The University of Georgia
Center for Food Safety

Study Title

Inactivation of Feline Calicivirus and Murine Norovirus After Exposure to Atmospheric Ozone (20 ppm) with Humidity Control (80%) using the ZONOsanitech Portable Ozone Generator.

Product Identity

On-site generated ozone (20 ppm) with humidity control (80%).

Data Requirement

Four log reduction of infectious virus using the human norovirus surrogates, Feline Calicivirus (FCV) and Murine Norovirus (MNV-1).

Author

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Study Completion Date

February 11, 2011

Testing Facility

The Center for Food Safety at the University of Georgia
1109 Experiment St. Melton Bldg. Griffin, GA 30223

Laboratory Project Number

ZONO.3

The University of Georgia

Center for Food Safety

STUDY REPORT

Study Title: Inactivation of Feline Calicivirus and Murine Norovirus After Exposure to Atmospheric Ozone (20 ppm) with Humidity Control (80%) using the ZONOsanitech Portable Ozone Generator.

Sponsor:
Walter Mann
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Johns Creek, GA 30022

Test Facility:
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1109 Experiment St.
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Griffin, GA 30223

Test substance identification

Test substance name: 20 ppm ozone
Lot/Batch number(s): Not applicable
Description of test substance: on-site generated
Chemical characterization: O_3

Study initiation date: December 9, 2009
Experimental start date: September 23, 2010
Experimental end date: October 16, 2010
Study completion date: February 11, 2011

Study objective: To determine the inactivation of feline calicivirus (FCV) and murine norovirus (MNV) {surrogates for human norovirus inactivation studies} upon exposure to a 20 ppm atmospheric ozone with 80% relative humidity, generated by a portable ozone generator (ZONOsanitech).

Test method: CONFIRMATORY VIRUCIDAL EFFECTIVENESS TEST Using Feline Calicivirus As Surrogate for Norovirus. Antimicrobials Division, U.S. Environmental Protection Agency.

Test system/strains: FCV (strain F-9; ATTC # VR-782) and MNV (a gift from Herbert "Skip" Virgin, Washington University) were propagated in confluent monolayers of Crandel Reese Feline Kidney (CRFK) cells (ATCC # CCL-94) or RAW 264.7 (ATCC # TIB-71) cells, respectively.

STUDY MATERIALS

Media: To determine the infectious titer of MNV and FCV, standard plaque assay techniques were employed. Briefly, cells were dispensed in 60 mm diameter cell culture plates at a density of 2×10^6 cells per plate and grown to 80-90% confluence in complete Dulbecco's Modified Eagles Medium (DMEM) containing penicillin (100 U/ml), streptomycin (100 µg/ml), 100 mM HEPES, and 1 mM sodium pyruvate and 10% low endotoxin fetal bovine serum (FBS) (HyClone, Logan, UT) or FBS (Atlanta Biologicals, Atlanta, GA), for MNV and FCV, respectively. Immediately preceding infection, the cell culture media was replaced with 0.5 ml of complete MEM without phenol red (Cellgro, Mediatech, Inc), supplemented with either (a) 5% low endotoxin FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) with 100 mM HEPES, and 10 mM sodium pyruvate for MNV or (b) 4% FBS (Atlanta Biologicals), 1% L-glutamine and 1% non-essential amino acids for FCV. Ten-fold serial dilutions of virus were prepared in phosphate buffered saline (PBS) pH 7.5 and cell monolayers were infected in duplicate with 0.1 ml of each virus dilution, and 0.5ml of complete MEM (see below) for 1 h at 37 °C and 5% CO_2 with gentle rocking every 15 min. Subsequently, the cells were overlaid with complete MEM (without phenol red) (Cellgro) supplemented as described above but also containing 0.5% agarose (SeaKem GTG, Lonza, Rockland, ME). Viruses were incubated for 48 h at 37 °C and 5% CO_2. Plaques were subsequently counted 5-8 h after a second agarose overlay (0.5% agarose diluted in deionized water and including 1% neutral red solution (Sigma-Aldrich, St. Louis, MO)) was added. Plates with 5 to 50 plaques were used to determine the virus titer in plaque forming units (PFU).
Neutralizing media consisted of DMEM supplemented with 5% FBS (Atlanta Biologicals).
Reagents: Serial dilutions of treated virus or untreated controls were performed in complete MEM prepared as described above.
Equipment: ZONOsanitech Portable Ozone Generator (ZONOsanitech, LLC. Johns Creek, GA), Ecosensors SM4 meter, NUV106 L Ozone Monitor by 2B Technologies (Boulder, Colorado).

