Study Report

Study Title
Quantitative Suspension Time Kill Test of R-Water test substance against *Clostridium difficile* endospores at ambient temperature.

Study Identification Number
NG1071

Test Microorganism(s)
Clostridium difficile ATCC 43598

Study Sponsor
Rayne Guest with R-Water, LLC

Testing Facility
Microchem Laboratory, LLC
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Round Rock, Texas 78664

Lead Scientist
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Study Completion Date
12 MAY 2015

Study Objective
The purpose of this study was to measure the ability of a test substance to kill *C. difficile* endospores in suspension at ambient temperature.

Study Conclusion
The test substance displayed a 5.47 $\log_{10}$ reduction against *C. difficile* endospores at a 1 minute contact time at ambient temperature.

Study Summary

One lot of test substance was evaluated for sporicidal activity in an ASTM 2315 suspension time-kill procedure against *C. difficile.* In this study, 0.10 ml of culture was added to a 9.90 ml volume of test substance. After a 1.0 minute exposure time, 1.00 ml of the reaction mixture was removed and added to a 9.00 ml volume of D/E (Dey Engley) neutralization recovery medium. Serial dilutions were made from this neutrallized suspension and plated on brain-heart infusion (BHI) agar supplemented with defibrinated blood and sodium taurocholate. The plates were incubated anaerobically for 96±2 hours at 36±1°C and then observed for growth and colonies counted.

To determine the baseline concentration of test microorganism before exposure to test substance, 9.90 ml of an inert control substance (PBS) was evaluated by inoculation with 0.100 ml of the same test culture and then immediately transferring 1.00 ml of this solution to 9.00 ml of D/E (Dey Engley) neutralization recovery medium. The unexposed "time zero" enumeration controls showed 3×10^{6} CFU/ml within the inoculum pre-exposure. The test samples showed 1.00×10^{1} CFU/ml after a 1 minute exposure to test substance. Under these conditions, the test substance demonstrated a 99.9997% rate of kill against *C. difficile* endospores, equivalent to a 5.47 $\log_{10}$ reduction relative to initial numbers control.

Alongside the test and enumeration control plates, sterility was confirmed for the D/E neutralization recovery medium, phosphate buffered saline (PBS), and the plating media itself. Neutralization validation of the tested solution relative to an untested control solution indicated success of the D/E neutralizer and confirmation of a true one minute contact time.

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Materials Used in the Study

- Pure culture of test system (*Clostridium difficile* ATCC 43598).
- Sufficient quantity of clean, sterile 100×15 mm Petri dishes.
- Sufficient quantity of 50 ml centrifuge tubes containing 9 ml sterile neutralizing recovery medium (D/E Broth).
- Sufficient quantity of microcentrifuge tubes containing 0.90 ml Phosphate Buffered Saline (PBS).
- Incubator capable of sustaining 36±1 °C incubation temperatures.
- Sufficient quantity of brain-heart infusion (BHI) agar supplemented with defibrinated blood and sodium taurocholate, for confirmation of growth of the test microorganism.
- Micropipettes and a sufficient quantity of appropriately sized sterile micropipette tips.
- Certified digital timer.
- Anaerobic chamber.
- Anaerobic sachets.

Procedure and Parameters

Preparation of Bacterial Culture

- The test microorganism was prepared by growth in liquid culture medium and the final spore suspension was confirmed to have an endospore purity exceeding 90% when comparing endospores to total CFU.

Preparation of Test Substance

- Test substance arrived ready to use from Sponsor.

Enumeration Control ("Time Zero")

- The test culture was assayed to determine the original number of CFU in the reaction tube. 100 µl of *C. difficile* inoculum was added to 9.90 ml PBS and mixed on a vortex mixer. 1.00 ml was then transferred to 9.00 ml D/E to simulate the neutralization step.
- A serial dilution was initiated with 0.1 ml removed from the solution and added to 0.9 ml PBS. Appropriate dilutions were plated on BHI agar, and incubated at 36±1°C for 96±2 hours.
- *C. difficile* colonies were counted and multiplied by the appropriate dilution factor to determine the number of CFU originally in the reaction tube.

Test Procedure

- 100 µl of *C. difficile* inoculum suspension was added to a 9.90 ml volume of test substance within a 50 ml tube and the solution was mixed on a vortex mixer. After an exposure time of 1.0 minute at ambient temperature, 1.00 ml of the test solution was removed and added to 9.00 ml D/E neutralizer. A ten-fold serial dilution was then initiated into PBS.
- The dilutions were plated on BHI agar and plates were incubated anaerobically for 96±2 hours at 36±1°C. *C. difficile* colonies were counted and multiplied by the appropriate dilution factor to determine the number of colony forming units (CFU) per milliliter.

Neutralization Validation

- A 1.00 ml aliquot of the test substance was neutralized in 9.00 ml D/E and then inoculated with a dilute *C. difficile* suspension targeting 100 CFU/ml.
- A 1.00 ml aliquot of PBS control was transferred to a separate 9.00 ml volume of D/E and then was inoculated with a dilute *C. difficile* suspension targeting 100 CFU/ml.
- Both vessels were plated on BHI agar and subsequently incubated anaerobically for 96±2 hours at 36±1°C. *C. difficile* colonies were counted and multiplied by the appropriate dilution factor to determine the number of colony forming units (CFU) per milliliter.
- It was confirmed that the treated neutralization test sample displayed at least 70% of the growth observed on the untreated neutralization control plates.

