6.2.04

AOAC Official Method 955.15
Testing Disinfectants against *Staphylococcus aureus*

Use-Dilution Method
First Action 1955
Final Action 1959
Revised 2006

(Applicable to testing disinfectants with H_2O to determine maximum dilutions effective for practical disinfection. These microbiological methods are technique-sensitive methods in which careful adherence to the method with identified critical control points, good microbiological techniques, and quality controls is required for proficiency and validity of results. These methods have been validated using distilled water only without soil challenge; *see* **A**(**c**) for detailed information on H_2O.)

Notes: (*1*) All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in the institutional biosafety regulations. Use the equipment and facilities indicated for the test organism. For recommendations on safe handling of microorganisms refer to the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories manual. (*2*) Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Personal protective clothing or devices are recommended during the handling of these items for purpose of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment may be employed when appropriate during 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. (*3*) References to water (H_2O) mean reagent grade, except where otherwise specified. (*4*) Commercial dehydrated media made to conform to the specified recipes may be substituted.

A. Reagents

(**a**) *Culture media for stock and test cultures*.—(*1*) *Nutrient broth*.—Boil 5 g beef extract (Difco; paste or powder), 5 g NaCl, and 10 g peptone (Anatone, peptic hydrolysate of pork tissues, manufactured by American Laboratories, Inc., Omaha, NE 68127, USA) in 1 L H_2O 20 min and dilute to volume with H_2O; adjust to pH 6.8 ± 0.1. (If colorimetric method is used, adjust broth to give dark green with bromothymol blue.) Filter through paper (Whatman No. 4, or equivalent), place 10 mL portions in 20   150 mm test tubes, and steam sterilize 20 min at 121 C. Use this broth for daily transfers of test cultures.

(*2*) *Synthetic broth*.—*Solution A*.—Dissolve 0.05 g L-cystine, 0.37 g DL-methionine, 0.4 g L-arginine HCl, 0.3 g DL-histidine, 0.85 g L-lysine HCl, 0.21 g L-tyrosine, 0.5 g DL-threonine, 1.0 g DL-valine, 0.8 g L-leucine, 0.44 g DL-isoleucine, 0.06 g glycine, 0.61 g DL-serine, 0.43 g DL-alanine, 1.3 g L-glutamic acid HCl, 0.45 g L-aspartic acid, 0.26 g DL-phenylalanine, 0.05 g DL-tryptophan, and 0.05 g L-proline in 500 mL H_2O containing 18 mL 1 N NaOH.

Solution B.—Dissolve 3.0 g NaCl, 0.2 g KCl, 0.1 g $MgSO_4$ 7 H_2O, 1.5 g KH_2PO_4, 4.0 g Na_2HPO_4, 0.01 g thiamine HCl, and 0.01 niacinamide in 500 mL H_2O.

Mix Solutions A and B, final pH should be 7.1 ± 0.1, dispense in 10 mL portions in 20   150 mm tubes, and steam sterilize 20 min at 121 C. Before using for daily transfers of test cultures, aseptically add 0.1 mL sterile 10% glucose solution per tube. Grow cultures with tube slanted.

(*3*) *Nutrient agar*.—Dissolve 1.5% Bacto agar (Difco) in nutrient broth and adjust to pH 7.2–7.4 (blue-green with bromothymol blue) or in synthetic broth, tube, steam sterilize, and slant.

(*4*) *Subculture media*.—Use (i), (ii), or (iii).

(i) *Nutrient broth*.—Described in (**a**)(*1*).

(ii) *Fluid thioglycolate medium USP*.—Mix 0.5 g L-cystine, 0.75 g agar, 2.5 g NaCl, 5.5 g glucose H_2O, 5.0 g H_2O soluble yeast extract, and 15.0 g pancreatic digest of casein with 1 L H_2O. Heat on water bath to dissolve, add 0.5 g Na thioglycolate or 0.3 g thioglycolic acid, and adjust with 1 N NaOH to pH 7.1 ± 0.2. If filtration is necessary, reheat without boiling and filter hot through moistened filter paper. Add 1.0 mL freshly prepared 0.1% Na resazurin solution, transfer 10 mL portions to 20   150 mm tubes, and steam sterilize 20 min at 121 C. Cool at once to 25 C and store at 20–30 C, protected from light.