TEST METHOD:

Preparation of test substance: Twenty parts per million (ppm) ozone was generated in the ZONOsanitech chamber during each experimental trial. Ozone concentrations inside the chamber were measured using a NUV106 L Ozone Monitor by 2B Technologies (Boulder, Colorado) and controlled by manual operation of an on/off switch.

Preparation of test system/strains:

Inoculum Preparation: FCV (strain F-9; ATTC # VR-782) and MNV (a gift from Herbert "Skip" Virgin, Washington University) were propagated in confluent monolayers of Crandel Reese Feline Kidney (CRFK) cells (ATCC # CCL-94) or RAW 264.7 (ATCC # TIB-71) cells, respectively. To generate high titer virus stocks, partially purified cell culture lysates were ultracentrifuged (500,000 x g) for 1 hr at 4°C and pellets were suspended in 0.1 M Phosphate Buffered Saline (PBS) containing 5% Fetal Bovine Serum (FBS). Aliquots of virus stocks were stored at -70°C until use.

Carrier Preparation: Two hundred µl of FCV or MNV stock solutions (6.87-7.71 $\log_{10}$ plaque forming units (pfu) / ml) were spread uniformly onto glass Petri dishes using a cell scraper. Virus

was allowed to dry for 30-40 min (until visibly dry). The physical conditions of the drying process are indicated in **Table 1**. Three treatment plates and one recovery control plate was prepared for each of three replicate experiments (for a total of n = 9 for treatment plates and n = 3 for recovery plates). One

un-inoculated Petri dish was also included in each experimental replicate and served as a negative control for subsequent testing of test agent neutralization and cytotoxicity.

Table 1: Record of conditions during drying for each experimental trial (Set A, Set B and Set C).

	Set A	Set B	Set C
Temp (°C)	27.2	26.4	27.4
Time (min)	30.0	30.0	30.0
Relative Humidity	39.0%	42.0%	41.0%

Exposure Conditions: For each experimental trial (replicate) virus-coated and un-inoculated control Petri dishes were exposed to 20 ppm atmospheric ozone for 18 min. Relative humidity (RH) inside the ZONOsanitech chamber were measured by a Ecosensors SM4 meter. Average RH measurements and standard deviations are listed for each experimental trial in **Table 2**.

Test system recovery:

Test substance neutralization and virus dilution: After a 10 min decontamination period, during which time ozone levels dropped to $\leq$ 0.1 ppm, Petri plates were removed and immediately transferred to a laminar flow biological safety hood. Appendix 1 (redacted for confidentiality) indicates ozone concentrations within the chamber at each exposure time. For neutralization of test agent, 2 ml of DMEM containing 10% FBS was immediately added to each Petri dish. Viruses were mixed into the neutralization buffer by agitation of the Petri dish surface with a cell scraper. Neutralized samples and controls were stored at -70°C undiluted until analysis. Just before analysis, 10X serial dilutions were prepared for each sample and control in cell culture media containing 5% FBS.

Table 2: Relative humidity inside the ZONOsantitech chamber during each experimental treatment (Set A, Set B, Set C).

