Study Report

Study Title
ASTM E1153 Surface Time Kill

Study Identification Number
NG1153

Test Microorganism(s)
Aspergillus brasiliensis ATCC 16404

Test Substance
TK-60

Study Sponsor
Rayne Guest

Testing Facility
Microchem Laboratory, LLC
16713 Picadilly Court
Round Rock, TX 78664

Lead Scientist
Shannon Von Gerichten, B.S.

Study Completion Date
27 NOV 2015

Study Objective

The purpose of this study was to evaluate the efficacy of R-Water's TK-60 ready to use test substance against *Aspergillus brasiliensis* using the standard E1153 surface time kill method.

Study Conclusion

After a 1 minute contact time at ambient conditions, the test substance demonstrated a 99.54% reduction (2.34 $\log_{10}$ reduction) against *Aspergillus brasiliensis*. After a 5 minute contact time at ambient conditions, the test substance demonstrated a 99.94% reduction (3.23 $\log_{10}$ reduction) against *Aspergillus brasiliensis*.

Materials Used in the Study

- Pure culture of test system (*Aspergillus brasiliensis* ATCC 16404)
- Sufficient quantity of sterile Phosphate Buffered Saline supplemented with 0.1% Triton X-100
- Sufficient quantity of 50 ml centrifuge tubes
- Sufficient quantity of sterile, processed 1"x 3" glass slides
- Sufficient quantity of clean, sterile 100 x 15 mm Petri dishes
- Sufficient quantity of 50 ml centrifuge tubes containing 20 ml sterile neutralizing recovery medium (D/E Broth)
- Sufficient quantity of microcentrifuge tubes with 0.900 ml Phosphate Buffered Saline
- Incubator capable of sustaining 30±2°C incubation temperatures
- Sufficient quantity of sterile potato dextrose agar (PDA)
- Bunsen burner, microbiological incinerator, or micro-torch as appropriate to ensure rapid and complete flame-sterilization of forceps
- Micropipettes and a sufficient quantity of appropriately sized sterile micropipette tips
- Vortex Mixer
- Forceps
- Certified digital timer

Procedure and Calculations

Preparation of Culture

- A loop from the microbial frozen stock was transferred to each of 5 sterile PDA slants and slants were incubated at 30±2 °C for 6-10 days.
- Spores were eluted via washing each slant with 10 ml of PBS supplemented with 0.1% Triton X-100 and spores were collected into sterile 50 ml conical vials before being vortexed thoroughly.
- The ~50 ml spore culture suspension was filtered through a sterile syringe containing ~1 cc of glass wool into a new 50 ml conical vial.

Preparation of Test Carriers

- Before the test, 1"x 3" glass slide carries were soaked in 95% ethanol and then thoroughly rinsed using multiple tap-water rinses followed by a double distilled water rinse.
- Carriers were autoclave-sterilized and allowed to cool to room temperature prior to use in the study.

Storage and Handling of Test Substance

- The received test substance was used imediately upon receipt.

Preparation of Test Substance

- Test substance arrived ready to use from Sponsor. The test substance total chlorine concentration was determined to be 180 ppm using an iodometric titration technique.

Contamination of Carriers with Test Culture

- Test carriers were inoculated with 0.020 ml of the prepared test inoculum and evenly spread across an ~ 1" x 2" area within 1/8 inch of the carrier perimeter.
- Inoculated carriers were dried at 36±1°C for 30 minutes, or until visibly dry.

Enumeration of Time Zero Controls

- Following the conclusion of the dry time, three untreated carriers were assayed immediately prior to conducting the test by transferring individual carriers to 20 ml D/E (Dey Engley) neutralization broth.
- The neutralized control carriers were sufficiently vortex-mixed and the resulting suspension was serially diluted and plated using standard pour-plating techniques.
- Enumeration plates were incubated for 48±6 hours at 30±2 °C.

Exposure of Carriers to Prepared Test Substance

- Carriers were treated with the prepared test substance by pipetting a 5.0 ml volume of the prepared test substance onto the surface of each inoculated carrier.
- Treated test carriers were allowed to sit undisturbed for the duration of the 1 minute or 5 minute contact time.
- Upon completion of the contact time, the test carriers were harvested in 20 ml D/E (Dey Engley) neutralization broth and vortex mixed.
- Suspensions were enumerated using standard dilution and plating techniques to determine number of surviving microorganisms.
- Enumeration plates were incubated for 48±6 hours at 30±2 °C.

