Sagewash® Disinfectant Study, March – May 2010

Protocol:

Inoculum preparation:

Actively growing cultures of the bacteria, *Escherichia coli, Clostridium perfringens* and *Clostridium difficile* were propagated on enriched, non-selective media incubated at 35-37^{0}C in anaerobic (*Clostridium* spp) or aerobic (*E.coli*) atmospheres. *E. coli* was grown for 18-24 hrs on Trypticase Soy Agar containing 5% sterile defibtinated sheep blood, *C. perfringens* was grown on CDC Anaerobe Blood Agar for 24 hrs and *C. difficile* was grown for 48 hrs on CDC Anaerobe Blood Agar. Bacteria harvested from plates were suspended in PBS and the heavy suspensions (OD > #4 McFarland opacity standard) were adjusted such that 1:10 dilutions from the suspensions gave spectrophotometer (DensiCheck, Biomerieux) readings corresponding to the #1 McFarland opacity standard, which for E. coli was estimated to give the target inoculum concentrations of approximately 1x10^9 cfu/ml. Inocula were prepared fresh for each experiment and used within 30 min of preparation. A separately prepared inoculum was used for each test replicate. The results of replicate titrations performed by the plate count method with separately prepared inocula were as follows:

Organism	**Replicate number**	**Date**		**Inoculum concentration**
E. coli	1	4/27/10	1.3 x 10^9	
	2	4/27/10	1.7 x 10^9	
	3	4/28/10	1.3 x 10^9	
	4	4/28/10	1.4 x 10^9	
				Average 1.4 x 10^9
C. perfringens	1	4/27/10	8.0 x 10^8	
	2	4/29/10	6.8 x 10^8	
	3	4/29/10	6.5 x 10^8	
	4	4/29/10	7.6 x 10^8	
				Average 7.2 x 10^8
C. difficile	1	5/24/10	1.1 x 10^9	
	2	5/24/10	6 x 10^8	
	3	5/26/10	3.4 x 10^8	
	4	5/26/10	4.2 x 10^8	
				Average 6.1 x 10^8

Test solution preparation:

Prior to preparation and testing chlorine levels, all containers were rinsed with 3 volumes of ultrapure water (18.1 Megohm/cm). Jacketed Sagewash® caplets were stored in the original container in a chemical fume hood at ambient room temperature (ca. 70^{0}F). For preparation of the Sagewash® test solution, a portion of the plastic jacket was removed from the caplet and a section ca. 1.5cm to 2.0 cm deep was broken off with a hammer and screw driver to expose a fresh surface. The newly exposed surface was scraped with a scalpel blade into a mortar dish. The Sagewash® material was ground into a powder with mortar and pestal before weighing on a pan balance. A 2x concentration was achieved by adding 0.16 gm of Sagewash® powder to 950 ml of ultrapure water. An additional 50 ml of ultrapure water was added after using it to rinse the weigh boat. The 2x Sagewash® test solutions were mixed

with a stir bar for ca 30 min. prior to testing free chlorine levels using a standard swimming pool test kit. Concentration test range for the kit was 0.2 ppm to 5.0 ppm. For testing, the 2X Sagewash® solution was diluted 1ml in 200ml. The test kit tube was rinsed with 3 volumes of the 1:200 dilution prior to adding test reagent. The resulting free chlorine levels measured were consistently in the 0.6 ppm to 1.0 range, thus indicating that the 2x Sagewash® test solution contained 120 ppm to 200 ppm of free chlorine. The pH of these solutions as measured with pH range 6.0 to 8.0 Hydrion paper was consistently pH 6.4 to pH 6.8.

A control test solution was prepared as a 150µl : 100 ml dilution of a commercial household bleach solution (Chlorox®). This bleach solution was tested as above and consistently produced free chlorine levels and pH measurements identical to those of the Sagewash® test solutions (ie. 2x control bleach solution had free chlorine levels of 120 ppm to 200 ppm and pH measurements of ph 6.4 to pH 6.8.).

