Study Report

Study Title
AOAC Germicidal and Detergent Sanitizing Action of Disinfectants Test of R-Water Test Substance against *Listeria monocytogenes* at Ambient Temperature.

Study Identification Number
NG1061

Test Microorganism(s)
Listeria monocytogenes ATCC 49594

Study Sponsor
Rayne Guest

Testing Facility
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Study Completion Date
02 APR 2015

Study Objective
The purpose of this study was to evaluate the antimicrobial efficacy of R-water's Test Substance against L. monocytogenes in accordance with the AOAC Germicidal and Detergent Sanitizing Action of Disinfectants test method.

Study Conclusion
The received Test Substance achieved a >5 $\log_{10}$ reduction against *L. monocytogenes* within 30 seconds when tested at ambient temperature.

Study Summary

The purpose of this study was to measure the antimicrobial efficacy of the received R-water Test Substance against *L. monocytogenes* in suspension at ambient temperature. In this study, 1.0 ml of *L. monocytogenes* test culture was added to 99.0 ml of the received Test Substance at ambient temperature. After observing the 30 second contact time, 1.0 ml of the test suspension was removed and neutralized in 9.0ml D/E (Dey Engley) neutralization broth. The neutralized test suspension was sufficiently vortex-mixed and standard dilution and pour plating techniques were used to enumerate the test suspension for survivors. All enumerations were incubated for 24±6 hours at 36±1°C. Colonies were counted and multiplied by appropriate dilution factors to determine the number of surviving colony forming units (CFU) of *L. monocytogenes* after the specified contact time.

The PBS control substance numbers control yielded a microbial baseline concentration of 1.65×10^8 CFU/ml of *L. monocytogenes*. After the 30 seconds contact time, 7.90×10^2 CFU/ml surviving *L. monocytogenes* was recovered from the Test Substance. Neutralization by the recovery method was validated as indicated by the results from the Neutralization Validation Control.

The received Test Substance achieved a >5 $\log_{10}$ reduction of *L. monocytogenes* relative to the Control Substance numbers control within 30 seconds at ambient temperature.

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Materials Used in the Study

- Pure culture of test system (*L. monocytogenes* ATCC No. 49594).
- Sufficient quantity of Test Substance provided by the Study Sponsor.
- Sufficient quantity of clean, sterile 100 x 15 mm Petri dishes.
- Sufficient quantity of micro centrifuge tubes containing 0.9 ml sterile Phosphate Buffered Saline (PBS), for dilution of microbial suspensions prior to plating.
- Sufficient quantity of 50 ml capacity centrifuge tubes containing 9.0 ml sterile D/E neutralization broth.
- Sufficient quantity of 15 ml capacity centrifuge tubes containing 10.0 ml sterile Brain Heart Infusion Broth.
- Sufficient volume of sterile Brain Heart Infusion Agar, for enumeration of diluted microbial suspensions.
- One wire inoculating loops with a 4mm inner diameter for inoculation of test culture.
- Bunsen burner, microbiological incinerator, or micro-torch as appropriate to ensure rapid and complete flame-sterilization of forceps and/or loops.
- Micropipettes and a sufficient quantity of appropriately sized sterile micropipette tips.
- Sufficient number of test tube racks.
- Incubator capable of sustaining 36±1°C incubation temperatures.
- Certified digital timer.

Procedure and Parameters

Preparation of Bacterial Culture

- A culture of the test microorganism was initiated from the monthly working stock into 10.0 ml Brain Heart Infusion broth and incubated at 36±1C for 24±6 hours.

Preparation of Disinfectant

- The Test Substance arrived as ready-to-use from the Study Sponsor. The Test Substance was delivered by the Study Sponsor on 31MAR2015 and was labeled as being at ~218 ppm.

Test Method

- 99.0 ml of the received Test Substance was added to a sterile 250 ml Erlenmeyer flask.
- The flask loaded with the Test Substance was gently swirled, stopping just before 1.0 ml of the *L. monocytogenes* test culture was added. Flasks were swirled in such a way to achieve enough residual motion to mix contents of the flask well.
- 30 seconds after the addition of the test culture, one 1 ml portion of the test suspension was neutralized by transfer into 9.0 ml D/E neutralization broth.
- The neutralized Test Substance was then sufficiently vortex mixed and a serial 1:10 dilution series was performed before diluted test suspension aliquots were plated in duplicate using pour-plating techniques.
- Plates were incubated at 36±1°C for 24±6 hours before being counted and multiplied by the appropriate dilution factor to determine the number of colony forming units (CFU).

Test Microorganism Numbers Control

- In order to determine the baseline microbial concentration of the test microorganism, 1.0 ml of *L. monocytogenes* test culture was added to 99 ml sterile PBS in the same manner as the treated Test Substance.
- 30 seconds after the addition of the test culture, one 1.0 ml portion of the control suspension was added to 9.0 ml D/E neutralization broth.
- The harvested Control Substance was then sufficiently vortex mixed and a serial 1:10 dilution series was performed before diluted control suspension aliquots were plated in duplicate using pour-plating techniques.
- Plates were incubated at 36±1°C for 24±6 hours before being counted and multiplied by the appropriate dilution factor to determine the number of colony forming units (CFU).

Neutralization Validation Control

- One 1.0 ml aliquot of the Test Substance was transferred to 9.0 ml neutralizing recovery broth. The tube was vortex-mixed and then inoculated with 0.1 ml of dilute test microorganism targeting 100 CFU/ml.

