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Rapid Inactivation of SARS-like Coronaviruses

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ABSTRACT

Chemical disinfection and inactivation of viruses is largely understudied, but is very important especially in the case of highly infectious viruses. The purpose of this LDRD was to determine the efficacy of the Sandia National Laboratories developed decontamination formulations against Bovine Coronavirus (BCV) as a surrogate for the coronavirus that causes Severe Acute Respiratory Syndrome (SARS) in humans. The outbreak of SARS in late 2002 resulted from a highly infectious virus that was able to survive and remain infectious for extended periods. For this study, preliminary testing with *Escherichia coli* MS-2 (MS-2) and *Escherichia coli* T4 (T4) bacteriophages was conducted to develop virucidal methodology for verifying the inactivation after treatment with the test formulations following AOAC germicidal methodologies. After the determination of various experimental parameters (i.e. exposure, concentration) of the formulations, final testing was conducted on BCV. All experiments were conducted with various organic challenges (horse serum, bovine feces, compost) for results that more accurately represent field use condition. The MS-2 and T4 were slightly more resistant than BCV and required a 2 minute exposure while BCV was completely inactivated after a 1 minute exposure. These results were also consistent for the testing conducted in the presence of the various organic challenges indicating that the test formulations are highly effective for real world application.

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FIGURES

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INTRODUCTION

In general, virucidal activity and mechanism of action of various disinfectants is largely understudied. Standards for virucidal testing are being developed, however none are mandated at this time for verifying virus inactivation. It is largely accepted and recommended to follow Association of Official Analytical Chemists (AOAC) germicidal methods for virucidal efficacy testing (1). The major structural targets of disinfectants against viruses include the viral envelope (predominantly lipid), the capsid (predominantly protein), and the genome (nucleic acid material) (3, 8, 9). According to the Klein-DeForest Scheme, mechanism of action of various chemical disinfectants against viruses is largely due to the presence or absence of a viral envelope, with the latter typically being more resistant (3, 9). Viruses are typically categorized into groups A (lipophilic with envelope), B (hydrophilic, non-enveloped), or C (intermediate solubility, nonenveloped) viruses (3, 9, 10). The structural target on the virus is dependent on the type of disinfectant used. Because coronaviruses are an enveloped virion, they fall into the Klein-DeForest susceptibility group A. Group A is susceptible to compounds including halogens, aldehydes, Quaternary Ammonium Compounds (QAC), phenolics, alcohols, peroxide, proteases, and detergents (10). It is important to note that the susceptibility is referring to the ability of the disinfectant to disrupt surface properties typically important in attachment to cellular receptors, and does not always result in complete degradation and loss of replication functionality of the nucleic acid (10).

In this research, BCV was used as a surrogate for the virus that causes SARS to study inactivation. Since there are few published protocols for determining virucidal activity of disinfectants, it was important to develop methodology in which exposure duration could be controlled by neutralizing, or quenching, the antiviral effects. Due to the necessity of an *in vitro* cell culture for propagation of BCV, the subsequent removal of the toxicity due to the nature of the chemicals present in the test formulations was necessary to conduct the virucidal efficacy tests. Disinfectant activity is often reduced in the presence of organic materials due to cross reactivity with compounds in the organic load effectiveness (4). In order to be representative of environmental conditions, the test formulations were also challenged in the presence of organic material including horse serum (for MS-2 and T4) or bovine feces and compost (for BCV).

MATERIALS AND METHODS

Part I: Inactivation of Bacteriophage Viruses

MS-2 *E. coli and T4 E. coli* Bacteriophage Inactivation. Virus stock solutions were prepared according to the instructions from the American Type Culture Collection (ATCC) for both the MS-2 (ATCC 15597b) and T4 (ATCC 11303b) bacteriophages. This procedure involved adding 0.5 ml of the rehydrated phage into a centrifuge tube containing 5 ml of soft agar (Tryptic Soy Broth TSB, Difco with 0.5% agar) with a 24-hour level of appropriate host. Samples were incubated as slants to allow for plaque formation. After incubation period, tubes were centrifuged and supernatant was filtered through a 0.22 µm filter to remove the host and develop the virus stock solution.

For the efficacy testing, 2 ml of the virus stock suspension was added to a sterile tube. Then 2 ml of the test formulation was added and mixed thoroughly, diluting the test formulation in half. Samples were taken after 30 seconds, 1 minute, and 2 minutes by taking

1 ml of sample and adding to 9 ml of the previously validated neutralizer solution (data not shown).

