US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for Germicidal Spray Products as Disinfectants (GSPT): Testing of Staphylococcus aureus, Pseudomonas aeruginosa, and Salmonella enterica

SOP Number: MB-06-08

Date Revised: 09-22-14

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Title	Germicidal Spray Products as Disinfectants (GSPT): Testing of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*
Scope	Describes the germicidal spray products test methodology (see 15.1) to determine efficacy of spray formulations against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica* on hard surfaces. Additional testing guidance is provided for Carbapenem Resistant *Klebsiella pneumoniae* – refer to Attachment 5 for details.
Application	The methodology described in this SOP is used to evaluate the performance of spray formulations (pump, trigger, aerosols) against the prescribed test microbes.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	Abbreviations/definitions are provided in the text.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOP EQ-01, EQ-02, EQ-03, EQ-04 and EQ-05 for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. Any disruption of the *Pseudomonas aeruginosa* pellicle resulting in the dropping or breaking of the pellicle in culture before or during its removal renders that culture unusable. 2. Prior to inoculation, ensure that the carriers are dry (inside Petri dishes). Moisture can interfere with the concentration and drying of the inoculum on the glass slide carrier. 3. Any inoculated carrier that is wet at the conclusion of the carrier drying period should not be used.
8. Non-conforming Data	1. Sterility and/or viability controls do not yield expected results. 2. The mean log density for control carriers falls outside the specified range. Note: The prescribed minimum and maximum carrier counts also account for the addition of 5% organic soil to the inoculum. a. The mean *TestLD* for carriers inoculated with *S. aureus* and *P. aeruginosa* must be at least 5.0 (corresponding to a geometric mean density of 1.0×10^5) and not above 6.5 (corresponding to a geometric mean density of 3.2×10^6); a mean *TestLD* below 5.0 and above 6.5 invalidates the test, except for two retesting scenarios (outlined in the study protocol). b. The mean *TestLD* for carriers inoculated with *S. enterica* must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 5.5 (corresponding to a geometric mean density of 3.2×10^5); a mean *TestLD* below 4.0 and above 5.5 invalidates the test, except for two retesting scenarios (outlined in the study protocol). 3. No contamination is acceptable in the test system.

	4. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. There are time sensitive steps in this procedure including the use periods of the inoculated carriers and the test chemical. 2. Verify the volume of dilution blanks, neutralizer tubes, and subculture tubes in advance and adjust accordingly.
11. Special Apparatus and Materials	1. *Subculture media* (e.g., letheen broth, fluid thioglycollate medium, and Dey/Engley broth). Note: Commercial media made to conform to the recipes provided in AOAC Method 961.02 may be substituted. 2. *Test organisms*. *Pseudomonas aeruginosa* (ATCC No. 15442), *Staphylococcus aureus* (ATCC No. 6538) and *Salmonella enterica* (ATCC No. 10708) obtained directly from ATCC. 3. *Culture media*. Note: Commercial media (e.g., synthetic broth) made to conform to the recipes provided in AOAC Method 961.02 may be substituted. a. *Synthetic broth (10 mL tubes).* Use for daily transfers and final test cultures of *S. aureus, P. aeruginosa* and *S. enterica.* b. *Nutrient broth (10 mL tubes).* Alternatively, use for daily transfers and final test cultures of *P. aeruginosa.* 4. *Trypticase soy agar (TSA).* For use in propagation of the test organism to generate frozen cultures and as a plating medium for carrier enumeration. Alternately, TSA with 5% sheep blood (BAP) may be used. 5. *Sterile water*. Use reagent-grade water 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. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water. 6. *Carriers*. Glass Slide Carriers, 25 mm × 25 mm (or comparable size) borosilicate glass cover slips with number 4 thickness. Refer to SOP MB-03, Screening of Stainless Steel Cylinders, Porcelain Cylinders and Glass Slide Carriers Used in Disinfectant Efficacy Testing. 7. *Specialized glassware.* For cultures/subcultures, use autoclavable 38 × 100 mm medication tubes (Bellco Glass Inc., Vineland, NJ). Cap tubes with closures before sterilizing. For glassware used to prepare test chemical, refer to SOP MB-22.