	Set A	Set B	Set C
Average	80.92%	80.20%	80.33%

Standard deviation	0.99%	0.84%	0.58%

Infection, cell maintenance and infectivity assays: Cells (CRFK and RAW 264.7) were dispensed in 60 mm diameter cell culture plates at a density of 2 x 10^6 cells per plate and grown to 80-90% confluence in complete DMEM. Immediately preceding infection, the cell culture media was replaced with 0.5 ml of complete MEM without phenol red (Cellgro, Mediatech, Inc), supplemented with either (a) 5% low endotoxin FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) with 100 mM HEPES, and 10 mM sodium pyruvate for MNV or (b) 4% FBS, 1% L-glutamine and 1% non-essential amino acids for FCV. One hundred µl portions of selected ten-fold serial dilutions of the neutralized virus/test agent mixtures and controls were added to cell culture monolayers in duplicate. Cells were incubated for 1 h at 37 °C and 5% CO_2 with gentle rocking every 15 min to allow virus adherence to monolayers. The liquid medium was subsequently removed from each Petri plate and cell monolayers were overlaid with complete MEM (without phenol red) (Cellgro) supplemented as described above but also containing 0.5% agarose (SeaKem GTG, Lonza, Rockland, ME). Viruses were incubated for 48 h at 37 °C and 5% CO_2. Plaques were subsequently counted 5-8 h after a second agarose overlay (0.5% agarose diluted in deionized water and including 1% neutral red solution (Sigma-Aldrich, St. Louis, MO)) was added. Plates with 5 to 50 plaques were used to determine the virus titer in plaque forming units (PFU).

Protocol deviations: None.

CONTROLS

Preparation of controls: For each experimental replicate, the following controls were subjected to identical conditions as the test samples; 1. Cell viability control: consisting of cell culture media only (no virus) used to measure viability of the cells throughout the course of the plaque assay; 2. Virus stock titer: consisting of selected serial dilutions of the virus stock to ensure viability of the virus and susceptibility and permissiveness of the host cells; 3. Plate recovery control (PRC): used to measure the recovery efficiency of the method used to elute viruses from the surface of the Petri dishes and measure any detrimental impacts of drying the virus on the surface of the Petri dishes, these controls were held in the biological safety hood for the duration of each test agent exposure time and processed identically to the treated samples. Log_{10} reductions in virus titers due to test agent treatment were determined by comparison to the PRC; 4. Neutralizer effectiveness control (NEC): to determine the effectiveness of the neutralization

procedure, these controls were prepared as follows; virus inoculation media (PBS containing 5% FBS) without virus was coated on a Petri dish, exposed to the test agent, neutralized, and processed identically to the test agents. After neutralization, 10-fold diluted (in PBS) virus added to each NEC, held at ambient temperature for the test agent exposure time (18 min) before serial dilution and analysis by plaque assay; 5. Cytotoxicity control (CT): used to measure cell viability after exposure to neutralized test agent, this control is identical to the NEC but without the addition of virus.

STUDY ACCEPTANCE CRITERIA

Study requirements:

1) Control, neutralization and strain quantification requirements follow the CONFIRMATORY VIRUCIDAL EFFECTIVENESS TEST Using Feline Calicivirus As Surrogate for Norovirus". (Antimicrobials Division, U.S. Environmental Protection Agency) and are outlined above.
2) A minimum of 4-$\log_{10}$ reduction in infectious virus titers (using Feline Calicivirus and Murine Norovirus model viruses) as per the U.S. EPA's requirements for making Virucidal claims for a surface disinfectant as outlined in "CONFIRMATORY VIRUCIDAL EFFECTIVENESS TEST Using Feline Calicivirus As Surrogate for Norovirus". Antimicrobials Division, U.S. Environmental Protection Agency. According to the EPA, the test agent is acceptable if a minimum of 4-log reduction in CPE of FCV (complete inactivation of the virus occurs at all dilutions tested) is demonstrated compared to PRC. When cytotoxicity is present, at least a 3-log reduction from the PRC must be demonstrated beyond the cytotoxic level with complete inactivation of the virus at all dilutions tested.

