegar, and Bioxy-S sanitizer were all fully effective in inactivating EBOV. This result disagrees with results against VACV, where Calla 1452 cleaner was not found to be fully effective in 10 min. A 30 min contact time was not tested against VACV.

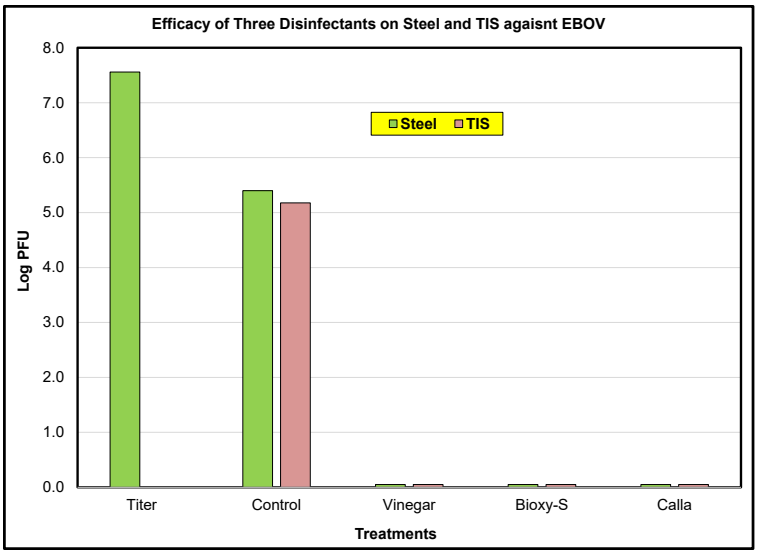

Figure 8. Efficacy of three disinfectants against EBOV on steel and TIS surfaces.

4.6 Efficacy of Disinfectants on FCV

The same six chemicals were evaluated against the nonenveloped FCV. The results summarized in Figure 9 show varying degrees of efficacy, which can be separated into three distinct classes: (a) highly effective (5–6 log reduction), including the Peridox and PureGreen 24 disinfectants; (b) partially effective (3.5–4.5 log reduction), including the Spic and Span cleaner and the Micro-Chem plus detergent disinfectant; and (c) poorly effective (1–2 log reduction), including the Zep Aviation RTU cleaner and the vinegar. It is interesting that the two surrogates selected for enveloped and nonenveloped viral pathogens exhibited different sensitivities to the virucidal chemicals. The two chemicals that were effective on the steel surface, the Peridox and PureGreen 24 disinfectants, were challenged against FCV on the other four surfaces (fabric, nylon, anti-skid material, and CARC-painted steel). The results summarized in Figure 10 show the Peridox disinfectant's effectiveness on all four surfaces. PureGreen 24 disinfectant was only partially effective on the four test surfaces. Efficacy ranged between a 2 and 4 log reduction.

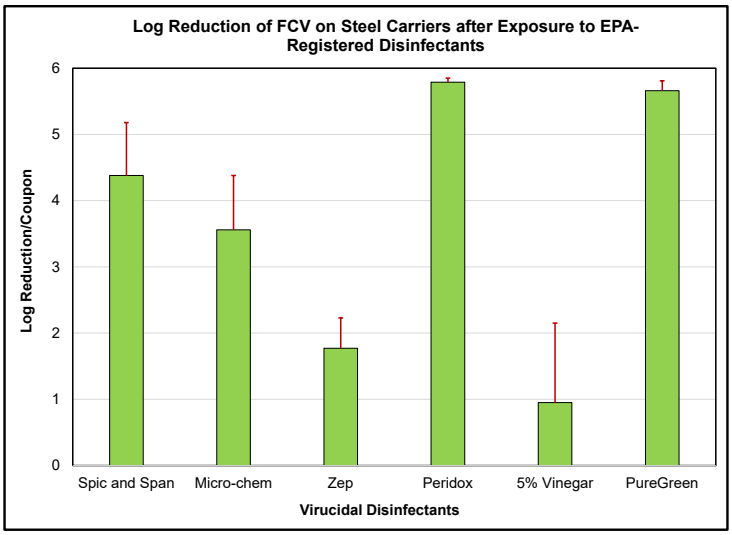

Figure 9. Efficacy of six virucidal chemicals against FCV on steel.

