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DISINFECTION OF *CLOSTRIDIUM DIFFICILE* SPORES

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ABSTRACT

C. difficile, a major nosocomial pathogen, is a serious challenge for infection control due to the high stability and microbicide resistance of its spores. We compared the indoor survival of *C. difficile* and *Bacillus subtilis* spores. The spores of three strains of *C. difficile* and one of *C. sporogenes* were also tested for their resistance to three chlorine-based formulations and one product with accelerated H₂O₂ (AHP; 70,000 ppm) at 22±2°C using the second tier of the quantitative carrier test (QCT-2) – now an ASTM standard (E-2197).

The spores remained viable on metal disks for at least nine months. AHP and bleach (5,000 ppm Cl₂) reduced the spore viability of all clostridia tested by ≥6 log₁₀ in about 12 min. Acidified bleach (5,000 ppm Cl₂), chlorine dioxide (1000 ppm Cl₂) and bleach with 3000 ppm Cl₂ were also generally effective, but with contact times ranging from 3-30 min. Bleach with 1000 ppm Cl₂ required an even wider range of 15->60 min to inactivate all the strains tested.

The same sporulation/recovery media could not be used due to differences in the growth characteristics of the strains tested, and this may reflect, to some degree, in the results obtained. Nevertheless, the findings are a good guide to selection of microbicide when rapid and reliable sporicidal action is needed against *C. difficile*.



INTRODUCTION

- *C. difficile* is a major nosocomial pathogen (Riley 1998).
- Its spores, excreted in feces, are highly stable and also relatively resistant to microbicides (Rutala *et al*, 2001).
- Changing demographics and chemotherapy further promote susceptibility to this pathogen (Riley 1998).
- Viable spores of *C. difficile* have been isolated from the hospital environment (Fekety *et al*, 1981).
- Therefore, proper environmental decontamination can reduce the risk of spread of *C. difficile*.
- We compared the indoor survival of *C. difficile* and *Bacillus subtilis* spores.
- The spores of three strains of *C. difficile* and one of *C. sporogenes* were also tested for their resistance to three chlorine-based formulations and one product with accelerated H₂O₂ (AHP; 70,000 ppm) at 22±2°C.
- A quantitative test (Springthorpe & Sattar, 2003) with metal disk carriers was used to assess the microbicides.

MATERIALS AND METHODS

Table 1. *Bacteria Tested*

Strain (ATCC #)	Source
<i>C. difficile</i> (9689)	ATCC*
<i>C. difficile</i>	Clinical isolate from a general hospital (THG)
<i>C. difficile</i>	Clinical isolate from a children's hospital (CHEO)
<i>C. sporogenes</i> (7955)	ATCC
<i>Bacillus subtilis</i> (19659)	ATCC

*AMER. TYPE CULTURE COLLECTION, MANASSAS, VA.

Bacterial Culture/Recovery

- The spores of ATCC and THG strains of *C. difficile* were produced using Columbia Broth with 5% sheep blood and 1.5% agar (CBSBA; QueLab Labs, Montreal, QC). For the CHEO isolate of *C. difficile*, *C. sporogenes* and *B. subtilis* Columbia Broth without blood was used.
- All *C. difficile* spores were washed with 1/10 brain-heart infusion broth (BHI; Oxoid); distilled water used for *B. subtilis*. The suspensions were then heated at 70°C for 10 min. to kill vegetative cells.
- All *C. difficile* spores were recovered using GS-BHI-Agar (Gebel *et al*, 2002). Anaerobe Basal Agar was used for *C. sporogenes* (MP0610, Oxoid). For *B. subtilis*, trypticase soy agar (TSA; QueLab) was used.
- The plates with membrane filters were incubated for up to five days for colony forming unit (CFU) counts.



Microbicides Tested

Table 2 shows the microbicides tested. They were selected for their potential to kill bacterial spores in a relatively short contact time. Water with 400 ppm as CaCO₃ was used as the diluent to prepare use-dilutions when required.

Metal Disks as Carriers

Disks (1 cm diam.; 0.7 mm thick) of magnetized/brushed stainless steel (AISI-430; Muzeen & Blythe, Winnipeg, MB) were washed and autoclaved (Springthorpe & Sattar 2003). Teflon vials (Cole Parmer, Vernon Hills, IL; cat. # PK-08936-30) were used for holding the carriers.

