

CHEMICAL INACTIVATION OF VIRUSES

by

JILL M. BIEKER

B.S., Kansas State University, 2000

M.S., Kansas State University, 2002

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2006

Abstract

Viruses differ in their susceptibility and resistance to disinfectants and their ability to be inactivated based predominantly on structural and size differences. The virucidal methodology followed in this study was consistent with the U.S. Environmental Protection Agency guidelines for such efficacy testing. Various disinfectant chemistries were effective in rapid and complete inactivation of bovine coronavirus (BCV) including 25, 50, and 100% DF-200d, 10% bleach, 1% Virkon® S, and 70% ethanol following 10 min treatments. Efficacy was slightly reduced in the presence of organic challenge material (feces, soil). Treatment with 100% DF-200d or 10% bleach resulted in the degradation of BCV RNA. Treatment with 50 or 100% DF-200d, 10% bleach, or 1% Virkon® S resulted in the degradation of BCV proteins as detected by western blot analysis. Various disinfectants were effective in the complete inactivation of both mammalian and avian influenza test strains and included DF-200d, 10% bleach, 1% Virkon® S, and 70% ethanol following 10 min exposure but was reduced in the presence of organic challenge. Treatment with DF-200d or 10% bleach resulted in significant degradation of influenza RNA. Effective treatments against foot-and-mouth disease virus (FMDV) and bovine enterovirus-2 (BEV-2) included 10% bleach, 4% sodium carbonate, 2% sodium hydroxide, and Sandia DF-200. Treatment with 5% acetic acid was effective against FMDV, but not BEV-2. Treatment with 70% ethanol was not effective for inactivating FMDV or BEV. Additionally, 10 min treatment with 10% bleach, 2% sodium hydroxide, and Sandia DF-200 degraded FMDV and BEV-2 RNA. FMDV inoculated surfaces were exposed to 5% acetic acid, 10% bleach, 70% ethanol, 4% sodium carbonate, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, or 1% Virkon® S. All treatments except 70% ethanol were effective following 10 and 20 min contact on rubber and stainless steel. No treatments were shown to be effective for concrete, due to a low recovery of virus from this surface. Additionally, 10 and 20 min treatments with 10% bleach, 2% sodium hydroxide, and Sandia DF-200 degraded FMDV RNA on the surfaces evaluated.

CHEMICAL INACTIVATION OF VIRUSES

by

JILL M. BIEKER

B.S., Kansas State University, 2000
M.S., Kansas State University, 2002

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2006

Approved by:

Major Professor
Dr. Richard Oberst

Abstract

Viruses differ in their susceptibility and resistance to disinfectants and their ability to be inactivated based predominantly on structural and size differences. The virucidal methodology followed in this study was consistent with the U.S. Environmental Protection Agency guidelines for such efficacy testing. Various disinfectant chemistries were effective in rapid and complete inactivation of bovine coronavirus (BCV) including 25, 50, and 100% DF-200d, 10% bleach, 1% Virkon® S, and 70% ethanol following 10 min treatments. Efficacy was slightly reduced in the presence of organic challenge material (feces, soil). Treatment with 100% DF-200d or 10% bleach resulted in the degradation of BCV RNA. Treatment with 50 or 100% DF-200d, 10% bleach, or 1% Virkon® S resulted in the degradation of BCV proteins as detected by western blot analysis. Various disinfectants were effective in the complete inactivation of both mammalian and avian influenza test strains and included DF-200d, 10% bleach, 1% Virkon® S, and 70% ethanol following 10 min exposure but was reduced in the presence of organic challenge. Treatment with DF-200d or 10% bleach resulted in significant degradation of influenza RNA. Effective treatments against foot-and-mouth disease virus (FMDV) and bovine enterovirus-2 (BEV-2) included 10% bleach, 4% sodium carbonate, 2% sodium hydroxide, and Sandia DF-200. Treatment with 5% acetic acid was effective against FMDV, but not BEV-2. Treatment with 70% ethanol was not effective for inactivating FMDV or BEV. Additionally, 10 min treatment with 10% bleach, 2% sodium hydroxide, and Sandia DF-200 degraded FMDV and BEV-2 RNA. FMDV inoculated surfaces were exposed to 5% acetic acid, 10% bleach, 70% ethanol, 4% sodium carbonate, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, or 1% Virkon® S. All treatments except 70% ethanol were effective following 10 and 20 min contact on rubber and stainless steel. No treatments were shown to be effective for concrete, due to a low recovery of virus from this surface. Additionally, 10 and 20 min treatments with 10% bleach, 2% sodium hydroxide, and Sandia DF-200 degraded FMDV RNA on the surfaces evaluated.

Table of Contents

| | |
|---|-----|
| List of Figures | vii |
| List of Tables | ix |
| Acknowledgements | xi |
| CHAPTER 1 - Introduction to the Chemical Inactivation of Viruses | 1 |
| Virucidal Methodologies | 1 |
| Chemical Disinfectants | 7 |
| Acids | 7 |
| Alkalis | 8 |
| Alcohols | 8 |
| Oxidizing Agents | 8 |
| Phenols | 9 |
| Surface-active Agents | 10 |
| Novel Chemistries | 10 |
| Influential Factors Affecting Disinfection | 12 |
| CHAPTER 2 - Inactivation and Mechanism of Action of Chemical Disinfectants against Coronavirus | 14 |
| Abstract | 14 |
| Introduction | 14 |
| Materials and Methods | 17 |
| Results | 23 |
| Discussion | 28 |
| Figures and Tables | 34 |
| CHAPTER 3 - Virucidal Activity of Biocides against Influenza A Viruses | 40 |
| Abstract | 40 |
| Introduction | 40 |
| Materials and Methods | 43 |
| Results | 48 |
| Discussion | 50 |

| | |
|---|-----|
| Figures and Tables | 57 |
| CHAPTER 4 - Inactivation of Foot and Mouth Disease Virus and Bovine Enterovirus . | 63 |
| Abstract..... | 63 |
| Introduction..... | 63 |
| Materials and Methods..... | 66 |
| Discussion..... | 73 |
| Figures and Tables | 77 |
| CHAPTER 5 - Inactivation of Foot and Mouth Disease Virus on Environmental | |
| Surfaces..... | 86 |
| Abstract..... | 86 |
| Introduction..... | 86 |
| Materials and Methods..... | 89 |
| Results..... | 91 |
| Discussion..... | 93 |
| Figures and Tables | 97 |
| CHAPTER 6 - Conclusions | 103 |
| References..... | 109 |

List of Figures

| | |
|--|----|
| Figure 2.1 Infectivity of BCV as measured by TCID ₅₀ following 1 min exposure to test disinfectants in the presence of no organic challenge, 10% soil, and 10% feces. | 34 |
| Figure 2.2 Infectivity of BCV as measured by TCID ₅₀ following 10 min exposure to test disinfectants in the presence of no organic challenge, 10% soil, and 10% feces. | 35 |
| Figure 2.3 Quantitative <i>r</i> RT-PCR following 1 min exposure to test disinfectants in the presence of no organic challenge, 10% soil, or 10% feces. | 36 |
| Figure 2.4 Quantitative <i>r</i> RT-PCR following 10 min exposure to test disinfectants in the presence of no organic challenge, 10% soil, or 10% feces. | 37 |
| Figure 2.5 Western blot analysis of BCV virus following various treatments with test disinfectants based on the monoclonal 8F2 antibody targeting the nucleocapsid protein. | 38 |
| Figure 3.1 Infectivity of H1N1 and H5N8 as measured by TCID ₅₀ /ml following 1 and 10 min exposure to chemical biocides with no organic challenge. | 57 |
| Figure 3.2 Infectivity of H1N1 and H5N8 as measured by TCID ₅₀ /ml following 1 and 10 min exposure to chemical biocides in the presence of 10% feces. | 58 |
| Figure 3.3 Infectivity of H1N1 and H5N8 as measured by TCID ₅₀ /ml following 1 and 10 min exposure to chemical biocides in the presence of 50% feces. | 59 |
| Figure 4.1 The percentage survival rate for BEV-2 and FMDV as recovered by TCID ₅₀ following 10 min exposure to test disinfectants in the presence of no organic challenge. | 77 |
| Figure 4.2 The percentage survival rate for BEV-2 and FMDV as recovered by TCID ₅₀ following 20 min exposure to test disinfectants in the presence of no organic challenge. | 78 |
| Figure 4.3 The percentage virus recovered by TCID ₅₀ or quantitative <i>r</i> RT-PCR for BEV-2 and FMDV following 10 min exposure to test disinfectants in the presence of no organic challenge. | 79 |
| Figure 4.4 The percentage virus recovered by TCID ₅₀ or quantitative <i>r</i> RT-PCR for BEV-2 and FMDV following 20 min exposure to test disinfectants in the presence of no organic challenge. | 80 |

| | |
|---|----|
| Figure 5.1 The fraction of viable FMDV (%) as recovered by TCID ₅₀ following 5 min exposure to test disinfectants on test surfaces..... | 97 |
| Figure 5.2 The fraction of viable FMDV (%) as recovered by TCID ₅₀ following 10 min exposure to test disinfectants on test surfaces..... | 98 |
| Figure 5.3 The fraction of viable FMDV (%) as recovered by TCID ₅₀ following 20 min exposure to test disinfectants on test surfaces..... | 99 |

List of Tables

| | |
|--|-----|
| Table 1.1 Summary of National and International Virucidal Efficacy Standards | 13 |
| Table 2.1 Test sensitivities for various BCV detection assays by infectivity, nucleic acid-based, and protein-based diagnostic procedures. | 39 |
| Table 3.1 Quantitative <i>r</i> RT-PCR reported as log ₁₀ RNA/TCID ₅₀ units following 1 or 10 minute exposure to test disinfectants in the presence of no organic challenge..... | 60 |
| Table 3.2 Quantitative <i>r</i> RT-PCR reported as log ₁₀ RNA/TCID ₅₀ units following 1 or 10 minute exposure to test disinfectants in the presence of 10% organic challenge. | 61 |
| Table 3.3 Quantitative <i>r</i> RT-PCR reported as log ₁₀ RNA/TCID ₅₀ units following 1 or 10 minute exposure to test disinfectants in the presence of 50% organic challenge. | 62 |
| Table 4.1 Average pH values for test disinfectants. | 81 |
| Table 4.2 Infectivity of BEV-2 and FMDV as measured by TCID ₅₀ following 10 min exposure to test disinfectants in the presence of test organic concentrations of bovine feces (0%, 10%, 50%). | 82 |
| Table 4.3 Infectivity of BEV-2 and FMDV as measured by TCID ₅₀ following 20 min exposure to test disinfectants in the presence of test organic concentrations of bovine feces (0%, 10%, 50%). | 83 |
| Table 4.4 Amount of BEV-2 and FMDV RNA (log ₁₀) based on a standard curve generated from RNA extracted from predetermined levels of both viruses (quantified by TCID ₅₀) as measured by quantitative <i>r</i> RT-PCR following 10 min exposure to test disinfectants in the presence of test organic concentrations of bovine feces (0%, 10%, 50%). | 84 |
| Table 4.5 Amount of BEV-2 and FMDV RNA (log ₁₀) based on a standard curve generated from RNA extracted from predetermined levels of both viruses (quantified by TCID ₅₀) as measured by quantitative <i>r</i> RT-PCR following 20 min exposure to test disinfectants in the presence of test organic concentrations of bovine feces (0%, 10%, 50%). | 85 |
| Table 5.1 The percentage of FMDV RNA as recovered by quantitative <i>r</i> RT-PCR following 5 min exposure to test disinfectants on test surfaces. | 100 |

Table 5.2 The percentage of FMDV RNA as recovered by quantitative *r*RT-PCR following 10 min exposure to test disinfectants on test surfaces. 101

Table 5.3 The percentage of FMDV RNA as recovered by quantitative *r*RT-PCR following 20 min exposure to test disinfectants on test surfaces. 102

Acknowledgements

I would like to acknowledge many people in this dissertation, but first and foremost, I thank God. I would like to thank my parents, Sheila and Francis, for their unconditional love and support throughout my life. They have been the best parents in the world and I try to follow in their remarkable footsteps! I would also like to acknowledge rest of my family including my sister Jackie, my brother Tony, and my twin Wes for their love and support. I am truly blessed to be surrounded by so many other caring and loving people in addition to my family, whom although not related by blood, I consider family as well. David, Joey, Tiffany, Christie, Anthony, and my precious Gillian Oakes have known me since I was a kid and have loved and helped me in so many ways, and I love them dearly. Ann, Alice, Jessica, Amy, and of course Mike White have been another family who have been with me through this entire ‘ride’, and without their love and support, I couldn’t have made it. Those White girls will always be my ‘pseudo-children.’ Caroline Souza has helped me not only by being such a wonderful and spectacular friend, but by offering her technical support through every phase of this work, and I can’t thank her enough. The diagnostic virology laboratory group including Heather Wisdom, Joe Anderson, Deb StCyr, Jessica Jewell, Pilar Guntar, and Aron Shuler are also not only amazing people who helped me throughout this process, but are each a special friend whom I will always keep in touch with. I would also like to thank the Forensics group at Plum Island, including Meri Rosco, Jessica Rowland, Tammy Beckham, Bill ‘Turtle’ Hurtle, Kate Schuman, and August Eberling, who all helped me in the process and turned out to be amazing friends. Finally I would like to acknowledge my major advisor, Dr. Richard Oberst and the other committee members including Dr. Bob Rowland, Dr. Lorena Passarelli, and Dr. Mark Tucker. You have each been positive mentors in this academic endeavor, and I thank you each for your guidance and continued support.

CHAPTER 1 - Introduction to the Chemical Inactivation of Viruses

Virucidal Methodologies

Chemical disinfectants are an important component in disrupting the transmission of viral infectious disease and aiding in containment and eradication efforts. While there is considerable published information and disinfection efficacy data regarding bacteria and fungi, the efficacy of chemical disinfectants against viruses has not been well studied. Existing literature in the area of virucidal disinfectant efficacy is of limited value by virtue of the diverse testing methodologies used.

Methodologies for evaluating disinfectants for virucidal efficacy exist both nationally and internationally in the form of guidelines and published standards, although these differ quite drastically. Among these methodologies, the two predominant methods for evaluating virucidal efficacy of disinfectants are suspension and carrier tests. Suspension tests evaluate the virus as liquid inoculum while carrier tests are designed for evaluating virus inoculated onto various surfaces. Many disinfectants are assumed to be effective against viral targets without proper validation and simply on the basis of their performance against other microbial targets such as bacteria or other groups of viruses.

Regardless of the inactivation method used, important considerations for evaluating virucidal activity of disinfectants should include the use of a representative test virus (ie. the specific virus or closely related virus) and the use of test protocols that mimic, as best as possible, the environmental conditions associated with anticipated use (58). Validation of disinfectant efficacy can be more challenging than bactericidal testing, since assays such as virus enumeration and detection rely on the development and maintenance of a viable host cell system.

Because the testing procedure relies on a viable cell culture system, any toxicity from the chemical treatments must be adequately removed prior to infection with treated viral samples.

To better understand the approach of existing national and international guidelines or standards on virus disinfection, it is important to understand some of the differences among virus groups with regard to their reaction to chemical disinfection. Viruses are divided into several subgroups with regard to their resistance to disinfectants (Table 1), based largely on the presence or absence of an envelope and the size of the virus particle. According to the Klein and Deforest schematic (47) for dividing viral groups, the least resistant to disinfectants are the enveloped viruses (ie. influenza, coronavirus); those with moderate resistance are the large non-enveloped viruses (ie. adenovirus); and those with the most resistance are the small non-enveloped viruses (ie. picornavirus, parvovirus). These subgroups of viruses are often termed category A, C, and B, respectively. Category A and C are the least resistant, and disinfectants such as hypochlorite, alkalis, oxidizing agents, alcohols and aldehydes are all very effective in relatively short contact times (57). Category B viruses are the most resistant viruses and great care must be taken to ensure complete inactivation. Because of these differences in resistance to disinfectants among the various groups of viruses, it is important to evaluate specific groups of a test virus (ie. enveloped versus non-enveloped) when establishing the efficacy of a particular disinfectant.

A set of prescribed and standardized testing protocols for evaluating virucidal efficacy of disinfectants does not currently exist in the U.S. However, national testing guidance can be found within the U.S. EPA (34) and the American Society for Testing and Materials (ASTM) (8-11), as well as international sources (24), including the Association Francaise de Normalisation (AFNOR) in France and the Department of Environment , Food and Rural Affairs (DEFRA) in the United Kingdom. Regardless of the testing protocol, most of the methods are described in

detail for either suspension tests, in which a liquid viral inoculum is evaluated, or carrier tests, in which virus is dried onto various surfaces and further treated with disinfectants. All tests rely on adequate removal of toxicity from the chemical disinfectant to allow for post-treatment enumeration in an appropriate viable host cell system. Many of these tests also consider additional organic challenge in the test protocols in order to better represent the disinfection environment that would be encountered in actual use. Most protocols recommend the inclusion of 5-10% organic compounds (such as serum, feces, soil, etc) in the evaluation.

Exposure length and virus recovery techniques are also described for infectious virus detection. Exposure length is dependent on the chemical nature of the disinfectant. In general, shorter exposure lengths are ideal, especially for surface disinfection on sensitive materials susceptible to corrosion effects from the disinfectants. In most cases, virus detection and quantification is based on endpoint dilution assays in suitable assay systems as described in *Fields Virology, Volume 1* (25). Depending on the ratio of virus inoculum to disinfectant, the presence or absence of organic challenge, as well as the exposure time and method for toxicity removal, the reported ID₅₀ values following disinfection can vary greatly between studies, making comparison difficult or sometimes impossible. Such difficulties warrant the need for standardized efficacy testing procedures in the validation of virucidal compounds.

Table 1.1 summarizes EPA guidelines, ASTM testing standards, and a few international standards available for evaluating the efficacy of chemical disinfectants using suspension and carrier-based methodologies. The EPA published guidelines outline efficacy testing data requirements for the registration of virucidal disinfectants (34). To summarize, these guidelines require that data be generated by generally recognized virological techniques which are technically sound and simulate as closely as possible the intended use and environment. The

EPA guidelines further require that a disinfectant must be validated for each individual organism for which disinfection efficacy claims will be made. Examples of accepted surrogate viruses in current EPA testing guidelines include duck hepatitis B virus for hepatitis B virus, bovine diarrhea virus for hepatitis C virus, feline calicivirus for norovirus (34, 83). More recently, virucidal efficacy claims against avian influenza A could be applicable for efficacy claims against the highly pathogenic avian influenza H5N1 (4). The Association of Analytical Communities (AOAC) Use-Dilution Method and the AOAC Germicidal Spray Products Test are both referenced by the EPA and require modification for virucidal testing, since these test procedures are developed predominantly for bacterial and fungal use (6).

Under the EPA guidelines, virus recovery using endpoint dilution assay must include four replicate analyses for each dilution in the specific assay system (ie. cell culture, embryonated egg, animal infection). The efficacy testing data package submitted to the EPA for chemical registration must include the following specific items: cytotoxicity controls; germicide activity (effectiveness); methods for increasing viral titer and/or removing residual germicide toxicity; initial infective dose-50 (ID-50) values; and reduction of ID-50 following treatment with test germicide expressed as \log_{10} concentrations. For EPA acceptance, the testing data must demonstrate complete inactivation of the virus at all dilutions, or if cytotoxicity is present, at least a 3-log reduction beyond the cytotoxic level (34). EPA guidelines further specify that the virucidal claim can only be made against entities actually tested. Additional standards for evaluating the efficacy of biocides against viral agents are described by the American Society for Testing and Methods.

There are several different international methods for evaluating virucidal activity, but there is no universal international standard or protocol (56). Both the German DVV and the

French AFNOR agencies require 4 log reduction in virus titer, but there are discrepancies among the various protocols with regard to protein content, removal of chemical disinfectant, and concentration of disinfectant (15). Most of the current accepted methods are limited by the selection of the test agent. Both the DVV and AFNOR standards include Polio 1 as a test virus; however, this virus is presently restricted for use in research as a result of the ongoing poliovirus eradication effort of the World Health Organization.

In the published literature, virucidal efficacy data is typically generated by quantitative assays for determining virus titer using generally recognized methods such as endpoint dilution (most commonly as TCID₅₀). The lack of standardized protocols makes data interpretation and comparison with other published testing results problematic. For example, starting concentrations of viral inoculum often vary, so the amount of virus remaining following disinfection can vary drastically even with similar levels of total log reduction. If a 4 log₁₀ reduction with an initial inoculum level of 9 log₁₀ occurs, there are still 5 log₁₀ remaining while if the starting level was 6 log₁₀ of virus then only 2 log₁₀ would remain.

Many detection assays available for quantifying viral titers differ greatly in sensitivity, sometimes by several logs, and some assays such as nucleic acid amplification, do not directly measure loss of infectivity. These assays also differ in what is used to confer diagnosis with regard to the structure and functionality of the virus. Depending on the diagnostic test, different components of the virus are used for detection. For example, endpoint assay is the ability of the virus to attach, enter, and replicate inside of a viable host culture system while an ELISA-based test requires the presence of a reactive epitope on the virus surface for a positive diagnosis. Nucleic acid amplification relies on successful extraction of nucleic material from the sample followed by a very specific polymerase chain reaction (PCR) or reverse transcriptase polymerase

chain reaction (RT-PCR) procedure for amplification. With insensitive tests, a negative reaction could be misinterpreted as complete virus inactivation when in fact the test may not be capable of detecting the virus that is still present.

Surrogate viruses are often necessary because of various factors including the inability to propagate a virus in a cell culture system or if working with a particular virus is difficult due to infectivity risk or other regulatory restrictions (84). Surrogate test viruses are typically related to the virus family of interest, in an effort to maintain similar reactivity. Fundamentally, surrogates are only a model system, and validation with the actual virus of interest should be conducted prior to making any specific claims regarding efficacy (58). As mentioned previously, the EPA does not allow for surrogate virus testing in place of the disinfection claim, except for the non-culturable viruses previously mentioned. In this setting, surrogate testing might be advantageous for preliminary testing and optimization of a testing protocol prior to final testing against the viral target of interest.

The mechanism of chemical inactivation against bacterial and fungal targets has been widely studied (43, 52, 53, 59). Viruses are quite different than bacterial and fungal organisms with regard to more simplex structures (55, 57, 68, 75). In general, the lipid envelope (if present), structural proteins such as the capsid or surface receptors, and the nucleic acid (DNA or RNA) can all be targets of attack by chemical biocides (75). Inactivation can occur by degradation or disruption of any one of these or by all of them, depending on the chemistry of the disinfectant. The following section includes information on several different groups of chemical disinfectants and includes hypothesized and tested theories for mechanism of virus inactivation.

Chemical Disinfectants

Several groups of chemical disinfectants have been shown to have virucidal activity. Chemical disinfectant classes include the following groups: acids, alkalis, alcohols, surface active agents, phenols, oxidizing agents, and novel chemicals (75). Investigations of the mechanisms of virucidal activity by chemical agents are not widely published but hypotheses do exist (52, 53, 55, 57, 59). The relative efficacy of disinfectants depends largely on whether or not the target virus is enveloped or non-enveloped.

Acids

Acetic acid (commonly referred to as vinegar) is a common disinfectant which shows efficacy against acid sensitive organisms most likely due to the undissociated molecule, H^+ (43). (13). Citric acid is described as a chelating agent that permeabilizes the outer membrane of lipophilic bacteria (62), and likely has virucidal activity against lipophilic (enveloped) viruses. High concentrations of citric acid are toxic and care should be taken when handling. Citric acid can also be corrosive against metals and long exposure times should be avoided. Citric acid is frequently recommended for decontamination of personnel and clothing material (7). Lactic acid is not as functional as a liquid disinfectant, but has shown efficacy as an air disinfectant functioning (43). Peracetic acid (PAA) is rapidly becoming a popular disinfectant among the peroxygens class of disinfectant. Peracetic acid is a fact-acting oxidizing agent that has been shown to be highly effective against bacteria, viruses, molds, yeasts, and bacterial spores (13, 62). This compound is active at relatively low concentrations, remains effective in the presence of organic material, and can be deployed as a liquid or vapor. The active ingredient in PAA is speculated to be the generation of organic radicals such as CH_3CO_2 or CH_3CO , which have shown greater longevity than the hydroxyl radical (20).

Alkalis

Sodium hydroxide (NaOH) is an alkali with strong disinfectant properties, unless aluminum is present (13). Due to the high pH of this compound, it is quite caustic to the eyes, skin, and mucous membranes (13) and should be handled with care and proper protective equipment. NaOH is very inexpensive and is highly effective against viral targets in the presence of organic material, making this disinfectant suitable for use in an agricultural setting.

Sodium carbonate (NaCO₃) is another alkali disinfectant which is caustic to the eyes and skin and should be handled with care. This disinfectant is described as being highly effective, even in the presence of organic material (7, 13). Similarly, NaCO₃ is also relatively low cost and is ideal for the agricultural setting.

Alcohols

At a 70% ethanol concentration, most enveloped viruses are readily inactivated, even in the presence of serum or other organic challenge. Non-enveloped viruses, on the other hand, are very resistant to inactivation by ethanol. The mechanism of inactivation by alcohol occurs by denaturing protein structure and inducing conformational changes; however the effects are dependent on concentration (53).

Oxidizing Agents

Sodium hypochlorite (household bleach) and *N*-chloro (organic chlorine) compounds are both oxidizing agents and frequently used for disinfection purposes. These compounds are popular because of their broad range of applicability, low cost, and low toxicity to humans. On the other hand, agents in this chemical class are irritants and corrosive to materials such as metal (13). The stability of chlorine solutions is dependent on the following factors: concentration, presence of catalysts/reducing agents, pH, temperature, organic material, and ultraviolet radiation

(33). Organic load and pH greatly reduces the effectiveness of chlorine disinfectants. The optimum pH range for hypochlorite disinfectants is a pH value of 6-9.

In general, the mechanism of action of chlorine based compounds begins with the disassociation of hypochlorous acid from the chlorine in solution. Dissociated hypochlorous acid has been speculated to interact with cell membranes thus altering metabolic processes, denaturing proteins, and the formation of toxic complexes (33). These compounds have also been shown to oxidize thiol groups of cysteine residues which are important to structure determinants of proteins (53). Chlorine disinfectants are affected by factors including pH, organic material, and light (71). Chlorine is a corrosive disinfectant (71) and can result in the production of disinfection by-products (DBP) which include chloroform, bromodichloromethane, dibromochloromethane, and bromoform (33).

Hydrogen peroxide, another oxidizing agent, generates free hydroxyl radicals which can break DNA (and RNA) structures (43). Free hydroxyl radicals have been described as the strongest oxidants known, and can attack membrane lipids, DNA, and other essential cellular components (20). These compounds have also been shown to oxidize thiol groups of cysteine residues which are important as structural determinants of proteins (53). In the bacterial cell, this typically results in metabolic inhibition of the cell (53) while in viruses it likely produces a disruption in virus capsid structure. The use of hydrogen peroxide results in few environmental side effects, since it breaks down into oxygen and water (62).