A control sample was prepared by adding 2 ml of sterile deionized water to the virus stock solution in place of the test formulation. Serial dilutions were made using 0.1% peptone blanks. Samples were enumerated by adding 100 µL of exposed virus to a pre-poured Tryptic Soy Agar (TSA, Difco) plate (Figure 1). Then approximately 5 ml of soft agar containing the appropriate host (*E. coli* 15597 for *MS-2* and *E. coli* 11303 for *T4*) was added to sample and swirled to mix thoroughly. Plates were incubated at 37°C for 24-48 hours for the enumeration of plaque forming units (PFU). The clearings or PFU's are caused from the infection and ultimate lysing of the host organism due to the viable viral agent.

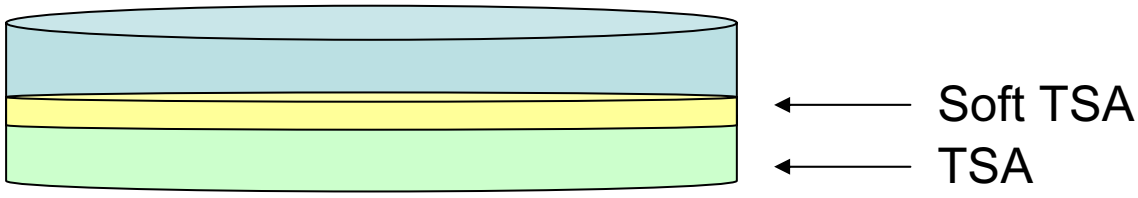

Figure 8: Schematic of viral overlay for enumeration of plaque forming units

Sandia National Laboratories decontamination formulations. The various test decontamination formulations include Sandia's DF-200D and the two licensee formulations: *Modec* MDF-200, and *Envirofoam Technologies, Inc.* EasyDecon-200. All of these formulations include QAC and various concentrations of peroxide. The formulations were mixed according to manufacturers' instructions prior to use. For proprietary reasons, the various components of the test formulations are noted as part A, B, and C. The final pH of the various formulations is approximately 9.8. Testing was also conducted with 5% horse serum as an organic challenge for testing with MS-2 and T4. For BCV inactivation, 10% bovine feces or 10% compost was assessed as an organic challenge in the efficacy testing.

DF-200D
7.29 g Part A + 0.2 g Part C + 2.5 g Part B
Modec *MDF-200*
15 ml Part A + 0.6 ml Part C + 14.4 ml Part B
***EFT* Easy Decon Solution**
16.5g Penetrator (Part A) + 0.6g Booster (Part C) + 16.5 g Fortifier (Part B)

Part II: BCV Inactivation

Virus and cells. BCV Wisconsin S1k (BCV) was propagated in Human Rectal Tumor 18 cells (HRT-18) with trypsin and pancreatin in the culture medium (5,6). Inoculum titer was assessed using plaque assay and hemagglutinin activity (HA). After samples were treated with the various decontamination formulations, samples were washed via ultracentrifugation. Pellets were reconstituted in 1 ml of MEM (Minimal Essential Media) containing 0.2% trypsin and 0.05% pancreatin. 50 µl of this sample was used to inoculate HRT 18 cells for the development of cytopathic effect (CPE) indicated by syncytia. After 24-48 hours of incubation at 37°C with 5% CO_2, samples were assessed for HA to determine the presence of BCV.

Virus Inactivation Protocol. BCV was added to sterile 25 ml ultracentrifuge tubes in 1 ml aliquots. Samples were diluted with 1 ml of MEM (for non-organic challenge) or 1 ml of 10% bovine feces or 10% compost wt/volume in 0.01M Phosphate Buffer Solution (PBS) (for organic challenge). Then, 2 ml of the test formulation was added to each tube using the following dilutions made with 0.01M PBS of the recommended concentration: 50%, 25%, or 12.5%. Samples were mixed thoroughly and exposed for either 1 or 3 minutes. After exposure, samples were neutralized using 4 ml neutralizer containing 50 µl 1% solution of Bovine Liver catalase to quench the antiviral activity and control exposure time. Tubes were then brought to volume with sterile 0.01M PBS. Samples were ultracentrifuged at 100,000 x G force for 1 hour at 4°C. After ultracentrifugation, supernatant was removed and pellet was reconstituted in 1 ml MEM containing 0.2% trypsin and 0.05% pancreatin.