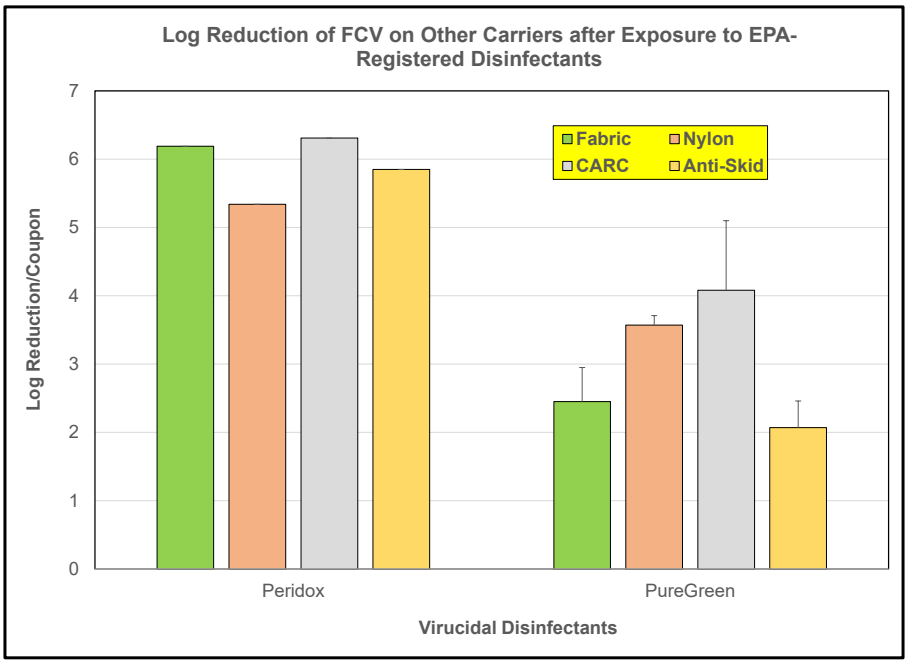

Figure 10. Efficacy of Peridox and PureGreen 24 disinfectants against FCV on four surfaces.

The overall efficacy results are summarized in Table 2. Highly effective virucidal chemicals are shaded in green, partially effective chemicals are shaded in yellow, and poorly effective chemicals are shaded in pink. In addition to the six EPA-registered chemicals, vinegar and an experimental technology, Bioxy-S powder, were evaluated. Bioxy-S sanitizer generates hydrogen peroxide and PAA at neutral pH. A 0.5% solution was tested and was found to be effective against all three viruses (VACV, EBOV, and FCV). It is noteworthy that a number of these chemicals are not effective against either enveloped or nonenveloped viruses. Lastly, based on the efficacy data, VACV may be a more conservative surrogate choice, given that three disinfectants (Calla 1452 cleaner, vinegar, and Bioxy-S sanitizer) were equally effective against

EBOV, but only vinegar and Bioxy-S sanitizer were effective against VACV. Calla 1452 cleaner was less effective against the surrogate VACV.

4.7 Comparative Sensitivity of VACV and Phi 6

A few reports have suggested use of Phi 6 as a possible surrogate for EBOV. Phi 6 is a bacteriophage for *P. syringae* and is enveloped, but it lacks any other similarity to EBOV. A limited set of tests was performed, and the effects of wet and dried blood was also investigated in this series of experiments. Three chemicals (bleach, Peridox disinfectant, and PAA at 0.2 and 0.5% concentrations) were evaluated. The disinfection results summarized in Figure 11 show that, in the absence of blood, all three chemicals were highly effective against Phi 6. The log reduction value was approximately 6.5 log. The presence of wet blood did not affect the efficacy of any of the three chemicals (Figure 12). The efficacy of Peridox disinfectant and bleach was not affected by the presence of dried blood, but the log reduction values for 0.2% PAA were slightly reduced, by 1 log value. It is confounding that bacteriophage sensitivity would be affected by dried blood.

