EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Determining Carrier Counts

SOP Number: MB-04-02

Date Revised: 09-09-02

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Controlled Copy No.: _____

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1.0 SCOPE AND APPLICATION:

1.1 The bacterial load on seeded carriers used in disinfectant efficacy testing is a critical test parameter. The carrier load serves as the challenge inoculum. This protocol describes the procedures used to enumerate test microbes on seeded carriers.

2.0 DEFINITIONS:

2.1 CFU = Colony Forming Units

2.2 TNTC = Too Numerous to Count

2.3 AOAC =AOAC International

2.4 DI = De-ionized water

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organisms are required to be performed in accordance with biosafety practices stipulated in SOP MB-01, Lab Biosafety.

4.0 CAUTIONS:

4.1 Sonication or vortexing of seeded carriers will be performed immediately after extraction.

4.2 Dilution plating is performed within 6 hours after carrier sonication or vortexing is complete.

4.3 The Autoplate 4000 Automated Spiral Plater can not be used to conduct carrier counts for *Mycobacterium bovis* (BCG) and *Bacillus subtilis*.

5.0 INTERFERENCES: None

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be

found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Branson Model 2200 Bath Sonicator or equivalent

7.2 A water bath set at 45-50°C

7.3 Incubator at 37±1°C or another temperature suitable for growth of the target organism

7.4 Refrigerator set at 2-5°C

7.5 Twenty 20 mm x 100 mm petri dishes (total plating area of 58.1 cm^2)

7.6 Plate spinner

7.7 Trypticase Soy Agar (TSA) or Middlebrook 7H9 Agar (M7H9)

7.8 Letheen Broth or Modified Proskauer Beck Medium (MPB)

7.9 Leica Darkfield Quebec Colony Counter

7.10 Autoplate 4000 Automated Spiral Plater (Spiral Biotech)

8.0 INSTRUMENT OR METHOD CALIBRATION: Not applicable

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Sonicated or vortexed tubes (see sections 10.1.3, 10.2.1 and 10.3.2) can be stored at 2-5°C until the dilution plating is performed. Plating must be completed within six hours of the sonication/vortexing step.

10.0 PROCEDURE AND ANALYSIS:

10.1 AOAC Use-Dilution Method with *Staphylococcus aureus* and *Pseudomonas aeruginosa*: On a typical test day, one preparation of inoculum is required to seed 72 stainless steel carriers necessary to test one product sample. Six of the 72 seeded carriers are used to estimate the carrier bacterial load.

10.1.1 Prior to testing, one carrier is randomly extracted from each of 6 petri dishes (12 carriers/dish).

10.1.1.1 Place each inoculated carrier into a 20 mm X 150 mm tube containing 10 mL of letheen broth.

10.1.1.2 Place all tubes with carriers into an appropriately sized beaker and fill the beaker with tap water to the level of letheen broth in the tubes.

10.1.1.3 Hold the beaker in the sonicator so that the water level in the beaker is even with the water level fill line on the sonicator tank and fill the tank up with tap water to the water level fill line. Be sure that the water level in the tank never falls below one inch from the top of the tank.

10.1.1.4 Hold the beaker in the sonicator tank so that it is not touching the bottom and so that all three liquid levels (inside the test tubes, inside the beaker and the sonicator tank) are the same.

10.1.1.5 Using an official timer, sonicate carriers inoculated with *P. aeruginosa* and *S. aureus* for 1 minute.

10.1.2 Serial ten-fold dilutions of the sonicated carrier tubes are made in 9 mL dilution blanks (20 mm X 150 mm tubes of phosphate buffered dilution water) (see 16.1).

10.1.3 If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5°C until this step can be done. The dilution and plating must be performed within 6 hours after sonication.

10.1.4 For plating, each dilution tube is vortexed briefly prior to

removing an aliquot from the tube. For *S. aureus* and *P. aeruginosa,* dilutions in the range of 1 x 10^{-2} to 1 x 10^{-5} are plated to achieve counts between 30-300 CFU per plate. One mL aliquots are plated for spread plating and pour plating methods. Dilutions are plated in duplicate.

10.1.5 If the spread plate method is used for bacterial enumeration, Trypticase soy agar (TSA) plates are prepared in advance and are refrigerated until needed.

10.1.5.1 Allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the surface of the agar, tilt the plate back and forth or, if necessary, use a glass spreading rod and plate spinner.

10.1.5.2 Alternatively, the Autoplate 4000 Spiral Plater may be used for bacterial enumeration. Dilutions are prepared as outlined in 10.1.3 and are spread on the TSA agar plates with the Spiral Plater (see ref. 15.3)

10.1.6 If the pour plate method is used for bacterial enumeration, the TSA is prepared and tempered after autoclaving (approx. 1 hr) to 45-50°C in a water bath prior to use. Tempered agar is added to each plate after the addition of the appropriate dilution and swirled to uniformly disperse the inoculum.

10.1.7 Incubate cultures at 37±1°C for 24-48 hrs.

10.1.8 Colonies may be counted by hand or with the aid of a plate counter. Plates yielding between 30 and 300 CFU are used for enumeration. Plates that have colony counts over 300 will be reported as TNTC.

