MICROBIOLOGICAL METHODS

Improving the AOAC Use-Dilution Method by Establishing a Minimum Log Density Value for Test Microbes on Inoculated Carriers

Stephen F. Tomasino and Rebecca M. Pines
U.S. Environmental Protection Agency, Office of Pesticide Programs, Microbiology Laboratory, Environmental Science Center, Fort Meade, MD 20755-5350
Martin A. Hamilton
Big Sky Statistical Analysts, Bozeman, MT 59715

The AOAC Use-Dilution methods, 955.14 (*Salmonella enterica*), 955.15 (*Staphylococcus aureus*), and 964.02 (*Pseudomonas aeruginosa*), are used to measure the efficacy of disinfectants on hard inanimate surfaces. The methods do not provide procedures to assess log density of the test microbe on inoculated penicylinders (carrier counts). 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 method deficiency. Based on carrier count data collected by four laboratories over an 8 year period, a minimum log density value is proposed to qualify the test results. Carrier count data were collected concurrently with 242 Use-Dilution tests. The tests were conducted on products bearing claims against *P. aeruginosa* and *S. aureus* with and without an organic soil load (OSL) added to the inoculum (as specified on the product label claim). Six carriers were assayed per test for a total of 1452 carriers. All 242 mean log densities were at least 6.0 (geometric mean of 1.0 10^6 CFU/carrier). The mean log densities did not exceed 7.5 (geometric mean of 3.2 10^7 CFU/carrier). For all microbes and OSL treatments, the mean log density (SEM) was 6.7 (0.07) per carrier (a geometric mean of 5.39 10^6 CFU/carrier). The mean log density for six carriers per test showed good repeatability (0.29) and reproducibility (0.32). A minimum mean log density of 6.0 is proposed as a validity requirement for *S. aureus* and *P. aeruginosa*. The minimum level provides for the potential inherent variability that may be experienced by a wide range of laboratories and the slight effect due to the addition of an OSL. A follow-up report is planned to present data to support the carrier count procedure and carrier counts for *S. enterica*.

The United States Environmental Protection Agency (EPA) regulations specify that product performance data must be submitted to support the registration of antimicrobial products bearing claims to control microorganisms that pose a threat to human health. The AOAC Use-Dilution methods (1–3) **955.14** (*Salmonella enterica*), **955.15** (*Staphylococcus aureus*), and **964.02** (*Pseudomonas aeruginosa*) are accepted by the EPA to substantiate bactericidal claims of hospital disinfectants for treating hard surfaces. With the exception of the test microbe and the preparation of test culture, the three Use-Dilution methods are essentially the same. In the Use-Dilution method, stainless steel carriers (penicylinders) are inoculated with the test microbe by submerging the carriers in a suspension of test culture; the carriers are dried in an incubator and are used to deliver the test microbe into a liquid disinfectant. Following the contact time, carriers are removed from the disinfectant and placed into a neutralizer/recovery medium. Survival of inoculum is determined qualitatively by a growth or no growth assessment.

Due to its continued use as a regulatory method, the EPA has worked closely with AOAC INTERNATIONAL and the user community over the years to enhance the method through the official AOAC editorial revision process. As a result, revised versions of the three Use-Dilution methods were published in the AOAC *Official Methods of Analysis* in 2006 (1–3). The editorial revisions focused on updating sources of reagents and supplies, and reducing ambiguity of the procedures by providing concise, easy to follow steps without extensive cross-referencing. The revisions allow users to perform the methods more consistently, without interpretation of the steps. The revised methods also include guidance on conducting neutralization confirmation, quantifying inoculum from carriers, and preparing hard water

Submitted for publication April 17, 2009.
The recommendation was approved by the Methods Committee on Antimicrobial Efficacy Testing as a method modification. *See* “Official Methods Program Actions,” (2009) *Inside Laboratory Management*, May/June issue.
Corresponding author’s e-mail: tomasino.stephen@epa.gov

Table 1. Summary for the mean log densities, averaged for six carriers per test, for each combination of microbe, OSL, and laboratory

Microbe	OSL	Laboratory	No. of tests	Mean	SEM	S_r
Pseudomonas aeruginosa	Absent	1	7	6.737	0.105	0.279
	Absent	2	27	6.926	0.059	0.306
	Absent	3	9	6.923	0.065	0.196
	Absent	4	6	7.198	0.030	0.073
	Present	1	12	6.635	0.100	0.348
	Present	2	31	6.389	0.030	0.164
	Present	3	20	6.846	0.042	0.187
	Present	4	14	6.711	0.036	0.133
Staphylococcus aureus	Absent	1	7	6.871	0.089	0.235
	Absent	2	23	6.751	0.057	0.275
	Absent	3	12	6.942	0.064	0.223
	Absent	4	5	6.763	0.039	0.087
	Present	1	10	6.615	0.105	0.331
	Present	2	30	6.487	0.037	0.203
	Present	3	15	6.932	0.070	0.271
	Present	4	14	6.474	0.029	0.109

and the organic soil load. The additional information is currently not considered an official part of the method. Although the editorial revisions should provide a more "level playing field" across laboratories, targeted procedural changes and the introduction of additional guidance will further enhance the methods.

