

Executive Summary by Allen Johnston - Chief Technology Officer, EcoQuest International

Kansas State University Testing

Biological reduction on surfaces through photocatalysis and ozone with ActivePure RCI technology.

Summary:

Testing has been performed at the Kansas State Food Science Institute in the Department of Animal Sciences & Industry, Kansas State University in Manhattan Kansas under the direction of Dr. James Marsden, Regent's Distinguished Professor of Meat Science. Kansas State is one of America's foremost Universities for animal science and Dr. Marsden is known around the world as one of the top researchers and experts in food safety.

Ten of the most deadly forms of mold, fungi, bacteria

and virus were subjected to a new and innovative Photocatalytic Reactor called Radiant Catalytic Ionization (ActivePure). These ten organisms were placed on a piece of stainless steel inside a test chamber and the ActivePure cell was turned on for 24 hours. Test results showed a 24-hour reduction ranging from 96.4% to 100%.

This testing validates the effectiveness and speed which RCI is able to treat the indoor surface environment using a natural process at safe levels of oxidation.

Discussion:

With most indoor airborne contaminants originating on surfaces, any efforts to control biological contamination in the indoor environment must address surfaces.

Microorganisms such as Mold, Bacteria and Viruses thrive on surfaces in the presence of moisture, and for this reason the food industry has focused on controlling and eliminating pathogens in food contact areas.

Dr. Marsden has dedicated his life to improving food safety through understanding and controlling the spread of biological contamination. Marsden's research has recently focused on the use of advanced photocatalysis, a technology which develops oxidizers which actively reduce airborne and surface pathogens.

Ten microorganisms were chosen for analysis. Three samples of each microorganism were prepared and placed on a stainless steel surface, allowing analysis at 2 hours, 6 hours and 24 hours of exposure.

The test organisms included:

- Staph (Staphylococcus aureus)
- MRSA (Methicillin Resistant Staphylococcus aureus)
- E-Coli (Escherichia coli)
- Anthrax family (Bacillus spp.)
- Strep (Streptococcus spp.)
- Pseudomonas aureuginos
- Listeria monocytogenes
- Candida albicans
- Black Mold (Stachybotrys chartarum)
- Avian Influenza H5N8

