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Inactivation of Various Influenza Strains to Model Avian Influenza (Bird Flu) With Various Disinfectant Chemistries

J. M. Bieker, C. A. Souza, and R. D. Oberst

Prepared by
Sandia National Laboratories
Albuquerque, New Mexico 87185 and Livermore, California 94550

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J. M. Bieker, C. A. Souza, and R. D. Oberst
Chemical and Biological Systems Department
Sandia National Laboratories
P.O. Box 5800
Albuquerque, NM 87185-0734
jmbieke@sandia.gov

Abstract

Due to the grave public health implications and economic impact possible with the emergence of the highly pathogenic avian influenza A isolate, H5N1, currently circulating in Asia we have evaluated the efficacy of various disinfectant chemistries against surrogate influenza A strains. Chemistries included in the tests were household bleach, ethanol, Virkon S[®], and a modified version of the Sandia National Laboratories developed DF-200 (DF-200d, a diluted version of the standard DF-200 formulation). Validation efforts followed EPA guidelines for evaluating chemical disinfectants against viruses. The efficacy of the various chemistries was determined by infectivity, quantitative RNA, and qualitative protein assays. Additionally, organic challenges using combined poultry feces and litter material were included in the experiments to simulate environments in which decontamination and remediation will likely occur. In all assays, 10% bleach and Sandia DF-200d were the most efficacious treatments against two influenza A isolates (mammalian and avian) as they provided the most rapid and complete inactivation of influenza A viruses.

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Inactivation of Various Influenza Strains to Model Avian Influenza (Bird Flu) With Various Disinfectant Chemistries

Introduction

Currently, there is a 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 (7) translate into elevated risk of this disease emergence in our nation. High pathogenic avian influenza, H5N1, initially emerged in 1996, with 18 total human cases. Six of these cases caused fatalities in Hong Kong in 1997 (9). Since that time, the virus has moved into avian and human populations in Viet Nam, Thailand, Cambodia, Indonesia, and again in China. H5N1 virus has also recently (2005) been detected in birds in Russia, Kazakhstan, and Mongolia (9).

Human treatment will likely rely on vaccine development and production as well as the use of available antiviral drugs. Many experts are not certain if enough vaccine protecting against H5N1 will be approved or available for use. Neuraminidase inhibitors, including oseltamivir (Tamiflu) and zanamir (Relenza) are recommended for use but are only effective if administered early. M2 inhibitors, including amantadine and rimantadine, are also recommended but are more susceptible to resistance mechanism of the virus (8). In an effort to aid in halting or minimizing disease spread, stringent sanitation and decontamination have been recommended for affected premises, including transport vehicles, clothing, cages, housing, and other potential contaminated areas (7). 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 may occur under a variety of conditions such as on relatively clean surfaces or surfaces that contain a very high organic matter. A disinfectant able to withstand the challenge of organic load while still maintaining user and environmental friendliness would be most ideal for inactivating highly pathogenic influenza.

Documentation of the effectiveness of viral disinfectants against viruses is minimal, and even less information is available on mechanism of action and efficacy in the presence of organic challenge. In addition to the lack of efficacy data, the data that are available in the literature are difficult to interpret and compare against other data due to lack of standardized testing protocols for the inactivation of viruses. To date, there are no standards for evaluating disinfectants against viruses in the U.S., however the EPA does provide guidelines for efficacy testing (1). The EPA guidelines require that a disinfectant must be validated for each individual organism for which it claims effective disinfection. Under these guidelines, the disinfection evaluation can be conducted as suspension or surface carrier tests, and must result in a 4 log₁₀ reduction in titer (1). Because viruses require a viable host to determine viability, the cytotoxicity (toxicity affecting the host cells) from the disinfectants must be completely removed prior to infecting the cells with disinfectant-treated virus.

The objective of this research was to evaluate various test disinfectants for efficacy against various isolates of influenza A including a mammalian strain (H1N1, A/WSN/33) and a low pathogenic avian influenza strain (H5N8, isolated from turkey) to be used as surrogates for the

high pathogenic avian influenza strain, H5N1. Physiochemical groups of viruses are believed to react similarly to disinfectants, due to the presence of similar structural targets such as structural surface proteins, capsid proteins, or the presence of a lipid envelope (3). On this basis, the surrogate strains (H1N1 and H5N8) of influenza A were deemed appropriate for evaluating the efficacy of disinfectants that could potentially be used against the H5N1 high pathogenic avian influenza A. Preliminary studies were conducted to determine infectious titer following various treatments with the test disinfectants after both 1 and 10 minute contact times and with various levels of organic challenge following existing EPA guidelines (1).

