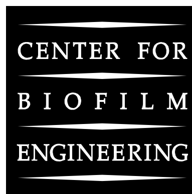

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Quantitative Carrier Test for Sporicides:
Statistical Analysis of the Collaborative Study
by
Martin Hamilton, Todd DeVries, and Nicola Tilt

Summary: *A collaborative study involving 15 laboratories was carried out to determine the repeatability and reproducibility of the Quantitative Carrier Test for Sporicides. The log reduction in viable spore numbers was used as the measure of efficacy. One negative control formulation and three concentrations of four active formulations were tested. Nine formulation×concentration combinations were tested as masked duplicates. The results showed that the repeatability and reproducibility depended on the efficacy of the tested formulation. For chemical disinfectants that produced mean (averaged over all laboratories) log reduction values between 7.5 and 7.9, the repeatability standard deviation of the test was 0.2, the reproducibility standard deviation was 0.5, and variability among laboratories contributed 76% of the total variability of test results. For disinfectants that produced mean log reduction values between 3.8 and 6.0, the repeatability standard deviation was 0.8, the reproducibility standard deviation was 1.3, and variability among laboratories contributed 60% of the total variability of test results. For strong disinfectants having mean Log Reduction values of at least 7.0, the reproducibility standard deviation was 0.5. Lab-to-lab variability contributed 76% of the total variability.*

INTRODUCTION

The Quantitative Carrier Test for Sporicides is a microbial assay for measuring the efficacy of a sporicidal chemical when applied to spores immobilized in a dried organic film on a glass surface. The standard operating procedure for the test is described in Springthorpe and Sattar (1995). In this study, 15 laboratories each conducted the test 13 times, using chemical formulations that were prepared, coded, and shipped from the University of Ottawa. Masked duplicates were included among the formulations sent to each laboratory. In all, thirteen distinct formulation×concentration combinations were tested, eight of them in duplicate. The thirteen comprised four sporicidal chemicals, each at three concentrations, and one negative control formulation.

The active formulations were commercial products, designated here by the generic names: Hydrogen peroxide, Glutaraldehyde 1, Glutaraldehyde 2, and Chlorine dioxide. A character *a, b,* or *c* was

added to a formulation name to indicate the concentration level, with *a* being most concentrated and *c* being most dilute. The 15 laboratories were randomly assigned single letter code names, A-O.

Each laboratory recorded the test results on standardized forms that were mailed to the Department of Microbiology and Immunology, University of Ottawa, where the data were entered into computer spreadsheet files. The spreadsheet files were sent to the Center for Biofilm Engineering, Montana State University, for statistical analysis.

DATA ANALYSIS

The primary goal of the data analysis was to calculate estimates of the repeatability and reproducibility. The estimates were based on the calculated Log Reduction values and each Log Reduction was based on the numbers of viable spores observed on each of the three control carriers and ten test carriers. The number of viable spores per carrier was calculated from the numbers of colony forming units (cfu) counted on filters, each filter holding spores for one dilution of a series of dilutions. The dilution series protocol called for filtering the following volumes, expressed as fractions of a test carrier suspension: 0.99, 0.0099, 0.000099, 0.000001. In this report, we denote the corresponding cfu counts by an ordered vector of integers (a, b, c, d). It is expected that
$a > b > c > d$.

The number of viable spores per carrier (density) and the Log Reduction (LR) were calculated using the statistical procedures described in Hamilton and DeVries (1996). Some laboratories used their in-house dilutions series method instead of following the recommended protocol. Each laboratory reported its dilution and filtering methodology and that information was used to compute the density. In this collaborative study, counts for more than 2500 dilution series were analyzed.

Initial Statistical Adjustments

For some of the tests, the LR was incalculable, either because the dilution series did not capture countable numbers of cfus or because a step in the calculation required taking the logarithm of zero. For those tests it was necessary to invoke the TNTC Rule or the Zeros Rule, which will now be described.