Study Controls

- Sterility controls were plated for D/E neutralizer, PBS, and supplemented BHI agar.
- A viability control was plated on BHI agar from the *C. difficile* suspension to confirm both viability of the culture and confirm anaerobic conditions during incubation.

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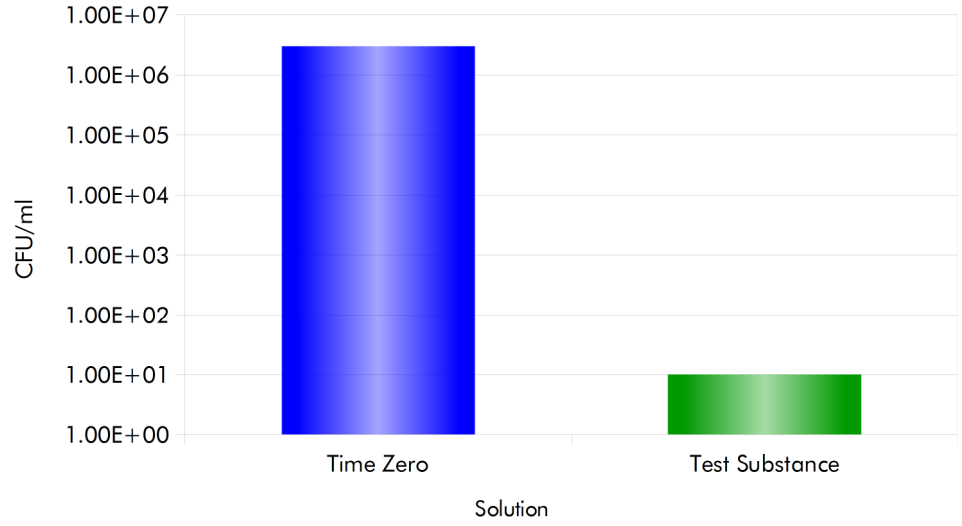
Results

Table 1. R-Water test substance tested against *C. difficile*.

Test Microorganism	Test Substance	Contact Time	Replicate CFU/Carrier	Percent Reduction Compared to Time Zero Control	Log_{10} Reduction Compared to Time Zero Control
C. difficile ATCC 43598	Time Zero		3.00E+06	N/A	
	Test Substance	1 minute	1.00E+01	99.9997%	5.47

Figure 1. Test substance tested against *C. difficile.*



Photos

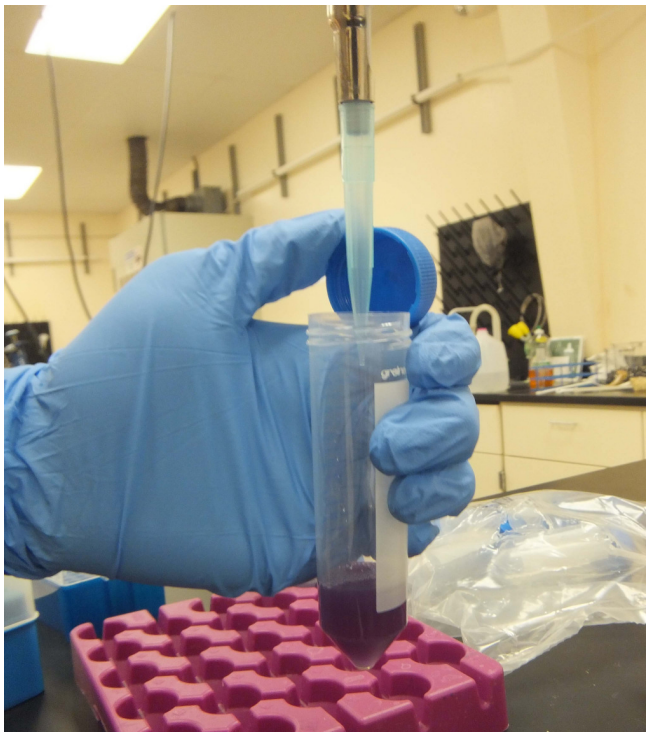


Figure 1. Transferring 1.00 ml of the mixed 9.90 ml PBS and 0.10 ml test inoculum enumeration control into 9.00 ml D/E neutralization broth.

Test Substance and Record Retention

Study Record Retention

The study report and corresponding data sheets will be held at Microchem Laboratory for at least 2 years after the date of the final report.

Test Substance Retention

The test substance may be returned to the Study Sponsor at Sponsor's request and expense within 30 days of study completion. If the Study Sponsor does not request the sample, it may be destroyed 30 days after study completion.

Disclaimer

**Microchem Laboratory makes no warranty or guarantee of material resistance to disinfectants in the field. The laboratory's study report is intended for use by the Study Sponsor to draw its own conclusions.

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