Designation: E 2414 – 05

Standard Test Method for Quantitative Sporicidal Three-Step Method (TSM) to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapor or Gases on Contaminated Carrier Surfaces[1]

This standard is issued under the fixed designation E 2414; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method determines the efficacy of sporicidal agents on microorganisms dried on the surface of solid carriers.

1.2 This test method can be used to evaluate sporicidal products (or decontaminant, disinfectant, and so forth), suspected, claimed, or assumed to have sporicidal activity. This test method allows:

1.2.1 Establishing the sporicidal efficacy of different disinfectants;

1.2.2 Identifying the effect, if any, of the surface materials on sporicidal efficacy; and

1.2.3 Comparing the relative resistance to disinfection of different microbial species or strains.

1.3 The values stated in SI units are to be regarded as the standard.

1.4 Strict adherence to the protocol is necessary for the validity of the test results.

1.5 *Follow all the safety guidance of the institution in which the testing is being conducted. This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:* [2]

E 1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

3. Summary of Test Method

3.1 This test method determines the efficacy of disinfectants on spores of *Bacillus subtilis* dried on carriers according to a general technique first described by J. L. Sagripanti and A. Bonifacino (**1, 2**)[3].

3.1.1 Spores of *B. subtilis* can be and have been replaced by similar amounts of spores of *Bacillus anthracis* to substantiate claims as may be needed in biodefense.

3.1.2 The material of the carrier is selected according to claims or intended use, or both, of the disinfectant product. General claims made to decontaminate metallic and polymeric materials are tested on carriers made of stainless steel and silicone medical rubber, respectively. Flat coupons (0.5 by 0.5 cm) are preferable. As may be required by claims or intended use, the test method can be accurately used also on a variety of carriers with diverse geometrical characteristics (additional examples of materials were reported by Sagripanti and Bonifacino (**1, 2**).

3.1.3 Although the test method described herein refers specifically to liquid disinfectants, the same procedure can be used to assess sporicidal activity of vapors and gaseous sporicidal agents, provided adequate containment is accomplished (see 10.4.5).

4. Significance and Use

4.1 The quantitative micromethod described herein was designed to fulfill the following specifications:

4.1.1 To be quantitative (the number of viable spores loaded into carriers is determined by the spores quantitatively recovered in the controls),

4.1.2 Sensitive (sporicidal activity can be accurately measured up to 7 Log_{10} inactivation, see Section 12),

4.1.3 Reproducible (standard deviation of spore killing is smaller than 1 Log and usually closer to 1/10 of a Log, see Section 12),

[1] This test method is under the jurisdiction of ASTM Committee E54 on Homeland Security Applications and is the direct responsibility of Subcommittee E54.03 on Decontamination.

Current edition approved June 15, 2005. Published July 2005.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

4.1.4 Rapid (except for the overnight culture, it can be completed within 4 h),

4.1.5 Economical (being a micromethod, it uses carriers, dishes, and plastic wares that are small, inexpensive, and disposable), and

4.1.6 Environmentally friendly (using a microlitre volume of disinfectant agent, the test method can be considered for all practical purposes as nondestructive).

5. Apparatus

5.1 *List of Equipment*—Make and models are provided as examples. Use the same or equivalents.

5.1.1 *Microcentrifuge*, rated to 12 000 g with rotor to hold 1.5-mL conical microcentrifuge tubes.

5.1.2 Autoclave.

5.1.3 Vortex mixer.

5.1.4 *Sonicator*, low-power water bath type, rated to 400 to 500 W (generally used for cleaning small objects by immersion).

5.1.5 Class II biosafety cabinet.

5.1.6 *Balance*, accurate to 1 mg.

5.1.7 *Refrigerator*, able to maintain 0 to 5°C.

5.1.8 *Freezer*, able to keep −80°C.

5.1.9 *Incubator*, able to maintain 37 ± 1°C (usually with a range from room temperature to 60°C).

5.1.10 Colony counter.

5.1.11 *Photomicroscope*, providing 1000× maximal magnification to control spore quality.

5.1.12 *Micropipets*, with corresponding sterile tips able to deliver volumes in the ranges of 10, 20, 100, 200, and 1000 µL.

5.1.13 *Timer*, any certified timer/watch/clock that can display time in seconds.

5.1.14 *Rotator*, able to provide 15 to 20 rpm (of the type used in hematology chemistry) with a rack to hold 1.5-mL microcentrifuge tubes.

5.1.15 *Shaker*, able to control speed and inside temperature.

5.1.16 *Anaerobic Jar*, if testing gases.

