# KILL-TIME STUDIES
Antimicrobial Activity of EO $H_2O$
Using *Clostridium difficile* spores
Test Solutions: Acidic $H_2O$
Submitted December 12, 2011

December 20, 2011

PREPARED FOR:

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I. PURPOSE.

The purpose of this study was to determine the antimicrobial activity of acidic EO water on *Clostridium difficile* ATCC 43598 spores. This was accomplished by performing a standard kill-time suspension test using 0.5, 1, and 2 minute contact times.

II. MATERIALS AND METHODS.

A. Test organism.

A test suspension containing endospores from *Clostridium difficile* (ATCC # 43598) was prepared from a culture grown for nine days at 37 °C on CDC anaerobic blood agar (0.04% Tryptic Soy agar, 0. 05% yeast extract, 0.004% L-cystine, 0.005% Hemin, 0.001% Vitamin $K_1$, 5.0% sheep blood). The suspension was placed at 70 °C for 5 min to kill vegetative organisms, and then centrifuged to pellet the spores. Spores were resuspended in sterile physiological saline solution (PSS) with 0.1% Tween and allowed to set overnight at 4 °C. This washing/setting process was repeated a total of three times. The final spore suspension was examined for purity using phase-contrast microscopy and stored at 4 °C until used.

B. Neutralizers.

The Neutralizer solution consisted of 9ml tubes of 12.7% Tween 80, 6.0% Tamol, 1.7% lecithin, 1% Peptone, 1.0% Cysteine and 500 mM Tris (pH 7.0).

C. Kill-Time Procedure.

1. 9.9 ml of acidic EO $H_2O$ was added to a 50 ml polypropylene sterile centrifuge tube, which was equilibrated in a 20 °C water bath. Then, 0.1 ml of the *C. difficile* spore suspension was added at time zero.
2. At the end of the specified contact times (30 sec, 1 min, 2 min), 1 ml of this mixture was added to 9 ml of neutralizer. The tube was mixed thoroughly.
3. After two min, the neutralized suspension was serially diluted in 9 ml blanks of physiological saline solution (PSS).
4. The number of viable organisms in selected dilution tubes was assayed by membrane filtration. One ml aliquots were plated in duplicate. The membranes were washed with about 100 ml of sterile PSS and removed to *C. difficile* fructose agar (4% Proteose Peptone, 0.5% $Na_2PO_4$, 0.1% $KHPO_4$, 0.2% NaCl, 0.01% $MgSO_4$, 0.6 fructose, 7% sheep blood, 0.1% sodium taurocholate) plates. The plates were incubated anaerobically using an Anoxomat system (Spiral Biotech, Inc.), at 37 °C for 6 days.
5. The number of colonies on each filter was counted and log reduction and percent kill values were computed.

D. Controls.

1. A titer of the test suspension was computed by performing membrane filtration assays on selected 1:10 dilutions in PSS of the test suspension.

2. A neutralizer control for each disinfectant was performed by inoculating a mixture of 9.0 ml of neutralizer and 1 ml of disinfectant with 0.1 ml of the $1:1x10^5$ dilution of the titer. This produced about 104 CFU / ml in the tube, which was allowed to stand for 20 minutes prior to dilution and assay by membrane filtration using duplicate 1 ml samples.

III. RESULTS.

***C. difficile* spore suspension:**
**Titer.**

| | Dilution: | | |
|---|---|---|---|
| | $1:1x10^6$ | $1:1x10^7$ | $1:1x10^8$ |
| Number of colonies: | TNC | 105 | 12 |
| | TNC | 106 | 13 |

**Acidic EO $H_2O$:**
(Received 12/12/11)
Exposure Dilution of *C. difficile* spores/disinfectant suspension:

| Time | $1:1x10^1$ | $1:1x10^2$ | $1:1x10^3$ | $1:1x10^4$ | $1:1x10^5$ |
|---|---|---|---|---|---|
| 30 sec | | | 0 | 0 | 0 |
| | | | 1 | 0 | 0 |
| 1 min | 31 | 1 | 0 | 0 | |
| | 42 | 2 | 0 | 0 | |
| 2 min | 0 | 0 | 0 | | |
| | 3 | 0 | 0 | | |

| **Neutralization Control** | | **Expected Counts**: | | **Percent of Expected:** |
|---|---|---|---|---|
| Undiluted | 1:10 | Undiluted | 1:10 | 134 |
| 146 | 13 | 104 | 10 | |
| 135 | 17 | | | |

**Sterility Controls:**

| Material | Counts |
|---|---|
| PSS | 0, 0 |
| Acidic water | 0, 0 |
| Neutralizer | 0, 0 |
| *C. difficile* fructose Agar | 0, 0 |

