Use of Purified *Clostridium difficile* Spores To Facilitate Evaluation of Health Care Disinfection Regimens[▽][†]

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Clostridium difficile is a major cause of antibiotic-associated diarrheal disease in many parts of the world. In recent years, distinct genetic variants of *C. difficile* that cause severe disease and persist within health care settings have emerged. Highly resistant and infectious *C. difficile* spores are proposed to be the main vectors of environmental persistence and host transmission, so methods to accurately monitor spores and their inactivation are urgently needed. Here we describe simple quantitative methods, based on purified *C. difficile* spores and a murine transmission model, for evaluating health care disinfection regimens. We demonstrate that disinfectants that contain strong oxidizing active ingredients, such as hydrogen peroxide, are very effective in inactivating pure spores and blocking spore-mediated transmission. Complete inactivation of 10^6 pure *C. difficile* spores on indicator strips, a six-log reduction, and a standard measure of stringent disinfection regimens require at least 5 min of exposure to hydrogen peroxide vapor (HPV; 400 ppm). In contrast, a 1-min treatment with HPV was required to disinfect an environment that was heavily contaminated with *C. difficile* spores (17 to 29 spores/cm^2) and block host transmission. Thus, pure *C. difficile* spores facilitate practical methods for evaluating the efficacy of *C. difficile* spore disinfection regimens and bringing scientific acumen to *C. difficile* infection control.

Clostridium difficile is a Gram-positive, spore-forming, anaerobic bacterium that is a major cause of health care-acquired infections and antibiotic-associated diarrhea (2). In recent years, several genetic variants of *C. difficile* have emerged as important health care pathogens (6). Perhaps most notable is the "hypervirulent" variant, commonly referred to as PCR ribotype 027/restriction endonuclease analysis (REA) group BI, that produces elevated levels of toxins TcdA and TcdB (17, 19). Other virulent ribotypes that display extensive heterogeneity among their toxin protein sequences (26) and gene activities (8) have emerged. Using whole-genome sequencing, we demonstrated that there are broad genetic differences between the entire genomes of several common variants, including ribotype/REA group variants 012/R, 017/CF, and 027/BI used in this study (12, 27, 31). In contrast, phylogeographic analysis of 027/BI isolates from Europe and the United States demonstrates that this clade is extremely clonal and implies recent transcontinental spread of hypervirulent *C. difficile* (12).

C. difficile is distinct from many other health care pathogens because it produces highly infectious spores that are shed into the environment (25, 28). *C. difficile* spores can resist disinfec-

tion regimens that normally inactivate other health care pathogens, such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, therefore challenging current infection control measures (2). A multifaceted approach is normally used to control *C. difficile* in health care facilities (32). Interventions include antimicrobial stewardship, increased clinical awareness, patient isolation (11), and enhanced environmental disinfection regimens based on hydrogen peroxide (H_2O_2) vapor (HPV) (4). While attempts to break the spore-mediated infection cycle and interrupt these efficient routes of transmission are important for infection control measures, there is little quantitative evidence indicating which interventions are most effective (7). Here we describe the exploitation of pure *C. difficile* spores (16) and a murine transmission model (15) in simple, practical methods to quantitatively monitor the impact of health care disinfection regimens on *C. difficile* viability. These methods can be used to optimize disinfection regimens targeted at *C. difficile*.

MATERIALS AND METHODS

***C. difficile* strains and spore purification.** *C. difficile* spores were purified as previously described (16) by using the human-virulent *C. difficile* strains 630 (*tcdA*+ *tcdB*+; ribotype 012/REA group R) (27), R20291 (*tcdA*+ *tcdB*+; ribotype 027/REA group BI) (31) and M68 (*tcdA tcdB*+; ribotype 017/REA group CF) (8). The genomes of these strains have been sequenced and are available at http://www.sanger.ac.uk/Projects/C_difficile/.