5.1.17 *Cooler*, able to maintain temperature at 21 ± 3°C

6. Reagents and Materials

6.1 *Spores of B. subtillis (Strains ATCC 1031 or ATCC 9372), Bacillus globigii (Renamed B. atrophaeus SB 512), or B. anthracis (Pathogenic Strains Albia BA 1029, Ames, NCTC 1087, or Vollum or Nonpathogenic Strains Sterne, Delta-Sterne, or Pasteur BC3132)*—Stock suspensions are prepared under the appropriate biosafety containment and produced at a concentration between 1×10^9 and 5×10^9 colony-forming units/mL. A variety of media are available to grow spores of Bacillus species. It is recommended the use of sporulation media Media S whose formulation is described in Appendix X1. Preparations (made as suggested in Appendix X1) are accepted for use when consisting in more than 95 % spores as determined by both microscopic observation and acid resistance. Microscopic observation of spores stained with trypan blue should reveal less than 10 vegetative cells (shaped as rods) during the observation of 1000 spores (round shape). Testing spores for acid resistance is described in Appendix X2 and Appendix X3. Both tests for spore quality follow techniques published previously (**1**, **2**, **4**).

6.2 *Sterile Luria*—Bertani broth (LB) (if in powder form, prepared as recommended by the manufacturer).

6.3 HPLC quality sterile water and sterile phosphate-buffered saline (PBS).

6.4 Nutrient agar plates.

6.5 Laboratory glassware, graduated cylinders, calibrated volumetric flasks.

6.6 Cupric chloride ($CuCl_2 \cdot 2H_2O$); *L*-ascorbic acid, and hydrogen peroxide if using cupric ascorbate as a positive control.

6.7 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[4]

6.8 *Carriers*:

6.8.1 *Spotting/Flat*—Preferable glass, but also rubber, stainless steel, polymeric plastics, or other materials cut in squares of 0.5 by 0.5 cm.

6.8.2 *Dipping*—Carriers that do not hold liquid and need to be contaminated by immersion, for example, screws, cylinders, and tubing. The size and shape of carriers should allow their introduction inside the 1.5-mL microcentrifuge tubes to be immersed in the 400 µL of liquid and have a total volume between 50 to 200 µL.

6.9 *Supplies*:

6.9.1 *Forceps/Tweezers*, to handle carriers.

6.9.2 *Sterile Disposable Petri Dishes*, 100 by 15 mm.

6.9.3 *Sterile Disposable Petri Dishes*, 47 mm in diameter (preferable to test sprays).

6.9.4 Sterile, disposable 1.5-mL polypropylene microcentrifuge tubes.

6.9.5 Sterile, disposable 15- and 50-mL conical-bottom centrifuge tubes.

6.9.6 Sterile, disposable spreaders.

6.9.7 Nonsterile, latex examination gloves.

7. Hazards

7.1 All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in the institutional biosafety regulations. Use equipment and facilities at the biosafety level indicated for the test microorganism. For recommendations on safe handling of microorganisms refer to Ref (**5**).

7.2 Sporicidal products may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, phenol, halogen-containing substances, quaternary ammonium compounds, or any other reagent suspected, claimed, or assumed to have sporicidal activity. Gloves and personal protective clothing or devices are worn during the handling of these

[4] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

materials. A chemical fume hood or other containment equipment is used when performing tasks with hazardous chemical products.

8. Sample Handling and Storage

8.1 Disinfectants are stored following manufacturer's instructions or at room temperature (21 ± 3°C) if the product label does not indicate special storage conditions. Disinfectants requiring dilution or activation (or pH adjustment) before use are diluted or activated and tested within the time period specified by the manufacturer or within the shelf-life time after activation or dilution, if known for the particular disinfectant. If no information is available from the manufacturer or shelf-life after dilution or activation is unknown, then test within 4 h after dilution or activation.

8.2 Stocks of spores are stored refrigerated between 2 and 5°C.

9. Calibration and Standardization

9.1 Refer to the laboratory equipment calibration and maintenance standard operation procedures (SOPs) for details on test methods and frequency of calibration.

10. Procedure

10.1 *Brief Summary*—This method recovers spores by differential elution (in Fractions A, B, and C) by sequentially applying treatments of increasing dislodging strength. The forces to dislodge spores in each fraction are different and not interchangeable (loosely released by washing in A, sonication in B, and incipient germination in C). These steps were selected after trying many of possible combinations to maximize the recovery and accountability of spores.

NOTE 1—This procedure is to be performed by personnel trained in microbiology and with experience in the use of all laboratory equipment listed. No standard or description can replace necessary training and experience. Accordingly, the procedures in this test method are described at a level of detail that should allow their understanding and correct execution by anybody minimally proficient in microbiology and in the use of general laboratory equipment.

