MICROBIOLOGICAL METHODS

Comparative Evaluation of Two Quantitative Test Methods for Determining the Efficacy of Liquid Sporicides and Sterilants on a Hard Surface: A Precollaborative Study

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Two quantitative carrier-based test methods for determining the efficacy of liquid sporicides and sterilants on a hard surface, the Standard Quantitative Carrier Test Method—ASTM E 2111-00 and an adaptation of a quantitative micro-method as reported by Sagripanti and Bonifacino, were compared in this study. The methods were selected based on their desirable characteristics (e.g., well-developed protocol, previous use with spores, fully quantitative, and use of readily available equipment) for testing liquid sporicides and sterilants on a hard surface. In this paper, the Sagripanti-Bonifacino procedure is referred to as the Three Step Method (TSM). AOAC Official Method 966.04 was included in this study as a reference method. Three laboratories participated in the evaluation. Three chemical treatments were tested: (*1*) 3000 ppm sodium hypochlorite with pH adjusted to 7.0, (*2*) a hydrogen peroxide/peroxyacetic acid product, and (*3*) 3000 ppm sodium hypochlorite with pH unadjusted (pH of approximately 10.0). A fourth treatment, 6000 ppm sodium hypochlorite solution with pH adjusted to 7.0, was included only for Method 966.04 as a positive control (high level of efficacy). The contact time was 10 min for all chemical treatments except the 6000 ppm sodium hypochlorite treatment which was tested at 30 min. Each chemical treatment was tested 3 times using each of the methods. Only 2 of the laboratories performed the AOAC method. Method performance was assessed by the within-laboratory variance, between-laboratory variance, and total variance associated with the log reduction (LR) estimates generated by each quantitative method. The quantitative methods performed similarly, and the LR values generated by each method were not statistically different for the 3 treatments evaluated. Based on feedback from the participating laboratories, compared to the TSM, ASTM E 2111-00 was more resource demanding and required more set-up time. The logistical and resource concerns identified for ASTM E 2111-00 were largely associated with the filtration process and counting bacterial colonies on filters. Thus, the TSM was determined to be the most suitable method.

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In response to the intentional release of spores of *Bacillus anthracis* in 2001, and the associated need for determining the performance of sporicidal and sterilant chemicals for use in the decontamination of buildings, the U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) initiated a research program to evaluate and improve laboratory efficacy test methods for sporicides and sterilants. Currently, no sporicidal and sterilant products are registered by the EPA for control of *B. anthracis*; however, for emergencies, the EPA may grant an exemption from a registration as specified under Section 18 of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). In response to the attacks with *B. anthracis*, OPP was inundated with requests for emergency exemptions that would permit the use of specific antimicrobial chemicals for decontamination of buildings and their contents. Applicants used numerous test methodologies and surrogates of *B. anthracis* in generating the efficacy data necessary to support the emergency requests.

For registration of sporicides and sterilants, the EPA currently accepts AOAC Method **966.04** (1), "Sporicidal Activity of Disinfectants," for generating efficacy data for

sporicides and sterilants. Method **966.04** provides a qualitative measure of product efficacy against spores of *B. subtilis* and *Clostridium sporogenes* dried on 2 types of carriers, porcelain penicylinders, and silk suture loops. Sixty carrier tests on 3 lots of product are required for an EPA registration—all carriers must show no growth to support a sporicidal claim. The quality and consistency of efficacy data generated with Method **966.04** has been considered problematic for many years. Potential sources of variability have been previously published, and several authors have proposed corrective solutions, including designing quantitative approaches to efficacy testing (2–6). The most significant concerns are associated with the qualitative nature of the method, the use of raw garden soil extract as a source of minerals for spore production, the carrier materials (unglazed porcelain and silk suture loops), the lack of a standardized procedure for enumeration of spores, spore wash-off, and the long incubation time (21 days). Due to the current limitations and deficiencies associated with Method **966.04**, a key priority related to EPA's Homeland Security efforts has been the development of quantitative methodology to replace or augment Method **966.04,** with emphasis on the regulatory aspects of verifying the performance of chemicals used in building decontamination.

Through funding provided by EPA's Office of Research and Development (National Homeland Security Research Center, Safe Buildings Program) and technical consultation with a federal Interagency Expert Panel on *B. anthracis*, a multitiered research plan was developed. In Tier-1, research was initiated on 2 fronts: comparative evaluation of quantitative test methods for liquid sporicides and sterilants, and collaborative testing of selected modifications to improve Method **966.04**. Modifying Method **966.04** is considered a short-term/interim approach. Replacing or augmenting Method **966.04** with a quantitative test is a long-term goal. In Tier-2, studies will include the evaluation of surrogates of *B. anthracis* using a quantitative method. A multilaboratory validation study of a quantitative method and surrogate is a Tier-3 objective.