(iii) *Letheen broth*.—Dissolve 0.7 g lecithin (American Lecithin Co., Oxford, CT 06478, USA) and 5.0 g polysorbate 80 (Tween 80, or equivalent) in 400 mL hot water, and boil until clear. Add 600 mL solution of 5.0 g beef extract (Difco; paste or powder), 10 g peptone (Anatone), **a**(*1*), and 5 g NaCl in H_2O and boil 10 min. Adjust with 1 N NaOH and/or 1 N HCl to pH 7.0 ± 0.2 and filter through coarse paper; transfer 10 mL portions to 20   150 mm tubes, and steam sterilize 20 min at 121 C.

(iv) *Other subculture media*.—Use (*4*)(ii) with 0.7 g lecithin (Alcolec Granules, American Lecithin Co.) and 5.0 g polysorbate 80 (Tween 80, or equivalent) added; or suspend 29.8 g dehydrated fluid thioglycolate medium (Difco), 0.7 g lecithin and 5.0 g polysorbate 80 in 1 L H_2O, and boil until solution is clear. Cool, dispense in 10 mL portions in 20   150 mm tubes, and steam sterilize 20 min at 121 C. Store at 20–30 C. Protect from light.

(**b**) *Test organism*, *Staphylococcus aureus*.—ATCC 6538. Maintain stock culture on nutrient agar slants by monthly (30 ± 2 days) transfers. Every 10 to 12 months, initiate new stock cultures from lyophilized culture obtained directly from a reputable supplier (ATCC or equivalent). Initiate cultures according to supplier recommendations or equivalent. For stock cultures, streak inoculate nutrient agar slants (inoculum taken from the initial reconstituted culture) and incubate at 36 ± 1 C for 48 ± 2 h. Every 30 ± 2 days inoculate a new set of stock culture tubes from a current stock culture tube. Incubate the new stock cultures 36 ± 1 C for 48 ± 2 h. Following incubation, store the cultures at 2–5 C for 30 ± 2 days. Repeat cycle for 1 year.

(**c**) *Sterile water*.—Prepare stock supply of H_2O in 1 L flasks with closures, sterilize 20 min at 121 C, and use to prepare dilutions of the test substance. Use reagent grade water, which should be free of substances that interfere with analytical methods. Any method of preparation of reagent grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent grade water when used in the proper arrangement. *See* Standard Methods for the Examination of Water and Wastewater for details on reagent grade water.

(**d**) *Sodium hydroxide solution*.—Approximately 1 M (4%). (For cleaning metal carriers before use.)

B. Apparatus

(**a**) *Pipets and glassware*.—(*1*) *Volumetric pipets and volumetric flasks*.—Various volumes for disinfectant preparation.

(*2*) *Test tubes*.—For disinfectant, autoclavable 25 150 mm or 25 100 mm (Bellco Glass Inc., Vineland, NJ 08360-3493, USA); reusable or disposable 20 150 mm (for cultures/subcultures). Cap with closures before sterilizing.

Sterilize all glassware 2 h in hot air oven at 180 C or steam sterilize for a minimum of 20 min at 121 C with drying cycle.

(**b**) *Water bath*.—Constant temperature, relatively deep water bath capable of maintaining 20 ± 1 C.

(**c**) *Racks or other tube holding device*.—Any convenient style.

(**d**) *Transfer loops and hooks or equivalent*.—(*1*) *Transfer loop*.—Make 4 mm id single loop at end of 50–75 mm (2–3 in.) Pt or Pt alloy wire No. 23 B&S gage or 4 mm loop fused on 75 mm (3 in.) shaft (available from Johnson Matthey, West Chester, PA 19380, USA). Fit other end in suitable holder. Bend loop at 30 angle with stem. Volumetric transfer devices may be used instead of transfer loops. (*2*) *Hooks*.—Make 3 mm right angle bend at end of 50–75 mm nichrome wire No. 18 B&S gage. Have other end in suitable holder.