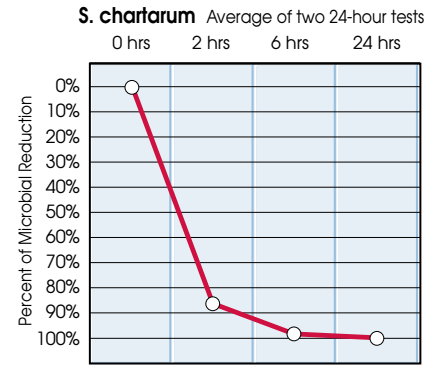
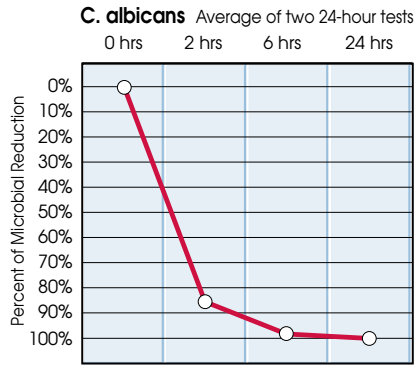
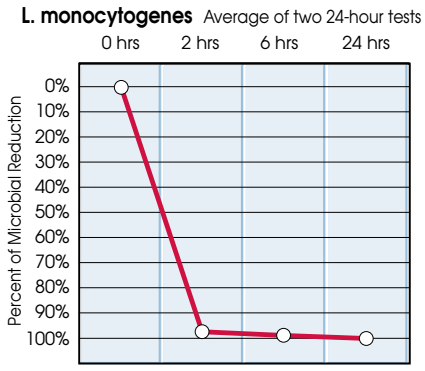
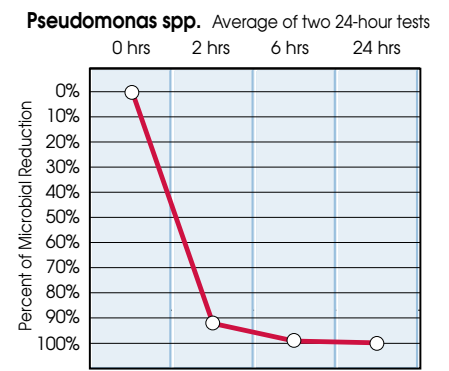
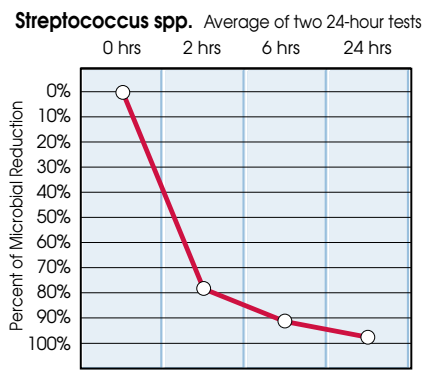
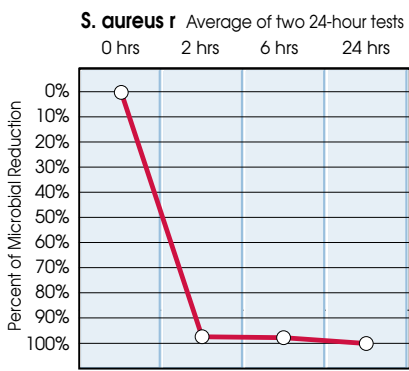
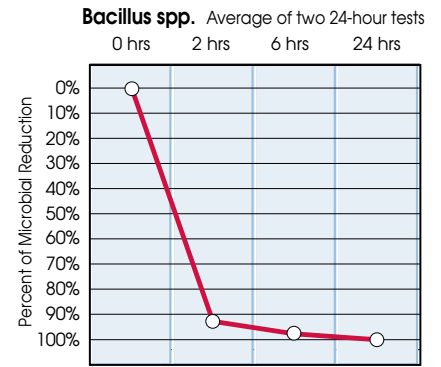
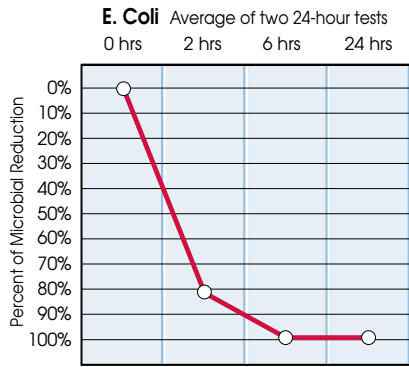
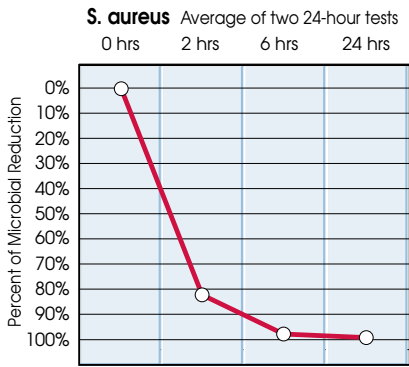
These organisms were subjected to air which was circulating through a proprietary photo catalytic reactor called Radiant Catalytic Ionization or RCI (ActivePure). Multiple parameters were monitored including temperature and humidity. The UV Lamp in the photo catalytic cell was positioned in the supply duct to insure there was no effect from the UVGI produced by the lamp. Understanding that Ozone is one of the oxidizers produced in this Photocatalytic process and the health concerns from exposure to excessive levels of ozone, the ozone level was monitored and never exceeded 20 parts per billion, well below EPA maximum level for continuous exposure.

In addition to the test chamber treated with ActivePure and the corona discharge ozone generator, a control chamber was set up to account for natural decay of the test organisms. Because some biological pathogens die-off on their own when exposed to air, any reputable study must account for such reductions. The test results shown in the report are the reductions in viable organisms with respect to the control sample.

The test results were astounding. After 24 hours of exposure the nine organism's viability was reduced between 96.4% and 100%. It should be noted that the double blind study accounted for natural decay. What was even more surprising to the researchers was how fast ActivePure reduced the pathogens. At the 2-hour sample the average reduction was well over 80%. At the 6-hour sample the average reduction was well over 90%.

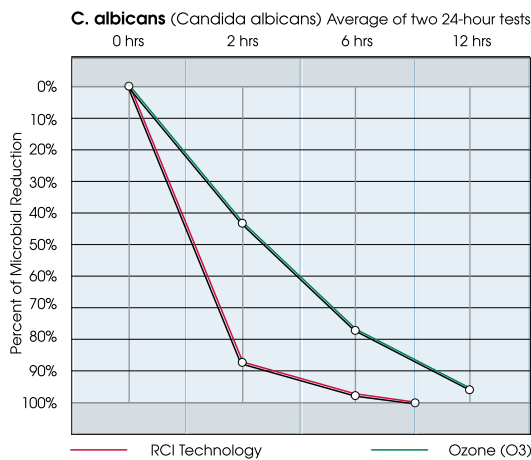
Effects of ActivePure (RCI) Technology

on reducing common bacteria and fungi on **surfaces*** in 24-hour testing.



Comparing The Effects of ActivePure (RCI) Technology and Ozone Technology

on reducing common bacteria and fungi on **surfaces*** in 24-hour testing.



Testing by Kansas State University. Field results may vary based on environmental conditions.