HA for detection of BCV. Hemagglutinin assay (HA) testing for BCV was conducted by the microtiter method as described elsewhere (11). 25 µl of diluent (0.1% bovine serum albumin in PBS) was added to each well of a V-bottom, polystyrene microtiter plate 1 (Dynex Technologies, Chantilly, VA). Control and treated samples in 25 µl volumes were added to their respective wells. Fresh erythrocytes from BALB/c mice were diluted to 1% in bovine serum albumin PBS diluent, and 25 µl was added to each well. The plates were incubated for 90 minutes at 4°C. The presence of BCV antigen was indicated by the dispersal of erythrocytes in a lattice across the bottom of the well. The formation of a button at the tip of the V-bottom well indicates the absence of BCV. Hemagglutination titers were recorded as the reciprocals of the highest dilution showing complete agglutination.

Direct fluorescent-antibody test. To demonstrate the presence or absence of BCV, HRT 18 cells were infected for up to 48 hours with samples that had been treated with the various test formulations in 48 well microtiter plates as previously described (7). The monolayers were washed with PBS (pH 7.6) and fixed with 80% acetone at 4°C for 30 to 60 minutes. After the monolayers were air dried, 100 µl of 8F2 labeled with fluorescent marker, a monoclonal antibody (MAb) that reacts with the nucleoprotein of BCV, was added to each well. The plates were incubated for 30 minutes at 37°C with 5% CO_2 and 95% humidity. After incubation, extra stain is poured off and plates were washed 2-3 times with PBS (pH 7.6) prior to viewing with a fluorescent microscope.

Electron microscopy of samples. BCV was exposed to the various test formulations or to 0.01M PBS as a control. Samples were prepared for electron microscopy as previously described by ultracentrifugation at 100,000 x G force for 1 hour at 4°C to pellet the virus particles (6). The supernatant was carefully removed. The pellet was carefully scraped and reconstituted in 20 drops of deionized water in a 24 well microtiter plate with 4 drops of 4% phosphotungstic acid and 1 drop of 0.1% bovine serum albumin. The suspension was mixed carefully and nubulized onto prepared grids.

RESULTS

Part I: Inactivation of Bacteriophage Viruses

MS-2 *E. coli and T4 E. coli* Bacteriophage Inactivation

The efficacy testing conducted with DF200D, EFT-200, and MDF-200 without additional organic challenge (5% horse serum) resulted in complete inactivation of both MS-2 and T4 *E. coli* bacteriophages as shown in Figure 2 and 3. The efficacy of the various test formulations was slightly decreased with the addition of the 5% horse serum challenge (figures 4 and 5) for MS-2 and T4, respectively.