Neutralization Verification Control

- Sterile carriers were treated as detailed above and transferred to 20 ml D/E neutralization broth and vortex mixed.
- The test microorganisms were diluted (by serial dilution in PBS) and neutralized carriers (test and control) were inoculated with a target concentration of 100 CFU/ml of the appropriate test microorganism.
- In parallel a 20 ml aliquot of D/E neutralization broth was similarly inoculated with a target concentration of 100 CFU/ml of the appropriate test microorganism to serve as a comparative viability control.
- An aliquot of each neutralized carrier was plated to Petri plates in double replicate.
- Enumeration plates were incubated for 48±6 hours at 30±2 °C before test carrier CFU counts were assessed for comparable levels of test microorganisms recovered relative to the respective comparative viability control.

Calculations

Percent Reduction:

$$= [(B - A) / B] \times 100$$

where,
B = Number of viable test microorganisms on the control carriers at time zero
A = Number of viable test microorganisms on the test carriers after treatment

Log Reduction:

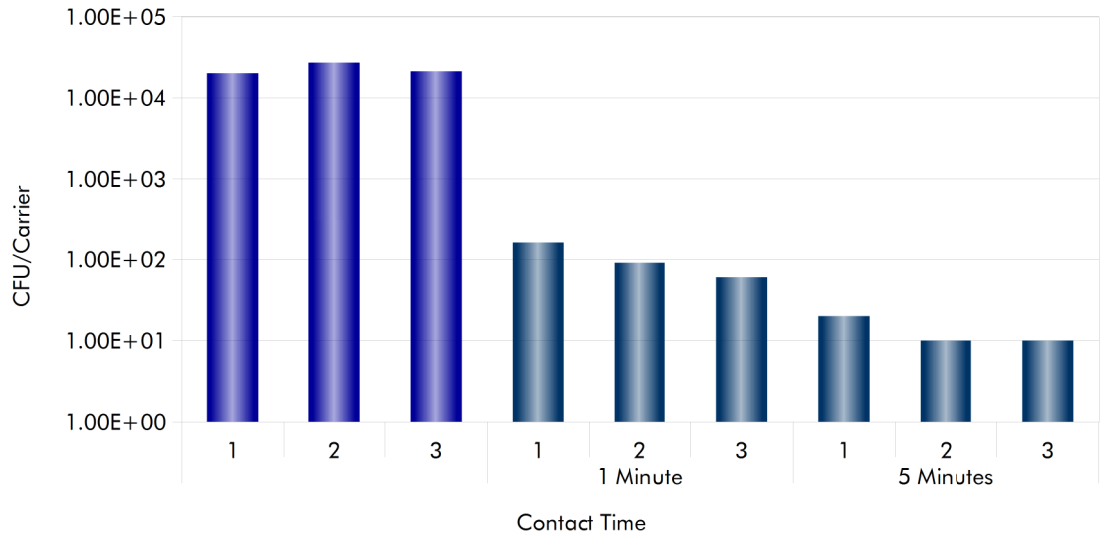
$$= \text{Log } (B/A)$$

where,
B = Number of viable test microorganisms on the control carriers at time zero
A = Number of viable test microorganisms on the test carriers after treatment

Study Results

Test Substance	Contact Time	Replicate	Replicate CFU/Carrier	Average CFU/Carrier	Percent Reduction vs. Time Zero Control	Log_{10} Reduction vs. Time Zero Control
Time Zero		1	2.00E+04	2.27E+04	N/A	
		2	2.70E+04			
		3	2.10E+04			
TK-60	1 Minute	1	1.60E+02	1.03E+02	99.54%	2.34
		2	9.00E+01			
		3	6.00E+01			
	5 Minutes	1	2.00E+01	1.33E+01	99.94%	3.23
		2	1.00E+01			
		3	1.00E+01			

Aspergillus brasiliensis ATCC 16404



Study Photos

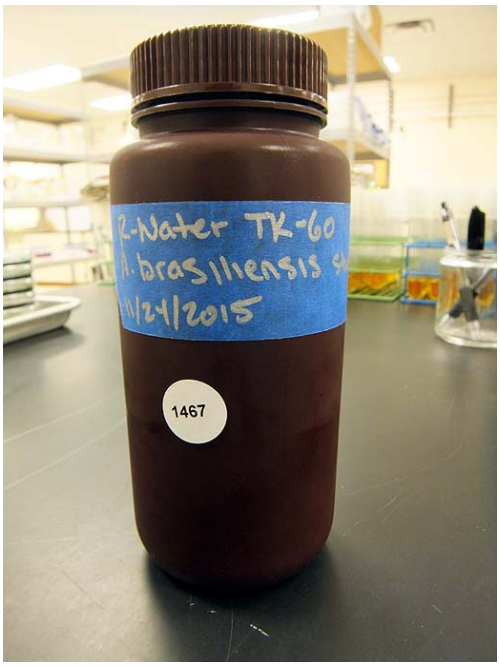


Image 1: Ready to use test substance TK-60.

Image 2: Inoculation of carrier with 0.020 ml of Aspergillus brasiliensis.