*Scientific testing has demonstrated the use of EcoQuest's ActivePure technology to substantially reduce microbial populations on surfaces – including but not limited to Escherichia coli, Listeria monocytogenes, Streptococcus spp., Pseudomonas aeruginosa, Bacillus spp., Staphylococcus aureus, Candida albicans, and S. chartarum. Field results may vary based on environmental conditions. No claim with respect to airborne microbials is made based on these results. These results have not been evaluated by the FDA. This product is not a medical device intended to diagnose, treat, cure, or prevent any disease.

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Efficacy of EcoQuest Radiant Catalytic Ionization (ActivePure) Cell and Breeze AT Ozone Generators at Reducing Microbial Populations on Stainless Steel Surfaces

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Summary and Implications

This study was conducted to determine the potential use of EcoQuest Radiant Catalytic Ionization (ActivePure) Cell for the inactivation of *Escherichia coli*, *Listeria monocytogenes*, *Streptococcus* spp., *Pseudomonas aeruginosa*, *Bacillus* spp., *Staphylococcus aureus*, *Candida albicans*, and *S. chartarum*, on stainless-steel surfaces at diverse contact times in a controlled airflow cabinet. In addition, the EcoQuest Breeze AT Ozone generator was evaluated under the same conditions for the inactivation of *Candida albicans* and *S. chartarum*. Better disinfection technologies for food contact surfaces are needed to control food borne pathogens in processing environments. Ozone technologies have only recently been approved for use on food contact surfaces. This study evaluated the application of gaseous ozone and other oxidative gases on stainless-steel surfaces against the microorganisms listed above. Both technologies reduced populations of all microorganisms tested on stainless-steel surfaces by at least 90% after 24 h exposure. The Radiant Catalytic Ionization (ActivePure) Cell was more effective at reducing microbial counts for shorter exposure times than was the Breeze AT Ozone Generator.

INTRODUCTION

The food and beverage industries face a number of issues when it comes to producing a safe, wholesome product. Foodborne pathogens such as *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. have been a growing concern throughout the years. Processors are also concerned about spoilage microorganisms that shorten shelf life and cost companies millions every year in spoiled product. Industries impacted include the meat, seafood, poultry, produce, baking, canned foods, dairy, and almost all other segments of the market.

The U.S. Department of Agriculture estimates the costs associated with food borne illness to be about \$5.5 to \$22 billion a year. This doesn't include the billions lost every year due to spoiled product, which must be disposed of or sold as a lesser valued product. Better disinfection and microbiological control measures are needed in almost every area of the food industry.

As a disinfectant, ozone has a tremendous ability to oxidize substances. It's thousands of times faster than chlorine and disinfects water three to four times more effectively. As it oxidizes a

substance ozone will literally destroy the substance's molecule. It can oxidize organic substances such as bacteria and mildew, sterilize the air, and destroy odors and toxic fumes. Ozone has been used by industry for many years in numerous applications such as odor control, water purification, and as a disinfectant (Mack, 1993). Recent government approval of ozone for use with foods and food contact surfaces has opened the door to many more exciting possibilities for this technology.

In June 2001, the U.S. Food and Drug Administration approved the use of ozone as a sanitizer for food contact surfaces, as well as for direct application on food products. Prior to that time, chlorine was the most widely used sanitizer in the food industry. Ozone may be a better choice for disinfection of surfaces than chlorine. Chlorine is a halogen-based chemical that is corrosive to stainless steel and other metals used to make food-processing equipment. Chlorine can also be a significant health hazard to workers; when mixed with ammonia or acid cleaners, even in small amounts, a toxic gas can form.

Chlorine is a common disinfect used in meat processing and is effective and safe when used

at proper concentrations. However, chlorine is far less effective than ozone and can result in the production of chloroform, carbon tetrachloride, chloroacetic acid, and tri-halomethanes. In contrast, ozone leaves no residual product upon its oxidative reaction.

An important advantage of using ozone in food processing is that the product can be called organic. An organic sanitizer must be registered as a food contact surface sanitizer with the U.S. Environmental Protection Agency (EPA). Ozone has such an EPA registration, and is approved by FDA as a sanitizer for food contact surfaces and for direct application on food products.

Ozone has become more accepted for use in food processing in recent years and is being used in more than just surface applications. A recent U.S. FDA recommendation (2004) stated that "ozone is a substance that can reduce levels of harmful microorganisms, including pathogenic *E. coli* strains and *Cryptosporidium*, in juice. Ozone is approved as a food additive that may be safely used as an antimicrobial agent in the treatment, storage, and processing of certain foods under the conditions of use prescribed in 21 CFR 173.368."