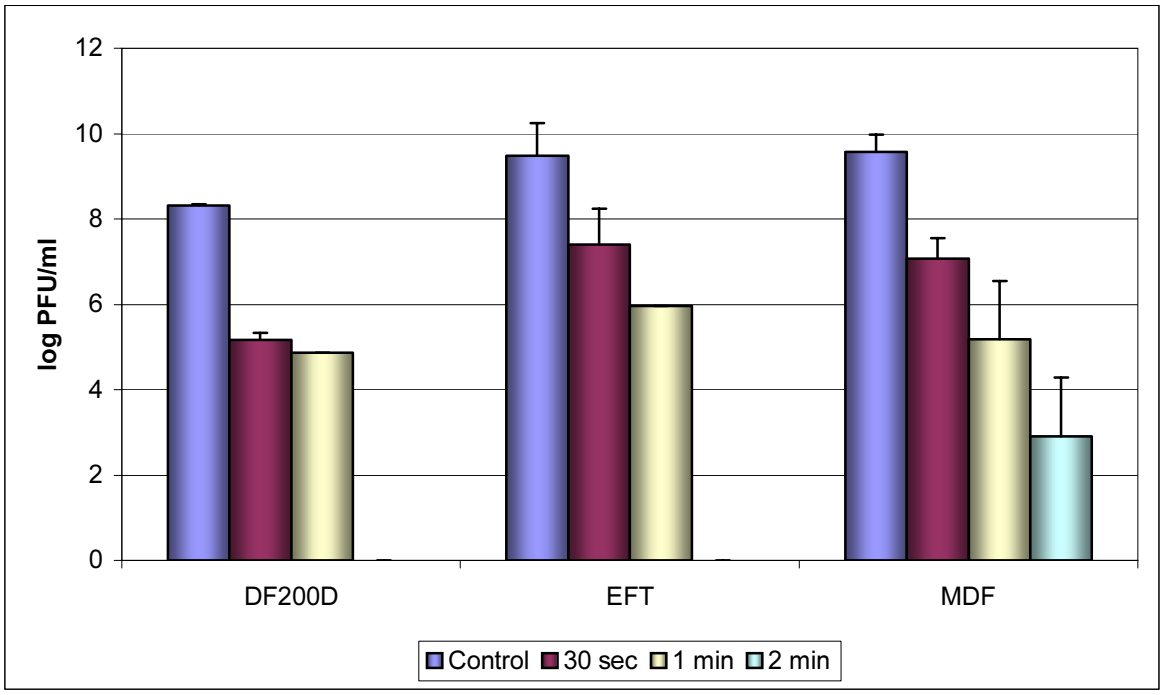

Figure 9: MS-2 recovered after various contact with test formulations without organic challenge.

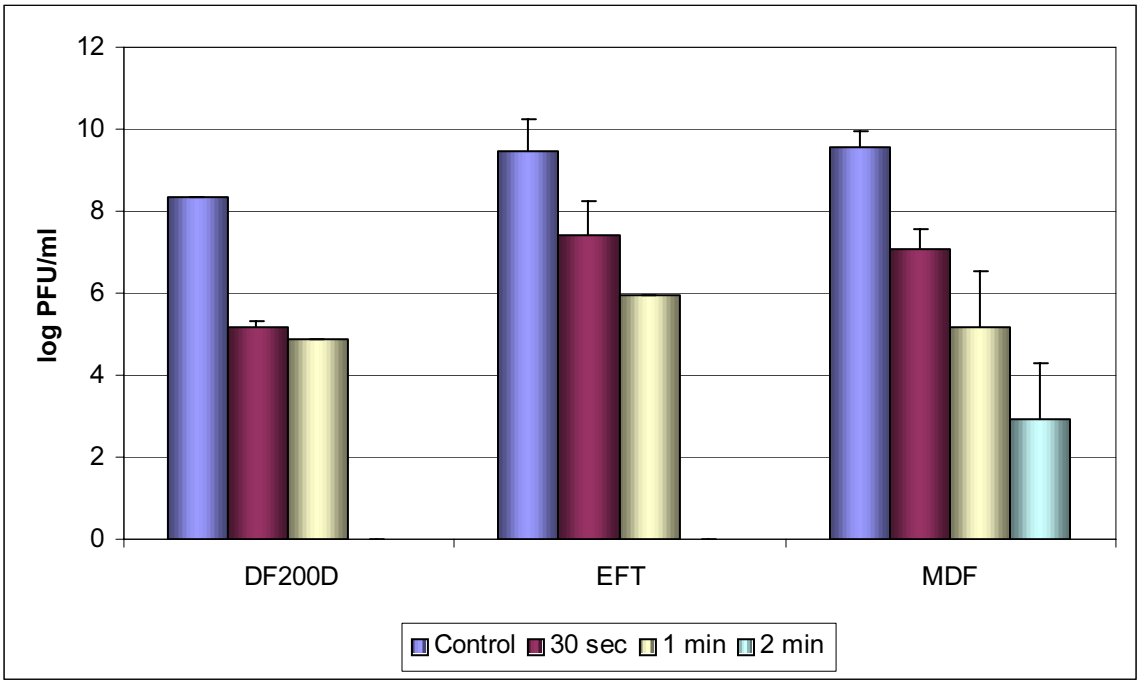

Figure 10: T4 recovered after various contact with test formulations without organic challenge.