TNTC Rule: A test carrier was excluded if TNTC (for "Too Numerous To Count") or NA (for "Not Available") was recorded at each dilution for that carrier. For Glutaraldehyde 1c and for one of the Negative Control duplicates at Lab F, all 10 test carriers were excluded by this rule; therefore, those two assays were eliminated from the analysis.

Zeros Rule: An artificial cfu count of 1 was inserted in place of 0 if all subsequent recorded dilutions produced cfu counts of 0 or NA. This adjustment was required because the logarithm of zero is mathematically incalculable. Examples are:

- The vector of cfu counts (0,0,0,0) was analyzed as if it were (1,0,0,0);
- The vector of cfu counts (TNTC,TNTC,0,0) was analyzed as if it were (TNTC, TNTC,1,0); and
- The vector of cfu counts (TNTC,0,0,NA) was analyzed as if it were (TNTC,1,0,NA).

Editing and Excluding Data

Some of the dilution series produced anomalous cfu counts that required adjustment. One of the LR values was an influential outlier. For these reasons, two separate statistical analyses were performed, an analysis of "Unedited Data" and an analysis of "Edited Data." The investigators believe that the Edited Data provide the best possible indication of repeatability and reproducibility; the Results section is based on analyses of the Edited Data. The Appendix shows results for the Unedited Data. The Edited Data were created by applying the following rules:

Rule i: For any carrier, whenever a count of 0 was followed by positive counts at higher dilutions, those positive counts were replaced with NA. The cases where this rule applied were anomalous counts suggestive of contamination. Examples are:

- The vector of cfu counts (0,0,29,0) was analyzed as if it were (1,0,0,0);
- The vector of cfu counts (TNTC,TNTC,0,1) was analyzed as if it were (TNTC, TNTC,1,0); &
- The vector of cfu counts (TNTC,0,0,6) was analyzed as if it were (TNTC,1,0,NA).

These corrections for anomalous counts had no practical effect on repeatability and reproducibility calculations.

Rule ii: The data for Lab F was excluded because a large fraction of counts in Lab F showed anomalous patterns suggestive of contamination. Application of this rule had no practical effect on the repeatability and reproducibility calculations.

Rule iii: For the Chlorine dioxide 1a assay at Lab O, one test carrier was excluded because the count was an obvious outlier. The excluded spore count was greater than 8 logs when the other 9 test carriers in the same assay contained ≤1 spore. Application of this rule had no practical effect on the repeatability and reproducibility calculations.

Rule iv: The Chlorine dioxide 1a assay at Lab J was excluded because the results for that assay were clearly incorrect. The Principle Investigators of this study suspect that Lab J was not really testing Chlorine dioxide 1a, possibly because of an error when the formulations were prepared, packaged,

coded, and shipped to Lab J. **This is the only editing rule that significantly affected the repeatability and reproducibility values.**

In summary, of the 195 assays submitted for analysis, two assays conducted by Laboratory F were unusable and were excluded for the Unedited Data analysis. For the Edited Data analysis, the remaining 11 assays conducted by Laboratory F and one outlying assay conducted by Laboratory J were also excluded, and some of the cfu counts were edited to correct for anomalies.

Statistical Analyses of the LR values

Tables and plots were constructed to display the variability of LR values among and within laboratories. The mean Log Reduction across laboratories was calculated for each formulation. Then the formulations were ordered according to their mean Log Reduction values and partitioned into three groups, called G_I, G_{II}, and G_{III}. The seven G_I Disinfectants had Mean Log Reductions (averaged across laboratories) less than 1.0; the two G_{II} Disinfectants had Mean Log Reductions of 3.04 and 6.02, and the four G_{III} Disinfectants had Mean Log Reductions between 7.49 and 7.85. For each group, a mixed effects analysis of variance was conducted to calculate laboratory-to-laboratory variation, within-laboratory variation (repeatability), and total variation (reproducibility). The within-laboratory variation was partitioned into day-to-day variation and inherent assay variation. The assay variability was estimated from the variability among carriers within an assay. Day-to-data variability was the variation, additional to assay variation, between independent assays of the same formulation within a laboratory. The statistical model for the analysis of variance is described in Appendix B. All calculations were performed using the statistical computer packages SAS© (SAS Institute, Inc. 1988) and Minitab© (Minitab, Inc. 1995).