MATERIALS AND METHODS

Preparation of Cultures:

The following bacteria and fungi cultures were used for the study: *Bacillus globigii* (ATCC # 31028, 49822, 49760), *Staphylococcus aureus* (ATCC # 10832D, 25178, 11987), *Candida albicans* (ATCC # 96108, 96114, 96351), *Stachybotrys chartarum* (ATCC # 18843, 26303, 9182), *Pseudomonas aeruginosa* (ATCC# 12121, 23315, 260), *Escherichia coli* (ATCC# 27214, 19110, 67053), *Streptococcus pneumoniae* (ATCC# 27945, 29514, 10782), and *Staphylococcus aureus* - methicillin resistant (ATCC# 33591). Cultures were revived using ATCC recommended instructions.

Bacteria, yeast, and mold strains were individually grown in trypticase soy broth (TSB; Difco Laboratories, Sparks, MD) and YM broth

(Difco Laboratories), respectively, to mid-exponential phase followed by a wash and re-suspension in 0.1% peptone water. The cultures were combined by species type to ca. 10^8 CFU/ml.

Preparation of Samples and Ozone Treatment:

The microbial species used to validate the ozone generators were tested as microbial cocktails inoculated onto 6.3 x 1.8 cm on #9 finish stainless-steel coupons (17.64 cm² double sided area). Four stainless steel coupons were dipped per microbial inoculum and vortexed 15 sec to optimize microbial dispersion. Using sterile binder clips, stainless steel coupons were suspended on a cooling rack contained inside a laminar flow cabinet for 1 h to dry. The initial microbial populations attached to the stainless steel coupons ranged from 5 to 6 log CFU/cm². The inoculated stainless steel coupons were transferred to a controlled airflow test cabinet (Mini-Environmental Enclosure, Terre Universal, Anaheim, CA) at 26°C and 46% relative humidity (ambient conditions), and treated using the EcoQuest Radiant Catalytic Ionization Cell for 0, 2, 6, and 24 h. The EcoQuest Breeze AT Ozone generator was evaluated separately for treatment periods of 0, 2, 6 and 24 h. Ozone levels were monitored throughout the study (Model 500, Astroqual, New Zealand).

Sampling:

At the end of the ozone contact time the coupons were vortexed for 30 sec in 30 ml of 0.1% peptone water. Samples inoculated with bacterial cultures were serially diluted, plated on trypticase soy agar (TSA; Difco Laboratories), and incubated for 24 h at 35°C. After preparing serial dilutions, samples inoculated with yeast were plated on potato dextrose agar (PDA; Difco Laboratories) and those inoculated with mold cultures were plated on cornmeal plates. Both PDA and cornmeal plates were incubated 30°C for 5 days. Following incubation, data for each microorganism were reported as colony-forming units per square centimeter (CFU/cm²).

RESULTS AND DISCUSSION

Reductions in microbial populations on #8 finish stainless steel coupons following 0, 2, 6, and 24 h exposure to the EcoQuest Radiant Catalytic Ionization Cell are presented in Figure 1. Exposure to ozone levels of 0.02 ppm for 2 h reduced all microbial populations tested by at least 0.7 log CFU/cm². Longer exposure times resulted in greater reductions, with the greatest reductions found after 24 h exposure. After 24 h exposure, mean microbial reductions for each organism were as follows: *S. aureus* (1.85 log CFU/cm²), *E. coli* (1.81 log CFU/cm²), *Bacillus* spp. (2.38 log CFU/cm²), *S. aureus* metr (2.98 log CFU/cm²), *Streptococcus* spp. (1.64 log CFU/cm²), *P. aeruginosa* (2.0 log CFU/cm²), *L. monocytogenes* (2.75 log CFU/cm²), *C. albicans* (3.22 log CFU/cm²), and *S. chartarum* (3.32 log CFU/cm²). Reductions in microbial populations following treatment of stainless steel coupons with the EcoQuest Breeze AT Ozone generator are shown in Figure 2. Reductions of at least 0.2 and 0.4 log CFU/cm² were observed after 2 and 6 h of ozone exposure, respectively. After 24 h exposure, mean reductions for *C. albicans* and *S. chartarum* were 1.48 and 1.32 log CFU/cm², respectively.

The EcoQuest Radiant Catalytic Ionization (ActivePure) Cell and EcoQuest Breeze AT Ozone generators reduced microbial populations on stainless steel surfaces within 2 h under ambient conditions, with greater reductions associated with longer exposure times. The Radiant Catalytic Ionization (ActivePure) Cell was more effective than the Breeze AT Ozone Generator at reducing microbiological populations at shorter exposure times of 2 and 6 hours. This study demonstrated that ozone gas has the potential to be an effective surface disinfectant for use in food processing applications. Testing is currently ongoing to evaluate non-treated controls. Phase II of the project, scheduled to be completed by the end of this year, will evaluate the effectiveness of the system for eliminating airborne contamination using the same microorganisms and oxidative technologies.