Spore inactivation assay. The concentration of the spore preparation was determined by visual enumeration with a light microscope and culturing on brain heart infusion (BHI) broth supplemented with 0.25% taurocholate (16). To test for disinfectant susceptibility/resistance, ~10^6 pure spores were placed in the

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TABLE 1. Summary of health care disinfectants and active ingredients tested against pure *C. difficile* spores

Disinfecting agent	Use(s)	Active ingredient	*C. difficile* spore inactivation[a]
70% ethanol	Surface	Alcohol	N
Spirigel	Hand	Alcohol	N
HiBiScrub	Hand/preoperative	Chlorhexidine gluconate	N
Flash (no bleach)	Surface	Benzisothiazolinone	Moderate
Steri-7	Surface	Isothiazolin-benzalkonium chloride	Moderate
Virusolve	Surface	Alkyl triamine/bromine	Y
3% H_2O_2	Surface	H_2O_2	Y
1% Virkon	Surface	Potassium peroxymonosulfate	Y
10% H_2O_2	Surface	H_2O_2	Y
Spor-Klenz	Surface	H_2O_2-peroxyacetic acid	Y
1% sodium hypochlorite	Surface	Sodium hypochlorite	Y
Chlor-Clean	Surface	Sodium dichloroisocyanurate	Y

[a] Based on data from Fig. 1a. N, no; Y, yes.

indicated disinfectant in a total volume of 0.5 ml for 1, 10, or 20 min. The following disinfectants (Table 1) were prepared as described by the manufacturer and used at a 100% concentration unless otherwise indicated: 70% ethanol (VWR, United Kingdom), Spirigel (Ecolab Ltd., Leeds, United Kingdom), HiBiScrub (Reagent Medical, Manchester, United Kingdom), Flash (Proctor and Gamble, Surrey, United Kingdom), Steri-7 (Steri-7 Worldwide [Cyprus] Ltd., Surrey, United Kingdom), Virusolve (Amity International, Barnsley, United Kingdom), H_2O_2 (Sigma, United Kingdom), Virkon (Antec International, Suffolk, United Kingdom), Spor-Klenz (Steris Ltd., St. Louis, MO), sodium hypochlorite (Adams Healthcare, Leeds, United Kingdom), and Chlor-Clean (Guest Medical, Kent, United Kingdom). After treatment, spores were pelleted with centrifugation and washed 2 times in sterile water to remove the disinfectant. Viable spores were enumerated as previously described (16). To compare the efficacies of the treatments, the number of viable spore counts was logarithmically (base 10) transformed, and then the data were fitted to analysis of variance models containing both treatment and ribotype/REA type effects. Spores were processed and transmission electron microscopy (TEM) images taken as previously described (16).

Spore strip assay. *Geobacillus stearothermophilus* spore strips (10^6 spores/strip) were purchased from Raven Biological Laboratories, Inc. (Cheshire, United Kingdom). Pure *C. difficile* M68 spores were suspended in sterile phosphate-buffered saline (PBS) at 10^7 spores/ml. From this stock, 0.1 ml (10^6 spores) was absorbed onto a sterile piece of Whatman filter paper (4 cm by 1 cm) and aseptically placed in a sterile bijou tube (7 ml) and capped. HPV treatment was performed by placing the uncapped tubes with spore strips inside in a biosafety cabinet fitted with a Bioquel Claris HPV generator and an HPV detector. HPV was administered to the cabinet at a level of 400 ppm and maintained for the indicated time prior to purging of the cabinet to remove that HPV. After exposure to HPV, *G. stearothermophilus* was cultured from spore strips and test results were interpreted based on the manufacturers' protocols. The *C. difficile* spore strips were cultured in 4 ml BHI (Oxoid, Cambridge, United Kingdom) broth supplemented with 0.25% taurocholate under anaerobic conditions for 72 h. Tubes that became turbid were tested for *C. difficile* by streaking on CCEY agar plates (Bioconnections, Wetherby, United Kingdom) (scored positive), whereas tubes that remained clear were scored negative for *C. difficile* growth.

Murine experiments. Transmission experiments and infective dose experiments were performed as previously described (15, 16). Cages were contaminated with the indicated numbers of pure M68 spores (infective dose experiment) or with feces from donor mice that were shedding high levels ($>10^7$ spores/gram feces) of *C. difficile* M68 spores (transmission experiment). Transmission occurred if mice were shedding *C. difficile* 4 days after exposure to a spore-contaminated environment (16). To estimate the environmental spore load after fecal contamination, 10 ml of sterile water was added to each cage and the cage bottoms were scrubbed with sterile cotton swabs. After extensive scrubbing, the water was collected, diluted, and plated on CCEY agar and incubated anaerobically at 37°C overnight as previously described (15). *C. difficile* colonies were counted and expressed as spores per cage or spores per cm^2 (cage bottom is 800 cm^2) in Fig. 3.

