DISINFECTANTS

Procedural Revision to the AOAC Germicidal Spray Products as Disinfectants Test Method: Establishment of Minimum and Maximum Log Density Values for Test Microbes on Inoculated Carriers

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The AOAC Germicidal Spray Products as Disinfectants test method (AOAC *Official Method* 961.02) is used to measure the efficacy of spray products on hard inanimate surfaces; however, the method does not provide procedures to determine the population of the test microbe on inoculated glass slide carriers (e.g., carrier counts reported as CFU/carrier). Without a method to measure and monitor carrier counts, the associated efficacy data may not be reliable and repeatable. This report provides a standardized procedure to address this issue and, based on carrier count data collected by four laboratories from 2000 to 2010, proposes a specific range for the mean log density per carrier as a requirement. Laboratory-based carrier count data were collected concurrently with 116 Method 961.02 efficacy tests conducted on spray products bearing claims against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. For many of the tests a soil load (SL) was added to the inoculum (as specified on the product label claim). Six carriers were assayed per test for a total of 696 carriers. All but two of the 116 mean log densities were at least 5.0 (a geometric mean of 1.0×10^5 CFU/carrier). Across the four combinations of microbes and SL treatments, the mean *TestLD* (mean log density across all enumerated carriers in a test) ranged from approximately 6.0 (a geometric mean of 0.9×10^6 CFU/carrier) to 6.3 (a geometric mean of 2.0×10^6 CFU/carrier). Across all microbes and SL treatments, the mean log density (±SEM) was 6.2 (±0.07) per carrier (a geometric mean of 1.5×10^6 CFU/carrier). The mean log density for six carriers per test showed good repeatability (0.32) and reproducibility (0.34). The proposed requirement for *S. aureus* tests and *P. aeruginosa* tests is a mean log density (across six carriers) between 5.0 and 6.5. A separate 2009 study at three laboratories was conducted to evaluate the persistence of *P. aeruginosa*, *S. aureus*, and *Salmonella enterica* on glass carriers. Based on the persistence data, a 2 h use period is proposed for using the inoculated carriers post drying. The persistence data set was also used to assess the carrier counts for *S. enterica.* The carrier counts were approximately one log lower for *S. enterica* compared to *S. aureus* and *P. aeruginosa*; a range of 4.0 to 5.5 logs is proposed as a requirement for *S. enterica* tests.

The AOAC Germicidal Spray Products as Disinfectants test method (AOAC *Official Method* **961.02**) is accepted by the U.S. Environmental Protection Agency (EPA) to verify bactericidal claims for disinfectant spray products (e.g., trigger sprays and aerosols). EPA's Office of Pesticide Programs (OPP), Biological and Economic Analysis Division, Microbiology Laboratory Branch, Fort Meade, MD, has worked closely with AOAC INTERNATIONAL and the user community to enhance the method through the official AOAC editorial revision process. As a result, a revised version of the method was published in the AOAC *Official Methods of Analysis* in 2009 (1). The editorial revisions focused on increasing the clarity of the method and eliminating the extensive cross-referencing to other methods, thereby creating a stand-alone procedure. Although the editorial revisions should provide more consistent test results across laboratories, targeted procedural changes will further enhance the method.

Currently, the Germicidal Spray Products as Disinfectants Test (GSPT) method lacks a standard procedure for the enumeration of the test microbe on untreated, dried inoculated glass slide carriers (referred to in this paper as "carrier counts"), and associated validity requirements (i.e., minimum and maximum levels of the test microbe). Without a method to measure and monitor control carrier counts, the associated efficacy data may not be as repeatable within a laboratory or reproducible between laboratories. Thus, we are proposing a set of procedural revisions to address these issues, namely, the establishment of a procedure to enumerate bacteria from inoculated control

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Table 1. Number of tests conducted in each laboratory for each combination of microbe and SL value