	8. *Spray Disinfectant Apparatus*. Refer to Attachment 4. 9. *Micropipettes*. For performing culture transfers and serial dilutions. 10. *Positive displacement pipette.* Use with corresponding sterile tips able to deliver 0.01 mL. 11. *Timer*. For managing timed activities, any certified timer that can display time in seconds. 12. *Electronic Plate Scanning Device*. 3M™ Petrifilm™ Plate Reader, 3M Food Safety, St. Paul, MN, USA, Cat. No. 6499, or equivalent. 13. *Vitek 2 Compact*. Alternative for microbe confirmation.
12. Procedure and Analysis	Prior to testing, perform the neutralization assay to determine if secondary subculture tubes are necessary (refer to SOP MB-17, Neutralization Confirmation). Refer to Attachment 5 for details on testing with Carbapenem Resistant *Klebsiella pneumoniae.* The AOAC Germicidal Spray Products Test Processing Sheet (see section 14) must be used for tracking testing activities.
12.1 Test Culture Preparation	a. Defrost a single cryovial at room temperature and briefly vortex to mix. Add 10 µL of the thawed frozen stock (single use) to a tube containing 10 mL of culture medium (Synthetic broth is used for *S. aureus, P. aeruginosa* and *S. enterica.* Nutrient broth may be used for *P. aeruginosa*.). Vortex, and incubate at 36 ± 1°C for 24 ± 2 h. One daily transfer is required prior to the inoculation of a final test culture. Daily cultures may be subcultured for up to 5 days; each daily culture may be used to generate a test culture. For *S. aureus* and *S. enterica* only, briefly vortex the 24 h cultures prior to transfer. b. For the final subculture transfer, inoculate a sufficient number of 20 × 150 mm tubes containing 10 mL growth medium (e.g., synthetic broth or nutrient broth) with 10 µL per tube of the 24 h culture then vortex to mix. Incubate 48-54 h at 36 ± 1°C. Do not shake the 48-54 h test culture. Record all culture transfers on the Organism Culture Tracking Form (see section 14).
12.2 Carrier Inoculation for *S. aureus, P. aeruginosa*, and *S. enterica*	a. Inoculate approximately 80 carriers; 60 carriers are required for testing, 6 for control carrier counts, and 1 for the viability control. For *P. aeruginosa*, remove the pellicle from the broth either by decanting the liquid aseptically into a sterile tube, by gently aspirating the broth away from the pellicle using a pipette, or by vacuum removal. Avoid harvesting pellicle from the bottom of the tube. Transfer test culture after pellicle removal into sterile 25 × 150 mm test tubes (up to approximately 20 mL per tube) and visually inspect for pellicle fragments. Presence of pellicle in the final culture

	makes it unusable for testing. Proceed as below in 12.2c. b. For *S. aureus* and *S. enterica*, using a vortex-style mixer, mix 48-54 h test cultures 3-4 s and let stand 10 min at room temperature before continuing. Remove the upper portion of each culture (e.g., upper ¾ or approximately 7.5 mL), leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. c. For *S. aureus, P. aeruginosa* and *S. enterica*, using a vortex-style mixer, mix 48-54 h test cultures 3-4 s and let stand 10 min at room temperature before continuing. Remove the upper portion of each culture (e.g., upper ¾ or approximately 7.5 mL), leaving behind any debris or clumps, and pool culture into a sterile flask; swirl to mix. Measure and record the OD at 650 nm. Use sterile broth medium to calibrate the spectrophotometer. Note: To achieve mean carrier counts within the appropriate range (see section 8), the final test culture may be diluted (e.g., one part culture plus one part sterile broth) prior to the addition of the OSL to the inoculum using the sterile culture medium used to generate the final test culture (e.g., synthetic broth). Use the diluted test culture for carrier inoculation within 30 min. d. Add appropriate amount of organic burden if required. Swirl to mix. e. Vortex-mix the inoculum periodically during the inoculation of carriers. Use a calibrated positive displacement pipette to transfer 0.01 mL of the culture to the sterile test carrier in the Petri dish. Immediately spread the inoculum uniformly using a sterile loop. Do not allow the inoculum to contact the edge of the glass slide carriers. Cover dish immediately. f. Dry carriers in incubator at 36 ± 1°C for 30-40 min. Record the timed carrier inoculation activities on the AOAC Germicidal Spray Products Test Processing Sheet (see section 14). Perform efficacy testing within two hours of drying.
12.3 Enumeration of viable bacteria from carriers (control carrier counts)	a. Assay dried carriers in 2 sets of three carriers, one set immediately prior to conducting the efficacy test and one set immediately following the test. Randomly select 6 inoculated carriers for carrier count analysis prior to efficacy testing. b. Place each of the inoculated, dried carriers in a 38 × 100 mm culture tube or sterile 50 mL polypropylene conical tube containing 20 mL of letheen broth. Vortex immediately – 60 ± 5 seconds for *P. aeruginosa* or 120 ± 5 seconds for *S. aureus* and *S. enterica.* Record the time of vortexing on the AOAC Germicidal Spray Products Test Processing Sheet (see section 14). c. After vortexing, briefly mix and make serial ten-fold dilutions in 9 mL