Surface disinfection was performed by adding 15 ml (covering the cage floor) of the indicated disinfectant to the contaminated cage for 10 min (see Fig. 3) as previously described (15). Disinfectants were then removed and the surface was patted dry with sterile paper towels before naïve mice were placed into the cage.

HPV disinfection of contaminated cages (see Fig. 3) was performed as described above. All animal infections were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986.

RESULTS

Oxidizing disinfectants effectively inactivate *C. difficile* spores. We previously described a protocol to purify infectious and highly resistant *C. difficile* spores (16). The ability to prepare pure spores allowed us to accurately measure the sensitivity of *C. difficile* spores to disinfectants in the absence of contaminating vegetative cells and their extracellular products, such as an S-layer (9) and capsule (3), that can interfere with spore-disinfectant interactions (20). We tested pure *C. difficile* spores against a panel of disinfectants that are commonly used in health care facilities (Fig. 1a; Table 1). The active ingredient and intended application for each disinfectant are provided in Table 1 because these products are often sold under different commercial names in different countries. We chose three wild-type strains of human-virulent *C. difficile* that represent the genotypes (PCR ribotype/REA type) 012/R, 017/CF, and 027/BI. The genome of each of these strains has been sequenced (12). In each experiment, we treated 10^6 spores, as complete inactivation of 10^6 microorganisms, commonly referred to as a six-log reduction, is considered a standard measure for stringent disinfection regimens (20).

C. difficile spores efficiently germinate and form colonies on nutrient agar supplemented with taurocholate (Fig. 1a) (30, 36). We found that exposure to 70% ethanol, Spirigel, and HiBiScrub had no obvious or reproducible effect on the viability of the *C. difficile* spores compared to those immersed in sterile H_2O ($F = 1.02$; $P = 0.41$) (Fig. 1a). The commonly used commercial detergents Flash and Steri-7 similarly ($F = 3.47$; $P = 0.10$) reduced the spore viability ~10-fold, which is statistically different from the value for the water treatment ($F = 898.8$; $P < 10^{-16}$) (Fig. 1a). Virkon, 3% hydrogen peroxide, and Virusolve inactivated the majority of spores, whereas Spor-Klenz, sodium hypochlorite, Chlor-Clean, and 10% hydrogen peroxide completely inactivated the spores in a manner that was significantly better than those of the other treatments ($F = 134.0$; $P < 10^{-16}$) (Fig. 1a). For example, we were not able to culture *C. difficile* (detection limit of 2 CFU/ml) when spores were treated with an appropriate concentration of Spor-
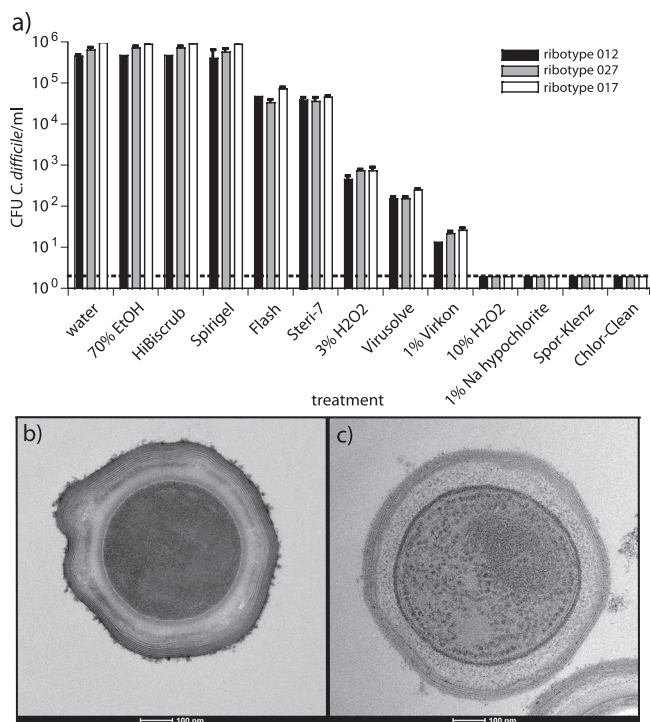
FIG. 1. Efficacy of health care disinfectants in inactivating pure *C. difficile* spores. (a) Viability of spores derived from distinct *C. difficile* genetic variants after exposure to various health care disinfectants. All *C. difficile* strains are virulent to humans. A summary of the disinfectants is listed in Table 1. Data are representative of 5 separate experiments. Error bars indicate standard deviations. The broken horizontal line indicates the detection limit of 2 CFU/ml. EtOH, ethyl alcohol. (b) TEM image of an untreated, viable spore. (c) TEM of a spore that was inactivated by 10% hydrogen peroxide exposure for 10 min.
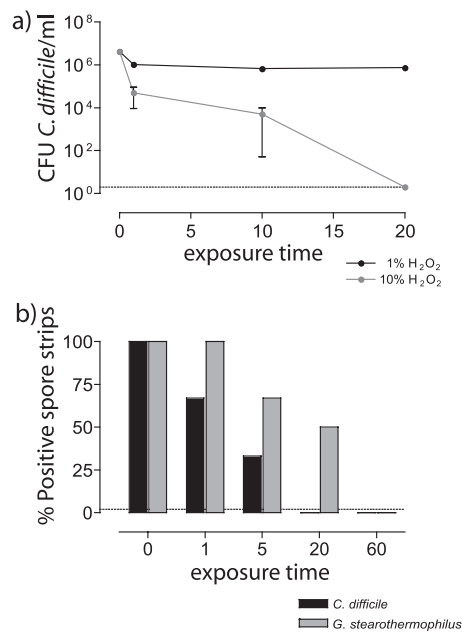