	Pseudomonas aeruginosa			*Staphylococcus aureus*		
	SL			SL		
Lab	Absent	Present	Total	Absent	Present	Total
1	2	1	3	5	1	6
2	6	6	12	6	6	12
3	3	5	8	4	5	9
4	20	12	32	20	14	34
Total	31	24	55	35	26	61

carriers, a minimum and maximum control carrier count range to qualify the efficacy results, and a use period for inoculated carriers. A precedent for such changes was set in 2009, when a similar set of procedural modifications was approved for the use-dilution method (2). Establishing minimum and maximum carrier counts, in conjunction with the published editorial revisions, will further standardize the GSPT and provide increased confidence in product efficacy results within and between laboratories. Most of the data presented in this report are associated with *Staphylococcus aureus* and *Pseudomonas aeruginosa* due to their importance in the EPA post-registration surveillance testing program designed to monitor the efficacy of disinfectant products. The present investigation uses an extensive, multilaboratory data set to support the proposed method modifications. In addition, a smaller data set associated with a microbe persistence study was used to establish a carrier count range for *Salmonella enterica* (subsp. *enterica* serovar Choleraesuis).

Methods

Historical Data Collection

The data were generated by four laboratories over an 11-year period (2000–2010) following the bacterial enumeration procedure described in this paper. Under the Antimicrobial Testing Program, OPP's Microbiology Laboratory Branch and three state Department of Agriculture laboratories (Michigan, Ohio, and North Carolina) collected the laboratory-based carrier count data concurrently while performing the efficacy evaluations. Product testing was conducted by trained analysts in accordance with EPA's Federal Insecticide, Fungicide, and Rodenticide Act Good Laboratory Practice Standards (3).

The laboratories strictly adhered to the published GSPT method for the stock culture initiation and maintenance, test culture preparation, and carrier inoculation steps. In all tests, no dilution of the final test cultures was performed. A total of 116 tests, distributed across the four laboratories, as shown in Table 1, were conducted on spray products bearing claims against *P. aeruginosa* and *S. aureus* with and without a soil load (SL) added to the inoculum (based on the product label). Nutrient broth was used as the growth medium for daily and final test cultures. Horse serum was used as the SL in most of the tests; fetal bovine serum was used in a few. SL was added at 5% (v/v) to the inoculum before carrier inoculation. In each of the 116 tests, six carriers were analyzed per test day, for a total of 696 total carriers.

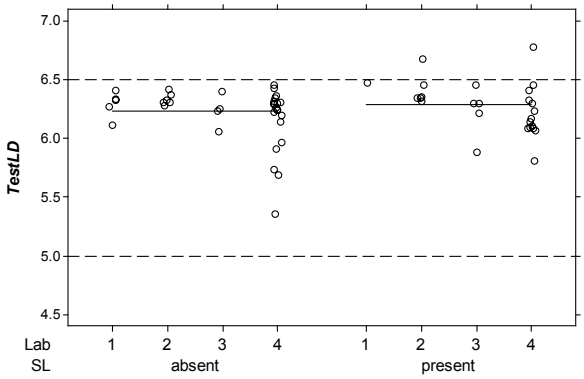

Figure 2. *TestLD* values for tests with *S. aureus*. Each point is a *TestLD*. Each solid line denotes the mean *TestLD* for the specified SL level. The dashed lines are at 5.0 and 6.5, the proposed limits of validity for *TestLD*.

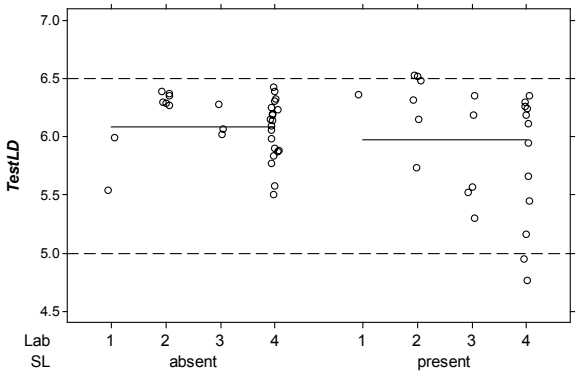

Figure 1. *TestLD* values for tests with *P. aeruginosa*. Each point is a *TestLD*. Each solid line denotes the mean *TestLD* for the specified SL level. The dashed lines are at 5.0 and 6.5, the proposed limits of validity for *TestLD*.