A secondary objective of this project was to study possible mechanisms of action of the test disinfectants by evaluating the effect on viral RNA using real time reverse transcriptase polymerase chain reaction (RT-PCR) and the effect on viral proteins using sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. SDS-PAGE is a method used to separate proteins based on size within a gel. Western blot analysis is used to detect the protein (based on a colorimetric reaction) using antibodies against the protein of interest. A minimum of three replications were conducted for all assays used in this study. Statistical evaluation of the amount of influenza A recovered following the various assays was determined by comparing 1-way, 2-way, and 3-way interactions between disinfectant treatment, time (exposure duration), and organic challenge level. The least square of means was calculated to determine if significant differences were observed in the amount of virus recovered by TCID₅₀ or quantitative RT-PCR.

Materials and Methods

Virus and Cells

Influenza A/WSN/33 (H1N1) or low pathogenic avian influenza H5N8 (H5N8) was propagated in 10 day embryonated hen eggs or Madin-Darby Canine Kidney (MDCK) cells to approximately $10^7 \log_{10}$ TCID₅₀. For cell propagation, infectivity media was made with Minimal Essential Medium (MEM) with Earle's salts, L-glutamine, and 2.2 g/L sodium bicarbonate (Fisher Scientific, Pittsburgh, PA) with the addition of trypsin TPK treated as previously described (10). Cells were maintained in Minimal Essential Medium (MEM) with Earle's salts, L-glutamine, and 2.2 g/L sodium bicarbonate (Fisher Scientific, Pittsburgh, PA) 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. Inoculum titer was assessed using endpoint dilution in tissue culture infective dose 50 (TCID₅₀) with MDCK cells.

Test Disinfectants

The disinfectants tested include ethanol (EtOH, 70%), bleach (1, 10%), Virkon® S (potassium peroxymonosulfate, Antec International, Ltd., 1%), and Sandia National Laboratories developed decontamination formulation, DF-200 (6), diluted by a factor of 2. This formulation was designated as DF-200d and tested at 25, 50, 100% strength. 70% EtOH was prepared by adding 70 ml 100% EtOH to 30 ml sterile deionized water. 10% bleach was prepared by adding 10 ml concentrated household bleach (5.25% sodium hypochlorite) to 90 ml sterile deionized water.

DF-200d was prepared by mixing 0.2 ml part 3 (propellant) to 2.5 ml of part 2 (hydrogen peroxide). This component was then added to 7.3 ml part 1 (surfactant). 1:2 serial dilutions were prepared in sterile deionized water.

Virus Inactivation

Virus inactivation was conducted with 0%, 10%, and 50% organic challenge (using a 10% poultry feces/liter liquid solution made by adding 10 g feces/litter to 90 ml sterile water). The poultry feces/litter material was provided generously by the Poultry Science Unit at Kansas State University, Manhattan, KS. For 0% organic challenge, 10 ml of test virus (H1N1 or H5N8) at approximately a $10^7 \log_{10}$ TCID₅₀/ml concentration was added to 10 ml test disinfectant (25-100% DF200d, 1-10% bleach, 1% Virkon® S, 70% EtOH) 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/litter 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/litter and exposed for 1 or 10 minutes. After exposure, samples were washed via ultracentrifugation at 100xG for 1 hour at 4°C. Pellets were reconstituted with 1 ml of virus infectivity media.

Infection Challenge

The titers of infectious virus were determined as TCID₅₀ in cell culture using a 96-well format. 96-well plates containing monolayer MDCK cells were washed once with infectivity media containing trypsin TPCK (0.2%). 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. Plates were then fed with 75 µl infectivity media into 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) visually observed due to damage of the host cells indicative of viral infection. The Reed-Muench method was calculated in a 96-well format to determine the dilution of virus resulting in infection of fewer than 50% replicate wells.

Effect on Viral RNA

Real time reverse transcription PCR was used to evaluate the effect of the various test disinfectants on the influenza viral RNA. RNA was extracted from disinfectant samples using a Qiagen Mini Viral RNA extraction kit. Following RNA extraction, real time RT-PCR was performed on the samples using 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 probe (5' TGC AGT CCT CGC TCA CTG GGC ACG 3'). Primer and probe sequences were provided by Ruben Donis, Chief Molecular Genetics Section Influenza Branch, at the Centers for Disease Control and Prevention in Atlanta, GA. Analysis of real time RT-PCR product amplification was determined and quantified by FAM fluorophor fluorescence using Cepheid SmartCycler system. A standard curve was made using known amounts of viral RNA to determine a quantitative measure of the amount of RNA in each sample.