	dilution blanks of PBDW. Briefly vortex and plate 0.1 mL aliquots of appropriate dilutions in duplicate on TSA or BAP using spread plating. Plate appropriate dilutions to achieve colony counts in the range of 30-300 colony forming units (CFU) per plate. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation. If the serial dilutions are not made and plated immediately, keep the tubes at 2-5°C until this step can be done. Complete the dilutions and plating within 2 h after vortexing. Alternatively, pool the letheen broth from the tubes with the carriers and briefly vortex. Serially dilute and plate 0.1 mL aliquots of the pooled media (60 mL). The average carrier count per set will be calculated. Refer to the AOAC GSPT Carrier Counts Form (see section 14). d. Incubate plates (inverted) at 36 ± 1°C for up to 48 ± 2 h. e. Count colonies. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the AOAC Germicidal Spray Products Test Carrier Counts Form (see section 14). See section 13 for data analysis. f. Alternatively, Petrifilm™ may be used for enumeration of bacterial organisms. Follow manufacturer's instructions for preparation and incubation of Petrifilm cards. *Note*: A culture purity check should be conducted on one dilution of one carrier.
12.4 Disinfectant Sample Preparation	a. Prepare disinfectant sample per SOP MB-22.
12.5 Test Procedure	a. After the required carrier drying time, spray the slides sequentially for a specified time, distance, and number of pumps at timed intervals (typically 30 seconds) with the carriers in a horizontal position. Use a certified timer to time the spray interval. b. Spray the slide within ±5 seconds of the specified time for a contact time of 1-10 minutes or within ±3 seconds for contact times <1 minute. After spraying, maintain the carriers in a horizontal position. Treated carriers must be kept undisturbed during the contact time. c. After the last slide of a set (typically 20 slides) has been sprayed with the disinfectant, and the exposure time is complete, sequentially transfer each slide into the primary subculture tube containing the appropriate neutralizer within the ±5 second time limit. Drain the excess disinfectant from each slide prior to transfer into the neutralizer tube. Drain carriers without touching the Petri dish or filter paper. Perform transfers with flame sterilized or autoclaved forceps.

	d. The slide can touch both the interior sides of the Petri dish and the subculture tube during the transfer, but avoid this contact as much as possible. e. After the slide is deposited, recap the subculture tube and shake culture thoroughly. f. Incubate at 36 ± 1°C for 48 ± 2 h. g. If a secondary subculture tube is deemed necessary to achieve neutralization, then transfer the carrier from the primary tube to a secondary tube of sterile medium after a minimum of 30 ± 5 min at room temperature from the end of the initial transfer. Within 25-60 min of the initial transfer, transfer the carriers using a sterile forceps to a second subculture tube containing 20 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 for both tubes (a carrier set) after this time. h. Record timed events on the AOAC Germicidal Spray Products Test Time Recording Sheet for Carrier Transfers (see section 14).
12.6 Sterility and viability controls	a. Viability controls. Place 1 (or 2) dried inoculated untreated carrier(s) into separate tubes of the neutralizing subculture broth (if primary and secondary media are different). Incubate tubes with the efficacy test. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity. Growth should occur in both tubes. Record results on AOAC Germicidal Spray Products Test Results Sheet (see section 14). b. Sterility controls. Place one sterile, uninoculated carrier into a tube of neutralizing subculture broth. Incubate tube with the efficacy test. Report results as + (growth), or 0 (no growth) as determined by presence or absence of turbidity. Growth should not occur in the tube. Record results on AOAC Germicidal Spray Products Test Results Sheet (see section 14).
12.7 Results	a. Gently shake each tube prior to recording results. Record results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity, on the AOAC Germicidal Spray Products Test Results Sheet (see section 14). b. If secondary subculture tubes are used, the primary and secondary subculture tubes for each carrier represent a “carrier set.” A positive result in either the primary or secondary subculture tube is considered a positive result for a carrier set.

	c. Specialized neutralizer/subculture medium such as Dey/Engley broth will not show turbidity; rather the presence of pellicle at the surface of the medium (for *P. aeruginosa*) or a color change to the medium (yellow for growth of *S. aureus* or *S. enterica*) must be used to assess the results as a positive or negative outcome. i. Use viability controls for comparative determination of a positive tube. ii. If the product passes the performance standard, a minimum of 20% of the remaining negative tubes will be assayed for the presence of the test microbe using isolations streaks on TSA or BAP. Record preliminary results and conduct isolation streaks at 48 ± 2 h; however, continue to incubate negative tubes for up to an additional 24 hours to confirm the results.[1]
12.8 Confirmatory Steps for Test Microbes	a. Confirm a minimum of three positive carrier sets per test. If there are less than three positive carriers, then confirm each carrier. If secondary subculture tubes are used and both tubes are positive in a carrier set, select only the tube with the carrier for confirmatory testing. b. For a test with greater than 20 positive carrier sets, confirm at least 20% by Gram staining, and a minimum of 4 positive carrier sets by Gram staining, solid media, and appropriate biochemical and antigenic analyses to ensure the identity of the organism. c. See Attachment 1 for Gram stain reactions, cell morphology, and colony characteristics on solid media. d. For additional confirmation steps refer to the appropriate Confirmation Flow Chart for *S. aureus, P. aeruginosa,* and *S. enterica* (see Attachment 3). e. If confirmatory testing determines that the identity of the unknown was not the test organism, annotate the positive entry (+) on the results sheet to indicate a contaminant was present. f. Alternatively, the Vitek 2 Compact may be used for confirmation of bacterial organisms. Follow manufacturer's instructions for use of the Vitek 2 Compact.
13. Data Analysis/ Calculations	Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Both electronic and hard copies of the spreadsheet will be retained. Counts from 0 through 300 and their associated dilutions will be included in the calculations.

