MICROBIOLOGICAL METHODS

In vitro Production of *Clostridium difficile* Spores for Use in the Efficacy Evaluation of Disinfectants: A Precollaborative Investigation

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Clostridium difficile **is a strict anaerobic spore-forming bacterium, and an increasingly common nosocomial pathogen. The U.S. Environmental Protection Agency (EPA) is responsible for the registration of disinfectants, including products designed to treat environmental surfaces contaminated with spores of *C. difficile*. Product efficacy data are required for registration; however, there is a lack of methodology for generating high-quality spore suspensions for evaluating product performance. As such, a study was carried out to select a suitable *C. difficile* strain and to develop a stand-alone method to prepare a spore suspension that meets specific criteria necessary for quantitative testing of disinfectants. The criteria are: (*1*) a spore titer of >8 $\log_{10}$/mL, (*2*) 90% spores to vegetative cells, and (*3*) resistance of spores (determined by viability) to 2.5 M hydrochloric acid (HCl). Several strains of *C. difficile* (toxigenic and nontoxigenic) were grown on various media (solid and liquid) for varying lengths of time to determine the best combination of incubation conditions and media to optimize spore production and quality. Once the spore production procedure was optimized, a toxigenic strain of *C. difficile* [American Type Culture Collection (ATCC) 43598] was selected for use in trials to verify repeatability from one production run to the next. The spore suspension was initiated by spreading vegetative cells of *C. difficile* (ATCC 43598) on CDC anaerobic 5% sheep blood agar plates and incubating for 7–10 days at 36 1 C under anaerobic conditions. Spores were harvested when 90% of the cells converted to spores as determined by observation using phase-contrast microscopy. The spores were washed three times with saline–Tween-80, resuspended in cold deionized water, heated to 70 C for 10 min, evaluated microscopically for quality, and enumerated on cycloserine-cefoxitin-fructose agar containing horse blood and taurocholate. The spore suspension was used to inoculate brushed stainless steel carriers (1 cm in diameter) with and without a soil load in accordance with the Standard Quantitative Carrier Disk Test Method (ASTM E-2197-02) to determine carrier load. Once it was determined that >6 $\log_{10}$ spores/carrier could be recovered, spores were evaluated for resistance to HCl. The sporulation method presented in this report is simple and repeatable and results in spore suspension of high titer (>8 $\log_{10}$/mL) and quality (90% spores to vegetative cells) that met acid resistance criteria (spores were resistant to 2.5 M HCl for 10 min). In addition, recovery from brushed stainless steel carriers with and without soil load was >6 $\log_{10}$ spores/carrier. A 6 $\log_{10}$ performance standard was set forth in the EPA's interim guidance for generating data to support a label claim for effectiveness against *C. difficile* spores on hard, nonporous surfaces. This precollaborative investigation successfully demonstrated the use of a methodology for in vitro production of *C. difficile* spores (ATCC 43598) necessary for conducting efficacy tests. A proposal will be submitted to the AOAC INTERNATIONAL Methods Committee on Antimicrobial Efficacy Testing for a collaborative study; *see* Appendix.**

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Clostridium difficile, a Gram-positive, strict anaerobic spore-forming bacterium, was first identified and described in the mid-1930s (1). For the next 40 years, there were infrequent reports of *C. difficile* as a causal agent of disease. However, in 1978, *C. difficile* was implicated as the primary cause of pseudo membranous colitis and was shown to be a primary isolate from the feces of patients undergoing clindamycin treatment (2). Over the next several decades,

C. difficile was identified as the principal causative agent of nosocomial diarrhea, causing a clinical spectrum of disease ranging from mild diarrhea to life-threatening colitis, leading to an increased length of hospitalization and an estimated 3.2 billion dollars in healthcare costs annually (3). Outbreaks of *C. difficile* infection (CDI) are not only common, but recent studies also suggest that the incidence, severity, and relapse rate may be increasing (4). Human CDI is responsible for 25% of all cases of antibiotic-associated diarrhea (5). Only toxigenic strains of *C. difficile* have been associated with CDI. During vegetative growth inside the human body, *C. difficile* cells produce two major toxins, TcdA (enterotoxin) and TcdB (cytotoxin), which are responsible for inflammation, fluid and mucus secretion, and mucosal damage, thus leading to diarrhea or colitis (6). More recently, widespread outbreaks have been associated with a previously uncommon *C. difficile* strain (North American Pulse-Field Gel Electrophoresis Type 1, also known as PCR Ribotype 027) in the United States, Canada, and Europe (7). The recent epidemic strain is responsible for approximately 24 confirmed deaths/million of infected cases in the United States in 2004 and has been reported to produce 16 times more TcdA and 23 times more TcdB than other strains of *C. difficile* (8, 9). There have been reports that epidemic strains have a greater sporulation rate than nonepidemic strains, and that sporulation might be closely related to cytotoxin production (10–13). These findings suggest that some strains of *C. difficile* have an increased inclination to cause disease and, possibly, increased propensity to persist as spores in the environments in healthcare institutions (14).

The spores of *C. difficile* have been found on floors, sinks, toilets, and other high-touch contaminated surfaces in rooms of symptomatic and asymptomatic patients and may persist in the hospital environment for many months (15, 16). Contaminated environmental surfaces and transient hand carriage by healthcare workers and patients have been described as sources of *C. difficile* spread in the hospital environment (14). The *C. difficile* spores, supported by a thickened cell wall, are protected from drying and acidic environments, and show resistance to many commonly used disinfectants, including chlorhexadine, vesphene, 70% isopropyl alcohol, 95% ethanol, 3% hydrogen peroxide, a disinfecting spray containing 65% ethanol and 0.6% quaternary ammonium compounds, 10% povidone iodine, and 0.5% hydrogen peroxide (17, 18). These disinfectants, however, were not sporicides. In fact, a few nonsporicidal disinfectant compounds, when applied in hospital environments, appeared to even encourage the sporulation rate of *C. difficile* (11, 14). It was suggested that the increased sporulation rate could be attributable to the environmental stress created by the sub-inhibitory concentrations of disinfectants (14). More data are needed to support routine environmental decontamination of *C. difficile* spores in the hospital environment by the use of either disinfectants or detergent-based formulas. In fact, there is a need for studies on the efficacy of disinfectants against *C. difficile* spores, particularly on environmental surfaces. However, no standardized sporulation protocol is available as of yet to produce *C. difficile* spores of high titer and quality for use in performing efficacy evaluations of disinfectants.

The U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) has responsibility for regulating antimicrobial products, including sporicides and sterilants that are used to control pathogenic bacteria, viruses, and other microorganisms on inanimate surfaces as well as the gas sterilants used on critical and semicritical medical devices and instruments. The U.S. Food and Drug Administration (FDA) Center for Devices and Radiological Health regulates liquid antimicrobial chemicals (e.g., liquid chemical sterilants) used to process critical and semicritical medical devices. The AOAC Sporicidal Activity of Disinfectants, AOAC *Official Method*^{SM} **966.04** (19), is accepted by the EPA and FDA for generating efficacy data to support the registration of sporicides (sterilants). AOAC Method **966.04** is a carrier-based test that provides a qualitative measure of product efficacy against spores of *Bacillus subtilis* (ATCC 19659) and *C. sporogenes* (ATCC 3584). Prior to 2008, no disinfectant products were registered to treat hard nonporous surfaces in healthcare settings contaminated with spores of *C. difficile.* In addition, current efficacy methods for *B. subtilis* and/or *C. sporogenes* do not address *C. difficile*. In response to growing needs and inquiries by registrants for generating data to register disinfectants with a sporicidal claim against *C. difficile*, the EPA issued interim guidance (20) in 2009 for the efficacy evaluation of disinfectants labeled for use to treat hard nonporous surfaces in healthcare settings contaminated with spores of *C. difficile.* The guidance recommends that the efficacy evaluation should be conducted using one of the following test methods: (*1*) AOAC Method **966.04**: Sporicidal Activity of Disinfectants Test, Method I (19); (*2*) AOAC Method **2008.05**: Efficacy of Liquid Sporicides Against Spores of *Bacillus subtilis* on a Hard Nonporous Surface, Quantitative Three Step Method (21); (*3*) ASTM International (ASTM; West Conshohocken, PA) E 2414-05: Standard Test Method for Quantitative Sporicidal Three Step Method (22); and (*4*) ASTM E 2197-02: Standard Quantitative Carrier Disk Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Liquid Chemical Germicides (23).

For a qualitative assessment by AOAC Method **966.04**, carriers (porcelain penicylinders) inoculated with spores of *C. difficile* must meet an acceptable acid resistance standard, and mean control carrier counts must be approximately 1 10^5 to 1 10^6 spores/carrier. For quantitative assessments, control carrier counts >6 $\log_{10}$ spore/carrier are required for AOAC Method **2008.05**, ASTM E 2414-05, and ASTM E 2197-02. Since the sporulation of *C. difficile* is not as rapid or efficient as in other species (e.g., *B. subtilis* on solid medium), it has been a challenge to generate *C. difficile* spores of high titers to demonstrate acceptable levels of sporicidal activity.