- As a comparative control, One 1.0 ml aliquot of the Control Substance (PBS) was transferred to 9.0 ml neutralizing recovery broth. The tube was vortex-mixed and then inoculated with 0.1 ml of dilute test microorganism targeting 100 CFU/ml.
- The plates were incubated at 36±1°C for 24±6 hours. Similar CFU recoveries on neutralization test and neutralization control plates confirms neutralization.

Calculations

$$\text{Percent Reduction} = \left(\frac{B-A}{B}\right) \times 100$$

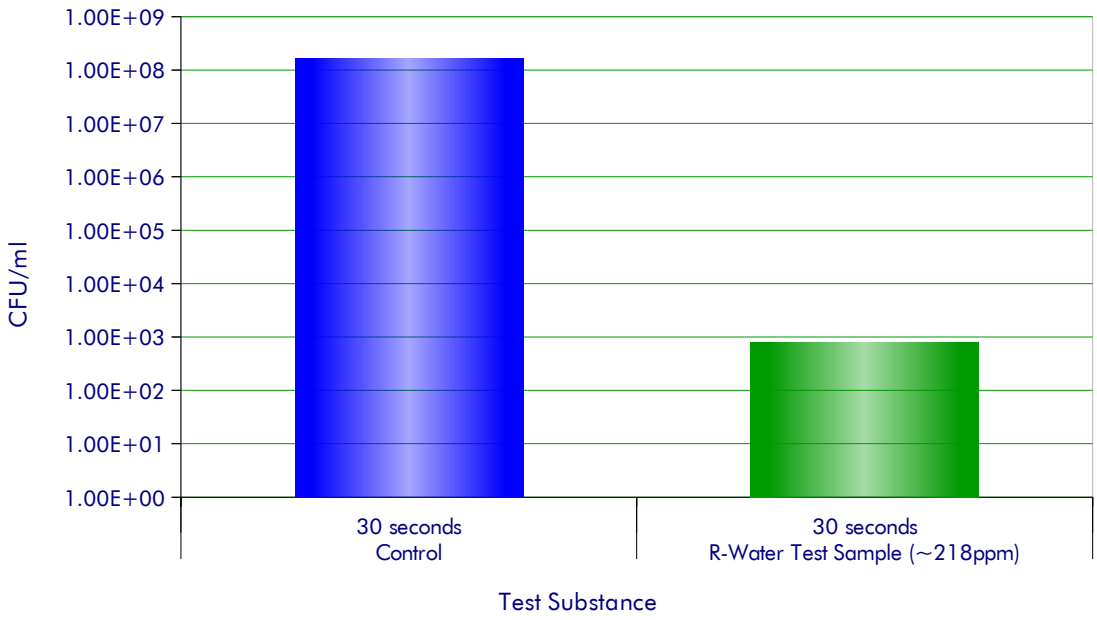
Where:
B = Number of viable test microorganisms in the control substance after the contact time
A = Number of viable test microorganisms in the test substance after the contact time

$$\text{Log}_{10}\text{Reduction} = \text{Log}\left(\frac{B}{A}\right)$$

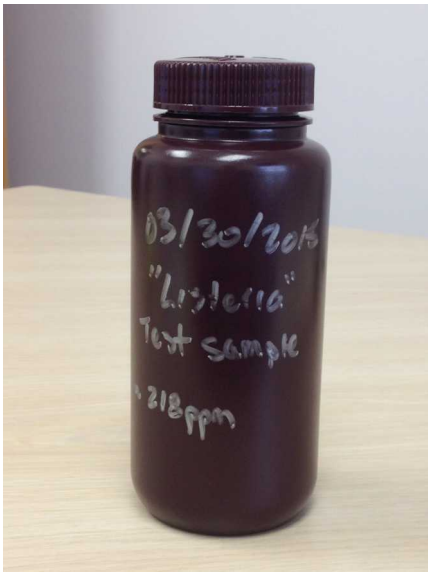
Where:
B = Number of viable test microorganisms in the control substance after the contact time
A = Number of viable test microorganisms in the test substance after the contact time

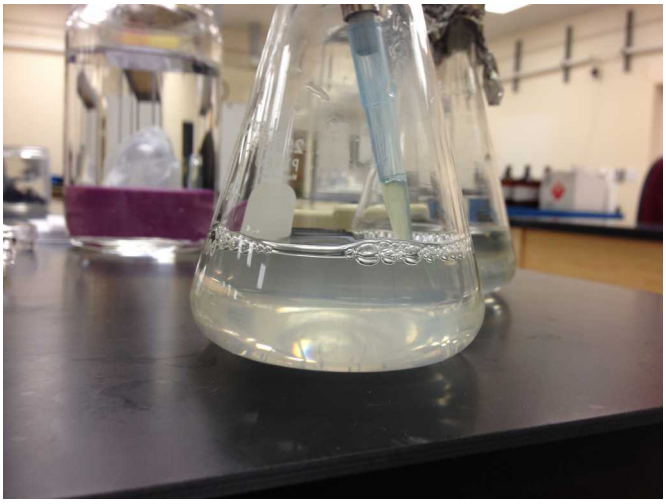
Study Results

Test Microorganism	Test Substance	Contact Time	Replicate CFU/ml	Percent Reduction Compared to Control	Log_{10} Reduction Compared to Control
L. monocytogenes 49594	Control	30 seconds	1.65E+08	N/A	
	R-Water Test Sample (~218ppm)	30 seconds	7.90E+02	99.9995%	5.32



Study Photos





Left: The Test Substance was received on 31MAR2015 and was labeled as being at ~218 ppm.
Right: Test culture suspension was added midway between center and inner edge of the flask with tip of pipet slightly immersed in Test Substance.