Table 2. Summary of Virucidal Chemical Efficacy against VACV, EBOV, and FCV

Virucidal Chemical	Contact Time (min)	Efficacy (Log Reduction)		
		VACV	FCV	EBOV
Peridox RTU disinfectant	10	>5–6	>5	Not done
Lysol Brand Clean & Fresh multi-surface cleaner	10	1–2	Not done	Not done
Micro-Chem plus detergent	10	1–2	3–4	Not done
Zep Aviation RTU disinfectant	10	1–2	1–2	Not done
Spic and Span All Purpose Spray-RTU	10	1–2	3–4	Not done
PureGreen 24 disinfectant	10	Not done	>5	Not done
White vinegar 5%	10/10/30	>5–6	1–2	4–5
Bioxy-S sanitizer	10/10/30	>5–6	>5–6	4–5
Calla 1452 cleaner	10/30	3–4	Not done	4–5

Note: Green shading indicates highly effective virucidal chemicals, yellow shading indicates partially effective chemicals, and pink shading indicates poorly effective chemicals.

The efficacy of the same disinfectants against VACV in the absence and presence of wet and dried blood was investigated. The results are summarized in Figure 13. Figure 13 shows that, although efficacy of Peridox disinfectant and bleach was not affected by the presence of wet blood, the efficacy of PAA was reduced to less than 50% by the presence of wet blood. In the presence of dried blood, the efficacy of Peridox disinfectant was not affected, but the efficacy of bleach was inhibited marginally (~1 log), and the efficacy of PAA was inhibited more than 50% (3.5–4 log). The results with VACV partly mimicked those of EBOV.

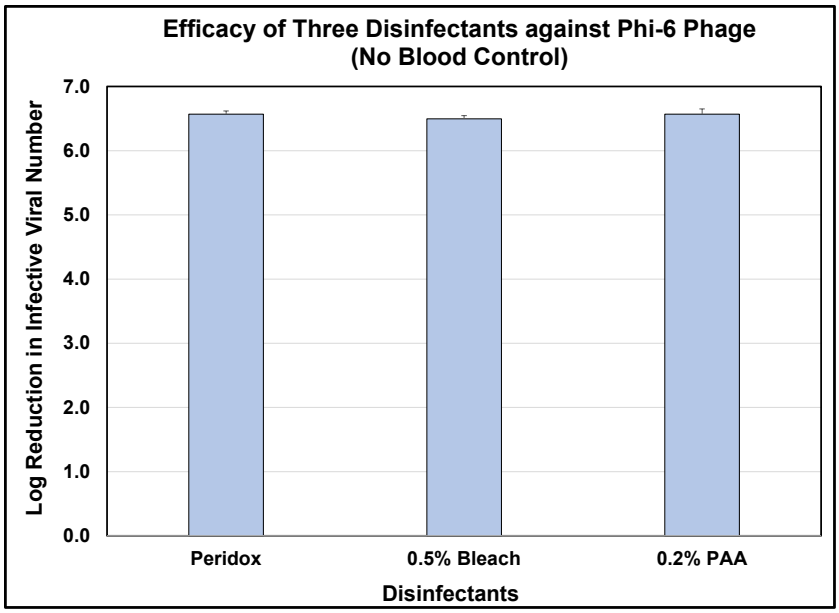

Figure 11. Efficacy of three disinfectants against Phi 6 in the absence of blood.