This paper reports the development of an easy to perform method for preparing a spore suspension from a suitable *C. difficile* strain (ATCC 43598) that meets specific criteria necessary for efficacy testing of disinfectants. The criteria are:

(*1*) a spore titer of >8 $\log_{10}$/mL, (*2*) 90% spores to vegetative cells, and (*3*) resistance of spores to 2.5 M HCl. The paper also proposes the method for consideration as a stand-alone AOAC *Official Method* for in vitro production of *C. difficile* spores for use in the efficacy evaluation of disinfectants upon successful completion of a collaborative study.

Experimental

The study was carried out at the EPA OPP Microbiology Laboratory, Fort Meade, MD, under quality assurance (QA) procedures consistent with EPA's Good Laboratory Practice Standards (24). The study was performed to determine the most suitable combination of microbe, spore production medium, spore enumeration medium, and incubation conditions in an effort to develop a method to prepare a high-quality spore suspension from *C. difficile*. Once the spore production procedure was optimized, independent trials were conducted by two other analysts to demonstrate in-house repeatability from one production run to the next. The spore suspension was then used to inoculate brushed stainless steel carriers, with and without a soil load, followed by drying and enumeration (carrier counts) to determine if >6 $\log_{10}$ spores/carrier could be recovered following a standard quantitative disinfectant carrier test (ASTM E2197-02). Four independent carrier enumeration experiments were conducted to observe in-house repeatability with carrier enumeration. Once acceptable recovery from carriers was determined, resistance testing using HCl was conducted.

Media and Reagents

(**a**) *Culture media.*—(*1*) *Reinforced clostridial medium (RCM).*—For use in rehydrating lyophilized/frozen vegetative culture of test organism (manufactured by Oxoid, Hampshire, UK, distributed by Remel, Lenexa, KS; Cat. No. 6614920). Suspended 38 g RCM (powder) in 1 L deionized water. Brought to boil to dissolve completely. Sterilized by autoclaving at 121 C for 15 min. Dispensed 25 mL aliquot into sterile 125 mL Erlenmeyer flask aseptically.

(*2*) *RCM plus 15% glycerol as cryoprotectant.*—For use as a maintenance and cryopreservation medium for vegetative frozen stock (VFS) cultures. Prepared RCM according to manufacturer's instructions and added 15% sterile glycerol (Cat. No. 228220; Becton, Dickinson and Co., Sparks, MD).

(*3*) *CDC anaerobic 5% sheep blood agar* (*CABA*).—Commercially available as prereduced (i.e., manufactured and sterilized in an oxygen-free environment and packaged individually in air-tight sealed pouches or bags) from Anaerobe Systems, Morgan Hill, CA (Cat. No. AS-646).

(*4*) *Egg yolk agar (EYA).*—Commercially available (prereduced) from Anaerobe Systems (Cat. No. AS-2140).

(*5*) *Cycloserine-cefoxitin fructose agar with horse blood and taurocholate (CCFA-HT).*—Commercially available (prereduced) from Anaerobe Systems (Cat. No. AS-2136).

(*6*) *Columbia broth (CB).*—Suspended 35 g powder (Becton, Dickenson and Co.) in 1 L deionized water. Mixed thoroughly. Heated with frequent agitation and boiled for 1 min to dissolve the powder completely. Distributed into bottles and autoclaved at 121 C for 15 min. Dispensed 25 mL aliquot into sterile 125 mL Erlenmeyer flask aseptically.

(*7*) *Brain heart infusion broth (BHIB).*—Suspended 37 g BHI powder in 1 L deionized water. Mixed thoroughly. Heated with frequent agitation and boiled for 1 min to dissolve the powder completely. Distributed into bottles and autoclaved at 121 C for 15 min. Dispensed 25 mL aliquot into sterile 125 mL Erlenmeyer flask aseptically.

(*8*) *Cooked meat medium (CMM).*—Added 1.9 g CMM (pellets) and 15 mL 5 g/mL $MnSO_4$ H_2O solution in deionized water to individual test tubes. Soaked pellets in the solution for approximately 15 min. Autoclaved for 15 min at 121 C. Pooled 25 mL into sterile 125 mL Erlenmeyer flask aseptically.

(*9*) *Tryptic soy agar with blood (TSAB).*—Trypticase soy agar II with 5% sheep blood. Commercially prepared and available from Becton, Dickinson and Co. (Cat. No. 221239).

(*10*) *Columbia agar (CA).*—CA with 5% sheep blood. Commercially available (prereduced) from Becton, Dickinson and Co. (Cat. No. 221263).

(**b**) *Phosphate buffered saline (PBS).*—Prepared 10× solution PBS by dissolving 492 g PBS powder (Fisher Scientific, Pittsburgh, PA; Cat. No. BP661-50) in 5 L deionized water. Diluted desired amount to 1 , distributed into bottles, and autoclaved for 20 min at 121 C.

(**c**) *Phosphate buffered dilution water (PBDW).*—Added 3.4 g potassium phosphate monobasic to 100 mL deionized water to prepare 0.25 M phosphate buffered stock solution (PBSS, pH 7.2); then added 1.25 mL 0.25 M PBSS to 1 L deionized water to prepare PBDW.

(**d**) *PBS containing 0.1% Tween 80 (ST80).*—Added 2.0 mL polysorbate (Tween 80, or equivalent) to 2.0 L PBS (1) solution in 2 L volumetric flask. Brought solution to volume with PBS. Distributed into bottles and autoclaved for 20 min at 121 C.

(**e**) *HCl.*—Prepared 2.5 M HCl from 5 M HCl (1:2 dilution in deionized water).

(**f**) *HistoDenz* .—Prepared a 50% (w/v) solution of HistoDenz, a nonionic density gradient medium (Cat. No. D2158; Sigma-Aldrich, St. Louis, MO).

(**g**) *Ethanol.*—Added 5 mL sterile water to 95 mL of 100% ethanol to prepare 95% (v/v) ethanol.

(**h**) *Soil load containing yeast extract, bovine serum albumin (BSA), and mucin.*—The soil load consisted of a mixture of the following stock solutions in phosphate buffer (pH 7.2): (*1*) added 0.5 g yeast extract to 10 mL phosphate buffer (5%, w/v); (*2*) added 0.5 g BSA to 10 mL phosphate buffer (5%, w/v); and (*3*) added 0.04 g bovine mucin to 10 mL phosphate buffer (0.4%, w/v). Prepared the solutions separately and sterilized by passage through a 0.22 m pore diameter membrane filter, aliquoted, and stored at 4 2 C.

(**i**) *Neutralizer*.—Added 1.25 mL 1 M NaOH to 98.75 mL PBS containing 0.1% ST80.

(**j**) *Water*.—Used sterile deionized water supplied by a Barnstead B-pure Pressure Cartridge System (Barnstead/Thermolyne Corp., Dubuque, IA) located inside

the Environmental Science Center facility (Ft. Meade, MD). The B-pure Pressure Cartridge System uses pretreatment, prefilters, deionization cartridges, and final filters to prepare high-quality deionized water.

(**k**) *Test organism.*—*C. difficile* (toxigenic ATCC 700792, ATCC 43598, ATCC 43599, and ATCC 9689; and nontoxigenic ATTC 700057) obtained directly from a commercial supplier [e.g., American Type Culture Collection (ATCC), Manassas, VA].

Apparatus

(**a**) *Certified biosafety cabinet (BSC; Type II, Class II).*—Recommended for use to maintain an aseptic work environment.

(**b**) *Sterile centrifuge tubes.*—Polypropylene, 15 mL (Fisher Scientific; Cat. No. 05-538-53D) and 50 mL (Fisher Scientific; Cat. No. 05-538-49) graduated plastic centrifuge tubes with conical bottoms.

(**c**) *Centrifuge*.—To allow sedimentation of spores for washing and/or concentration using a swinging bucket rotor (Eppendorf AG, Hamburg, Germany).

(**d**) *Micropipet*.—Calibrated.

(**e**) *Positive displacement pipet*.—To inoculate steel carriers with spores (Gilson Inc., Middleton, WI).

(**f**) *Vacuum desiccators*.—For carrier drying and storage (Thermo Scientific Nalgene, Rochester, NY).

(**g**) *Certified timer*.—Any certified timer that can display time in seconds (VWR International LLC, Arlington, Heights, IL).

(**h**) *Test tubes*.—Reusable or disposable 20 150 mm for cultures/subcultures (Fisherbrand Fisher Scientific, Springfield, NJ).