(**e**) *Carriers*.—Polished stainless steel cylinders (penicillin cups), 8 ± 1 mm od, 6 ± 1 mm id, length 10 ± 1 mm, of type 304 stainless steel, SS 18-8 (S&L Aerospace Metals, Maspeth, NY 11378, USA, or Fisher Scientific, e.g., Cat. No. 7-907-5 as of January 2006). Discard carriers that are visibly damaged (dull, chipped, dented, or gouged). Before the stainless steel carriers can be used for use-dilution testing, each individual carrier must be screened biologically. This is accomplished by performing an AOAC use-dilution test on each carrier using a 48–54 h old culture of *Staphylococcus aureus* (ATCC 6538) and 500 ppm alkyl dimethyl benzyl ammonium chloride with alkyl chain distribution C14, 50%, C12, 40%, C16, 10% (e.g., BTC-835 Stepan Co., Northfield, IL 60093, USA). Dilute chemical in water to 500 ppm and use as the test disinfectant. Test at 20 ± 1 C with a 10 min exposure period. Discard those carriers giving positive results. In subsequent testing of samples, carriers in tubes showing growth must be rescreened and may not be reused unless screen tests result in no growth.

(**f**) *Positive displacement pipet*.—With corresponding sterile tips able to deliver 10 L.

(**g**) *Timer*.—Any certified timer that can display time in seconds.

(**h**) *Petri dishes*.—Matted with 2 layers of S&S No. 597 or Whatman No. 2, 9 cm filter paper.

C. Operating Technique

(**a**) *Carrier preparation*.—Visually screen carriers. Discard cylinders that are visibly damaged (dull, chipped, dented, or gouged). Soak carriers overnight (approximately 12 h) in 1 N NaOH, rinse several times with tap water. Collect a portion of the last rinse water and add 2–3 drops of 1% phenolphthalein; if any NaOH remains, the phenolpthalein turns pink, indicating the need for additional rinsing. Continue to rinse the carriers until the addition of phenolpthalein does not produce a color change, then rinse twice more with H_2O. Place cleaned carriers in 25 150 mm test tubes with closures, cover with water, sterilize 20 min at 121 C, cool, and hold at room temperature up to 3 months; then reclean and sterilize prior to use.

(**b**) *Test culture preparation*.—Initiate test culture by inoculating a 10 mL tube (20 150 mm) of nutrient broth or synthetic broth from a stock slant. Transfer one 4 mm id loopful (or use a 10 L certified transfer loop or a calibrated positive displacement pipet to deliver 10 L) of inoculum from the stock culture into the broth. Make at least 3 consecutive 24 ± 2 h transfers (use one 4 mm id loopful, or a 10 L certified transfer loop, or a calibrated positive displacement pipet to deliver 10 L) in 10 mL nutrient broth or synthetic broth incubated at 36 ± 1 C. Up to 30 ± 2 total transfers are allowed. If only one of the consecutive 24 h transfers has been missed, it is not necessary to repeat the previous 3-day sequence prior to the inoculation of the 48–54 h test culture. For this final subculture step, inoculate for the test procedure, a sufficient number of 25 150 mm tubes containing 20 mL nutrient or synthetic broth; incubate 48–54 h at 36 ± 1 C. Using a Vortex-style mixer, mix nutrient broth test cultures 3–4 s and let stand 10 min at room temperature before continuing. Remove the upper portion of each culture, leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. Aliquot 20 mL portions into sterile 25 150 mm test tubes.

Using a sterile hook, aseptically transfer 20 carriers prepared as above into each of the tubes containing the test culture. Drain the water from the carriers by tapping them against the side of the tube before transferring. Multiple carriers may be transferred on a single wire hook. The test culture must completely cover the carriers. If a carrier is not covered, gently shake the tube, or reposition the carrier within the tube with a sterile wire hook. Be sure to inoculate a sufficient number of carriers for the test. (Alternately, the water may be siphoned off the carriers and the 20 mL test culture added directly to the carriers without transferring.)

After 15 ± 2 min contact period, remove carriers using flamed nichrome wire hook; shake carrier vigorously against side of the tube to remove excess culture, and place on end in vertical position in sterile Petri dish matted with 2 layers of Whatman No. 2 (or equivalent) filter paper, making sure that carriers do not touch to prevent improper drying. Place no more than 12 carriers in a Petri dish. Carriers that touch or fall over cannot be used for testing and must be removed and recleaned. Once all of the carriers have been transferred, cover and place in incubator at 36 ± 1 C and let dry 40 ± 2 min. Inoculated carriers must be used on day of preparation.