Phenols

Phenols bind to amino acid residues of cytoplasmic and membrane proteins that result in enzyme inhibition, membrane damage, and denaturation of cytoplasmic proteins (39, 43). Phenols are highly toxic, have a strong odor, and are irritants to skin and eyes (13). Phenol

groups are effective against a wide variety of bacteria and often used as fungicides in the paper and cardboard industries. Phenols cannot be used in food processing or with materials that will be used for food. These groups of disinfectants are dated and not used as commonly in today's disinfection practices (13). In general, phenols are not very effective against non-enveloped viruses (39).

Surface-active Agents

Surface-active agents, also known as surfactants or quaternary ammonium compounds (QAC), are compounds comprised of a hydrophilic and a lipophilic portion (60). Their mode of action against bacteria is largely described as cell membrane disruption and loss of potassium ions (60). At present, little information is available on virus susceptibility to surfactant-based disinfectants. Suspected mechanisms of action on viral systems include a primary effect on the lipid envelope with subsequent degradation of viral capsid proteins (58). Viruses, specifically hydrophilic non-enveloped, are less susceptible to QACs than bacteria or fungi possibly due to a high amount of resistant structural protein (60). Quaternary ammonium compounds remain active against lipophilic-enveloped viruses. The mechanism of action of QACs involves protein denaturation and dissociation of enzymes (60) and interaction with lipids (53). Both of these mechanisms could impact on both enveloped and non-enveloped viruses.

Novel Chemistries

Novel chemistries include a group of disinfectants which have been developed containing more than one active ingredient for a synergistic mechanism of action. Many of these disinfectants have also been optimized for efficacy in the presence of organic material and for shorter contact times. Oxy-Sept® 333 is an oxidizing disinfectant manufactured by Ecolab® with EPA registration (No. 1677-129) (2). The active ingredient is peroxyacetic acid (5.8%,

undiluted) and hydrogen peroxide (27.5%, undiluted), as well as a surfactant. Oxy-Sept® 333 is described as a stabilized peroxyacetic acid formulation tolerant, including water hardness, cold temperatures, and pH (2).

Sandia Decon Foam-200 (Sandia DF-200) is a unique blend of quaternary ammonium compounds and peroxide for a synergistic mechanism of action that was developed at Sandia National Laboratories. It is an aqueous-based disinfectant formulation which contains a cationic surfactant, buffers, a low concentration of hydrogen peroxide (<2% by weight), and a novel peroxide activator. DF-200 formulation has been demonstrated to be highly effective at neutralizing and/or killing chemical and biological warfare agents, and is in use by the U.S. military (91). DF-200 is commercially available from EnviroFoam Technologies, Inc (www.envirofoam.com) and Modec, Inc. (www.deconsolutions.com). The disinfection mechanism of DF-200 against viruses is most likely a result of the dual synergy between the surfactant oxidizing properties. In addition to these chemistries, peracetate is also produced and further acts as a strong oxidizer. Rapid degradation of capsid proteins and viral RNA has been observed in relatively short contact times (17, 18).

Virkon® S is a powdered disinfectant manufactured by Antec International, Suffolk, UK (5). The active ingredient in Virkon® S is potassium peroxymonosulfate, characterized as a strong oxidizing agent and a high level of surfactant (7). This compound is toxic to the eyes, skin, and mucous membranes, and special care is required when handling. A 1% solution of Virkon® S is recommended to disinfect walls, ceilings, floors, decks, container surfaces, vehicles, wheels, and other livestock equipment, although metal surfaces should not be exposed for more than 10 minutes due to its corrosiveness. The recommended surface application level for Virkon® S is 300 ml/m² (13).

Influential Factors Affecting Disinfection

Various environmental factors including temperature, humidity, pH, and organic load can all greatly impact the efficacy of disinfectants against viruses (15, 58, 59, 68, 71, 75). Warmer temperatures can increase the rate of the chemical reaction, but very high temperatures can affect the stability of the disinfection agent. Cold temperatures will slow down chemical reactions, and very cold temperatures can freeze liquid based disinfectants. Factors such as pH have the greatest impact on acidic- and alkaline-based disinfectants. These disinfectants require specific pH ranges (acidic for the acid based disinfectants and basic for the alkaline compounds) and are sometimes affected by the presence of heavy organic soiling (68, 71).

Other factors can come into play based on the intended application of the disinfecting agent. Corrosive properties of some disinfectants can have undesirable effects on materials being treated. The corrosion of metal surfaces that are treated with bleach is a primary example (71). Exposure time of the disinfectant on sensitive surfaces or materials will also influence the degree of corrosion. Furthermore, chemical disinfectants possess their own inherent stability properties and disinfectant product shelf life can also be an important issue.

| Agency/ Organization | Test Title | Summary | Reference |
|-------------------------|---|---|---|
| US EPA | Efficacy Data Requirements: Virucides | Can be conducted as suspension or surface tests, minimum 10 ⁴ test titer, must consider cytotoxicity | (26) http://www.epa.gov |
| ASTM | E1052-96 Efficacy of Antimicrobial Agents Against Viruses in Suspension | Method for evaluating efficacy of disinfectants against specific viruses in liquid suspension designed for cell culture host systems. Includes cell culture control, virus control, virucidal test, cytotoxicity control, and neutralization control. | (8) http://www.astm.org |
| ASTM | E1053-97 Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces | Method for evaluating disinfectants as liquid, aerosol, or trigger spray on inanimate surfaces. Includes a cell culture control, virus control, virucidal test, cytotoxicity control, and a neutralization control. | (9) http://www.astm.org |
| ASTM | E1482-04 Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations | Method utilizes a gel filtration technology to separate the treated virus from the test disinfectant following the desired exposure duration. | (1) http://www.astm.org |
| ASTM | E2197-02 Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporocidal Activities of Liquid Chemical Germicides | Method for evaluation liquid disinfectants for efficacy against various microbial targets in the presence of a soil load on disk carriers representing environmental surfaces or medical devices. Test viruses include Human adenovirus, hepatitis A, Canine parvovirus, human rhinovirus, and human rotavirus. | (7) http://www.astm.org |
| AFNOR | Association Francaise de Normalisation | Evaluates disinfectants at 2x concentration mixed to an equal volume of test virus (Polio 1*, Adenovirus, Vaccinia). Organic challenge is not used, and virus is recovered by host culture following dilution and washing to remove cytotoxicity. Efficacy claim must result in at least a 4 log ₁₀ reduction. | (16) http://www.afnor.fr |
| DVV | Deutsche Verinigung zur Bekämpfung der Viruskrankheiten | Evaluates disinfectants at final use concentration and includes challenge with BSA or FCS (0.2-10%) against test virus (Polio 1*, Adenovirus, Vaccinia, SV40). Virus is recovered by host culture following dilution to remove cytotoxicity. Efficacy claim must result in at least a 4 log ₁₀ reduction. | (24) Guidelines of Bundesgesundheitsamt |

Table 1.1 Summary of National and International Virucidal Efficacy Standards

CHAPTER 2 - Inactivation and Mechanism of Action of Chemical Disinfectants against Coronavirus

Abstract

Viruses differ in their susceptibility and resistance to disinfectants and inactivation based predominantly on structural and size differences. The virucidal methodology followed in this study was consistent with the U.S. Environmental Protection Agency guidelines for such efficacy testing. We report that various disinfectant chemistries were effective in rapid and complete inactivation of bovine coronavirus (BCV) including 25, 50, and 100% DF-200d, 10% bleach, 1% Virkon® S, and 70% ethanol. Efficacy of all test disinfectants was slightly reduced in the presence of organic challenge material (feces, soil). Additionally, the use of various diagnostic assays dependent on the intact presence of various viral structures (nucleic acid, protein). Treatment with 100% DF-200d or 10% bleach resulted in the degradation of BCV RNA. Treatment with 50 or 100% DF-200d, 10% bleach, or 1% Virkon® S resulted in the degradation of BCV proteins as observed by western blot analysis.

Introduction

Viruses are responsible for nearly 60% of human infections can have widespread disease occurrence and devastating economic impact to animal health (70). In addition to vaccine and antiviral disease control strategies, chemical inactivation of viruses aid in the disruption of the infectious life cycle and disease transmission to aid in overall containment and control efforts (57, 75). A more thorough and in depth understanding of both the efficacy and mechanism of action of different antiviral chemistries is necessary for effective disease control strategies.

Viruses, due to their simplicity, present limited targets for chemical mechanisms of inactivation. These targets include the lipophilic envelope (if an enveloped virus), proteins (capsid, structural), and nucleic acid (RNA or DNA) (57, 67). Viruses have been classified into three different groups to predict susceptibility to chemical disinfectants based on lipophilic properties and virus size (47, 67). Group A viruses that have lipoprotein envelopes are the most susceptible to disinfectants. Group B viruses are small, non-enveloped viruses that are the least susceptible to disinfectants. Group C viruses are large, non-enveloped viruses which are intermediately susceptible to disinfectants. In addition to the Klein and Deforest classification scheme, other factors including the infectious life cycle conditions of a particular virus (ie. enteric) can allow for resistance (to conditions such as acidity) to occur (70). The Klein and Deforest classification is a predictive value but actual efficacy testing with specific compounds is necessary for proper validation measures.

While there is considerable published information and disinfection efficacy data pertaining to bacteria and fungi, the efficacy of chemical disinfectants against viruses has not been well studied. Disinfection is one of the leading measures for preventing infectious virus infections (57, 84). Documentation of the effectiveness of viral disinfectants for emerging viruses is minimal, and is often assumed to occur based on existing bacteriological testing or without proper validation on specific viruses and in the presence of appropriate environmental conditions (84). Published material is available in the general area of viral disinfection, but efficacy data on specific viruses or group of viruses isn't readily available in existing literature and is difficult to interpret due to a lack of universal standardized virucidal testing procedures. Testing methodologies exist both nationally and internationally for evaluating disinfectant

efficacy against viruses in the form of guidelines or published standards but present considerable variability in test viruses required and in the degree of detail among these sources (16).

In the U.S., the Environmental Protection Agency (EPA) provides guidelines for efficacy testing (34, 67). The EPA guidelines require that a disinfectant must be validated for each individual organism it claims to be effective against. It is unclear whether or not surrogate viruses are suitable for use in place of a closely related virus, or if the evaluation of one virus among a particular viral family can provide data useful for other viruses in the same family. Under the EPA testing guidelines, the disinfection evaluation can be conducted as suspension or surface carrier tests (based on the recommended use of the disinfectant) and the recovery of the virus must be obtained from a minimum of 4 determinations per each dilution assay system (74) (tissue culture, embryonated egg, animal infection infecting dose 50), and must result in complete inactivation of virus at all dilutions or at least a 3-log reduction in titer beyond the cytotoxic level (of the dilution assay system) (34).

The purpose of this study was to evaluate the efficacy and mechanism of action of various chemical disinfectants against bovine coronavirus (BCV). Disinfectant efficacy was determined following the EPA guidelines (34) with virus recovery using tissue culture infective dose 50 (TCID₅₀) format. To evaluate mechanism of inactivation of the disinfectant chemistries tested against BCV, reverse transcriptase polymerase chain reaction (RT-PCR) and protein identification assays were utilized to detect residual viral nucleic acid or protein, respectively. Chemical disinfectants used in this study included bleach, ethanol, Virkon[®] S (5), and DF-200d (91). To determine the most effective disinfectants against BCV statistical analysis of the various disinfectant treatments were evaluated measuring the viral response (as measured by

TCID₅₀ and quantitative BCV RNA concentration) to differences in time of exposure to the treatment, and the presence of different organic sample matrices.

Materials and Methods

Detection assays. Analytical sensitivity was determined by evaluating various BCV diagnostic methods and included identifying BCV infectivity, BCV nucleic acid, and BCV protein using predetermined quantities of reference virus. Endpoint titration (TCID₅₀) for infectivity, RT-PCR for nucleic acid detection, and hemagglutination or electrophoresis/western blot analysis for protein detection were evaluated. Assays resulting in the greatest analytical sensitivity for the detection of BCV were selected for further use in the disinfection efficacy and mechanism of action.

BCV infectivity assay

Virus and cells. Bovine coronavirus isolate Wisconsin 1SK was propagated in Human Rectal Tumor-18 (HRT) cells as previously described (45). Culture media was made by adding Minimal Essential Medium with Earle's salts and L-glutamine (Invitrogen Corporation, Carlsbad, CA) and 2.2 g/L sodium bicarbonate collectively referred to as MEM with the addition of trypsin (5 µg/ml) and pancreatin (5 µg/ml) (Fisher Scientific, Hampton, NH) (44). Cells were maintained in MEM containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) supplemented with antibiotics [2.5 mg/L amphotericin B; 0.67 g/L streptomycin; and 0.3 g/L penicillin G (all from Fisher Scientific)].

Tissue culture infective dose 50 (TCID₅₀). To determine BCV infectious viral titers, tissue culture infective dose 50 (TCID₅₀) were determined. The Reed-Muench method (74) was calculated in a 96-well format to determine the dilution of virus resulting in infection of fewer than 50% replicate wells. Nunc 96-well cell culture microtiter plates (Fisher Scientific) were

seeded with HRT cells and incubated (approximately 24 hrs, at 37°C with 5% CO₂). Once cells were monolayered, the media was removed and plates were washed once with 0.01 M phosphate buffered saline (PBS, pH 7.2). Tissue culture-adapted BCV was then serially diluted (ten fold) in culture media to a 10⁻⁹ dilution. The microtiter plates were inoculated by adding 25 µl of each virus dilution to each well. Negative controls were inoculated with 25 µl of uninfected culture media. An additional 75 µl culture media was added to each well and the plate was incubated (48 hrs, 37°C with 5% CO₂). Infectivity was determined by cytopathic effect (CPE) as indicated by the presence of large syncytial sloughing of infected cells and TCID₅₀ was calculated using the Reed-Muench method for estimating 50% endpoints of infectivity (74).

BCV nucleic acid detection

Real-time reverse transcription polymerase chain reaction (rRT-PCR). Viral RNA was recovered using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Quantitative detection of the extracted BCV RNA was conducted using rRT-PCR using a fluorescently labeled TaqMan probe, and qualitative detection was visualized by agarose gel electrophoresis and Southern blot analysis. A fluorescently labeled TaqMan probe was designed to be used with existing forward and reverse primers for the rRT-PCR amplification and detection of BCV RNA (90). The TaqMan probe corresponded to the BCV nucleocapsid sequence (5'-TTT GGC ATG CGG TCC TGT TCC AAG AT-3') and was synthesized and labeled (6-FAM™ at the 5'-end and Iowa Black Fluorescent Quencher at the 3'-end, Integrated DNA Technologies, Coralville, IA). For each 25 µl reaction, 2.5 µl of the RNA sample was added to 22.5 µl of master mix that contained 5 µl OneStep buffer, 1 µl dNTP, 1 µl enzyme (all from Qiagen's OneStep RT-PCR kit), and 0.5 µl each of the forward and reverse primers (1 µM concentration), 2 µl probe (5 µM concentration), and 12.5 µl RNase-free water. The SmartCycler thermocycler was set with the

following settings: Stage 1 hold (42°C, 1800 s without optics), Stage 2 hold (95°C, 900 s without optics), Stage 3 was repeated 35 times using a 3-temperature cycle (94°C, 15 s without optics; 58°C, 15 s without optics, and 72°C, 30 s with optics on). The detection threshold for successfully detecting BCV RNA was a FAM fluorescence signal ≥ 3 using the SmartCycler.

Analysis of *rRT-PCR* amplification products were completed by agarose gel electrophoresis. Briefly, aliquots from *rRT-PCR* were loaded onto 1% agarose gels containing ethidium bromide and electrophoresed (90 V, 45 min). A successful BCV *rRT-PCR* followed by electrophoresis and ethidium bromide staining results in the visualization of a 407 bp amplicon (90).

Southern hybridizations were also completed on the *rRT-PCR* samples following electrophoresis. The DIG System – Nonradioactive and Highly Sensitive Detection of Nucleic Acids (Roche Diagnostics, Indianapolis, IN) was used in our study and included all washing and blocking buffers required (49). Gels were prepared for Southern hybridizations by washing in denaturing buffer for 30 min. Denature buffer was removed, and gels were washed in neutralizing buffer for 30 min. The DNA from the gel was transferred to a nylon membrane after these wash steps overnight and ultra violet (UV) cross-linked. Following UV cross-linking, the DIG System protocol for colorimetric detection were completed (49). The probe sequence used for the Southern hybridization was 5'- TTT GGC ATG CGG TCC TGT TCC AAG AT- 3' labeled with digoxigenin (DigN) at the 3' end. A successful Southern blot of the BCV *rRT-PCR* was interpreted as the visualization of a dark blue band of the 407 bp amplicon.

Inhibition of *rRT-PCR*. To determine if reverse transcriptase (RT) or the subsequent PCR activity were inhibited by possible residual carry-over of chemical disinfectants in the extraction process, the test disinfectants were evaluated. The test disinfectants were prepared as

described and then the various chemistries were extracted using the QIAamp Viral RNA Mini Kit. Following this extraction process (which didn't have any RNA present) 1.25 µl from each of the extracted test disinfectants was inoculated with 1.25 µl of tissue culture derived extracted BCV RNA to obtain the total 2.5 µl volume required for the 25 µl reaction (described previously) to conduct the *r*RT-PCR amplification assay. To determine if there was any carryover of residual disinfectant chemistry causing inhibition or interference of the *r*RT-PCR from any of the test disinfectants, a positive *r*RT-PCR (based on a FAM fluorescence signal ≥ 3 using the SmartCycler) result was necessary. A negative result would indicate that there was some residual disinfectant chemistry following the extraction process which inhibited or interfered with the *r*RT-PCR assay.

BCV protein detection

Hemagglutination activity. Hemagglutination activity (HA) of the samples as a correlation to amount of BCV present was also evaluated for determining BCV virus using rodent erythrocytes. The microtiter method was used to detect BCV hemagglutinin activity using washed mouse erythrocytes in PBS (pH 7.2) (85). Virus was diluted 1:2 in 0.01 M PBS for a total volume of 100 µl in each well, and 100 µl of a 1:100 dilution of washed mouse erythrocytes was added to each well and the plates were incubated (30 min, 4°C). The wells showing a lattice formation were recorded as a positive HA, while those with a button formation were recorded as negative HA.

Electrophoresis and western blot. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis was used to determine intact BCV structural proteins. Viral samples were prepared for western blot analysis by adding 40 µl of each sample to 6 µl SDS 4x loading buffer (ISC BioExpress, Kaysville, UT) and mixing.

Samples were heat-denatured (boiled, 5 min) and 25 μ l were loaded onto a 10% SDS- PAGE Express Gel (ISC BioExpress) and electrophoresed (80 min, 100 volts). Following electrophoresis the gel was blotted to nitrocellulose membranes (60 min, 100 volts). The nitrocellulose membrane was removed from the transfer apparatus and incubated (24 hours, 4°C) with 10% non-fat milk block (Bio-Rad, Hercules, CA). A monoclonal antibody (8F2) (27) targeting the BCV nucleocapsid protein was added at a 1:100 dilution to the 10% non-fat milk block and incubated (60 min, room temp). The membrane was washed (3 times, 10 min each) in PBS containing 0.05% Tween 20 (PBS/Tween). A horse radish peroxidase-labeled secondary anti-mouse antibody (KPL, MA) was added at a 1:10,000 dilution in PBS/Tween and was incubated (60 min, room temp). The membrane was washed as previously described. The presence of intact or residual BCV nucleoprotein was determined by colorimetric detection by adding 20 ml SureBlue™ TMB Peroxidase Substrate solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and the subsequent visualization of blue bands of a 50-60 kDa nucleocapsid protein (50).

Disinfectant efficacy testing

Test disinfectants. Ethanol was prepared by adding 70 ml 100% anhydrous ethanol (Fisher Scientific) to 30 ml sterile deionized water for a final use concentration of 70%. Bleach was prepared by adding 10 ml concentrated house hold bleach to 90 ml sterile deionized water for a final concentration of 10%. DF-200d, a reduced concentration of Sandia DF-200 (91), was supplied by Sandia National Laboratories (Albuquerque, NM) and was prepared by mixing 7.3 ml of part 1 (surfactants and fatty acids) to 0.2 ml part 3 (a diacetin-based propellant). Finally, 2.5 ml of part 2 (hydrogen peroxide, 7.99%) was added and the mixture was thoroughly mixed to yield the 100% test concentration of DF-200d. The 100% DF-200 was serially diluted (two-fold

dilutions) in deionized water to obtain the 50% and 25% test concentrations. Virkon® S was purchased from the DuPont Animal Health Solutions (Antec®, Suffolk, UK) and diluted to 1% by adding 0.1 g to 10 ml deionized water.

Organic matrices. The organic matrices used in this study included a 10% weight/volume (wt/vol) solution of bovine feces and a 10% wt/vol solution of soil. The bovine feces were prepared by rectally collecting fecal material from healthy cattle, weighing out 10 g of the feces, and adding 90 ml 0.01 M PBS and mixing thoroughly. The soil used in this study was provided by the Soil Testing Laboratory (Department of Agronomy, Kansas State University, Manhattan, KS) and was prepared by removing most of the plant/root material via a sifting process. Soil (10 g) was then added to 90 ml of 0.01 M PBS and mixed thoroughly.

Virus inactivation. Ten milliliters of tissue culture propagated BCV (approximately 10^{6-7} log₁₀ TCID₅₀/ml) was added to 10 ml test disinfectant (25, 50, and 100% DF200d, 1 and 10% bleach, 1% Virkon® S, 70% ethanol) and were incubated (1 and 10 min, room temperature). For each organic challenge, 10 ml of test virus was added to 9 ml of test disinfectants containing 1 ml of a 10% bovine fecal slurry or 10% soil slurry and were incubated (1 and 10 min, room temperature). Resulting samples were washed by ultracentrifugation (100,000xg, 1 hour, 4°C) and pellets were reconstituted with 1 ml of MEM and stored at -80°C.

Post-treatment infectivity determination. The titers of infectious virus were determined by TCID₅₀ as previously described. Nunc 96-well plates containing confluent HRT monolayers were washed once with MEM. Wells were filled with 25 µl of each disinfectant sample and their dilutions. Then, 75 µl media were added to each well and plates were incubated (24-48 hours, 37°C, 5% CO₂). The TCID₅₀ was evaluated by visual CPE and calculated using the Reed-Muench method for estimating 50% endpoints of infectivity.

Post-treatment RNA determination. Viral BCV RNA was recovered from the test samples following 1 or 10 min treatments with each test disinfectant using the QIAamp Viral RNA Mini Kit, as previously described. Quantitative detection of BCV RNA was determined by *r*RT-PCR and qualitative detection was visualized by electrophoresis/ethidium bromide staining or Southern blot hybridization assay as previously described.

Post-treatment protein recovery. The effect of the various test disinfectants on the ability to detect BCV nucleocapsid proteins was determined by western blot analysis of the samples treated with each disinfectant using a monoclonal antibody (8F2) with specificity for the nucleocapsid protein. Disinfectant samples were prepared for SDS-PAGE and western blot analysis as previously described.

Statistical analysis. Statistical evaluation comparing of the level of BCV recovered (for infectivity by TCID₅₀ or for RNA by quantitative *r*RT-PCR) following the experimental treatment parameters was determined by comparing 1-way, 2-way, and 3-way interactions between disinfectant treatment, time (1, 10 min), and organic challenge (no organic, 10% soil, 10% feces). The least square of means was calculated to determine if significant differences were observed in the untreated (positive control) amount of BCV recovered compared to the level of BCV recovered post treatment. A significant difference resulted in a P-value < 0.05.

Results

Detection assay. The analytical sensitivity of the various diagnostic BCV assays were evaluated and included virus isolation of infectious BCV as assessed by endpoint dilution (TCID₅₀), specific amplification of BCV RNA by RT-PCR (detected by TaqMan probe, ethidium bromide, or Southern blot hybridizations), and detection of BCV proteins (HA analysis of BCV hemagglutinin esterase structural protein and western blot analysis of BCV nucleocapsid protein)

(Table 2.1). Detection of BCV RNA using the *r*RT-PCR assay as detected using a TaqMan probe was the most sensitive assay detecting 10^2 TCID₅₀/ml BCV. Infectivity titration based on endpoint dilution (TCID₅₀) in HRT cells resulted in a detection sensitivity of 10^3 TCID₅₀/ml BCV. The RT-PCR product as detected by ethidium bromide staining and Southern blot analysis resulted in a detection sensitivity of 10^4 TCID₅₀/ml BCV. Detection of BCV hemagglutinin resulted in a detection sensitivity of 10^6 TCID₅₀/ml BCV while detection of BCV nucleoprotein using western blot analysis with the monoclonal antibody (8F2) resulted in detection sensitivity of 10^5 TCID₅₀/ml BCV.

The most sensitive detection assays (Table 2.1) were selected for a quantitative recovery of BCV virus post disinfectant treatment to evaluate the disinfectant efficacy. These assays included the infectivity titration of BCV based on endpoint dilution (TCID₅₀) in HRT cells and detection of BCV RNA using *r*RT-PCR as detected using a TaqMan probe. Although not quantitative, detection of BCV nucleoprotein using western blot analysis with the monoclonal antibody (8F2) was selected as a qualitative recovery of BCV protein following disinfectant treatments.

Post-treatment infectivity determination. No significant 3-way interactions were observed between disinfectant treatment, time, or organic challenge ($P > 0.05$). The exposure time and organic challenge parameters resulted in a significant 2-way interaction ($P < 0.05$). Challenge with 10% feces resulted in a greater loss of disinfectant efficacy than challenge with 10% soil following 1 min treatment, however these organic challenge effects resulted in no significant difference following 10 min treatment. Without the addition of organic challenge, there were no significant differences between the 1 or 10 min exposure times in the amount of BCV recovered. However, the addition of 10% soil or 10% feces challenge resulted in

significantly reducing the efficacy of all test disinfectants following 1 min compared to treatment with 10 min based on the amount of infectious BCV recovered post-treatment ($P < 0.05$).

The amount of untreated (positive control) BCV ranged between 6.64 to 8.85 \log_{10} TCID₅₀/ml for all organic challenge matrices (0%, 10% soil, 10% feces) evaluated. The addition of 10% soil or 10% feces reduced the efficacy of all test disinfectants for both 1 and 10 min treatments (Figures 2.1 and 2.2). All test disinfectants resulted in a significant reduction in the amount of BCV recovered following 1 and 10 min treatments when compared to the positive control ($P < 0.05$). There were three categories of significant differences observed among the test disinfectants with regard to the level and extent of inactivation. Overall, 100% DF-200 and 10% bleach were consistently the most effective treatments. The next most effective grouping of disinfectant treatments was 25% and 50% DF-200 and 1% Virkon S. The least effective grouping of disinfectant treatments was 1% bleach and 70% ethanol.