The EPA is considering the adoption of a quantitative efficacy test method for measuring the performance of antimicrobial products; however, the development and implementation of new methodology and performance standards will likely require considerable time to complete. Thus, as an interim measure, efforts to improve and further standardize the Use-Dilution method by introducing procedural changes are justified.

Relative to the subject of this paper, the lack of an enumeration procedure to assess and monitor the viable test microbe population dried on inoculated penicylinders (referred to here as "carrier counts") of the Use-Dilution method is addressed. Without a method to measure and monitor carrier counts, the associated efficacy data may not be reliable and repeatable. Although an enumeration process was not provided in the original Use-Dilution method, most, if not all, laboratories that conduct the Use-Dilution method on a regular basis measure carrier counts. This report provides a standardized procedure to address this deficiency and establishes a minimum level of viable cells per carrier to qualify the efficacy results. We recognize that other aspects of the Use-Dilution methods may also require enhancement and standardization; however, we feel that monitoring the carrier counts is a priority.

A procedure used to determine carrier counts of untreated dried carriers has been used for several years by the EPA and collaborating laboratories to monitor the quality and consistency of test cultures and for charting purposes. Previously, we reported on the use of the carrier count protocol and presented data generated from a single laboratory (EPA) over a 7 year period; a proposal to incorporate the carrier count procedure for *Staphylococcus* and *Pseudomonas,* and an expected range in viable cells were also presented in the report (4). In the single-laboratory study, a mean log density of 6.6 (approximately $3.7 \quad 10^6$ CFU/carrier) was shown for both microbes and the presence/absence of an organic soil load; 95% of the mean carrier counts fell within 6.0–7.0 logs. To verify the results, data from three additional laboratories that also performed the carrier count procedure over a comparable time period were collected, organized in a database, combined with the single laboratory data and analyzed. Due to the limited amount of testing of *S. enterica* over the data collection period, only preliminary, single-laboratory data for *S. enterica* are presented here. Multilaboratory data collection for *S. enterica* carrier counts is underway using the same enumeration procedure. A follow-up report is planned to present data to support the carrier count procedure and minimum carrier counts for *S. enterica*.

Previously, AOAC Method **966.04** (Sporicidal Activity of Disinfectants), a qualitative procedure similar to the Use-Dilution method, was modified to include a carrier count procedure to assess spore populations on dried inoculated porcelain carriers and a minimum spore load of 5 logs per carrier (5, 6). Thus, a relevant example exists for the

Table 2. Observed log density for *S. aureus* with OSL absent[a]

Lab	Test	Mean	SD
1	1	7.07906	0.26997
1	2	7.03689	0.31090
1	3	7.05490	0.32960
1	4	7.03513	0.41379
1	5	6.53156	0.15172
1	6	6.75328	0.11078
1	7	6.60597	0.09378
2	1	6.99689	0.20906
2	2	6.54048	0.11704
2	3	7.14490	0.19661
2	4	7.27669	0.16573
2	5	6.66076	0.15269
2	6	6.80355	0.25850
2	7	6.97394	0.12250
2	8	7.07605	0.21075
2	9	6.23508	0.14732
2	10	6.74121	0.08366
2	11	6.66286	0.11999
2	12	6.80581	0.08533
2	13	6.53132	0.21142
2	14	6.68293	0.07669
2	15	6.66163	0.18486
2	16	6.55518	0.19877
2	17	6.74585	0.10532
2	18	6.81355	0.10025
2	19	6.83291	0.10005
2	20	6.12620	0.06514
2	21	6.87851	0.09915
2	22	7.05115	0.15397
2	23	6.47117	0.05933
3	1	6.85453	0.18097
3	2	7.07432	0.26911
3	3	7.19897	0.17502
3	4	6.90838	0.38827
3	5	7.24423	0.10909
3	6	7.20054	0.17007
3	7	7.01114	0.16891
3	8	6.83297	0.23615
3	9	6.86677	0.20724
3	10	6.48566	0.09365
3	11	6.68204	0.14564
3	12	6.94022	0.23326
4	1	6.77421	0.07396
4	2	6.77331	0.16400
4	3	6.61543	0.10631
4	4	6.84322	0.08541
4	5	6.80798	0.10780

[a] The mean and SD of six log densities are provided for each of the 47 tests.

modification of a qualitative method to quantify carrier-associated bacteria. The present investigation uses the extensive, multilaboratory data set to support the adoption of an inoculum enumeration procedure and the establishment of a minimum carrier count as official modifications to Methods **955.15** and **964.02**. The inclusion of these modifications, along with the recently published editorial changes, will vastly improve the Use-Dilution method. Carrier counts can be used to assess the quality and consistency of the microbial test system from test to test and to establish validity of the efficacy test. In addition, if recorded, carrier counts can be used along with the frequency of the number of positive and negative carriers to estimate the log reduction per the P/N formula (5), thereby providing the Use-Dilution method with a conventional quantitative measure of disinfectant product efficacy.