(**i**) *Inoculating loop*.—VWR International LLC.

(**j**) *COY anaerobic chamber*.—Supported by a gas mixture consisting of 10% hydrogen, 5% CO_2, and 85% N_2 (COY Laboratory Products, Inc., Grass Lake, MI).

(**k**) *Anaerobic incubator*.—Anaerobic incubator (COY Laboratory Products, Inc.) at 36 1 C placed inside the COY anaerobic chamber to support the growth of the organism when plated onto solid media or liquid broth. Placed pan/container filled with deionized water inside the anaerobic incubator to ensure adequate moisture level (70%) to prevent/reduce dehydration of agar plates during extended incubation. Plates must be incubated in an anaerobic incubator at 36 1 C for growth to occur.

(**l**) *Microscope with 10× eyepiece and 40 and 100 oil objectives with phase contrast option.*—For phase-contrast microscopy (Nikon Americas Inc., Melville, NY).

(**m**) *Vortex mixer*.—Genie 2 Mixer/G560 (Scientific Industries, Inc., Bohemia, NY).

(**n**) *Serological pipets.*—Sterile, single-use pipet of 10.0, 5.0, and 1.0 mL capacity (VWR International LLC).

(**o**) *Cell scraper*.—Used to scrape plates gently to remove spores for harvesting (Techno Plastic Products, Trasadingen, Switzerland; Cat. No. 99002).

(**p**) *Plate spreader*.—Used to spread inoculum on plates to create a uniform lawn on top of plate (manufactured by VWR International, West Chester, PA; Cat. No. 30002-112).

(**q**) *Microcentrifuge tubes*.—Sterile 1.5 mL low retention (siliconized) microcentrifuge tubes (Fisher Scientific; Cat. No. 02-681-320).

(**r**) *Carriers*.—Brushed stainless steel disks as per ASTM E2197-02, 1 cm diameter and approximately 0.7 mm thick (manufactured by Drummond Scientific, Broomall, PA; Cat. No. TP430#3).

(**s**) *Cryovials*.—Sterile 2.0 mL cryovials (Corning Inc., Corning, NY; Cat. No. 430659).

Preparation of Frozen Vegetative and Spore Stock Cultures

Strains of *C. difficile* were obtained from ATCC, received both in lyophilized or frozen states, and processed in accordance with ATCC specifications. When received as lyophilized cultures, the contents of the lyophilized culture vial were reconstituted with 1–1.5 mL sterile RCM in an anaerobic environment. After rehydration, the entire contents were aseptically transferred to a tube of sterile RCM (4 1 mL) and mixed gently by vortexing. When received as frozen cultures, frozen vials were thawed at room temperature; the entire contents were transferred to a tube of sterile RCM, total volume 4 1 mL, in an anaerobic environment. Tubes were mixed gently by vortexing. The suspension from the lyophilized or frozen vial was used to inoculate CABA plates as follows: to generate working stock cultures of vegetative cells, five CABA plates were spread with the ATCC culture (100 L/plate). Plates were inverted and incubated anaerobically at 36 1 C for 48 4 h. To generate working stock cultures of spores, five CABA plates were spread with the reconstituted/thawed ATCC culture (100 L/plate). Plates were inverted and incubated anaerobically at 36 1 C for 5 1 days. After incubation, 2 mL cryoprotectant (cryoprotectant = RCM + 15% glycerol, v/v) was added to each CABA plate. Using a sterile cell scraper, culture was scraped from the surface of the open plate, aspirated with a pipet, and transferred to a 15 mL conical tube. This process was repeated for the remaining plates. The pooled culture was mixed thoroughly and aliquoted into cryovials (1–1.5 mL/cryovial). The cryovials were stored at –70 C with appropriate labeling for long-term preservation. These tubes were labeled either vegetative frozen stock culture (VFSC; predominantly vegetative cells) or spore frozen stock culture (SFSC; predominantly spores).

Optimization of Spore Production

Several media were initially evaluated to determine spore production potential: (*1*) RCM, (*2*) CABA, (*3*) TSAB, (*4*) CMM, (*5*) CB, and (*6*) BHIB. Attempts were made to generate spores from frozen spore stock and compare with spores generated from frozen vegetative stock. After thawing a frozen vegetative or spore stock, initially 100 L was added into an Erlenmeyer flask containing 25 mL broth (CMM, CB, RCM, and BHIB), and 100 L was spread-plated onto each of five prereduced CABA and five TSAB plates. The above method, however, was later modified as follows: three CABA plates were streaked using a frozen vegetative stock for

isolation, two were incubated anaerobically at 36 1 C for 48 4 h, and the third plate was incubated aerobically at 36 1 C for 48 4 h. No growth was expected from the plate incubated aerobically. Purity and typical colony characteristics (gray, flat, and irregular) were observed on plates incubated anaerobically. Ten milliliters prereduced RCM was inoculated with a colony and incubated anaerobically at 36 1 C for 24 2 h. Five CABA plates and 25 mL prereduced broth (CMM, CB, RCM, and BHIB) were inoculated, each with 100 L overnight (RCM) broth culture (presumably in log phase). All inoculated broth cultures were prereduced in an anaerobic environment for 24 2 h. All broth cultures and plates, excluding CMM, were incubated anaerobically at 36 1 C for 7–10 days. Plates were initially kept unwrapped for 24 2 h and after (day 2 and onward during incubation) wrapped with parafilm to prevent dehydration during extended incubation. CMM was incubated aerobically at 36 1 C for 7–10 days. During the incubation, growth from broth and plates was collected periodically between 2–6 days and daily between 7–10 days to inspect the culture and to estimate the approximate ratio of spores to vegetative cells using phase-contrast microscopy. Spores appeared bright and ovular while vegetative *C. difficile* cells appeared dark and rod-shaped. This step was designed to determine the optimal time for harvesting to achieve 90% spores to vegetative cell ratio. From broth, a 10 L suspension was collected after vortexing and placed on a sterile glass slide, while growth from agar-based plates was collected with a sterile inoculating loop on a glass slide containing 10 L deionized water and mixed to make a suspension prior to observing by phase-contrast microscopy.

At the end of the incubation period (when the spores appeared to reach 90% spores to vegetative cell ratio), cultures were harvested from each plate by adding 5 1 mL PBS (1 solution) + 0.1% ST80 to each plate and gently scraping the surface of the plate with a cell scraper to dislodge the growth. The dislodged culture, containing predominantly spores, vegetative cells, and cell fragments, was collected using a sterile serological pipet and pooled into a sterile 50 mL plastic centrifuge tube. Each plate was washed with an additional 1–2 mL ST80 to collect residual growth. As much residual culture as possible was recovered from the plates.

Spore suspensions from each of the BHI, RCM, and CB broths were independently collected using a serological pipette into separate sterile 50 mL plastic centrifuge tubes. The spore suspension from CMM was filtered through moistened glass wool and collected into a sterile 50 mL plastic centrifuge tube. Different harvesting techniques (e.g., using swab, sterile rod, loop, or cell scraper) with various washing reagents [e.g., cold, 2–5 C, deionized water; PBDW (pH 7.3); and PBS (1) containing 0.1% ST80] were attempted in an effort to ensure greater recovery of spores from solid media and broth. All tubes containing spore suspensions were centrifuged at 4500 *g* at 4 C for 15 min and washed three times with cold (2–5 C) PBS containing 0.1% ST80. The final pellet was resuspended in approximately 5 mL ST80. The harvested spore suspension typically consisted of predominantly spores along with live/dead vegetative cells and cell fragments. In an effort to inactivate the viable vegetative cells, spore suspensions were heated at 65 C for 10 min in a heating block. In addition, a comparison was made between heating at 65 C for 10 min and treating spore suspensions with 95% (v/v) ethanol (1 part spore suspension + 1 part 95% ethanol) for 1 h at room temperature (J. Noble-Wang, CDC, personal communication).