10.1.1 Each clean and sterile carrier (either flat 0.5 by 0.5 cm or dipping) receives 10 µL of a spore suspension containing between 1×10^9 and 5×10^9 organisms/mL (resulting in a microbial load between 1 to 5×10^7 spores per carrier), with or without organic load[5] and dried for 10 to 20 h at 20 to 25°C. The carrier loaded with spores is placed inside of a 1.5-mL microcentrifuge tube (labeled A). The sporicidal product being tested, is added to this tube, assuring that the inoculum in the carrier is completely submerged in the fluid (see 10.4.3). Exposure to different temperatures other than room temperature (21 ± 3°C) can be achieved by equilibrating the microcentrifuge tubes at the desired temperature within a laboratory cooler. Control carriers do not receive disinfectant, but instead receive an equal volume of sterile distilled water. The exposure time and temperature may vary according to manufacturer's specifications. After incubation with the disinfectant, ice-cold[6] LB medium with or without neutralizer is added. The carrier is immediately transferred to a new 1.5-mL microcentrifuge tube (labeled B) containing sterile distilled water at room temperature (21 ± 3°C) and sonicated for 5 min. Ice-cold LB medium is added and the carrier is transferred to a new 1.5-mL microcentrifuge tube (labeled C) with LB medium. The Tubes C are incubated in a rotator inside of an incubator at 37°C for 30 min. Ice-cold LB is added and the carrier discarded. The fluid contained in Tubes A, B, and C correspond to Fractions A, B, and C. The surviving spores in each fraction are enumerated by serial dilution and spread on petri dishes containing nutrient agar medium. Culture plates are incubated overnight (at least 12 h) at 37 ± 1°C and colonies are counted. Total spores surviving treatment with disinfectant are calculated by adding the spores counted in Fraction A, plus spores in Fraction B, plus spores in Fraction C. The sporicidal effect of a disinfectant is calculated by subtracting $\log_{10}$ of the total number of spores surviving the treatment with disinfectant from the $\log_{10}$ of the total number of spores in the controls incubated with sterile water. A summary of the method is shown in Figs. 1 and 2.

10.1.2 Use and change frequently nonsterile examination latex gloves for all handling during the procedure to avoid spore carryover.

10.2 *Carrier Inoculation*:

10.2.1 *Carrier Inoculation by Spotting*:

10.2.1.1 Prepare a suspension of spores in sterile distilled water at 5×10^9 spores/mL with or without organic burden.

10.2.1.2 The spores can be concentrated by centrifugation or diluted in water if required. Resuspend spores thoroughly before spotting on carrier.

10.2.1.3 For general sporicidal claims, use glass carriers in 0.5 by 0.5-cm squares (cut from microscope slides). For specific claims, carriers can be made of, for example, silicone medical rubber, light metal armor, building materials, or any material that reflects intended use of the tested disinfectant and that can be cut into 0.5 by 0.5-cm squares, or a combination thereof. Wash carriers three times with sterile distilled water and rinse once with 95 % ethanol. The carriers can be sterilized by autoclaving or other procedures which will not affect the properties of the carrier material.

10.2.1.4 Place the carriers flat inside the lower plate of a sterile plastic petri dish and load each carrier with 10 µL of the 5×10^9 spore/mL suspension as prepared in 10.2.1.1. The fluid must remain on the carrier or the carrier is discarded. Replace the cover of the petri dish and let the carriers dry 10 to 20 h (overnight) inside a biosafety hood.

10.2.2 *Carrier Preparation by Dipping*—If odd-shaped material is to be tested (screws, tubing, and so forth), each carrier device is immersed in a separate microtube with 50 µL of spore suspension, such that each carrier binds 1 to 5×10^7 spores (for experimental examples, see Refs **1, 2**). After immersing the carrier for 30 min in the spore suspension, remove each carrier with sterile forceps and let the carriers dry for 10 to 20 h at 20 to 25°C. Depending on the shape of the carrier, they are placed to dry in any holder that prevents contact with the

[5] Organic load, as bovine serum albumen, horse serum at 5 % (v/v) final concentration (for details on the effect of organic burden refer to Ref (**3**)).

[6] Ice-cold is 0°C. LB is equilibrated in an ice-containing waterbath.