In this paper, we present the results of a multilaboratory study designed to compare 2 quantitative test methods, the Standard Quantitative Carrier Test Method—ASTM E 2111-00 (7) and an adaptation of a quantitative micro-method as reported by Sagripanti and Bonifacino (8), a Tier-1 activity. The scope of this study was limited to measuring the efficacy of liquid sporicides and sterilants applied to a hard surface using *B. subtilis* as the test microbe. The main objective was to evaluate method performance by generating and comparing efficacy data and assessing the degree and source(s) of variability associated with the data. The generation of baseline efficacy data using Method **966.04** is useful to the development of performance standards for a new quantitative method, i.e., the development of pass/fail criteria, and was included in this study. Figure 1 illustrates the basic method attributes (e.g., carrier size and material and amount of sporicide/carrier). The quantitative method deemed most acceptable will be used for the evaluation of surrogates of *B. anthracis* and will be subjected to a full collaborative study to validate the method. Ultimately, quantitative efficacy data may be used in the development of regulatory guidelines, including new performance standards for sporicidal and sterilant products specifically designed for the treatment of surfaces contaminated with spores of *B. anthracis*.

Experimental

Quality Management

Prior to initiating the research, an on-site quality control readiness review was conducted at each laboratory to ensure compliance with an EPA Quality Assurance Project Plan (9). Each laboratory was required to establish and perform a set of quality control activities consistent with EPA Good Laboratory Practices. In addition, hands-on training was provided for each test method to ensure consistency of expertise from laboratory to laboratory. To reduce sources of variability, several experimental components were standardized across laboratories, e.g., use of one production lot of each test chemical, certified reagents for preparing test chemicals, and use of one commercial source of spore inoculum and inoculated penicylinders. In advance of conducting the study, the test chemicals were evaluated for formulation chemistry and each fell within the certified limits established for the product. Each laboratory was provided with a set of method protocols, data sheets, and media preparation sheets.

Safety Precautions

All manipulations of the test organism (*B. subtilis*) were required to be performed in accordance with standard

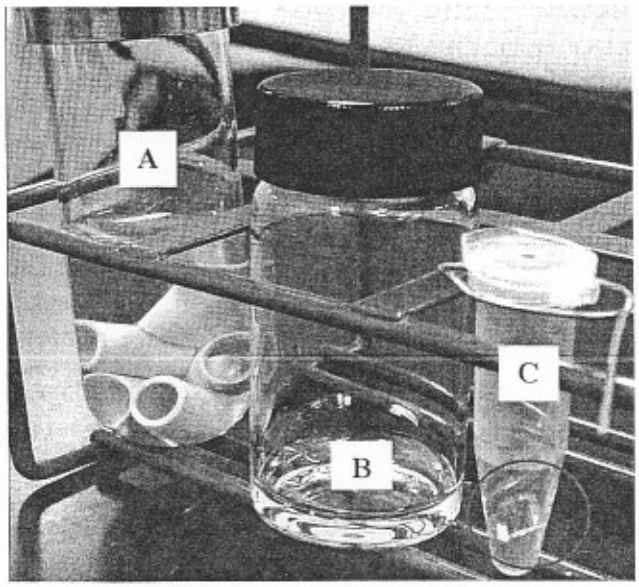

Figure 1. Carrier type and volume of sporicide or sterilant tested for AOAC Method 966.04 (A), ASTM E 2111-00 (B), and TSM (C). Circle in C indicates carrier. Volume is 10 mL/5 carriers, 1 mL/carrier, and 400 μL/carrier for AOAC Method 966.04, ASTM E 2111-00, and TSM, respectively.

biosafety practices (10). Sporicidal and sterilant agents contain active ingredients such as sodium hypochlorite, hydrogen peroxide, and peroxyacetic acid. Latex gloves and other personal protective clothing or devices were worn during the handling of these items for purposes of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment was used during tasks with concentrated products. Some sporicidal and sterilant chemicals were not tested together due to reactivity. Material Safety Data Sheets (MSDSs) were reviewed prior to testing.

Spore Preparation

Spores of *B. subtilis* (ATCC No. 19659) for each test method evaluated in this study were generated according to Method **966.04** (i.e., harvested from soil extract nutrient broth). Presque Isle Cultures (Erie, PA) supplied the spore preparation (spore suspension) and inoculated porcelain penicylinders. Porcelain penicylinders were inoculated according to Method **966.04**, and a concentrated spore suspension was used to inoculate carriers for the quantitative tests. The concentrated spore suspension was generated in the following manner: a filtered spore suspension prepared according to Method **966.04** was washed 3 times in cold sterile deionized water using a refrigerated centrifuge set at 14 000 × *g* for a maximum of 5 min. The centrifuged pellets were resuspended in 10% aqueous ethanol. The quality and purity of the resuspended spores were rechecked using phase contrast microscopy and staining (malachite green). When complete, the preparation contained at least 95% individual separated spores. Vegetative cells and germinated spores were not more than 5% of the total. If the preparation was less than 95% individual separated spores, it was re-rinsed with cold deionized water and centrifuged, and the supernatant was removed to clean and concentrate the spores. The pellet was resuspended in 10% ethanol and checked for quality and purity of the spores. This step was repeated as necessary. In this study, the final concentration of the spore suspension was 5.1×10^9 colony forming units (CFU/mL) as determined by serial dilution and plating on trypticase soy agar (TSA). The purity of the spore preparation exceeded 95% as viewed under phase contrast microscopy. The spore preparation was stored at 2–5°C. Horse serum was added to the spore suspension prior to inoculation of all carriers at an effective concentration of 5% (v/v). The spore suspension was mixed on a Vortex mixer prior to carrier inoculation to evenly distribute the spores. During the study, confirmation testing for the identification of the test microbe was performed on individual colonies from plates or filters by isolation streaks on TSA and Gram staining.