[1] Step not contained in the AOAC standard method 961.02.

14. Forms and Data Sheets	1. Attachment 1: Typical Growth Characteristics of strains of *P. aeruginosa, S. aureus,* and *S. enterica.* 2. Attachment 2: Culture Initiation Flow Chart for *S. aureus, P. aeruginosa,* and *S. enterica.* 3. Attachment 3: Confirmation Flow Charts for *S. aureus, P. aeruginosa* and *S. enterica.* 4. Attachment 4: Photographs of spray apparatus. 5. Attachment 5: Confirmatory Testing Against Carbapenem Resistant *Klebsiella pneumoniae* (ATCC# BAA-1705) 6. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record — MB-06-08_F1.docx Organism Culture Tracking Form (Frozen Stock Cultures) — MB-06-08_F2.docx Test Microbe Confirmation Sheet (Quality Control) — MB-06-08_F3.docx AOAC Germicidal Spray Products Test Carrier Counts Form — MB-06-08_F4.docx AOAC Germicidal Spray Products Test Time Recording Sheet for Carrier Transfers — MB-06-08_F5.docx AOAC Germicidal Spray Products Test Information Sheet — MB-06-08_F6.docx AOAC Germicidal Spray Products Test Results Sheet (1°/2°) — MB-06-08_F7.docx AOAC Germicidal Spray Products Test Results Sheet (1°) — MB-06-08_F8.docx Test Microbe Confirmation Sheet — MB-06-08_F9.docx Carrier Count Spreadsheet MS Excel spreadsheet: Carrier Count Template_GSPT_v3 — MB-06-08_F10.xlsx AOAC Germicidal Spray Products Test Carrier Counts Form (Pooled Carriers) — MB-06-08_F11.docx AOAC Germicidal Spray Products Test Processing Sheet — MB-06-08_F12.docx
15. References	1. Official Methods of Analysis. Revised 2013. AOAC INTERNATIONAL, Gaithersburg, MD, (Method 961.02). 2. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD.

	P. aeruginosa p. 164, *S. enterica* p. 447. 3. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. *S. aureus* p. 1015. 4. Package Insert – Gram Stain Kit and Reagents. Becton, Dickinson and Company. Part no. 882020191JAA. Revision 07/2011. 5. Package Insert – Catalase Reagent Droppers. Becton, Dickinson and Company. Part no. L001237. Revision 06/2010. 6. Package Insert – Staphaurex Plus*. Remel. Part no. R30950102. Revised 11/23/07. 7. Package Insert – Oxidase Reagent Droppers. Becton, Dickinson and Company. Part no. L001133. Revision 06/2010. 8. Package Insert – Wellcolex* Colour Salmonella. Remel. Part no. R30858301. Revised 10/17/07 9. CDC Protocol: Modified Hodge Test for Carbapenemase Detection in Enterobacteriaceae. Download: http://www.ndhealth.gov/microlab/Uploads/HodgeTest.pdf

Attachment 1

Typical Growth Characteristics of strains of *P. aeruginosa, S. aureus,* and *S. enterica* (see ref. 15.2 and 15.3).