Treatments for 1 min without organic challenge using 100% DF-200 or 10% bleach resulted in the complete inactivation of BCV, to the detection limit of this assay (10^3 TCID₅₀/ml BCV) (Figure 2.1). The addition of 10% soil or 10% feces reduced the efficacy of these disinfectants and resulted in the recovery of 0.86 to 4.10 \log_{10} TCID₅₀/ml BCV. Treatment for 1 min without organic challenge using 25% DF-200, 50% DF-200 or 1% Virkon S resulted in the recovery of 0.57 to 1.44 \log_{10} TCID₅₀/ml BCV (Figure 1). The addition of 10% soil or 10% feces reduced the efficacy of these disinfectants and resulted in the recovery of 2.32 to 5.43 \log_{10} TCID₅₀/ml BCV. Treatments for 1 min without organic challenge using 1% bleach or 70% ethanol resulted in the recovery of 1.3 to 1.64 \log_{10} TCID₅₀/ml BCV (Figure 2.1). The addition of 10% soil or 10% feces reduced the efficacy of these disinfectants and resulted in the recovery of 4.68 to 6.43 \log_{10} TCID₅₀/ml BCV.

Treatments for 10 min without organic challenge using 25%, 50%, and 100% DF-200, 1% Virkon S and 10% bleach resulted in the complete inactivation of BCV, to the detection limit of this assay (10^3 TCID₅₀/ml BCV) (Figure 2.2). The addition of 10% soil or 10% feces reduced the efficacy of these disinfectants and resulted in the recovery of 0.70 to 3.76 log₁₀ TCID₅₀/ml BCV. Treatments for 10 min without organic challenge using 1% bleach or 70% ethanol resulted in the recovery of 1.64 to 1.97 log₁₀ TCID₅₀/ml BCV (Figure 2.2). The addition of 10% soil or 10% feces reduced the efficacy of these disinfectants and resulted in the recovery of 1.86 to 4.10 log₁₀ TCID₅₀/ml BCV.

Post-treatment RNA determination. Inhibition of the *r*RT-PCR assay was evaluated to verify that carry-over of test disinfectant chemistries was not interfering or inhibiting the *r*RT-PCR reaction. Inhibition of *r*RT-PCR did not occur for any of the test disinfectants used in this study based on a positive reaction occurring after inoculating the extracted disinfectant samples with purified BCV RNA (based on a FAM fluorescence signal ≥ 3 using the SmartCycler). Based on the demonstration of no interference or inhibition occurring with any of the test disinfectants, any reduction in the amount of BCV RNA recovered post-treatment is due to the mechanism of the disinfectant degrading the nucleic acid as opposed to chemical interference or inhibition of the *r*RT-PCR assay reaction.

There were no significant 3-way interactions observed between disinfectant treatment, time, or organic challenge ($P > 0.05$) in the amount of BCV RNA recovered by *r*RT-PCR. Disinfectant treatment and exposure time resulted in a significant 2-way interaction ($P < 0.05$). Challenge with 10% feces resulted in a greater loss of efficacy than challenge with 10% soil when compared to treatment without organic challenge for both time exposures. Organic challenge with 10% soil was not significantly different when compared to treatment without the

addition of organic challenge ($P > 0.05$). There were also no significant differences observed in the amount of BCV RNA recovered post-treatment between 1 or 10 min exposure except with treatments using 50% DF-200 and 1% Virkon S ($P < 0.05$).

The amount of untreated (positive control) BCV RNA units recovered ranged between 6.47 to 7.42 \log_{10} based on a known starting amount of cell-culture derived BCV (titrated as \log_{10} TCID₅₀/ml) and was calculated as the fluorescence intensity of the FAM reporter divided by the Ct (cycle threshold) value (RNA FAM/Ct). The efficacy of all test disinfectants for both 1 and 10 min treatments was significantly reduced by the addition of 10% feces ($P < 0.05$), but not with the addition of the 10% soil ($P > 0.05$), (Figures 2.3 and 2.4).

Treatment with 100% DF-200 and 10% bleach were the most effective in RNA degradation resulting in complete degradation (0% organic or 10% soil) and significant from the remaining treatments, $P < 0.05$, (Figure 2.3). The addition of the 10% feces reduced the efficacy resulting in the recovery of 1.08 to 2.58 \log_{10} RNA FAM/Ct. The next most effective treatment was with 50% DF-200, resulting in the recovery of 0.91 and 2.97 \log_{10} RNA FAM/Ct with 0% organic or 10% soil challenge, respectively. The addition of the 10% feces reduced the efficacy resulting in the recovery of 4.76 \log_{10} RNA FAM/Ct. Treatment with 25% DF-200 resulted in the recovery of 4.62-5.43 \log_{10} RNA FAM/Ct with all organic challenges evaluated. The least effective treatments included 1% bleach, 1% Virkon S, and 70% ethanol resulting in the recovery of 6.11 to 7.30 \log_{10} RNA FAM/Ct with all organic challenges evaluated.

Treatment with 50% DF-200, 100% DF-200 and 10% bleach were the most effective in RNA degradation resulting complete degradation (0% organic or 10% soil) and significant from the remaining treatments, $P < 0.05$, (Figure 2.4). The addition of the 10% feces reduced the efficacy resulting in the recovery of 1.11 to 2.31 \log_{10} RNA FAM/Ct. The second most effective

treatments were 25% DF-200 and 1% Virkon S resulting in the recovery of 2.79 to 5.66 log₁₀ RNA FAM/Ct with all organic challenges evaluated. The least effective treatments were 1% bleach and 70% ethanol resulting in the recovery of 4.05 to 7.10 log₁₀ RNA FAM/Ct with all organic challenges evaluated and were not significantly different from the amount of BCV RNA recovered from the untreated positive control, $P > 0.05$.

Post-treatment protein recovery. Following a 1 min exposure and western blot using the monoclonal 8F2 antibody (Figure 2.5), treatment with 50% DF-200d, 100% DF-200d and 10% bleach completely degraded BCV nucleoprotein (based on a negative presence of the appropriate band). BCV protein was still recovered from samples treated with 25% DF-200d, 1% bleach, 1% Virkon® S, and 70% ethanol. Following a 10 min exposure, 50-100% DF-200, 10% bleach, and 1% Virkon® S completely degraded BCV nucleoprotein. BCV nucleoprotein was still recovered from samples treated with 25% DF-200d, 1% bleach, and 70% ethanol. A 3 min time exposure was also conducted and resulted in the same recovery results as with the 10 min exposure, indicating that the 1% Virkon® S required at least a 3 minute contact to degrade this viral protein.

Discussion

In order to increase the understanding of the virucidal efficacy and mechanism of action of various disinfectant chemistries, we evaluated the chemical inactivation of various chemical treatments against BCV based on infectivity assays to be compliant with the current EPA virucidal testing guidelines as well as other detection assays. Disinfectant efficacy was determined following the EPA guidelines (34) with exposure at two times (1 or 10 min) with virus recovery using TCID₅₀ format. To represent possible environmental or less than ideal conditions, we included two types of organic challenge with soil or fecal material. The 1 min

treatments with 25, 50, and 100% DF-200d, 10% bleach, 1% Virkon® S, and 70% ethanol all resulted in the complete inactivation of BCV. These treatments were all affected by the presence of both organic challenges consisting of 10% soil or 10% feces. Additional molecular and serological assays were included to evaluate the possible mechanism of action of the test disinfectants against virus targets such as the nucleic acid or protein. Treatment with 100% DF-200d or 10% bleach resulted in the degradation of BCV RNA. Treatment with 50 or 100% DF-200d, 10% bleach, or 1% Virkon® S resulted in the degradation of BCV proteins as detected by western blot analysis.

The enveloped (category A) viruses are likely the least resistant due to relatively easy degradation and breakdown of the unstable lipid envelope rendering the virus particles replication defective, due to the compromised attachment and entry processes. Following disruption of the lipid envelope (which likely results in complete inactivation), the nucleocapsid protein of the virus is exposed and remains susceptible to the activity of chemical denaturants including quaternary ammonium compounds (QAC), chlorine compounds, phenols, acids, bases, and some alcohols (67). The viral RNA also becomes susceptible to the activity of nucleic acid denaturants, including various oxidants, acids, and bases (67).

The preliminary evaluation of various detection assays for the recovery of BCV was important to determine the most sensitive assays for detecting how much BCV is present in a sample. Although not required within the EPA guidelines for virucidal testing, it is helpful to understand the limitations with regard to test sensitivity of diagnostic assays, especially when trying to determine if a reduction in titer has occurred. We found endpoint dilution using TCID₅₀ and quantitative *r*RT-PCR using fluorescent detection to be the most sensitive assays for detecting BCV. The greatest challenge for both of these assays to be used for virucidal testing

was the potential for chemical carry-over inducing cytotoxicity issues in the infectivity assay (16, 34, 67) or inhibition or interference of the *r*RT-PCR assay resulting in a possible false negative. We showed that none of the test disinfectants used in our evaluation resulted in either cytotoxicity of the cell culture recovery process (TCID₅₀) or in the inhibition or interference of the *r*RT-PCR assay. The addition of an ultracentrifugation step in our methodology to purify the virus and remove the chemical component of the samples resulted in the removal of cytotoxicity for most disinfectants evaluated (except for DF-200d and Virkon S). The use of gel filtration in special centrifuge tubes has also been shown to be effective for the adequate removal of chemical disinfectants and cytotoxicity concerns (79). Although the DF-200d and Virkon S were cytotoxic even post-ultracentrifugation, all dilutions beyond the first serial 1:10 dilution for both of these disinfectants were non-cytotoxic and met the requirements of the EPA guidelines “showing a minimum of a 3-log reduction in titer beyond the cytotoxic level” (34).

The RNA recovery procedure used in the experiments adequately removed detectable residual RT-PCR inhibitory chemicals. Within the current EPA testing guidelines, the RNA recovery procedure validation process is not included as a necessary requirement to determine if inhibitory chemicals are present because infectious titer recovery is the recommended procedure. Because some viruses such as the noroviruses (31, 32) are not able to be cultivated in a host system, the use of molecular or serological assays for virus recovery would have to be used and such preliminary validation would be very important to ensure that inhibition or interference is not occurring.

We showed that the amount of BCV recovered using TCID₅₀ demonstrated similar efficacy of each test disinfectant for both time exposures (1 or 10 min) in the absence of organic challenge. This relatively short and efficacious contact time for an enveloped virus is consistent

with the Klein and Deforest scheme predicting virus susceptibility to chemical inactivation (47). Other work comparing the efficacy of feline viruses resulted in similar conclusions showing greater susceptibility of enveloped viruses against chemical disinfectants when compared to non-enveloped test strains after a 10 min exposure (79). In separate inactivation research efforts conducted using the Severe Acute Respiratory Syndrome (SARS) virus, also a coronavirus, the virus was found to be sensitive to many chemical treatments resulting in loss of infectivity (28, 73, 92). In one study, chemical treatments with pH conditions of 1 and 3 or 12 and 14 were shown to completely inactivate the SARS virus at 25 and 37°C for 1 h, however formaldehyde and glutaraldehyde at 1:1000 were found to be effective only after 2-3 days (28). In another experiment, the SARS virus was reported to be inactivated by various fixative procedures including 2-isopropanol (70%), Sterillium (45% 2-propanol, 30% 1-propanol), formaldehyde (0.7 and 1%), or glutardaldehyde (0.5%) (73), however the total reduction in titer for most of these was not greater than the 4 log₁₀ infectious titer reduction required in the EPA virucidal guidelines. In a third example, free residue chlorine over 0.5 mg/L for chlorine or 2.19 mg/L for chlorine dioxide were shown to effectively inactivate the SARS virus following a 30 min exposure (92). Most of these experiments were conducted in ideal conditions without the addition of any environmental or organic substrates to challenge the efficacy parameters.

In the presence of two organic challenge matrices (10% soil and feces) used in our evaluation to represent environmental or less than ideal conditions, the virucidal efficacy was reduced in all test disinfectants used. This observed reduction is consistent with current literature suggesting that the presence of organic material likely disrupts the mechanism and efficacy of chemical disinfectants by directly neutralizing the reaction or by serving as a protective barrier disrupting the penetration to the virus particle (57, 67, 76). The current EPA

guidelines for conducting virucidal efficacy testing do not require the addition of any organic challenge even though it is likely that most chemical disinfectants will be negatively affected in their presence and should be included to enhance the overall evaluation.

The amount of BCV RNA recovered post-treatment with the test disinfectants evaluated in the absence of organic challenge did not vary significantly whether exposed for short contact (1 min) or long contact (10 min), indicating that any mechanism occurring was fairly rapid. Not all test disinfectants evaluated in this study degraded nucleic acid. Treatment with 100% DF-200d or with 10% bleach resulted in the greatest amount of BCV RNA degradation. This is likely due to the presence of strong oxidizing agents (hydrogen peroxide in DF-200d or dissociated hypochlorous acid in bleach) which have both been shown to degrade nucleic acid (20, 33). Treatment with 25% or 50% DF-200d also resulted in some degradation of BCV RNA, but not as much as observed with the full strength 100% DF-200d, probably due to the dilution of the oxidizing agent. Treatments which did not appear to degrade BCV RNA at all included 1% bleach, 1% Virkon S, and 70% ethanol. The 1% bleach most likely did not degrade BCV RNA due to the reduced concentration of the reactive dissociated hypochlorous acid, as was observed with the reduced concentrations of DF-200d. Potassium peroxymonosulfate is the active ingredient in 1% Virkon S and is also an oxidizing agent. It is not fully understood why this treatment didn't degrade the BCV RNA, but could be due to the highly acidic nature of this chemistry (ca. 2.0) (5, 52, 57). Viral inactivation has been described as fully occurring when the viral nucleic acid has been rendered non-infectious (52), especially in the case of a positive sense RNA genome such as that of the coronavirus. Although it might be unlikely for RNA to remain infectious in the environment, it is useful to understand the mechanism of action against the limited viral targets (envelope, protein, nucleic acid) as such diagnostic assays based on the

intact presence of these targets could be designed for more rapid determination of viral inactivation.

The recovery of infectious BCV in this study was based on endpoint titration (TCID₅₀) in HRT cells and resulted in a greater than 4 log₁₀ reduction in titer by the Sandia National Laboratories DF-200d (25, 50, 100%), 10% bleach, and 1% Virkon® S following 1 min exposure. Based on the EPA guidelines and criteria for determining virucidal efficacy (34), these disinfectants show complete inactivation meet the criteria for making virucidal claims against BCV. It is likely that these disinfectants would also be effective against the closely related SARS virus based on existing reports using surrogate viruses (84).

In conclusion, we report that various disinfectant chemistries were effective in rapid and complete inactivation of BCV including 10% bleach, 25, 50, and 100% DF-200d, and 1% Virkon® S. Efficacy of all test disinfectants evaluated was reduced in the presence of organic challenge material (feces, soil). The virucidal methodology followed in this study was consistent with the US EPA guidelines for such efficacy testing. Additionally, the use of various diagnostic assays dependent on the intact presence of various viral structures (nucleic acid, protein) provided useful data on possible mechanism of action of the chemical disinfectants evaluated in this study.

Figures and Tables

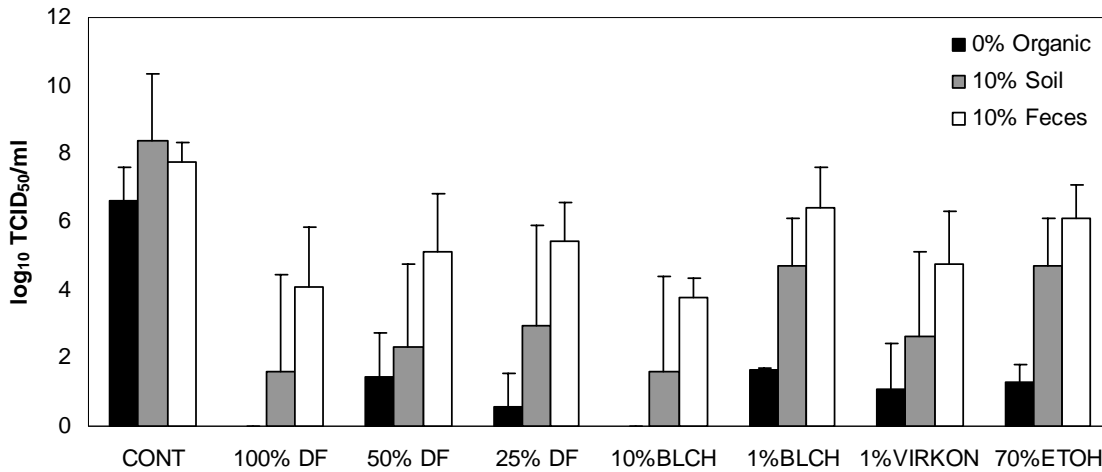


Figure 2.1 Infectivity of BCV as measured by TCID₅₀ following 1 min exposure to test disinfectants in the presence of no organic challenge, 10% soil, and 10% feces.

Error bars indicate 1 standard deviation (n = 3). CONT, control sample using 0.01 M PBS in place of disinfectant; DF, DF-200d; BLCH, bleach; VIRKON, Virkon® S; ETOH, ethanol.

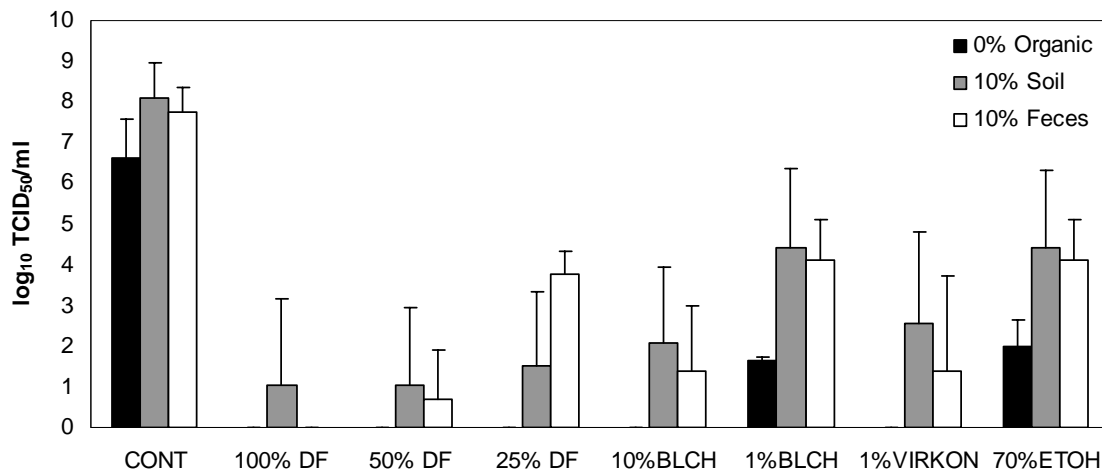


Figure 2.2 Infectivity of BCV as measured by TCID₅₀ following 10 min exposure to test disinfectants in the presence of no organic challenge, 10% soil, and 10% feces.

Error bars indicate 1 standard deviation (n = 3). CONT, control sample using 0.01 M PBS in place of disinfectant; DF, DF-200d; BLCH, bleach; VIRKON, Virkon® S; ETOH, ethanol.

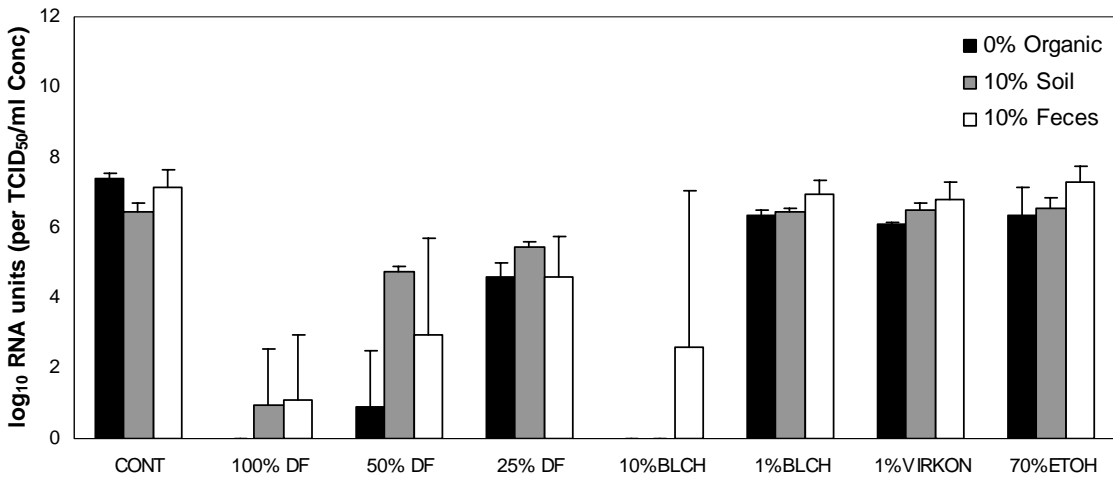


Figure 2.3 Quantitative *r*RT-PCR following 1 min exposure to test disinfectants in the presence of no organic challenge, 10% soil, or 10% feces.

Error bars indicate 1 standard deviation (n = 3). CONT, control sample using 0.01 M PBS in place of disinfectant; DF, DF-200d; BLCH, bleach; VIRKON, Virkon® S; ETOH, ethanol.

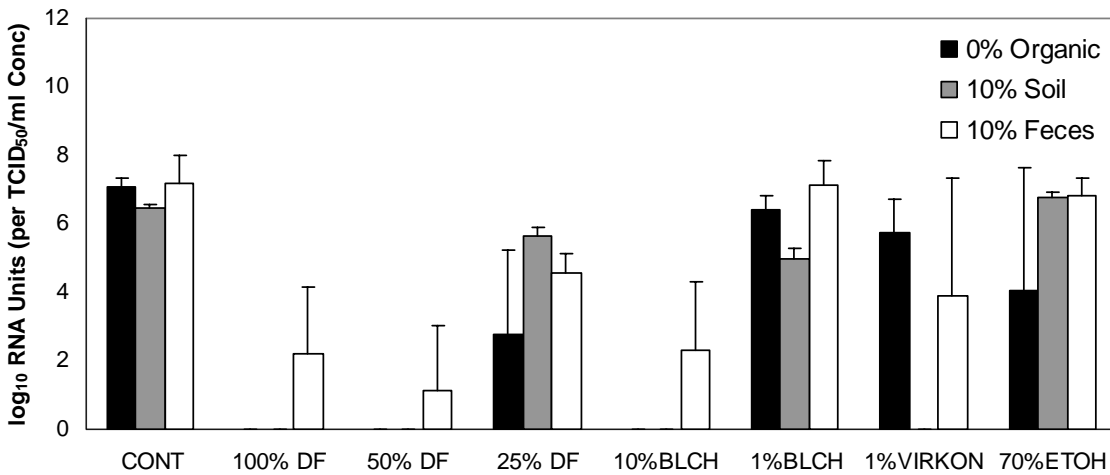


Figure 2.4 Quantitative *r*RT-PCR following 10 min exposure to test disinfectants in the presence of no organic challenge, 10% soil, or 10% feces.

Error bars indicate 1 standard deviation (n = 3). CONT, control sample using 0.01 M PBS in place of disinfectant; DF, DF-200d; BLCH, bleach; VIRKON, Virkon® S; ETOH, ethanol.

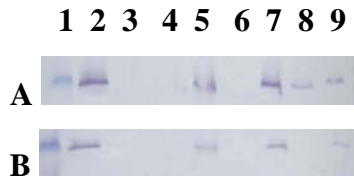


Figure 2.5 Western blot analysis of BCV virus following various treatments with test disinfectants based on the monoclonal 8F2 antibody targeting the nucleocapsid protein.

Samples were treated for 1 min (A) or 10 min (B). Sample order is as follows: 1. 50 kDa protein marker, 2. positive control (BCV); 3. 100% DF-200d; 4. 50% DF-200d; 5. 25% DF-200d; 6. 10% bleach; 7. 1% bleach; 8. 1% Virkon® S; 9. 70% EtOH.

| BCV Conc ^a | Infectivity | | Nucleic Acid | | | Protein | | |
|--------------------------|---------------------------|---------------------------------|-------------------|-------------------|-----------------------|--------------------|-------------------------|-----------------|
| | Log Titer | TCID ₅₀ ^b | EtBr ^c | SYBR ^d | Southern ^e | Probe ^f | 8F2 Mab ^g | HA ^h |
| 1.00E+06 | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) |
| 1.00E+05 | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (1/3) |
| 1.00E+04 | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (0/3) | (0/3) |
| 1.00E+03 | (3/3) ⁱ | (0/3) | (0/3) | (3/3) | (3/3) | (3/3) | (0/3) | (0/3) |
| 1.00E+02 | (2/3) | (0/3) | (0/3) | (0/3) | (3/3) | (0/3) | (0/3) | (0/3) |
| 1.00E+01 | (0/3) | (0/3) | (0/3) | (0/3) | (1/3) | (0/3) | (0/3) | (0/3) |
| 1 | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) |
| ≤ 10 | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) |

Table 2.1 Test sensitivities for various BCV detection assays by infectivity, nucleic acid-based, and protein-based diagnostic procedures.

^a BCV Conc, obtained from tissue culture derived BCV titrated by endpoint dilution and diluted serially to obtain test dilutions.

^b TCID₅₀, tissue culture infecting dose 50.

^c EtBr, ethidium bromide.

^d SYBR, SYBR green.

^e Southern, Southern blot hybridization.

^f Probe, TaqMan probe.

^g 8F2 Mab, monoclonal BCV nucleoprotein (27).

^h HA, hemagglutinin activity

ⁱ Most dilute level of virus resulting in three positive results out of three replicate samples was bolded.

CHAPTER 3 - Virucidal Activity of Biocides against Influenza A Viruses

Abstract

Influenza viruses, namely the avian H5N1 subtype, pose a great threat for pandemic status. Disruption of the transmission cycle could aid in minimizing disease spread with the use of chemical biocides in contaminated environments. In addition, the use of safer less pathogenic influenza viruses in place of the H5N1 could be conducted to safely generate efficacy data. This research indicates that no significant differences existed when comparing disinfectant efficacy data between mammalian or avian influenza, suggesting the use of a less pathogenic influenza A virus in place of a more pathogenic one. We also report that various biocides were effective in rapid and complete inactivation of both mammalian and avian influenza test strains and included DF-200d, 10% bleach, 1% Virkon® S, and 70% ethanol following 10 min exposure. Efficacy of all biocides was reduced in the presence of organic challenge using 10 or 50% poultry feces. Treatment with DF-200d or 10% bleach resulted in significant degradation of viral RNA. The virucidal procedures conducted in this study were consistent with the U.S. Environmental Protection Agency guidelines for virucidal efficacy claim testing.