Experimental

Data Collection

The data were generated by four laboratories over an 8 year period (1999–2006) according to the enumeration procedure described by Tomasino et al. (4). Under a federal Antimicrobial Testing Program, a program designed to verify the performance of registered hospital disinfectants, the EPA Office of Pesticide Programs Microbiology Laboratory, Fort Meade, MD, and three state laboratories (Michigan Department of Agriculture, Ohio Department of Agriculture, and North Carolina Department of Agriculture) collected the carrier count data concurrently with performing the efficacy evaluations. Product testing was conducted in accordance with EPA's Federal Insecticide, Fungicide, and Rodenticide Act Good Laboratory Practice Standards (7). Trained analysts conducted the assays. The testing program prioritized the evaluation of product claims against *S. aureus* and *P. aeruginosa*; thus, the data are predominantly associated with Methods **955.15** and **964.02**. The laboratories strictly adhered to the published Use-Dilution methods. Most important to this study, the stock culture initiation and maintenance, test culture preparation, and carrier inoculation steps detailed in the Use-Dilution methods were closely followed. In all studies, no dilution of the test cultures was performed. Each laboratory followed the specified protocol for determining carrier counts.

Although the Use-Dilution methods provide an option for the use of nutrient broth or synthetic broth for the preparation of test cultures, only nutrient broth was used in this study. Thus, it was necessary to generate supplemental carrier count data using synthetic broth for growing test cultures; the data are provided in this report.

The carrier count data presented here are associated with 242 individual Use-Dilution method tests. The efficacy tests were conducted on products bearing claims against *P. aeruginosa* and *S. aureus* with and without an organic soil load (OSL) added to the inoculum (depending on the specific product label claim). Horse serum was used as the OSL in the majority of the tests. OSL was added at 5% (v/v) to the inoculum prior to

Table 3. Observed log density data for *S. aureus* with OSL present[a]

Lab	Test	Mean	SD
1	1	7.16250	0.40096
1	2	6.82221	0.18978
1	3	6.73028	0.14848
1	4	6.76398	0.33086
1	5	7.01891	0.08064
1	6	6.19803	0.10966
1	7	6.28747	0.17253
1	8	6.38727	0.10457
1	9	6.33373	0.11472
1	10	6.44259	0.20351
2	1	6.93728	0.21412
2	2	6.48796	0.45331
2	3	6.65426	0.04678
2	4	6.43325	0.11277
2	5	6.54141	0.05296
2	6	6.59223	0.13638
2	7	6.65746	0.16588
2	8	6.77383	0.18637
2	9	6.53247	0.22629
2	10	6.61468	0.24764
2	11	6.64796	0.17439
2	12	6.38209	0.15862
2	13	6.84182	0.20669
2	14	6.50723	0.20011
2	15	6.63594	0.20049
2	16	6.28049	0.14819
2	17	6.63227	0.14625
2	18	6.28976	0.13029
2	19	6.21934	0.19253
2	20	6.13919	0.19978
2	21	6.19949	0.12003
2	22	6.20108	0.05593
2	23	6.61825	0.05901
2	24	6.39871	0.16492
2	25	6.18549	0.19505
2	26	6.48761	0.20524
2	27	6.60011	0.13118
2	28	6.36038	0.28436
2	29	6.36409	0.21745
2	30	6.38079	0.22229
3	1	6.91370	0.10121
3	2	6.52580	0.20529
3	3	6.59062	0.14449
3	4	6.98719	0.22036
3	5	7.29882	0.18469
3	6	7.11015	0.17826
3	7	7.20533	0.17638
3	8	7.05523	0.18239
3	9	7.38594	0.26431
3	10	7.05331	0.26338
3	11	6.85811	0.15162
3	12	6.87342	0.40131
3	13	6.80767	0.20154
3	14	6.87113	0.18758
3	15	6.44572	0.16722
4	1	6.48554	0.14145
4	2	6.56618	0.12960
4	3	6.27281	0.04693
4	4	6.56922	0.13349
4	5	6.44439	0.07575
4	6	6.41549	0.09100
4	7	6.55879	0.06038
4	8	6.35296	0.12617
4	9	6.55871	0.07750
4	10	6.43649	0.07709
4	11	6.61681	0.05634
4	12	6.29458	0.08430
4	13	6.57128	0.09394
4	14	6.49042	0.11033

