CleanSpray TECHNOLOGIES

TESTING PROCEDURE AND FINDINGS

Initial Disinfectant Kill Test

To assess the advertise features of four commercially available disinfectants, an experiment was designed to determine the ability of each to kill Salmonella.

Bacterial Culture

Salmonella javiana 312 is a highly virulent, antibiotic-resistant serovar that is known to infect horses, frequently resulting in death in younger animals. It can cause disease in humans and other animals and be difficult to treat due to the multi-drug resistance. For this experiment, a single colony was picked to 5 ml of Brain Heart Infusion broth, placed in a 37 C shaking incubator, and grown overnight. The culture was then precipitated by centrifugation at 4,510 x g in a benchtop Sorval ST 16R refrigerated centrifuge. Spent media was removed and the bacterial pellet resuspended in 10 ml sterile 1x phosphate buffered saline (PBS) to approximate a McFarland std of 0.5. The resuspended culture had approximately 5 x 10^7 CFU/ml and was stored in the refrigerator until needed.

Experimental Test and Design

Four disinfectants were tested in this experiment: A) Disinfectant 1 (D-1), B) Disinfectant 2 (D-2), C) Disinfectant 3 (D-3), and D) Disinfectant 4 (D-4). For each disinfectant, 15 ml tubes were labeled with the disinfectant name and dilution of the compound ranging from 100% to 0.5%. A total of 15 tubes were used per disinfectant (see Table 1). In order to stress the system, growth media was used as a diluent to encourage growth in the event of solution failure.

Once all dilutions were made, 100 µl of 5 x 10^7 CFU/ml Salmonella in PBS was added to each tube and all tubes placed in a 37 C shaking incubator overnight. After 20 h of incubation, a 10 µl aliquot from each tube was transferred to an SB/amp agar plate fitted with grid lines and a number corresponding to the tube number. Plates were incubated overnight at 37 C (20 h) and growth/no growth was recorded.

Table 1. The dilutions listed below were replicated for each disinfectant.

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Disinfectant	100	90	80	70	60	50	40	30	20	10	0	50	25	10	5
Media	0	10	20	30	40	50	60	70	80	90	100	950	975	990	995

Results

Of the compounds tested, only D-3 and D-4 yielded no growth through the first 10 dilutions. Tube 11 for each solution served as a positive control, while tubes 12 – 15 diluted the disinfectant beyond its capacity to kill the Salmonella. D-2 yielded inconsistent results by producing growth at some dilutions and not at others, even if the product was more concentrated. In addition, it is not believed to contain nanoparticle as verbally advertised. Some of this growth could be due to a precipitant occurring in dilutions with higher media concentrations, which may have removed the substances needed for pathogen killing. The most surprising results were obtained with D-4, in that no growth inhibition was observed even in the most concentrated form of the product. Based on these results, we made the decision to remove D-4 from future testing.

Table 2. Killing effect of disinfectants at different concentrations. + = growth, - = no growth, U = undetermined due to precipitation in the tube.

Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D-1	-	-	-	-	-	-	-	-	-		+	+	+	+	+
D-2	-	-	+	-	+	-	-	-	-	+	+	+	+	+	+
D-3	-	-	-	-	-	-	-	-	-	-	+		+		+
D-4	+	+	U	U	U	U	U	U	U	U	+	U	U	U	U

Bus Seat test using three disinfectants

In order to determine the killing ability of D-1, D-2, and D-3, an experiment was set up using *Salmonella javiana* as the test organism on a typical vinyl covered bus seat, the type found on school buses used for K-12 transportation. The experiment was designed to test the killing ability of disinfectants on the bus seat, when exposed to Salmonella, and Salmonella on the bus seat followed by disinfectant treatment.

Test organism

First, a 5 ml culture of *S. javiana* in brain-heart-infusion broth was grown at 37 C overnight in a shaking incubator. The culture was then precipitated by centrifugation at 4,510 x g in a benchtop Sorval ST 16R refrigerated centrifuge. Spent media was removed and the bacterial pellet resuspended in sterile 1x phosphate buffered saline (PBS). The resuspended culture had approximately 5 x 10^7 CFU/ml and was stored in the refrigerator until needed.