Introduction

Currently, more than 30 countries have experienced outbreaks of the highly pathogenic avian influenza (HPAI) A virus subtype H5N1 in poultry and other avian species (63). Due to the widespread status of this virus, there is a relatively high risk that an avian influenza pandemic outbreak could occur throughout the world. Increased globalization and the avian influenza virus' natural reservoir among migratory waterfowl (93) translate into a continuing risk for this disease to emergence in the U.S. The use of chemical biocides may aid in the disruption of the

transmission cycle of the influenza virus and aid in containment measures to minimize disease spread and devastation (72, 75).

Efficacy data specific for the H5N1 subtype is not widely available in existing literature. Virucidal data which does exist is often difficult to interpret or compare due to lack of standardized procedures for evaluating chemical biocides. Even less information is available on mechanism of action of various disinfectants as well as the efficacy in the presence of organic challenge. In the U.S., the Environmental Protection Agency (EPA) provides guidelines for efficacy testing (34, 67). The EPA guidelines require that a disinfectant must be validated for each individual organism it claims to be effective against, although this is somewhat vague with regard to interpretation and is unclear whether this includes for example all influenza A viruses versus species specific or actual strains to obtain a product registration. Due to the BSL-3 status of the H5N1 subtype, it is difficult to rapidly generate efficacy data during an outbreak. The EPA currently maintains a list of chemical disinfectants approved for the use against avian influenza A viruses but there are no antimicrobial products registered specifically against the H5N1 subtype (3). This document also states that the U.S. EPA believes that “based on available scientific information that the currently registered avian influenza A products, when applied in strict accordance with label directions, will be effective against the H5N1 strain.” Viruses belonging to the same family are believed to react similarly to chemical inactivation (47, 75), but the use of a surrogate should be validated before making claims on the basis of surrogate virus efficacy testing.

Stringent sanitation and decontamination efforts have been recommended for facilities or premises contaminated in the event of an avian influenza outbreak including transport vehicles, clothing, cages, housing, and other potential contaminated areas (22, 93). Due to the zoonotic

nature of avian influenza, affected premises could range from poultry production housing and growing facilities to human hospital emergency rooms. Therefore, decontamination operations and procedures may occur under a variety of conditions such as on relatively clean surfaces or surfaces that contain a very high organic matter. A pre-cleaning wash step for the removal of organic material is typically recommended for decontamination to occur due to the possible physical protective effect of the virus particles and or the chemical neutralization of the biocide mechanism (23, 75).

Influenza viruses are characterized as having an envelope and containing a segmented negative-stranded RNA genome, with a non-symmetrical shape approximately 80-120 nm in diameter belonging to the family, *Orthomyxoviridae* (51). The influenza virus is composed of approximately 1% nucleic acid (RNA), 70% protein, 20% lipid, and 5-8% carbohydrate which all present as potential targets to the mechanism of inactivation of chemical biocides (51, 75, 88). Virus inactivation is typically accomplished by destroying the lipid envelope (if present), viral proteins such as the structural receptors or capsid, or the viral nucleic acid. Enveloped viruses are generally not considered to be resistant to chemical inactivation due to the relatively easy disruption of the lipid envelope by most chemical biocides (47), but peer-reviewed efficacy data is not widely available in existing literature (8-11, 34). Additionally, parameters including disinfectant concentration, pH, temperature, contact time, virus clumping, and the presence of organic material can severely impact the overall efficacy (67, 72, 75, 82, 88). Efficacy data which is available is often difficult to interpret or compare due to the diversity in methodology for conducting such experiments by the lack in a universally accepted standard for virucidal testing.

The purpose of this study was to evaluate the efficacy of various chemical disinfectants against two strains of influenza A, mammalian H1N1 and low pathogenic avian influenza H5N8, referred to as H1N1 or H5N8, respectively. The purpose of including both test strains in this research was to evaluate the validity for using closely related viruses as surrogates in place of more pathogenic viruses, such as the H5N1, by conducting a statistical comparison of the efficacy results obtained. To evaluate the possible use of surrogate viruses for disinfectant efficacy in place of the more infectious HPAI, statistical analysis comparing the quantitative TCID₅₀ and RNA responses post-treatment between the H1N1 and H5N8 strains was also included. Biocide efficacy was determined following the EPA guidelines for evaluating virucidal efficacy (34). Virus was recovered using tissue culture infective dose 50 (TCID₅₀) in an appropriate host cell line. To evaluate a possible mechanism of inactivation against the viral RNA, a quantitative universal real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay was included post-treatment. Chemical disinfectants used in this study included bleach, ethanol, Virkon[®] S (5), and DF-200d (91). To determine the most effective disinfectants against the influenza A test strains, statistical analysis of the various disinfectant treatments were evaluated measuring the amount of infectious virus recovered post-treatment (as measured by TCID₅₀ and quantitative influenza A RNA concentration) to differences in time of exposure to the treatment, and the presence of different organic sample matrices.

Materials and Methods

Virus and cells. Mammalian influenza A/WSN/33 or low pathogenic avian influenza H5N8 (H5N8, provided generously by the Centers for Disease Control and Prevention, Atlanta, GA) was propagated in Madin-Darby Canine Kidney (MDCK, ATCC CCL-34[™], Manassas, VA) cells to approximately 10⁷ log₁₀ (based on endpoint titration using TCID₅₀). MDCK cells

were maintained in Minimal Essential Medium with Earle's salts and L-glutamine (Invitrogen Corporation, Carlsbad, CA) with the addition of 2.2 g/L sodium bicarbonate (Fisher Scientific, Hampton, NH) collectively referred to as MEM containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) supplemented with 2.5 mg/L amphotericin B, 0.67 g/L streptomycin, and 0.3 g/L penicillin G (all from Fisher Scientific). Infectivity media was made using MEM media containing antibiotics with the addition of 0.1% TPCK treated trypsin (Fisher Scientific).

To determine infectious viral titers, an endpoint method tissue culture infective dose 50 (TCID₅₀) was completed. The Reed-Muench method (74) was calculated in a 96-well format to determine the dilution of virus resulting in infection of fewer than 50% replicate wells. Nunc 96-well cell culture microtiter plates (Fisher Scientific) were seeded with MDCK cells and incubated (approximately 24 hrs, at 37°C with 5% CO₂). Once cells were monolayer, the media was removed and plates were washed once with 0.01 M phosphate buffered saline (PBS, pH 7.2). Tissue culture-derived virus (H1N1 and H5N8) was then serially diluted (ten-fold) in culture media to a 10⁻⁹ dilution. The microtiter plates were inoculated by adding 25 µl of each virus dilution to each well. Negative controls (culture media) were inoculated with 25 µl of uninfected culture media. An additional 75 µl culture media was added to each well and the plate was incubated (48 hrs, 37°C with 5% CO₂). Infectivity was determined by cytopathic effect (CPE) as indicated by the presence of rolling or sloughing of infected cells and TCID₅₀ was calculated using the Reed-Muench method for estimating 50% endpoints of infectivity (74).

Test Disinfectants. Disinfectants previously evaluated against coronavirus (18) were included in this study and included the following: 1 and 10 % bleach, 70% ethanol, Sandia DF-

200 (91), and 1% Virkon® S (Antec®, Suffolk, UK) The pH values of each of the test disinfectants were also measured prior to use.

Organic Matrices. Poultry feces was obtained from healthy (disease free) animals provided generously by the Poultry Science Unit at Kansas State University, Manhattan, KS. Fresh fecal samples were collected from the cage pans and weighed to 10 g and added to 100 ml 0.01 M PBS and mixed thoroughly to prepare the poultry feces solution used for organic challenge. Test disinfectants were challenged with 0, 10, or 50% levels by adding 0, 1, or 5 ml poultry feces to 10, 9, or 5 ml test disinfectant.

Virus Inactivation. Virus inactivation was conducted with 0%, 10%, and 50% organic challenge (poultry feces). For 0% organic challenge, 10 ml of test virus (H1N1 or H5N8) at approximately a 10^7 log₁₀ TCID₅₀/ml concentration was added to 10 ml test disinfectant and exposed for 1 or 10 minutes. For 10% organic challenge, 10 ml of test virus was added to 9 ml of test disinfectants containing 1 ml of 10% poultry feces and exposed for 1 or 10 minutes. For 50% organic challenge, 10 ml of test virus was added to 5 ml of test disinfectants containing 5 ml of 10% poultry feces and exposed for 1 or 10 minutes. After exposure, samples were washed via ultracentrifugation at 100 x G for 1 hr at 4°C. Pellets were reconstituted with 1 ml of virus infectivity media.

Post-treatment Recovery of Infectious Virus. The titers of infectious virus were determined as TCID₅₀ in cell culture using a 96-well format as previously described. The disinfectant samples were serially diluted (1:10) in infectivity media and then 25 µl of each dilution was added in quadruplicate to the 96 well microtiter plates containing confluent MDCK cells. Then, 75 µl infectivity media was added to each well and placed in an incubator for 24-48 hours at 37°C with 5% CO₂. TCID₅₀ was determined by evaluating cytopathic effect (CPE)

typical for influenza viruses as indicated by the presence of rolling or sloughing of infected cells and TCID₅₀ was calculated using the Reed-Muench method (74).

Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR). Viral RNA was recovered using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Quantitative detection of the extracted influenza RNA was conducted using rRT-PCR using a fluorescently labeled TaqMan probe. The rRT-PCR primer and probe sequences were provided generously by the Molecular Genetics Influenza Branch, Centers for Disease Control and Prevention in Atlanta, GA and included a forward primer (5'- CAT GGA RTG GCT AAA GAC AAG ACC -3'), reverse primer (5'- AGG GCA TTT TGG ACA AAK CGT CTA -3') and a fluorescently labeled probe (5' TGC AGT CCT CGC TCA CTG GGC ACG 3') with 6-FAM™ at the 5'-end and Iowa Black Fluorescent Quencher at the 3'-end (Integrated DNA Technologies, Coralville, IA).

For each 25 µl reaction, 2.5 µl of the RNA sample was added to 22.5 µl of master mix was that contained 5 µl OneStep buffer, 1 µl dNTP, 1 µl enzyme (all from Qiagen's OneStep RT-PCR kit), and 0.5 µl each of the forward and reverse primers (1 µM concentration), 2 µl probe (5 µM concentration), and 12.5 µl RNase-free water. The SmartCycler thermocycler was set with the following settings: Stage 1 hold (42°C, 1800 s without optics), Stage 2 hold (95°C, 900 s without optics), Stage 3 was repeated 35 times using a 3-temperature cycle (94°C, 15 s without optics; 58°C, 15 s without optics, and 72°C, 30 s with optics on). The detection threshold for successfully detecting influenza RNA was a FAM fluorescence signal ≥ 3 using the SmartCycler.

Inhibition of rRT-PCR. To determine if the reverse transcriptase (RT) or polymerase chain reaction (PCR) activity were inhibited by residual or carry-over of the active chemicals

present in the test disinfectants in the RNA recovery process, the test disinfectants were evaluated. The test disinfectants were prepared as previously described and then the samples were extracted using the QIAamp Viral RNA Mini Kit (Qiagen). Following this extraction process (in which no RNA is present) 1.25 μ l from each of the extracted test disinfectants was inoculated with 1.25 μ l purified tissue culture derived H1N1 or H5N8 RNA (extracted using the same process) to obtain the total 2.5 μ l volume required for the 25 μ l *r*RT-PCR reaction. A positive *r*RT-PCR (based on a FAM fluorescence signal ≥ 3 using the SmartCycler) result would indicate that no inhibition or interference was occurring. A negative result would indicate that there was inhibition or interference occurring to the *r*RT-PCR assay.

Post-treatment Recovery of Viral RNA. A standard curve using the *r*RT-PCR assay was developed by extracting known starting amounts of TCID₅₀ derived H1N1 and H5N8. Serial 1:10 dilutions of the extracted RNA were prepared in RNAase-free water (Qiagen). Then, *r*RT-PCR was conducted on these samples to generate cycle threshold (Ct) values. A standard curve was generated based on these Ct values using the SmartCycler software. The slope of the standard curve was calculated and used to quantify the amount of viral RNA recovered post-treatment.

Post-treated viral RNA was recovered from the test samples using the QIAamp Viral RNA Mini Kit. Quantitative detection of the RNA was determined by a FAM fluorescence signal ≥ 3 using the SmartCycler. Ct values obtained were converted to a quantitative measure of RNA based on the standard curve generated using known amounts of starting virus (reported as log₁₀ RNA/TCID₅₀ titer).

Statistical Analysis. Statistical evaluation comparing of the level of test influenza (H1N1 or H5N8) recovered (for infectivity by TCID₅₀ or for RNA by quantitative *r*RT-PCR) following

the experimental treatment parameters was determined by comparing 1-way, 2-way, and 3-way interactions between disinfectant treatment, time (1, 10 min), and organic challenge (0%, 10%, 50% poultry feces). The least square of means was calculated to determine if significant differences were observed in any of the experiments within each test virus as well as comparing H1N1 to results obtained with H5N8 (for potential validation of a surrogate). A significant difference resulted in a P-value < 0.05.

Results

Post-treatment Recovery of Infectious Virus. The average range of untreated (positive control) virus recovered by TCID₅₀ ranged from 6.68 to 7.27 (H1N1) or 6.72 to 7.55 (H5N8) log₁₀ TCID₅₀/ml (Figures 3.1-3.3). No significant statistical differences were observed when comparing the amount of infectious H1N1 recovered versus the amount of infectious H5N8 recovered for the various experimental parameters (treatment, time, organic challenge) in this study, P > 0.05 (Figures 3.1-3.3) suggesting that these viruses are suitable surrogates within the orthomyxovirus family. Following a 1 min treatment without the addition of organic challenge, the most effective disinfectants were DF-200d and 10% bleach resulting in > 4.0 log₁₀ reduction in titer required by EPA for virucidal efficacy claims (Figure 3.1). Following a 10 min treatment, all treatments except for 1% bleach and 70% ethanol resulted in a > 4 log₁₀ TCID₅₀/ml reduction in viral titer, (Figure 3.1). The addition of 10% organic challenge significantly reduced the efficacy of all test disinfectants except for DF-200d or 10% bleach evaluated for both 1 and 10 min treatments (Figure 3.2), P < 0.05. The addition of 50% organic challenge significantly reduced the efficacy of all test disinfectants bleach evaluated for both 1 and 10 min treatments (Figure 3.3), P < 0.05.

Post-treatment Recovery of Viral RNA. None of the test disinfectants evaluated in this research demonstrated inhibition or inhibition of the *r*RT-PCR assay based on a positive reaction following the initial inhibition evaluation. This finding is necessary to determine that any reduction which does occur in the amount of H1N1 or H5N8 RNA recovered post-disinfectant treatment is most likely explained by some mechanism of that particular disinfectant degrading the viral nucleic acid as opposed to inhibition or interference of the reaction.

A standard curve was generated for both H1N1 and H5N8 RNA based on the subsequent Ct values obtained from the purified RNA and dilutions (extracted from pre-determined TCID₅₀ values of infectious virus) using the SmartCycler system. To generate the quantitative efficacy data, Ct values obtained with the disinfection samples for both H1N1 and H5N8 were calculated by the following equation:

$$y = m(x) + b; y = \text{quantitative RNA unit/per TCID}_{50} \text{ value, } m = -0.254 \text{ (H1N1) or } -0.246 \text{ (H5N8), } x = \text{Ct value, } b = 11.459 \text{ (H1N1) or } 9.888 \text{ (H5N8)}$$

The amount of untreated (positive control) viral RNA recovered ranged between 6.44 to 6.88 (H1N1) or 5.75 to 5.99 (H5N8) log₁₀ RNA/TCID₅₀ calculated as the fluorescence intensity of the FAM reporter divided by the Ct value (RNA FAM/Ct). No significant statistical differences were observed when comparing the amount of H1N1 RNA recovered versus the amount of H5N8 RNA recovered for the experimental parameters (treatment, time, organic challenge) in this study, $P > 0.05$ (Tables 3.1-3.3), regardless of organic challenge amount. Without the addition of organic challenge, treatment with DF-200d or 10% bleach resulted in significant reduction in the amount of viral RNA recovered post-treatment for 1 and 10 min contact times. The degradation of viral RNA by DF-200 or 10% bleach was significantly

reduced for both 1 or 10 min in the presence of 10% and 50% organic challenge (Tables 3.2 and 3.3), $P < 0.05$.

Discussion

It is not well documented how the influenza A virus is inactivated by chemical biocides. In order to better understand the chemical inactivation of disinfectants against the influenza virus, the efficacy of various test disinfectants was studied using two strains of influenza A (mammalian H1N1 and low pathogenic avian influenza H5N8). The use of the two closely related influenza strains was included to determine how similar the inactivation results are for different subtypes of the same family of virus and to determine if a less pathogenic virus (such as LPAI H5N8) could be evaluated in place of a more infectious one (like HPAI H5N1). No significant differences were observed in the efficacy data obtained for the mammalian H1N1 isolate or the LPAI H5N8 isolate suggesting that these viruses are well suited as surrogates for one another. It would be expected that the HPAI H5N1 virus would react similarly to chemical biocides due to its genetic relatedness to each of these test viruses and being in the same viral family, *Orthomyxoviridae* (95). The chemical inactivation of the influenza test isolates was evaluated based on infectivity assays (using TCID₅₀ titration) following the current EPA testing guidelines (34) to determine the efficacy compared to untreated positive control samples. Two time contacts were included in this study (1 or 10 min) as well as three levels of organic challenge consisting of poultry feces (0, 10, or 50%). DF-200 and 10% bleach were the most efficacious with a short contact time (1 min) for inactivating both influenza test strains by $> 4.0 \log_{10}$ reduction in titer. Following a longer contact time (10 min), all test disinfectants except for 1% bleach were efficacious against both influenza test strains by $> 4.0 \log_{10}$ reduction in titer. The efficacy of all test disinfectants was reduced in the presence or organic challenge. A

quantitative *r*RT-PCR assay was included in this study to determine if degradation of viral nucleic acid was occurring as a possible mechanism of action of the chemical disinfectants. Treatment with DF-200d or 10% bleach resulted in significant reduction with regard to the amount of viral RNA recovered post-treatment for 1 and 10 min contact times and were the only treatments found to degrade nucleic acid. The degradation of RNA was significantly reduced in the presence of organic challenge.

Due to the high level of containment required for the H5N1 virus, the less pathogenic mammalian H1N1 and avian H5N8 were evaluated in this study to determine their potential use as surrogate viruses for efficacy testing based on the similarity in efficacy. It is expected that viruses among the same viral family will react similarly to chemical inactivation (47, 57, 68, 83). Infectious titer results obtained from the TCID₅₀ assays in this study showed similar efficacy results with no significant differences when comparing both influenza test strains (H1N1 or H5N8) for all experimental parameters including time contact and organic challenge. Examples of accepted surrogate viruses in current EPA testing guidelines include duck hepatitis B virus for hepatitis B virus, bovine diarrhea virus for hepatitis C virus, feline calicivirus for norovirus (34, 83). Bovine coronavirus or avian infectious bronchitis virus have also been suggested as safe surrogate viruses for disinfectant assays for the SARS virus based on the general expectation that viruses belonging to the same virus family react similarly to chemical disinfection (83). These surrogates are generally used if the intended virus is not culturable or when high level of containment is necessary and less pathogenic viruses are acceptable.

In this research, determination of infectious titer post disinfectant treatment was conducted based on endpoint dilution in TCID₅₀ format. The greatest challenge for this assay was the potential for chemical carry-over which would cause cytotoxicity to the MDCK cells

(16, 67) and ultimately prevent the recovery of any residual infectious virus. According to current EPA virucidal guidelines, disinfectants which still have some cytotoxic effect must “show a minimum of a 3-log reduction in titer beyond the cytotoxic level” (34). To overcome this issue, samples were washed via ultracentrifugation to pellet the virus and remove the chemical biocide. DF-200d and Virkon S both remained cytotoxic at the least dilute sample in the TCID₅₀ recovery process even post-ultracentrifugation, but resulted in negative CPE and cytotoxic effects for the 3-log₁₀ dilution requirement included in the current EPA guidelines. Gel filtration in addition to centrifugation has been shown to remove potential cytotoxicity prior to infectivity recovery processes (79). Other examples to overcome cytotoxicity issues in virucidal testing include neutralization of the chemical mechanism or dilution of the disinfectant (40), but reduce the detection limit of the assay depending on how much diluting is required.

The influenza viruses used in this study were inactivated by > 4.0 log₁₀ reduction in titer following a 1 min exposure. This rapid response to chemical inactivation is consistent with the Klein and Deforest scheme (48) for virus susceptibility predictions indicating that enveloped viruses are most susceptible to chemical inactivation compared to non-enveloped viruses (67). This is most likely a result of the relatively easy disruption of the lipid envelope by the biocides used in this study, and not necessarily the disruption of other viral targets including the protein or nucleic acid (59, 75, 88). Reduced efficacy in the presence of 10 and 50% levels of organic challenge was observed with all biocides evaluated. The presence of organic material is known to disrupt biocide mechanism and overall efficacy by a few mechanisms (57, 67, 72, 75, 88). Direct neutralization of the chemical activity of the biocide by the compounds of the organic material could disrupt efficacy. Organic material might also disrupt the efficacy of viral inactivation by diluting the disinfectant and lowering the overall effect. Another theory is that

the organic material provides a physical protection of the viral particles which prevents the interaction of the biocide and viral target.

The recovery of the influenza test strains used in this study based on endpoint titration (TCID₅₀) in MDCK cells indicate that the infectious virus was reduced by > 4 Log₁₀ following 1 min treatment with DF-200d and 10% bleach. High standard deviations were apparent for efficacy results using 1% Virkon® S and 70% ethanol at this time exposure. Treatment for 10 min resulted in > 4 Log₁₀ reduction in titer for all biocides except for 1% bleach, and could therefore be labeled with virucidal claims according to the current EPA guidelines (34). The addition of organic challenge reduced the efficacy for all biocides evaluated in this testing. Treatment for 10 min with DF-220d or 10% bleach for both 10 and 50% organic challenge levels still maintained a > 4.0 log₁₀ reduction in virus titer, and were the least effected by the disruption mechanisms in the presence of organic material (57, 68, 72, 77). Treatment with either DF-200d or 10% bleach would be recommended for virucidal clean-up of a contaminated poultry facility based on these findings and the likelihood for organic soiling in such an environment.

Another challenge in this research was the potential for inhibition or interference of the *rRT-PCR* assay due to chemical carry-over from the test biocides in the RNA recovery procedure for evaluating the possible effect of the chemical biocides on the nucleic acid. A preliminary evaluation of the test biocides was conducted to verify that no inhibition was occurring (based on a positive *rRT-PCR* reaction) after inoculating the samples with purified viral RNA to verified that the RNA recovery procedure used in this testing successfully removed any inhibitory or interfering chemicals. Treatment with DF-200d or 10% bleach were the only two biocides resulting in a significant effect degrading viral RNA. The amount of H1N1 or H5N8 RNA recovered post-treatment did not vary significantly when comparing responses

obtained following short contact (1 min) or long contact (10 min), suggesting that most of the degradation occurs rapidly. DF-200d and 10% bleach are characterized as strong oxidizing agents with hydrogen peroxide being the active ingredient in DF-200d or dissociated hypochlorous acid as the active ingredient in bleach (20, 33, 59). The nucleic acid degradation was significantly minimized in the presence of organic challenge, likely due to neutralizing, diluting, or protecting mechanisms discussed previously (57, 67, 68, 72, 77). Treatment with 1% Virkon S or 70% ethanol did not result in the degradation of the viral RNA. Therefore, efficacy results from these treatments is most likely a result of disrupting the viral envelope or structural proteins necessary for successful viral replication (47, 52, 55, 57, 59, 88, 95).

The recovery of influenza RNA based on *r*RT-PCR resulted in significant nucleic acid degradation occurring following treatment with 10% bleach and 100% DF-200d. The degradation of viral RNA is most likely a result of the strong oxidizing mechanism from the dissociation of hypochlorous acid from the 10% bleach solution (33) or the hydrogen peroxide and peracetate acid formation occurring in the chemistry of the 100% DF-200d (20). The other test disinfectants evaluated in this research (25 and 50% DF-200d, 1% bleach, 1% Virkon S, 70% ethanol) did not demonstrate any degradation occurring when compared to the non-treated positive control samples. It is likely that any loss of infectivity occurring from these treatments was a result of action against the viral envelope or structural proteins (47, 52, 55, 57, 68, 95).

Separate disinfectant studies with influenza viruses have been conducted but are difficult to compare due to differences in the test methodologies used for determining the virucidal efficacy. In one report, an LPAI H7N2 virus was found to be completely inactivated by exposure to 70% ethanol for approximately 30 min (54). This study also showed that H7N2 was sensitive to an acidic pH value (of 2) and relatively high temperature (56°C) after approximately

30 min exposure. Shorter contact times were not evaluated, so inactivation could have occurred faster than what was actually evaluated. In another report, virucidal evaluation was conducted using a formaldehyde-containing disinfectant and an organic acid-based disinfectant against an LPAI H7N1 isolate following the guidelines of the German Veterinary Association to address efficacy at reduced temperatures and in the presence of organic material using fetal calf serum (96). This study found that both disinfectants were efficacious if applied at 20°C, but efficacy was significantly reduced at 10 or 4°C and in the presence of organic material. Disinfectant efficacy has also been reported against a nonpathogenic H7N2 avian isolate with the addition of antifreezing compounds to four biocides (phenol, quaternary ammonium, quaternary ammonium combined with formaldehyde, and sodium hypochlorite) to address efficacy at reduced temperatures (29). The study was conducted following a modification of the use-dilution analysis of the Association of Official Agricultural Chemists for bacteria and found that the antifreezing compounds reduced the efficacy for the quaternary ammonium combined with formaldehyde and the sodium hypochlorite disinfectants but not the phenol or quaternary ammonium compounds.

Influenza viruses have also been shown to persist on various surfaces, which could aid in disease spread if not properly decontaminated. In an environmental study conducted using influenza A and B viruses, infectious virus could be recovered from non-porous surfaces (such as metal or plastic) for up to 48 h and from materials such as cloth, paper, or tissue for up to 12 h with 35-40% humidity at 28°C (14). In addition to surface decontamination, influenza virus has also been shown to spread via aerosols, large droplets, or contact with infectious secretions or fomites (87), further emphasizing the need for stringent sanitation and disinfection regimens to be in place to aid in containment efforts (94).

Due to the potential effect that chemical biocides might have on viral nucleic acid, one study was conducted to evaluate this effect on avian influenza H7N2 detected by real time RT-PCR (86). In this study phenol, quaternary ammonium, Virkon S, and sodium hypochlorite were evaluated using a 10 min or 1 hr contact time to determine if degradation of viral RNA was occurring. Results reported for the 1 hr contact time indicate that all disinfectants were found to be effective at inactivating the virus by an infectivity assay, but only treatment with Virkon S and sodium hypochlorite were found to degrade the RNA. The data on degrading nucleic acid was not reported for the 10 min exposure. In our study, the viral RNA was degraded significantly by 10% sodium hypochlorite, but not significantly by Virkon S. Based on these results, it's likely that degradation of the nucleic acid does occur with Virkon S but requires the longer contact time.