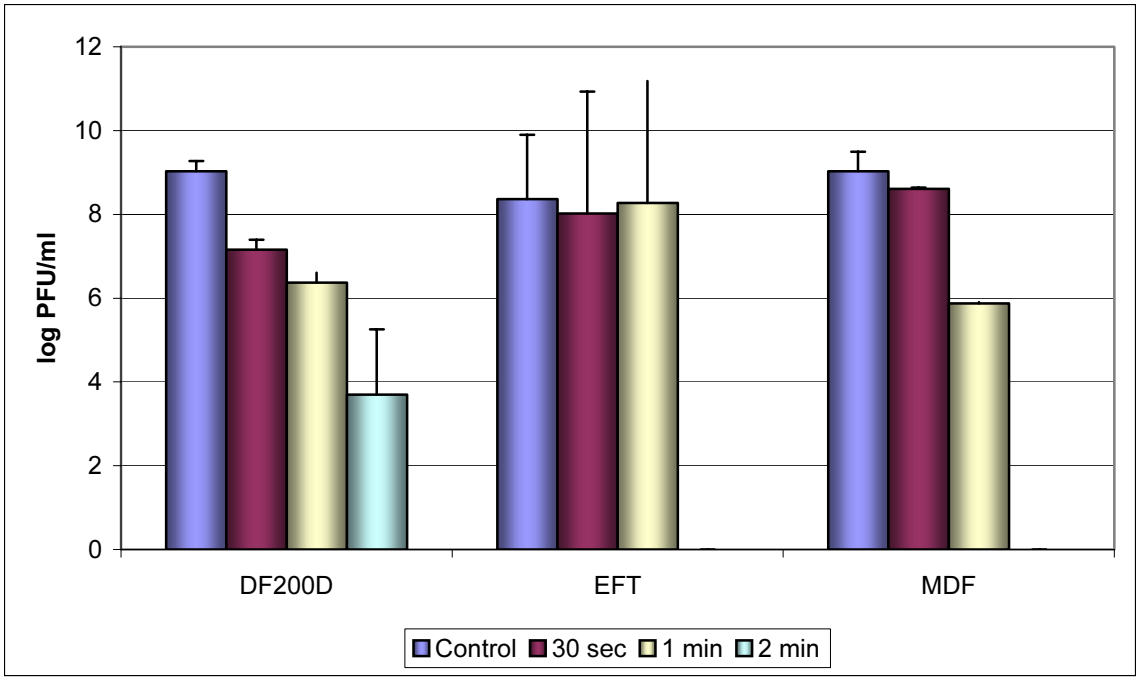

Figure 11: MS-2 recovered after various treatments with test formulations in the presence of 5% horse serum.

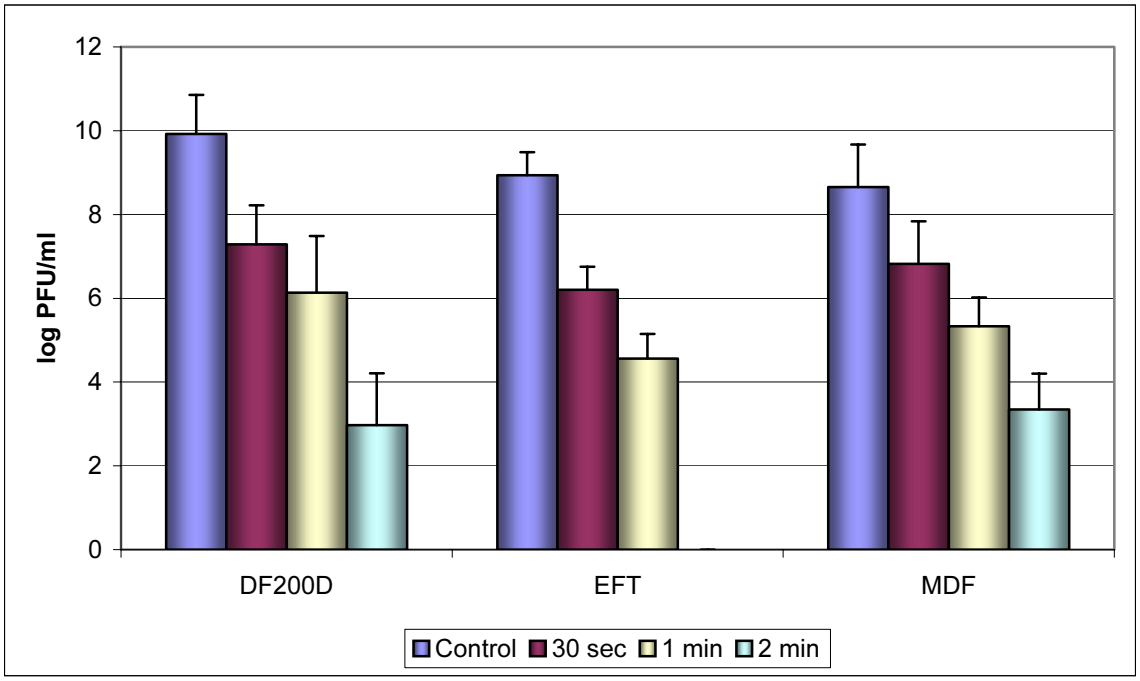

Figure 12: T4 recovered after various treatments with test formulations in the presence of 5% horse serum.