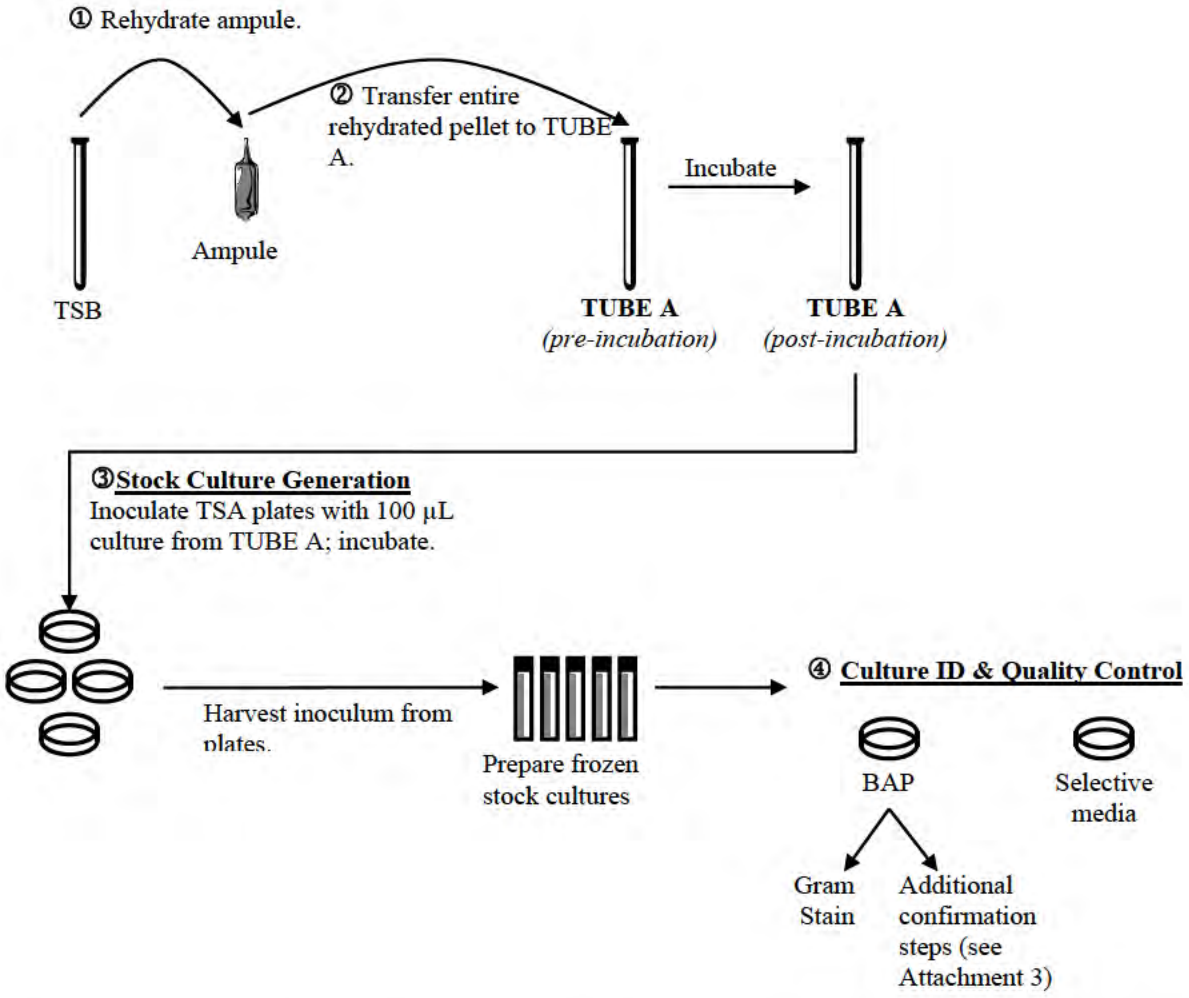
	*P. aeruginosa**	*S. aureus**	*S. enterica**
Gram stain reaction	(-)	(+)	(-)
Typical Growth Characteristics on Solid Media			
Mannitol Salt	No Growth	circular, small, yellow colonies, agar turning fluorescent yellow	N/A
Cetrimide	circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth	N/A
Xylose lysine deoxycholate (XLD) agar	N/A	N/A	Round, clear red colonies with black centers
Blood agar (BAP)	flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	small, circular, yellow or white, glistening, beta hemolytic	entire, glistening, circular, smooth, translucent, low convex, non-hemolytic
Typical Microscopic Characteristics			
Cell dimensions	0.5-1.0 µm in diameter by 1.5-5.0 µm in length*	0.5-1.5 µm in diameter*	0.7-1.5 µm in diameter by 2.0-5.0 µm in length*
Cell appearance	straight or slightly curved rods, single polar flagella, rods formed in chains	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	straight rods, peritrichous flagella

*After 24±2 hours

(1) Test organism may display three colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading, and translucent. Pyocyanin is not produced.

Attachment 2

Culture Initiation and Stock Culture Generation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica*



A1. Preparation of Frozen Stock Cultures. Refer to SOP MB-02 for establishment of the organism control number.

a. Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), and *Salmonella enterica* (ATCC 10708) from ATCC within 18 months.

b. Open ampule of freeze dried organism as indicated by ATCC. Using a tube containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth designated as "TUBE A". Mix well.

c. Incubate broth culture (TUBE A) at 36 ± 1ºC for 24 ± 2 hours. Record all

manipulations on the Organism Culture Tracking Form (see section 14).

d. Using a sterile spreader, inoculate a sufficient number of TSA plates (e.g., 5 to 10 plates per organism) with 100 µL each of the culture. Incubate plates at 36 ± 1°C for 24 ± 2 h.

e. Following incubation, add 5 mL cryoprotectant solution (TSB with 15% v/v glycerol) to the surface of each agar plate. Re-suspend the cells in this solution using a sterile spreader or a sterile swab and aspirate the cell suspension from the surface of the agar. Transfer the suspension into a sterile vessel. Repeat by adding another 5 mL of cryoprotectant to the agar plates, re-suspend the cells, aspirate the suspension and pool with the initial cell suspension.

f. Mix the pooled contents of the vessel thoroughly. Immediately after mixing, dispense approximately 1.0 mL aliquots into cryovials (e.g., 1.5 mL cyrovials). Perform QC of stock cultures concurrently with freezing (see section A2: QC of Stock Cultures).

g. Place and store the cryovials at -70°C or below; these are the frozen stock cultures. Store stock cultures up to 18 months. These cultures are single-use only.