FIG. 2. *C. difficile* spores are inactivated by prolonged exposure to high-level hydrogen peroxide. (a) Viability of *C. difficile* M68 spores after exposure to 1% or 10% hydrogen peroxide for 1, 10, and 20 min. Data are representative of three separate experiments. Error bars indicate standard deviations. The broken horizontal line indicates the detection limit of 2 CFU/ml. (b) Spore strips containing either 10^6 *C. difficile* M68 or *G. stearothermophilus* spores were exposed to 400 ppm hydrogen peroxide vapor for 1, 5, 20, or 60 min prior to culture of the bacteria to determine the efficacy of disinfection. Positive growth strip indicates that the spores were not inactivated by HPV. Six spore strips were tested per time point.

Klenz, sodium hypochlorite, Chlor-Clean, or 10% hydrogen peroxide for 20 min, corresponding to a six-log reduction in viability (Fig. 1a). Importantly, spores derived from distinct genetic variants of *C. difficile*, specifically ribotypes 012, 017, and 027, responded in similar manners to each disinfectant.

Untreated, viable spores have an electron-dense outer spore coat with distinct striated layers that encompasses a lighter layer referred to as the cortex (Fig. 1b). Encased within the cortex is the core, defined by a continuous membrane that surrounds the nuclear DNA and ribosomes (Fig. 1b). After 10% H_2O_2 treatment, the spore coat showed significant decreases in intensity and banding patterns, while the inner layers appeared more separated and diffuse (Fig. 1c). The core staining was less uniform than seen in untreated spores, and this core staining appeared to be comparatively expanded (Fig. 1c). Therefore, *C. difficile* spores are resistant to many commonly used health care disinfectants but are inactivated and rendered nonviable by disinfectants that contain oxidizing active ingredients.

Kinetics of pure *C. difficile* spore inactivation by hydrogen peroxide. The level of spore inactivation is likely to depend to a significant extent on the contact time for *C. difficile* spores and the disinfectant, as sufficient time is required to allow the disinfecting agent to access and damage the cellular target (20). Thus, we evaluated the kinetics of spore inactivation (10^6

spores) after independent exposure to two concentrations of a hydrogen peroxide solution (Fig. 2a). Treatment of *C. difficile* spores with 1% hydrogen peroxide resulted in an immediate (i.e., 1-min) inactivation of 75% of the spores without subsequent inactivation over the following 19 min (Fig. 2a). In contrast, a 1-min exposure to 10% hydrogen peroxide resulted in inactivation of >99% of the spores, followed by a gradual reduction in viable spores over the next 19 min when the spores were fully inactivated (Fig. 2a).