All test solutions were used fresh on the same day they were prepared.

Experimental Procedure:

The 2x Sagewash® test solutions and control bleach solutions were mixed with equal volumes of the respective bacterial inocula and held at room temperature for 10 min. Immediately after the contact period, the mixtures were serially diluted in a buffered neutralizing medium (one-half strength Tryptic Soy Broth with 0.6% sodium thiosulfate) and viable organisms were determined by plate count. Negative controls consisted of the same procedure with bacterial inocula and sterile water. Bacterial inocula and 1.5% household bleach solutions were used as positive controls.

Test results were as follows:

Test	Replicate number	Date	Bacterial conc. after 10 min contact (cfu)
E. coli + water	1	5/11/10	1.3×10^9
E. coli + Sagewash® soln	1		<20
E. coli + bleach soln	1		< 20
E. coli + water	2	5/11/10	1.7×10^9
E. coli + Sagewash® soln	2		<20
E. coli + bleach soln	2		<20
E. coli + water	3	5/12/10	1.3×10^9
E. coli + Sagewash® soln	3		< 20
E. coli + bleach soln	3		< 20
E. coli + water	4	5/12/10	1.4×10^9
E. coli + Sagewash® soln	4		< 20
E. coli + bleach soln	4		< 20
		Average inoculum conc	1.4×10^9
		average conc reduction Sagewash soln	8 $\log_{10}$
		Average conc reduction bleach soln	8 $\log_{10}$

Test	Replicate number	Date	Bacterial conc. after 10 min contact
C. perf. + water	1	5/18/10	5.9×10^8
C. perf+ Sagewash® soln	1		<20
C. perf. + bleach soln	1		<20
C. perf. + water	2	5/18/10	5.0×10^8
C. perf+ Sagewash® soln	2		<20
C. perf. + bleach soln	2		<20
C. perf. + water	3	5/19/10	3.6×10^8
C. perf+ Sagewash® soln	3		<20
C. perf. + bleach soln	3		<20
C. perf. + water	4	5/19/10	4.8×10^8
C. perf+ Sagewash® soln	4		<20
C. perf. + bleach soln	4		<20
		Average inoculum conc	4.8×10^8
		average conc reduction Sagewash soln	7 $\log_{10}$
		Average conc reduction bleach soln	7 $\log_{10}$

Test	Replicate number	Date	Bacterial conc. after 10 min contact
C. diff. + water	1	5/24/10	5.6×10^8
C. diff. +Sagewash® soln	1		3.8×10^3
C. diff. + bleach soln	1		5.9×10^3
C. diff. + water	2	5/24/10	3.0×10^8
C. diff. +Sagewash® soln	2		3.8×10^3
C. diff. + bleach soln	2		1.5×10^4
C. diff. + water	3	5/26/10	1.7×10^8
C. diff. +Sagewash® soln	3		3.6×10^3
C. diff. + bleach soln	3		2.7×10^3
C. diff. + water	4	5/26/10	2.1×10^8
C. diff. +Sagewash® soln	4		1.2×10^2
C. diff. + bleach soln	4		1.5×10^3
		Average inoculum conc	3.1×10^8
		average conc reduction Sagewash soln	4 $\log_{10}$
		Average conc reduction bleach soln	4 $\log_{10}$

The conclusion from these experiments is that the Sagewash ® solution, with free chlorine level equivalent to that achieved during normal product use, caused a significant reduction in bacterial