The carriers were randomly removed from the inoculated set after the drying step (36 ± 1°C for 40 ± 2 min). Processing of carriers after drying was conducted within 2 h of drying. Control carrier counts, expressed as CFU/carrier, were entered into a Microsoft Access database; the data were sorted by laboratory, organism, date, and presence/absence of SL. The data were peer-reviewed by EPA laboratory staff and verified by the Quality Assurance Unit before statistical analysis. If an unusual observation was discovered during the peer review or statistical analysis process, the data were discussed with the appropriate laboratory personnel and necessary adjustments made. For one *P. aeruginosa* test, it was determined that the cell density for one of the six carriers was unusually large, and was the result of an error. That test was deleted from the data set before analysis and is not numbered among the 116 tests. No other adjustment to the submitted data set was made. Data for *P. aeruginosa* are provided in Appendix 1 (*see* Supplemental Material on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac for Appendix 1–5), data for *S. aureus* in Appendix 2.

Microbe Persistence on Glass Slide Carriers

To augment the carrier count data, a study was conducted by the same three state laboratories over several months in 2009

Table 2. Summary of the *TestLD* values (mean log densities, averaged across six carriers per test) for each microbe and SL combination

Microbe	SL	Mean *TestLD*	SEM	S_r	S_R	Geometric mean of *TestLD*
P. aeruginosa	Absent	6.09	0.10	0.231	0.291	1.22×10^6
	Present	5.97	0.15	0.492	0.534	9.37×10^5
S. aureus	Absent	6.23	0.05	0.227	0.234	1.70×10^6
	Present	6.29	0.06	0.208	0.222	1.96×10^6
Overall		6.17	0.07	0.321	0.342	1.47×10^6

to evaluate the persistence of *P. aeruginosa*, *S. aureus*, and *S. enterica* on glass slide carriers over a 6 h period at room temperature using the same carrier count procedure. On each test day, the laboratories enumerated the viable inoculum on carriers with and without a 5% (v/v) SL in a side-by-side fashion over four time periods post drying (15, 120, 240, and 360 min). Three replicate tests were performed for each organism/SL/dry time combination with four carriers evaluated per treatment. At one of the three laboratories, the tests with a SL were performed 1 to 2 days after the corresponding experiment without a SL, except in one instance (for *S. aureus*) where there was a 7-day gap. Data for *P. aeruginosa* are provided in Appendix 3, data for *S. aureus* in Appendix 4, and data for *S. enterica* in Appendix 5.

Statistical Analysis

On each carrier, the density of viable bacteria (CFU/carrier) was measured and $\log_{10}$-transformed to form the log density (LD). The term *TestLD* is used to denote the mean LD across all enumerated carriers in a test. All statistical calculations were performed on the carrier LD values.

The historical data, over 11 years, were partitioned into four subsets, one for each combination of microbe and SL. An analysis of variance (ANOVA) was fit to each subset with random effects due to lab and "tests nested within lab." This model provided estimates of the reproducibility SD (S_R), which measures total variability among the four laboratories of the *TestLD*, and the repeatability SD (S_r), which measures the within-laboratory variability of the *TestLD*. The test for a linear time trend of *TestLD* values was conducted for each combination of microbe and SL by pooling the slopes across the four laboratories using an analysis of covariance (ANCOVA) with lab as a random effect and time as a covariate. Because there were four laboratories in the study, all reported *P*-values were calculated using three degrees of freedom.