DATA ANALYSIS

Calculations: Virus titers for each experimental replicate and replicates within each experiment are expressed in $\log_{10}$ pfu which represent the average virus titers from duplicate plates in the plaque assay. Reported virus titers represent the average of all replicates combined (n = 9 for treatments and n = 3 for controls). Log_{10} reductions in infectivity were calculated by subtracting average virus titers after treatment from PRC virus titers.

Statistical analysis: All differences among treatment and control groups were determined with Analysis of Variance (SAS/English statistical package version 9.2) and comparison of the average log transformed values of the responses (log pfu reduction of virus infectivity) to that of the untreated controls. Significant differences were determined at the α =0.05 level.

STUDY RETENTION

Data Retention: Electronic files are maintained on a protected folder on the University of Georgia, Center for Food Safety network drive. Files are password protected for access only by approved personnel in the Cannon lab.

Specimen retention: Specimens are retained in a -70°C freezer at the University of Georgia, Center for Food Safety (Cannon lab- room 121, Melton Bldg.).

STUDY RESULTS:

Experimental results for treatment and control samples are presented in **Table 3**. Virus stock titers were 6.87 and 7.71 for FCV and MNV, respectively. There was no visible loss of cell viability following incubation of cells with neutralization buffer (complete DMEM). There was no apparent cytotoxicity following incubation of neutralized test agent on cell cultures. Addition of virus to neutralization controls resulted in average virus stock titers of 6.79 and 6.57 $\log_{10}$ pfu / ml, indicating sufficient neutralization of test agent. Plate recovery controls were 5.17 $\log_{10}$ pfu / ml for FCV and MNV but not viable virus was detected in samples treated with 20 ppm ozone. Therefore $\log_{10}$ reductions of virus titer (pfu / ml) were 5.17 for both FCV and MNV.

Table 3: Average virus titers ($\log_{10}$ pfu / ml) of treatment and control samples. Standard deviations are indicated in parentheses.

	FCV titer (pfu/ml)	MNV titer (pfu/ml)
Virus stock titer	6.87	7.71
Test results	0.00 (±0.00)	0.00 (±0.00)
Plate recovery control	5.17 (±1.25)	5.17 (±0.38)
Neutralization control	6.79 (±0.32)	6.57 (±0.11)
Cytotoxicity control	0	0
Cell viability control	0	0

STUDY CONCLUSIONS:

A 4 $\log_{10}$ reduction of two norovirus surrogates was achieved using the ZONOsanitech device, indicating this technology meets the performance criteria necessary to make efficacy claims for virucical activity against norovirus, as per the U.S. EPA's requirements for making Virucidal claims for a surface disinfectant as outlined in "CONFIRMATORY VIRUCIDAL EFFECTIVENESS TEST Using Feline Calicivirus As Surrogate for Norovirus". Antimicrobials Division, U.S. Environmental Protection Agency.

DISCUSSION:

Noroviruses are the leading cause of acute gastroenteritis and the second leading cause of severe gastroenteritis in children ≤ 5 years old. They are easily transmissible from person to person or through contact with contaminated items touched by an ill person. Unlike influenza virus, they are non enveloped (they do not have a lipid bilayer membrane (envelope) surrounding the virus structure). This property - makes them well suited to survival on surfaces (i.e. door knobs, hand rails, toys, table tops) for long periods of time and infers them greater resistant to many common surface disinfectants (i.e. ethanol, quaternary ammonium, or phenols).

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Results from our studies indicate that atmospheric ozone conditions generated inside the ZONOsanitech apparatus (20 ppm ozone, for 18 min, at ambient temperature and 80% relative humidity) are effective for inactivating noroviruses. This has been determined by observation of at least a 4 log10 reduction in virus titers (using Feline Calicivirus and Murine Norovirus model viruses) after treatment. This meets EPA's requirements for making Virucidal claims for a surface disinfectant.

Report Submitted by:

Study Director

Feb. 11, 2011

Study Completion Date

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