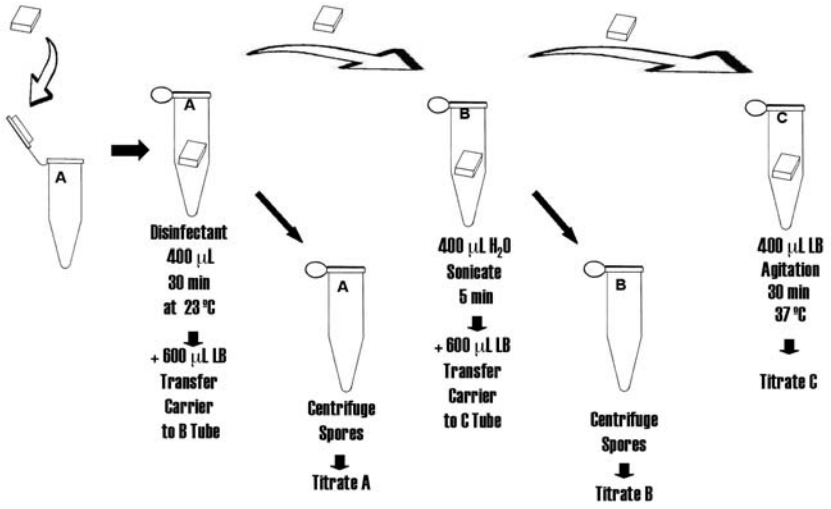

FIG. 1 Scheme of Test Using Carriers Loaded by Spotting

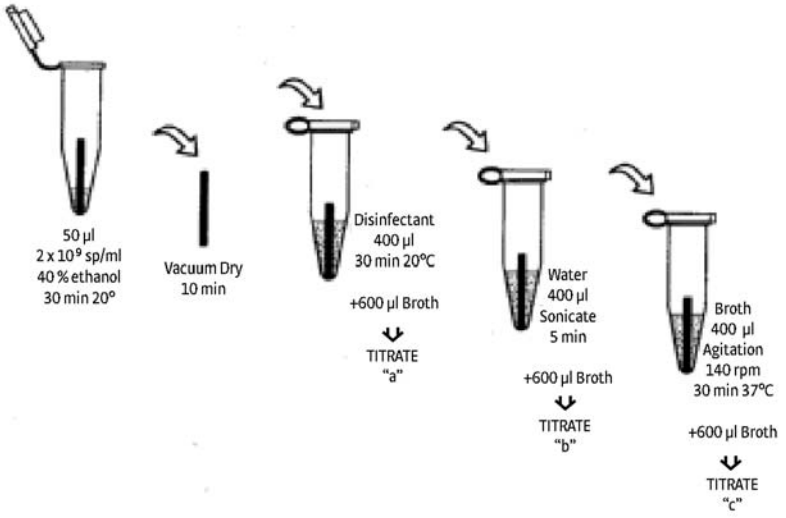

FIG. 2 Scheme of Test Using Carriers Loaded by Dipping

contaminated end (holes drilled in a 6 to 13-mm thick acrylic plate work well to hold small cylindrical items with the contaminated end upward). The dried carriers are placed inside a 1.5-mL microcentrifuge tube until use as described in 10.4.

10.3 *Disinfectant Sample Preparation*:

10.3.1 Before opening the container, shake the container and clean the area around the cap with sterile 95 % ethanol. Remove the cap after the surface is dried, avoiding any touching of the inside of the cap. Remove any container seal attached with a sterile cutting instrument (that is, razor blade, scissor, or forceps).

10.3.2 Pour an appropriate aliquot of the sample into a 50-mL sterile conical bottom centrifuge tube. Do not introduce any instrument or pipet inside the container and close the cap tightly after use.

10.3.3 Ready-to-use products are tested without dilution.

10.3.4 For products requiring dilution, prepare all dilutions with the diluent recommended by the manufacturer or with sterile distilled or HPLC grade water using sterile standardized volumetric glassware.

NOTE 2—It is not mandatory, but highly recommended, to monitor the overall reproducibility of the test method by including cupric ascorbate as a positive control of (intermediate) sporicidal activity. Prepare fresh same-day stock solutions for each of the three ingredients with the following concentrations: 1.5 % wt/vol of cupric ions [as cupric chloride ($CuCl_2 \cdot 2H_2O$)]; 0.3 % wt/vol of *L*-ascorbic acid, and 0.009 % hydrogen peroxide, pH 2.9. Mix equal volumes of each three stock solutions before running a test and use cupric ascorbate as a disinfectant product (**1-4**)).

10.4 *Test Procedure*:

10.4.1 Number all 1.5-mL microcentrifuge tubes on the top. Use letters (A, B, C) to indicate fraction and numbers to identify tube (controls, disinfectants, and replicates). Analyze products and controls by triplicate.

10.4.2 With clean, sterilized tweezers, place one inoculated carrier inside each 1.5-mL microcentrifuge tube labeled A. Avoid touching the inoculated area of the carrier and the sides of the tube. One carrier is added per A tube. Use the same tweezers to set up all A tubes.

10.4.3 *Liquids*—Add 400 µL of disinfectant or control, making sure the carriers are completely submerged in the fluid. Testing tubes contain the sporicidal product or products. All conditions are tested in triplicate. Disinfectant negative controls consist of contaminated carriers exposed to 400 µL of sterile distilled water under the same conditions (time and temperature) as carriers exposed to disinfectants. The exposure conditions are the working conditions specified in the instructions of use provided by the manufacturer. The time that contaminated carriers are exposed to disinfectant is an important variable that should be controlled and monitored with a certified timer. The order in which tubes are processed until the disinfecting reaction is stopped by dilution or neutralization must be kept constant to assure the same exposure interval for spores in each carrier.