(**c**) *Disinfectant sample preparation*.—Equilibrate the water bath and allow it to come to 20 1 C or the temperature specified (1 C). Prepare the disinfectant dilutions within 3 h of performing the assay unless test parameters specify otherwise. Ready-to-use products are tested as received; no dilution is required.

Aseptically prepare disinfectant samples as directed. Prepare all dilutions with sterile standardized volumetric glassware. For diluted products, use ≥1.0 mL or 1.0 g of sample disinfectant to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Round to 2 decimal places toward a stronger product. Dispense 10 mL aliquots of the diluted disinfectant or ready-to-use product into 25 100 mm (or 25 150 mm) test tubes, one tube per carrier. Place tubes in the equilibrated water bath for approximately 10 min to allow test solution to come to specified temperature.

(**d**) *Test procedure*.—After the required drying time, the carriers are sequentially transferred from the Petri dish to the test tubes containing the disinfectant at appropriate intervals. Use a certified timer to time the transfers. Modify intervals to accommodate exposure times other than 10 min. (*Note*: Proper execution of

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transfer step is one of the most critical, technique-sensitive areas of method. False positives will result if sides of tube are touched.)

One carrier is added per tube. Immediately after placing carrier in the test tube, briefly swirl tube before placing it back in the bath. The carrier must be deposited in the tube within 5 s of the prescribed drop time. Using alternating hooks, flame the hook and allow it to cool after each carrier transfer. When lowering the carriers into the disinfectant tubes, neither the carrier itself nor the tip of the wire hook can touch the interior sides of the tube. Individual manipulation of carriers is required; the use of semi-automated ring carrier is prohibited. (*Note*: Above step is one of the most critical, technique-sensitive areas of method. False positives can result from transfer of live organisms to sides of tubes due to contact or aerosol formation.) If the side is touched, mark or note the tube; the tube is not counted if it yields a positive result.

After the carriers have been deposited into the disinfectant, and the exposure time is complete, the carriers are then transferred in a sequentially timed fashion into the subculture tubes containing the appropriate neutralizer (10 mL in 20 150 mm tubes). The carrier is removed from the disinfectant tube with a sterile hook, tapped against the interior sides of the tube to remove the excess disinfectant and transferred into the subculture tube. Flame hook after each carrier transfer. The remaining carriers are moved into their corresponding subculture tubes at the appropriate time. As with the transfers to the disinfectant tubes, transfers into subculture tubes should be within 5 s of the actual transfer. Contact of the carrier to the interior sides of the subculture tube during transfer should be avoided as much as possible.

After the carrier is deposited in the subculture tube, recap the subculture tube and shake thoroughly. Place subculture tubes into 36 1 C incubator and incubate for 48 2 h.

The subculture medium (primary subculture tube) must serve as a suitable neutralizer for the test substance as well as an adequate growth medium which must be confirmed in advance or concurrently with the use-dilution test. Report results as + (growth), or – (no growth) as determined by presence or absence of turbidity. Growth in tubes should be checked by Gram stain to ensure that no contamination is present. Check ≥20% of positive tubes. In the event that there are positive carriers present in the test, the test may be repeated in order to confirm the outcome. Once the results are recorded, it is important that the carriers be reprocessed before use in another study.

Note: If a secondary subculture tube is deemed necessary to achieve complete neutralization, then transfer carrier from the primary tube to a secondary tube of sterile medium after a minimum of 30 ± 5 min from the end of the initial transfer. Transfer the carriers using a sterile wire hook to a second subculture tube containing 10 mL of the appropriate subculture medium which may contain a suitable neutralizer. Move the carriers in order but the movements do not have to be timed. Thoroughly shake the subculture tubes after all of the carriers have been transferred. Incubate both the primary and secondary subculture tubes 48 2 h at 36 1 C. Record the results from both tubes (a carrier set) after this time.