In conclusion, we report that various disinfectant chemistries were effective in rapid and complete inactivation of both mammalian influenza H1N1 and LPAI H5N8 including 10% bleach, 50 and 100% DF-200d, and 1% Virkon® S following a 10 min exposure based on the current EPA guidelines for evaluating the efficacy of chemical disinfectants for virucidal activity (34). There were no significant differences in the efficacy results obtained between the two test strains indicating efficacy of all test disinfectants evaluated was reduced in the presence of organic challenge due to both a neutralizing effect of the chemical mechanism as well as a physical protective effect of the matrix for the virus particles.

Figures and Tables

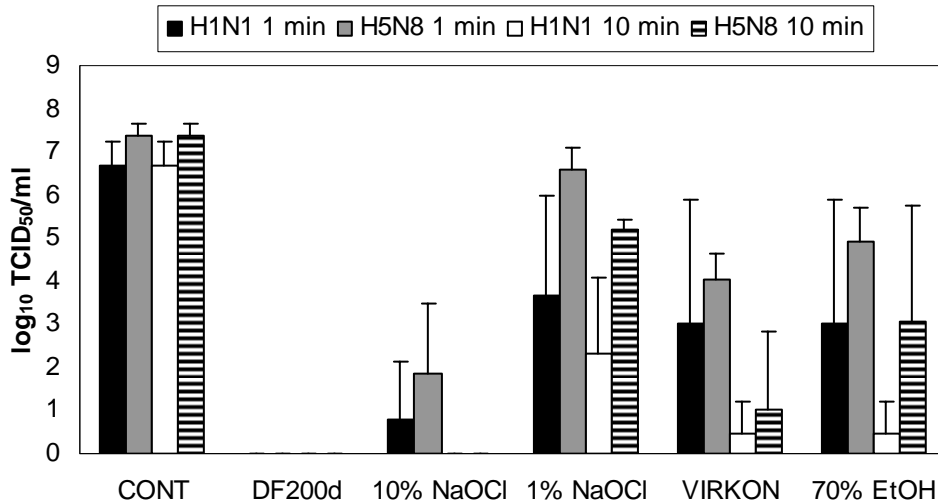


Figure 3.1 Infectivity of H1N1 and H5N8 as measured by TCID₅₀/ml following 1 and 10 min exposure to chemical biocides with no organic challenge.

CONT, Control (0.01 M PBS in place of disinfectant); DF-200d, Decontamination Formulation; 10% NaOCl, 10% sodium hypochlorite; 1% NaOCl, 1% sodium hypochlorite; VIRKON, 1% Virkon S; 70% EtOH, 70% ethanol

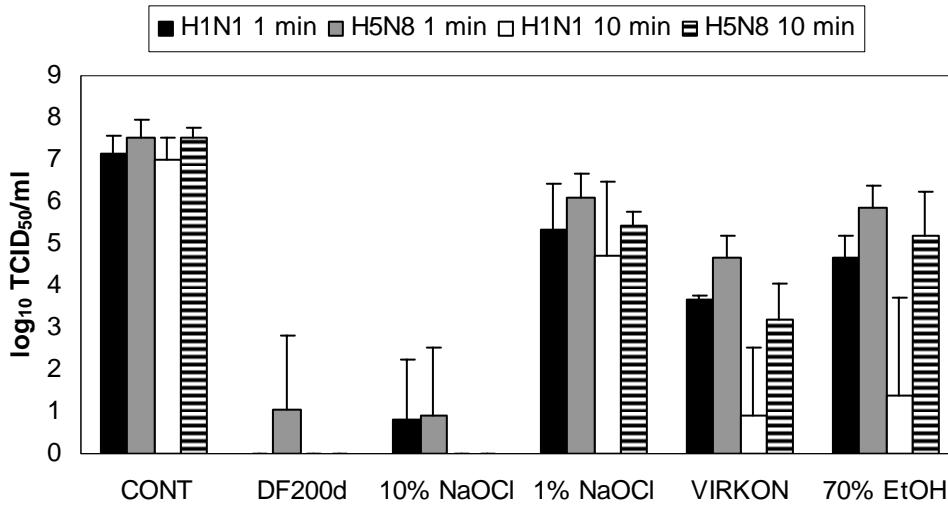


Figure 3.2 Infectivity of H1N1 and H5N8 as measured by TCID₅₀/ml following 1 and 10 min exposure to chemical biocides in the presence of 10% feces.

CONT, Control (0.01 M PBS in place of disinfectant); DF-200d, Decontamination Formulation; 10% NaOCl, 10% sodium hypochlorite; 1% NaOCl, 1% sodium hypochlorite; VIRKON, 1% Virkon S; 70% EtOH, 70% ethanol

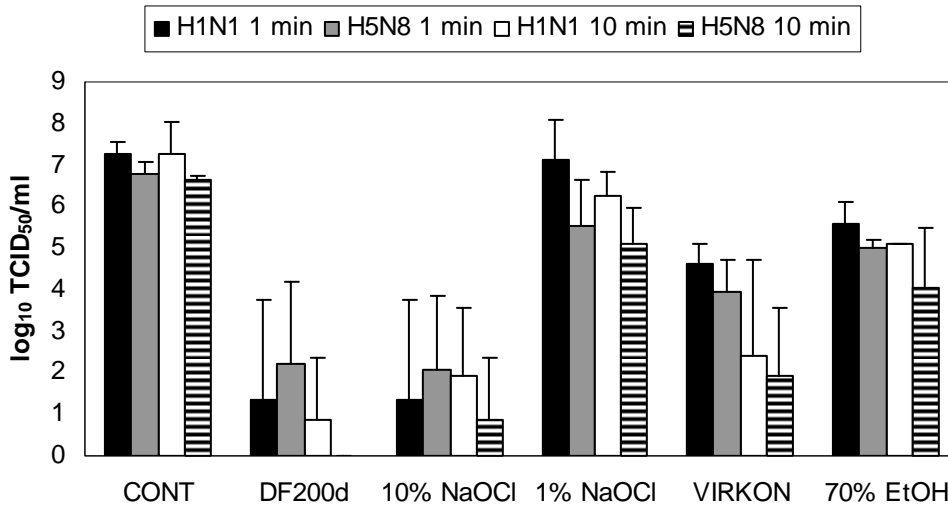


Figure 3.3 Infectivity of H1N1 and H5N8 as measured by TCID₅₀/ml following 1 and 10 min exposure to chemical biocides in the presence of 50% feces.

CONT, Control (0.01 M PBS in place of disinfectant); DF-200d, Decontamination Formulation; 10% NaOCl, 10% sodium hypochlorite; 1% NaOCl, 1% sodium hypochlorite; VIRKON, 1% Virkon S; 70% EtOH, 70% ethanol

| Treatment | 1 min | | 10 min | |
|------------------------|-------|------|--------|------|
| | H1N1 | H5N8 | H1N1 | H5N8 |
| Cont ^a | 6.88 | 5.86 | 6.70 | 5.89 |
| DF200d ^b | 1.33 | 2.05 | 0.00 | 0.62 |
| 10% NaOCl ^c | 0.86 | 0.00 | 0.00 | 0.96 |
| 1% NaOCl ^d | 5.85 | 5.37 | 6.68 | 5.86 |
| VIRKON ^e | 5.92 | 5.47 | 4.54 | 5.25 |
| 70% EtOH ^f | 5.69 | 5.88 | 6.91 | 5.74 |

Table 3.1 Quantitative *r*RT-PCR reported as log₁₀ RNA/TCID₅₀ units following 1 or 10 minute exposure to test disinfectants in the presence of no organic challenge.

^a Cont, Control (0.01 M PBS in place of disinfectant)

^b DF-200d, Decontamination Formulation

^c 10% NaOCl, 10% sodium hypochlorite

^d 1% NaOCl, 1% sodium hypochlorite

^e VIRKON, 1% Virkon S

^f 70% EtOH, 70% ethanol

| Treatment | 1 min | | 10 min | |
|------------------------|-------|------|--------|------|
| | H1N1 | H5N8 | H1N1 | H5N8 |
| Cont ^a | 6.78 | 5.99 | 6.55 | 5.91 |
| DF200d ^b | 3.05 | 3.44 | 1.09 | 1.54 |
| 10% NaOCl ^c | 0.00 | 0.00 | 0.00 | 0.85 |
| 1% NaOCl ^d | 6.65 | 5.84 | 6.41 | 6.06 |
| VIRKON ^e | 6.57 | 5.30 | 6.42 | 4.91 |
| 70% EtOH ^f | 6.66 | 5.83 | 6.49 | 5.39 |

Table 3.2 Quantitative *r*RT-PCR reported as log₁₀ RNA/TCID₅₀ units following 1 or 10 minute exposure to test disinfectants in the presence of 10% organic challenge.

^a Cont, Control (0.01 M PBS in place of disinfectant)

^b DF-200d, Decontamination Formulation

^c 10% NaOCl, 10% sodium hypochlorite

^d 1% NaOCl, 1% sodium hypochlorite

^e VIRKON, 1% Virkon S

^f 70% EtOH, 70% ethanol

| Treatment | 1 min | | 10 min | |
|------------------------|-------|------|--------|------|
| | H1N1 | H5N8 | H1N1 | H5N8 |
| Cont ^a | 6.44 | 5.75 | 6.47 | 5.88 |
| DF200d ^b | 5.82 | 4.77 | 5.80 | 4.87 |
| 10% NaOCl ^c | 6.01 | 5.51 | 6.26 | 5.23 |
| 1% NaOCl ^d | 5.84 | 5.66 | 5.94 | 5.81 |
| VIRKON ^e | 6.19 | 5.49 | 6.37 | 5.40 |
| 70% EtOH ^f | 6.47 | 5.42 | 6.94 | 5.79 |

Table 3.3 Quantitative *r*RT-PCR reported as log₁₀ RNA/TCID₅₀ units following 1 or 10 minute exposure to test disinfectants in the presence of 50% organic challenge.

^a Cont, Control (0.01 M PBS in place of disinfectant)

^b DF-200d, Decontamination Formulation

^c 10% NaOCl, 10% sodium hypochlorite

^d 1% NaOCl, 1% sodium hypochlorite

^e VIRKON, 1% Virkon S

^f 70% EtOH, 70% ethanol

CHAPTER 4 - Inactivation of Foot and Mouth Disease Virus and Bovine Enterovirus

Abstract

Foot and Mouth Disease virus (FMDV) is recognized as one of the most economically important diseases of food animals. This virus is highly contagious and can persist in the environment for extended periods, making containment and eradication efforts difficult. Chemical decontamination of affected premises could aid in disease containment and facility restoration. The purpose of this study was to evaluate the chemical inactivation of FMDV and a closely related virus, bovine enterovirus (BEV), to determine if surrogate efficacy testing is valid for FMDV. In this study, 10 min treatment with 5% acetic acid, 10% bleach, 4% sodium carbonate, 2% sodium hydroxide, and Sandia DF-200 were effective at inactivating FMDV. Treatment with 5% acetic acid was not effective for inactivating BEV. Treatment with 70% ethanol was not effective for inactivating FMDV or BEV. Additionally, 10 min treatment with 10% bleach, 2% sodium hydroxide, and Sandia DF-200 were shown to degrade the nucleic acid (RNA) of both test viruses.

Introduction

Foot and Mouth disease virus (FMDV) is a highly infectious disease occurring in cloven-hoofed animals and is known to spread by direct contact from infected animals, contaminated animal products such as feces, urine, milk, semen, by the airborne route, and by mechanical means from contaminated surfaces, people, and by vehicles (1, 78). Consequently, FMDV is considered one of the most infectious diseases known to exist (41) and rapid containment and eradication efforts are necessary to minimize animal suffering and economic loss.

Decontamination of the infected premises during an outbreak is recommended during containment and eradication efforts before naïve animals can be reintroduced.

Existing literature in the area of disinfectant efficacy specific for FMDV is of limited value by virtue of the diverse testing methodologies used. Furthermore, much of the published data was produced in the late 1950's to late 1970's (12, 26, 35-37, 66, 81), and precedes current standardization and guidelines issued by various agencies for virucidal testing (8-11, 15, 24, 30, 34). Additionally, strong evidence exists to support the survival and persistence of FMDV in the environment if inadequate decontamination measures are in place (26, 37, 38, 42, 66, 71, 80, 89), further supporting the need to have virucidal data available for FMDV. The U. S. Environmental Protection Agency (EPA) has published guidelines for evaluating disinfectants for virucidal claims and also recommends that testing be conducted against a specific viral target (34). In some cases, disinfectant efficacy testing with surrogate viruses is allowed in the case of non-culturable pathogens including duck hepatitis B virus for hepatitis B virus, bovine diarrhea virus for hepatitis C virus, and feline calicivirus for norovirus (34, 83). Due to the difficulty in working with FMDV in the U. S. (which can only be conducted at the Plum Island Foreign Animal Disease Center in Greenport, NY), the potential use of a surrogate virus for disinfectant efficacy testing is highly desired.

Strong evidence exists to support the survival and persistence of FMDV in the environment making containment and eradication difficult (26, 37, 38, 42, 66, 71, 80, 89). Although culling, slaughter, and vaccination programs are often used as the major eradication efforts for FMDV outbreaks, affected premises cannot be released to susceptible populations of animals until adequate decontamination and restoration efforts are completed and verified due to the persistence of this virus. FMDV belongs to the *Picornaviridae* family, and as a non-

enveloped virus is more resistant than enveloped viruses to chemical inactivation according to the Klein and Deforest virus susceptibility prediction scheme (47). Because FMDV does not have a lipid envelope, chemical biocides must degrade or denature the capsid proteins and or the nucleic acid material to effectively inactivate the virus (75). In addition to the resistance of this virus, the presence of organic material such as feces, urine, blood, or soil in the affected premises can also disrupt the mechanism of the chemical biocide by diluting, neutralizing, or preventing contact to the target (72).

Viruses are divided into several subgroups with regard to their resistance to disinfectants. Differences observed are based largely on the presence or absence of a viral envelope and the size of the virus particle. According to the Klein and Deforest (47) schematic for dividing viral groups, the least resistant to disinfectants are the enveloped viruses (ie. influenza, coronavirus); those with moderate resistance are the large non-enveloped viruses (ie. adenovirus); and those with the most resistance are the small non-enveloped viruses (ie. picornavirus, parvovirus) including FMDV. These subgroups of viruses are often termed category A, C, and B, respectively. Category A and C are the least resistant, and disinfectants such as hypochlorite, alkalis, oxidizing agents, and aldehydes are all very effective (7). Category B viruses, including FMDV, are the most resistant viruses and great care must be taken to ensure complete inactivation.

The purpose of this study was to evaluate the efficacy of various chemical disinfectants against bovine enterovirus (BEV) under BSL2 conditions and against FMDV under BSL3 conditions to consider the use of BEV as a possible surrogate virus against FMDV for generating virucidal efficacy data. Suspension tests were used to evaluate several chemical biocides following published EPA virucidal testing methods. Statistical analysis was conducted

comparing the quantitative tissue culture infective dose TCID₅₀ and quantitative RNA responses resulting for BEV and FMDV following treatment with the test disinfectants. Virus was recovered using the TCID₅₀ method in an appropriate host cell line. A quantitative real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay specific for BEV or FMDV was also evaluated to determine if disinfectant chemistry resulted in significant degradation of viral RNA. In both recovery systems, statistical analysis was conducted to determine if the disinfectant treatment resulted in significant reduction in viral titer when compared to non-treated positive control samples. Chemical disinfectants used in this study included acetic acid, bleach, ethanol, sodium carbonate, sodium hydroxide, and novel chemistries including Sandia DF-200 (91), Oxy-Sept® 333 (2), and Virkon® S (5). Sandia DF-200 is a unique blend of quaternary ammonium compounds and peroxide for synergistic mechanism of action. Oxy-Sept® 333 is an oxidizer with surfactant ingredients to aid in surface penetration and to resist breakdown in the presence of organic compounds. Virkon® S is an oxidizer with surfactant ingredients to aid in surface penetration, but initial cleaning is recommended to remove heavy organic material. Disinfectants were exposed for 10 or 20 min and challenged in the presence of 0, 10, or 50% bovine feces to represent organic soiling of an infected premise.

Materials and Methods

Virus and cells. BEV-2 (ATCC VR-754, Manassas, VA) was propagated in Madin Darby Bovine Kidney (MDBK, ATCC CCL-22) cells. FMDV type O1 strain Brugge was propagated in Baby Hamster Kidney 21 (BHK-21, ATCC CCL-10) cells. MDBK and BHK-21 cells were propagated in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT)

supplemented with 2.5 mg/L amphotericin B, 0.67 g/L streptomycin, and 0.3 g/L penicillin. MDBK cells were infected with BEV-2 without the addition of 10% FBS. BHK-21 cells were infected with FMDV using 4% FBS. Inoculum titer was assessed using tissue culture infective dose 50, TCID₅₀ and calculated by the Reed-Muench method (74).

Test Disinfectants. A 5% solution of acetic acid (Fisher Scientific, Hampton, NH) was prepared by adding 5 ml acetic acid to 95 ml sterile deionized water. A 10% solution of bleach was prepared by adding 10 ml concentrated Clorox® (6.15% sodium hypochlorite, Oakland, CA) to 90 ml sterile deionized water. A 70% solution of ethanol (100% anhydrous ethanol, Fisher Scientific) was prepared by adding 70 ml 100% ethanol to 30 ml sterile deionized water. A 4% solution of sodium carbonate (Fisher Scientific) was prepared by adding 4 g sodium carbonate to 100 ml sterile deionized water. A 2% solution of sodium hydroxide (Fisher Scientific) was prepared by adding 2 g sodium hydroxide to 100 ml sterile deionized water. Decon Foam (Sandia DF-200, Albuquerque, NM) (91), was supplied by Sandia National Laboratories and was prepared by mixing 2.45 ml of Part 1 (surfactants and fatty acids) to 0.1 ml Part 3 (a diacetin-based propellant) and then 2.45 ml of Part 2 (hydrogen peroxide, 7.99%) was added and mixture was thoroughly mixed to yield the 100% test concentration of DF-200. Oxy-Sept® 333 was purchased from Ecolab® (St. Paul, MN) and a 0.4% recommended solution was prepared by adding 400 µl Oxy-Sept® 333 to 100 ml sterile deionized water. Virkon® S was purchased from DuPont Animal Health Solutions (Antec®, Suffolk, UK) and a 1% solution recommended by the manufacturer was prepared by adding 0.1 g to 10 ml sterile deionized water. Test disinfectants were prepared fresh each time prior to use. The pH values of each of the test disinfectants were also measured and recorded prior to use.

Organic Matrices. Bovine feces was collected from healthy (disease free) animals. A 10-g portion of fresh fecal sample was to 100 ml 0.01 M PBS, mixed thoroughly and centrifuged (400 RCF, 10 min) to clarify the fecal solution to be used for organic challenge. Test disinfectants were challenged with 0, 10, or 50% levels by adding 0, 1, or 5 ml bovine fecal solution to 10, 9, or 5 ml test disinfectant.

Virus Inactivation. Virus inactivation was conducted with 0%, 10%, and 50% organic challenge (bovine feces) using current EPA virucidal testing guidelines (34). For 0% organic challenge, 10 ml of test virus (BEV-2 or FMDV) at approximately a $10^6 \log_{10} \text{TCID}_{50}/\text{ml}$ concentration was added to 10 ml test disinfectant and exposed for 10 or 20 min. For 10% organic challenge, 10 ml of test virus was added to 9 ml of test disinfectants containing 1 ml bovine feces and exposed for 10 or 20 min. For 50% organic challenge, 10 ml of test virus was added to 5 ml of test disinfectants containing 5 ml bovine feces and exposed for 10 or 20 minutes. Following exposure, disinfectant samples were washed via ultracentrifugation at 100 x G for 1 hr at 4°C. Pellets were reconstituted with 1 ml infectivity media.

Post-treatment Recovery of Infectious Virus. The titers of infectious virus were determined as TCID_{50} in cell culture using a 96-well format. The disinfectant samples were serially diluted (1:10) in infectivity media and then 25 μl of each dilution was added in quadruplicate to the 96 well microtiter plates containing confluent MDBK or BHK-21 cells for BEV-2 or FMDV, respectively. Then, 75 μl infectivity media was added to each well and plates were placed in an incubator for 24-48 hours at 37°C with 5% CO_2 . TCID_{50} was determined visually by evaluating cytopathic effect (CPE) typical for BEV-2 and FMDV as indicated by the presence of rolling or sloughing of infected cells and TCID_{50} was calculated using the Reed-Muench method (74).

Real-Time Reverse Transcription Polymerase Chain Reaction (*rRT-PCR*). BEV-2 or FMDV RNA was recovered using the RNeasy Kit (Qiagen, Valencia, CA). Quantitative detection of the extracted RNA was conducted using *rRT-PCR* using a fluorescently labeled TaqMan probe. The *rRT-PCR* and probe sequences for BEV-2 were obtained from Onodera and Melcher, 2002 (64) and for FMDV obtained from Callahan et al., (2002) (21).

For BEV-2 *rRT-PCR*, the OneStep RT-PCR kit (Qiagen) was used. For FMDV *rRT-PCR*, the GeneAmp® Ez rTth RNA PCR kit (Applied Biosystems, Foster City, CA). For each 25 µl reaction, 2.5 µl of the RNA sample was added to 22.5 µl of master mix prepared following the manufacturers' recommendation for the OneStep RT-PCR kit or the GeneAmp® Ez rTth RNA PCR kit. The SmartCycler® thermocycler conditions are shown in Table 2 for BEV-2 and FMDV.

Inhibition of *rRT-PCR*. To determine if inhibition or interference was occurring in the *rRT-PCR* assays for either BEV-2 or FMDV, the test disinfectants were prepared as control solutions as previously described and then were extracted using the RNeasy Mini Kit. Following this extraction process (in which no RNA is present) 1.25 µl from each of the extracted test disinfectants was inoculated with 1.25 µl purified tissue culture derived BEV-2 or FMDV RNA (extracted using the same process) to obtain the total 2.5 µl volume required for the 25 µl *rRT-PCR* reaction. The *rRT-PCR* assay was conducted for each sample and a positive *rRT-PCR* based on a FAM fluorescence signal ≥ 3 would indicate that no inhibition or interference was occurring (18). A negative result would indicate that there was inhibition or interference occurring to the *rRT-PCR* assay.

Post-treatment Recovery of Viral RNA. In order to have a quantitative *rRT-PCR* for BEV-2 or FMDV, a standard curve was generated using the SmartCycler®. BEV-2 or FMDV

were titrated using TCID₅₀ and RNA was purified using the RNeasy Mini Kit as previously described. Serial 1:10 dilutions of the extracted RNA were prepared in RNAase-free water (Qiagen). Then, *r*RT-PCR was conducted on both viral samples to generate subsequent Ct values for each dilution. A standard curve was generated based on these Ct values using the SmartCycler® software by incorporating the TCID₅₀ derived titers for the resulting Ct value. The slope of the standard curve was calculated and used to quantify the amount of viral RNA recovered post-treatment.

Post-treated viral RNA was recovered from the test samples using the RNeasy Mini Kit. Quantitative detection of the RNA was determined by a FAM fluorescence signal ≥ 3 using the SmartCycler® using *r*RT-PCR assays for BEV-2 or FMDV. Ct values obtained were converted to a quantitative measure of RNA based on the standard curve generated using known amounts of starting virus (reported as log₁₀ RNA/TCID₅₀ titer).

Statistical Analysis. An analysis to assess the statistical differences in the level of virus (BEV-2 or FMDV) recovered by TCID₅₀ or by quantitative *r*RT-PCR following the experimental treatment parameters was carried out by comparing 1-way, 2-way, and 3-way interactions between disinfectant treatment, time (10, 20 min), and organic challenge (0%, 10%, 50% feces). The least square of means was calculated to determine if significant differences were observed comparing BEV-2 efficacy results to FMDV efficacy results. A significant difference between treatments is inferred with a P-value ≤ 0.05 .

RESULTS

Post-treatment Recovery of Infectious Virus. The average infectious titer (non-treated, positive control) was 6.04 or 6.50 log₁₀ TCID₅₀/ml for BEV-2 or FMDV, respectively (Tables 4.2- 4.3). The pH values of each test disinfectant are shown in Table 4.1. Acidic disinfectants

included 5% acetic acid, 0.4% Oxy-Sept® 333, and 1% Virkon® S. Basic disinfectants included 10% bleach, 4% sodium carbonate, 2% sodium hydroxide, and Sandia DF-200. 70% Ethanol was considered neutral. Effective treatments were inferred by observations of a statistically significant reduction ($P \leq 0.05$) in infectious titer of each test virus when compared to the non-treated positive control sample. In addition to statistical significance, the total reduction in infectious titer must be $\geq 4.0 \log_{10}$ TCID₅₀/ml to meet the current requirement for virucidal efficacy in the U. S. EPA guidelines (34). If no infectious virus could be recovered in the TCID₅₀ assay based on the absence of CPE, then the detection limit ($1.80 \log_{10}$ TCID₅₀/ml) was reported.

Following 10 min exposure with the experimental disinfectants, effective treatments (statistically significant and resulting in a $> 4.0 \log_{10}$ reduction in titer) for both viruses included 10% bleach, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S (Table 4.2). Ten-min treatments with 5% acetic acid or 4% sodium carbonate were effective against FMDV, but not BEV-2 (Table 4.2). Treatment with 70% ethanol did not result in a significant reduction in titer for BEV-2 or FMDV, $P > 0.05$. There was no significant effect of the presence of organic material (10 or 50% bovine feces) disrupting the mechanism of action of the experimental disinfectants in this study ($P > 0.05$) as shown in Table 4.2.

Following 20 min exposure with the experimental disinfectants, effective treatments (statistically significant and resulting in a $> 4.0 \log_{10}$ reduction in titer) for both viruses included 10% bleach, 4% sodium carbonate, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S (Table 4.3). Twenty-min treatment with 5% acetic acid was effective against FMDV, but not BEV-2 (Table 4.3). Twenty min treatment with 70% ethanol did not result in a significant reduction in titer for BEV-2 or FMDV ($P > 0.05$). As with the 10 min

recovery results, there was no significant effect of the presence of organic material (10 or 50% bovine feces) disrupting the mechanism of action of the experimental disinfectants in this study ($P > 0.05$) as shown in Table 4.3.

Post-treatment Recovery of Viral RNA. The average amount of non-treated positive control viral RNA recovered was 5.65 or 7.04 \log_{10} as measured from predetermined $TCID_{50}/ml$ titers for BEV-2 or FMDV, respectively (Tables 4.4, 4.5). Effective disinfectant treatments are indicated by a statistically significant reduction ($P \leq 0.05$) in the amount of RNA detected for both viruses when compared to the amount of RNA recovered from the non-treated positive control samples. Only results obtained with 0 and 10% bovine feces are reported in Tables 4.4 and 4.5 as the organic challenge with 50% bovine feces was found to be inhibitory of the overall *rRT-PCR* assay.