A2. QC of Stock Cultures.

a. Conduct QC of the pooled culture concurrently with freezing. Streak a loopful on a plate of BAP. In addition, for *S. aureus* and *P. aeruginosa*, streak a loopful onto both selective media (MSA and Cetrimide); for *S. enterica*, streak a loopful onto XLD. Incubate all plates at 36 ± 1ºC for 24 ± 2 hours.

b. Following the incubation period, record the colony morphology as observed on the BAPs and selective media plates (including the absence of growth) and Gram stain. See Attachment 1 for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.

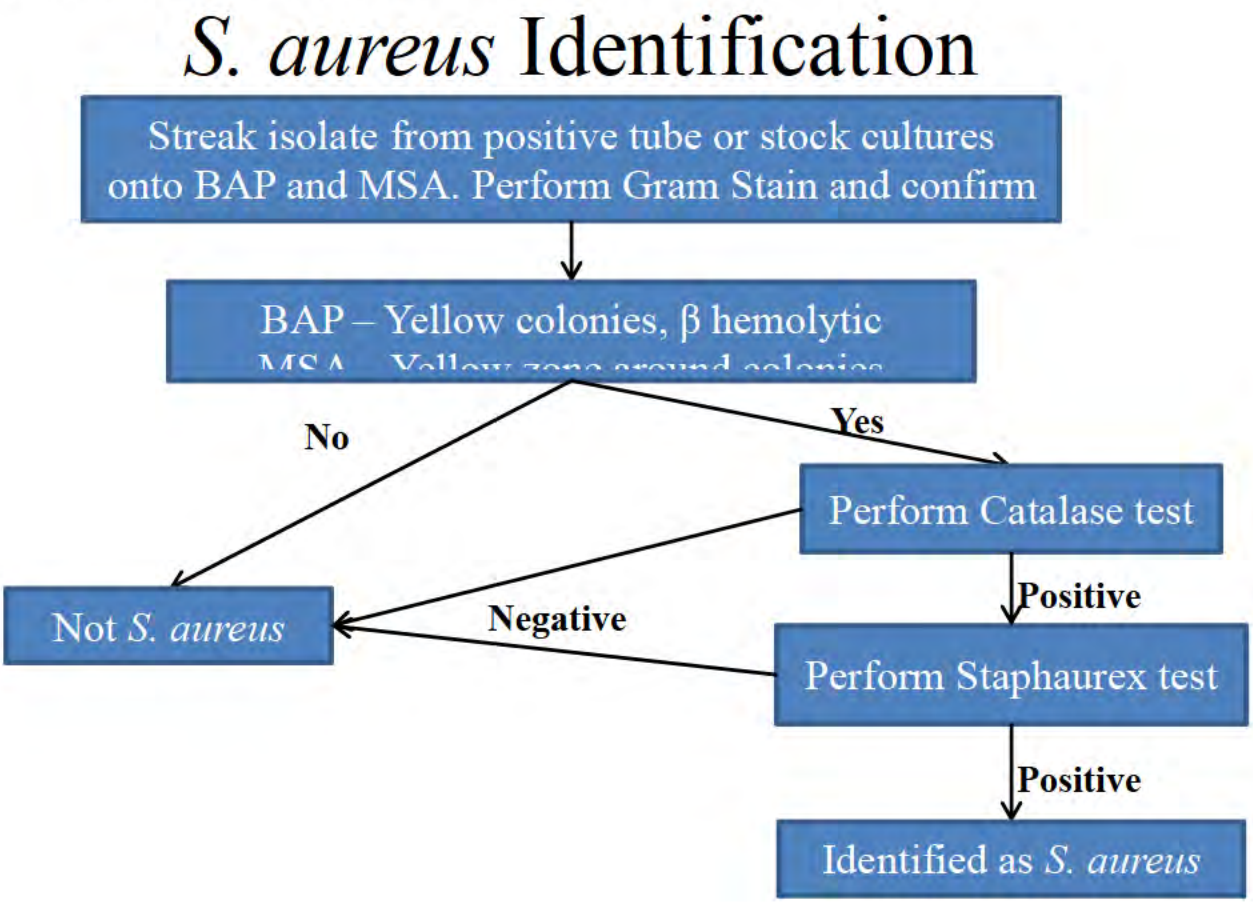
c. For each organism, perform a Gram stain (refer to 15.5) from growth taken from the BAPs according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).

d. For additional confirmation steps refer to the appropriate Confirmation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica* (see Attachment 3). Refer to 15.6-15.9 for instructions.

e. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).