Spore Purification

In the event that the spore suspensions required further purification to remove vegetative cells and cell fragments, the spore suspension was subjected to an additional step, a density-gradient purification by HistoDenz. For this purpose, a protocol was adapted from other investigators (25, 26) to purify *C. difficile* spore suspensions by separating the remaining vegetative cells and cell fragments from spores using HistoDenz, with minor modifications. A 50% (w/v) solution of HistoDenz was prepared in deionized water, 1 mL spore suspensions in ST80 were gently layered on top of 5 mL 50% (w/v) HistoDenz in each of two 15 mL centrifuge tubes, and centrifuged at 4500 *g* for 10 1 min using a swinging bucket rotor. (*Note*: Use of a swinging bucket rotor is essential for proper layer removal and spore retention.) Spores were collected in the bottom pellet and surrounding cloudy layer (4th layer) directly above (3–4 mm) the pellet. The top three layers, including the upper clear layer, middle interface dense layer, and lower clear layer, were carefully removed. These three layers contained vegetative cells and cell fragments. The pellet was resuspended into remaining cloudy layer 3–4 mm above the pellet, and approximately 1 mL was transferred to a siliconized microcentrifuge tube. Tubes were centrifuged at 16 000 *g* for 5 min. The supernatant was discarded, and the pellet was resuspended with 1–1.5 mL cold (2–5 C) ST80. Tubes were mixed by vortexing to disaggregate the pellet entirely and centrifuged at 16 000 *g* for 2 min. The supernatant was discarded, and the pellet resuspended with 1–1.5 mL cold (2–5 C) ST80. Pellets were mixed by vortexing to disaggregate pellet entirely. This step was the first wash. The cap was placed tightly prior to vortexing. (*Note*: Resuspended contents can be combined after the pellet has been disaggregated in one or more tubes.) Pellets were washed two additional times with ST80 and mixed each time by vortexing. The supernatant was discarded, and each pellet resuspended with 0.5 mL of sterile deionized water per microcentrifuge tube. Each tube was mixed by vortexing to disaggregate the pellet entirely. The resuspended spores were pooled. This is the final working suspension (semi-purified). The semi-purified spore suspension is maintained at 4 C for routine use or at –20 C for long-term storage.

Recovery Media for Spore Enumeration

Several agar-based plates were evaluated for enumeration of spores in an effort to find the most efficient medium for spore recovery: (*1*) CABA, (*2*) EYA, (*3*) CCFA-HT, (*4*) TSAB, and (*5*) CA. Spore suspensions were diluted and plated on agar plates inside a BSC. More specifically, a set of

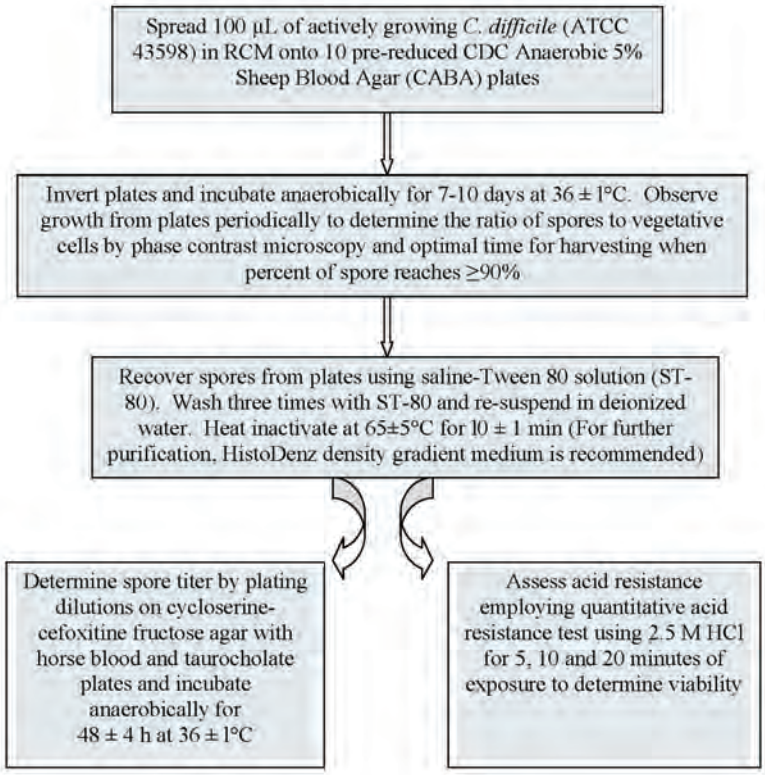

Figure 1. Flow chart of major steps of an in vitro spore production method for *C. difficile*.

dilution tubes were prepared by adding 9 mL deionized water (DW) in six sterile 15 mL centrifuge tubes. Spore suspensions were vortexed vigorously. One milliliter spore suspension was immediately withdrawn and added to the first tube containing 9 mL DW (10^{-1} dilution) and vortexed. Serial dilutions were done out to 10^{-6}. One hundred microliters was withdrawn from 10^{-5} and 10^{-6} dilutions each and spread onto duplicate plates of prereduced CABA, EYA, CCFA-HT, TSAB, and CA using a sterile disposable spreader. It was ensured that each plate was dried during spreading. Plates were inverted and incubated anaerobically at 36 1 C for 1–2 days. Colonies were counted and recorded.

Development and Testing of an Optimized Protocol for In Vitro Production of C. difficile Spores

Once the spore production procedure was finalized (Figure 1), two other analysts independently, in separate experiments, repeated the production of spores of ATCC strain 43598 more than 8 times to confirm repeatability of in vitro production of spore suspensions of high titer and quality.

Carrier Enumeration Test

ASTM E2197-02 specifies to inoculate carriers with inoculum containing a three-part soil load comprising yeast extract, BSA, and mucin. In the EPA Interim Guidance for *C. difficile*, soil load is not to be added to the spore inoculum since all products carry a precleaning step. However, for the purpose of this study, carriers were inoculated with and without a soil load.

To obtain 500 L inoculum, 160 L three-part soil load (35 L 5% yeast extract, 25 L 5% BSA, and 100 L 0.4% mucin) was added to 340 L spore suspension. Smaller volumes of inoculum with soil load were prepared as needed.

The spore suspension (with and without soil load) was vortexed to evenly distribute spores. Ten microliters of the spore suspension was transferred with a positive displacement pipet and applied at the center of a disk carrier. For consistency, the same pipet tip was used throughout the inoculation of a batch of carriers. The inoculum was allowed to dry for no more than 1 h inside a BSC. The length of the drying period varied depending on spore concentration and humidity level inside the laboratory. The dried inoculum was observed on each carrier, and any carrier in which the inoculum had run off the surface of the disk was discarded. The disk carriers were dried in a desiccator under vacuum for an additional 2 h.

Three randomly selected carriers were assayed. Each inoculated carrier was placed in a vial with the inoculum side facing up; 50 L sterile PBS was placed to cover the entire inoculum for a 10 min contact time, and then 9.95 mL sterile PBS was added. Vials were vortexed vigorously three times for 30 s each. Following vortexing, spore suspensions were diluted by transferring a 1 mL aliquot to tubes containing 9 mL PBDW. Spore suspensions were diluted to 10^{-5}, and 0.1 mL from each of the dilutions (10^{-3}–10^{-5}) was plated on

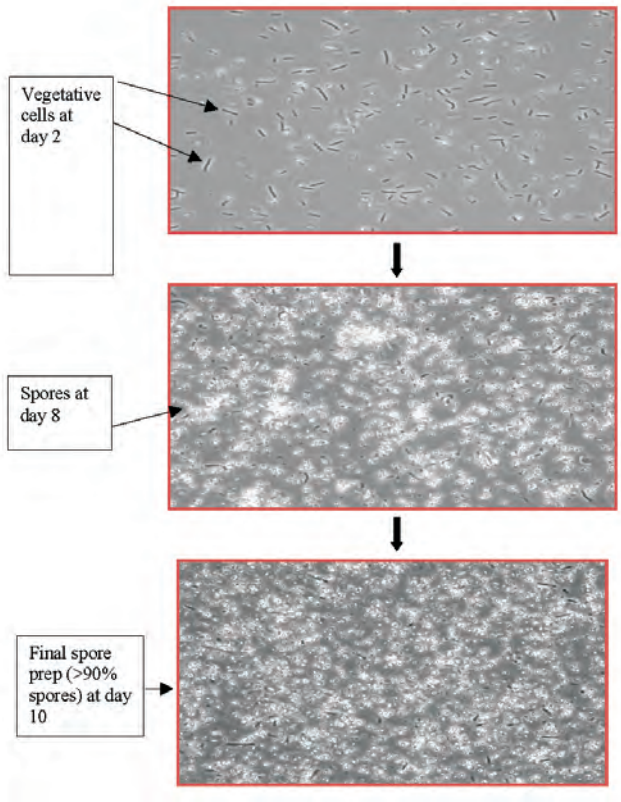

Figure 2. Monitoring percent sporulation of *C. difficile* (ATCC 43598) during incubation at 36 ± 1 C under phase-contrast microscopy (magnification 1000).

duplicate plates of CCFA-HT and spread until dried. Plates were inverted and incubated for 24 to 48 h at 36 1 C under anaerobic conditions. Plate counts were recorded to determine spore/carrier. The mean carrier counts for each carrier were required to be $>10^6$ spores/carrier.

