



## ***Clostridium difficile* Test Exposure to iHP™ (ionized Hydrogen Peroxide) Decontamination Technology**

### **Executive Summary:**

SixLog's iHP™ (ionized Hydrogen Peroxide) decontamination technology was used to kill *Clostridium difficile* in a test chamber under conditions approximating use in a hospital room. The data show killing of greater than  $6 \times 10^{13}$  spores per square meter when the product is used as recommended.

### **Introduction:**

*Clostridium difficile* has become an increasingly significant health problem impacting the operations of hospitals, ambulatory care facilities and nursing homes. SixLog has developed a decontamination system that uses activated hydrogen peroxide to kill bacteria and other microorganisms on surfaces. We have performed extensive testing of the iHP technology ability to kill vegetative bacteria and bacterial spores and are expanding these studies to examine the effects of our treatment on specific high concentration spore preparations.

### **Objective:**

Test the SixLog iHP technology in a closed chamber with *C. difficile* spore preparations to determine the extent of inactivation of the samples.

### **Procedures:**

*C. difficile* spore samples on stainless steel coupons will be exposed to the iHP technology for 5 minutes followed by a 5-minute dwell period in a closed chamber. After exposure to the iHP technology, the *C. difficile* spore samples will be recovered by washing the inoculated stainless steel coupons with sterile deionized water. The recovered spores will then be diluted with sterile deionized water and aliquots plated in triplicate onto *C. difficile* selective media. The plate will be incubated anaerobically for five days at 37 degrees Celsius to ensure spore germination and accurate quantification.

#### Procedure:

1. Grow *C. difficile* strain ATCC 9689 using both solid and liquid media under strict anaerobic conditions ("Laboratory Maintenance of *Clostridium difficile*" J.A. Sorg and S.S. Dineen, Current Protocols in Microbiology 9A1.1-9A1.10, Feb. 2009)..
2. Allow liquid *C. difficile* cultures sufficient time to sporulate.
3. Produce 75-100 ml of vegetative cell-free spore stocks.
4. Determine concentration of spore suspensions by serial dilution and subsequent plating onto selective media.
5. Spread 10 microliters or 50 microliters of the spore suspension onto individual stainless steel coupons.
6. Allow coupons to air dry in the biological safety cabinet and then store for subsequent treatment and quantification.
7. Treat inoculated coupons with iHP
8. Recover spores from coupons
9. Plate serial dilutions of recovered spores onto selective media
10. Incubate plates for five days under anaerobic conditions to ensure maximal sporulation
11. Compare counts from plates with spores from treated coupons to plates with spores from positive control coupons.

### **Experiment # 1 conducted September 1, 2009**

The stainless steel coupons containing 10 microliters of the dried *C. difficile* spores were exposed to the iHP mist for five minutes followed by a “dwell” period of five minutes. Each treatment was performed on a minimum of three coupons. Following the iHP treatment recovered spores were diluted and plated, in triplicate, onto *C. difficile* selective agar (Blood Heart Infusion Broth with Yeast extract/ 0.1% sodium taurocholate agar (BHIST)). Control coupons, inoculated and dried, but not exposed to iHP, were used as positive control samples. Uninoculated coupons were used as negative controls.

Positive control plates showed growth at all dilutions tested. The average count from the positive controls show that there were  $1.33 \times 10^4$  CFU associated with the 10 ul of dried suspension. Negative control plates showed no growth. In stark contrast to the positive controls, plates with spores from iHP treated coupons had no growth at any dilution.

### **Conclusions:**

There was significant effect of the iHP exposure on the *C. difficile* spores. Similar to results with *Geobacillus stearothermophilus* spores, all of the *C. difficile* spores treated were killed. Considering that the 10 microliters of the spore suspension yielding  $1.33 \times 10^4$  viable CFU's was applied to a relatively small surface area (roughly 20 sq mm), the *C. difficile* killing ability of iHP approaches  $6.6 \times 10^{13}$  spores per square meter.

### **Experiment # 2 conducted September 8, 2009**

Stainless steel coupons containing 50 microliters of the dried *C. difficile* spores were exposed to the iHP mist for five minutes followed by a “dwell” period of five minutes. Each treatment was performed on a minimum of three coupons. Following the iHP treatment recovered spores were diluted and plated, in triplicate, onto *C. difficile* selective agar (BHIST agar). Control coupons, inoculated and dried, but not exposed to iHP, were used as positive control samples. Uninoculated coupons were used as negative controls.