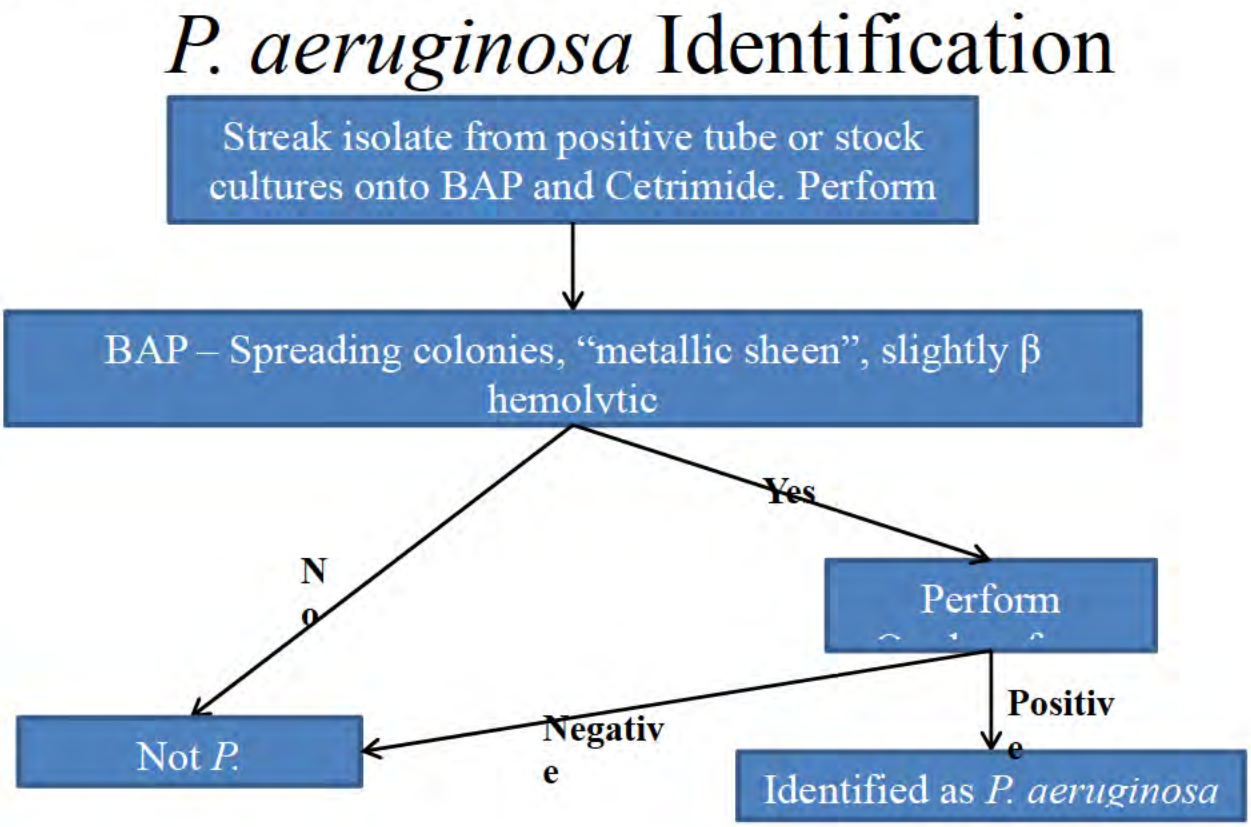
Attachment 3

Confirmation Flow Chart for *S. aureus*



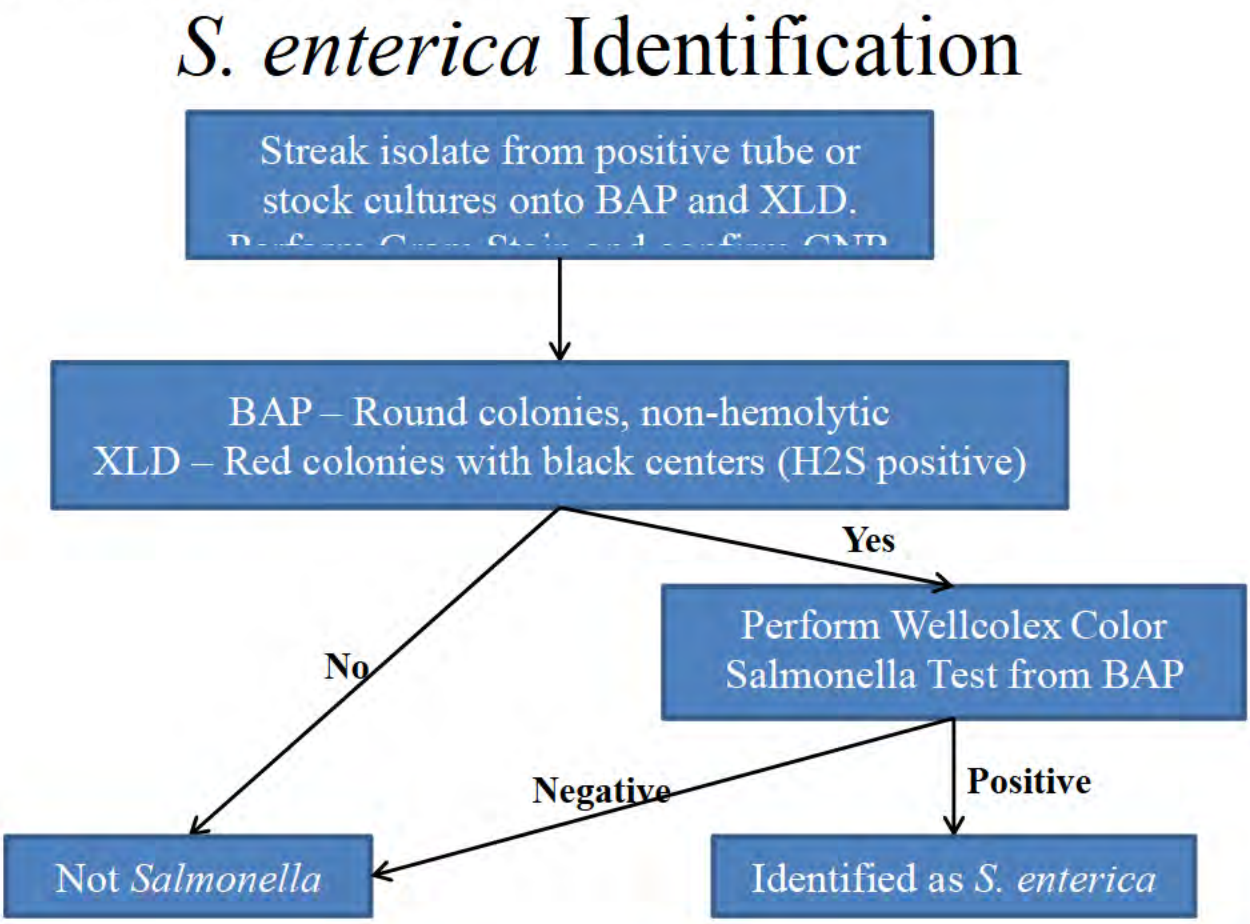
Attachment 3 (cont.)

Confirmation Flow Chart for *P. aeruginosa*



Attachment 3 (cont.)

Confirmation Flow Chart for *S. enterica*



Attachment 4

Attachment 5 – Testing Against Carbapenem Resistant *Klebsiella pneumoniae* (ATCC# BAA-1705). The following modifications to this SOP are used when testing Carbapenem Resistant *K. pneumoniae*:

Stock cultures are prepared according to Attachment #2 using tryptic soy broth (TSB) to rehydrate the lyophilized culture.

Test cultures are prepared according to Section 12.1; no pellicle is formed with *K. pneumoniae.*

Section 8, #2: The mean *TestLD* for carriers inoculated with *Klebsiella pneumoniae* must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 5.0 (corresponding to a geometric mean density of 1.0×10^5); a mean *TestLD* below 4.0 and above 5.0 invalidates the test, except for two retesting scenarios (outlined in the study protocol).

Section 12.2: Inoculate approximately 15-20 carriers; 10 carriers are required for testing, 3 for control carrier counts, and 1 for the viability control.

Section 12.2, c: Dilution of the final test culture may be required to achieve the target control counts.