Quantitative Acid Resistance Test

Since ASTM E2197-02 does not specify an HCl resistance test, the methodology for an HCl resistance test was adapted, in part, from AOAC Method **2008.05** for the spores of *B. subtilis*, with modifications, to provide a standard for spore resistance. Five brushed stainless steel carriers were inoculated as per ASTM E-2197-02 as stated in the *Carrier Enumeration Test* section above. During each replication, inoculated carriers were removed from vacuum desiccation and placed into plastic Nalgene vials using flamed forceps, ensuring that forceps did not contact inoculum on the carrier. Fifty microliters 2.5 M HCl was added to four test carriers sequentially at 1 min intervals, ensuring the entire inoculum was covered with acid. For a viability control, 50 L sterile PBS was added to a control carrier, ensuring PBS covered the entire inoculum. After 2, 5, 10, and 20 min of contact time, 9.95 mL neutralizer (ST80 + 1.25% NaOH) was added to each acid-treated vial; the vial was closed and rotated. Neutralizer was also added to the control vial 20 min after inoculation of the carrier. After neutralizer was added, each vial was vigorously vortexed three times for 30 s each, allowing the vial to rest in between each vortex. HCl-treated spores were diluted by transferring 1 mL aliquots to tubes containing 9 mL PBDW. Spore suspensions were diluted out to 10^{-4}, and 0.1 mL was spread plated from dilutions 10^{-2} to 10^{-4} in duplicate plates of CCFA-HT. Plates were dried and incubated inverted for 24–48 h at 36 1 C under anaerobic conditions. Colonies were enumerated (viable spores) per carrier, and $\log_{10}$ reduction was determined upon HCl treatment as compared to control carrier.

Results

A wide range of sporulation rates were observed (data not shown) for the five strains evaluated (ATCC 700792, 43598, 43599, 9689, and 700057) in this study. Among all of the strains tested, ATCC strain 700792 exhibited the highest sporulation capability (approximately 7 $\log_{10}$/mL). It was followed by ATCC strain 43598, which had approximately 6 $\log_{10}$/mL. To minimize variation among strains of *C. difficile*, strain 43598 was advanced for further evaluation as this strain was used more commonly by industry to generate data for efficacy testing of disinfectants with a sporicidal claim against *C. difficile* spores. The procedure used for generation of vegetative and frozen stock was successful. The titer was >8 $\log_{10}$/mL after cryopreservation at –70 C.

CABA was selected as the most efficient medium for spore production as it consistently produced a greater concentration of spores when compared to CB, BHIB, or RCM and TSAB. Incubation for 7–10 days at 36 1 C in an anaerobic chamber while maintaining an adequate humidity level of 70% was optimal. Wrapping plates with parafilm on day 2 of incubation ensured that plates were protected from dehydration during extended incubation. Adequate sporulation did not occur prior to day 7 of incubation on agar-based medium. Monitoring the spore to vegetative cell ratio by phase-contrast microscopy

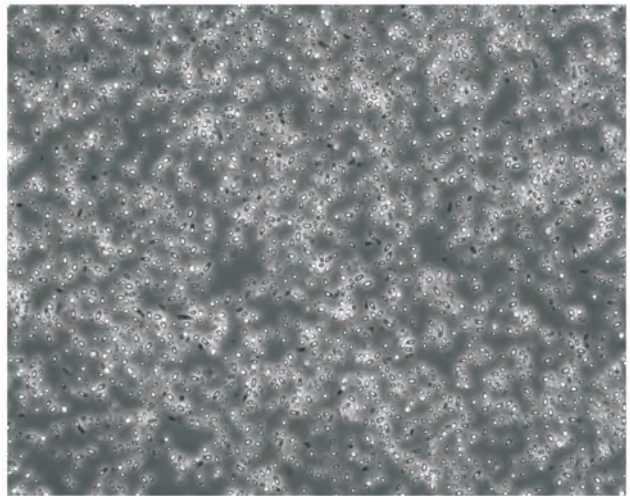

Figure 3. Purified *C. difficile* spores (ATCC 43598) using a density gradient medium (HistoDenz) exhibiting 99% purity (free from vegetative cells and cell fragments).

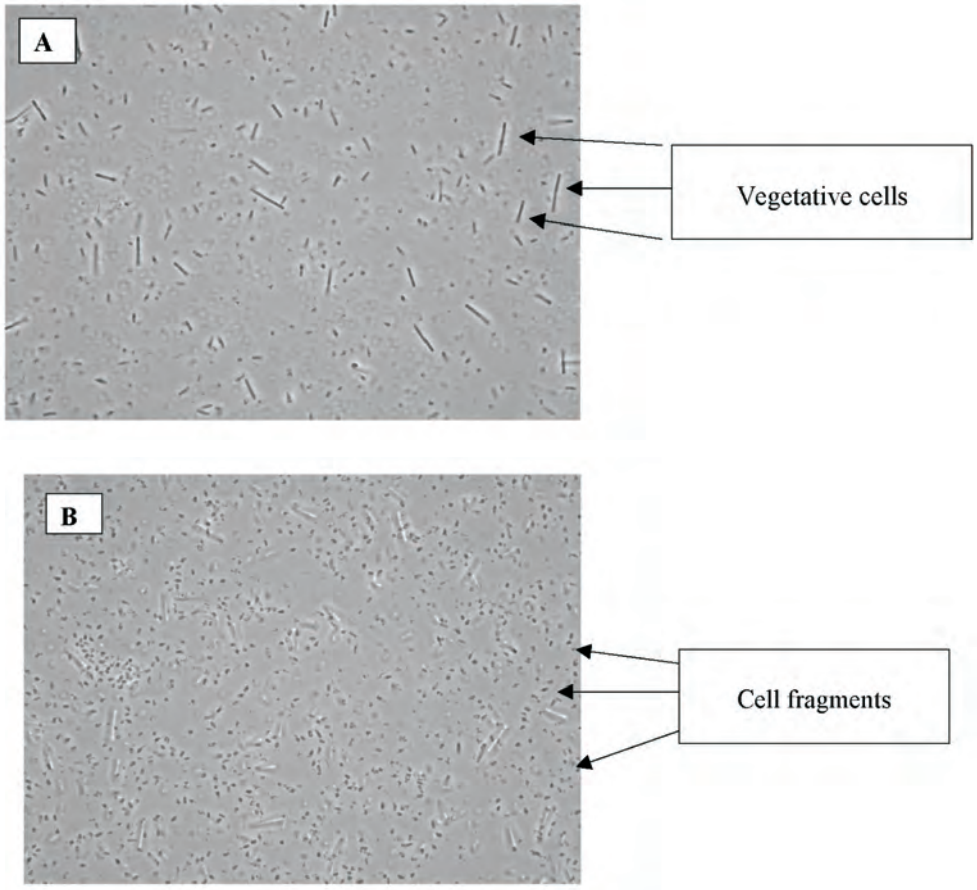

Figure 4. Separation of (A) vegetative cells and (B) cell fragments from *C. difficile* spores (ATCC 43598) upon purification by HistoDenz, a density gradient medium.

during incubation for 7–10 days was critical to determine the optimal time for harvesting to ensure that 90% vegetative cells have been converted to spores (Figure 2). Since 90% spores consistently occurred within 7–10 days of incubation, the impact of incubation beyond 10 days was not investigated in this experiment.

Harvesting with a cell scraper dislodged spores more efficiently than swabs, sterile rods, or loops. Initial attempts to wash with cold water (2–5 C) or only PBS exhibited varying levels of spore clumping. However, washing with ST80 yielded maximum recovery of dispersed spores without any spore clumps. It was noticed that spores generated from frozen vegetative stock (predominantly vegetative cells) produced less clumping upon washing when compared to spores generated from frozen spore stock (predominantly spores).

Although no difference was noticed in inactivating vegetative cells with heat and alcohol treatments, heat at 70 C for 10 min was chosen for routine use as this method is easy and effective.

A spore suspension (approximately 90%) was used throughout the course of the study; however, the purification step using HistoDenz-based density gradient medium, used late in the study, yielded 99% pure spore suspension as evidenced by the phase contrast microscopy (Figure 3). The use of HistoDenz significantly reduced the amount of vegetative cells and cell fragments (Figure 4) in the final spore suspension.

CCFA–HT containing 0.1% sodium taurocholate, a known spore germinant, was selected as the most efficient medium for spore enumeration. Recovery of spores on CCFA-HT was consistently 1.5–2.5 logs higher as compared to CABA. The number of enumerated spores obtained from TSAB, EYA, and CA was lower than that of CABA or CCFA-HT.

Upon the development of the spore production protocol, when two other analysts independently, in separate experiments, repeated the in vitro production of spores of *C. difficile* (ATCC 43598) more than eight times, each analyst repeatedly and consistently produced spore suspensions that met quantitative (>8 $\log_{10}$/mL) and qualitative (90% spores) criteria.

Using a preparation of spore suspension, all four replications for carrier enumeration using brushed stainless steel disks as per ASTM E2197-02 (with and without soil load) resulted in recovery of >6 $\log_{10}$ spores/carrier (Figure 5).