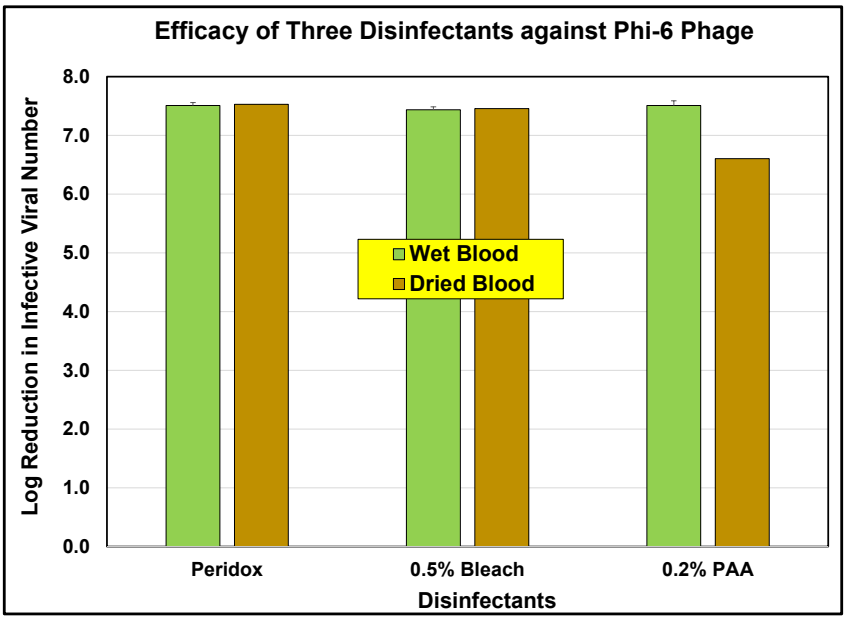

Figure 12. Effects of wet and dried blood on efficacy of three disinfectants against Phi 6.

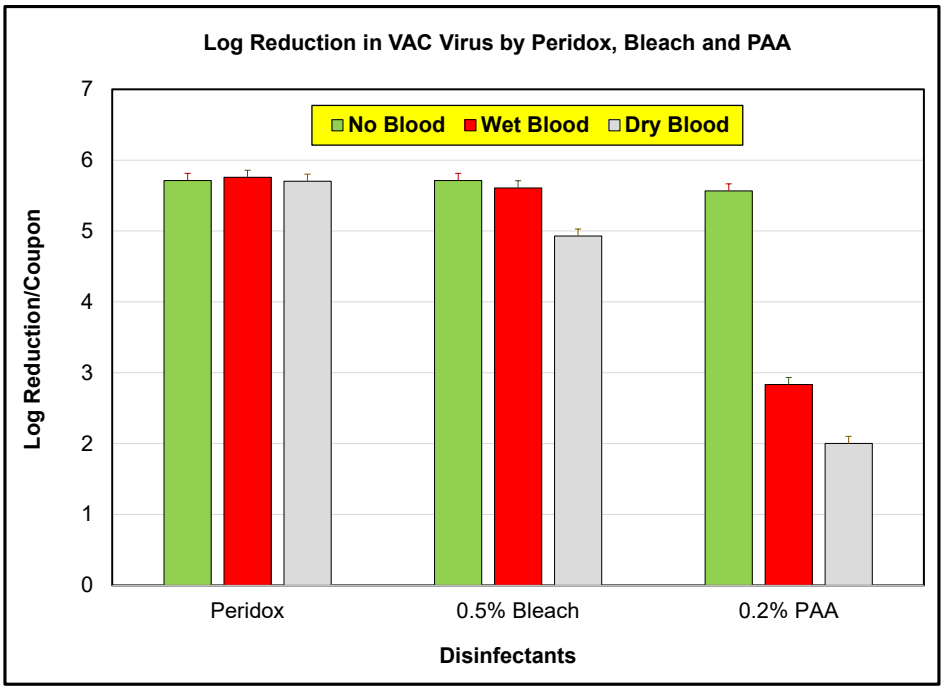

Figure 13. Effects of wet and dried blood on efficacy of three disinfectants against VACV.

5. DISCUSSION AND CONCLUSIONS

This study was completed in three phases. In phase 1, the use of VACV (in the BSL-2) as a suitable surrogate for the EBOV was evaluated. Comparative efficacy data against the two viruses on four surfaces was generated. Although the propensity for environmental persistence of EBOV has yet to be fully elucidated, this critical information is needed to make informed decisions on disinfection policies in the event of an outbreak. Recently, Nikiforuk and coworkers (2017) reported that EBOV persisted for 72 h or less at 80% relative humidity (RH). This deviance in EBOV persistence could be due to high RH; relatively low RH (30–40%) was used in our study. As for VACV, previous studies (Wood et al., 2013) and the present study clearly confirm its infectivity and persistence over a three to four week period. In the present study, infective EBOV was not detected after 24 h of drying on either of the two environmental surfaces (steel and TIS). Importantly, live EBOV decayed quickly (between 2 and 18 h) on both stainless steel and TIS when tested at ambient temperature on a 12 h light/dark cycle. In previously published reports, EBOV persistence was observed on glass carriers for 5.9 days when the carriers were protected from light exposure at 20–25 °C and 30–40% RH (Sagripanti et al., 2010). Although a direct comparison between the two studies cannot be made due to experimental differences in the viral recovery method and the environmental conditions, it is intriguing to note the potential role of light on inactivation of the virus, particularly in a field setting. This is further corroborated by a study showing that both dried EBOV and EBOV suspended in liquid were fully inactivated upon exposure to UV light (Sagripanti et al., 2011). For the current study, EBOV was not supplemented with an organic load, which is known to increase the robustness of the virus (Cook et al., 2015; Piercy et al., 2010). Therefore, future testing should include differing biological matrices (i.e., blood or saliva). Other environmental factors, such as humidity (high RH), temperature, and light, can also be included to better predict