10.2 AOAC Germicidal Spray Products Test and Germicidal Towelette Product Test with *S. aureus* and *P. aeruginosa*: On a typical test day, one preparation of inoculum is required to seed 72 glass slide carriers. Six of the 72 carriers will be assayed for carrier counts for each inoculum

preparation. Slide carriers are randomly selected for carrier counts.

10.2.1 Prior to testing, place each of the seeded, dried carrier in a 38 mm x 100 mm culture tube containing 20 mL of medium (e.g. letheen broth). Vortex immediately. Vortexing time is 60 seconds for *P. aeruginosa*, and 120 seconds for *S. aureus*.

10.2.2 Serial ten-fold dilutions of the vortexed carrier tubes are made in 9 mL dilution blanks (20 mm x 150 mm tubes of phosphate buffered dilution water) (see 16.1).

10.2.3 If the serial dilutions are not made and plated immediately, the tubes are kept at 2-5°C until this step can be performed. The dilution and plating must be performed within 6 hours after vortexing of carriers.

10.2.4 Each dilution tube is vortexed briefly prior to removing an aliquot from the tube. For *S. aureus* and *P. aeruginosa,* dilutions in the range of 1×10^{-2} to 1×10^{-5} are plated to obtain counts between 30-300 CFU per plate. One mL aliquots are plated for spread plating and pour plating methods. Plate each dilution in duplicate.

10.2.5 For spread plate method, see section 10.1.5.

10.2.6 For pour plate method, see section 10.1.6.

10.2.7 Incubate cultures at 37±1°C for 24-48 hrs.

10.2.8 Colonies may be counted by hand or with aid of a plate counter. Plates yielding between 30 and 300 CFU are used for enumeration. Plates that have colony counts over 300 will be reported as TNTC.

10.3 <u>AOAC Confirmatory Tuberculocidal Test with *M. bovis* (BCG)</u>: Three porcelain carriers per inoculum preparation are assayed. For tuberculocidal testing, a total of 36 carriers are inoculated on a typical test day (see SOP MB-07, Confirmatory Tuberculocidal Method). The 36 carriers are distributed in 3 petri dishes (12 carriers/dish). For the carrier

count assay, randomly select one carrier from each of the three petri dishes. The test parameters for product testing may or may not require the addition of organic soil load (OSL).

10.3.1 Place each seeded carrier in a 20 mm x 150 mm culture tube containing 10 mL of Modified Proskauer Beck (MPB). The tubes are then placed in a beaker containing tap water up to the level of media in the tubes. Likewise, the level of water in the sonicator bath is adjusted to the same level as the liquid in the tubes and beaker.

10.3.2 The beaker of tubes is then sonicated for 10 minutes. These tubes are referred to as the "sonicated carrier tubes."

10.3.3 Serial ten-fold dilutions of the sonicated carrier tubes are made in 9 mL dilution blanks (20 mm x 150 mm tubes of phosphate buffered dilution water).

10.3.4 If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5°C until this step can be done (within 6 hours after sonication of carriers).

10.3.5 Each dilution tube is vortexed briefly prior to removing an aliquot from the tube. Dilutions in the range of 1×10^{-1} to 1×10^{-4} are plated in duplicate to achieve counts between 30-300 CFU per plate. One (1) mL aliquots are plated for spread plating and pour plating methods. Dilutions are plated in duplicate.

10.3.6 If the <u>spread plate method</u> is used for bacterial enumeration, Middlebrook 7H9 agar plates are prepared in advance and are refrigerated prior to use. Allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the surface of the agar, tilt the plate back and forth or, if necessary, use a glass spreading rod and plate spinner.

10.3.7 If the <u>pour plate method</u> is used for bacterial enumeration, the Middlebrook 7H9 (M7H9) agar is prepared and tempered (approx. 1 hr) to 45-50°C in a water bath prior to use.

Tempered M7H9 agar is added to each plate after the addition of the appropriate dilution and swirled to uniformly disperse the inoculum.

10.3.8 Incubate cultures at 37±1°C for a minimum of 21 days (up to 25 days).

10.3.9 Colonies may be counted by hand or with aid of a plate counter. Dilutions yielding between 30 and 300 CFU per plate are used for enumeration. Plates that have colony counts over 300 will be reported as TNTC.

10.4 AOAC Germicidal Spray Products Test and Germicidal Towelette Product Test with *M. bovis* (BCG): On a typical test day, one inoculum preparation is used to seed carriers for testing (15 carriers per sample and two samples per product). Three glass slide carriers are evaluated per inoculum preparation per test day.

10.4.1 Prior to testing, place each seeded, dried carrier in a 38 mm x 100 mm tube, containing 20 mL of Modified Proskauer Beck (MPB) broth. Carriers are selected at random.

10.4.2 Vortex each tube for 15 seconds. These tubes are referred to as "vortexed carrier tubes." Follow steps listed in 10.3.4 thru 10.3.9 for preparing dilutions and plating.