Study Design and Test Conditions

Three laboratories participated in the evaluation: EPA OPP Microbiology Laboratory, Fort Meade, MD; U.S. Food and Drug Administration, Denver District Laboratory, Denver, CO; and U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD. For this study, treatments (test chemicals) and associated test conditions were selected to provide a range of efficacy, thus allowing for a comparative evaluation of surviving spores. If all treatments resulted in few to no survivors, the data would have limited value in a comparative analysis. The test chemicals were experimental components and were not being examined to verify registered sporicidal claims. Two commercially available products were used as test chemicals. The first product, commercial bleach, contained 6.0% sodium hypochlorite, and the second product contained a mixture of 0.8% hydrogen peroxide and 0.06% peroxyacetic acid. Treatments were: 1:20 dilution (to achieve ca 3000 ppm) of the sodium hypochlorite product with pH adjusted to 7.0; hydrogen peroxide/peroxyacetic acid product (no dilution); 1:20 dilution of the sodium hypochlorite product with pH unadjusted (pH of ca 10.0); and 1:10 dilution (to achieve ca 6000 ppm) of the sodium hypochlorite product with pH adjusted to 7.0). The contact time was 10 min for all chemical treatments except the 1:10 sodium hypochlorite treatment, which was tested at 30 min. The 1:10 sodium hypochlorite treatment was only used in Method **966.04** as a positive control (highly efficacious); the concentration/contact time has been previously shown to be a highly effective treatment, although occasional positive carriers were possible due to the shortened contact time (i.e., minimum of 60 min recommended for complete kill). The sodium hypochlorite solutions were prepared using the following formula and conditions:

(**a**) *1:20 Overall dilution of 6.0% sodium hypochlorite with unadjusted pH.*—One part sodium hypochlorite + 19 parts sterile high-performance liquid chromatography (HPLC) grade water, unadjusted pH of ca 10.0, tested within 1 h of preparation.

(**b**) *1:20 Overall dilution of 6.0% sodium hypochlorite with adjusted pH.*—One part sodium hypochlorite + 0.6 parts 5% acetic acid + 18.4 parts sterile HPLC grade water, pH adjusted to 7.0 ± 0.5, tested within 3 h of preparation.

(**c**) *1:10 Overall dilution of 6.0% sodium hypochlorite with adjusted pH.*—One part sodium hypochlorite + 0.6 parts 5% acetic acid + 8.4 parts sterile HPLC grade water, pH adjusted to 7.0 ± 0.5, tested within 3 h of preparation.

No dilution was required for the hydrogen peroxide/peroxyacetic acid; the product was tested within 3 h of dispensing. Due to the logistical challenges and resources necessary to perform efficacy testing, the treatments were evaluated using one method/day; 3 replications (same method in a 3-day sequence) were performed by each laboratory. The order of testing (test method) and the order of chemicals (treatments) were randomized for each laboratory; however, only 2 laboratories performed the AOAC method. Each method protocol was followed as published at the time of the study except the following modifications: the spore production protocol used for the quantitative tests, the addition of 5% horse serum to the spore inoculum as the organic burden, and the use of glass coupons for the Three Step Method (TSM).

Acceptable HCl resistance (spores must resist 2.5 M HCl for ≥2 min to be qualified as resistant test spores) and a minimum of 2.0×10^5 spores/carrier were required for the

inoculated porcelain carriers. Carrier counts for the AOAC method were determined using the following procedure: Place inoculated carrier into a 20 × 150 mm tube containing 10 mL sterile deionized water. Sonicate for 5 min (±30 s). Mix on a Vortex mixer 2 min (±5 s). Serially dilute spore suspensions and plate in duplicate on TSA. Invert plates, incubate for 24–48 h at 37 ± 1°C, and count colonies. Dilutions yielding between 30 and 300 CFU/plate were used for enumeration. Each laboratory evaluated 5 to 7 randomly selected carriers from the inoculated carrier set prior to initiating the study.

Based on guidance taken from ASTM Method E 2111-00, the target carrier load established for the quantitative tests was 10^7 spores/carrier or 7.0 logs/carrier—a level suitable for measuring a log reduction (LR) of ≥6 logs. *See* the discussion on processing control carriers for each quantitative method.

ASTM E 2111-00—Standard Quantitative Carrier Test Method

Reagents and Culture Media

(**a**) *Sterile saline.*—For rinsing during the filtration process, dilution blanks, and for exposing control carriers.

(**b**) *Horse serum.*—Gibco (Cat. No. 16050-122; Invitrogen Corp., Carlsbad, CA); sterile, for preparation of 5% (v/v) organic burden, added to inoculum prior to inoculation of carriers.

(**c**) *7X Cleaning solution.*—ICN Biomedicals (Cat. No. 76-670-94; Irvine, CA); for preparation of 1% solution for cleaning carriers, inserts, and caps.

(**d**) *Water.*—HPLC grade, sterile; diluent for sodium hypochlorite.

(**e**) *Acetic acid.*—5%, used to adjust pH of sodium hypochlorite solutions; filter-sterilize prior to use.