infectivity of residual virus following drying on a broad range of DoD-relevant surfaces. In the event of an outbreak or intentional release, such a body of information on multi-factorial environmental persistence would be critical to mounting an appropriate level of disinfection treatment response.

Although the EPA's List L contains several virucidal products for use against EBOV, no disinfectant chemistries with a specific label claim for EBOV are listed by the WHO. The WHO recommends the use of 0.5% available chlorine to disinfect contaminated surfaces (Cook et al., 2015). Vinegar, Bioxy-S sanitizer, and Calla 1452 cleaner could completely inactivate EBOV on stainless steel and TIS with a 30 min contact time. Commercially available white distilled vinegar typically contains 3.5–8% acetic acid and has been shown to be an effective disinfectant for enveloped viruses, including influenza, but not for nonenveloped viruses (Lombardi et al., 2008). In addition, 3% acetic acid was previously shown to inactivate EBOV when added to blood samples containing the virus (Mitchell and McCormick, 1984). Bioxy-S powder generates PAA at a neutral pH when resuspended in water. It has been shown to neutralize Gram-positive and -negative bacteria, but there are no reports on its efficacy against viruses. Calla 1452 cleaner is a pH-neutral disinfectant designed to be used on hard, nonporous surfaces for inactivation of numerous Gram-positive and -negative bacteria and viruses. Bioxy-S sanitizer, vinegar, and Calla 1452 cleaner are attractive disinfectant compounds for use during an outbreak because of their stability and cost-effectiveness. Bioxy-S powder has obvious transport advantages for large-area disinfection. Other disinfectant compounds are being tested to prove a spectrum of potential disinfectant chemistries, to be used for future outbreaks or potential exposure to our armed forces. It should be stressed that EBOV was included only in phase 1, and the rest of the experiments were focused on the nonenveloped VACV and FCV.

Interestingly, of the six chemicals assessed in phase 2, only Peridox disinfectant was found to be highly effective against both VACV (enveloped) and FCV (nonenveloped). Vinegar was also effective against VACV, and PureGreen 24 disinfectant was effective against FCV. All of the other chemicals (Lysol cleaner, Micro-Chem detergent disinfectant, Zep Aviation RTU disinfectant, Spic and Span cleaner), were all either partially effective or noneffective against the two test viruses. These virucidal disinfectants were selected from the approved EPA List L. Based on these results, we strongly recommend that the DoD must have its own list that is based on efficacy studies for enveloped and nonenveloped viruses. Such a list would be essential for responding to high exposure risks to Warfighters or an outbreak (endemic or pandemic). Such a database would immensely help in the selection of appropriate disinfectants that are suited to different scenarios and surfaces.

Finally, although no phylogenetic or taxonomic relationship exists between EBOV and VACV, extended persistence and comparable disinfection efficacy are consistent with the conclusion that VACV is an appropriate surrogate for EBOV. Persistence of EBOV was reported for up to seven days under laboratory conditions (21 °C and 40% RH) and up to four days in the presence of dried blood under West African conditions (27 °C and 80% RH) (Fischer et al., 2015). Although liquid or wet blood appears to extend EBOV persistence for up to 14 days, dried blood did not appear to extend viral persistence. One disinfectant, Calla 1452 cleaner, was fully effective against EBOV, but only partially effective against VACV; this suggests that VACV is a more conservative surrogate choice.

Future VACV and FCV studies are strongly recommended in the three following areas:

a. persistence of the two viruses in the presence of matrices, such as blood and saliva;
b. persistence of the two viruses on additional porous and nonporous surfaces, under varying environmental conditions (RH and temperature);
c. kill kinetics with the same and additional disinfectants in the presence of matrices such as blood or saliva to determine the minimal time required for complete inactivation of the virus with a backdrop of body fluids.