Indicator organisms are commonly used to evaluate the efficacy of environmental decontamination regimens, particularly in environments such as purpose-built bacterial containment facilities. *Geobacillus stearothermophilus* spore strips are available as commercial kits and are considered the gold standard. Consequently, we exposed both standard *G. stearothermophilus* spore strips (10^6 spores) and custom-made *C. difficile* spore strips (10^6 pure spores on sterile filter paper) to 400 ppm hydrogen peroxide vapor for 1, 5, 20, and 60 min before attempting to culture the bacteria (Fig. 2b). *G. stearothermophilus* spore strips required a 20- to 60-min exposure to HPV to render the bacteria nonviable. In contrast, the *C. difficile* spore strips required a 5- to 20-min exposure to render the bacteria completely nonviable (Fig. 2b). Thus, prolonged exposure with HPV is required to achieve a six-log reduction of bacterial spores and *C. difficile* spores are significantly more sensitive to HPV than *G. stearothermophilus* spores.
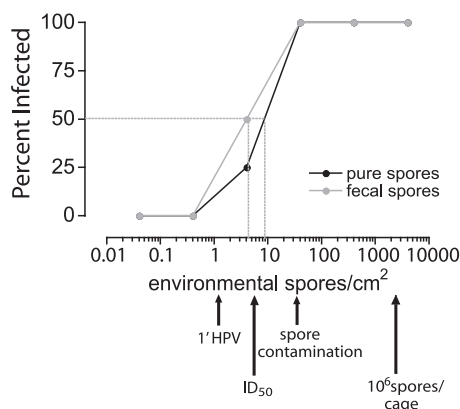
FIG. 3. Environmental *C. difficile* spores are highly infective. Infective dose curves of pure and fecally derived *C. difficile* M68 spores in mice (4 per dose) after exposure to environmental spore contamination. Based on the Reed-Muench formulation (24), the environmental spore dose to infect 50% (ID_{50}) of the mice with a 1-hour exposure is 5 spores/cm² for fecal spores and 10 spores/cm² for pure spores, as indicated by the gray vertical dashed lines and the black arrow below the graph. Data are representative of two independent experiments. As a reference, the vertical arrow below the graph labeled "spore contamination" indicates the level of spores that was retrieved from the cage after fecal contamination by the infected mice corresponding to Fig. 4. The vertical arrow labeled "10^6 spores/cage" indicates the level of spores that is equivalent to that on the indicator strips corresponding to Fig. 2.

Oxidizing disinfectants block spore-mediated transmission. It is difficult to extrapolate *in vitro* disinfection results directly to an applied setting, as during host transmission, organic/fecal materials that can interfere with interactions between the disinfectant and spore may be present (20). Murine fecal-oral transmission models are valuable surrogates for the study of host and pathogen factors that are involved in transmission (14, 33, 35) and for the evaluation of the ability of disinfectants to block spore-mediated transmission (15). Here we used a murine transmission model to evaluate the abilities of various health care disinfectants to block spore-mediated transmission. With our transmission model, we found that mice that are exposed to an environment contaminated with pure *C. difficile* M68 spores for 1 h become colonized in a dose-dependent

manner (Fig. 3), which is consistent with previous results from a study using *C. difficile* 630 spores (16). Based on these experiments, we are able to estimate that the environmental spore load required to infect 50% of those mice exposed for 1 h is 5 to 10 spores/cm² (Fig. 3).

Heavy contamination of cages by mice shedding high levels of *C. difficile* M68 spores (average 23 ± 6.1 spores/cm² of cage bottom; $n = 4$ cages) facilitates efficient spore-mediated transmission to naïve mice (Fig. 4a). Next, we tested the efficiencies of various health care disinfectants in blocking *C. difficile* transmission. As expected, a 10-min surface disinfection with 70% alcohol, HiBiScrub, and the detergents Flash and Steri-7 had minimal to moderate impacts on the reduction of transmission (Fig. 4a). In contrast, the oxidizing disinfectants Virusolve and Chlor-Clean were the most effective treatments for reducing host-to-host transmission (Fig. 4a). Enhanced cleaning of hospital wards is routinely accomplished with HPV (5). We next tested the efficacy of HPV (400 ppm) in blocking spore-mediated transmission. HPV treatment proved to be dramatically efficient, with a 1-min pulse reducing environmental spore contamination sufficiently to completely eliminate transmission (Fig. 4b). Thus, oxidizing disinfectants effectively inactivate environmental spores below the dose required to infect mice.

DISCUSSION

Disinfection of the hospital environment with hypochlorite solution (11, 18, 21, 34) and hydrogen peroxide dry mist or vapor (5, 29) reduces the incidence of *C. difficile* disease. However, it is difficult to evaluate the direct impact of these disinfectants alone, as during use of enhanced disinfection regimens, there is generally an increased sense of awareness among hospital staff that may also contribute to improved infection control (7, 34). Thus, the availability of pure *C. difficile* spores and a murine transmission model offer the unique opportunity to quantitatively evaluate and monitor the efficacy of *C. difficile* disinfection regimens in a controlled manner.