(**f**) *Nutrient agar (NA).*—In plates as the recovery medium.

(**g**) *Letheen broth.*—Neutralizer for hydrogen peroxide/peroxyacetic acid.

(**h**) *Letheen broth with 0.1% (w/v) sodium thiosulfate.*—Neutralizer for sodium hypochlorite.

Apparatus

(**a**) *Positive displacement pipet.*—Used for uniform delivery of spore inoculum to carriers.

(**b**) *Sterile threaded stir bars.*—15 × 4 mm; with TFE–fluorocarbon surface to dislodge inoculum from the carrier surface.

(**c**) *Magnet.*—For holding stir bar in place in the glass vial while liquid is being poured out into the filter.

(**d**) *Sterile glass inserts.*—To be placed inside the glass carriers during carrier inoculation.

(**e**) *Glass vials.*—Sterile, 28 × 58 mm, flat-bottomed; used as carriers.

(**f**) *Sterile dispenser.*—For rinsing filter unit.

(**g**) *Desiccator.*—For storing inoculated carriers.

(**h**) *Filter unit.*—47 mm, disposable, sterile, 150 mL unit with 0.22 µm membrane (Cat. No. 130-4020; Nalgene, Thermo Fisher Scientific, Waltham, MA).

(**i**) *Recirculating chiller.*—Neslab RTE-221 (Thermo Fisher Scientific), or equivalent.

(**j**) *Test tubes.*—20 × 100 mm, sterile.

(**k**) *Petri dishes.*—Sterile, plastic (100 × 20 mm).

(**l**) *Incubator.*—Set at 37 ± 1°C.

(**m**) *Certified timer.*—For managing timed activities; any certified timer that can display time in seconds.

Procedure

(*1*) Use 28 × 58 mm flat-bottomed glass vials as carriers.

(*2*) Inoculate the inside bottom of each vial with 10 µL spore suspension with a positive displacement pipet. Use a glass insert (sleeve) with a septate cap during inoculation to reduce potential contamination caused by micro-aerosols.

(*3*) Allow inoculum to dry 1 h in a biological safety cabinet; then overnight in a desiccator. Remove glass inserts and septate caps after drying. Replace septate caps with regular, sterile screw caps.

(*4*) Test 10 carriers for each test chemical and 3 for controls. Process all carriers together.

(*5*) Add 1 mL test chemical over dried inoculum.

(*6*) At the end of the contact time, add 9 mL neutralizing solution [letheen broth with 0.1% (w/v) sodium thiosulfate for sodium hypochlorite, and letheen broth for hydrogen peroxide/peroxyacetic acid] to the vial to inactivate the sporicidal agent.

(*7*) Add a sterile stir bar to the carrier and recover spores using stirring (30 s); then mix on a Vortex mixer (30 s).

(*8*) Pour liquid into the filter apparatus holding the stir bar in place with a magnet. Pass the liquid–spore mixture through a filter membrane. Rinse each filter with saline and place on NA.

(*9*) Incubate membranes 3–5 days at 37 ± 1°C. Enumerate colonies with the aid of a colony counter.

(*10*) Process control carriers, 3 per test day, using 1 mL saline per vial instead of test chemical, and 9 mL saline added as the "neutralizer." At the end of the contact time, recover spores by stirring for 1 min and mixing on a Vortex mixer 30 s.

(*11*) Compare control carrier counts to the treated carriers. Determine the level of efficacy by calculating $\log_{10}$ reduction.

Three Step Method

Reagents and Culture Media

(**a**) *Luria-Bertani broth (Difco).*—(*1*) Becton Dickinson (Sparks, MD); with 0.1% (w/v) sodium thiosulfate is used as a neutralizer for sodium hypochlorite. (*2*) Used as neutralizer for hydrogen peroxide/peroxyacetic acid.

(**b**) *Water.*—HPLC grade, sterile, filter-sterilized prior to use as diluent.

(**c**) *Sodium thiosulfate.*—Sigma S-7026 (St. Louis, MO).

(**d**) *Nutrient agar.*—In plates.

(**e**) *Horse serum.*—5% (v/v), sterile.

Apparatus

(**a**) *Microcentrifuge tubes.*—Sterile, 1.5 mL (Thermo Fisher Scientific; Cat. No. 05-408-129, or equivalent).

(**b**) *Petri dishes.*—Sterile, plastic (100 × 20 mm).

(**c**) *Recirculating chiller.*—Neslab (Thermo Fisher Scientific) RTE-221, or equivalent, or Nalgene Labtop Cooler (Cat. No. 5115-0032, or equivalent).

(**d**) *Drying oven.*—Yamato Scientific America, Inc. (Santa Clara, CA) mechanical convection oven (No. DKN 810), or equivalent.

(**e**) *Filter forceps.*—Sterile; for transfer of inoculated carriers from Petri dish to microcentrifuge tube (Cat. No. 30033-042; VWR, West Chester, PA, or equivalent).

(**f**) *Dissecting forceps.*—Sterile; for transfer of carrier between fractions (VWR; Cat. No. 25607-195, or equivalent).

(**g**) *Positive displacement pipet.*—For carrier inoculation (Gilson Microman, Gilson Inc., Middleton, WI, or equivalent).