Effect on Viral Protein

To determine the effect of the disinfectant against viral proteins, samples were heat denatured by boiling for 5 minutes and loaded onto a 10% SDS-polyacrylamide gel Express Gel (ISC BioExpress, Kaysville, UT). Gels were transferred onto a nylon membrane and western blot analysis was conducted using a monoclonal antibody specific for the nucleoprotein influenza A isolates (CDC, Atlanta GA).

Results

Infection Challenge

Infectious titer was determined in TCID₅₀ format following treatment with the various test disinfectants. The Reed-Muench method (5) was calculated in a 96-well format to determine the dilution of virus resulting in infection of fewer than 50% replicate wells. There were no significant differences ($p>0.05$) between the infectious titers of the H1N1 mammalian isolate (Figures 1-2) and the H5N8 low pathogenic avian influenza isolates (Figure 3 and 4) in comparing the least square of means. Treatment with DF-200d (100%) or bleach (10%) were the most effective resulting in nearly complete inactivation for all exposure times and organic challenge levels. With a 50% organic challenge, only 1 log₁₀ and 2 log₁₀ infectious viruses were recovered following 10 min treatment with DF-200d (100%) or bleach (10%), respectively. Treatment with DF-200d (25-50%) resulted in nearly complete inactivation, with less than 1 log₁₀ recovered following 1 or 10 min treatment with up to 10% organic challenge. With 50% organic challenge, infectious titer was reduced to 3 or 2 log₁₀ following 1 or 10 min treatment, respectively. Treatment with 1% Virkon S or 70% EtOH was most effective following a 10 min exposure. Infectious titer was reduced to less than 1 log₁₀ with 0% organic challenge, less than 2 log₁₀ with 10% organic challenge, and less than 3 log₁₀ with 50% organic challenge (Virkon S only) following 10 min exposures. Treatment with 70% EtOH resulted in 2 log₁₀ reduction at the 50% organic challenge following 1 or 10 min treatment. In all cases, treatment with 1% bleach was the least effective disinfectant. With 0% organic challenge, 2.5 log₁₀ virus was still recovered following 10 min treatment. There was also a decrease in efficacy with 10% and 50% organic challenges.

Figure 1: Infectious titer of mammalian test isolate A/WSN/33 H1N1 determined by TCID₅₀ following 1 minute exposure. CONT, Positive control; DF 100%, DF-200d (100%); DF 50%, DF-200d (50%); DF 25%, DF-200d (25%); BLCH 10%, bleach (10%); BLCH 1%, bleach (1%); VIRKON 1%, Virkon S (1%); EtOH 70%, ethanol (70%)

Figure 2: Infectious titer of mammalian test isolate A/WSN/33 H1N1 determined by TCID₅₀ following 10 minute exposure. CONT, Positive control; DF 100%, DF-200d (100%); DF 50%, DF-200d (50%); DF 25%, DF-200d (25%); BLCH 10%, bleach (10%); BLCH 1%, bleach (1%); VIRKON 1%, Virkon S (1%); EtOH 70%, ethanol (70%)

Figure 3: Infectious titer of avian test isolate H5N8 determined by TCID₅₀ following 1 minute exposure. CONT, Positive control; DF 100%, DF-200d (100%); DF 50%, DF-200d (50%); DF 25%, DF-200d (25%); BLCH 10%, bleach (10%); BLCH 1%, bleach (1%); VIRKON 1%, Virkon S (1%); EtOH 70%, ethanol (70%)

Figure 4: Infectious titer of avian test isolate H5N8 determined by TCID₅₀ following 10 minute exposure. CONT, Positive control; DF 100%, DF-200d (100%); DF 50%, DF-200d (50%); DF 25%, DF-200d (25%); BLCH 10%, bleach (10%); BLCH 1%, bleach (1%); VIRKON 1%, Virkon S (1%); EtOH 70%, ethanol (70%)

Effect on Viral RNA

Prior to analyzing any of the viral RNA treated with various test disinfectants, preliminary experiments were conducted to ensure that inhibition, due to possible chemical carry-over during extraction, was not resulting in false negatives. None of the test disinfectants used in these experiments resulted in inhibition, based on positive RT-PCR reactions after spiking the extractions with influenza RNA. Therefore, the observed degradation is due to disinfectant degradation of nucleic acid as opposed to chemical inhibition of the assay itself.