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Fig. 1 Decontamination of highly polished stainless steel surfaces using the EcoQuest Radiant Catalytic Ionization (ActivePure) Cell

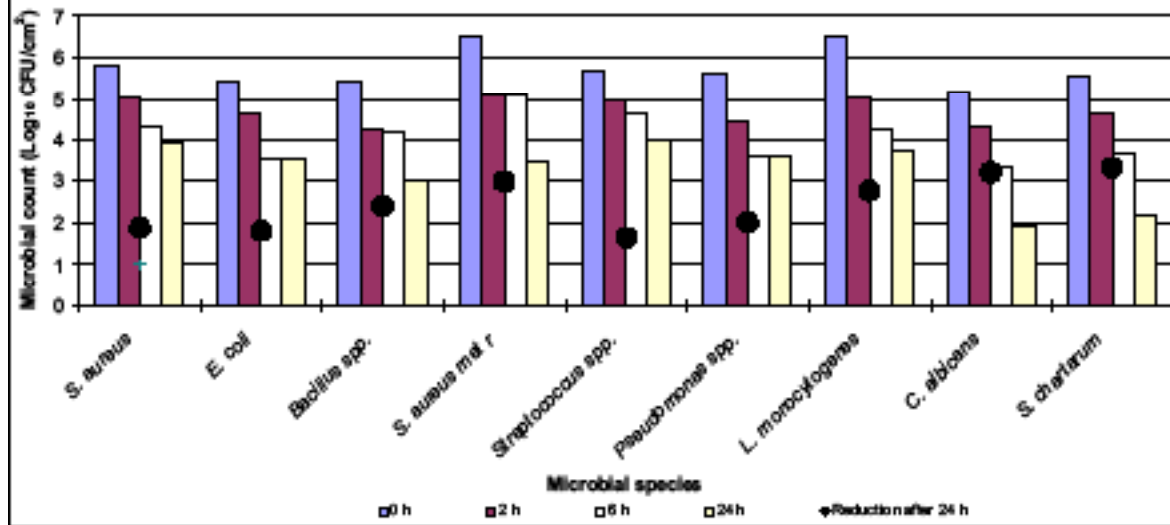
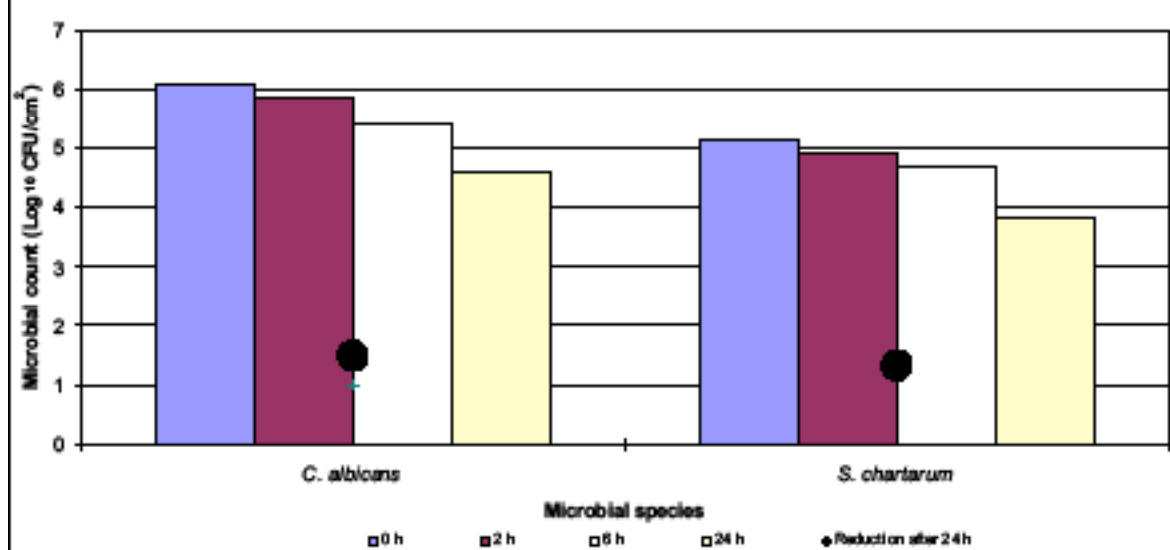


Fig 2. Ozone decontamination on highly polished stainless steel surfaces using the EcoQuest Breeze AT Ozone generator



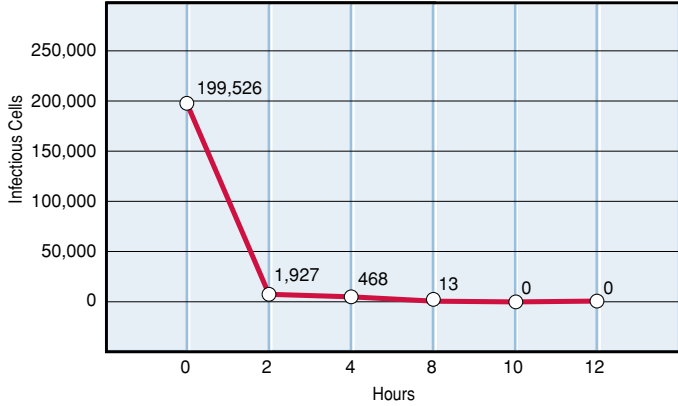
Effects of ActivePure (RCI) Technology

on reducing Avian Influenza A (H5N8) on **surfaces*** in 12-hour testing.