Part II: BCV Inactivation

HA for detection of BCV

Due to the nature of the chemicals present in the test formulations and the neutralizer, it was necessary to include washing steps of the samples prior to inoculating the HRT 18. Washing with PBS followed by ultracentrifugation to pellet the virus particles was sufficient to remove the toxicity for cell culture (data not shown).

BCV titers of 5-6 log levels as demonstrated by $TCID_{50}$ (neutralization titration in HRT 18 cells) and HA were completely inactivated after 1 and 3 minute exposure to all test formulations including the Sandia National Laboratories DF200D, Modec MDF-200, and Easy Decon-200 at 50%, 25%, and 12.5% the recommended concentration based on the absence of CPE in the HRT 18 cells and negative HA reaction after subsequent transfer and analysis. BCV was also completely inactivated in the presence of the 10% bovine feces and 10% compost organic challenges verified by lack of CPE in HRT 18 cells and negative HA results after exposure for 1 and 3 minutes to all test formulations at all test concentrations.

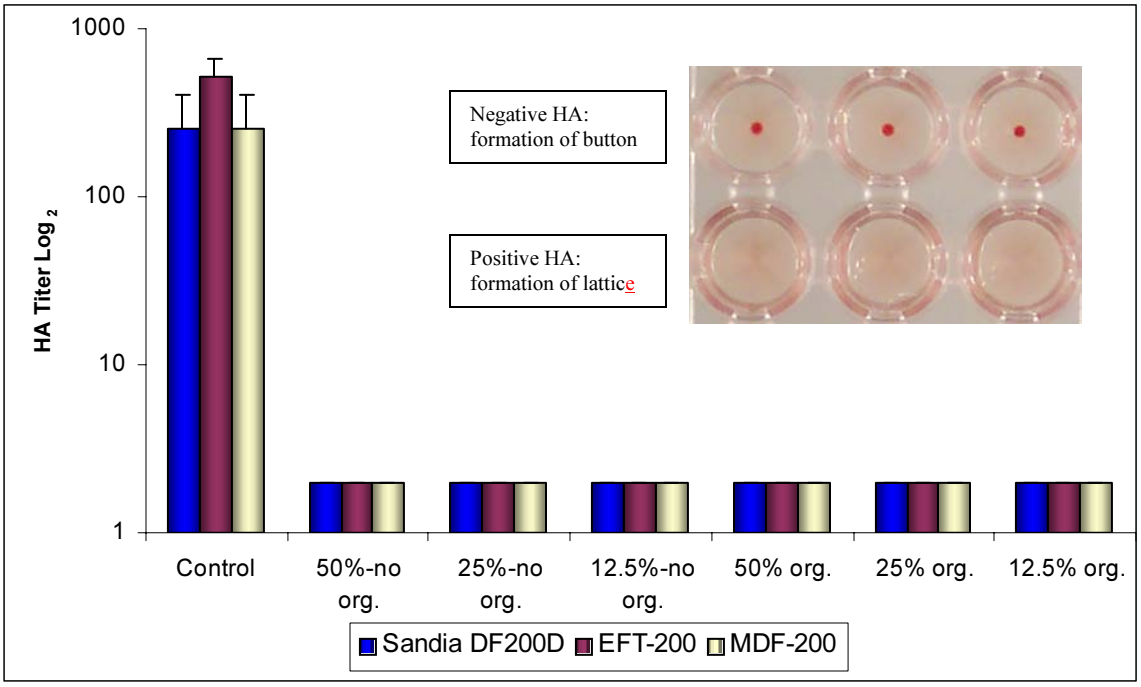

Figure 13: BCV recovered after various treatment with Sandia DF-200D, EFT-200, and MDF-200 with and without organic challenge (bovine feces or compost, 10%) by HA titration following infection in HRT 18 cells.

Direct fluorescent-antibody test

The results of the electron microscopy visually demonstrate that the non-treated isolate of BCV maintained visible structural characteristics including the envelope, spike glycoprotein, and shorter hemagglutinin protein. The treated samples were not visible by microscopy indicating the possibility that the test formulations were dissolving the viral envelope and potentially releasing the other structural proteins.