RESULTS

For Each Disinfectant: The Typical Value and Variability Across Laboratories

Table 1 lists the LR values upon which the results are based. Figure 1 displays the spread of LR values for each formulation×concentration; each plotted point is a single laboratory. Each vertical chain of points is for a single formulation×concentration. Blind duplicates were tested on the negative control formulation and on the two strongest concentrations of each of the active formulations. The duplicate LR values for a formulation×concentration are shown in a separate, adjacent column in Table 1 and as an adjacent chain of points in Figure 1.

Table 1 shows the mean Log Reduction values (Mean LR) across labs and the Standard Deviation of LR (StdDev LR) across labs. The means vary from nearly zero to nearly 8. As the formulations become more dilute, the Mean LR values decrease (Table 1 and Figure 1). A small standard deviation (Table 1) and a short chain of points (Figure 1) indicate good reproducibility. The LR values are less reproducible for G_{II} disinfectants than for G_I or G_{III} disinfectants.

Repeatability and Reproducibility

Figure 2 is a plot of the LR values for each pair of duplicate formulations observed in the study. Each point plotted corresponds to a single laboratory. A point near the line of equality indicates good repeatability; that is, the laboratory arrived at nearly the same LR when blindly testing that formulation a second time. Good reproducibility is indicated when the points are clustered near each other along the line of equality. Good repeatability and reproducibility are exhibited for group G_{III} disinfectants (designated by circles). Poor repeatability and poor reproducibility are shown for the two G_{II} disinfectants (designated by open and solid squares).

Table 2 presents the repeatability and reproducibility results. It also shows how the total variance (reproducibility squared) can be partitioned into percentages for Lab-to-Lab variability, Day-to-day variability, and Assay variability. Note that for G_{III} disinfectants, the percentages contributed to the total variance are 76% due to Lab-to-Lab variability, 19% due to Day-to-Day variability, and 5% due to Assay variability.

Concentration-Response Relationships

Figure 3 shows the observed concentration-response curve for each individual lab and each active formulation. The concentration-response curve is reproducible but less so as the curve passes through the G_{II} disinfectant range, $3.8 \leq LR \leq 6.0$. Note that each concentration was expressed numerically as a fraction of the highest concentration and plotted on a log scale.

DISCUSSION

In this collaborative study, as is true for all antimicrobial tests, there is no "gold standard" LR value; that is, there is no formulation for which the true LR is known. It is conventional practice to define the true, unknown LR as the mean across all laboratories that could potentially test the formulation. The true LR is estimated by the mean values shown in Table 1.

Because lab-to-lab variability is the main contributor to the reproducibility standard deviation, the only way to generate an improved estimate of LR is to conduct the test at multiple laboratories and take

the average. Stated another way, expending more effort to test a formulation at a single laboratory will provide an excellent LR for that laboratory, but will provide a sample size of just one for estimating the mean across all laboratories. These results are in agreement with other collaborative studies of antimicrobial carrier assays (Bloomfield et al. 1993, Bloomfield et al. 1994).

The Quantitative Carrier Test for Sporicides proved to be more reproducible and repeatable for G_I and G_{III} formulations than for G_{II} formulations. We think the main reason for this result is that G_{II} formulations are located on the steep part of the concentration-response curve, and therefore small variations in the inoculation, dilution, or activation steps cause large changes in the response.