The data from the persistence study in 2009 were partitioned into 24 subsets corresponding to the 24 combinations of microbe, time, and SL. For each of these subsets, an ANOVA model was fit, with lab and "tests nested within lab" as random effects. If all counts at the plated dilutions were zero, then a substitution rule of 0.5 was used at the lowest dilution for only one of the plates. This study was conducted using four carriers per test. In order to compare the results of these four-carrier tests with the historical data analysis presented in this report (which used six carriers per test), the S_r and the S_R were calculated from the formula for six carriers per test. To assess the effect of time on each of the six combinations of microbe and SL, an ANCOVA

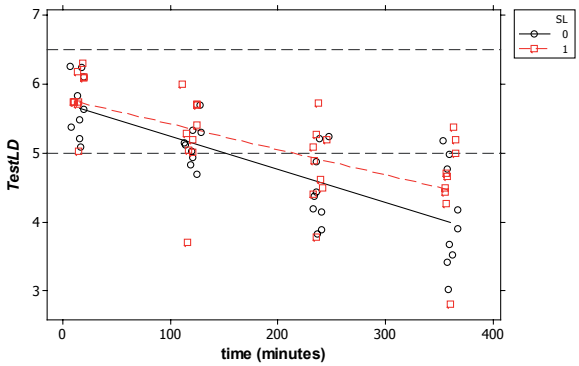

Figure 3. Persistence of *P. aeruginosa* on glass slide carriers. Each point is a *TestLD* for a specific SL. Although sampling only occurred at 15, 120, 240, and 360 min, the data have been jittered in the horizontal direction for ease in viewing. The regression lines indicate the decreasing trend over time for each SL level. The horizontal dashed lines are at 5.0 and 6.5, the proposed limits of validity for *TestLD*.

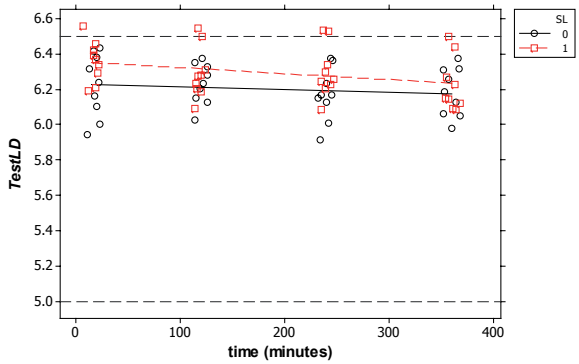

Figure 4. Persistence of *Staphylococcus aureus* on glass slide carriers. Each point is a *TestLD* for a specific SL. Although sampling only occurred at 15, 120, 240, and 360 min, the data have been jittered in the horizontal direction for ease in viewing. The regression lines indicate the decreasing trend over time. The horizontal dashed lines are at 5.0 and 6.5, the proposed limits of validity for *TestLD*.

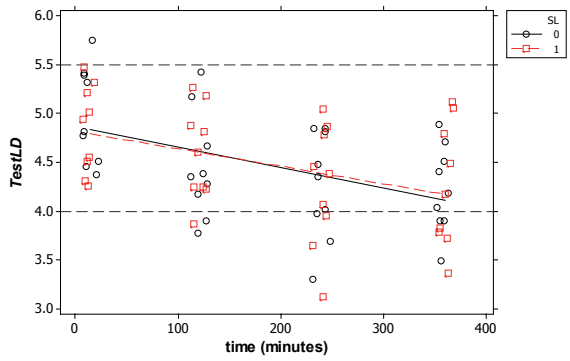

Figure 5. Persistence of *S. enterica* on glass slide carriers. Each point is a *TestLD* for a specific SL. Although sampling only occurred at 15, 120, 240, and 360 min, the data have been jittered in the horizontal direction for ease in viewing. The regression lines indicate the decreasing trend over time. The horizontal dashed lines are at 4.0 and 5.5, the proposed limits of validity for *TestLD*.