Following 10 min exposure with the experimental disinfectants, effective treatments for both viruses included 10% bleach, 2% sodium hydroxide, and Sandia DF-200 (Table 4.4). Ten min treatment with 4% sodium carbonate was effective against FMDV RNA, but not BEV-2 RNA (Table 4.4). Treatments with 5% acetic acid, 70% ethanol, 0.4% Oxy-Sept® 333, or 1% Virkon® S did not result in a significant reduction of BEV-2 or FMDV RNA ($P > 0.05$). With most disinfectants, there was a significant effect in the presence of 10% organic material against the mechanism for degrading viral RNA ($P \leq 0.05$) as shown in Table 4.4.

Following 20 min exposure with the experimental disinfectants, effective treatments for both viruses included 10% bleach, 2% sodium hydroxide, and Sandia DF-200 (Table 4.5). Twenty min treatment with 4% sodium carbonate or Oxy-Sept® 333 was effective against FMDV RNA, but not BEV-2 RNA (Table 4.5). Treatments with 5% acetic acid, 70% ethanol, or 1% Virkon® S did not result in a significant reduction of BEV-2 or FMDV RNA ($P > 0.05$). A

significant effect in the presence of 10% organic material against the mechanism for degrading viral RNA was observed for most disinfectants evaluated ($P \leq 0.05$) as shown in Table 4.5.

Discussion

Available data on virucidal efficacy of chemical biocides against FMDV suffers from diverse or non-standardized methodology used for generating such data and is somewhat outdated, with much of this work being conducted during the late 1950s through the 1970s (35, 36, 66, 81). To better understand the virucidal efficacy of chemical biocides against FMDV, the efficacy of various disinfectants against FMDV type O1 strain Brugge was evaluated. To evaluate the use of a possible surrogate virus for generating efficacy data under BSL-2 conditions, bovine enterovirus (BEV) was also evaluated as a member of the *Picornaviridae* virus family. Biocide efficacy was determined using the EPA guidelines requiring a total reduction in infectious titer $\geq 4.0 \log_{10}$ TCID₅₀/ml (18, 34). Infectious virus was recovered and quantified by TCID₅₀. Treatment with 10% bleach, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S exposed for 10 min resulted in the inactivation of both viruses. Treatment with 5% acetic acid or 4% sodium carbonate was effective against FMDV, but not BEV-2. Treatment with 70% ethanol was not effective against BEV-2 or FMDV. Treatment with 10% bleach, 4% sodium carbonate, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S exposed for 20 min resulted in the inactivation of both viruses. A quantitative *rRT-PCR* assay specific for BEV or FMDV was also evaluated to evaluate the effect on viral RNA post-treatment with the chemical biocides. BEV-2 and FMDV RNA was significantly reduced following 10 min exposure to 10% bleach, 2% sodium hydroxide, and Sandia DF-200, with 4% sodium carbonate being efficacious against FMDV RNA, but not BEV-2 RNA. Effective treatments following a 20 min exposure were the same as

for the 10 min, except treatment with 0.4% Oxy-Sept® 333 was also effective against FMDV RNA, but not BEV-2 RNA.

It is generally accepted that viruses closely related or sharing characteristic features such as being enveloped or non-enveloped will react similarly to chemical inactivation, and is the basis for the Klein and Deforest prediction schemes for virus susceptibility to disinfectants (47, 68). In this study, BEV-2 was evaluated as a possible surrogate for FMDV in an effort to generate efficacy data under less stringent BSL-2 conditions. In addition, FMDV can only be handled at the Plum Island Foreign Animal Disease Center in Greenport, NY, making it even more difficult to generate disinfectant efficacy results. Our results showed that FMDV was consistently inactivated by 5% acetic acid at both 10 and 20 min exposure, but that BEV-2 was not. This is most likely explained due to the enteric nature of BEV-2, which must be able to survive in the acidic conditions of the gastric environment during the course of its infectious life cycle (65). FMDV is characterized as being unstable at a pH less than 6.5 (72), so inactivation by acidic compounds is not surprising. BEV-2 was shown to be inactivated by two of the acidic disinfectants including 0.4% Oxy-Sept® 333 and 1% Virkon® S, even though it was resistant to 5% acetic acid. This is most likely a result of the novel chemistries of these two disinfectants including a surfactant which likely aided in the oxidative efficacy regardless of pH. An important consideration for acidic or basic disinfectants is the stability of the pH in the presence of organic material. As long as the active pH is maintained, the mechanism for inactivation against pH-sensitive viruses such as FMDV should still occur (66, 81).

In this study, the presence of organic material at 10 or 50% bovine feces did not appear to disrupt the mechanism of action of the test disinfectants evaluated. This is not consistent with previous observations evaluating the inactivation of bovine coronavirus (18), or with information

widely available in the literature regarding virus inactivation (15, 42, 47, 57, 68, 71, 75). The presence of organic material has been described as breaking down the chemical mechanism for inactivation by dilution, neutralization, and physical protection of the virus particles (71). It is not fully clear why the organic material levels evaluated in this study did not appear to have any significant effect on the observed efficacy. It is possible that if a reduced contact time (less than 10 min) which would most likely not result in complete virus inactivation by most disinfectants was evaluated, that some effect of the organic material might be more apparent or significant.

Degradation of viral RNA as a mechanism of inactivation was shown to occur with 10 min treatment with 10% bleach, 2% sodium hydroxide, and Sandia DF-200 for both viruses. Following a 20 min treatment, 4% sodium carbonate and 0.4% Oxy-Sept® 333 appeared to degrade FMDV RNA, but not BEV-2 RNA. The use of the quantitative *r*RT-PCR assays for the recovery of BEV-2 and FMDV RNA were similar to infectious titer recovery by TCID₅₀ assay for 10% bleach, 2% sodium hydroxide, or Sandia DF-200. Although 4% sodium carbonate, 0.4% Oxy-Sept® 333, and 1% Virkon® S all resulted in significant reductions of infectious titer, there was not a significant reduction in the amount of viral RNA present. This finding suggests that these disinfectants are efficacious through the denaturing or breakdown of the capsid protein which would prevent the infectious life cycle. The use of *r*RT-PCR as a measure of the efficacy of a decontamination effort could only be applied following treatment with 10% bleach, 2% sodium hydroxide, or Sandia DF-200.

In conclusion, several disinfectants were found to be effective for inactivating FMDV following the current EPA guidelines for virucidal efficacy claims and included 5% acetic acid, 10% bleach, 4% sodium carbonate, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S if exposed for 10 min. BEV-2, a closely related member in the same

viral family as FMDV, showed elevated resistance to the acid 5% acetic acid. Due to the differences in pH stability, BEV-2 or any other enteric virus would most likely not be a suitable surrogate for a pH-sensitive agent such as FMDV. Other non-enteric viruses among the *Picornaviridae* virus family should be considered for possible use as an appropriate surrogate.

Figures and Tables

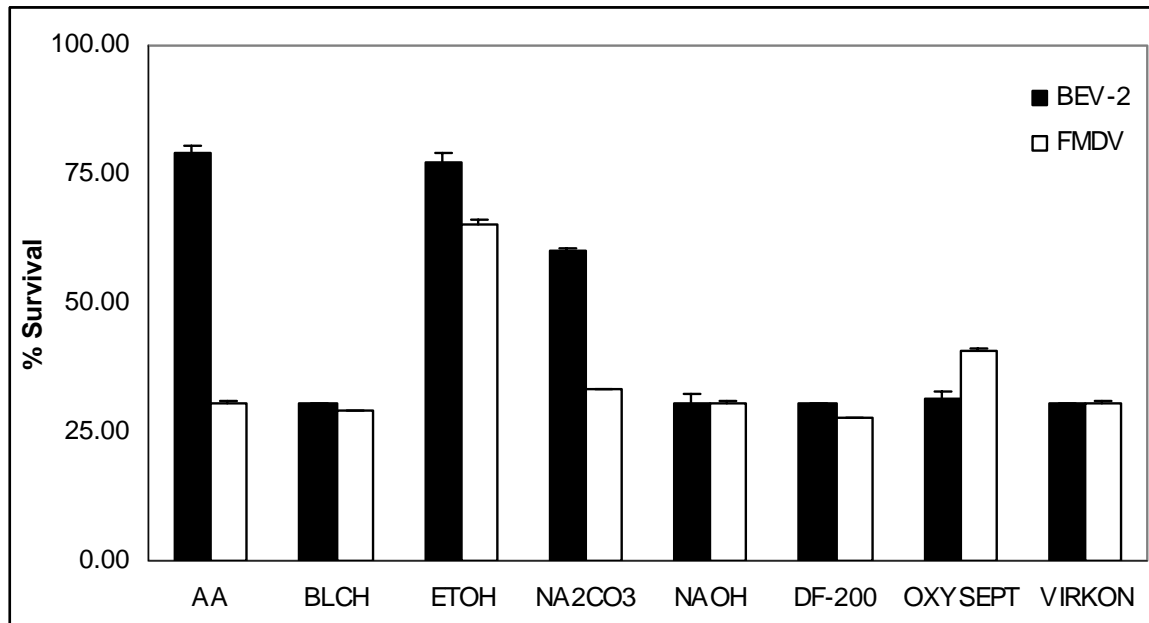


Figure 4.1 The percentage survival rate for BEV-2 and FMDV as recovered by TCID₅₀ following 10 min exposure to test disinfectants in the presence of no organic challenge.

Error bars indicate 1 standard deviation (n = 3). AA, 5% acetic acid; BLCH, 10% bleach; ETOH, 70% ethanol; NA₂CO₃, 4% sodium carbonate; NAOH, 2% sodium hydroxide; DF-200, Sandia Decon Foam; OXYSEPT, 0.4% Oxy-Sept® 333; VIRKON, 1% Virkon® S.

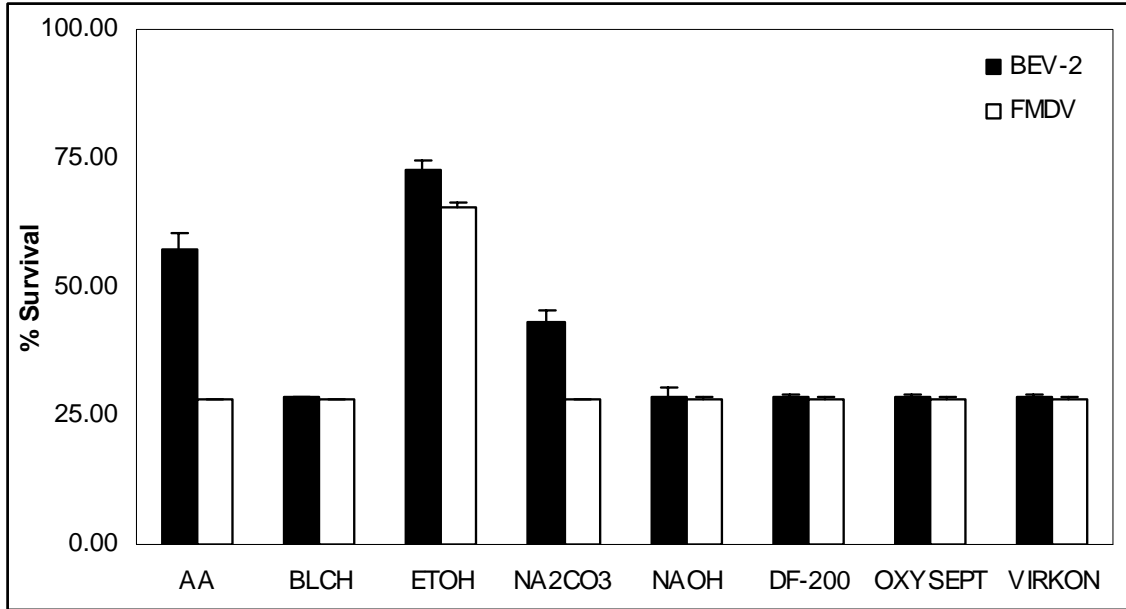


Figure 4.2 The percentage survival rate for BEV-2 and FMDV as recovered by TCID₅₀ following 20 min exposure to test disinfectants in the presence of no organic challenge.

Error bars indicate 1 standard deviation (n = 3). AA, 5% acetic acid; BLCH, 10% bleach; ETOH, 70% ethanol; NA₂CO₃, 4% sodium carbonate; NAOH, 2% sodium hydroxide; DF-200, Sandia Decon Foam; OXYSEPT, 0.4% Oxy-Sept® 333; VIRKON, 1% Virkon® S.

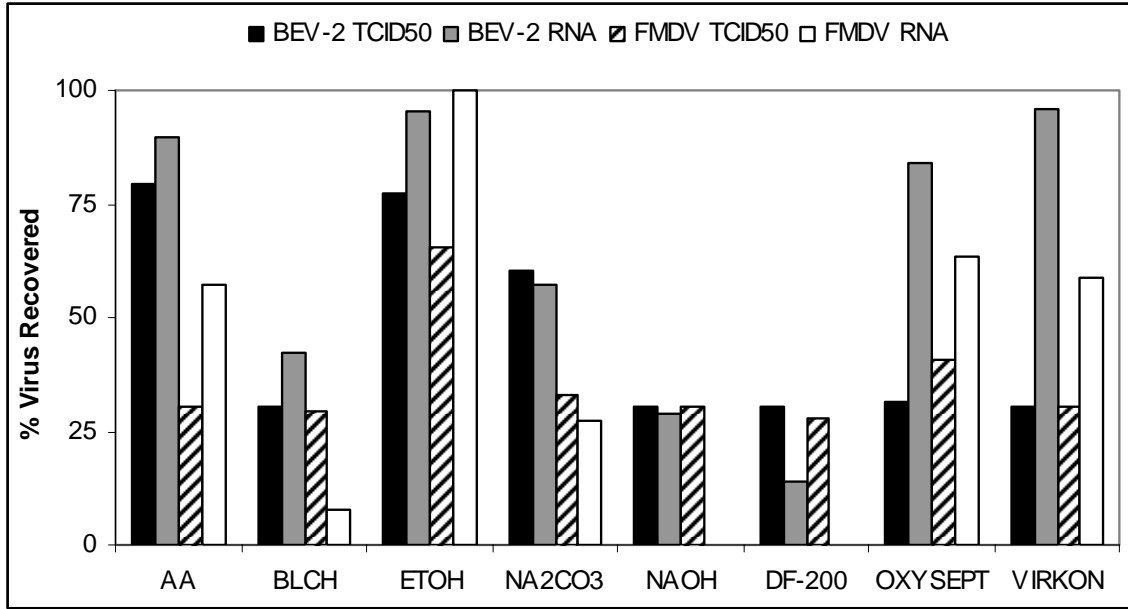


Figure 4.3 The percentage virus recovered by TCID₅₀ or quantitative *rRT-PCR* for BEV-2 and FMDV following 10 min exposure to test disinfectants in the presence of no organic challenge.

AA, 5% acetic acid; BLCH, 10% bleach; ETOH, 70% ethanol; NA₂CO₃, 4% sodium carbonate; NAOH, 2% sodium hydroxide; DF-200, Sandia Decon Foam; OXYSEPT, 0.4% Oxy-Sept® 333; VIRKON, 1% Virkon® S.

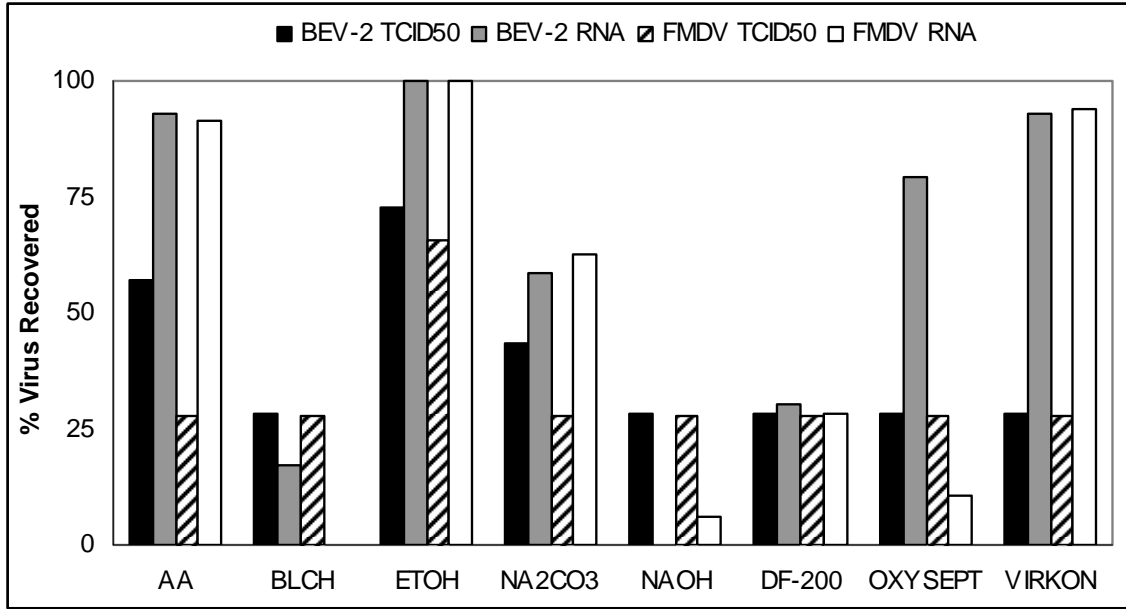


Figure 4.4 The percentage virus recovered by TCID₅₀ or quantitative *rRT-PCR* for BEV-2 and FMDV following 20 min exposure to test disinfectants in the presence of no organic challenge.

AA, 5% acetic acid; BLCH, 10% bleach; ETOH, 70% ethanol; NA₂CO₃, 4% sodium carbonate; NAOH, 2% sodium hydroxide; DF-200, Sandia Decon Foam; OXYSEPT, 0.4% Oxy-Sept® 333; VIRKON, 1% Virkon® S.

| Disinfectant | pH Value |
|---------------------|-----------------|
| 5% acetic acid | 2.3 |
| 10% bleach | 11.54 |
| 70% ethanol | 6.68 |
| 4% sodium carbonate | 11.71 |
| 2% sodium hydroxide | 12.01 |
| Sandia DF-200 | 9.95 |
| 0.4% Oxy-Sept® 333 | 2.44 |
| 1% Virkon® S | 2.45 |

Table 4.1 Average pH values for test disinfectants.

| Treatment | BEV-2 | | | FMDV | | |
|--|-----------------|-------------|-------------|-------------|-------------|-------------|
| | 0% ^a | 10% | 50% | 0% | 10% | 50% |
| POS ^b | 5.88 | 5.99 | 5.77 | 6.44 | 6.43 | 6.64 |
| AA ^c | 4.66* | 3.62 | 4.99 | 1.97 | 1.97 | 1.88 |
| BLCH ^d | 1.80 | 1.80 | 1.80 | 1.88 | 1.88 | 2.05 |
| ETOH ^e | 4.54 | 4.66 | 4.66 | 4.22 | 4.47 | 5.38 |
| NA ₂ CO ₃ ^f | 3.55 | 3.25 | 3.10 | 2.13 | 2.20 | 1.80 |
| NAOH ^g | 1.80 | 1.80 | 1.96 | 1.97 | 2.17 | 2.05 |
| DF-200 ^h | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 |
| OXYSEPT ⁱ | 1.85 | 2.88 | 2.77 | 2.63 | 2.30 | 1.88 |
| VIRKON ^j | 1.80 | 1.80 | 1.85 | 1.97 | 2.13 | 1.80 |

Table 4.2 Infectivity of BEV-2 and FMDV as measured by TCID₅₀ following 10 min exposure to test disinfectants in the presence of test organic concentrations of bovine feces (0%, 10%, 50%).

^a % Bovine feces

^b POS, Positive untreated control sample

^c AA, 5% acetic acid

^d BLCH, 10% bleach

^e ETOH, 70% ethanol

^f NA₂CO₃, 4% sodium carbonate

^g NAOH, 2% sodium hydroxide

^h DF-200, Sandia decon foam

ⁱ OXYSEPT, 0.4% Oxy-Sept® 333

^j VIRKON, 1% Virkon® S

* Bolded numbers are significantly different between BEV-2 and FMDV

| Treatment | BEV-2 | | | FMDV | | |
|--|-----------------|-------------|-------------|-------------|-------------|-------------|
| | 0% ^a | 10% | 50% | 0% | 10% | 50% |
| POS ^b | 6.33 | 5.99 | 6.25 | 6.44 | 6.43 | 6.64 |
| AA ^c | 3.62* | 3.73 | 3.52 | 1.80 | 1.80 | 1.80 |
| BLCH ^d | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 |
| ETOH ^e | 4.61 | 4.88 | 4.88 | 4.22 | 6.97 | 5.55 |
| NA ₂ CO ₃ ^f | 2.73 | 2.93 | 2.88 | 1.80 | 1.80 | 1.80 |
| NAOH ^g | 1.80 | 1.80 | 1.96 | 1.80 | 1.80 | 1.80 |
| DF-200 ^h | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 |
| OXYSEPT ⁱ | 1.80 | 1.80 | 1.96 | 1.80 | 1.80 | 1.80 |
| VIRKON ^j | 1.80 | 1.80 | 1.85 | 1.80 | 1.80 | 1.80 |

Table 4.3 Infectivity of BEV-2 and FMDV as measured by TCID₅₀ following 20 min exposure to test disinfectants in the presence of test organic concentrations of bovine feces (0%, 10%, 50%).

^a % Bovine feces

^b POS, Positive untreated control sample

^c AA, 5% acetic acid

^d BLCH, 10% bleach

^e ETOH, 70% ethanol

^f NA₂CO₃, 4% sodium carbonate

^g NAOH, 2% sodium hydroxide

^h DF-200, Sandia decon foam

ⁱ OXYSEPT, 0.4% Oxy-Sept® 333

^j VIRKON, 1% Virkon® S

* Bolded numbers are significantly different between BEV-2 and FMDV

| Treatment | <u>BEV-2</u> | | <u>FMDV</u> | |
|--|-----------------|-------------|-------------|-------------|
| | 0% ^a | 10% | 0% | 10% |
| POS ^b | 5.48 | 5.65 | 6.43 | 7.91 |
| AA ^c | 4.93 | 5.08 | 3.69 | 4.93 |
| BLCH ^d | 2.33* | 4.73 | 0.48 | 3.44 |
| ETOH ^e | 5.24 | 5.11 | 6.63 | 6.76 |
| NA ₂ CO ₃ ^f | 3.14 | 4.01 | 1.76 | 4.73 |
| NAOH ^g | 1.59 | 2.22 | 0.00 | 4.45 |
| DF-200 ^h | 0.76 | 1.55 | 0.00 | 2.43 |
| OXYSEPT ⁱ | 4.61 | 4.79 | 4.07 | 5.38 |
| VIRKON ^j | 5.25 | 4.86 | 3.78 | 5.24 |

Table 4.4 Amount of BEV-2 and FMDV RNA (log₁₀) based on a standard curve generated from RNA extracted from predetermined levels of both viruses (quantified by TCID₅₀) as measured by quantitative *r*RT-PCR following 10 min exposure to test disinfectants in the presence of test organic concentrations of bovine feces (0%, 10%, 50%).

^a % Bovine feces

^b POS, Positive untreated control sample

^c AA, 5% acetic acid

^d BLCH, 10% bleach

^e ETOH, 70% ethanol

^f NA₂CO₃, 4% sodium carbonate

^g NAOH, 2% sodium hydroxide

^h DF-200, Sandia decon foam

ⁱ OXYSEPT, 0.4% Oxy-Sept® 333

^j VIRKON, 1% Virkon® S

* Bolded numbers are significantly different between BEV-2 and FMDV

| Treatment | <u>BEV-2</u> | | <u>FMDV</u> | |
|--|-----------------|-------------|-------------|-------------|
| | 0% ^a | 10% | 0% | 10% |
| POS ^b | 5.48 | 5.98 | 5.91 | 7.91 |
| AA ^c | 5.11 | 4.94 | 5.39 | 5.37 |
| BLCH ^d | 0.93 | 2.53 | 0.00 | 0.48 |
| ETOH ^e | 5.76 | 5.26 | 6.63 | 7.23 |
| NA ₂ CO ₃ ^f | 3.21 | 3.43 | 3.71 | 4.78 |
| NAOH ^g | 0.00 | 1.89 | 0.37 | 4.45 |
| DF-200 ^h | 1.66 | 3.30 | 1.68 | 3.44 |
| OXYSEPT ⁱ | 4.34 | 3.05 | 0.64 | 5.38 |
| VIRKON ^j | 5.10 | 5.22 | 5.54 | 7.68 |

Table 4.5 Amount of BEV-2 and FMDV RNA (log₁₀) based on a standard curve generated from RNA extracted from predetermined levels of both viruses (quantified by TCID₅₀) as measured by quantitative *r*RT-PCR following 20 min exposure to test disinfectants in the presence of test organic concentrations of bovine feces (0%, 10%, 50%).

^a % Bovine feces

^b POS, Positive untreated control sample

^c AA, 5% acetic acid

^d BLCH, 10% bleach

^e ETOH, 70% ethanol

^f NA₂CO₃, 4% sodium carbonate

^g NAOH, 2% sodium hydroxide

^h DF-200, Sandia decon foam

ⁱ OXYSEPT, 0.4% Oxy-Sept® 333

^j VIRKON, 1% Virkon® S

* Bolded numbers are significantly different between BEV-2 and FMDV

CHAPTER 5 - Inactivation of Foot and Mouth Disease Virus on Environmental Surfaces

Abstract

Foot and Mouth Disease virus (FMDV) is of great economic importance and highly contagious. This virus is known to persist in the environment and survive on various surfaces for extended periods, making containment and eradication efforts difficult. Chemical decontamination and restoration of contaminated facilities is necessary prior to the re-introduction of susceptible animals. The purpose of this study was to evaluate the virucidal efficacy of chemical biocides using US EPA testing guidelines against FMDV inoculated onto concrete, rubber, or stainless steel. Experimental exposures (5, 10, 20 min) were evaluated with 5% acetic acid, 10% bleach, 70% ethanol, 4% sodium carbonate, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, or 1% Virkon® S. All treatments except 70% ethanol were effective under EPA requirements following 10 and 20 min contact on rubber and stainless steel. No treatments were shown to be effective under EPA requirements for concrete, due to a low recovery of virus from this surface. Additionally, 10 and 20 min treatment with 10% bleach, 2% sodium hydroxide, and Sandia DF-200 were shown to degrade the viral nucleic acid (RNA) based on a quantitative *r*RT-PCR assay.

Introduction

Foot and Mouth Disease virus (FMDV), which infects cloven-hoofed animals, is considered one of the most infectious diseases known to exist and is undoubtedly one of the most economically important diseases (41). Prospects for disease control include culling, slaughter, and vaccination to prevent the spread of virus by contact with infected animals, animal products,

fomites, or aerosol (46). Decontamination and facility restoration of the contaminated premises during an FMDV outbreak would aid in disease containment and eradication and is required before naïve animals can be reintroduced.