Another reason for less reproducibility when testing G_{II} formulations is that they exhibited greater inherent variability than G_I or G_{III} formulations. The data showed that carrier densities were more variable for test carriers than for control carriers when testing G_{II} formulations but not when testing G_I or G_{III} formulations. Our explanations for this observation are two-fold. First, because G_I formulations were essentially negative controls, the test carriers showed the same low variability as control carriers. Second, for G_{III} formulations, every assay produced low carrier densities on all test carriers, and therefore the variance among test carriers was reduced.

CONCLUSIONS

The collaborative study has shown that the reproducibility standard deviation of the Quantitative Carrier Test for Sporicides is approximately 0.5 (half a log) when the sporicide achieves a LR greater than 7. The test is capable of elucidating the shape of concentration-log reduction curves, although the middle of the curve is less reproducibly estimated. Lab-to-lab variation is much more important than within-laboratory variation. Testing a product at n laboratories will provide a more reliable LR estimate than n repetitions of the assay within a single laboratory.

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Tables and Figures

Table 1. Log Reduction (LR) values for Edited Data

Lab	Hydrogen Peroxide					Glutaraldehyde 1					Glutaraldehyde 2				
	a	a	b	b	c	a	a	b	b	c	a	a	b	b	c
A	7.15	7.78	7.67			7.49		4.47	4.52		0.94		0.48		
B	7.69		7.69	7.90		7.53		6.11			1.45		0.26		0.05
C	7.70		7.80			7.34		6.33		0.02	1.08	0.79	0.46		
D	7.40		7.29	7.33		7.49		3.55			0.60		0.25		0.40
E	8.12	8.23	8.09			7.86		6.60	7.31		2.15		1.74		
F															
G	8.56	7.73	7.92			5.23		6.39	8.31		0.72		0.44		
H	7.86		7.96		0.73	7.93	7.94	5.75			1.50		0.50	0.73	
I	7.84		7.85		0.70	7.92	7.68	6.43			2.06		0.46	0.51	
J	7.68		7.83	7.91		7.79		7.76			2.96		1.78		0.64
K	7.18		7.22			6.68		5.34		-0.15	1.71	1.18	0.98		
L	7.63	7.56	7.78			7.74		5.68	5.69		1.36		0.41		
M	8.11		8.15		1.13	8.11	8.07	7.76			2.41		0.56	0.58	
N	7.95		8.12			8.03		5.82		0.12	0.85	1.00	0.39		
O	7.42		8.67	8.14		7.84		4.91			1.09		0.21		1.16
Mean	7.735	7.825	7.860	7.820	0.853	7.499	7.897	5.921	6.457	-.003	1.491	0.990	0.637	0.607	0.563
Std Dev	0.384	0.286	0.360	0.345	0.240	0.747	0.199	1.147	1.683	0.137	0.694	0.195	0.510	0.112	0.466

Lab	Chlorine dioxide					Negative Control	
	a	a	b	b	c		
A	7.69		1.91		0.69	-0.12	0.15
B	7.61	7.93	2.95			-0.01	0.07
C	7.81		2.96	5.29		-0.14	0.11
D	7.51	7.69	3.53			0.09	0.03
E	8.13		4.43		0.24	1.88	1.79
F							
G	7.64		6.48		2.05	0.10	0.55
H	7.87		4.08			0.06	0.02
I	8.15		3.95			-0.01	-0.01
J		6.21	3.28			-0.06	0.02
K	7.18		2.12	2.67		0.09	-0.24
L	7.46		2.32		0.77	0.04	-0.16
M	8.16		5.06			-0.70	-0.06
N	7.34		5.76	5.93		-0.01	0.17
O	8.13	8.21	3.43			-0.05	-0.10
Mean	7.745	7.510	3.733	4.630	0.937	0.083	0.167
Std	0.330	0.892	1.346	1.727	0.777	0.555	0.502

Table 2. Analysis of Variance Summary for the Data of Table 1, After Grouping the Disinfectants According to Mean LR (averaged across laboratories).