(**h**) *Eppendorf pipets.*—Calibrated.

(**i**) *Pipet calibration system.*—PCS 2 Pipette (Artel, Westbrook, ME); for the calibration of Eppendorf pipets.

(**j**) *Vortex adapters.*—Thermo Fisher Scientific (Cat. Nos 1281161 and 1281211), or equivalent.

(**k**) *Microcentrifuge.*—Microfuge 18 (Beckman Coulter, Fullerton, CA), or equivalent.

(**l**) *Ultrasonic cleaner.*—Branson Model 1510 bath sonicator (Danbury, CT), or equivalent.

(**m**) *Hematology rotator.*—Hematology Chemistry Mixer 346 (Thermo Fisher Scientific, or equivalent).

(**n**) *Vortex mixer.*—Genie 2 Mixer/G-560 (Scientific Industries, Inc., Bohemia, NY).

(**o**) *Incubator.*—Set at 37 ± 1°C.

(**p**) *Certified timer.*—For managing timed activities; any certified timer that can display time in seconds.

(**q**) *Glass carrier.*—Sterile, single use, 5 × 5 × 1 mm (Erie Scientific Co., Portsmouth, NH; custom order Part No. EPA-1101).

Procedure

(*1*) Use 5 × 5 × 1 mm glass carriers.

(*2*) Inoculate carriers with 10 µL (1 spot) spore suspension. Allow inoculated carriers to dry overnight in biological safety cabinet before use. Carriers are held in sterile Petri dish for inoculation and drying.

(*3*) Test 12 total carriers, 3 for each of the 3 test chemicals and 3 for control set. Process all carriers together.

(*4*) Carefully place inoculated carriers into 1.5 mL microcentrifuge polypropylene tubes without touching inside wall of tubes. Label as Fraction A.

(*5*) Add 400 µL disinfectant (test carriers) or 400 µL sterile HPLC grade water (control carriers) to an appropriate microcentrifuge tube (in triplicate). Allow contact of carriers to disinfectant or water in Fraction A tubes for appropriate exposure period. Following exposure period, add 600 µL appropriate ice-cold (0–5°C) neutralizer (e.g., Luria-Bertani broth) to each disinfectant and control Fraction A tube.

(*6*) Process control carriers in same manner as treated carriers.

(*7*) Following exposure, remove each carrier with sterile forceps from Fraction A tubes and place into separate 1.5 mL microcentrifuge tube containing 400 µL ice-cold (0–5°C) sterile HPLC grade water. Label as Fraction B. Fluid remaining in Fraction A tubes contains spores dislodged from carrier by exposure to disinfectant or water control.

(*8*) Centrifuge liquid in Fraction A tubes 6 min at 13 000 rpm. Carefully remove 950 µL supernatant and discard without disturbing the pellet.

(*9*) Add 950 µL ice-cold Luria-Bertani broth. Repeat centrifugation and wash 2 more times. After third centrifugation and supernatant removal, add 100 µL ice-cold Luria-Bertani broth to pellet in Fraction A tubes. Mix on a Vortex mixer 5 min to resuspend pellets. Add 850 µL ice-cold Luria-Bertani broth to each.

(*10*) Sonicate Fraction B tubes 5 min.

(*11*) Add 600 µL ice-cold Luria-Bertani broth to each Fraction B tube. Mix on a Vortex mixer 1 min.

(*12*) Using sterile forceps, transfer carriers from Fraction B tubes into separate 1.5 mL microcentifuge tubes containing 400 µL ice-cold Luria-Bertani broth. Label as Fraction C. Fluid remaining in Fraction B tubes contains spores dislodged from carrier by sonication.

(*13*) After sonication, centrifuge Fraction B tubes once at 13 000 rpm for 6 min. Remove 950 µL supernatant and discard. Add 100 µL Luria-Bertani broth to each tube and mix on a Vortex mixer 5 min. Add 850 µL Luria-Bertani broth to each tube.

(*14*) Place Fraction C tubes in a hematology rotator inside a 37 ± 1°C incubator for 30 min and mix tubes on a Vortex mixer for 1 min.

(*15*) Add 600 µL ice-cold Luria-Bertani broth to each tube. Carriers remain in Fraction C tubes. Fluid remaining in Fraction C tubes contains spores dislodged from carrier by shaking for 30 min.

(*16*) Serially dilute (10-fold) Fractions A–C for test and control carriers.

For each carrier, direct plate 100 µL of sufficient dilutions onto NA to ensure obtaining counts within target range of 30–300 CFU/plate. Incubate all plates at 37 ± 1°C for minimum of 24 h.

Obtain total number of spores/fraction by dividing number of colonies counted in each fraction by its dilution, and account for volume plated. Obtain total number of spores/carrier by adding total number of viable spores/fraction for Fractions A–C. Determine log density (LD) of total number of viable spores/carrier by taking $\log_{10}$ (total number of spores/carrier). Determine LR of test carriers by subtracting LD of test carriers from LD of control carriers. Determine average LD and LR for each disinfectant.

Method 966.04

Reagents and Culture Media

(**a**) *Fluid thioglycollate medium (FTM).*

(b) *Letheen broth.*—With 0.1% (w/v) sodium thiosulfate.