Two predominant methods for evaluating virucidal efficacy of disinfectants exist in the form of guidelines and include suspension and carrier tests (67, 75, 82). Suspension tests evaluate the test virus as liquid inoculum while carrier tests are designed for evaluating virus inoculated and dried onto environmental surfaces (or carriers). Existing efficacy literature specific for FMDV includes suspension and carrier based procedures, but is of limited value due to the inconsistent methodology and the majority of this information precedes the current virucidal standardization and guidelines existing for virucidal testing (8-11, 15, 24, 30, 34). Strong evidence exists to support the survival and persistence of FMDV in the environment and on various fomites (26, 37, 38, 42, 66, 71, 80, 89), further supporting the need to have carrier efficacy evaluations available for FMDV. Literature also indicates that it is typically more difficult to decontaminate dried inoculum on surfaces when compared to the same inoculum in liquid suspension (82).

The Klein and Deforest virus susceptibility scheme (47) is based predominantly on the physical features of viral groups, categorizing into enveloped, large non-enveloped, and small non-enveloped. Limitations within this classification scheme exist, and viruses with similar grouping or even belonging to the same viral family do not always react similarly to chemical inactivation. This was shown previously comparing efficacy between FMDV and bovine enterovirus, also a member of the *Picornaviridae* family (19). Other factors, including biocide concentration, pH, contact time, and temperature should also be considered for more accurate predictions (56). For surface disinfection, the ability of a disinfectant to adequately cover a

surface and maintain effectiveness in the presence of organic material is also very important for virucidal efficacy.

The U. S. Environmental Protection Agency (EPA) has published guidelines for evaluating disinfectants for virucidal claims including both suspension and carrier assays (34). The guidelines require a modification of the AOAC Use-Dilution Method (6) which was prepared for bactericidal methodology, but provide very little information on the type of surface to be used, how long to allow the inoculum to dry, or how to remove cytotoxicity from the samples. There is also no guidance on how to standardize the amount of virus or how much virus to inoculate a surface with, but a recoverable virus titer of at least 10^4 is required from non-treated positive control samples.

The purpose of this study was to evaluate the efficacy of standard and novel chemistries against FMDV inoculated onto environmental surfaces following the current EPA guidelines. Virus was enumerated by a quantitative recovery of infectious titer (as determined by tissue culture infective dose 50) or by a quantitative real-time reverse transcriptase polymerase chain reaction (*rRT-PCR*) assay specific for FMDV. In both recovery systems, statistical analysis was conducted to determine if the disinfectant treatment resulted in significant reduction in viral titer when compared to non-treated positive control samples. Chemical biocides used in this study included acetic acid, bleach, ethanol, sodium carbonate, sodium hydroxide, and novel chemistries including Sandia DF-200 (91), Oxy-Sept® 333 (2), and Virkon® S (5) previously evaluated in a suspension based study (19). Experimental surfaces included concrete, rubber, and stainless steel and were exposed for 5, 10 or 20 min.

Materials and Methods

Virus and cells. FMDV type O1 strain Brugge was propagated in Baby Hamster Kidney 21 (BHK-21, ATCC CCL-10) cells as previously described (19). BHK-21 cells were maintained in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) supplemented with antibiotics previously described (19). FMDV titer was determined using tissue culture infective dose 50, TCID₅₀ and calculated by the Reed-Muench method (74).

Test Disinfectants. Test disinfectants were prepared as previously described (19) and included the following: 5% acetic acid, 10% bleach, 70% ethanol, 4% sodium carbonate, 2% sodium hydroxide, 100% Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S. Test disinfectants were prepared fresh each time prior to use. The pH values were measured and recorded prior to use to ensure proper mixing.

Test Surfaces. Test surfaces included concrete, rubber, and stainless steel. Quikcrete (The Home Depot, Albuquerque, NM) was purchased and mixed according to the manufacturer's instructions. Sterile 50 ml conical tubes (Fischer, Hampton, NH) were filled with the Quikcrete mixture to approximately the 10 ml volume. Samples were dried in a sterile hood for approximately 30 days. Prior to use, samples were exposed to a UV light in a sterile hood for 1 hour. Butyl rubber sheets (McMaster Carr, Atlanta, GA) were cut into round coupons having a surface area of 4.15 cm². Stainless steel type 304 sheets (McMaster Carr) were cut into round coupons having a surface area of 4.15 cm². The rubber and stainless steel coupons were autoclaved prior to use.

Virus Inactivation. Test coupons were inoculated with 100 µl FMDV (propagated to approximately 10⁷ in BHK-21 cells) and dried in a sterile biosafety class II cabinet for

approximately 30 min. After samples were completely dried, 500 μ l test disinfectant (or MEM containing 4% FBS for non-treated positive control samples) was added to each surface and exposed for 5, 10 or 20 min. Following exposure, surfaces were transferred to sterile 50 ml conical tubes and 5 ml MEM containing 4% FBS was added. Samples were vortexed for 1 min to release virus from surface.

Post-treatment Recovery of Infectious Virus. The titers of infectious virus were determined as TCID₅₀ in cell culture using a 96-well format as previously described (19). TCID₅₀ was determined visually by evaluating cytopathic effect (CPE) typical for FMDV as indicated by the presence of rolling or sloughing of infected cells. TCID₅₀ titer was determined using the Reed-Muench method for estimating endpoints (74).

Post-treatment Recovery of FMDV RNA using *r*RT-PCR. FMDV RNA was recovered using the RNeasy Kit (Qiagen, Valencia, CA) and quantitative detection of the extracted RNA was conducted using *r*RT-PCR assay as previously described (19). The *r*RT-PCR primer and probe sequence for FMDV was obtained from Callahan et al, 2002 (21). A standard curve was generated using the SmartCycler® using purified FMDV RNA from a predetermined amount of infectious FMDV (determined by TCID₅₀). The slope of the standard curve generated was calculated and used to quantify the amount of viral RNA recovered post-treatment. Post-treated viral RNA was recovered from the test samples using the RNeasy Mini Kit. Quantitative detection of the RNA was determined by a FAM fluorescence signal ≥ 3 using the SmartCycler® using *r*RT-PCR.

Statistical Analysis. An analysis to assess the statistical differences in the level of virus recovered by TCID₅₀ or by quantitative *r*RT-PCR following the experimental treatment parameters was carried out by comparing 1-way, 2-way, and 3-way interactions between

disinfectant treatments, time. The least square of means was calculated to determine if significant differences were observed in amount of infectious virus or viral RNA recovered compared to non-treated positive control samples. A significant difference between treatments is inferred with a P-value ≤ 0.05 .

Results

Post-treatment Recovery of Infectious Virus. The average infectious titer recovered from the test surfaces was 4.83, 6.17, or 6.25 \log_{10} TCID₅₀/ml for concrete, rubber, or stainless steel, respectively (Figure 5.1- 5.3). Effective disinfectant treatments were inferred by observations of a statistically significant reduction ($P \leq 0.05$) in infectious titer of each test virus when compared to the non-treated positive control sample and must be $\geq 4.0 \log_{10}$ TCID₅₀/ml to meet the EPA requirement for virucidal efficacy claims. If no infectious virus could be recovered in the TCID₅₀ assay based on the absence of CPE, then the detection limit of the assay was reported (1.75 \log_{10} TCID₅₀ which equals 28.36% of viable virus).

Following 5 min exposure with the test biocides, there were no effective treatments for FMDV resulting in a $\geq 4.0 \log_{10}$ reduction in titer on any of the test surfaces. Most treatments resulted in the recovery of approximately 40-53% viable virus, while 70% ethanol resulted in the recovery of 81-100% viable virus for all surfaces evaluated (Figure 5.1).

Following 10 min exposure with the test biocides, there were no effective treatments for FMDV resulting in a $\geq 4.0 \log_{10}$ reductions in titer on concrete, as the positive control sample was not greater than 4.0 \log_{10} above the detection limit of the assay (1.75 \log_{10} TCID₅₀). Most treatments resulted in the recovery of approximately 44-53% viable virus, while 70% ethanol resulted in the recovery of 84% viable virus (Figure 5.2). Effective treatments ($P \leq 0.05$) for FMDV resulting in a $\geq 4.0 \log_{10}$ reductions in titer on rubber and stainless steel included all

disinfectants evaluated except for 70% ethanol, which resulted in the recovery 93-100% viable virus (Figure 5.2).

Following 20 min exposure with the test biocides, there were no effective treatments for FMDV resulting in a $\geq 4.0 \log_{10}$ reductions in titer on concrete, as the positive control sample was not greater than $4.0 \log_{10}$ above the detection limit of the assay ($1.75 \log_{10}$ TCID₅₀). Most treatments resulted in the recovery of approximately 34-41% viable virus, while 70% ethanol resulted in the recovery of 82% viable virus (Figure 5.3). Effective treatments ($P \leq 0.05$) for FMDV resulting in a $\geq 4.0 \log_{10}$ reductions in titer on rubber and stainless steel included all disinfectants evaluated except for 70% ethanol, which resulted in the recovery 88-91% viable virus (Figure 5.3).

Post-treatment Recovery of FMDV RNA using rRT-PCR. The average amount of FMDV RNA recovered from the test surfaces was 3.95, 6.06, or 6.14 \log_{10} RNA copies per TCID₅₀/ml for concrete, rubber, or stainless steel, respectively (Tables 5.1-5.3). Effective disinfectant treatments were inferred by observations of a statistically significant reduction ($P \leq 0.05$) in FMDV RNA when compared to the non-treated positive control sample.

Following 5 min exposure (Table 5.1), effective treatments for degrading FMDV RNA on concrete were 10% bleach and Sandia DF-200. The other disinfectants resulted in the recovery of 31-46% of the viral RNA. The only effective treatment following 5 min exposure for degrading FMDV RNA on rubber was 2% sodium hydroxide. The remaining test treatments resulted in the recovery of 42-95% of the viral RNA. Effective treatments following 5 min exposure for degrading FMDV RNA on stainless steel included 10% bleach, 2% sodium hydroxide, and Sandia DF-200. The remaining test treatments resulted in the recovery of 43-62% of the viral RNA.

Following 10 min exposure (Table 5.2), effective treatments for degrading FMDV RNA on concrete were 10% bleach, 4% sodium carbonate and Sandia DF-200. The other disinfectants resulted in the recovery of 40-100% of the viral RNA. Effective treatments following 10 min exposure for degrading FMDV RNA on rubber included 10% bleach, 2% sodium hydroxide, and Sandia DF-200. The remaining test treatments resulted in the recovery of 41-96% of the viral RNA. Effective treatments following 10 min exposure for degrading FMDV RNA on stainless steel included 10% bleach, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S. The remaining test treatments resulted in the recovery of 66-93% of the viral RNA.

Following 20 min exposure (Table 5.3), effective treatments for degrading FMDV RNA on concrete included all treatments except 1% Virkon® S, which resulted in the recovery of 66% of the viral RNA. Effective treatments following 10 min exposure for degrading FMDV RNA on rubber included 10% bleach, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S. The remaining test treatments resulted in the recovery of 42-100% of the viral RNA. Effective treatments following 10 min exposure for degrading FMDV RNA on stainless steel included 10% bleach, 4% sodium carbonate, 2% sodium hydroxide, Sandia DF-200, 0.4% Oxy-Sept® 333, and 1% Virkon® S. The remaining test treatments resulted in the recovery of 77-89% of the viral RNA.

Discussion

Due to the persistence and stability of FMDV on various fomites and in the environment, the purpose of this study was to evaluate the efficacy of standard and novel chemistries against FMDV inoculated onto environmental surfaces. Virus was enumerated by a quantitative recovery of infectious titer (as determined by tissue culture infective dose 50) or by a

quantitative real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay specific for FMDV. In both recovery systems, statistical analysis was conducted to determine if the disinfectant treatment resulted in significant reduction in viral titer when compared to non-treated positive control samples. No effective treatments were observed on concrete surfaces for any of the time exposures evaluated. Effective treatments against FMDV resulting in a ≥ 4.0 \log_{10} reduction in titer on rubber and stainless steel included all disinfectants evaluated except for 70% ethanol following 10 and 20 min exposure. The amount of viral RNA that was degraded varied depending on the test surface, but treatment with 10% bleach, 2% sodium hydroxide or Sandia DF-200 resulted in significant reductions following 10 or 20 min treatment.

The selection of the test surfaces used in this study was to include materials (both porous and non-porous) commonly found in an agriculture setting. It is generally accepted that inanimate surfaces can play an important role in disease transmission, especially in the case of a highly infectious and persistent virus such as FMDV (71). Decontamination of contaminated surfaces such as animal pens, vehicles, equipment, and even personnel (71, 75) is necessary for disruption the transmission cycle of FMDV to aid in disease containment.

Efficacy results generated in this study for FMDV were similar to those generated in a similar study based on suspension tests (19), although 5 min contact times were not previously evaluated. In both studies, FMDV was inactivated by all treatments except for 70% ethanol following 10 min exposure. In the current study, most treatments (except 70% ethanol) resulted in a 50% reduction in titer following 5 min exposure. The drying step resulted in approximately a 1.0 \log_{10} reduction in starting titer on rubber and stainless steel, and approximately a 2.5 \log_{10} reduction in titer onto concrete. It is common for some virus to be lost in the drying step for carrier assays (56, 61, 69, 75). The amount of virus recovered from concrete was

consistently lower than the amount recovered from rubber and stainless steel. This is most likely due to the cytotoxicity occurring due to both the high pH (approximately 11) of the concrete and the particulate debris releasing from the surface during the sample vortexing step. In many cases, the amount of virus recovered from the concrete was not more than a 4.0 log₁₀ amount over the assay detection limit of 1.75 log₁₀, making the ≥ 4.0 log₁₀ reduction in titer impossible to achieve. The amount of viral RNA recovered from the concrete surface was also lower than that recovered from rubber and stainless steel. This could be explained in two ways. In the first example, virus particles are more protected in the rough surface of this material, and are more difficult to recover. The second possibility is likely due to a low level inhibition of the *r*RT-PCR assay occurring from the relatively basic pH of this material, and the presence of particulates in the sample solution post-vortexing.

Following the longer time exposures evaluated (10 or 20 min) efficacy results obtained from rubber and stainless steel were very similar and appeared to be easier to decontaminate than concrete. Concrete was consistently the most difficult surface to effectively decontaminate, probably due to the very rough surface associated with this material. The cracks and pores could potentially serve as physical barrier preventing the necessary contact of the test disinfectants to the virus targets. Viruses, especially the picornaviruses, which are approximately 30 nm in diameter (65), can be protected in scratches or imperfections on a surface (75, 82). The smoother surfaces of the rubber and stainless steel carriers were probably easier for the test disinfectants to penetrate and interact with the viral targets.

In conclusion, several disinfectants were found to be effective for inactivating FMDV following the current EPA guidelines for virucidal efficacy requirements resulting in a ≥ 4.0 log₁₀ reduction in titer on various environmental surfaces. Effective treatments against FMDV

on rubber and stainless steel included all disinfectants evaluated except for 70% ethanol following 10 and 20 min exposure. No effective treatments (by US EPA definition) were observed on concrete surfaces mostly because the amount of non-treated positive control virus recovered was very low. The difference in the amount of positive control virus recovered and the detection limit of the assay was not greater than 4.0 log₁₀, so it was impossible to achieve the required reduction in titer. The amount of viral RNA recovered from the test surfaces was lowest from concrete samples and higher from rubber and stainless steel. In general, treatment with 10% bleach, 2% sodium hydroxide or Sandia DF-200 resulted in a significant reduction in the amount of FMDV RNA following 10 or 20 min treatment and correlated with results obtained from TCID₅₀.

Figures and Tables

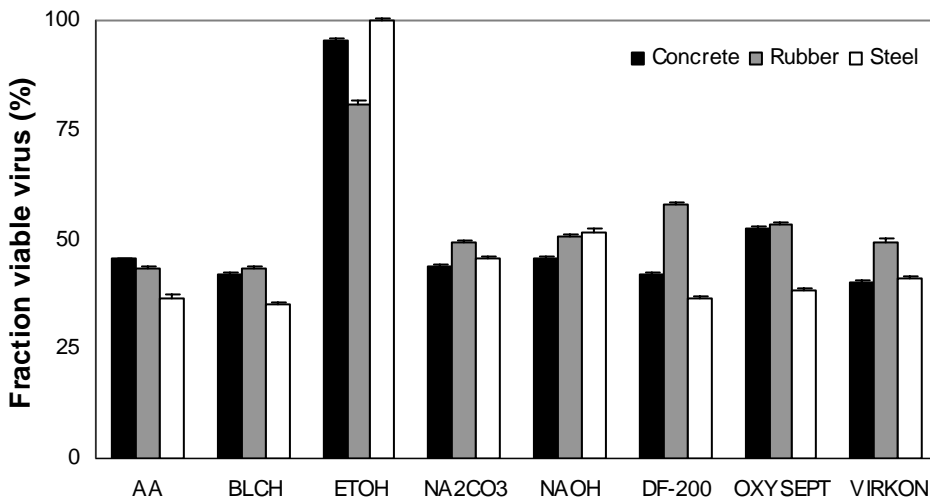


Figure 5.1 The fraction of viable FMDV (%) as recovered by TCID₅₀ following 5 min exposure to test disinfectants on test surfaces.

Error bars indicate 1 standard deviation (n = 3). AA, 5% acetic acid; BLCH, 10% bleach; ETOH, 70% ethanol; NA₂CO₃, 4% sodium carbonate; NAOH, 2% sodium hydroxide; DF-200, Sandia Decon Foam; OXYSEPT, 0.4% Oxy-Sept® 333; VIRKON, 1% Virkon® S.

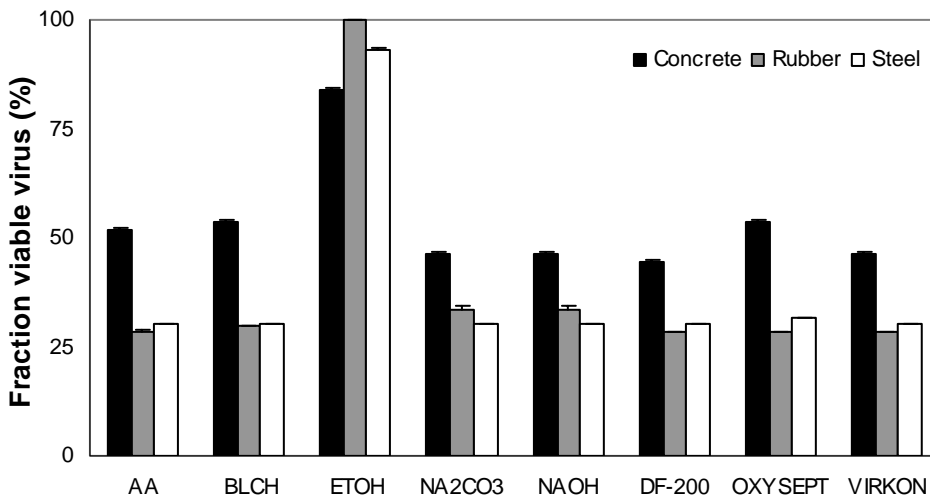


Figure 5.2 The fraction of viable FMDV (%) as recovered by TCID₅₀ following 10 min exposure to test disinfectants on test surfaces.

Error bars indicate 1 standard deviation (n = 3). AA, 5% acetic acid; BLCH, 10% bleach; ETOH, 70% ethanol; NA₂CO₃, 4% sodium carbonate; NAOH, 2% sodium hydroxide; DF-200, Sandia Decon Foam; OXYSEPT, 0.4% Oxy-Sept® 333; VIRKON, 1% Virkon® S.

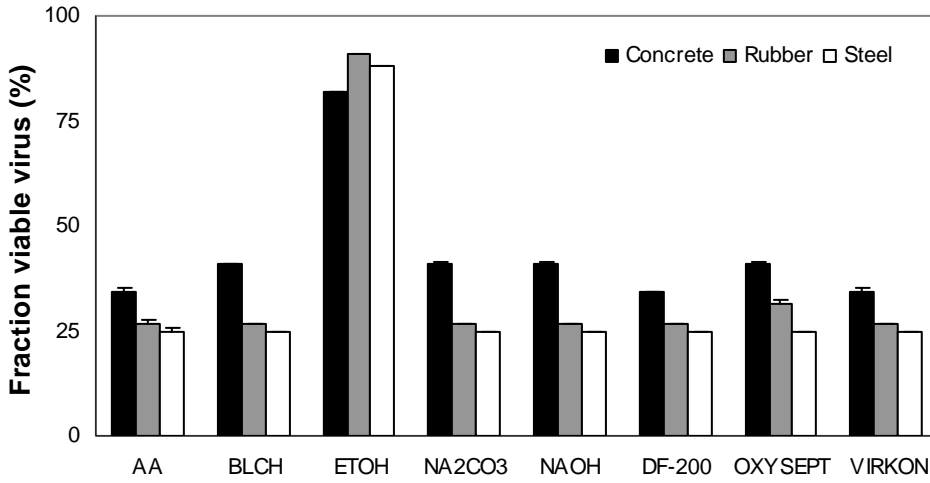


Figure 5.3 The fraction of viable FMDV (%) as recovered by TCID₅₀ following 20 min exposure to test disinfectants on test surfaces.

Error bars indicate 1 standard deviation (n = 3). AA, 5% acetic acid; BLCH, 10% bleach; ETOH, 70% ethanol; NA₂CO₃, 4% sodium carbonate; NaOH, 2% sodium hydroxide; DF-200, Sandia Decon Foam; OXYSEPT, 0.4% Oxy-Sept® 333; VIRKON, 1% Virkon® S.

| Treatment | Concrete | Rubber | Stainless steel |
|--|-----------------|---------------|------------------------|
| AA ^a | 36.57 | 59.70 | 61.98 |
| BLCH ^b | 0.00 | 85.41 | 22.15 |
| ETOH ^c | 38.23 | 95.19 | 99.17 |
| NA ₂ CO ₃ ^d | 31.02 | 44.44 | 47.11 |
| NAOH ^e | 31.02 | 0.00 | 19.01 |
| DF-200 ^f | 0.00 | 42.29 | 20.83 |
| OXYSEPT ^g | 45.71 | 68.66 | 51.07 |
| VIRKON ^h | 31.02 | 50.58 | 42.98 |

Table 5.1 The percentage of FMDV RNA as recovered by quantitative *r*RT-PCR following 5 min exposure to test disinfectants on test surfaces.

^a AA, 5% acetic acid

^b BLCH, 10% bleach

^c ETOH, 70% ethanol

^d NA₂CO₃, 4% sodium carbonate

^e NAOH, 2% sodium hydroxide

^f DF-200, Sandia decon foam

^g OXYSEPT, 0.4% Oxy-Sept® 333

^h VIRKON, 1% Virkon® S

| Treatment | Concrete | Rubber | Stainless steel |
|--|-----------------|---------------|------------------------|
| AA ^a | 70.22 | 88.37 | 86.02 |
| BLCH ^b | 0.00 | 44.03 | 16.74 |
| ETOH ^c | 111.86 | 95.97 | 93.39 |
| NA ₂ CO ₃ ^d | 0.00 | 73.33 | 65.59 |
| NAOH ^e | 40.19 | 17.21 | 34.41 |
| DF-200 ^f | 0.00 | 40.93 | 25.35 |
| OXYSEPT ^g | 92.98 | 53.18 | 35.18 |
| VIRKON ^h | 67.07 | 42.02 | 35.64 |

Table 5.2 The percentage of FMDV RNA as recovered by quantitative *r*RT-PCR following 10 min exposure to test disinfectants on test surfaces.

^a AA, 5% acetic acid

^b BLCH, 10% bleach

^c ETOH, 70% ethanol

^d NA₂CO₃, 4% sodium carbonate

^e NAOH, 2% sodium hydroxide

^f DF-200, Sandia decon foam

^g OXYSEPT, 0.4% Oxy-Sept® 333

^h VIRKON, 1% Virkon® S

| Treatment | Concrete | Rubber | Stainless steel |
|--|-----------------|---------------|------------------------|
| AA ^a | 28.22 | 84.56 | 77.44 |
| BLCH ^b | 33.58 | 21.75 | 0.00 |
| ETOH ^c | 34.31 | 107.02 | 88.72 |
| NA ₂ CO ₃ ^d | 28.22 | 41.93 | 18.46 |
| NAOH ^e | 0.00 | 0.00 | 0.00 |
| DF-200 ^f | 0.00 | 0.00 | 0.00 |
| OXYSEPT ^g | 36.98 | 24.39 | 19.32 |
| VIRKON ^h | 65.94 | 0.00 | 0.00 |

Table 5.3 The percentage of FMDV RNA as recovered by quantitative *r*RT-PCR following 20 min exposure to test disinfectants on test surfaces.

^a AA, 5% acetic acid

^b BLCH, 10% bleach

^c ETOH, 70% ethanol

^d NA₂CO₃, 4% sodium carbonate

^e NAOH, 2% sodium hydroxide

^f DF-200, Sandia decon foam

^g OXYSEPT, 0.4% Oxy-Sept® 333

^h VIRKON, 1% Virkon® S

CHAPTER 6 - Conclusions

In order to increase insight into the overall efficacy and mechanism for virucidal inactivation by chemical biocides, several studies were conducted using different types of viruses. It is widely recognized that different types of viruses vary with regard to their susceptibility to chemical inactivation and have been well characterized by Klein and Deforest (47). The US EPA has a published set of guidelines for producing virucidal efficacy claims on chemical disinfectants (34). Among these virucidal guidelines, information regarding initial review and labeling of the disinfectant, efficacy data requirements, and confirmatory efficacy requirements are all included. Under the efficacy data requirements for virucidal efficacy claims, it is recommended that a recoverable virus titer of at least 10^4 reported as infective dose 50 (ID₅₀) of the test system should be recovered from non-treated positive control samples. The ID50 assay used must have a minimum of four determinations per each dilution of test sample and cytotoxicity controls must be included. For the disinfectant to be accepted as virucidal, the product should demonstrate complete inactivation at all dilutions. In the case of cytotoxicity, a 3-log reduction in titer must be demonstrated beyond the cytotoxic level. The claim of virucidal efficacy is restricted to the virus which is evaluated, except in the case of the EPA accepted surrogate test viruses. It is unclear as to whether such claims can only be made with that particular isolate or the viral family in general.

In the first study conducted using bovine coronavirus and described in Chapter 2, the US EPA guidelines were followed to generate the virucidal efficacy data and also included two different types of organic challenge, which aren't required in the existing guidelines. The greatest challenge in this evaluation was cytotoxicity resulting from the chemical disinfectants in

the infectivity assay (TCID₅₀). The addition of an ultracentrifugation step in the methodology to purify the virus and remove the chemical component of the samples resulted in the removal of cytotoxicity for most disinfectants evaluated (except for DF-200d and Virkon® S). Although the DF-200d and Virkon® S were cytotoxic even post-ultracentrifugation, most of the cytotoxicity was removed and all dilutions beyond the first serial 1:10 dilution for both of these disinfectants were non-cytotoxic and fulfilled the requirements of the EPA guidelines “showing a minimum of a 3-log reduction in titer beyond the cytotoxic level” (34).