		Estimated Standard Deviation (Standard Error of Estimated SD)			**Sources of Variability** Percentage of Total		
Disinfectant Group (No. formulations)	**Mean LR**	**Lab-to-Lab**	**Repeat-ability**	**Reproduc-ibility**	**Lab-to-Lab**	**Day-to-Day**	**Assay**
$\mathbf{G_I}$ (7 formulations)	< 1.0	0.5471 (0.0601)	0.1935 (0.0303)	0.5803 (0.0566)	89%	8%	3%
$\mathbf{G_{II}}$ (2 formulations)	3.84 & 6.02	1.0134 (0.2775)	0.8357 (0.2109)	1.3135 (0.1915)	60%	37%	3%
$\mathbf{G_{III}}$ (4 formulations)	7.49 to 7.85	0.4412 (0.0639)	0.2461 (0.0487)	0.5052 (0.0511)	76%	19%	5%

Figure 1. Log Reduction (LR) values for the Edited Data of Table 1. Each point is for a single laboratory. the two lines of dots at a single concentration show the scatter for the two blind assessments. Concentrations a and b were tested by all laboratories, but the blind duplicates were tested at only 3 or 4 laboratories.

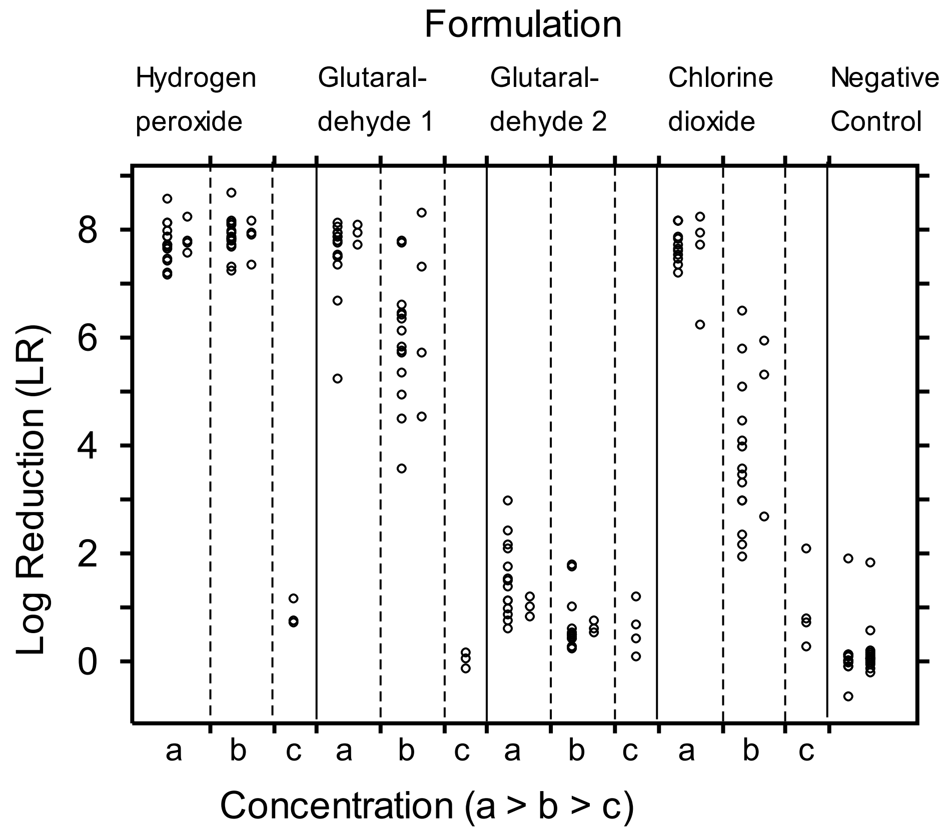


Figure 2. LR values for blind duplicate tests of formulations. Each point is a single laboratory. The $\mathbf{G_{III}}$ formulations are plotted as open circles, the two $\mathbf{G_{II}}$ disinfectants are plotted as squares (open for one disinfectant, closed for the other), and the $\mathbf{G_{I}}$ formulations are plotted as open triangles.