Eluent/diluent

Saline (pH 7.2-7.4) was the diluent for eluates before titrations for CFU. Saline with 0.1% (v/v) Tween-80 (Saline-T; pH of 7.2-7.4) with a neutralizer was used as eluent/diluent at the end of the contact time.



TABLE 2. MICROBICIDES TESTED

Formulation	Source	Actives	pH	Neutralizer
Domestic Bleach	Purchased locally	5.25% sodium hypochlorite (52,500 ppm chlorine)	11.0	1% sod. thio. + 0.1% Tween 80
Acidified bleach	Prepared locally	1 part bleach + 1 part 5% acetic acid + 8 parts water (5,250 ppm chlorine)	5.4	1% sod. thio. + 0.1% Tween 80
Chlorine dioxide	ERCO, Toronto, ON	25% sod. chlorite + 5.25% sod. hypochlorite + 12 N HCl (1000 ppm chlorine)	4.0	1% sod. thio. + 0.1% Tween 80
Accelerated hydrogen peroxide (AHP)	Virox Tech., Oakville, ON	7% H ₂ O ₂	2.0	Lethen Broth + 1% sod. thio.

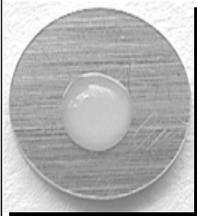
Soil load

For added soil load, each 340 µL of spores received 25 µL of 5% bovine albumin (cat #B-4287; Sigma), 100 µL of 0.4% bovine mucin (cat # M-4503; Sigma) and 35 µL of 5% Tryptone (cat #0123-01-1; Difco) prepared as stock solutions in phosphate buffer (Springthorpe and Sattar, 2003).

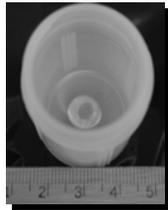
Quantitative carrier test (Flowchart)

The second tier of quantitative carrier test (QCT-2) – an ASTM standard (#E-2197) - was used to assess sporicidal activity (Springthorpe & Sattar 2003; 2004). Figure 1 and the Flowchart give the main steps in QCT-2.

FIGURE 1. MAIN STEPS IN QCT 2



Disk with 10 μ L inoculum



Disk with inoculum (dried) in Teflon vial



Disk with 50 μ L of microbicide



Disk with 9.95 mL of eluent/neutralizer



Vortexing for elution of organisms



Eluate being poured into filter holder



Filter and holder being rinsed



Filter being placed on recovery agar

FLOWCHART: MAIN STEPS IN QCT-2

Carrier received 10 μ L of test organism, dried and placed in a Teflon vial.



Test formulation (50 μ L) was placed on 5 carriers and 3 controls received saline; carriers held for required contact time at room temperature.



Each vial received 9.95 mL of normal saline + 0.1% Tween-80 (Saline-T) with suitable neutralizer and vortexed for 45-60 seconds.



Eluates, along with saline washes and any dilutions required, were membrane filtered separately.



Each filter placed separately on agar plate; colonies counted after 5 days.