The coronavirus study also evaluated the sensitivities of existing diagnostic assays for potential use to evaluate the efficacy of chemical disinfectants. Although ID₅₀ assays are required in the EPA guidelines, other assays can enhance the overall data by providing insight into the possible mechanism of inactivation. For example, the use of a diagnostic assay depending on the presence of nucleic acid could show if any degradation to this target was occurring. This could also be determined using a diagnostic assay depending on the presence of a structural protein receptor or capsid. Quantitative RT-PCR was found to be the most sensitive for determining the mechanism of action while the detection of structural proteins could only be included qualitatively and suffered in being less sensitive.

The second set of virucidal efficacy testing conducted in this research is described in Chapter 3 comparing a mammalian influenza A virus to an avian influenza A virus. The use of surrogate viruses for efficacy testing are a result of the Klein and Deforest susceptibility predictions (47) are the basis for the accepted surrogate viruses by the US EPA and is published in their current virucidal efficacy testing guidelines (34). Most of these accepted surrogates are the result of not having a cultivation method for the virus of interest, so a closely related surrogate virus is evaluated instead on the assumption that they will react similarly. Due to the

emergence of highly pathogenic and infectious diseases such as the highly pathogenic avian influenza, H5N1 or the threat of non-endemic diseases such as FMDV, which are not privy for cultivation in standard virology laboratories, the use of closely related surrogate viruses could result in the safe generation of efficacy data following the EPA guidelines under BSL-2 containment. In Chapter 3 efficacy results obtained for two closely related viruses, mammalian H1N1 and a low pathogenic avian H5N8 isolate, did not show any significantly different observations, suggesting that one influenza A virus could be a surrogate testing virus for another influenza A virus. This is an important finding as the US EPA is accepting the use of efficacy data for any avian influenza A virus to be applied to the H5N1 isolate (3), even though this has not been previously validated in existing literature.

Although the Klein and Deforest prediction scheme suggests that viruses among the same family will react similarly to chemical disinfectants, there are some examples in which this does not occur. An example of this is described in Chapter 4 comparing the virucidal efficacy of different disinfectant chemistries between FMDV and BEV-2, both members of the *Picornaviridae* family. Because BEV-2 and other enteroviruses in this family are enteric, they must remain viable in acidic environments to remain infectious. Viruses such as FMDV, although physically similar, do not require this resistance and were very susceptible to inactivation by acidic compounds. This outcome emphasizes the importance for not only considering the physical properties of a virus, but also the factors necessary for their infectious cycle, such as an acid resistance mechanism which might result in very different susceptibilities to chemical disinfectants.

The final study conducted and described in Chapter 5 was evaluating the inactivation of FMDV on environmental surfaces including concrete, rubber, and stainless steel. The current

US EPA virucidal efficacy testing guidelines recommend that disinfectants for use on surfaces should be evaluated using carrier tests and reference the AOAC Use-Dilution Method (6), which was actually prepared for bacterial agents. There is no guidance on how much virus to inoculate the surfaces with, or how to standardize the titer among various surfaces. The guidelines require a recoverable virus titer of at least $10^4 \log_{10}$ TCID₅₀ from each surface for non-treated positive control samples. In our study, we determined that inoculating the 4.15 cm² surfaces with 100 μ l FMDV (at a starting titer of 10^{7-8} TCID₅₀/ml) resulted in the consistent recovery of $> 10^4 \log$ virus titer for each surface. The drying step resulted in approximately a 1.0 \log_{10} reduction in starting titer on rubber and stainless steel, and approximately a 2.5 \log_{10} reduction in titer onto concrete and is common for some infectivity to be lost during the drying step (56, 61, 69, 75). Overall, the surface results were very similar to the suspension results described in Chapter 4. Most treatments, except for 70% ethanol, resulted in a greater than 4.0 \log_{10} reduction in titer.

Based on the results obtained from all of these studies, some general conclusions and next steps can be summarized. Enveloped viruses including corona and influenza A viruses evaluated were much less resistant than the non-enveloped picornaviruses evaluated in the various studies, and was consistent with the Klein and Deforest virus prediction scheme (47). In one case, two viruses within the same viral family (FMDV and BEV-2, *Picornaviridae*) were shown to have different susceptibility to acidic compounds, due largely on differences with regard to their infectious life cycle. All tests conducted followed the current US EPA guidelines for evaluating virucidal efficacy of chemical biocides. Cytotoxicity from the active chemicals in the disinfectants was the greatest challenge in the recovery assay to determine how much infectious virus was remaining. Cytotoxicity was significantly reduced by an ultracentrifugation step, and allowed the successful recovery of viable virus from each disinfectant evaluated. The

use of *r*RT-PCR assays for all test viruses provided insight into the mechanism of action occurring from degradation of RNA for some of the chemical biocides used, and could be used as a rapid assay for verifying loss of infectivity for these disinfectants. The biocides which did not show degradation of RNA most likely functioned by disruption of other viral targets such as the envelope (if present) or viral proteins. Both of these mechanisms likely prevent the attachment and entry of a virus particle into a cell resulting in the successful disruption of the transmission cycle.

In closing, these results show the importance for validating chemical disinfectants for virucidal efficacy against different groups of virus. As a current member of a working group with the US EPA, which also includes representatives from the U. S. Department of Agriculture (USDA), the Food and Drug Administration (FDA), the U. S. Department of Homeland Security (DHS), there is a strong need to standardize the current guidelines required for obtaining virucidal efficacy labeling claims. Such standards are already in place with the EPA for bactericidal, sporicidal, fungicidal, and other microbiocidal claims. Based on the results from the experiments in this study, I feel that there are a few issues which should be addressed for a standardized protocol. The current process requires the validation of every virus for which a claim is to be made. During an ongoing outbreak, it might be very difficult to generate such efficacy data, so perhaps the evaluation of several viruses representing the various groups of viruses likely to differ in susceptibility (according to Klein and Deforest classification) should be required to be evaluated in the standardized protocol. Specific detail including disinfectant concentration, exposure length, relative humidity, temperature, and organic challenge would all be included. Cytotoxicity controls would also be included for each host tissue culture required in the recovery process. Additionally, specific surfaces for carrier assays would be identified and

information regarding the volume of inoculum and time for drying would be specified to standardize the assay for all viruses evaluated. Having a set of standardized test viruses would provide useful data for chemical disinfectants and could be generalized with regard to efficacy for a particular virus in the case of an outbreak until specific efficacy data could be generated. This would be possible as long as the required standard viruses adequately represented the major groups among viruses. The standardized testing would also be useful for biosafety decontamination procedures in biological containment laboratories to ensure that viruses used in such facilities are being effectively inactivated.

References

1. **Anonymous.** 2003. Foot-and-mouth disease: scientific problems and recent progress 1st annual report (2003) prepared for DEFRA, Science Directorate. Department for Environment, Food and Rural Affairs.
2. **Anonymous** 2001, posting date. Oxy-Sept 333. [Online.]
3. **Anonymous.** 2006. Registered Antimicrobial Products with Label Claims for Avian (Bird) Flu Disinfectants. *In* U. S. E. P. Agency (ed.).
4. **Anonymous.** 2006. Registered Antimicrobial Products with Label Claims for Avian (Bird) Flu Disinfectants *In* U. S. E. P. Agency (ed.).
5. **Anonymous.** Virkon S Broad Spectrum Disinfectant
6. **AOAC.** 1960. Official Methods of Analysis for Germicidal and Detergent Sanitizing Action of Disinfectants. . Association of Official Analytical Chemists.
7. **ARMCANZ** 2000, posting date. AUSVET Decontamination Operational Procedures Manual. [Online.]
8. **ASTM.** 2002. Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Chemical Germicides. ASTM International.
9. **ASTM.** 2002. Standard Test Method for Efficacy of Antimicrobial Agents Against Viruses in Suspension Designation: E 1052 – 96 (Reapproved 2002). ASTM International.
10. **ASTM.** 2002. Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces Designation E 1053-97. ASTM International.
11. **ASTM.** 2004. Standard Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations Designation E 1482-04. ASTM International.
12. **Bachrach, H. L., S. S. Breese, Jr., J. J. Callis, W. R. Hess, and R. E. Patty.** 1957. Inactivation of foot-and-mouth disease virus by pH and temperature changes and by formaldehyde. *Proc Soc Exp Biol Med* **95**:147-52.
13. **Baird, B. E., and J. W. Savell.** 2004. Decontamination of Sites & Carcasses in Carcass Disposal: A Comprehensive Review. National Agriculture Biosecurity Center

- Consortium, USDA APHIS Cooperative Agreement Project, Carcass Disposal Working Group.
14. **Bean, B., B. M. Moore, B. Sterner, L. R. Peterson, D. N. Gerding, and H. H. J. Balfour.** 1982. Survival of Influenza Viruses on Environmental Surfaces. *The Journal of Infectious Diseases* **146**:47-51.
 15. **Bellamy, K.** 1995. A review of the test methods used to establish virucidal activity. *Journal of Hospital Infection* **30**:389-396.
 16. **Bellamy, K.** 1995. A review of the test methods used to establish virucidal activity. *J Hosp Infect* **30**:389-396.
 17. **Bieker-Hawkinson, J. Anderson, R. D. Oberst, and S. Kapil.** 2004. Presented at the American Biological Safety Association, San Antonio, TX, Oct. 14.
 18. **Bieker, J. M., R. D. Oberst, and S. K. Kapil.** 2006. Inactivation and Mechanism of Action of Chemical Disinfectants against Coronavirus. *Appl. Env. Micro.* *In Review*.
 19. **Bieker, J. M., A. Shuler, W. Einfeld, T. Beckham, and R. D. Oberst.** 2006. Inactivation of Foot and Mouth Disease Virus and Bovine Enterovirus. *Appl. Env. Micro.* *In Review*.
 20. **Block, S. S.** 2001. Peroxygen Compounds, p. 185-214. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation*, Fifth ed. Lippincott, Williams, & Wilkins, Philadelphia.
 21. **Callahan, J. D., F. Brown, F. A. Osorio, J. H. Sur, E. Kramer, G. W. Long, J. Lubroth, S. J. Ellis, K. S. Shoulars, K. L. Gaffney, D. L. Rock, and W. M. Nelson.** 2002. Use of a portable real-time reverse transcriptase polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *J. Am. Vet. Med. Assoc.* **220**:1636-1642.
 22. **Cardona, C.** 2006, posting date. Avian Influenza. [Online.]
 23. **Cardona, C.** 2006, posting date. Avian Influenza - Sources and Spread of Avian Influenza virus (AIV). [Online.]
 24. **CEN** 2004, posting date. The European Committee for Standardization [Online.]
 25. **Condit, R. C.** 2001. Principles of Virology, p. 19-51. *In* D. M. Knipe and P. M. Howley (ed.), *Fields Virology*, vol. 1. Lippincott, Williams, & Wilkins, Philadelphia.
 26. **Cottral, G. E.** 1969. Persistence of foot-and-mouth disease virus in animals, their products and the environment. *Bull Off Int Epizoot* **70**:549-68.

27. **Daginakatte, G. C., C. Chard-Bergstrom, G. A. Andrews, and S. Kapil.** 1999. Production, characterization, and uses of monoclonal antibodies against recombinant nucleoprotein of elk coronavirus. *Clin Diagn Lab Immunol* **6**:341-344.
28. **Darnell, M. E., K. Subbarao, S. M. Feinstone, and D. R. Taylor.** 2004. Inactivation of the coronavirus that induces severe acute respiratory syndrome, SARS-CoV. *J Virol Methods* **121**:85-91.
29. **Davison, S., C. E. Benson, A. F. Ziegler, and R. J. Eckroade.** 1999. Evaluation of Disinfectants with the Addition of Antifreezing Compounds Against Nonpathogenic H7N2 Avian Influenza Virus. *Avian Diseases* **43**:533-537.
30. **DEFRA** June 30, 2003 2003, posting date. LIST OF DISINFECTANTS APPROVED BY THE MINISTER OF AGRICULTURE, FISHERIES AND FOOD FOR THE PURPOSES OF THE DISEASES OF ANIMALS (APPROVED DISINFECTANTS) ORDER 1978 (AS AMENDED) SPECIFYING THE DILUTIONS AT WHICH THEY ARE RESPECTIVELY APPROVED. [Online.]
31. **Doultree, J. C., J. D. Druce, C. J. Birch, D. S. Bowden, and J. A. Marshall.** 1999. Inactivation of feline calicivirus, a Norwalk virus surrogate. *J Hosp Infect* **41**:51-7.
32. **Duizer, E., P. Bijkerk, B. Rockx, A. De Groot, F. Twisk, and M. Koopmans.** 2004. Inactivation of caliciviruses. *Appl Environ Microbiol* **70**:4538-43.
33. **Dychdala, G. R.** 2001. Chlorine and Chlorine Compounds, p. 135-158. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation*, Fifth ed. Lippincott Williams & Wilkins, Philadelphia.
34. **EPA** 2005, posting date. Antimicrobial Science Policies Disinfectant Technical Science Section. [Online.]
35. **Fellowes, O. N.** 1960. Chemical Inactivation of Foot-and-Mouth Disease Virus. *Annals of The New York Academy of Sciences* **83**:595-608.
36. **Fellowes, O. N., A. G. Edward, J. Tessler, G. C. Poppensiek, and J. B. Sharp.** 1959. The Inactivation of Foot-and-Mouth Disease Virus, Type A-119, with Beta-Propiolactone. *J. Vet. Res.* **20**:992-995.
37. **Gailunas, P., and G. E. Cottral.** 1966. Presence and persistence of foot-and-mouth disease virus in bovine skin. *J Bacteriol* **91**:2333-8.

38. **Gailunas, P., and G. E. Cottral.** 1967. Survival of foot-and-mouth disease virus in bovine hides. *Am J Vet Res* **28**:1047-53.
39. **Goddard, P. A., and K. A. McCue.** 2001. *Phenolic Compounds*, Fifth ed. Lippincott, Williams, & Wilkins, Philadelphia.
40. **Gordon, V., S. Parry, K. Bellamy, and R. Osborne.** 1993. Assessment of chemical disinfectants against human immunodeficiency virus: overcoming the problem of cytotoxicity and the evaluation of selected actives. *J. Virol. Meths.* **45**:247-257.
41. **Grubman, M. J., and B. Baxt.** 2004. Foot-and-Mouth Disease. *Clinical Microbiology Reviews* **17**:465-493.
42. **Haas, B., R. Ahl, R. Bohm, and D. Strauch.** 1995. Inactivation of viruses in liquid manure. *Rev. Sci. Tech. Off. Int. Epiz.* **14**:435-445.
43. **Hugo, W. B., and A. D. Russell.** 1999. Types of Antimicrobial Agents, p. 5-94. *In* A. D. Russell, W. B. Hugo, and G. A. J. Ayliffe (ed.), *Principles and Practice of Disinfection, Preservation and Sterilization*, 3rd ed. Blackwell Science, Oxford.
44. **Kapil, S., C. Chard-Bergstrom, P. Bolin, and D. Landers.** 1995. Plaque variations in clinical isolates of bovine coronavirus. *J Vet Diagn Invest* **7**:538-539.
45. **Kapil, S., K. L. Richardson, C. Radi, and C. Chard-Bergstrom.** 1996. Factors affecting isolation and propagation of bovine coronavirus in human rectal tumor-18 cell line. *J Vet Diagn Invest* **8**:96-99.
46. **Kitching, R. P.** 2005. Global Epidemiology and Prospects for Control of Foot-and-Mouth Disease, p. 133-148. *In* B. W. J. Mahy (ed.), *Foot-and-Mouth Disease Virus*. Springer, Berlin.
47. **Klein, M., and A. Deforest.** 1983. Principles of viral inactivation, p. 422-434. *In* Block (ed.), *Disinfection, Sterilization, and Preservation*, 3rd ed. Lea & Febiger, Philadelphia.
48. **Klein, M., and A. Deforest.** 1983. Principles of virus inactivation, p. 422-434. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation*, Third ed. Lea and Febiger, Philadelphia.
49. **Kruchen, B., and B. Rueger.** 2000. The DIG system - nonradioactive and highly sensitive detection of nucleic acids. *Biochemica* **3**:13-15.

50. **Lai, M. M. C., and K. V. Holmes.** 2001. Coronaviridae: The Viruses and Their Replication, p. 1163-1185. *In* D. M. Knipe and P. M. Howley (ed.), *Fields Virology*, Fourth ed, vol. 1. Lippincott, Williams, & Wilkins, Philadelphia.
51. **Lamb, R. A., and R. M. Krug.** 2001. Orthomyxoviridae: The Viruses and Their Replication, p. 1487-1531. *In* D. M. Knipe and P. M. Howley (ed.), *Fields Virology*, Fourth ed, vol. 1. Lippincott Williams & Wilkins, Philadelphia.
52. **Lambert, P. A.** 2004. *Mechanisms of action of biocides*, Fourth ed. Blackwell Publishing, Massachusetts.
53. **Lambert, P. A.** 2004. Mechanisms of action of biocides, p. 139-153. *In* A. P. Fraise, P. A. Lambert, and J.-Y. Mallaird (ed.), *Principles and Practice of Disinfection Preservation & Sterilization*. Blackwell Publishing Ltd, Oxford, UK.
54. **Lu, H., A. E. Castro, K. Pennick, J. Liu, Q. Yang, P. Dunn, D. Weinstock, and D. Henzler.** 2003. Survival of Avian Influenza Virus H7N2 in SPF Chickens and Their Environments. *Avian Diseases* **47**:1015-1021.
55. **Maillard, J.-Y.** 2001. Virus Susceptibility to Biocides: an understanding. *Reviews in Medical Microbiology* **12**:63-74.
56. **Maillard, J. Y.** 1999. Mechanisms of Viricidal Action, p. 207-221. *In* A. D. Russell, W. B. Hugo, and G. A. J. Ayliffe (ed.), *Principles and Practice of Disinfection, Preservation and Sterilization*. Blackwell Science, Oxford.
57. **Maillard, J. Y., and A. D. Russell.** 1997. Viricidal activity and mechanisms of action of biocides. *Sci Prog* **80 (Pt 4)**:287-315.
58. **Mallaird, J.-Y.** 2004. Viricidal activity of biocides, p. 272-323. *In* A. P. Fraise, P. A. Lambert, and J.-Y. Mallaird (ed.), *Principles and Practice of Disinfection Preservation & Sterilization*. Blackwell Publishing Ltd., Oxford, UK.
59. **Maris, P.** 1995. Modes of action of disinfectants. *Rev. Sci. Tech. Off. Int. Epiz.* **14**:47-55.
60. **Merianos, J. J.** 2001. Surface-Active Agents, p. 283-320. *In* S. S. Block (ed.), *Disinfection, Preservation, and Sterilization*, Fifth ed. Lippincott, Williams, & Wilkins, Philadelphia.

61. **Moce-Llivina, L., G. T. Papageorgiou, and J. Jofre.** 2006. A membrane-based quantitative carrier test to assess the virucidal activity of disinfectants and persistence of viruses on porous fomites. *J Virol Methods* **135**:49-55.
62. **Moore, S. L., and D. N. Payne.** 2004. Types of antimicrobial agents, p. 8-97. *In* A. P. Fraise, P. A. Lambert, and J.-Y. Mallaird (ed.), *Principles and Practice of Disinfection Preservation & Sterilization*, Fourth ed. Blackwell Publishing Ltd., Oxford, UK.
63. **OIE** June 20, 2006 2006, posting date. UPDATE ON AVIAN INFLUENZA IN ANIMALS (TYPE H5). [Online.]
64. **Onodera, K., and U. Melcher.** 2002. VirOligo: a database of virus-specific oligonucleotides. *Nucleic Acids Res.* **30**:203-204.
65. **Pallansch, M. A., and R. P. Roos.** 2001. Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and Newer Enteroviruses, p. 723-775. *In* D. M. Knipe and P. M. Howley (ed.), *Fields Virology*, 4th ed, vol. 1. Lippincott Williams & Wilkins, Philadelphia.
66. **Parker, J.** 1971. Presence and inactivation of foot-and-mouth disease virus in animal faeces. *Vet Rec* **88**:659-62.
67. **Prince, H. N., and D. L. Prince.** 2001. Principles of Viral Control and Transmission, p. 543-571. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation*, Fifth ed. Lippincott Williams & Wilkins, Philadelphia, PA.
68. **Prince, H. N., and D. L. Prince.** 2001. Principles of Viral Control and Transmission, p. 543-571. *In* S. S. Block (ed.), *Disinfection, Preservation, and Sterilization*, 5th ed. Lippincott, Williams, & Wilkins, Philadelphia.
69. **Quinn, P. J., and M. E. Carter.** 1999. Evaluation of Virucidal Activity, p. 197-206. *In* A. D. Russell, W. B. Hugo, and G. A. J. Ayliffe (ed.), *Principles and Practice of Disinfection, Perservation and Sterilization*. Blackwell Science, Oxford.
70. **Quinn, P. J., and B. K. Markey.** 1999. Activity Against Veterinary Viruses, p. 187-196. *In* A. D. Russell, W. B. Hugo, and G. A. J. Ayliffe (ed.), *Principles and Practice of Disinfection, Preservation, and Sterilization*, Third ed. Blackwell Science Ltd, Oxford.
71. **Quinn, P. J., and B. K. Markey.** 2001. *Disinfection and Disease Prevention in Veterinary Medicine*, Fifth ed. Lippincott Williams & Wilkins, Philadelphia.
72. **Quinn, P. J., and B. K. Markey.** 1999. *Disinfection and Disease Prevention in Veterinary Medicine*, Fifth ed. Lippincott Williams & Wilkins, Philadelphia.

73. **Rabenau, H. F., J. Cinatl, B. Morgenstern, G. Bauer, W. Preiser, and H. W. Doerr.** 2005. Stability and inactivation of SARS coronavirus. *Med Microbiol Immunol (Berl)* **194**:1-6.
74. **Reed, L. J., and H. Muench.** 1932. A simple method for estimating 50% endpoints. *Am J Hyg* **27**:493-497.
75. **Sattar, A. S., and S. Springthorpe.** 1999. Activity Against Human Viruses, p. 168-186. *In* A. D. Russell, W. B. Hugo, and G. A. J. Ayliffe (ed.), *Principles and Practice of Disinfection, Preservation, and Sterilization*, 3rd ed. Blackwell Science, Oxford.
76. **Sattar, A. S., and S. Springthorpe.** 2001. *Methods of Testing the Virucidal Activity of Chemicals*, Fifth ed. Lippincott Williams & Wilkins, Philadelphia.
77. **Sattar, S. S., V.S.; Adegbonrin, O.; Zafer, A.A., and Busa, M.** 2003. A disc-based quantitative carrier test method to assess the virucidal activity of chemical germicides. *Journal of Virological Methods* **112**:3-12.
78. **Schijven, J., G. B. Rijs, and A. M. de Roda Husman.** 2005. Quantitative risk assessment of FMD virus transmission via water. *Risk Anal* **25**:13-21.
79. **Scott, F. W.** 1980. Virucidal Disinfectants and Feline Viruses. *Am J Vet Res* **41**:410-414.
80. **Scott, F. W., G. E. Cottral, and P. Gailunas.** 1966. Persistence of Foot-and-Mouth Disease Virus in External Lesions and Saliva of Experimentally Infected Cattle. *Am J Vet Res* **27**:1531-1536.
81. **Sellers, R. F.** 1968. The Inactivation of Foot-and-Mouth Disease Virus by Chemicals and Disinfectants. *The Veterinary Record* **83**:504-506.
82. **Springthorpe, V. S., and S. A. Sattar.** 2005. Carrier Tests to Assess Microbicidal Activities of Chemical Disinfectants for Use on Medical Devices and Environmental Surfaces. *J. AOAC Internat.* **88**:182-201.
83. **Steinmann, J.** 2004. Surrogate viruses for testing virucidal efficacy of chemical disinfectants. *Journal of Hospital Infection* **56**:49-54.
84. **Steinmann, J.** 2004. Surrogate viruses for testing virucidal efficacy of chemical disinfectants. *J Hosp Infect* **56 Suppl 2**:S49-54.

85. **Storz, J., R. Rott, and G. Kaluza.** 1981. Enhancement of plaque formation and cell fusion of an enteropathogenic Coronavirus by trypsin treatment. *Infect Immun* **31**:1214-1222.
86. **Suarez, D. L., E. Spackman, D. A. Senne, L. Bulaga, A. C. Welsch, and K. Froberg.** 2003. The Effect of Various Disinfectants on Detection of Avian Influenza Virus by Real Time RT-PCR. *Avian Diseases* **47**:1091-1095.
87. **Tellier, R.** 2006. Review of Aerosol Transmission of Influenza A Virus. *Emerging Infectious Disease* **12**.
88. **Thurman, R. B., and C. P. Gerba.** 1988. Molecular mechanisms of viral inactivation by water disinfectants. *Adv Appl Microbiol* **33**:75-105.
89. **Tomasula, P. M., and R. P. Konstance.** 2004. The Survival of Foot-and-Mouth Disease virus in Raw and Pasteurized Milk and Milk Products. *J. Dairy Sci.* **87**:1115-1121.
90. **Tsunemitsu, H., D. R. Smith, and L. J. Saif.** 1999. Experimental inoculation of adult dairy cows with bovine coronavirus and detection of coronavirus in feces by RT-PCR. *Arch Virol* **144**:167-175.
91. **Tucker, M. D., C. V. Williams, M. E. Tadros, P. M. Bura, R. Betty, and J. Paul.** 2000. Aqueous foam for the decontamination and mitigation of chemical and biological warfare agents. Sand Report, Sandia National Laboratories:1419.
92. **Wang, X. W., J. S. Li, M. Jin, B. Zhen, Q. X. Kong, N. Song, W. J. Xiao, J. Yin, W. Wei, G. J. Wang, B. Y. Si, B. Z. Guo, C. Liu, G. R. Ou, M. N. Wang, T. Y. Fang, F. H. Chao, and J. W. Li.** 2005. Study on the resistance of severe acute respiratory syndrome-associated coronavirus. *J Virol Methods* **126**:171-7.
93. **WHO** 2004, posting date. Avian Influenza ("bird flu") and the significance of its transmission to humans. [Online.]
94. **WHO.** 2006. Nonpharmaceutical Interventions for Pandemic Influenza, International Measures. *Emerging Infectious Disease* **12**:81-87.
95. **Wright, P. F., and R. G. Webster.** 2001. Orthomyxoviruses, Fourth ed, vol. 1. Lippincott Williams & Wilkins, Philadelphia.
96. **Yilmaz, a., U. Heffels-Redmann, and T. Redmann.** 2004. Evaluation of the virucidal efficacy of two chemical disinfectants against avian influenza virus A at different temperatures. *Arch. Geflugelk.* **68**:50-56.