(**e**) *Viability controls*.—On the day of testing, place 2 dried inoculated carriers into separate tubes containing 10 mL neutralizing subculture broth. Incubate tubes for 48 ± 2 h at 36 ± 1 C. Positive growth in each tube validates test system viability.

(**f**) *Verification of positive carriers*.—Positive carriers are examined for test organism by inoculating onto TSA and selective media. Incubate TSA and selective media plates 18–24 h at 36 ± 1 C. Examine plates for colonial morphology characteristic to the test organism (conforming to the morphology in Bergeys manual). Growth from subculture media should be checked by Gram stain. Any suitable identification can also be done.

Maximum dilution of germicide which kills test organism on 10 carriers in 10 min interval represents presumed maximum safe use-dilution for practical disinfection.

Note: While killing in 10 of 10 replicates specified provides reasonably reliable index in most cases, killing in 59 of 60 replicates is necessary for confidence level of 95%.

References: *J. Bacteriol*. **49**, 526(1945).
Am. J. Vet. Res. **9**, 104(1948).
JAOAC **36**, 466(1953); **70**, 318(1987);
71, 117(1988); **72**, 116(1989).
ASTM International Method E 1054–Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents.
Standard Methods for the Examination of Water and Wastewater (2005) 21st Ed., American Public Health Association, Washington, DC, USA.
Biosafety in Microbiological and Biomedical Laboratories (1999) 4th Ed., U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health.

Additional Guidance

The information provided in this section is not considered a component of the official test; rather it serves as procedural guidance to augment use-dilution testing of specific antimicrobial products and specific test conditions as the need arises.

A. Neutralization Confirmation

A neutralization confirmation test must be performed in advance or in conjunction with the use-dilution test. Historical use of neutralizer media for specific active ingredients may also be taken in consideration. A neutralization confirmation procedure must demonstrate the recovery of a low level (e.g., 10–100 CFU) of the test organism in the subculture media. For example:

(**a**) At the conclusion of the incubation period, randomly select at least one negative tube for each 10 tubes tested to be used in neutralization confirmation. Dilute a 24–48 h culture of the test organism using phosphate buffer dilution water to achieve 100–1000 CFU/mL. Add 0.1 mL diluted suspension to each tube. Confirm number of cells in the suspension in duplicate by pour plate or spread plates. Incubate tubes and plates for 48 ± 2 h at 36 ± 1 C. Count colonies on plates to determine inoculum level. Examine tubes for growth. Growth in tubes indicates effective neutralization.

(**b**) In a separate assay to simulate actual test conditions, expose a sterile carrier to the test material and transfer to subculture medium (or both primary and secondary tubes if used in the efficacy test) as in the test procedure. Immediately following the transfer, inoculate the tube(s) with 10–100 CFU/tube of the specified culture and incubate 48 ± 2 h at 36 ± 1 C. Confirm number of cells in the suspension in duplicate by pour plate or spread plates. Count colonies on plates to determine inoculum level. Examine tubes for growth. Growth in tubes indicates effective neutralization.

(**c**) *See also* Method ASTM E 1054.

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B. Quantitation of Test Organism on Carriers

Perform count on at least 3 carriers for each set of carriers used in testing. Place each dried inoculated carrier into 10 mL neutralizing broth (e.g., letheen broth). Recover bacteria from the carrier by sonication for 30 s to 5 min or mixing on a Vortex mixer for 30 s. Alternatively, carriers may be pooled in neutralizing broth at the same 1 carrier per 10 mL ratio prior to sonication or mixing on a Vortex mixer. Prepare serial dilutions in phosphate buffer dilution water and plate in duplicate using standard plating procedures. Incubate at 36 ± 1 C for 24–48 h and record the number of colonies.

C. Hard Water

For products requiring hard water, *see* **960.09E** (*see* 6.3.03).

D. Organic Burden

For one-step cleaner disinfectants, mix on a Vortex mixer the 48–54 h test culture. Allow culture to stand for 10 min before using. For a 5% preparation, pipet 19 mL culture and 1 mL organic soil/serum into a 25 150 mm test tube (this volume will allow testing of 20 carriers), mix, or use 9.5 mL culture and 0.5 mL organic load/serum (this volume will allow testing of 10 carriers).

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