10.5 AOAC Sporicidal Activity Test with *B. subtilis*: Porcelain penicylinders and silk suture loops inoculated with spores of *Bacillus subtilis* may be purchased or prepared as specified in the AOAC Official Method 966.04, Sporicidal Activity of Disinfectants. For each carrier type (silk suture loops or porcelain penicylinders), determine the spore load on each lot of carriers. A lot is defined as those carriers prepared at the same time from a single preparation of *Bacillus subtilis* inoculum. For lots of carriers 300 or less, determine the spore load on a minimum of 3 carriers. For lots of carriers greater than 300, determine the spore load on a minimum of 1% of the total number of carriers (rounding up the nearest whole carrier, for example, a lot of 405 carriers would require enumerating the spore load on 5 carriers). Carrier counts need only be performed once on each lot of carriers and will apply to all tests using those carriers during the 60 day period in which the carriers are valid and will be independent of acid

resistance tests.

10.5.1 Place each inoculated carrier into a 20 mm X 150 mm tube containing 10 mL of sterile DI water.

10.5.2 Place all tubes with carriers into an appropriately sized beaker and fill the beaker with tap water to the level of sterile DI water in the tubes.

10.5.3 Hold the beaker in the sonicator so that the water level in the beaker is even with the water level fill line on the sonicator tank and fill the tank up with tap water to the water level fill line. Be sure that the water level in the tank never falls below one inch from the top of the tank.

10.5.4 Hold the beaker in the sonicator tank so that it is not touching the bottom and so that all three water levels (inside the test tubes, inside the beaker and the sonicator tank) are the same.

10.5.5 Using an official timer, sonicate the samples for 5 minutes.

10.5.6 Following sonication, vortex each tube for 2 minutes.

10.5.7 Dilute the spore suspensions by transferring 1 mL aliquots to tubes containing 9 mL sterile DI water. Dilute the spore suspension out to 10^{-7} and plate 1 mL dilutions 10^{-4} through 10^{-7}. Plate each dilution in duplicate.

10.5.8 To each plate, add molten TSA that has been tempered to 45-50°C. Swirl the plates to distribute spores evenly in the agar and allow to solidify.

10.5.9 Invert the plates and incubate for 24-48 hours at 37°C. Colonies may be counted by hand or with aid of a plate counter. Dilutions yielding between 30 and 300 CFU per plate are used for enumeration. Plates that have colony counts over 300 will be reported as TNTC. Record the results on the appropriate form.

11.0 DATA ANALYSIS/CALCULATIONS:

11.1 To calculate average CFU/mL per carrier, average the counts for all plates (those which fall within 30-300 CFU) and divide the count by its dilution. Counts are taken from one plate in the event that only one of the two plates fall within the 30-300 CFU range.

11.2 To calculate average CFU/carrier, multiply the CFU/mL per carrier by the volume of media used to suspend carrier for sonication or vortexing. Numbers are rounded and only two significant figures are used in calculating averages.

11.3 To calculate average CFU/carrier for all carriers tested, obtain by averaging the average CFU/carrier.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the Carrier Count Data Sheets. Completed forms are archived in notebooks kept in locked file cabinets in the file room D217. Only authorized personnel have access to the locked files. Archived data is subject to OPP's official retention schedule as stipulated in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 The OPP Microbiology Laboratory conforms to 40CFR Part 160, Good Laboratory Practices. Appropriate quality control measures are integrated into each SOP.

13.2 For quality control purposes, the required information is documented on the appropriate forms (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 If the average carrier count is less than 1.0×10^4 CFU/carrier for *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Mycobacterium bovis* (BCG) and less than 1.0×10^5 CFU/carrier for *Bacillus subtilis*, the associated efficacy test will be deemed invalid and will be repeated.

15.0 REFERENCES:

15.1 Official Methods of Analysis. 1990. 15th Ed., Association of Official Analytical Chemists, Arlington, VA. Method 966.04.

16.0 FORMS AND DATA SHEETS:

16.1 Serial Dilution/Plating Tracking Form

16.2 Carrier Count Data Sheet

Serial Dilution/Plating Tracking Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
EPA Reg. No.	
Name	
Sample No. (s)	
Test Date	
Organism	

Confirmed by:_____	Dilution Tube							
	1	2	3	4	5	6	7	8
Vol. In Dil. Tube prior to Addition								
Volume Added to Dil. Tube								
Overall Dilution in Dil. Tube								
Volume Plated								
Overall Dilution on Plate								
Number of Plates per Dilution								
Media Plated Onto								
Number of Carriers Analyzed								
Comments:								

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Carrier Count Data Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
EPA Reg. No.	
Name	
Sample No. (s)	
Test Date	
Organism	
SOP	

Check the appropri

RESULTS						
Date/Initials						
Plating method						
Volume of media in initial tube receiving carrier						
Carrier No. / Dilution	CFU per Dilution Plate (2)				Average CFU per mL per Carrier	Average CFU per Carrier†
					Avg. CFU per Carrier for all Carriers Tested:	
Comments:						

† Values reflect dilution factor for volume of media in initial tube receiving carrier.