[a] The mean and SD of six log densities are provided for each of the 69 tests.

carrier inoculation. The distribution of the tests conducted by the four laboratories is provided in Table 1. Six carriers per test day, for a total of 1452 total carriers, were analyzed for inoculum titer. Carriers were removed at random from the inoculated set after the 40 min drying step. Processing of carriers after drying was conducted as soon as possible, and generally within 2 h of drying. 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 OSL. Carrier count data (raw data and spreadsheets) were peer-reviewed by EPA laboratory staff and verified by the Quality Assurance Unit prior to the statistical analysis. If an unusual observation was discovered during the peer review or the statistical analysis, the data were discussed with the appropriate laboratory personnel. No data errors were discovered. All data were deemed valid and were included in the statistical analysis.

For the additional data on synthetic broth, test cultures of *P. aeruginosa* and *S. aureus* were prepared with synthetic broth per the Use-Dilution method. Twenty-four inoculated carriers (without OSL) were evaluated per organism; four carriers were evaluated per organism at 15 and 120 min after

Table 4. Observed log density data for *P. aeruginosa* with OSL absent[a]

Lab	Test	Mean	SD
1	1	6.5029	0.2309
1	2	6.5091	0.2196
1	3	6.5744	0.1842
1	4	6.4829	0.2431
1	5	6.9490	0.1626
1	6	7.0955	0.1831
1	7	7.0444	0.1915
2	1	7.2225	0.1717
2	2	7.3241	0.2999
2	3	6.9510	0.1809
2	4	6.8986	0.2257
2	5	6.6059	0.1884
2	6	6.9286	0.1289
2	7	6.7494	0.2440
2	8	6.7714	0.1305
2	9	6.0890[b]	0.4013
2	10	7.1064	0.1845
2	11	6.7994	0.1869
2	12	7.0907	0.1144
2	13	6.6971	0.1210
2	14	7.2960	0.2368
2	15	6.9213	0.2285
2	16	7.4795	0.2258
2	17	7.0503	0.0972
2	18	7.0066	0.2432
2	19	6.7028	0.1056
2	20	7.0394	0.1391
2	21	7.4534	0.0655
2	22	7.0027	0.0620
2	23	6.6803	0.2225
2	24	7.0247	0.2303
2	25	6.5442	0.2699
2	26	6.5219	0.1996
2	27	7.0473	0.0875
3	1	7.0975	0.1378
3	2	7.0103	0.2416
3	3	6.6905	0.1024
3	4	7.0592	0.2798
3	5	7.1941	0.1804
3	6	6.8998	0.9016
3	7	6.9902	0.1346
3	8	6.7164	0.1912
3	9	6.6466	0.1244
4	1	7.1548	0.1372
4	2	7.1806	0.1042
4	3	7.2422	0.0980
4	4	7.3161	0.1982
4	5	7.1903	0.0690
4	6	7.1054	0.1217

[a] The mean and SD of six log densities are provided for each of the 49 tests.

[b] Determined to be an outlier; however, no outliers were excluded from the data analysis and standard deviation estimates.

carrier drying with three replications (i.e., independent test cultures). A nutrient broth control was included in one of the three replications and served as baseline carrier count data.

Statistical Analysis

The density of viable bacteria (CFU/carrier) was measured on 1452 carriers, six carriers in each of 242 Use-Dilution method tests. Each density was $\log_{10}$-transformed to form the log density. All statistical calculations were performed on the log density values. The log densities were partitioned into 16 subsets, one for each combination of laboratory, microbe, and OSL. Each subset was submitted to analysis of variance (ANOVA) using a one-factor, random effects, linear statistical model to calculate the mean log density, the variance among Use-Dilution method tests (denoted by V_T), the variance among carriers within a test (denoted by V), and the repeatability SD (denoted by S_r). The S_rv alue pertains to the mean of the six carriers in a test, $S_r = [V_T + V/6]^{½}$.

For purposes of estimating the reproducibility standard deviation (denoted by S_R), the log density values were partitioned into four subsets, one for each combination of microbe and OSL. The entire data set and all four subsets were submitted to ANOVA using a two-factor, nested random effects, linear statistical model to estimate the mean log density, the variance among labs (denoted by V_L), the V_T, and the V. Let N denote the number of tests in the analysis. The standard error of the mean log density (SEM) was calculated using $SEM = [(V_L/4) + (V_T/N) + (V/(6N))]^{½}$, which has 3 degrees of freedom. The repeatability and reproducibility standard deviations pertain to the mean of the six carriers in a test, $S_r = [V_T + V/6]^{½}$ and $S_R = [V_L + V_T + V/6]^{½}$.