10.4.4 *Liquid Sprays*—Place each carrier (inoculated by spotting and dried as in 10.2.1) in the center of one sterile, empty 45 mm petri dish set horizontally. Use triplicate carriers for testing the disinfectant and another set of carrier controls (also in triplicate) sprayed with water. Locate the sprayer with the nozzle perpendicular to the carrier and petri dish at the distance either recommended by the manufacturer or at 30 cm. Make one (or more as recommended by the manufacturer) gentle spray application over each carrier in its own petri dish. Another set of carriers (also triplicate) is sprayed with sterile distilled water with a sprayer similar to the one used to spray the disinfectant. Allow the spray to act on the carriers for the exposure time and at the temperature recommended by the manufacturer or for 30 min at room temperature (21 ± 3°C). Remove carriers and process as in 10.4.6. Any spores mechanically dislodged by the spray jet are accounted for by adding 10 mL of ice cold LB to the petri dish, resuspending any dislodged spores in this recovery LB volume, and subsequently titrating any spore present by serial dilution. We observed that spores dislodged by low-pressure sprays (squeeze type) are dislodged in numbers low enough as to be counted by plating 100 µL directly from the 10 mL of the recovery LB without further dilution. Add any spore dislodged by the spray to total survival in fractions A + B + C as detailed in 11.2.

10.4.5 *Gases*—Place loaded carriers inside a (loosely) covered petri dish and then inside the rack of a vented anaerobic jar (see 5.1). Open stopcock and evacuate jar at or below 700 mm of mercury (as specified by jar manufacturer). Open valve to the gas source and fill the jar with the gas or vapor to be tested. Evacuate and fill the jar three times (as specified by the jar manufacturer). Expose carriers in the tested gas atmosphere for the duration and under temperature, pressure, and humidity conditions specified by the manufacturer of the gaseous sporicidal system or for 30 min at 21 ± 3°C at atmospheric pressure and ambient humidity. Carrier controls are incubated in the same conditions in another anaerobic jar but in the presence of air. After exposure, the jars are evacuated and each carrier is immersed in separate microtubes labeled A that contain 400 µL of ice-cold LB broth. After incubation of spores in broth for 30 min at room temperature (21 ± 3°C), the processing of these carriers continues identically as the processing of liquid-treated carriers in 10.4.6.

10.4.6 After exposing the carriers to the disinfectant (liquid as 10.4.3, sprays as 10.4.4, or gaseous as 10.4.5), add 600-µL ice-cold LB broth.

10.4.7 Transfer the carriers with sterile tweezers from each tube labeled A to a new 1.5-mL sterile microtube (labeled B) containing 400 µL of sterile distilled water.

10.4.8 Fluid remaining in Tube A and containing spores dislodged by incubation with water or disinfectant is considered Fraction A for data calculations.

10.4.9 The residual sporicidal activity of any liquid disinfectants remaining in Tube A after incubation is stopped by the addition of the cold LB broth added in 10.4.6. Dilution with cold LB has been shown to stop various disinfectants formulations and disinfecting chemicals (**1, 2, 3, 4**). This procedure does not add any additional reagent that can interact with spores or the following steps, and therefore, it should be the procedure of choice to stop any residual activity, when appropriate (see 10.4.10).

10.4.10 For those disinfectants whose residual activity would remain detectable after dilution as in 10.4.9, the manufacturer must indicate an appropriate neutralizer. Mixing one volume of concentrated neutralizer at twice the concentration recommended for use (2×) with one volume of LB broth results in the neutralizer at the proper concentration.

10.4.10.1 The neutralizer must be selected in accordance with Test Methods E 1054 and tested as described in Test Methods E 1054, or by using the method to establish neutralizing activity which is described in Appendix X4.

10.4.11 For disinfectants whose residual activity would remain detectable after dilution as in 10.4.9, and for which there is no known neutralizer as indicated in 10.4.10, instead of adding a neutralizer, the residual sporicidal activity is stopped by washing the residual disinfectant from spores through centrifugation. This is done by spinning the microtubes in a microcentrifuge (see 5.1) and aspirating 900 µL of supernatant containing the residual disinfectant without disturbing the spores in the pellet. It is convenient to position each tube in the centrifuge with the hinge oriented outwards in the rotor. This will aid locating the pellet and prevent its aspiration, even if the pellet is too small to visualize. The pellet is washed a second time by gently adding 900 µL of fresh cold LB broth, again without disturbing the pellet.

10.4.12 Sonicate Tube B for 5 min. Add 600 µL of ice-cold LB broth to Tube B and vortex. In large experiments, we suggest the use of adapters to allow vortexing multiple tubes at the same time.

10.4.13 Transfer the carrier with tweezers to a new 1.5-mL tube (labeled C) containing 400 µL of room temperature LB broth.

10.4.14 Fluid remaining in Tube B and containing spores dislodged from the carrier by sonication is considered Fraction B for data calculations.

10.4.15 Tubes labeled C with the carrier in 400 µL of LB broth are incubated for 30 min at 37°C in a rotator (similar results are obtained by incubating in a shaker operating at 140 rpm).