concentrations of Gram-negative and Gram-positive bacteria comparable to that achieved with a similar solution of household bleach. The bacteria chosen for the study represented a spectrum of Gram-negative, Gram-positive and endospore-forming bacterial pathogens frequently found in mammals and spread by fecal-oral transmission. The Gram-negative isolate, *E. coli*, was resistant to multiple antibacterial drugs. Bacteria with multiple antibiotic resistance may also show increased resistance to some disinfectants (eg. quaternary ammonium compounds). The *C. perfringens* isolate was a Gram-positive, sporeforming bacterium in vegetative cell form only (*C. perfringens* rarely sporulates under routine laboratory growth conditions). The *C. difficile* was a Gram-positive, sporeforming bacterium, which after 48 hr of growth was a mixture of predominantly vegetative cells and less than 10% endospores, as judged by Gram stain appearance. Bacterial endospores represent the most resistant state of a bacterial cell and are resistant to heat and several disinfectant classes. The viable growth observed from the *C. difficile* following treatment with Sagewash® and household bleach probably originated from endospores. The failure to kill endospores with the use dilutions (60 to 100 ppm free chlorine) and contact time tested in this study was not surprising, since EPA recommendations for bleach disinfection of *Bacillus anthracis* endospore contaminated hard surfaces employ fifty times greater chlorine concentrations (ca. 5000 ppm) and longer contact (ca. 60 min.).

As an addendum to this study, a suspension of *C. difficile* was made from plates incubated for 7 days. The suspension was stored in the refrigerator for 31 days and used for a disinfection experiment. At the time of testing the suspension appeared microscopically to contain > 95% endospores. Data for two replicates is presented below. Results confirm that neither Sagwash® nor household bleach, at the concentrations used, were effective disinfectants for *C. difficile* endospores.

Test	Replicate number	Date	Bacterial conc. after 10 min contact (cfu)
C. diff. spore suspension + water	1	5/25/10	1.7×10^3
C. diff. spore suspension + Sagewash® soln	1		9.5×10^3
C. diff. spore suspension + bleach soln	1		1.0×10^4
C. diff.spore suspension + water	2	5/25/10	1.0×10^3
C. diff. spore suspension + Sagewash® soln	2		8.8×10^3
C. diff. spore suspension + bleach soln	2		9.6×10^3
		Average inoculum conc	1.3×10^3
		average conc reduction Sagewash® soln	No reduction
		Average conc reduction bleach soln	No reduction

Non-Technical Summary:

This study was conducted to test the ability of Sagewash® to kill three different types of bacteria that cause disease in domestic animals and that often contaminate the environments they live in. Testing was performed by mixing equal proportions of solutions prepared from Sagewash® caplets with liquid suspensions containing high concentrations of the bacteria. The bacteria choosen were *Escherichia coli*, *Clostridium perfringens* and *Clostridium difficile*). The concentration of active chlorine ingredient in the Sagewash® solution was equivalent to that found during normal use of the product. After a contact exposure time of 10 minutes, the residual active chlorine ingredient in the mixture was neutralized and the number of remaining viable bacteria were determined. The number was then compared to that obtained in an equivalent test using sterile water instead of Sagewash® solution. Another comparison was made to the number obtained in a third equivalent test performed using a solution of commercial household bleach adjusted to the same chlorine concentration as the Sagewash® test solution. Each set of the three testing conditions were repeated 4 times to ensure reproducibility.

Results of the study show that the Sagewash® solution, at normal product usage concentration, readily killed high concentrations of active (vegetative) bacteria within the 10 minute contact time tested. Furthermore, the level of bacterial killing observed with the Sagewash® solution was equal to that observed with a comparable solution of commercial household bleach. One of the selected bacteria (*Clostridium difficile*) also produced an inactive, dormant (spore) form that was not destroyed by either of the test solutions. The latter was not a surprising finding, since bacterial spores are known to be highly resistant to many physical and chemical treatments. The U.S. Environmental Protection Agency (EPA) recommends that usage of household bleach solutions to kill similar bacterial spores should contain active chlorine levels 50 times higher than those tested in this study and that the solutions should be applied to contaminated surfaces for longer contact times (60 min.).

Conclusion: Sagewash® solutions containing active chlorine concentrations similar to those attained during normal product usage have bacterial disinfectant properties that are equivalent to those of household bleach solutions containing similar concentrations of active chlorine.

Respectfully submitted 2 June, 2010

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