Experimental Test and Design

A vinyl covered bus seat was divided into two, 12 grid sections, with each section measuring approximately 2" x 2". Each section was labeled either P (positive control), D-1, D-2, or D-3. The first 12 grids were treated as follows: three untreated control sections, three sections swabbed until wet with either Di1, D-2, or D-3. The grid was allowed to air dry for approximately 30 minutes.

The second grid was laid out identically to the first grid, but swabbed until wet with *S. javiana* suspended in PBS. The grid was allowed to air dry for approximately 30 minutes.

Product Application

The grid to which disinfectant was applied first was swabbed with *S. javiana* until wet, whereas the second grid was swabbed with the disinfectant appropriate to the section until wet. A separate, sterile, cotton tipped applicator was used for each grid section in order to avoid cross-contamination. Each grid was allowed 15 minutes of contact time to allow the disinfectant to kill on *S. javiana*.

Culture method

Each grid section was swabbed with a separate, sterile, cotton tipped applicator, which was then used to inoculate a superbroth agar plate supplemented with 100 mcg/ml ampicillin. Each plate was divided into 4 sections, and each ¼ was labeled with P, D-1, D-2, orD-3. The ¼ labeled with the corresponding test section was swabbed to check for Salmonella growth. Once plates were inoculated, they were placed in a stationary 37 C incubator overnight.

P	D-1	D-2	D-1
D-3	D-2	P	D-3
D-2	D-3	D-1	P

Results

Of the three disinfectants tested, D-1 did the best job of evenly wetting the vinyl; the other two tended to bead-up on the surface making even distribution difficult and likely resulted in "hot spots" of killing activity vs areas without killing activity. Based on the nature of the grid system,

it was not possible to use a spray method of application without overspray getting on the surrounding area. There is also a question of whether or not the swab removed too much of the Salmonella or disinfectant (depending on the grid being swabbed).
In spite of these issues, consistent data was obtained between the replicates for each treatment. Not surprisingly, applying the disinfectant over the Salmonella-contaminated seat yielded the best kill rate for all treatment groups. In the disinfectant-over-salmonella group, the D-1 and D-2 yielded the best kill rate after 15 minutes of exposure, with the D-3 performing the worst. D-1 still performed the best of the three, even in comparison to D-2, as measured by more numerous colonies in the D-2 quadrants. Each replicate (1/4 of a plate) was scored for growth or no growth. Growth was detected in the positive control (numerous colonies) and the D-3.
In the Salmonella over disinfectant treatments, D-1 significantly out-performed both D-2 and D-3, the latter two having numerous colonies in 2 of 3 replicates, similar to the positive control, after 15 minutes of exposure. Because of the experimental design and need to use sterile cotton-tipped swabs, an accurate, quantitative count could not be obtained as it could in the first experiment. In future experiments, this issue will be resolved and quantitative numbers obtained.

Discussion

The data presented above is preliminary in nature and designed to narrow the field of disinfectants to be tested; this objective was obtained with D-1 being chosen for further evaluation and formulation. This is a plant-derived, non-toxic formulation that does not have to be wiped off of a surface once applied. While the disinfectant-over-Salmonella provided the best kill rate as reported above, the Salmonella-over-disinfectant still performed very well with a 15 minutes of exposure time. This observation is significant due to the thick lipopolysaccharide capsule surrounding this highly virulent, multi-drug resistant *S. javiana*. If an organism this well protected from its environment can be killed so quickly, it is anticipated that most virues would be neutralized within seconds or no more than a few minutes after exposure.
Our theory is that improved application techniques (spray, instead of swab) will provide kill rates similar to the disinfectant-over-Salmonella. Future experiments are planned to determine how long the effective kill rate can be maintained using D-1 as a disinfectant; we will test grids daily for two weeks or until no kill efficacy is found when the D-1 is applied and challenged daily with Salomonella.

Testing performed by Dr. Richard Cooper on behalf of CleanSpray Technologies.