10.4.16 Remove and dispose of the carrier. (If aliquots from all fractions are titrated promptly as in 10.4.18, there is no need to remove the carrier from Tube C.)

10.4.17 Fluid-containing spores dislodged from the carrier by shaking 30 min at 37°C is labeled Fraction C.

10.4.18 The pellet in Fraction A is resuspended by vortexing, and spore survival in Fractions A, B, and C is immediately determined by serial dilution and plating onto nutrient agar plates.

10.4.19 The dilution yielding colonies within a countable range (neither too numerous to count nor absent, see 11.1) from each fraction will depend on the number of surviving spores from the inoculum. The sporicidal activity of disinfectants can vary from those killing spores beyond the limit of detection of the method (7 Logs) to those lacking activity and comparable to the water controls. Therefore, survival in each fraction must be determined by serial dilution. The following dilutions are provided only as a guide where adequate colony-forming units (CFU) have been obtained and not to replace serial dilutions. For disinfectants killing more than 5 Logs, countable plates are obtained by plating the total volume of resuspended Fractions A, B, and C. For disinfectants killing between 3.5 and 5 Logs, countable CFU are generally observed after plating 100 µL of Fraction A diluted 1:100, Fraction B diluted 1:10, and Fraction C undiluted, respectively.

10.4.20 Adequate CFU counts in the controls should be obtained after plating 100 µL of Fraction A diluted 1:40 000, Fraction B diluted 1:10 000, and Fraction C diluted 1:1000, respectively.

10.4.21 Dilution/concentrate, 100 µL, is spread onto nutrient agar petri dishes and incubated overnight (at least 12 h) at 37°C.

11. Calculation

11.1 Count colonies and calculate the total viable spores in each fraction (A, B, and C). For adequate accuracy, the colony count for the controls must fall between 20 and 200 colonies in each (100 by 15-mm) petri dish. Any colony is counted for the disinfectant treated plates.

11.2 *Calculate*—Total viable spores per each carrier is obtained by adding the total colonies in Fraction A + Fraction B + Fraction C. The spores recovered from the control carriers (exposed to water) should be within 10 % of the inoculated spores.

11.3 *Calculate*—Log_{10} of total viable count in each carrier. This log survival value in each carrier is called LS.

11.4 Calculate average and standard deviation of recovered spores in carriers exposed to water using the triplicates from the controls carriers (see 10.4). The log of the average in the controls is called LC.

11.5 For each carrier exposed to disinfectant, calculate the Log_{10} spore killing as:

$$LK = LC - LS \quad (1)$$

11.6 Calculate the LK (Log of Kill) for a disinfectant as the average of the three LK obtained from each triplicate carriers. Inoculating carriers with 1×10^7 to 5×10^7 spores (as described in 10.2), sporicidal activities can be measured from those killing spores up to the limit of detection of the method (7 logs) to those lacking activity and comparable to the water controls.

11.7 The standard deviation for LK is calculated also from the same three values of LK obtained from triplicate carriers.

11.8 A disinfectant is considered to pass the sporicidal three-step method (TSM) when it produces a log killing equal to or greater than the standard assurance level specified for its intended use.

11.9 To visualize the results and identify interactions between disinfectant and carrier surfaces, it is not mandatory, but very useful, to graph DECON treatment versus average survived spores ± standard deviation in each fraction (specific examples of plotting and interpretation can be found in (**1-4**)).

12. Precision and Bias

12.1 Precision is within 1 Log_{10}. The average standard deviation in 54 tests of several liquid and foamy disinfectants and decontaminating products on spores of 4 pathogenic and 3 nonpathogenic strains of *B. Anthracis*, plus spores of *B. globiggi*, *B. subtilis*, *B. turingensis*, and *B. cereus* was 0.33 of one Log_{10}. An independent and rigorous analysis of the performance of this method in three federal laboratories resulted in a mean Log_{10} spore counted in the controls of 7.493 $Logs_{10}$ and a standard error of 0.057.

13. Keywords

13.1 biodefense; biological protection; carrier surfaces; decontamination; disinfection; liquid sterilization; microbicidal effect; sporicidal efficacy

APPENDIXES

(Nonmandatory Information)

X1. PREPARATION OF BACILLUS SPORES IN LIQUID MEDIA

X1.1 *Equipment*:

X1.1.1 Incubator, 37 ± 1°C.
X1.1.2 Shaker incubator, 35 ± 1°C.
X1.1.3 Beckman J2-21 centrifuge (rotor JA 12).
X1.1.4 50-mL centrifuge tubes.
X1.1.5 Test tube racks for microcentrifuge, 15- and 50-mL tubes.
X1.1.6 Tube rotator for 15- and 50-mL tubes.
X1.1.7 Nitrile gloves.
X1.1.8 10-mL pipets.
X1.1.9 Pipeting device.
X1.1.10 Micropipet (Eppendorf type P20, P200, P1000).
X1.1.11 Pipet tubes.
X1.1.12 Hazardous waste disposal bag with support.
X1.1.13 Phase contrast microscope.
X1.1.14 Microscope slides.
X1.1.15 Microscope cover glass.
X1.1.16 Immersion oil.
X1.1.17 Permanent marker.
X1.1.18 Ice bucket with ice.