The Phi 6 bacteriophage was included in the third phase because a few investigators have suggested this BSL-1 phage as a surrogate for enveloped infectious viruses, including Ebola, SARS, and other RNA viruses. Phi 6 is a spherical virion of ~85 nm in diameter. It possesses a dsRNA within a lipid-containing envelope. The bacteriophage is not as persistent as VACV or EBOV, and it appears to survive better at low and high RH than at moderate RH (Prussin et al., 2018). EBOV survives better under hospital conditions (7 days) than under African climatic conditions (4 days), and in liquid blood, the virus persisted for beyond 8 days (Fischer et al., 2015). Sensitivity of Phi 6 to 0.5% sodium hypochlorite was not comparable to that of EBOV; EBOV was undetectable after a 5 min treatment, whereas Phi 6 was detectable for up to 10 min.

In closing, it should be reiterated that the objective of this study was to assess applicability of an enveloped virus (VACV) and a nonenveloped virus (FCV) as viral surrogates in response to EPA-registered virucidal disinfectants. Although EPA-registered disinfectants are backed by data on smooth, hard surfaces such as steel or glass, effectiveness and efficacy of the same disinfectants are extrapolated to other surface types with no experimental data. The intent of this study was to fill the data gap of select disinfectants on military-relevant surfaces. Furthermore, efficacy of the same disinfectants in the presence of biological matrices, such as blood, has not been documented. Other environmental factors such as humidity, temperature, and light could also be included to better predict infectivity of residual viral particles following drying on a broad range of DoD-relevant surfaces. In the event of an outbreak or intentional release, such a body of information on multi-factorial environmental persistence will be critical to identify whether disinfection of surfaces is needed. Furthermore, with the availability of a DoD database, an appropriate disinfection option, guided by data, could be selected without issue.

6. EPILOGUE

Since the preparation of this report, the world has faced a huge challenge, the COVID-19 pandemic. A novel coronavirus responsible for the ongoing pandemic, known as SARS-CoV-2, was first detected in Wuhan City, Hubei Province, China, in December 2019. In a little over six months (as of 20 July 2020), the number of confirmed cases has increased to 14.5 million with more than 606,000 deaths. The infection has spread from China to every continent. Within the United States, the number of SARS-CoV-2-positive cases has reached

3.83 million with more than 143,000 million deaths. Although SARS-CoV-2 is an enveloped virus, it appears that its persistence does not extend more than 3 to 4 days. Disinfection of contaminated facilities, surfaces, and personal protective equipment has become paramount for infection control. This report provides an important perspective on research studies related to viral persistence and disinfection efficacy.

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ACRONYMS AND ABBREVIATIONS

ATCC	American Type Culture Collection
BSL-1	biosafety level one
BSL-2	biosafety level two
BSL-4	biosafety level four
CARC	chemical agent resistant coating
COTS	commercial off-the-shelf
CPE	cytopathic effects
CRFK	Crandell–Rees feline kidney
DEVCOM CBC	U.S. Army Combat Capabilities Development Command Chemical Biological Center
DMEM	Dulbecco's modified Eagle's medium
dsRNA	double-stranded RNA
EBOV	Ebola virus
ECBC	U.S. Army Edgewood Chemical Biological Center
EHF	Ebola hemorrhagic fever
EID	emerging infectious disease
EMEM	Eagle's minimum essential medium
EPA	U.S. Environmental Protection Agency
EVD	Ebola virus disease
FBS	fetal bovine serum
FCV	feline calicivirus
HIV	human immunodeficiency virus
kbp	kilobase pair
LB	Luria–Bertani
MPN	minimum probable number
OECD	Organisation for Economic Co-operation and Development
PAA	peracetic acid
PFU	plaque-forming unit
RH	relative humidity
RNA	ribonucleic acid
rpm	rotations per minute
RTU	ready to use
ssRNA	single-stranded RNA
SARS	severe acute respiratory syndrome
TIS	transport isolation system
USAMRIID	U.S. Army Medical Research Institute of Infectious Diseases
VACV	vaccinia virus
WHO	World Health Organization

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