Table 3. Summary of the variability results associated with microbe persistence on glass slide carriers[a]

		Without SL			With 5% SL		
Organism	Time	Mean *TestLD*	S_r	S_R	Mean *TestLD*	S_r	S_R
S. aureus	15	6.22	0.1489	0.1859	6.36	0.0957	0.1213
	120	6.23	0.1050	0.1157	6.29	0.1285	0.1465
	240	6.17	0.1217	0.1527	6.30	0.1463	0.1463
	360	6.18	0.1368	0.1368	6.22	0.1480	0.1480
P. aeruginosa	15	5.69	0.2460	0.4854	5.84	0.3079	0.3880
	120	5.12	0.2395	0.3031	5.22	0.5050	0.6935
	240	4.47	0.1417	0.5963	4.83	0.4519	0.5971
	360	4.07	0.3138	0.8338	4.55	0.6180	0.7816
S. enterica	15	4.98	0.3302	0.5368	4.84	0.3336	0.4755
	120	4.46	0.3548	0.5763	4.59	0.3011	0.5044
	240	4.26	0.2225	0.6156	4.26	0.5980	0.5980
	360	4.22	0.2970	0.4573	4.26	0.3439	0.7021

[a] Although the actual tests were conducted with four carriers, these calculations show the predicted S_r and the predicted S_R for six carrier tests.

was fit, with lab and "tests nested within lab" as random effects and time as a covariate. Because experiments with and without SL were run side-by-side, SL differences in time trend were analyzed by first subtracting the paired SL responses from each other. The ANCOVA was fit to these differences. Because there were only three laboratories in the study, all reported *P*-values were calculated using 2 degrees of freedom.

For all data analyses, individual value plots, residual plots, normal probability plots, and the Anderson-Darling test for normality (4) were used to identify unusual data and to assess model fit. All ANOVA and ANCOVA models were implemented using the statistical software R, package *nlme* (5, 6). All statements of statistical significance are made with respect to a significance level of 5%.

Results and Discussion

Historical Data

The LD values for *S. aureus* and *P. aeruginosa* are plotted in Figures 1 and 2, and the summaries of the data are presented in Table 2. Across the four combinations of microbes and SL treatments, the mean *TestLD* ranged from approximately 6.0 (a geometric mean density of 0.9×10^6 CFU/carrier) to 6.3 (a geometric mean density of 2.0×10^6 CFU/carrier). All but two of the 116 *TestLD* values were at least 5.0 (a geometric mean of 1.0×10^5 CFU/carrier). The maximum observed *TestLD* was 6.53 among *P. aeruginosa* tests and 6.77 among *S. aureus* tests. The *TestLD* values per microbe and SL combination showed good repeatability (range of 0.208 to 0.492) and reproducibility (range of 0.222 to 0.534). Across microbes and SL treatments, the mean *TestLD* (±SEM, standard error of the mean) was 6.2 (±0.07) (a geometric mean of 1.5×10^6 CFU/carrier). The data presented here form the basis for the minimum and maximum carrier count.

Plots of *TestLD*s against date and experiment number (data not shown) did not produce an obvious time trend. The ANCOVA produced a small, not statistically significant ($P \geq 0.07$), positive linear slope. The homogeneity of variances assumption was not discredited by the data; for each of the four combinations of microbe and SL, the within-laboratory variances of *TestLD*s were not statistically significantly different among the laboratories. The normality assumption was discredited by the data; the Anderson-Darling test for normality for the residuals of the ANOVA model showed statistically significant deviations from normality due to an occasional unusually small LD value. We believe that those few unusual values represent the inherent variability of the GSPT, and that the number of experiments is sufficiently large that the statistical comparison of means is reliable even though the data do not conform to the normality assumption. Based on the analysis of the historical data set, we propose a minimum *TestLD* of 5.0 (a geometric mean of 1.0×10^5 CFU/carrier) and a maximum *TestLD* of 6.5 (a geometric mean of 3.2×10^6 CFU/carrier) as a requirement for *S. aureus* tests and *P. aeruginosa* tests. This range provides for the potential inherent variability that may be experienced across a wide range of laboratories and the slight effect due to the addition of an SL. Thus, any tests producing a mean log density lower than 5.0 or greater than 6.5 would be invalidated and would have to be repeated, except for two retesting scenarios (*see* description below).

