STUDY TITLE

Assessment of the Activity of ozonated water technology against Viruses in Suspension:
Testing against Testing against Cystovirus Phi6 (ATCC 21781-B1) as a representative Healthcare-Associated Pathogen

TEST ORGANISM

Cystovirus phi6 (ATCC 21781-B1))

TEST SAMPLE IDENTITY

Ozonated Water Technology

TEST Method

ASTM E1052-11

AUTHOR

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Study Director

STUDY COMPLETION DATE

Aug/17/20

PERFORMING LABORATORY

CREM Co. Labs. Units 1-2, 3403 American Dr., Mississauga, Ontario, Canada L4V 1T4

SPONSOR

Franke Kindred Canada
1000 Franke Kindred Road, Midland, Ontario L4R 4K9, Canada

STUDY NUMBER

FKC200625-01

GOOD LABORATORY PRACTICE STATEMENT

The study referenced in this report was not conducted in compliance with U.S. Environmental Protection Agency's Good Laboratory Practice (GLP) regulations set forth in 40 CFR Part 160.

Submitter: _____ Date: _____

Sponsor: _____ Date: _____

Study Director: _____ Date: _____

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title:	Assessment of the Activity of ozonated water technology against Viruses in Suspension:Testing against Testing against Cystovirus Phi6 (ATCC 21781-B1) as a representative Healthcare-Associated Pathogen
Test Substance	Ozonated Water Technology
Study Number:	FKC200625-01
Sponsor	Franke Kindred Canada, Midland, Ontario, Canada
Testing Facility	CREM Co Labs Unit 1-2, 3403 American Drive, Mississauga, ON, Canada L4V 1T4

TEST SUBSTANCE IDENTITY

Test Substance Name:

Ozonated Water 2ppm

TEST SYSTEM

1. **Test Microorganism**
   Cysovirus Phi6 (ATCC 21781-B1): Cysovirus Phi6 (ATCC 21781-B1) is an enveloped phage with a spherical virion of 85 nm in diameter. The virion has a double-capsid structure with a three-segmented dsRNA genome. It is a lytic bacteriophage of Gram-negative plant pathogenic bacteria (ICTV 2009). It belong to the family Cystoviridae.

   Structure and function in the lipid-containing Cystovirus phi6 have been well characterized (Jaalinoja et al. 2007). The size, relative molecular weight (Mr), buoyant density, protein content, overall complexity and structural composition appear to be similar to large enveloped viruses such as Coronavirus. Here, we use phi6 as a surrogate of coronaviruses such as 229E, SARS-1 and SARS-2 (19-nCOV). Unlike phi6, testing against 229E is expensive and time consuming, on the other hand SARS-1, SARS-2 and Middle-East Respiratory Syndrome (MERS) virus require Biosafety Level 3 labs. Therefore, Phi6 is used as surrogate for them to screen the activity of different technologies for infection prevention and control (IPAC) for finding the shortest contact time.

2. **Host Bacteria**

   Pseudomonas syringae (ATCC 19310). *Pseudomonas syringae* subsp. *syringae* (ATCC19310) is a Gram-negative plant pathogenic bacteria. *P. syringea* was grown aerobically in Loren broth (LB; Fisher) at 28±1°C for 18±2 h.

Preparation of Test Inocula
The suspension of phi6 was diluted 1000 times using normal saline. No soil load has been used in this study

TEST METHOD

1. **Preparation of Test Substance**
The ozone generated (Figure 1) was used to generate 2 ppm ozonised water. The concentration of ozone was validated using a HATCH Ozone Test Kit (OZ-2(2064400))

Figure 1: ozone generator

2. Test Procedure

The virus suspension was mixed thoroughly and one part (50 µL) of the suspension was added to nineteen parts (950 µL) of the test substance. The mixture was vortex mixed for 30 seconds and held at room temperature (22±2°C) for the rest of the contact time. Immediately at the end of the contact time, the microbicidal activity was neutralized by ten-fold dilution into a normal saline solution, i.e. 100 µL of the mixture of virus and test substance in 900 µL of diluent (dilution 10^0).

As a control, one part (50 µL) of diluted virus suspension was added to nineteen parts (950 µL) of EBSS. The contact time and temperature for the control samples were the same as those for the test samples.

Using normal saline, 10-fold dilutions were prepared for each test and control samples (up

to dilution 10^{-6}). All the samples were kept on ice during processing. LB agar plates with host bacteria cells were inoculated with 100 µL of the dilutions prepared from treated and control samples. The cultures were incubated at 30±1°C for 20-24 hrs before counting plaque-forming units (PFU).

DATA ANALYSIS

Calculation of Log_{10} Reduction

Log_{10} Reduction = Log_{10} of average PFU from control carriers – log_{10} of average PFU the test carriers.

STUDY ACCEPTANCE CRITERIA

No product acceptance criterion was specified for this range-finding study.

TEST RESULTS

Efficacy Test: Table 1 shows the result of virus inactivating activity of each sample tested.

Table 1: Log_{10} Reductions and Percentage Reduction of ozonated water against Cystovirus *phi6 (ATCC VR-740)*

Test Sample	Log_{10} Reductions	Percentage Reductions
Ozonated Water 2ppm	5.40	99.9996

CONCLUSIONS

No plaques were observed on any of the test plates. The sample demonstrate >5.40 Log_{10} reduction (99.9996% reduction).

APPENDIX

Result of suspension test on 2ppm ozonated water at 60 minutes contact time against Cystovirus phi6.

Contact Time	60 minutes			
Dilution	Control #1	Control #1	Treated #1	Control
10^{-0}	TNTC	TNTC	0	0
10^{-1}	TNTC	TNTC	0	0
10^{-2}	TNTC	TNTC	0	0
10^{-3}	112	94	0	0
10^{-4}	11	7	0	0
10^{-5}	2	2	0	0
10^{-6}	0	0	0	0

C= Control
TNTC= Too numerous to count

References

1. ASTM International. Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension (E1052-11). ASTM, West Conshohocken, PA.
2. Canadian Biosafety Standards and Guidelines (2015). Public Health Agency of Canada, Ottawa, ON, Canada. (http://canadianbiosafetystandards.collaboration.gc.ca/cbs-ncb/index-eng.php.).
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4. American Chemical Society, Reagent Chemicals, American Chemical Society Specifications, Washington, DC, 10th edition, 2006. (https://www.amazon.com/Reagent-Chemicals-Specifications-Procedures-Analytical/dp/0841239452).
5. Freshney, R.I., Culture of Animal Cells: A Manual of Basic Technique & Specialized Applications, 6th ed., 2010. Wiley-Blackwell, New York, NY. (http://ca.wiley.com/WileyCDA/WileyTitle/productCd-0470528125.html?productCd=0470528125).
6. Sattar, S.A., Springthorpe, V.S., Adegbunrin O., Zafer, A.A. & Busa M. (2003). A disc-based quantitative carrier test method to assess the virucidal activity of chemical germicides. J. Virol. Methods. 112: 3-12.