X1.2 *Reagents*:

X1.2.1 One tube (5 mL) with trypticase soy broth.
X1.2.2 500-mL flask containing 80-mL sporulation Medium S.
X1.2.3 Nutrient agar plates.
X1.2.4 Distilled water.
X1.2.5 20 % ethanol.

X1.3 *Sporulation Media (Medium S)*:

X1.3.1 Nutrient broth, 8 g.
X1.3.2 KCL (1.2*M*), 10 mL.
X1.3.3 NaOH (1*M*), 5 mL.
X1.3.4 $MgSO_4H_2 \cdot 7H_2O$ (1.2 % W/V), 10 mL.
X1.3.5 Distilled H_2O.
X1.3.6 Autoclave and then add the following sterile solutions:
X1.3.6.1 $Ca(NO_3)_2$ (0.25*M*), 30 mL.
X1.3.6.2 $FeCl_2$ (10*mM*), 0.1 mL.
X1.3.6.3 $MnCl_2$ (10*mM*), 1 mL.

X1.4 *Spore-Growing Procedure*:

X1.4.1 *Step 1: Preparation of Fresh Streak Plates (see Section 7)*—In a Biosafety Cabinet II (BSC II), scratch the surface of the frozen primary stock (PS) strain that is going to be produced with a sterile loop and stride an agar plate to generate isolated colonies. Leave at 37°C overnight.

X1.4.2 *Step 2: Preparation of the Inoculums*:

X1.4.2.1 Take a loopful of cells from an isolated colony of the bacillus strain fresh plate from Step 1.
X1.4.2.2 Inoculate 5 mL of Typticase Soy Broth (TSB).
X1.4.2.3 Place the label tube on the rotator at 37°C.
X1.4.2.4 Incubate about 6 h.

X1.4.3 *Step 3: Inoculation of Sporulation Media*:

X1.4.3.1 Use 500 µL of culture to inoculate 250 mL of sporulation Medium S modified in a 2-L flask at 36°C.
X1.4.3.2 Transfer the flask to a shaker incubator at 36°C and 230 rpm.
X1.4.3.3 Examine the spore preparation every day on the phase contrast microscope until spores are formed and sporangia has disintegrated (no vegetative cells, 2 to 3 days).

X1.5 *Harvest and Rinses*:

X1.5.1 Transfer the culture to 50-mL tubes. From this point on, always keep tubes on ice.
X1.5.2 Centrifuge spores at 4°C at 3000 rpm for 15 min in a Beckman J2-21 centrifuge rotor JA 12.
X1.5.3 Decant supernatant and resuspend the pellet of each tube in 40-mL cold (4°C) sterile water. Vortex to resuspend.
X1.5.4 Wash six to ten times with cold distilled water, each time centrifuging for 15 min at 3000 rpm, until clean spores and no more debris is observed under the microscope.
X1.5.5 Resuspend the pellet in 40-mL cold distilled H_2O and transfer to a new 50-mL tube.
X1.5.6 Observe under the microscope. Follow X1.5.7 only if some sporangia can be seen; in other cases, skip X1.5.7.
X1.5.7 Incubate overnight at 4°C in a rotating mixer or refrigerated shaker at 5 to 8°C.
X1.5.8 Centrifuge spores at 4°C at 3000 rpm for 15 min in a Beckman J2-21 centrifuge rotor JA 12.
X1.5.9 Resuspend the pellet in cold distilled H_2O (approximately four volumes of the pellet volume).
X1.5.10 Examine spores preparation on the contrast phase microscope and take a photograph for the records.
X1.5.11 Make aliquots of 1 mL or plate for titration and make aliquots of 1 to 5 × 10^9 spores/mL.

X2. QUALITY CONTROL AND TITRATION OF SPORE PREPARATIONS

X2.1 Quality control of spore preparations and criteria for accepting batches follows a technique described in Refs (**1, 4**).

X2.2 Set up biosafety cabinet with decontamination solution, liquid biohazardous waste receptacle, biohazardous waste bag, and absorbent pads. Gather Trypticase Soy Agar (TSA) plates and aliquot of spores for titration, 2.5 N HCl, 2.5 N NaOH, LB broth, and microcentrifuge tubes and racks. Place items in the biosafety cabinet.

X2.3 Label three sets of ten microcentrifuge tubes with dilution factor and organize in racks. One set is for titrating spores before freezing, the second set will be for titrating spores after HCl treatment as described in Appendix X3, and the third for titrating spores after they have been frozen. Starting with the first tube of the first set, transfer 10 µL of spores into 990 µL of LB broth. Make a 1:100 dilution in the subsequent tubes up to 10^{-8}.