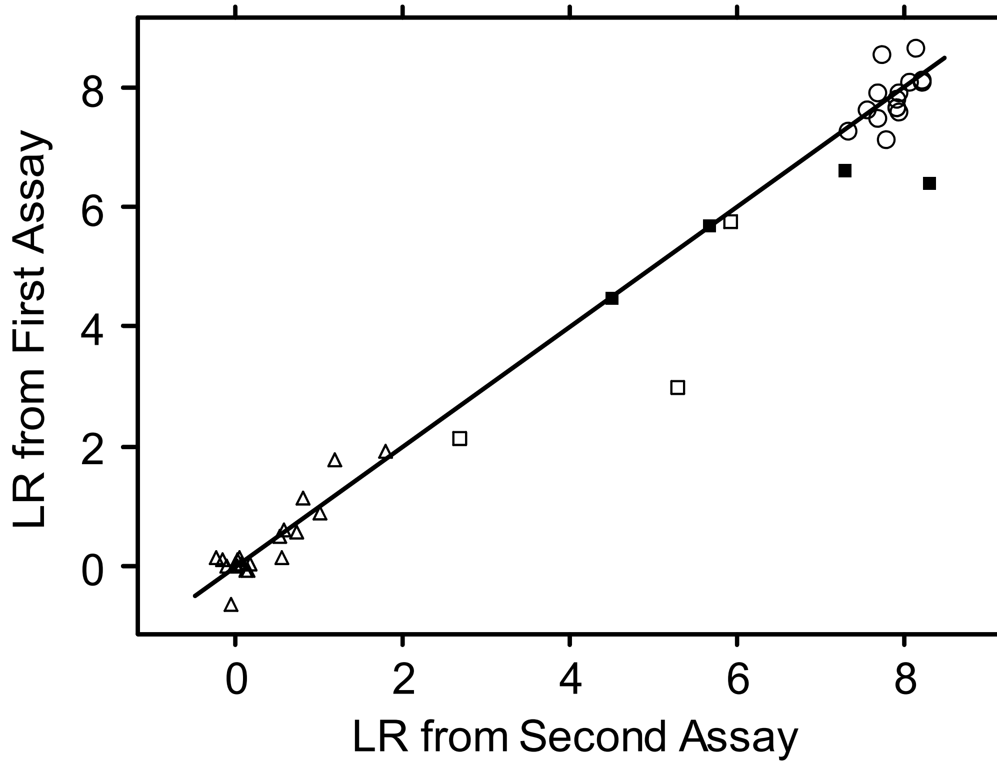
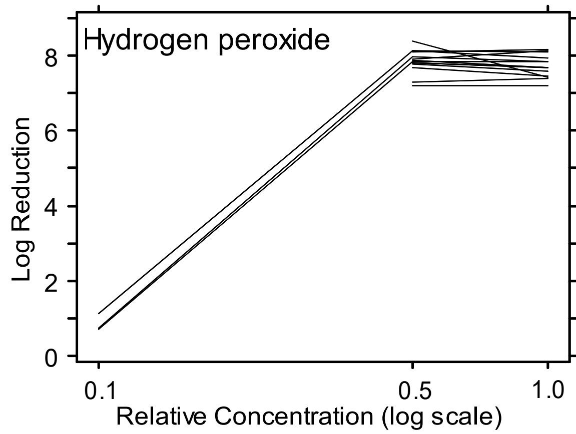
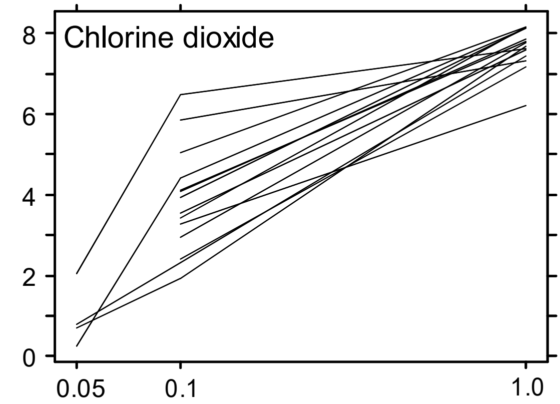
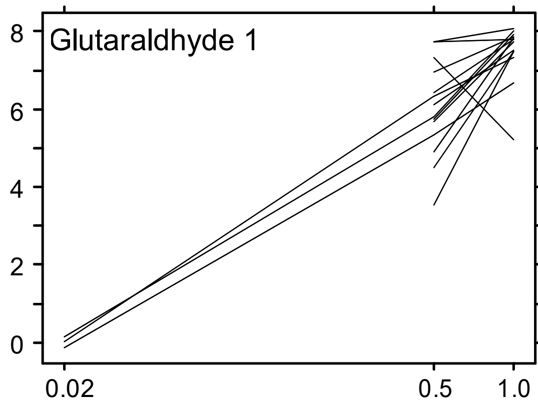
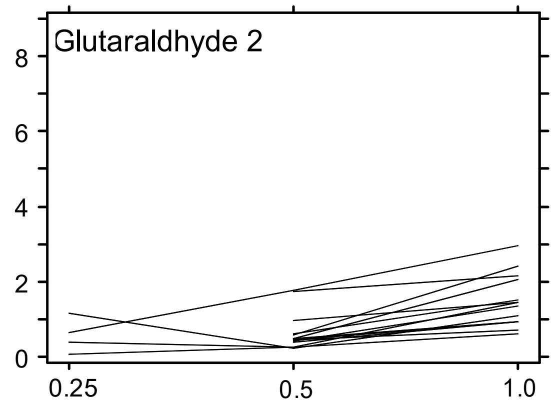