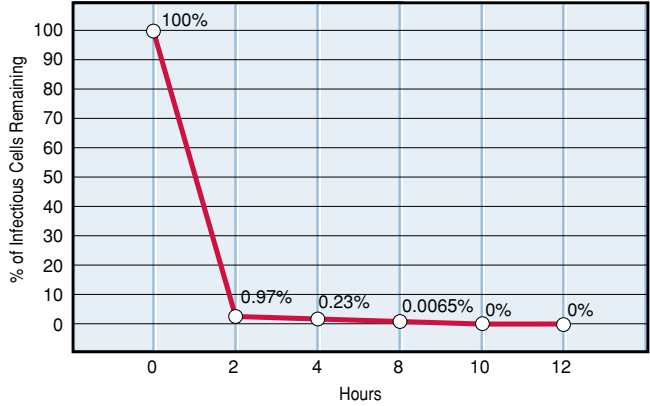
Testing by Kansas State University.



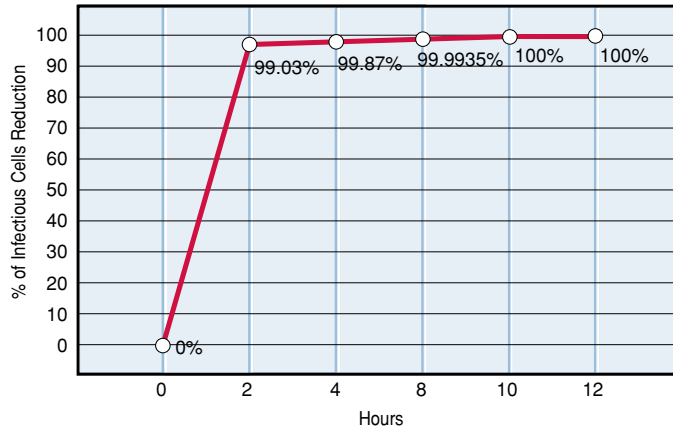
Avian Influenza A (H5N8) Inactivation with ActivePure (RCI)
Infectious Cells vs Time



Avian Influenza A (H5N8) Inactivation with ActivePure (RCI)
Percent of Infectious Cells Remaining vs Time



Avian Influenza A (H5N8) Inactivation with ActivePure (RCI)
Percent of Infectious Cells Reduced vs Time



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ActivePure (RCI) Inactivation of Avian Influenza

INTRODUCTION

The influenza virus, a member of the viral family Orthomyxoviridae, is characterized as being an enveloped single stranded negative sensed RNA virus (6) that can result in yearly endemic outbreaks and more severe world-wide pandemic outbreaks. Influenza A commonly infects human, swine, equine, and avian isolates. In the case of a pandemic outbreak, highly pathogenic avian influenza (H5N1) is currently the greatest threat due to current epidemic status in Asia, Europe, and Africa and continued threat for pandemic spread. Reassortment of genomic information of the influenza virus can result in a more pathogenic and infectious isolate is heightened during ongoing outbreaks, which could result in a devastating human-to-human transmissibility. Influenza virus is typically spread via aerosols, large droplets, or contact with infectious secretions or fomites (4).

Rapid containment of an outbreak is important for preventing further spread and minimizing the potential for reassortment to occur. Influenza has been shown to survive on nonporous surfaces for up to 48 hours and on material surfaces such as cloth, paper, or tissue for up to 12 hours after being deposited at approximately a 10⁵ TCID₅₀/ml level (1). In addition to surface sanitation and disinfection regimens, airborne inactivation of influenza virus is also vital to address predominant modes of transmission such as aerosol and large droplet (4). Environmental contamination with aerosolized droplets containing this pathogen can serve as a reservoir for infection and must be controlled by effective sanitation and disinfection protocols. Minimizing the degree of environmental contamination with highly effective decontamination measures would aid in the overall containment efforts of an outbreak.

The purpose of this study is to validate the complete inactivation of influenza A viruses using a low pathogenic avian influenza (H5N8) as a surrogate virus for the highly pathogenic avian influenza (H5N1) following exposure to the Radiant Catalytic Ionization-Cell™ (ActivePure-Cell™) system. The ActivePure-Cell™ system is an advanced oxidation tool which combines UV inactivation in the presence of hydroxical radicals so that synergy between two highly effective inactivation technologies occurs. Efficacy will be determined for dried inoculum on solid surfaces, in cell culture propagated inoculum, and nebulized in a controlled chamber. Efficacy will be determined by reduced or complete loss of infectivity in a cell culture system for treated samples compared to non-treated positive control samples.