(c) *Water.*—Sterile, HPLC grade; filter-sterilize prior to use as diluent.

Apparatus

(a) *Inoculated porcelain penicylinders.*—Presque Isle Cultures (Erie, PA).

(b) *Penicylinders.*—Porcelain, 8 ± 1 mm od, 6 ± 1 mm id, 10 ± 1 mm length (Thermo Fisher Scientific; Cat. No. 07-907).

(c) *Recirculating chiller.*—Neslab RTE-221, or equivalent.

(d) *Vacuum desiccator.*—For storing inoculated carriers.

(e) *Test tubes.*—Sterile, 20 × 100 mm.

(f) *Wire hook.*—For carrier transfer. Make 3 mm right angle bend at the end of 50–75 mm nichrome wire (No. 18 B&S gage). Have the other end in suitable holder.

(g) *Certified timer.*—For managing timed activities; any certified timer that can display time in seconds.

(h) *Ultrasonic cleaner.*—Branson Model 1510 Bath Sonicator, or equivalent.

Procedure

(*1*) Use porcelain carriers.

(*2*) Test 60 inoculated carriers, 5/tube for each chemical treatment. Test 20 inoculated carriers, 5/tube for control treatment.

(*3*) Using sterile hook, drop carriers at 2 min intervals in groups of 5 into tubes of sporicidal agent held at 20 ± 1°C.

(*4*) At completion of exposure period, remove carriers, one at a time, from sporicidal agent medication tube with sterile hook and tap against interior sides of tube to remove excess sporicidal agent.

(*5*) Transfer carriers in same sequential timed fashion into primary subculture tubes containing appropriate neutralizer: letheen broth with 0.1% (w/v) sodium thiosulfate for sodium hypochlorite and FTM for hydrogen peroxide/peroxyacetic acid. All 5 carriers must be transferred during each 2 min interval. Flame the hook between each carrier transfer.

(*6*) Transfer carriers into their corresponding neutralizer tubes at the appropriate time.

(*7*) After all transfers have been completed, transfer carriers again to fresh tubes of subculture media (FTM for sodium hypochlorite and hydrogen peroxide/peroxyacetic acid).

(*8*) Incubate both sets of tubes for 21 days at 37 ± 1°C. For tubes showing no growth after 21 day incubation period, heat-shock at 80 ± 2°C for ca 20 min and incubate an additional 72 ± 2 h at 37 ± 1°C.

(*9*) Report results as growth (+) or no growth (0). Growth in either primary (neutralizer) or secondary subculture tube is considered positive result for carrier set.

Statistical Analysis

Emphasis was placed on comparing method performance and not evaluating differences in chemical treatments. The analysis of variance (ANOVA) was based on a one-way, random effects linear statistical model, similar to the models used by Tilt and Hamilton (11). For each combination of test method and chemical evaluated with a quantitative method, the LR was the response variable, and the main effect was the laboratory. For the quantitative tests, method performance was assessed by measuring the within-laboratory variance (i.e., independent repeats of the same test), between-laboratory variance (i.e., the variability among laboratories in excess of what was expected based on the within-laboratory variability), and total variance associated with the LR estimates generated from each method. To meet these goals, an estimate of the repeatability standard deviation (square root of the within-laboratory variance), expressed as S_r, and the reproducibility standard deviation (square root of the total variance), expressed as S_R, were generated. Control counts are expressed as recoverable viable spores/carrier: $\log_{10}$ (CFU/carrier). The LR was based on the mean $\log_{10}$ (CFU/control carrier) – mean $\log_{10}$ (CFU/treated carrier). For LR calculations involving the ASTM E 2111-00 and TSM, 200 was substituted at the last dilution for plates or filters with colonies too numerous to count, and 0.5 was substituted at the first dilution for plates or filters with 0 colonies. Also, comparing the performance of the test chemicals across both types of methods, qualitative and quantitative, was a useful exercise to ascertain the difference in ease or difficulty in achieving spore kill due to the type of method and response being measured.

Results and Discussion

ASTM E 2111-00 and TSM

For ASTM E 2111-00, 10 treated (test chemical) and 3 control carriers were evaluated by each laboratory for each replication, respectively. Three treated (test chemical) and 3 control carriers/set of 3 test chemicals were analyzed by each laboratory with the TSM for each replication. Mean ± the standard error (SE) control carrier counts (LD) were 7.2 ± 0.13 for ASTM E 2111-00 and 7.5 ± 0.06 for the TSM; the mean densities were not significantly different (*t*-test; 2-tailed *P*-value = 0.14). These values were expected based on the titer of the concentrated spore suspension (5.1×10^9 CFU/mL) and the volume applied/carrier (10 µL). Control carrier count data are presented in Figure 2. Control carrier counts for both quantitative methods exhibited low S_r (0.22 for ASTM E 2111-00 and 0.18 for TSM) and S_R (0.29 for ASTM E 2111-00 and 0.18 for TSM), thus indicating good method performance for control carriers. The LD for each TSM carrier was the sum of 3 counts (Fractions A–C). On average, Fraction A of TSM contributed 95% of the total spores for the control carriers. Only a small percentage of the total variance was attributable to the variance among carriers within a test (14% for ASTM E 2111-00 and 16% for TSM). Consequently, analyzing 3 carriers/test proved sufficient for measuring the control counts.