Figure 3. Log Reduction versus Concentration curves for each of 4 formulations. Each line in a plot corresponds to a single laboratory.









APPENDIX A

RESULTS FOR THE UNEDITED DATA ANALYSIS.

Table A1. Log Reduction (LR) values based on the Unedited data

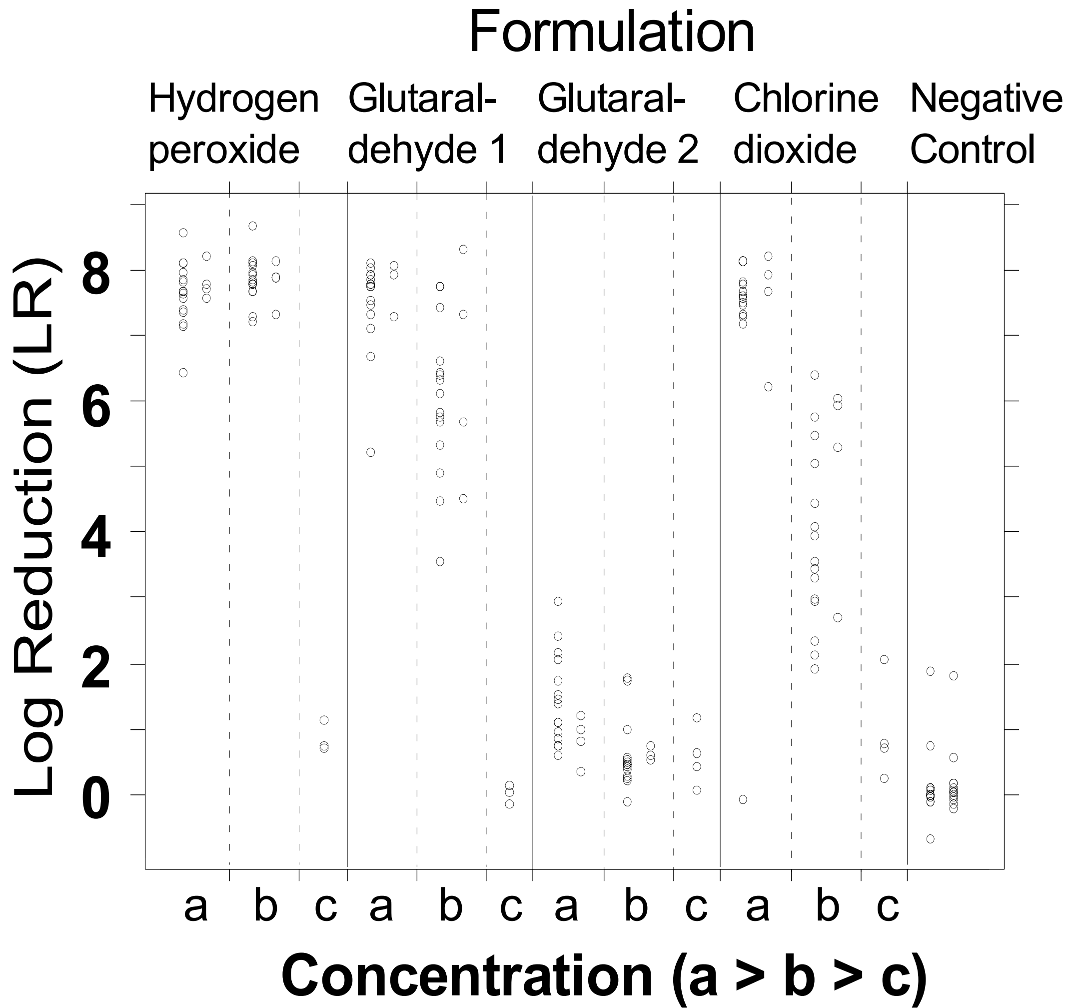
Lab	Hydrogen Peroxide					Glutaraldehyde 1					Glutaraldehyde 2				
	a	a	b	b	c	a	a	b	b	c	a	a	b	b	c
A	7.15	7.78	7.67			7.11		4.47	4.51		0.94		0.48		
B	7.69		7.69	7.90		7.53		6.11			1.45		0.26		0.05
C	7.56		7.80			7.34		6.33		0.02	1.08	0.79	0.46		
D	7.40		7.29	7.33		7.49		3.55			0.60		0.25		0.40
E	8.12	8.23	8.09			7.86		6.60	7.31		2.15		1.74		
F	7.83		7.77			7.78		7.42			0.72	0.33	-0.11		
G	8.56	7.73	7.92			5.23		6.39	8.31		0.72		0.44		