MATERIALS AND METHODS

Virus and cells. Low pathogenic avian influenza H5N8 (H5N8, provided generously by the Centers for Disease Control and Prevention, Atlanta, GA) was propagated in 10 day embryonated hen eggs (Kansas State University Department of Poultry Science, Manhattan, KS) to approximately 10⁷ log₁₀ TCID₅₀ (as determined in Madin Darby Canine Kidney, MDCK cells). Cells were maintained in Minimal Essential Medium with Earle's salts and L-glutamine (Invitrogen Corporation, Carlsbad, CA) and 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 antibiotics [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 by adding MEM with the addition of 0.1% TPCK treated trypsin (Fisher Scientific) and supplemented with antibiotics (2.5 mg/L amphotericin B; 0.67 g/L streptomycin; and 0.3 g/L penicillin G).

H5N8 inactivation. Type 302 stainless steel (McMasterCarr, Atlanta, GA) coupons (2 x 10 cm², thickness 0.8 mm) were sterilized by autoclaving for 15 min at 121 C. In a biosafety class II cabinet, 100 µl of egg propagated H5N8 was added to each test coupon and spread to cover the entire surface using the pipette tip and allowed to dry completely for approximately 10-15 min. Then, the inoculated coupons were placed into a sterile transport container and transported to the test chamber. The test coupons were then attached to clips within the test chamber so that all sides of the coupon would be exposed to the ActivePure-Cell™ treatment. One coupon was removed prior to starting the ActivePure-Cell™ treatment to be used as the initial control sample. The ActivePure-Cell™ device was then turned on and samples were taken at various intervals (2, 4, 8, 12, 24 hours) by removing a test coupon and preparing it for virus recovery as described below.

Virus Recovery. H5N8 virus was recovered from the stainless steel surfaces by adding the test coupon to a sterile 50 ml conical vial (Fisher Scientific) containing 5 ml infectivity media. Tubes were then vortexed for 1 min. Endpoint dilution titration was conducted in MDCK cells by adding 220 µl from the 5 ml infectivity media containing any suspended virus to the first dilution well in a minimum of 6 wells of a 96 well microtiter plate containing confluent MDCK cells. Then, serial 1:10 dilutions were prepared by adding 20 µl from the first well into the next 6 wells each containing 180 µl infectivity media. The final well contained only 200 µl infectivity media to serve as a negative cellular control. Plates were incubated at 37 C, 5% CO₂ for 48 hours. Cytopathic effect (CPE) was determined for each well and viral counts were reported as TCID₅₀/ml as calculated by Reed and Muench (3).

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. The detection threshold for successfully detecting influenza RNA was a FAM fluorescence signal ≥ 3 using the SmartCycler.

RESULTS

The average amount of H5N8 recovered from the stainless steel coupons in all experiments was 5.35 log₁₀ TCID₅₀/ml. Following treatment with the ActivePure-Cell™, the average log reductions of the H5N8 virus were 1.85, 2.79, 4.16, 5.35, and 5.35 log₁₀ TCID₅₀/ml following 2, 4, 8, 12, and 24 hour treatments (Figure 1) based on the recovery of infectious virus.

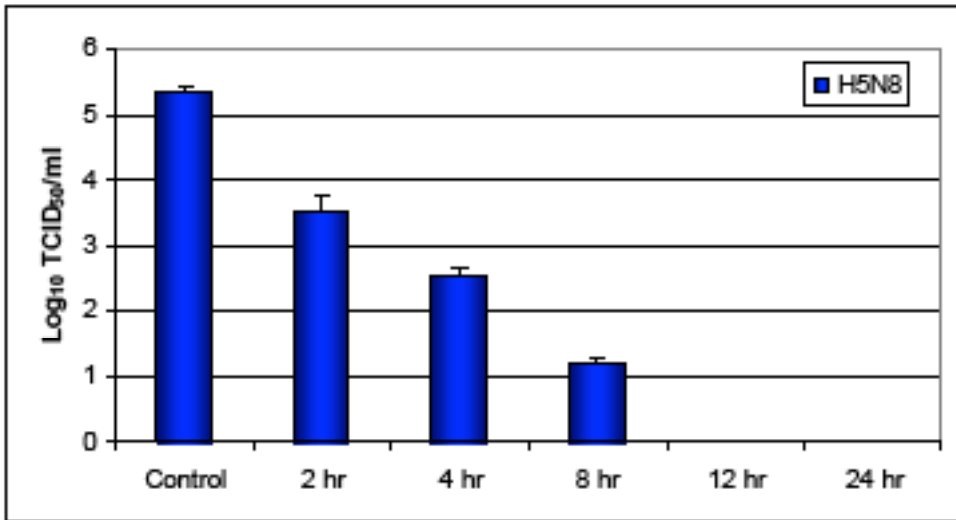


Figure 1: Recovery of H5N8 post-treatment with ActivePure-Cell™ based on TCID₅₀/ml in MDCK cells.