All ANOVA calculations were performed using the Minitab statistical computer package (Release 15, Minitab Statistical Software, http://www.minitab.com/products), which uses the same formulas as in the AOAC Guidelines (*Official Methods of Analysis* (2005), 18th Ed., Appendix D; http://www.aoac.org/stats/AOAC_BlindDup_v2-0.xls).

Results and Discussion

The *S. aureus* and *P. aeruginosa* data (mean log density) for each test are presented in Tables 2–6. After the initial review of

Table 5. Observed log density data for *P. aeruginosa* with OSL present[a]

Lab	Test	Mean	SD
1	1	6.9043	0.1990
1	2	6.7384	0.3517
1	3	7.3631[b]	0.2403
1	4	6.8579	0.4093
1	5	6.2849	0.2061
1	6	6.9746	0.2776
1	7	6.5376	0.1490
1	8	6.6540	0.0632
1	9	6.2631	0.0899
1	10	6.3446	0.1336
1	11	6.2216	0.0974
1	12	6.4713	0.2586
2	1	6.4920	0.2716
2	2	6.5785	0.0861
2	3	6.5079	0.0913
2	4	6.3677	0.2524
2	5	6.0176	0.0720
2	6	6.5477	0.2285
2	7	6.7269	0.1217
2	8	6.2313	0.1676
2	9	6.1255	0.1720
2	10	6.3318	0.1371
2	11	6.3054	0.1052
2	12	6.3219	0.0624
2	13	6.3377	0.0704
2	14	6.2872	0.1276
2	15	6.3847	0.1685
2	16	6.3407	0.1381
2	17	6.3220	0.1859
2	18	6.6483	0.5312
2	19	6.2364	0.1443
2	20	6.2333	0.0379
2	21	6.4644	0.2972
2	22	6.2341	0.1161
2	23	6.2847	0.1740
2	24	6.3745	0.0975
2	25	6.2225	0.0919
2	26	6.3483	0.0864
2	27	6.5608	0.1785
2	28	6.4650	0.0843
2	29	6.6413	0.0859
2	30	6.4943	0.1731
2	31	6.6105	0.1540
3	1	6.8849	0.3616
3	2	6.8114	0.1655
3	3	6.9741	0.1227
3	4	6.7374	0.3063
3	5	6.8937	0.2269
3	6	6.6833	0.1685
3	7	6.9024	0.0969
3	8	6.7610	0.3099
3	9	7.0015	0.6256
3	10	7.1585	0.5156
3	11	6.8490	0.1739
3	12	6.6581	0.3593
3	13	6.9318	0.2081
3	14	6.6538	0.0661
3	15	7.2153	0.3068
3	16	6.8254	0.1624
3	17	6.7398	0.1934
3	18	7.0347	0.2297
3	19	6.8186	0.1948
3	20	6.3862	0.4572
4	1	6.6865	0.2792
4	2	6.7473	0.0998
4	3	6.8045	0.1262
4	4	6.4809	0.0330
4	5	6.8776	0.0575
4	6	6.9184	0.0968
4	7	6.7714	0.0900
4	8	6.7946	0.0806
4	9	6.6693	0.0820
4	10	6.6998	0.0706
4	11	6.7433	0.0714
4	12	6.5213	0.1448
4	13	6.7452	0.1161
4	14	6.4970	0.3295

[a] The mean and SD of six log densities are provided for each of the 77 tests.

[b] Determined to be an outlier; however, no outliers were excluded from the data analysis and standard deviation estimates.

the data (data audit), two log densities were identified as potential outliers: 6.1 (Laboratory 2/*P. aeruginosa*/OSL absent) and 7.4 (Laboratory 1/*P. aeruginosa*/OSL present). However, based on further investigation, the data could not be invalidated and were included in the analysis.

Among the 242 mean log densities, the smallest was 6.0 (geometric mean of 1.0 10^{6} CFU/carrier) and the highest was 7.5 (geometric mean of 3.2 10^{7} CFU/carrier; Tables 2–5 and Figure 1). The mean log density across six carriers per test showed good repeatability (0.29) and reproducibility (0.32); *see* Table 7. For microbes and OSL treatments, the mean log

Table 6. Observed log density data for *S. aureus* and *P. aeruginosa* grown in synthetic broth at 15 min and 120 min post drying[a]

Lab	Test	Organism	Dry time (min)	Mean	SD
1	1[b]	*S. aureus.*	15	6.7990	0.0852
1	2	*S. aureus.*	15	6.8249	0.0766
1	3	*S. aureus.*	15	6.9955	0.3206
1	1[b]	*S. aureus.*	120	6.7160	0.1929
1	2	*S. aureus.*	120	6.9487	0.1292
1	3	*S. aureus*	120	6.8762	0.3745
1	1[c]	*P. aeruginosa*	15	7.0792	0.2297
1	2	*P. aeruginosa*	15	6.7282	0.2678
1	3	*P. aeruginosa*	15	6.7622	0.0802
1	1[c]	*P. aeruginosa*	120	6.8204	0.1577
1	2	*P. aeruginosa*	120	6.2189	0.2242
1	3	*P. aeruginosa*	120	6.8486	0.1895

[a] The mean and SD of four log densities are provided for each of the 12 tests.