Section 12.3, a: Three control carriers will be evaluated at the end of the test period.

Section 12.3, b: Vortex control carriers for 120 ± 5 seconds.

Section 12.8: Growth from <u>all</u> positive tubes (efficacy test) will be confirmed by Gram stain (Gram negative rods), growth on blood agar (large, mucoid, non-hemolytic colonies), and MacConkey agar (large, mucoid, lactose-fermenting pink colonies). See Table 1 below.

The carbapenem resistance of the organism shall be confirmed on cultures from <u>each</u> positive tube using the modified Hodge test (see Reference 9). The following additional items will be required to perform the modified Hodge Test:

1. 10 µg meropenem susceptibility disks
2. Mueller-Hinton agar
3. 0.85% saline
4. *Klebsiella pneumoniae* (ATCC# BAA-1706) for negative control
5. *Escherichia coli* (ATCC# 25922)

Table 1. Growth Characteristics of *K. pneumoniae* on Growth Media

K. pneumoniae[*]	
Gram stain reaction	(-)
Typical Growth Characteristics on Solid Media	
Blood agar (BAP)	Large, round, mucoid colonies. Non-hemolytic.
MacConkey Agar	Large, round, mucoid colonies. Lactose fermenter (pink colonies)
Cell dimensions	0.5-1.0 µm in diameter by 1.5-5.0 µm in length*
Cell appearance	Straight rods, capsule may be visible

*After 24±2 hours