The *C. difficile* spores met or exceeded the acid resistance criterion as set forth in AOAC Method **2008.05** for the spores of *B. subtilis*. No significant $\log_{10}$ reduction was seen at 2, 5, and 10 min exposure to 2.5 M HCl. A $\log_{10}$ reduction of approximately 1.2 was observed at 20 min of exposure. Table 1 exhibits averaged data of a typical acid resistance test using 2.5 M HCl on *C. difficile* spores.

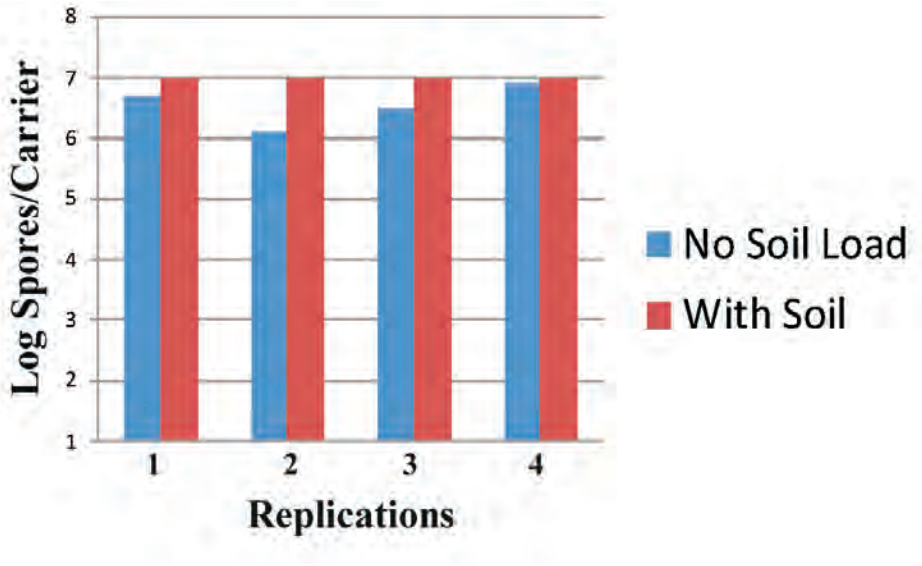

Figure 5. Carrier enumeration with and without soil load using a spore suspension of *C. difficile* (ATCC 43598).

Discussion

Selection of ATCC strain 43598, anaerobic incubation of 7–10 days at 36 1 C, CABA as the sporulation medium, and CCFA-HT as the recovery medium, coupled with routine monitoring of growth during sporulation to ensure appropriate time for harvesting, along with post-harvest washing steps with ST80, yielded an optimized protocol (Figure 1) for in vitro production of *C. difficile* spores of high titer and quality.

Sporulation capability varied between strains tested in our study. Although the titer of ATCC strain 43598 (positive for Toxin B only) was not as high as ATCC strain 700792 (positive for both Toxins A and B), it was decided to proceed with ATCC 43598 as this strain was used more commonly by industry in an effort to keep the variability of strain to a minimum.

CABA is a highly nutritious medium due to its content of peptones, yeast extract, hemin, vitamin K_1, and sheep blood. It was originally formulated by Dowell et al. (27) of the CDC and recommended to EPA by J. Noble-Wang, CDC (personal communication). Being nutritious and prereduced for obligate anaerobes, CABA demonstrated good growth as a bacterial lawn, and was consequently confirmed to be an efficient sporulation medium for ATCC strains 700792 and 43598. Both agar plates and broth-based media have been used in the past for *C. difficile* spore production (25, 26, 28–31). The spores of *B. subtilis*, for example, are also generated using the agar-based plate method in accordance with the AOAC Quantitative Three Step Method. The spore harvesting from agar appears to be more efficient and can be less of a biological hazard compared to the use of large volumes of broth. It has been suggested that many laboratory strains could not be sporulated in liquid medium (26). The lower production of spores observed in this study, when grown in broth, could be connected with the smaller volume (i.e., 25 mL) used for growth. A higher spore titer may have been generated from a broth culture that used a larger volume for growing the culture, followed by centrifugation and concentration of the culture.

Since some uncertainties, e.g., humidity level of the anaerobic chamber, final gas mixture, availability of nutrients and moisture during initial growth, initiation and completion of sporulation process, etc., may affect the optimal production of spores, monitoring of the ratio of spores to vegetative cells by phase-contrast microscopy was a critical step, as suggested earlier (26), for ensuring that 90% vegetative cells were converted to spores (Figure 2). In addition, wrapping the plates with parafilm reduced the dehydration of the media during the extended incubation period.

The inclusion of a known nonionic detergent, i.e., Tween 80 (0.1%) in the washing reagent (ST80) was found to resolve the issue of spore clumps when spores were generated from frozen vegetative stock. However, the washing reagent (ST80) was not effective in significant removal of spore clumping when spores were generated from frozen spore stock. When comparing various agar-based media for enumeration, recovery of spores on CCFA-HT containing 0.1% sodium taurocholate (a known spore germinant) was the highest, an observation reported by other investigators (4, 25, 26). The inclusion of 0.1% sodium taurocholate in an agar-based plate medium enhanced recovery of *C. difficile* spores, an observation first reported by Wilson et al. in 1982 (28) and subsequently by other investigators over the last three decades (25, 31). While both CABA for sporulation and CCFA-HT for enumeration are readily available from vendors in prereduced form, the CCFA-HT plates are a bit more expensive. A recent report claimed that an in-house made *C. difficile* brucella agar (CDBA), containing only 0.05% taurocholate, is as effective as CCFA-HT, but is less expensive (32). In response to recommendations by the AOAC INTERNATIONAL Expert Review Panel (ERP) on *C. difficile*, a precollaborative study by two federal laboratories and one industry partner has been completed to compare five spore recovery media side-by-side

Table 1. Mean log_{10} density/carrier and log_{10} reduction at 2, 5, 10, and 20 min exposure period with 2.5 M HCl to determine the acid resistance of *C. difficile* spores

	Exposure time, min	Mean log_{10} density/carrier	Mean log_{10} reduction
Treated with 2.5 M HCl	2	6.0	0.6
	5	6.5	0.1
	10	6.3	0.3
	20	5.4	1.2
Treated with water (control)	20	6.6	0

for enumeration of spores of *C. difficile*. The media are CCFA-HT; CDBA containing 0.05% taurocholate; brain heart infusion agar containing yeast extract, horse blood, and 0.1% taurocholate (BHIY-HT); cycloserine cefoxitin fructose agar with lysozyme (CCFA-L; Anaerobe Systems); and brain heart infusion agar containing yeast extract, lysozyme, and 0.1% taurocholate (BHIY-LT; S. Sattar, University of Ottawa, ON, Canada, personal communication). The data are currently under review by the ERP while this manuscript is in press.

In accordance with the AOAC Method **2008.05**, the spores of *B. subtilis* are considered acid resistant if the log_{10} reduction remains between 0–3 at 2 min exposure, and between 2–6 following 5 min exposure. On this basis, the *C. difficile* spores were qualified as acceptable resistant test spores as only approximately 0.3 (an average of 2, 5, and 10 min) log_{10} reduction was exhibited up to 10 min. A log_{10} reduction of 1.2, when spores were treated with 2.5 M HCl for 20 min, suggests that spores of *C. difficile* generated under this protocol are extremely resistant to 2.5 M HCl. When one preparation of spore suspension kept at 4 C in deionized water was retested for acid resistance after 90 days, no change with regard to resistance of spores was observed. In a related effort to establish a way to measure the resistance to sodium hypocholorite, when semipurified *C. difficile* spores were treated with sodium hypochlorite at a concentration of 1000 ppm available chlorine, spores showed approximately 2–3 log_{10} reduction in 10 min (33).

As recovery of >6 log spores/carrier (Figure 5) was consistently achieved, the observation of slightly higher carrier counts with soil load in all four replications could not be explained, especially when the addition of soil load dilutes the spore suspension by approximately 30%. Whether the addition of three protein-based soil load provides additional nutritional enhancement to *C. difficile* spores or the spores with soil load are recovered from the carriers more efficiently during vortexing remains to be confirmed.

The data on the development of methodology for in vitro production of *C. difficile* spores of high titer and quality for use in the efficacy evaluation of disinfectants have recently been presented at the 110th General Meeting of the American Society for Microbiology (34).

The spores used in this study to inoculate carriers in the carrier enumeration experiment were not purified by HistoDenz-based density gradient medium. However, due to the effectiveness of HistoDenz to remove vegetative cells and cell fragments from spore suspensions, thus increasing spore purity and reducing potential variables for efficacy evaluation (30), the authors recommend the use of HistoDenz in the final proposed method. The resulting purified spore suspension (99%), as observed in this study, was also demonstrated by Sorg and Dineen (26) when HistoDenz density gradient medium was used. The spore purification step is simple, user-friendly, and efficient. The low osmolality, high density, and low viscosity compared to sucrose make this nonionic density gradient medium a suitable candidate to purify *C. difficile* spores.