[b] Log density data with nutrient broth were 7.0 and 7.2 at 15 and 120 min, respectively.

[c] Log density data with nutrient broth were 6.7 and 7.1 at 15 and 120 min, respectively.

density (SEM) was 6.7 (0.07) per carrier (a geometric mean of 5.39 10^6 CFU/carrier); *see* Table 7. For the 16 combinations of laboratories, microbes, and OSL treatments, the mean log density ranged from 6.4 to 7.2 (Table 1). For OSL treatments, the mean log density for *P. aeruginosa* was higher by 0.07 compared to *S. aureus*; however, the difference was not statistically significant ($P = 0.48$). For microbes, the presence of OSL reduced the mean log density by 0.25 logs (6.89–6.64) per carrier, which is statistically significant ($P = 0.018$). We do not consider the difference of 0.25 to be of practical importance.

Carrier counts generated from the test cultures prepared using synthetic broth were comparable to those for nutrient broth (Table 6). The carrier counts were stable over the sampling period. For the two sample periods (15 and 120 min after carrier drying), the mean log density was 6.8 (range of 6.7–6.9) and 6.7 (range of 6.2–7.0) for *S. aureus* and *P. aeruginosa*, respectively. The corresponding nutrient broth controls exhibited mean log densities of 7.1 for *S. aureus* and 6.9 for *P. aeruginosa* across the sample periods; the values are comparable to the historical data set.

For *S. enterica*, preliminary research from a single laboratory (12 total carrier count tests) showed that mean log densities ranged from 6.4 for OSL present to 6.5 for OSL absent. Additional multilaboratory data will be generated to increase the statistical reliability of these estimates.

Other published reports (8–11) also provide carrier counts for the Use-Dilution method, and the data are comparable to those presented in this report. Specifically, Arlea et al. (11) discuss a four laboratory study involving proposed modifications to the Use-Dilution method and associated carrier count data. The log CFU/carrier for the undiluted *P. aeruginosa* test culture treatment was 7.06 with a range of 6.11–7.76. In addition, the AOAC Hard Surface Carrier Test (HSCT) Methods (**991.47**, **991.48**, and **991.49**) for evaluating efficacy of disinfectants provide procedures to standardize inoculum and specify an acceptable range for carrier counts for each microorganism. The average carrier counts required for a valid HSCT are 0.5–2.0 10^6 CFU/carrier for *S. enterica*(12), and 1–5 10^6 CFU/carrier for *S. aureus* (13) and *P. aeruginosa* (14). Thus, the carrier count data presented in this report are comparable to the accepted range established for the HSCT.

To optimize the predictability and utility of the carrier count data, it would be beneficial to add guidance on a timeframe for conducting the carrier counts assay, and to emphasize the need to perform efficacy testing as soon as possible after carrier drying. Currently, the method indicates that carriers must be used on the day of preparation. This use period is too long and should be revised. In the previous report (1), populations of *S. aureus* and *P. aeruginosa* dried on carriers demonstrated no loss in viability over a 2 h period under ambient conditions. The carrier count data presented in this report were generated from carriers assayed within 2 h after drying. It is advisable to synchronize, as much as possible, the conduct of the carrier count procedure and the efficacy component of the Use-Dilution method. Editorial revisions to encourage use of inoculated carriers as soon as possible after drying on the day of preparation and to avoid

Table 7. Summary of the mean log densities, averaged for six carriers per test, for each microbe and OSL combination

Microbe	OSL	No. of tests	No. of labs	Mean	S_r	S_R
Pseudomonas aeruginosa	Absent	49	4	6.946	0.268	0.296
	Present	77	4	6.645	0.204	0.300
Staphylococcus aureus	Absent	47	4	6.832	0.245	0.254
	Present	69	4	6.627	0.227	0.309
Microbe and OSL combined		242	4	6.731	0.290	0.317