We showed that purified spores derived from distinct genetic variants of human-virulent *C. difficile* are effectively and equally inactivated by a variety of disinfectants that contain strong oxidizing agents. Therefore, the spore structures of these variants have not genetically acquired altered resistance
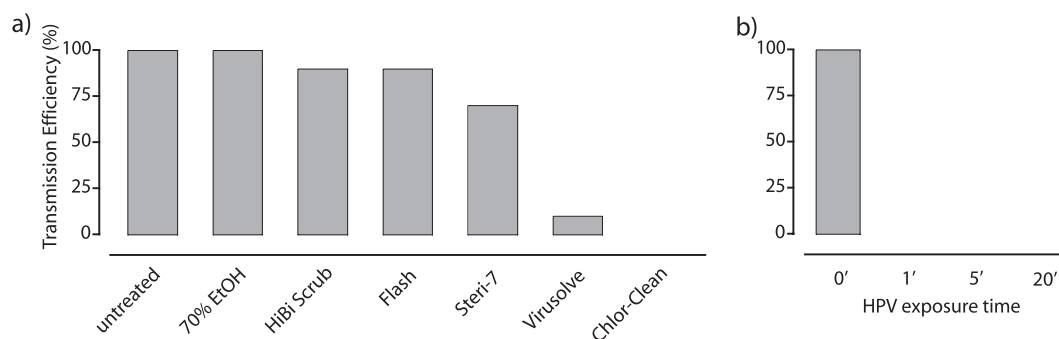

FIG. 4. Efficacies of health care disinfectants in reducing host transmission of *C. difficile* via environmental spore contamination. (a) Transmission efficiency of environmental *C. difficile* M68 spores to naïve mice after surface disinfection with the indicated agent (10 mice per agent) compared to that of the untreated group (18 mice untreated). (b) Effect of hydrogen peroxide vapor contact time on reducing transmission of *C. difficile* spores to naïve mice. Exposure time is defined as the time the HPV was at the peak level of 400 ppm. There were 10 mice corresponding to each time point (0, 1, 5, and 20 min). The estimated level of spore contamination was 23 ± 6 spores/cm².

to health care disinfectants. Overall, our observations are consistent with those from others who have successfully demonstrated that cultures of *C. difficile* containing a mixture of vegetative cells and spores are inactivated by chlorine-releasing disinfectants (10) and hydrogen peroxide dry mist (1). We also demonstrated that oxidation-based disinfectants efficiently block spore-mediated transmission between mice, whereas many commonly used health care disinfectants failed. The availability of a complete *C. difficile* spore proteome (16) will facilitate studies aimed at identifying the targets of the oxidizing disinfectants and could aid in the development of novel disinfectants that can be used on hands, potentially one of the most important mediators of transmission (23).

Environmental disinfection protocols with gaseous disinfectants (such as HPV or formaldehyde) commonly employ excessive treatment regimens to provide the greatest assurance of sterility. Generally, a six-log reduction of *G. stearothermophilus* spores on indicator strips is the desired endpoint (22). With our HPV disinfection protocol, it takes >20 min to fully inactivate 10^6 *G. stearothermophilus* spores but only 5 min to inactivate 10^6 *C. difficile* spores. Thus, *G. stearothermophilus* spores are likely to be inherently very resistant to disinfection (13). In contrast, we demonstrate that only a 1-min HPV treatment in a heavily contaminated environment was required to reduce viable *C. difficile* spore levels below the infectious dose and block transmission. Thus, disinfection regimens that completely inactivate either type of spore strip should indicate successful environmental *C. difficile* spore disinfection.

Since *G. stearothermophilus* spores are significantly more resistant than *C. difficile* spores, it may be prudent to include *C. difficile* spore strips to monitor the efficacy of disinfection with hydrogen peroxide vapor. This could be achieved by safely enclosing accurately measured quantities of *C. difficile* spores (possibly from a nontoxigenic strain) within strips that fully retain the spores but allow access of the inactivating vapors. Such an approach would facilitate the tailoring of HPV disinfection regimens to *C. difficile* and bring scientific acumen to *C. difficile* infection control.

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