Log₁₀ reductions calculated

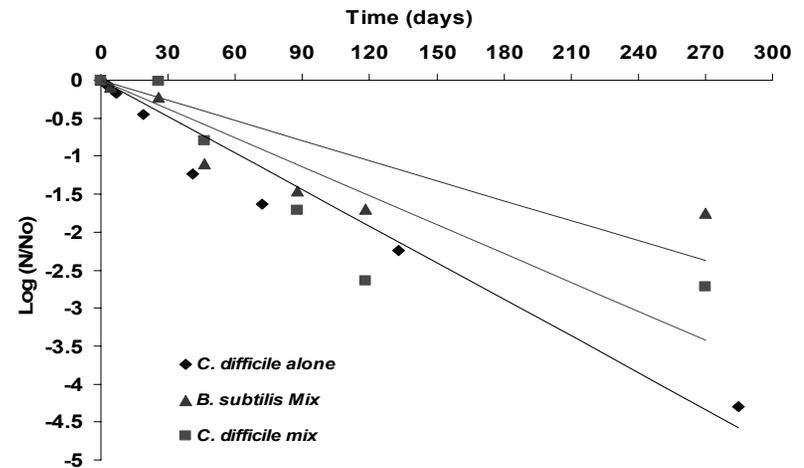
EXPERIMENTAL AND RESULTS

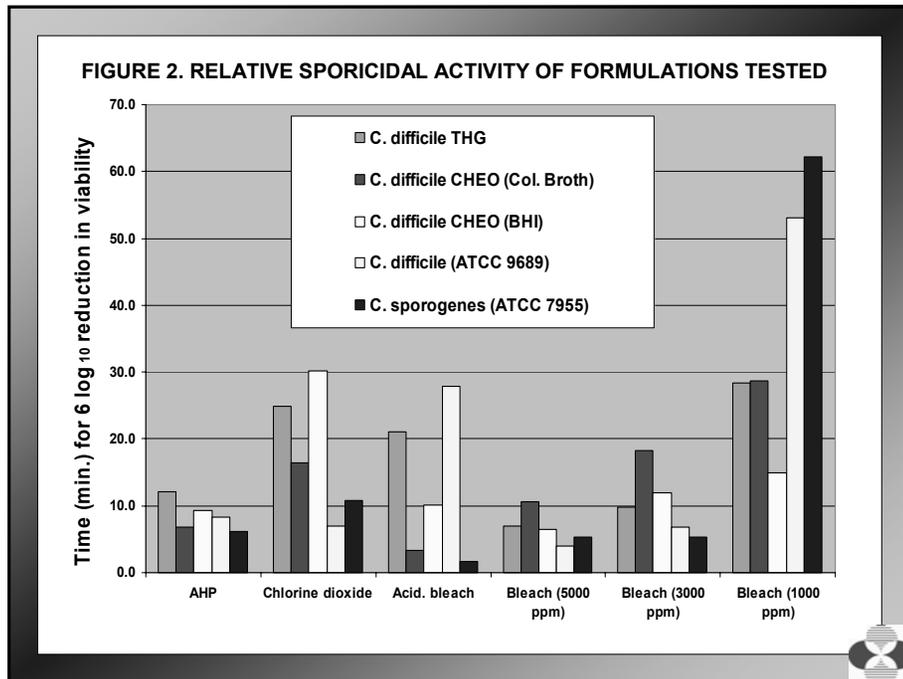
Survival of spores: The spores were suspended in a soil load. Metal disks with about 10^7 CFU each were held indoors >9 months to study the viability of *C. difficile* spores alone or combined with the spores of *B. subtilis*.

The survival of both types of spores was similar (Fig. 1), and, based on the rate constants of viability loss, their survival could continue for two years or longer.

Sporicidal activity: AHP and bleach (5,000 ppm Cl_2) reduced the spore viability of all clostridia tested by $\geq 6 \log_{10}$ in about 12 min. Acidified bleach (5,000 ppm Cl_2), chlorine dioxide (1000 ppm Cl_2) and bleach with 3000 ppm Cl_2 were also generally effective, but with contact times ranging from 3-30 min. Bleach with 1000 ppm Cl_2 required an even wider range of 15->60 min to inactivate all the strains tested.

FIGURE 1. SURVIVAL OF *C. DIFFICILE* & *B. SUBTILIS* SPORES INDOORS ON METAL DISKS





- CONCLUDING REMARKS**
- One ATCC and two clinical isolates of *C. difficile* were tested.
 - Spores of the strain of *C. sporogenes*, commonly used in testing sporicides, were included for comparison.
 - The AHP-based formulation was supplied ready-to-use.
 - Acidification of bleach (Kuroiwa *et al.* 2003) releases chlorine gas – caution!
 - The sod. chlorite solution was supplied by the manufacturer and mixed with sod hypochlorite and HCl before testing.
 - The same sporulation/recovery media could not be used due to differences in the growth characteristics of the strains tested, and this may reflect, to some degree, in the results obtained. Nevertheless, the findings are a good guide to selection of microbicide when rapid and reliable sporicidal action is needed against *C. difficile*. Human/environmental safety and materials compatibility must also be considered.
 - Weaker microbicides may promote *C. difficile* sporulation (Wilcox *et al.* 2003).

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