The LR estimates generated using both quantitative methods were similar for the 3 treatments evaluated and were not statistically different across methods (Table 1). The

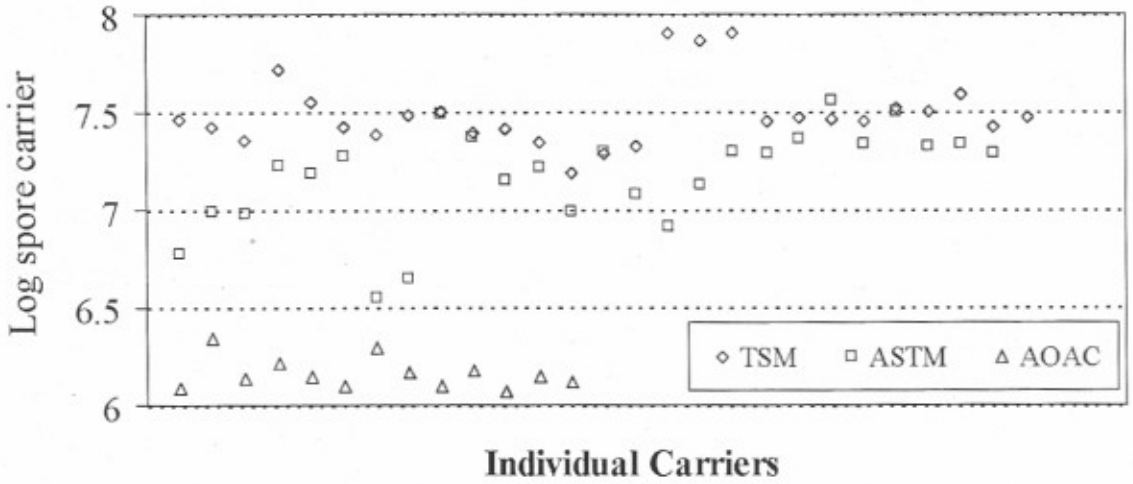

Figure 2. Individual control carrier counts (log spores/carrier) for 3 sporicide and sterilant test methods across laboratories. *n* = 27 for TSM (mean = 7.5); *n* = 26 for ASTM E 2111-00 (mean = 7.2); *n* = 12 for AOAC 966.04 (mean = 6.2).

difference for the inactive sodium hypochlorite treatment (3000 ppm with unadjusted pH) was not significant (*P*-value = 0.053). The expected difference in efficacy between adjusted and unadjusted pH sodium hypochlorite was exhibited in the LR values for both quantitative methods (7.1 and 7.5 for adjusted sodium hypochlorite and 3.6 and 1.2 for unadjusted sodium hypochlorite for ASTM E 2111-00 and TSM, respectively). The hydrogen peroxide/peroxyacetic acid treatment was slightly less effective than 3000 ppm adjusted sodium hypochlorite, with LR values of 6.7 for ASTM E 2111-00 and 7.3 for the TSM; the LR values between methods were not significantly different (*P*-value = 0.25). Across methods and treatments, the repeatability standard deviation (SD_r) ranged from 0.25 to 0.66, and the reproducibility standard deviation (SD_R) ranged from 0.26 to 1.12. This range of variability is an indicator of good method performance (11).

Overall, the quantitative methods performed similarly in this study. Due to similar method performance, additional attributes were used to select a method. A questionnaire was developed and submitted to the analysts from each laboratory that actually performed the tests. The questionnaire comprised 15 questions and asked for opinions on pertinent test method attributes related to ease of using the protocols, amount of training required, logistics for setting up the test, number of technique-sensitive steps, and the human resources required to perform the method. The majority of the questions required the analyst to simply select the method that best fit the answer to the question. Each laboratory responded independently. A subgroup of the federal Interagency Expert Panel on *B. anthracis* reviewed the responses and looked for trends and a set of the questions that might be used as an indicator for method selection, i.e., questions that were more direct and specific were weighed more heavily. The responses to the questionnaire indicated that compared to the TSM, ASTM E 2111-00 was more difficult to set up and required more set-up time, was more difficult to perform from start to finish, and required more human resources. The outcome of questions related to the human resources required to perform each test was completely favorable for the TSM. The logistical and resource concerns identified for ASTM E 2111-00 were largely associated with the filtration process and counting bacterial colonies on filters, and were seen as significant

Table 1. Mean log reduction (LR) values and method performance statistics for ASTM E 2111-00 and Three Step Method

	ASTM E 2111-00			Three Step Method			
Test chemical	LR	SD_r	SD_R	LR	SD_r	SD_R	P^a
Sodium hypochlorite (3000 ppm with adjusted pH)	7.1	0.36	0.39	7.5	0.27	0.48	0.28
Sodium hypochlorite (3000 ppm with unadjusted pH)	3.6	0.66	1.12	1.2	0.26	0.26	0.053
Hydrogen peroxide/peroxyacetic acid	6.7	0.45	0.52	7.3	0.25	0.75	0.25

[a] *t*-Test; 2-tailed *P*-value for comparison of mean LR values between test methods.