Similar to the infectious titer results, there was no significant difference ($p>0.05$) between the RT-PCR analysis results for the mammalian H1N1 (Figures 5 and 6) and the avian H5N8 (Figures 7 and 8) isolates.

Treatment with bleach (10%) or DF-200d (100%) with 0% organic challenge resulted in complete degradation of viral RNA following 10 min exposure. With 10% organic challenge, bleach (10%) remained highly efficacious resulting in complete degradation, while DF-200d resulted in 1 $\log_{10}$ recovery of viral RNA following 10 min exposure. For both bleach (10%) and DF-200d (100%), 50% organic challenge severely reduced disinfectant capability to degrade viral RNA, resulting in only a 1 $\log_{10}$ reduction from the starting concentration of virus. Treatment with DF-200d (25, 50%) or 1% Virkon S resulted in 2 to 4 $\log_{10}$ reduction following 10 min treatment with 0% organic challenge. Again, severe loss of mechanism for degrading viral RNA occurred with both 10% and 50% organic challenges. Treatment with bleach (1%) or 70% EtOH were the least effective at degrading viral RNA and resulted in similar $\log_{10}$ levels as the untreated positive control sample (figures 5-8)..

Figure 5: Recovered RNA from mammalian test isolate A/WSN/33 H1N1 determined by quantitative real time RT-PCR following 1 minute exposure. CONT, Positive control; DF 100%, DF-200d (100%); DF 50%, DF-200d (50%); DF 25%, DF-200d (25%); BLCH 10%, bleach (10%); BLCH 1%, bleach (1%); VIRKON 1%, Virkon S (1%); ETOH 70%, ethanol (70%)

Figure 6: Recovered RNA from mammalian test isolate A/WSN/33 H1N1 determined by quantitative real time RT-PCR following 10 minute exposure. CONT, Positive control; DF 100%, DF-200d (100%); DF 50%, DF-200d (50%); DF 25%, DF-200d (25%); BLCH 10%, bleach (10%); BLCH 1%, bleach (1%); VIRKON 1%, Virkon S (1%); ETOH 70%, ethanol (70%)

Figure 7: Recovered RNA from avian test isolate H5N8 determined by quantitative real time RT-PCR following 1 minute exposure. CONT, Positive control; DF 100%, DF-200d (100%); DF 50%, DF-200d (50%); DF 25%, DF-200d (25%); BLCH 10%, bleach (10%); BLCH 1%, bleach (1%); VIRKON 1%, Virkon S (1%); ETOH 70%, ethanol (70%)

Figure 8: Recovered RNA from avian test isolate H5N8 determined by quantitative real time RT-PCR following 10 minute exposure. CONT, Positive control; DF 100%, DF-200d (100%); DF 50%, DF-200d (50%); DF 25%, DF-200d (25%); BLCH 10%, bleach (10%); BLCH 1%, bleach (1%); VIRKON 1%, Virkon S (1%); ETOH 70%, ethanol (70%)

Effect on Viral Protein

Analysis by western blot assay using the monoclonal antibody is not very sensitive for detecting viral proteins (requiring 5 to 7 log₁₀ virus) and is not a quantitative assay. There are quantitative enzyme linked immunosorbant assays (ELISA) available, but many of the disinfectant chemistries interfered with the overall assay limiting its usefulness in this study. Due to the limited test sensitivity, western blot analysis was conducted for the test disinfectants with only the 0% organic challenge for 1 or 10 min treatment. Following a 1 min treatment, viral protein was visualized with the following treatments: DF-200d (25%), bleach (1%), 1% Virkon S, and 70% EtOH. Following 10 min exposure, viral protein was recovered from DF-200d (25%), bleach (1%), and 70% EtOH.

Figure 9: Western blot analysis of mammalian test isolate A/WSN/33 following various treatments with test disinfectants. Samples were treated for 1 min (A) or 10 min (B). Sample order is as follows: 1.) 50 kDa protein marker, 2.) positive control (H1N1); 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.

Discussion

Viruses are divided into several subgroups with regard to their resistance to disinfectants, as shown in 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 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) (2).