Direct fluorescent antibody (FA) was also used to demonstrate viral inactivation after various exposure times to the test formulations. FA verified that BCV treated with the

various test formulations at all test concentrations (50%, 25%, and 12.5%) for 1 and 3 minutes was completely inactivated. This was verified by examination with a fluorescent microscope on the microtiter plates containing HRT 18 cells infected with the various treated samples and the control samples. The HRT 18 cells infected with the control samples (treated with PBS in place of the test formulations) resulted in a positive FA reaction indicating that the BCV were intact and capable of infection. All of the treated samples, with and without organic challenge, resulted in a negative FA indicating that these samples were inactivated resulting in loss of infectivity.

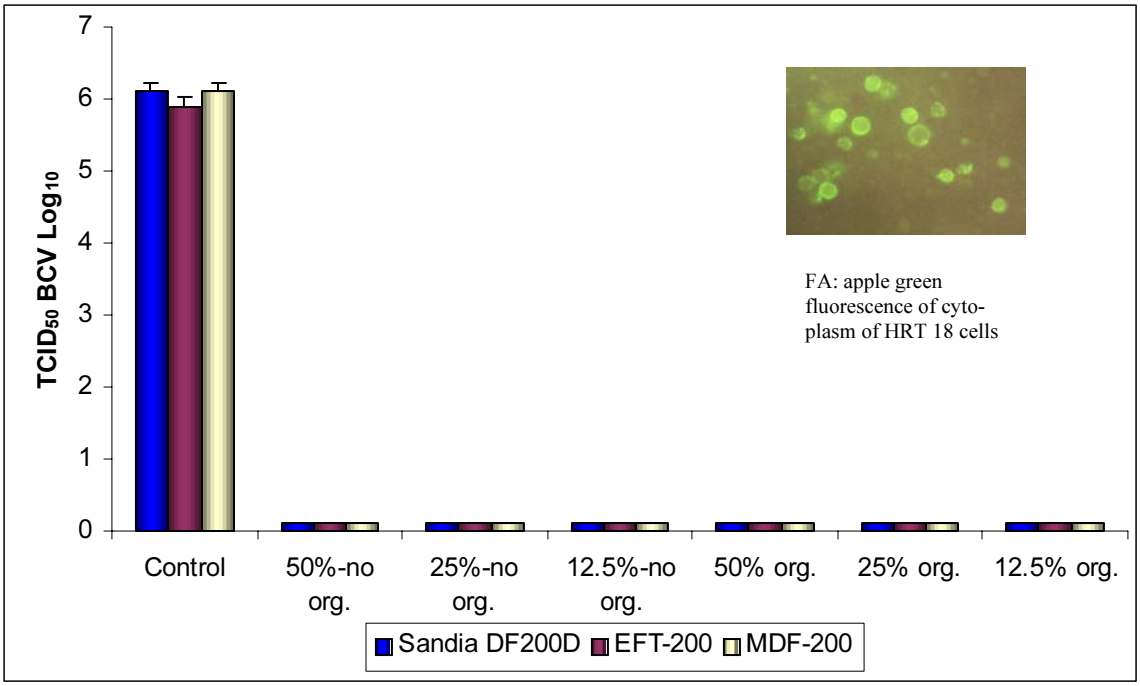

Figure 14: $TCID_{50}$ (calculated from FA) for untreated (control) and treated samples (various experimental parameters. Complete $TCID_{50}$ reduction was achieved for 1 and 3 minute exposure for all concentrations tested and in the presence of organic challenge.

Electron Microscopy of Samples

The results of the electron microscopy visually demonstrate that the non-treated isolate of BCV maintained visible structural characteristics including the envelope, spike glycoprotein, and shorter hemagglutinin protein. The treated samples were not visible by microscopy indicating the possibility that the test formulations were dissolving the viral envelope and potentially releasing the other structural proteins.