In order to reduce the potential of acquiring carrier counts higher than the upper limit, additional steps are recommended to allow for dilution (using sterile broth) of the final 48–54 h test culture. Conversely, over-dilution of the final test culture may result in carrier counts lower than the 5 log minimum. To adjust the microbial titer, the harvested inoculum may be diluted in sterile broth before the addition of a SL (if required). Although unlikely, concentration of the final test cultures may be necessary to increase the bacterial titer before carrier inoculation. Guidance for dilution of the test culture is provided in the proposed text for the method revision as presented below. Based on the product's efficacy, repeat testing scenarios when carrier counts fall above or below the established range must be addressed as revisions to the method, as well.

Persistence Data

The mean *TestLD* values are plotted as a function of time for each of *P. aeruginosa*, *S. aureus*, and *S. enterica* in Figures 3–5, respectively. Each figure depicts the linear trend for the *TestLD*s both with and without SL. When the SL levels were analyzed separately, only *S. enterica*, both with and without SL, exhibited a statistically significant decrease in the mean log density over time (P-value <0.05 for *S. enterica*; P-value <0.10 for *P. aeruginosa*; P-value >0.05 for *S. aureus*). However, when pooled across both SL levels, all three microbes exhibited significant negative trends (P-value <0.05). Pooling was appropriate since none of the microbes exhibited statistically significantly different trends between SLs (P-value ≥0.07). Per hour, the overall decreasing linear trend in the mean log density was 0.25 for *P. aeruginosa*, 0.02 for *S. aureus*, and 0.12 for *S. enterica*.

For each microbe and point in time, the mean *TestLD*, S_r, and S_R are provided in Table 3. After 2 h, there was a noticeable drop in the mean *TestLD* for *P. aeruginosa* and *S. enterica.* At 6 h, there was a marked increase in the S_R for *P. aeruginosa* both with and without a SL, and an increase in the S_R for *S. enterica* with a SL.

Based on these persistence study data, a minimum *TestLD* (calculated from six carriers) of 4.0 (a geometric mean of 1.0×10^4 CFU/carrier) and a maximum *TestLD* (calculated from six carriers) of 5.5 (a geometric mean 3.2×10^5 CFU/carrier) are recommended for carriers inoculated with *S. enterica.* Over all the data, 16 *TestLD*s fell below 4.0 and one was larger than 5.5 (Figure 5). However, when only the first 2 h of data are considered, only three *TestLD*s fell below 4.0.

The current GSPT indicates that carriers must be used on the day of preparation. This use period is too long, especially for *P. aeruginosa* and *S. enterica*, and should be revised. Based on the data generated during the persistence study, it is advisable to perform the efficacy testing and the carrier counts within 2 h after drying to mitigate a loss of viable inoculum.

AOAC *Official Method* **961.02** has been revised to include the procedural changes described in this report. The proposed carrier count procedure for the GSPT method is provided as follows:

*Carrier Count Procedure for AOAC Official Method **961.02** (to follow section C. Operating Technique.—***(i)** *Verification of positive carriers).*

(j) *Enumeration of viable bacteria from carriers (carrier counts).*—After the carriers have dried, assay carriers in two sets of three carriers, one set prior to conducting the efficacy tests and one set following the test. Place each of the inoculated, dried carriers in a 38 × 100 mm culture tube or sterile 50 mL polypropylene conical tube containing 20 mL letheen broth. Vortex immediately—60 ± 5 s for *P. aeruginosa* or 120 ± 5 s for *S. aureus* and *S. enterica*. After vortexing, make serial 10-fold dilutions in 9 mL phosphate-buffered dilution water. If the serial dilutions are not made and plated immediately, keep the vortexed tubes at 2–5°C until this step can be done. However, dilution and plating should be performed within 2 h of vortexing. Alternatively, the letheen broth tubes may be pooled after vortexing for each set of three carriers. An aliquot of the pooled media (60 mL) will be serially diluted and plated, and the average carrier count per set will be calculated.