The average amount of viral H5N8 RNA recovered from the stainless steel coupons in all experiments was 4.00 log₁₀ based on a quantitative RT-PCR available for influenza A viruses. Following treatment with the ActivePure-Cell™, the average log reductions of the H5N8 virus based on the amount of RNA recovered varied between 0.23 to 0.54 log₁₀ following all exposure times (2, 4, 8, 12, and 24 hour) indicating that the mechanism of action for loss of infectivity was more likely due to disruption of the lipid envelope or structural proteins than with degradation of the viral nucleic acid (Figure 2).

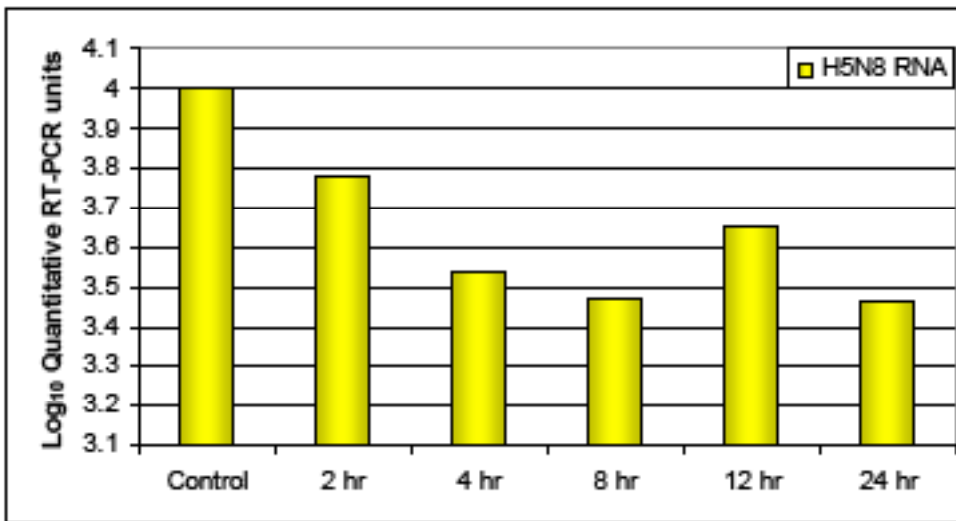


Figure 2: Recovery of H5N8 RNA post-treatment with ActivePure-Cell™ based on quantitative RT-PCR.

DISCUSSION

In an effort to better understand the inactivation of the influenza virus using the ActivePure-Cell™, the efficacy was evaluated using a low pathogenic avian influenza isolate, H5N8 inoculated onto stainless steel surfaces. Inactivation efficacy was determined following

the current EPA guidelines for determining virus disinfection (2) which allows the recovery of treated virus as endpoint dilution including a TCID50 recovery assay of infectious virus. In addition to the recovery of infectious virus, we wanted to determine if any disruption of viral RNA was occurring by using a quantitative RT-PCR assay specific for influenza A viruses in our experiments.

Based on the current EPA guidelines to achieve a $> 4.0 \log_{10}$ reduction in starting virus titer (2), ActivePure-Cell™ treatment for 8 hours or more resulted in the successful inactivation of the H5N8 isolate (Figure 1) for a starting contamination level of $5.35 \log_{10}$ TCID50/ml. Additional testing would be required to determine if lower exposure times would result in complete inactivation for contamination levels lower than $5.35 \log_{10}$ TCID50/ml, which might be more representative in a real outbreak (1, 5).

The quantitative RT-PCR results indicate that degradation of viral RNA (Figure 2) was not the major mechanism for viral inactivation, as the levels of RNA recovered after each treatment time were not significantly different from each other, $P > 0.05$. Other possible viral targets include the lipid envelope and structural proteins which were likely affected by the ActivePure-Cell™ treatment. The oxidative mechanism of this treatment likely disrupted the relatively susceptible envelope and could have resulted in denaturing the surface structural proteins of the influenza virus necessary for successful attachment and entry mechanism vital for infectivity.

The results obtained in this research experiment show that exposure to the ActivePure-Cell™ system for 8 hours results in the required level of inactivation of an avian influenza isolate, H5N8 which was used as a safe surrogate for the highly pathogenic H5N1 isolate. The mechanism of action of this technology is likely due to the oxidative chemistry resulting in both disruption of the lipid envelope and the denaturing effect on the structural viral proteins necessary for virus replication.

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