X2.4 Using an aliquot of spores from the same batch that has been frozen at least overnight, repeat X2.3, making the dilutions in the same way.

X2.5 Plate 100 µL of each dilution and incubate at 37°C overnight (at least 12 h). Count colonies and make calculations.

X2.6 Dispose of solid waste in the biohazardous waste bags. Decontaminate the biosafety cabinet using a 10 % bleach.

X3. HYDROCHLORIC TREATMENT

X3.1 Add 10 µL of Bacillus spore suspension to two microcentrifuge tubes.

X3.2 Add to one tube 90-µL sterile water as control.

X3.3 Add to the other tube 90 µL of 2.5 N HCl.

X3.4 Incubate precisely 5 min at room temperature mixing in a rotator.

X3.5 Stop reaction in tube with HCl by adding 810 µL of cold (4°C) LB plus 90 µL of 2.5 N NaOH.

X3.6 Add 900 µL of LB to the tube control.

X3.7 Proceed to a serial dilution as mentioned in X2.3.

X3.8 A spore preparation is accepted when: *(1)* microscopic observation of spores stained with trypan blue reveals 10 vegetative cells (shaped as rods) or fewer during the observation of 1000 spores (round shape), and *(2)* survival of spores after 5-min incubation in 2.5 N HCl is not significantly different than the number of spores in the water-treated control.

X4. NEUTRALIZER TESTING PROTOCOL

X4.1 *Materials*:

X4.1.1 Sterile microcentrifuge tubes, 1.5 mL.
X4.1.2 LB: Luria-Bertani broth.
X4.1.3 *Bacillus sp.* spores.
X4.1.4 Neutralizer prepared in LB at a working concentration such that when 600 µL are diluted to 1 mL, the final concentration corresponds to that specified for use.
X4.1.5 Disinfectant.
X4.1.6 Sterile distilled water.
X4.1.7 Nutrient agar plates.

X4.2 *Preparation of Materials*:

X4.2.1 Label microcentrifuge tubes with numbers 1 to 12 and place in a rack. These will be the reaction tubes in which the actual mixing and incubation is done.
X4.2.2 Label one to twelve dilution tubes with numbers 1 to 12 and add 990 µL of LB to each.
X4.2.3 Prepare nutrient agar plates as required for titration in X4.3.11.

X4.3 *Method*:

X4.3.1 Add to Tubes 1-6 400 µL of water.

X4.3.2 Add to Tubes 7-12 400 µL of disinfectant.

X4.3.3 Equilibrate Tubes 1-9 at 0°C in crushed ice for 10 min.

X4.3.4 Equilibrate Tubes 10-12 at the temperature specified by the manufacturer of the disinfectant or at 21 ± 3°C for 10 min.

X4.3.5 Add to Tubes 4-6 600 µL of neutralizer in ice-cold LB. These tubes with water and neutralizer will be the neutralizer controls.

X4.3.6 Add to Tubes 7-9 600 µL of ice-cold neutralizer in LB. These tubes with disinfectant and neutralizer (in LB) will measure the inhibition of killing by the neutralizer.

X4.3.7 Add to each tube (1-12), 10 to 50 millions *Bacillus sp.* spores, close tubes, vortex for 15 s.

X4.3.8 Start timing a 30-min incubation period with a calibrated watch.

X4.3.9 After incubation, add to Tubes 1-3 600 µL of ice-cold LB. These tubes with water and LB are the survival controls.

X4.3.10 Add to Tubes 10-12 600 µL of ice-cold LB. These tubes with disinfectant and LB will be the disinfectant controls.

X4.3.11 Transfer 10 µL from each Reaction Tubes 1-12 to corresponding dilution tubes readied in X4.2.2 and mix by vortexing.

X4.3.12 Titrate surviving spores in each of the dilution tubes by serial dilution and sampling 100 µL onto agar plates. Recommended dilutions are 1:10 000 for Tubes 1-9 (survival and neutralizer controls as well as for inhibition tubes). Depending on the activity of the disinfectants, we recommend to plate the disinfectant controls (Tubes 10-12) undiluted or 1:100, or both.

X4.3.13 Incubate agar plates between 12 to 20 h at 37°C.

X4.3.14 Count colonies.

X4.3.15 Spore survival in Tubes 1-3 and 4-6 must be high and similar. The neutralizer is deemed to have a sporicidal effect if survival in Tubes 4-6 (neutralizer controls) is significantly lower than in Tubes 1-3 (survival controls).

X4.3.16 Killing of spores in suspension by the disinfectant in tubes (10-12) should reduce colony-forming units typically by at least 6 Log (compared to that obtained in Tubes 1-6).

X4.3.17 A substance neutralizes the sporicidal activity of the disinfectant when spore survival in Tubes 7-9 (inhibition) is not significantly different than spore survival in Tubes 1-3 (survival controls).

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