Conclusions

Sporulation in *C. difficile* is not as rapid or efficient as in other species (26), and it is generally difficult to produce *C. difficile* spores of high titer in the laboratory (29). EPA guidance specifies that products with a proposed claim to inactivate *C. difficile* spores must meet strict performance requirements, e.g., 6 log_{10} reduction in spores with control carrier counts >6 log_{10} spore/carrier. Since a standard method is not available for producing *C. difficile* spores of high titer and quality, a method has successfully been developed and is being reported here for in vitro production of *C. difficile* spores for use in the efficacy evaluation of disinfectants. In summary, the method consists of several components: (*1*) preparation and maintenance of VFS culture; (*2*) isolation of pure colonies by streaking on CABA and inoculation of 10 mL prereduced RCM with an isolated colony for growth in log phase; (*3*) inoculation onto CABA with growth in log phase in RCM to initiate sporulation; (*4*) determination of spore-to-vegetative cell ratio by phase-contrast microscopy during sporulation to find out the optimal harvest time; (*5*) preparation of the working spore suspension and washing steps to partially purify and remove spore clumps; (*6*) further purification of spores using a density gradient medium to separate spores from the remaining vegetative cells and cell fragments to reduce potential variables; (*7*) microscopic examination of the spore preparation to evaluate purity and quality of spores; (*8*) determination of spore titer using CCFA-HT; and (*9*) assessment of spore quality by a quantitative acid resistance test.

The method consistently achieved the required criteria of concentration and quality of spores of *C. difficile* (>8 log_{10} spores/mL and spore quality of 90% spores) that met or exceeded the HCl resistance criterion. The spores generated under the protocol yielded carrier counts >6 logs/carrier using the ASTM E2197-02. The method is simple and repeatable and would be useful and feasible in meeting the requirements of EPA guidance for the quantitative efficacy assessment of products having label claims for *C. difficile* spores. The

proposed method is submitted here for consideration as a stand-alone AOAC *Official Method* for in vitro production of *C. difficile* spores for use in the efficacy evaluation of disinfectants upon successful completion of a collaborative study.

Acknowledgments

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Appendix: Proposed Method for Submission to the *Official Methods of Analysis of AOAC INTERNATIONAL*: In Vitro Production of *Clostridium difficile* Spores for Use in the Efficacy Evaluation of Disinfectants

Caution: The test organism (*Clostridium difficile*, ATCC 43598) must be initiated and incubated under strict anaerobic conditions and in accordance with biosafety practices stipulated by each institution. Processing of spores may be conducted under aerobic environment. However, all incubation must occur anaerobically. Use the equipment and facilities indicated for the test organism. For recommendations on safe handling of microorganisms, refer to the CDC/National Institutes of Health (NIH) Biosafety in Microbiological and Biomedical Laboratories (BMBL) manual (35). Pathogenic *Clostridium* species are classified as Biosafety Level II organisms by NIH. In addition, hydrochloric acid (2.5 M HCl) is a highly corrosive liquid and considered hazardous. Personal protective equipment (PPE) or devices are recommended during the handling of these items for purpose of activation, dilution, or efficacy testing. PPE and a chemical fume hood should be used when performing tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for the specific product/active ingredient to determine best course of action. References to water mean deionized sterile water (reagent grade), except where otherwise specified (36). Exact adherence to the method, good laboratory practices, and QC are required for proficiency and validity of the results.

A. Media and Reagents

(**a**) *Culture media.*—(*1*) *RCM.*—For use in rehydrating lyophilized/frozen vegetative culture of test organism. (yeast extract, 'Lab-Lemco' powder, peptone, glucose, soluble starch, sodium chloride, sodium acetate, cysteine hydrochloride, agar, manufactured by Oxoid). Suspend 38 g in 1 L deionized water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121 C for 15 mins.

(*2*) *RCM plus 15% glycerol as cryoprotectant.*—For use as maintenance and cryopreservation medium for vegetative frozen stock cultures. Suspend 3.8 g RCM (powder) in 85 mL deionized water. Bring to boil to dissolve completely. Add 15 mL glycerol (Difco). Sterilize by autoclaving at 121 C for 15 mins.

(*3*) *CABA.*—For generation of *C. difficile* spores. Commercially available from Anaerobe Systems as prereduced (i.e., they are manufactured, sterilized in an oxygen-free environment, and packaged individually in air-tight sealed pouches or bags).

(*4*) *CCFA-HT or BHIY-HT (Cat. No. AS-6463).*—For enumeration of *C. difficile* spores. Commercially available from Anaerobe Systems as prereduced.

(**b**) *PBS.*—Prepare 10 solution PBS by dissolving 492 g PBS powder 1 concentrate (Fisher Scientific, Cat. No. BP661-50) in 5 L deionized water. Dilute desired amount to 1 and distribute into bottles and autoclave for 20 min at 121 C.

(**c**) *PBDW.*—Add 3.4 g potassium phosphate monobasic to 100 mL deionized water to prepare 0.25 M phosphate buffered stock solution (PBSS; pH 7.2), and then add 1.25 mL of 0.25 M PBSS to 1 L deionized water to prepare PBDW.

(**d**) *PBS containing 0.1% ST80.*—To use as washing reagent. Add 2.0 mL polysorbate (Tween 80, or equivalent) to 2.0 L PBS (1) solution to a 2 L volumetric flask. Bring solution to volume with PBS. Distribute into bottles and autoclave for 20 min at 121 C.

(**e**) *Water.*—Use sterile deionized water.

(**f**) *Hydrochloric acid.*—Prepare 2.5 M HCl.

(**g**) *HistoDenz.*—Prepare a 50% (w/v) solution of HistoDenz (Sigma-Aldrich) in deionized water.

(**h**) *Test organism.*—*C. difficile* (ATCC No. 43598), a toxigenic strain (*tcdA*$^-$, *tcdB*$^+$) obtained directly from the ATCC. The strain produces Toxin B only (presence of *tcdB* gene by PCR). The organism is an obligate anaerobe and a Gram-positive spore forming bacterium, and produces flat, gray, and irregular colonies within 48 h on CABA.

B. Apparatus

(**a**) *Certified BSC (Type II, Class II).*—Recommended to maintain an aseptic work environment.

(**b**) *Sterile centrifuge tubes.*—Polypropylene, 15 and 50 mL (Fisher Scientific) graduated plastic centrifuge tubes with conical bottoms.

(**c**) *Centrifuge.*—To allow sedimentation of spores for washing and or concentration (Eppendorf 5804 R).

(**d**) *Micropipet.*—Calibrated.

(**e**) *Positive displacement pipet.*—To inoculate steel carriers with spores (Gilson Inc.).

(**f**) *Vacuum desiccators.*—For carrier drying and storage (Thermo Scientific).

(**g**) *Certified timer.*—Any certified timer that can display time in seconds (VWR International).

(**h**) *Test tubes.*—Reusable or disposable 20 150 mm for cultures/subcultures (Fisher Scientific).

(**i**) *Inoculating loop.*—VWR International.

(**j**) *Anaerobic chamber.*—Supported by a gas mixture consisting of 10% hydrogen, 5% CO_2, and 85% N_2 (e.g., COY). Alternatively, an activated anaerobic jar with gas pack can be used.

(**k**) *Anaerobic incubator.*—Anaerobic incubator at 36 1 C placed inside an anaerobic chamber (e.g., COY) to support the growth of the organism when plated onto solid media or liquid broth. Plates must be incubated in an anaerobic incubator (e.g., COY or activated anaerobic jar) at 36 1 C for growth to occur.

(**l**) *Microscope with 10 eyepiece and 40 and 100 oil objectives with phase contrast option.*—Nikon Americas Inc.

(**m**) *Vortex mixer.*—Genie 2 Mixer/G560, Scientific Industries, Inc. (VWR International).

(**n**) *Serological pipets.*—Sterile, single-use pipets of 10.0, 5.0, 1.0 mL capacity (VWR International).

(**o**) *Cell scraper.*—Use to gently scrape plates to remove spores for harvesting (Techno Plastic Products).

(**p**) *Plate spreader.*—Use to spread inoculums on plates to create a uniform lawn on top of plate (VWR International).

(**q**) *Microcentrifuge tubes.*—Sterile 1.5 mL low retention (siliconized) microcentrifuge tubes (Fisher Scientific).

(**r**) *Cryovials.*—Sterile 2.0 mL cryovials (Corning Inc.).