IV. DISCUSSION.

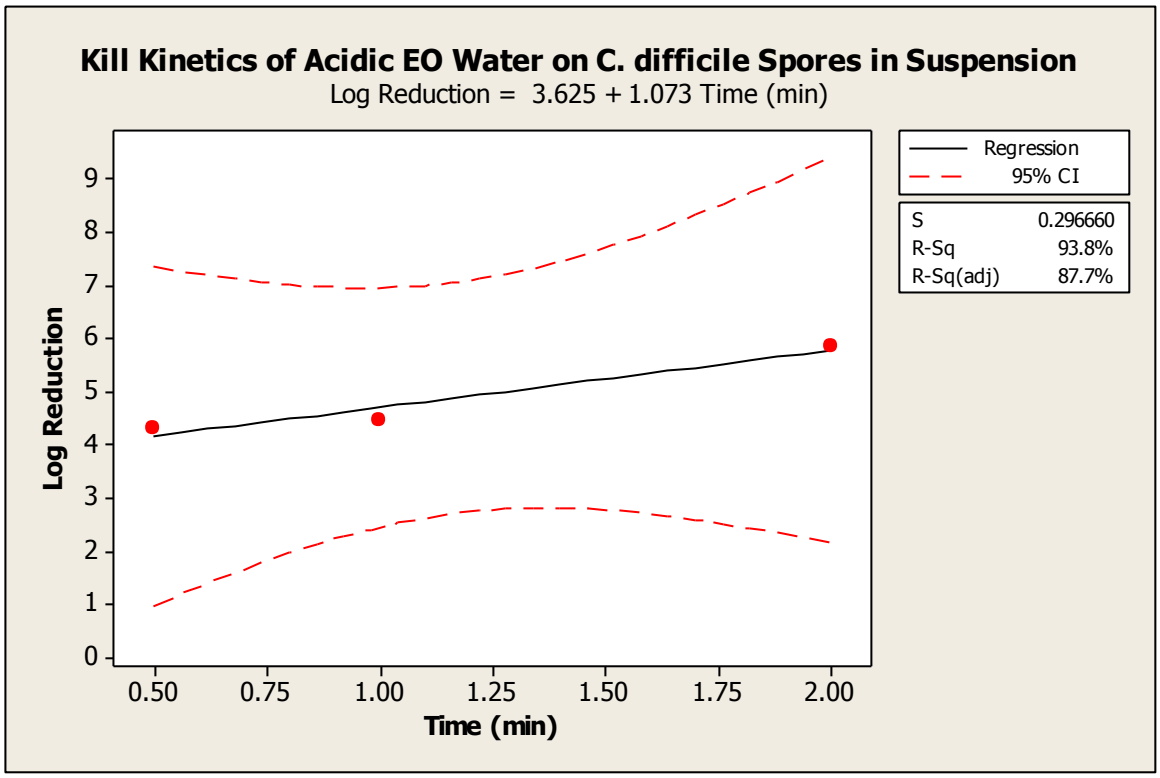
Results of the titer showed a viable *C. difficile* spore concentration of $1.06 \times 10^9$ organisms per ml in the test suspension. Inoculation of 9.9 ml of disinfectant with 0.1 ml of this suspension produced an initial concentration of $1.06 \times 10^7$ *C. difficile* spores per ml in the assay tube.

Results from these procedures allowed log reduction (LR) and percent kill (PK) values to be calculated using the formulas: 1) LR = -Log(S/So); where S = concentration of viable spores after the specified contact time; and So = the initial concentration of viable spores at time zero. 2) PK = (1 - (S/So)) × 100. These values are shown below.

| Solution | Contact Time | Log Reduction (LR) | Percent Kill (PK) |
|---|---|---|---|
| Acidic EO water | 0.5 min | ~4.32 | 99.9953 |
| | 1.0 min | 4.46 | 99.9965 |
| | 2.0 min | 5.85 | 99.99986 |

Neutralization control data revealed that the neutralizer was able to adequately neutralize the acidic EO water. Observed counts were 134% of those expected.

The acidic EO water submitted on 12/12/2011 exhibited rapid sporicidal activity on *C. difficile* spores. A regression fit of this data is shown on the following page. The adjusted $R^2$ value was only of 87.7%, probably reflecting inaccurate log reduction estimates at the 30 second and 2 minute contact times, due to very low counts. However, the data does show that the acidic EO water is capable of a very rapid kill of *C. difficile* spores, exhibiting a 4.46 log reduction within 1 minute. Extrapolation using the regression equation obtained from these data would predict a 6 log reduction (killing of 1 million *C. difficile* spores) in 2.2 minutes.


Kill Kinetics of Acidic EO Water on C. difficile Spores in Suspension
Log Reduction = 3.625 + 1.073 Time (min)
Log Reduction
Time (min)
0
1
2
3
4
5
6
7
8
9
0.50
0.75
1.00
1.25
1.50
1.75
2.00
Regression
95% CI
S 0.296660
R-Sq 93.8%
R-Sq(adj) 87.7%

Test Dates: December 13-20, 2011

Performed By:

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