Virus Type	Category	Distinguishing Features	Examples
Enveloped	A	Nucleic acid, capsid protein, lipid envelope	Influenza , SARS, Vaccinia virus, HIV
Small Non-enveloped	B	Nucleic acid, capsid protein	Polio, FMDV, Rhino, Coxsackie
Large Non-enveloped	C	Nucleic acid, capsid	Adenovirus, Rotavirus

Table 1: Virus Classifications According to Klein and DeForest Disinfection Resistance (2, 4)

Test results indicate, as expected, that enveloped influenza A isolates are relatively easy to inactivate. Overall, these results indicate either DF-200d (100%) or household bleach (10%) are the most effective disinfectants for inactivation of influenza viruses based on TCID₅₀ results and degradation of viral RNA. The use of bleach would likely be less expensive, but has toxicity and corrosivity issues. However, if toxicity and corrosivity are issues are paramount, DF-200d would be more suitable for sensitive environments and materials based on manufacturers claims (6). Testing of disinfectant effectiveness towards the highly pathogenic avian influenza strain, H5N1, is required before inactivation and disinfection claims towards that specific strain can be asserted, and to obtain appropriate EPA registration or waiver for disinfectant use during an outbreak (1).

Finally, the use of a real time quantitative RT-PCR could have very useful field application for determining decontamination effectiveness. Current standards rely on environmental sampling with subsequent virus isolation in appropriate cell culture, which can take 24 to 72 hours. Nucleic acid degradation and subsequent viral inactivation can be confirmed by real time RT-PCR assay and requires only a few hours for complete analysis. Such a field application would be appropriate for a presumptive analysis of the effectiveness of the decontamination effort, but full virus isolation would still be required for decontamination verification and site clearance.

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DISTRIBUTION:

M. M. Chengappa
Mosier Hall, KSU Vet School
1800 Denison Ave.
Manhattan, KS 66506

Richard D. Oberst
Mosier Hall, KSU Vet School
1800 Denison Ave.
Manhattan, KS 66506

David R. Franz
Director National Agricultural Biosecurity Center
365 West Patrick Street, Suite 223
Federick, MD 21701

R. W. Trewyn Ph. D.
Vice Provost for Research
108 Anderson Hall
Manhattan, KS 66506-0113

Nancy Jaax
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1 Fairchild Hall
Kansas State University
Manhattan, KS 66506

Ruben Donis Ph. D.
Section Chief, Molecular Genetics
Centers for Disease Control and Prevention
1600 Clifton Road MailStop G-16
Atlanta, GA 30333

Tammy Beckham DVM, Ph.D.
Chief Forensics/Disease Threat and Assessment
Department of Homeland Security
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Orient Point, NY 11944

Gregory R. Siragusa Ph. D.
Research Microbiologist
Agricultural Research Service - USDA
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950 College Station Road
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Distinguished Professor
Pennsylvania State University
115 Henning Building
University Park, PA 16802-3500

Peter Setlow
Dept. of Molecular, Microbial and Structural Biology
University of Connecticut Health Center
263 Farmington Avenue
Farmington, CT 06032

Carol Cardona, DVM, PhD, Dipl. ACPV
VM: Extension
Rm. 1383 Surge III
University of California, Davis
Davis, CA 95616

Dennis Smagac
Director of Business Development
Intelagard
590 Burbank Street
Suite 220
Broomfield, CO 80020

Roger Messick
Vice President, Operations
EnviroFoam Technologies
2903 Wall Triana Hwy., Suite 5B
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Brian J. Kalamanka
President/CEO
Modec Inc.
4725 Oakland Street
Denver, CO 80239

Neville P. Clarke
Director
National Center for Foreign Animal and Zoonotic Disease Defense
TAMUS 2129
College Station, TX 77843-2129

Kevin Irvine
Director of Operations
EnviroFoam Technologies
2901 Wall Triana Hwy, Suite 200
Huntsville, AL 35824

Jack Gelb, Jr, Ph.D.
Dept. of Animal and Food Sciences
College of Agriculture and Natural Resources
University of Delaware
531 South College Avenue
Newark, DE 19717 USA

Robert G. Webster, PhD
Rose Marie Thomas Chair
St. Jude Children's Research Hospital
Director World Health Organization Collaborating Center for Studies on the Ecology of
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St. Jude Children's Research Hospital
332 N. Lauderdale
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Washington, DC 20460

Francis (Frank) F. Busta
Director, The National Center for Food Protection and Defense
University of Minnesota - Twin Cities Campus
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