Plate 0.1 mL aliquots of appropriate dilutions in duplicate on tryptic soy agar (TSA) or TSA with 5% sheep blood using pour- or surface-spread plating; dilutions of 10^{-1} through 10^{-3} should result in plates with a countable range of colonies. Briefly mix each serial dilution tube on a vortex mixer before plating. For pour-plating, add molten TSA tempered to approximately 45°C to each plate. Swirl the pour-plates to distribute cells evenly and allow agar to solidify. Incubate plates (inverted) concurrently with the efficacy subculture tubes at 36 ± 1°C for up to 48 ± 2 h. Count the colonies by hand or with a colony counter. Use dilutions yielding counts up to 300 for enumeration; plate counts of 0 are to be included in the calculations.

Calculate the $\log_{10}$ density (LD) for each carrier by taking the $\log_{10}$ of the density (per carrier). The mean LD across carriers is the *TestLD* for the test. The *TestLD* 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) for *P. aeruginosa* and *S. aureus* tests. A *TestLD* below 5.0 or above 6.5 invalidates the test, except for two retesting scenarios (*see Retesting Guidance* below). For *S. enterica*, the *TestLD* 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 *TestLD* below 4.0 or above 5.5 invalidates the test, except for two retesting scenarios (*see Retesting Guidance* below).

Note: If the GSPT method is strictly followed, *TestLD*s of at least 5.0 (for *P. aeruginosa* and *S. aureus*) and 4.0 (for *S. enterica*) are expected; values lower than these levels may indicate a dilution error, poor media quality, interference by environmental parameters (e.g., carrier drying and culture incubation conditions), or lack of adherence to the method. The prescribed minimum count also accounts for the addition of 5% SL to the inoculum.

Note: For the purpose of achieving the carrier count range, dilution of the final test culture may be performed using the sterile culture medium used to generate the final test culture (e.g., nutrient broth). Dilution of the final test culture (e.g., one part culture plus one part sterile broth) should be made before addition of the SL to the inoculum. Although unlikely, concentration of the final test culture may be necessary if the bacterial titer in the final test cultures is too low. Concentration may be achieved using centrifugation (e.g., 5000 × *g* for 20 min) and resuspending the pellet in the appropriate volume of the sterile final test culture medium necessary to meet the carrier count range. In addition, the use of a spectrophotometer to measure optical density (OD 650 nm) is recommended to provide a tool (i.e., development of a standard curve) for assessing the need to concentrate or dilute the final test culture. Sterile broth medium should always be used to calibrate the spectrophotometer.

Retesting guidance.—For tests where the product passes and the *TestLD* value is above 6.5 for *S. aureus* and *P. aeruginosa* or above 5.5 for *S. enterica*, no retesting is necessary. For a test where the product fails and the *TestLD* is below 5.0 for *S. aureus* and *P. aeruginosa* or below 4.0 for *S. enterica*, no retesting is necessary. For tests where the product fails and the mean *TestLD* is above 6.5 for *S. aureus* and *P. aeruginosa* or above 5.5 for *S. enterica*, retesting may be conducted. These changes are only for the organisms specified in this report.

*Revision for AOAC Official Method **961.02**, section C. Operating Technique.*—**(e)** *Carrier inoculation.*—Delete the following statement: "Inoculated carriers must be used on

day of preparation." Insert the following statements: *"Note:* Use inoculated carriers for determining carrier counts, (**j**), and performing efficacy testing. Use inoculated carriers within 2 h of drying."

Acknowledgments

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