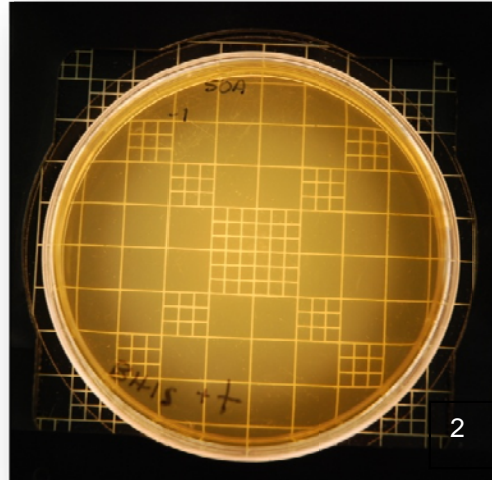
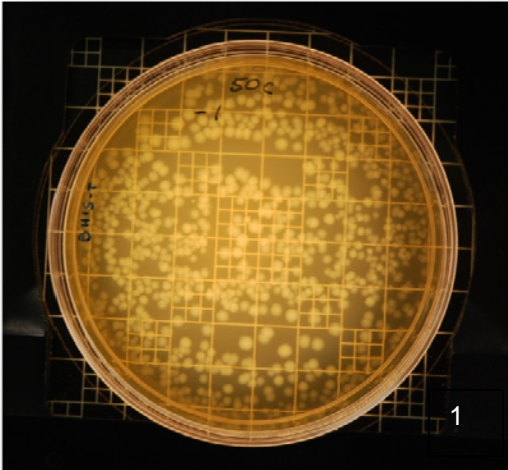
Positive control plates showed growth at all dilutions tested. The average count from the positive controls show that there were  $5.10 \times 10^4$  CFU associated with the 50 ul of dried suspension. Negative control plates showed no growth. Again, in contrast to the positive controls, plates with spores from iHP treated coupons had no growth at any dilution.

### **Conclusions:**

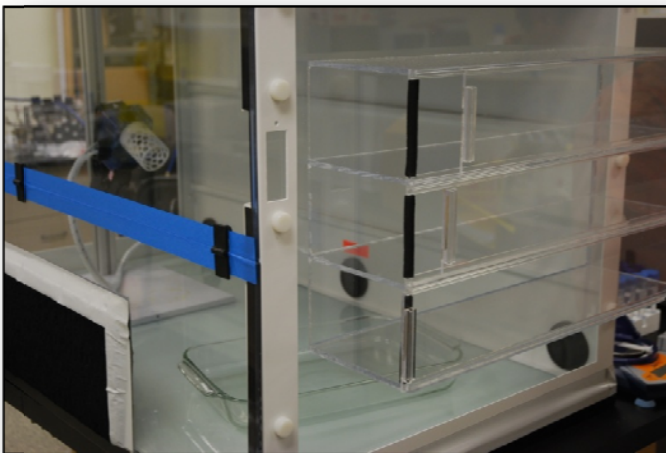
The primary difference between Experiment #1 and Experiment #2 was the concentration of the *C. difficile* spore suspension dried onto the stainless steel coupon. While we did examine a slightly higher concentration of spores, the primary intent was to determine the repeatability of the first experiment. Like the results from the previous experiment, all of the *C. difficile* spores were killed, the positive controls were positive for the expected level of growth and the negative controls were negative for growth.

The iHP test chamber is designed to generate an indirect spray and approximate the misting process when the commercial iHP technology is used in a hospital room. The high average concentration of hydrogen peroxide reaches just above 300 ppm at the beginning of the dwell period and falls slowly until the coupons are removed. The coupons are placed on a shelf that is above the line of spray from the iHP test unit and cantilevered away from the main body of the chamber resulting in an indirect path from the spray head to the test coupons.

These results are the first direct evidence of spore killing by hydrogen peroxide activated as a mist.



1. *C. difficile* positive control from 50 microliter inoculated positive control coupon. Spores were recovered from the stainless steel coupon in deionized water, diluted 1:10 and 100 ul of the 1:10 dilution was plated on BHIS+ agar and incubated anaerobically at 37 degrees Celsius.
2. *C. difficile* test coupon from 50 microliter inoculated coupon. Spores were recovered from the stainless steel coupon in deionized water, diluted 1:10 and 100 ul of the 1:10 dilution was plated on BHIS+ agar and incubated anaerobically at 37 degrees Celsius. No growth following iHP treatment.



SixLog test chamber. The inoculated stainless steel test coupons are placed just inside the center shelf, approximately 3 inches back from the main body of the chamber.