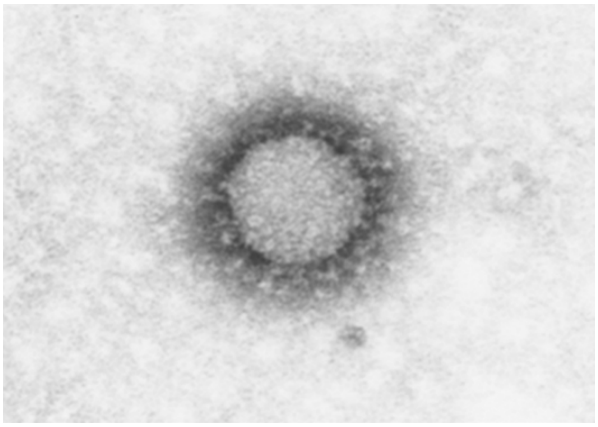

Figure 15: Electron Microscopy depicting intact, non-treated BCV. Treated samples

DISCUSSION

The purpose of these experiments was to determine the effectiveness of Sandia National Laboratories test decontamination formulations to inactivate SARS coronavirus by using a close relative, BCV that would react similarly. Physiochemical groups of viruses (ie. coronaviruses) are generally recognized to react similarly to each other with regard to sensitivity to chemical agents (9). The results indicate that all test formulations including Sandia DF-200D, *Modec* MDF-200, and *Envirofoam Technologies, Inc.* EasyDecon-200 were all highly effect in complete BCV inactivation in contact times as low as 1 minute even in the presence of various organic challenges. It is important to note that the term complete inactivation indicates the failure to recover the test virus, due to an inability to detect the last infective virus particle as defined by Chen, 1991 (3). The loss of infectivity of the treated viruses is a result of the disruption of important structures on the surface (namely the viral receptor cites) that physically prevent successful attachment and subsequent penetration into the host cell (9).

It was important to consider the control of various testing parameters in this research to determine the virucidal properties of the test formulations against coronaviruses. Because standard and official methods or protocols do not currently exist for testing disinfectants as virucides, factors including time of contact, organic load, tissue culture system, sterility, and temperature were all carefully controlled and monitored in this research (3, 9, 10). In general, methodology for AOAC Official Method 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants was followed and modified for specific testing using BCV (1). There are different host systems used to recover viruses including tissue culture, chick embryo, and murine models depending on the virus being studied. It is recommended to use the host system which results in the greatest level of infectivity for virucidal studies (3). With BCV, tissue culture using HRT 18 cells has been previously described for enhanced infectivity (5). Toxicity to the HRT 18 cells was resolved by a combination of dilution of the test formulation (50%, 25%, and 12.5% the recommended concentration), neutralization and washing/removal as recommended by Chen, 1991 (3).

Although the objectives in this research project did not include determining the mechanism of action of the various test formulation, it is likely that the lipid envelop of BCV is targeted and denatured by surfactant activity of the QAC and the oxidizer is acting on the nucleocapsid protein and possibly the genome. In general, viruses are deemed to be susceptible to various disinfectants based on certain structural components, largely the

presence or absence of a lipid envelope. Viruses have been classified into groups A, B, and C representing lipid enveloped, nonlipid non-enveloped, and nonlipid capsomeric lipophilicity, respectively (3, 9, 10). Enveloped viruses have been shown to be sensitive to lipophilic disinfectants, including cationic QAC and other broad spectrum disinfectants (3, 4, 9, 10). Enveloped viruses, including the coronaviruses, are considered to be lipophilic and are soluble in lipids which allow the penetration of lipophilic antimicrobials such as QAC to penetrate their viral envelope typically resulting in inactivation, which was the case in this research. Surprisingly, concentrations as low as 12.5% the recommended use for the test formulations with only a 1 minute contact time were effective in complete viral inactivation, even in the presence of the fecal and compost challenges. This is probably due to synergy of the peroxide oxidizer present in the test formulations, even if at greatly reduced concentrations. The hydroxyl radical ($OH^{\cdot}$) is believed to be the mechanism by which hydrogen peroxide effects the membrane lipids, nucleic acid materials, and proteins (2). It is possible that after the QAC has penetrated the lipid envelope, the oxidizer is able to have a denaturing effect on the nucleocapsid protein as well as the nucleic acid material held within. These findings however have not been proven and would need further analysis to verify.

CONCLUSION

In conclusion, the Sandia National Laboratories test formulations used in this study resulted in complete viral inactivation with relatively short contact times with reduced concentrations and even in the presence of organic materials. Results obtained in this study can be applied broadly to other lipophilic viruses within category A having a lipid envelope including Orthomyxoviridae (influenza) with regard to virucidal effectiveness. Further analysis for category B and C viruses (nonlipid non-enveloped and nonlipid capsomeric lipophilicity, respectively) would need to be conducted to verify the efficacy of the Sandia National Laboratories test formulations against these groups.

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