Table 2. Number of carriers scored as positive out of the number of carriers tested for AOAC Method 966.04

Chemical treatment	Lab 1 (n = 3)	Lab 2 (n = 3)
Sodium hypochlorite (3000 ppm with adjusted pH)	37/60; 31/60; 21/60	20/60; 23/60; 16/60
Sodium hypochlorite (3000 ppm with unadjusted pH)	59/60; 60/60; 56/60	60/60; 60/60; 60/60
Hydrogen peroxide/peroxyacetic acid	11/60; 5/60; 7/60	19/60; 28/60; 25/60
Sodium hypochlorite (6000 ppm with adjusted pH)	0/20; 5/20; 3/20	0/20; 0/20; 0/20

drawbacks to the method. However, the responses indicated that the TSM required more practice time and training.

*AOAC Method **966.04***

The mean (±SE) carrier counts [LD 6.2 (±0.03) spores/carrier] were within the established range for the study. Only one set of carrier counts was requested for the Method **966.04** for each laboratory; thus S_r was not calculated. Based on the number of positive carrier sets, Method **966.04** successfully measured a range of sporicidal efficacy across the treatments and the positive control (Table 2). For the 1:20 (3000 ppm) unadjusted pH sodium hypochlorite, 4 of 6 total tests resulted in 60/60 positives. For the 1:20 (3000 ppm) adjusted pH sodium hypochlorite, the number of positive carriers was lower, ranging from 16 to 37 positives out of 60. Hydrogen peroxide/peroxyacetic acid exhibited a range of 5 to 28 positives out of 60. The positive control, 1:10 (6000 ppm) pH adjusted sodium hypochlorite, showed fewer positive carriers overall, 4 of 6 tests showing 0 positive carriers out of 20. The occurrence of positives in this control was not entirely unexpected; it would have been a more effective control by increasing the contact time to 60 min to ensure complete kill of spores.

This initiative represents the first in a series of investigations designed to advance our knowledge of quantitative sporicide and sterilant efficacy testing. Two quantitative methods were selected and compared in this study, and attributes such as the existence of a well established, highly developed protocol; previous use for testing sporicides; readily available materials; clear method for enumeration of spores from control and treated carriers; neutralization procedure; flexible contact time; and the ability to incorporate organic burden were important aspects considered during the selection process. The use of multiple efficacy test methods for comparative research (e.g., surrogate studies) is impractical and makes the comparison of efficacy data very difficult; thus the selection of a single method was desirable. By conducting this study in a comparative, standardized manner across multiple laboratories, valuable statistical information on the performance of 2 quantitative methods was generated. Thus, the goals established for this study were achieved. In this study, only 2 chemical classes were examined; both were oxidizers, and this limitation is recognized. This study focused on testing liquids on a hard surface; thus, expanding testing to include multiple carrier materials, test chemicals, and formulation types is imperative.

The quantitative methods evaluated in this study displayed statistical similarities, and based on the LR data alone, the selection of a method "on this basis alone" was not possible. Compared to the variability associated with other antimicrobial test methods (11), both the ASTM E 2111-00 and the TSM exhibited small and acceptable S_r and S_R values. Based on the statistical parameters measured and the additional test method attributes related to logistics and human resources, the TSM was determined to be the most suitable method and will be recommended for future evaluation and validation testing. The flexibility in the TSM for incorporating coupon materials other than glass was considered as an advantage, and practice time and training issues can be resolved through the reorganization of the protocol and the use of a flowchart and process checklist.

The "too numerous to count" substitution rule was required for 20 out of 27 A fractions when TSM was used to test sodium hypochlorite at 3000 ppm with unadjusted pH. For this reason, the treated carrier counts were artificially similar, creating the prospect that the observed SD_r and SD_R values underestimate the true variability for that case. One can expect that future applications of the TSM to marginal chemicals such as sodium hypochlorite at 3000 ppm with unadjusted pH will exhibit SD_r and SD_R values that are at least as large as those observed here for the 2 active chemicals.

Although the study was not designed to compare differences in the efficacy of test chemicals between the quantitative methods and Method **966.04**, it appears that high LR values can be anticipated for products that pass (0 positive carriers) the AOAC method. In this study, LR values of 6.7 to 7.5 were generated by the quantitative methods for 1:20 sodium hypochlorite with adjusted pH and the hydrogen peroxide/peroxyacetic acid, but the same treatments failed Method **966.04** with numerous positive carriers. As the development and use of quantitative test methods for sporicides and sterilants proceed, additional studies will be necessary to develop meaningful and relevant performance standards (i.e., pass/fail criteria with a minimum LR).

Since the period of conducting this collaborative study, the TSM protocol has been refined by EPA scientists and has been used to generate data on surrogates of *B. anthracis* (*see* AOAC General Referee Report, 2005—Disinfectants). For example, the revised TSM protocol calls for the use of nutrient

agar amended with manganese sulfate as the spore production medium for *Bacillus*, and a series of flow charts and processing sheets have been included to make the method easier to understand. It should be noted that following the completion of this study, a similar version of the TSM was accepted and published by ASTM International (12). We anticipate that the TSM protocol will be submitted to AOAC INTERNATIONAL for official validation testing. The development of a validation protocol will be enhanced by the data generated and knowledge gained from this study.

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