C. Operating Techniques

(**a**) *Preparation and maintenance of frozen stock cultures.*—Cultures from ATCC are available in two forms: lyophilized or frozen. *Lyophilized culture from ATCC*: Reconstitute the contents of the vial with 1–1.5 mL RCM in an anaerobic environment. After rehydration, aseptically transfer entire contents to a tube of RCM (3–5 mL) and mix gently by vortex. The rehydrated suspension is used for inoculation of CABA plates to generate vegetative stock cultures. *Frozen culture from ATCC*: After thawing the frozen culture at room temperature, transfer the entire contents to a tube of sterile RCM, total volume 4–5 mL. Mix gently by vortex. The suspension is used for inoculation of CABA plates to develop vegetative stock cultures, as follows: Inoculate five CABA plates with the reconstituted/diluted ATCC culture (100 L/plate). Incubate plates inverted anaerobically at 36 1 C for 48 4 h. After incubation, add 2 mL cryoprotectant to each CABA plate. Using a sterile cell scraper, scrape culture from the surface of each plate, aspirate with a pipet and transfer to a 15 mL conical tube. Repeat this process for the remaining plates. Mix the pooled culture thoroughly and aliquot into cryovials (1–1.5 mL/cryovial). Store the cryovial tubes at –70 C with appropriate labeling. These tubes are frozen stock culture (FSC). It is recommended that after a stock culture is initiated and frozen for 7 1 day, an evaluation of frozen titer should be conducted as follows: thaw an FSC tube at room temperature inside an anaerobic chamber. After vortexing of thawed culture, remove 1 mL of culture and serially dilute 10-fold (e.g., out to 10^{-6}) in PBS. Plate appropriate dilutions on CCFA-HT or BHIY-HT in duplicate. Incubate plates anaerobically at 36 1 C for 48 4 h, record plate counts, and determine the titer/mL of predominantly vegetative cells. The titer should be >8 $\log_{10}$/mL to ensure that FSC can withstand long-term preservation at –70 C.

(**b**) *Preparation of a test spore suspension from FSC.*—As part of quality control step, streak three CABA plates for isolation from an FSC tube. Incubate two plates anaerobically and the third plate aerobically at 36 1 C for 48 4 h. Expect no growth from plate incubated aerobically. Observe purity and colony characteristics typical of the test organism. Inoculate 10 mL of prereduced RCM with a colony and incubate anaerobically at 36 1 C for 24 2 h. Inoculate a minimum of 10 CABA plates, each with 100 L of the RCM broth culture (presumably in log phase). Spread the inoculum evenly using a disposable sterile spreader to create a lawn. Place pan/container filled with deionized water inside the anaerobic incubator ensuring adequate moisture level (70%). In addition, cover agar plates with parafilm to prevent dehydration during the extended incubation. Invert plates and incubate anaerobically for 7–10 days at 36 ± l°C. Within 24 h of incubation, plates should be observed for confluent bacterial lawn. During the incubation period, samples of growth of *C. difficile* from agar plates should be wet mounted periodically (between 2–6 days) and daily (between 7–10 days) on a glass slide and observed under phase-contrast microscopy to inspect spores and to estimate the approximate ratio of spores to vegetative cells to determine the optimal time for harvesting. Spores appear bright and ovular while vegetative *C. difficile* cells appear dark and rod-shaped. When the percent of spore reaches 90%, harvest growth from each plate by adding approximately 5 mL PBS (1 solution) + 0.1% ST80 to each plate, and gently scraping the surface of the plate with a cell scraper to dislodge the growth. Use a sterile serological pipet to collect the culture, and pool in a sterile flask. Recover as much residual culture as possible from the plate. Transfer pooled spore suspension into sterile 50 mL plastic conical tubes. Cap the tubes tightly before placing them in the centrifuge.

Perform the following steps to wash excess media and debris by centrifugation: centrifuge tubes at 4500 *g* for 15 min using a swinging bucket rotor. Discard the supernatant and resuspend the pellet with 20–30 mL ST80. Mix by vortexing to disaggregate pellet entirely. This step is the first wash. Cap the tubes tightly prior to vortexing. Repeat the above washing step two more times. (*Note*: Resuspended contents can be combined in one tube if using more than one centrifuge tube after pellet has been disaggregated.) Mix by vortexing. After the third wash, discard the supernatant and resuspend the pellet with approximately 5 mL of sterile deionized water. Mix by vortexing to disaggregate pellet entirely. This is the working spore suspension. Heat-treat the spore stock suspension for l0 1 min at 65 5 C. Ensure that the contents inside the tube have reached 65 5 C prior to starting the timer by placing a tube (temperature control) with deionized water alongside the culture tubes; place a thermometer inside the control tube to monitor temperature. Once the temperature of the control tube has reached 65 5 C, the timer can be started. Allow it to return to room temperature.

Remove an appropriate sample (e.g., 10 L) of well-vortexed spores and evaluate titer (follow serial dilution scheme) to determine spores/mL after heat treatment. CCFA-HT or BHIY-HT plates are used for enumeration. Prepare a wet mount and observe under phase-contrast microscope for quality of spore suspension. The spore suspension should be of high quality, 90% of spores to vegetative cells. Aliquot of spore suspensions can be made for long-term preservation at –20 C.

To evaluate titer of the spore suspension, perform a serial dilution of the spore suspension in PBDW out to 10^{-6}. Spread plate 0.1 mL of the appropriate dilutions on CCFA-HT or BHIY-HT in duplicate. Invert plates and incubate anaerobically at 36 1 C for 48 4 h. Record plate counts. The titer should be $>10^8$ spores/mL in order to achieve the target carrier count of >10 spores/carrier.

(**c**) *Spore purification.*—Make a 50% (w/v) solution of HistoDenz (Sigma-Aldrich) in sterile deionized water. Layer 1 mL of spore suspension in ST80 on top of 5 mL 50% (w/v) HistoDenz in each of two 15 mL plastic centrifuge tubes. Centrifuge tubes at 4500 *g* for 10 min using a swinging bucket rotor. (*Note*: Use of a swinging bucket rotor is essential for proper layer removal and spore retention.) Spores are collected in the bottom pellet and surrounding cloudy layer (4th layer) directly above (3–4 mm) the pellet. Carefully remove top three layers including upper clear layer, middle interface dense layer, and lower clear layer. Resuspend pellet into remaining cloudy layer above 3–4 mm above the pellet and transfer approximately 1 mL to a siliconized microcentrifuge tube. Centrifuge tubes at 16 000 *g* for 5 min. Discard the supernatant and resuspend the pellet with 1–1.5 mL cold (2–5 C) ST80. Mix by vortexing to disaggregate pellet entirely. Centrifuge tubes at 16 000 *g* for 2 min. Discard the supernatant and resuspend the pellet with 1–1.5 mL cold (2–5 C) ST80. Mix by vortexing to disaggregate pellet entirely. This step is the first wash. (*Note*: Resuspended contents can be combined in one or more tubes after pellet has been disaggregated.) Wash two additional times with ST80. Discard the supernatant and resuspend the pellet with 0.5 mL sterile deionized water per microcentrifuge tube. Mix by vortexing to disaggregate pellet entirely. Pool the resuspended spore suspensions. This is the final working suspension (semipurified). Inspect the purity of the spores by phase-contrast microscopy. Spores should be 97 2% pure. Dilute spore suspensions in deionized water out to 10^{-6} and spread plate 0.1 mL from appropriate dilutions (10^{-5} and 10^{-6}) in duplicate on CCFA-HT or BHIY-HT. Invert plates and incubate for 48 4 h at 36 1 C under anaerobic conditions. Enumerate colonies (viable spores) to determine titer of semipurified spores of *C. difficile.*

(**d**) *HCl resistance.*—Place 990 L 2.5 M HCl into each of three 1.5 mL low-retention (siliconized) microcentrifuge tubes, and 990 L deionized water (control) into a 1.5 mL low-retention microcentrifuge tube. Using a positive displacement pipet, add 10 L spore suspension (with a spore titer of >8 $\log_{10}$/mL) to each microcentrifuge tube to achieve $>10^6$ spores/mL. Vortex each tube. Explore the acid-treated spores for 5, 10, and 20 min exposure periods and the control tube for 20 min. Following the exposure period, dilute/neutralize HCl-treated spores by transferring 0.1 mL to tubes containing 900 L PBDW. Dilute spore suspensions out to 10^{-4} and spread plate 0.1 mL from appropriate dilutions (10^{-2} to 10^{-4}) in duplicate on CCFA-HT or BHIY-HT. Dry plates and incubate inverted for 24–48 h at 36 1 C under anaerobic conditions. Enumerate colonies (viable spores) per carrier and determine $\log_{10}$ reduction upon HCl treatment as compared to control. Spores should resist HCl for 10 min to be qualified as acceptable resistant test spores. The spores are considered acid resistant if $\log_{10}$ reduction remains between 0 and 2 following 10 min exposure when compared with control counts.