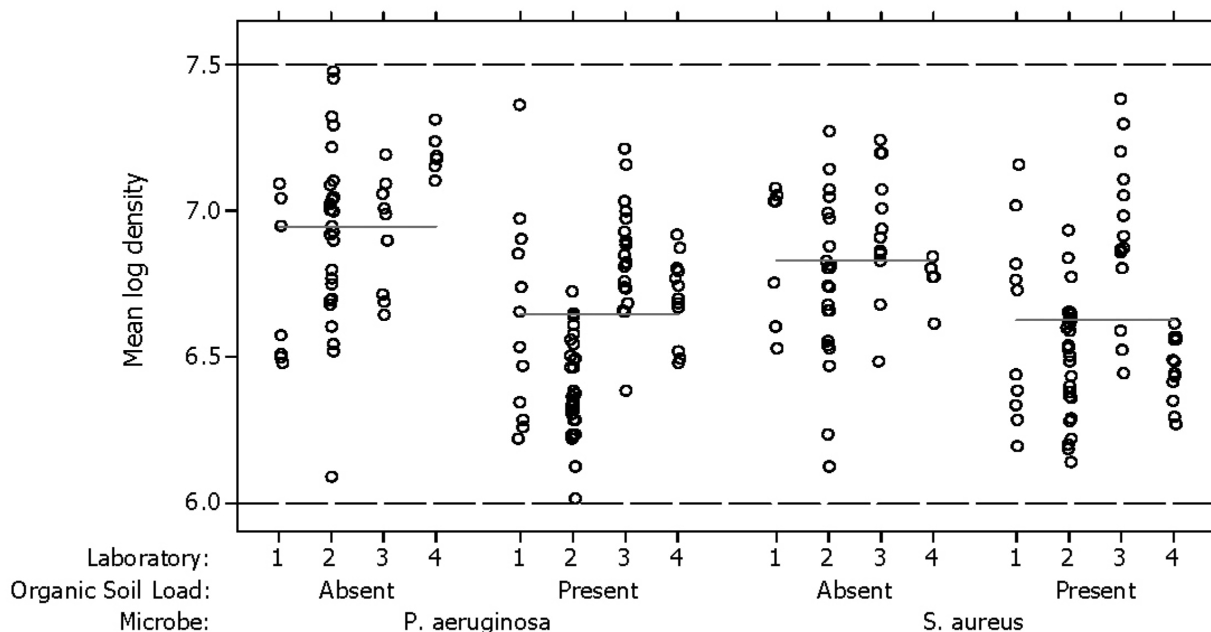

Figure 1. Each point is the mean log density for the six carriers in one of the 242 Use-Dilution method tests. The points aligned vertically are tests in the same laboratory for the specified combination of OSL and microbe. The points are jittered slightly in the horizontal direction to expose overlapping points. The horizontal solid lines are the means of all tests at the specified combination of OSL and microbe. The horizontal dashed lines are at 6.0 and 7.5 logs.

use of carriers that are stored for lengthy periods are justified and are provided in the proposed revision sections.

Conclusions

The AOAC Use-Dilution methods remain important for measuring the performance of hard surface disinfectants, both for pre- and post-registration product testing. The enumeration of viable bacteria dried on the test carriers and the associated charting of these data over the years have been essential to the quality assurance of EPA's post registration Antimicrobial Testing Program. The adoption of quantitative test methods to augment or possibly replace the current qualitative tests is being explored by the EPA and the international regulatory community. As an interim measure during this transition, further enhancements to the Use-Dilution methods are justified and beneficial. Furthermore, the P/N formula (5), which is used to convert qualitative growth/no growth data into log reduction, can be used in the development of a quantitative performance standard as quantitative procedures are introduced into the regulatory framework. A central component of the P/N formula is the mean log density for control carriers.

Although the Use-Dilution method requires carrier inoculation via submersion in the test culture, the resulting mean inoculum level per carrier is very predictable. Most importantly, these data also support the adoption of a minimum carrier count. Based on the data provided in this report, a geometric mean carrier count of approximately 5 10^6 CFU/carrier (mean log density of 6.7) is expected if the laboratory strictly follows the official method for the preparation and harvest of the test culture, including carrier inoculation and drying, and if trained analysts are conducting the tests. To further improve the method, a minimum log density value is recommended as a "qualifying" factor for the validity of the test. We propose a minimum mean log density of 6.0 (geometric mean of 1.0 10^6 CFU/carrier) as the validity requirement for *S. aureus* and *P. aeruginosa*. This level 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 OSL. We recognize that the Use-Dilution method does not call for an OSL to be added to the inoculum; however, as shown in this report, the majority (60%) of the tests were conducted with the addition of OSL to the inoculum (Table 1). Thus, any tests producing a mean log density lower than 6.0 would be invalidated and would have to be repeated. Although a maximum mean log density could be proposed (e.g., 7.5), establishment of a minimum level is more important (i.e., the stringency of the test may be diminished if the carrier counts are too low). Based on the supplemental data presented in this report, the scope of the modifications are applicable to test cultures grown in nutrient broth or synthetic broth. Furthermore, the methodology would be strengthened by the addition of an editorial revision to discourage the dilution or further manipulation of the test culture that may impact the carrier counts. Preliminary data for *S. enterica* also support the use of a minimum mean log density of 6.0; the *S. enterica* data will be fully described in an

upcoming report. Methods **955.15** and **964.02** have been revised to include the procedural changes described in this report (15, 16). The revisions are as follows:

*Carrier Count Procedure for Methods **955.15** and **964.02** (to follow section **C. Operating Technique** (**f**) Verification of positive carriers)*

(**g**) *Enumeration of viable bacteria from carriers (carrier counts)*.—After inoculated carriers have dried, randomly select one carrier from each Petri dish (e.g., six Petri dishes with 12 carriers per dish), see **C**(**b**). Carriers should be assayed for carrier counts within 2 h of drying. *Note*: The carrier count assay and efficacy testing should be synchronized as much as possible. Place each individual carrier in a tube of 10 mL letheen broth and sonicate the tube in an ultrasonic cleaner for 1 min 5 s. For sonication, place tubes into an appropriately sized glass beaker with tap water to the level of letheen broth in the tubes. Place beaker in an ultrasonic cleaner so that the water level in the beaker is even with the water level fill-line on the tank. Fill tank with tap water to water level fill-line. Manually hold beaker so that it does not touch the bottom of the tank, and all three liquid levels (inside test tubes, inside beaker, and tank) are approximately the same. After sonication, briefly mix the letheen broth tube with the carrier on a vortex mixer, and make serial 10-fold dilutions in 9 mL phosphate-buffered dilution water. If the serial dilutions are not made and plated immediately, keep the sonicated tubes at 2–5 C until this step can be done; however, dilution and plating should be performed within 2 h of sonication. Plate 0.1 mL aliquots of appropriate dilutions in duplicate on tryptic soy agar (TSA) using pour- or surface-spread plating; dilutions of 10^{-2} through 10^{-4} should result in plates with a countable range of colonies. Briefly mix each serial dilution tube on a vortex mixer prior to 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 24–48 h. Count colonies by hand or with colony counter. Use dilutions yielding counts up to 300 for enumeration; plate counts of 0 are to be included in the calculations.

To calculate CFU/mL (of broth), use the following equation, where 10^{-x}, 10^{-y}, and 10^{-z} are the dilutions plated:

$$\text{CFU/mL} = \frac{(avg.CFU\ for 10^{-x}) \quad (avg.CFU\ for 10^{-y}) \quad (avg.CFU\ for 10^{-z})}{10^{-x} \quad 10^{-y} \quad 10^{-z}}.$$

Use counts of 0–300 for calculation purposes. Score counts above 300 as TNTC (Too Numerous to Count). To calculate CFU/carrier, multiply the CFU/mL by the volume of letheen broth into which the bacteria were harvested from the carrier by sonication (10 mL).

Example calculation: Adjust dilutions for volume plated (0.1 mL). For average CFU of 115 at the 10^{-3} dilution, 15 at the 10^{-4} dilution, and 0 at the 10^{-5} dilution, the result is 1.17 10^5 CFU/mL (of broth). The viable bacterial density for the carrier is:

1.17 10^5 CFU/mL of broth 10 mL of broth/carrier = 1.17 10^6 CFU/carrier.

Calculate the log density for each carrier by taking the $\log_{10}$ of the density (per carrier). The mean log density across carriers is the mean log density for the test. Let M denote the mean log density. The 10^M is the geometric mean density for the test. The mean log density must be at least 6.0 (corresponding to a geometric mean density of 1.0 10^6); a mean log density below 6.0 invalidates the test. *Note*: If the Use-Dilution method is strictly followed, mean log densities of at least 6.0 are expected; values lower than 6.0 may be indicative of a dilution error, poor media quality, interference by environmental parameters (e.g., carrier drying and culture incubation conditions), contamination, or lack of adherence to the method. Concentration and/or dilution of the test culture are not required to achieve acceptable counts. The prescribed minimum count also accounts for the addition of 5% OSL to the inoculum.

*Revision for Methods **955.15** and **964.02**, section C. **Operating Technique** (**b**) Test culture preparation*

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 (**g**) and performing efficacy testing as soon as possible after drying on the day of preparation to avoid a reduction in microbial titer. Overnight or long-term storage of inoculated carriers is discouraged."

Acknowledgments

We thank Kiran Verma, Quality Assurance Officer for the EPA Microbiology Laboratory, for conducting the audit of data that appear in this report, and Michele Cottrill (EPA Microbiology Laboratory) for her work on *S. enterica*. We also thank Knoxley Japal (EPA Microbiology Laboratory) for providing the supplemental carrier count data for synthetic broth. We express our appreciation to the following laboratories for their participation in the EPA Antimicrobial Testing Program and generation of the carrier count data:

Michigan Department of Agriculture, Laboratory Division, William C. Geagley Laboratory, East Lansing, MI

Ohio Department of Agriculture, Consumer Analytical Laboratory, Reynoldsburg, OH

North Carolina Department of Agriculture, Constable Laboratory, Raleigh, NC

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