H	7.86		7.96		0.73	7.93	7.94	5.75			1.50		0.50	0.73	
I	6.42		7.85		0.70	7.92	7.30	6.43			2.06		0.46	0.51	
J	7.68		7.83	7.91		7.79		7.76			2.96		1.78		0.64
K	7.18		7.22			6.68		5.34		-0.15	1.71	1.18	0.98		
L	7.63	7.56	7.78			7.74		5.68	5.69		1.36		0.41		
M	8.11		8.15		1.13	8.11	8.07	7.76			2.41		0.56	0.58	
N	7.95		8.12			8.03		5.82		0.12	0.85	1.00	0.39		
O	7.35		8.67	8.14		7.74		4.91			1.09		0.21		1.16
Mean	7.633	7.825	7.854	7.820	0.853	7.485	7.770	6.021	6.455	-0.003	1.440	0.825	0.587	0.607	0.563
Std Dev	0.505	0.286	0.348	0.345	0.240	0.728	0.412	1.171	1.687	0.137	0.698	0.366	0.528	0.112	0.466

Lab	Chlorine dioxide					Negative Control	
	a	a	b	b	c		
A	7.69		1.91		0.69	-0.12	0.15
B	7.61	7.93	2.95			-0.01	0.07
C	7.81		2.96	5.29		-0.14	0.11
D	7.51	7.69	3.53			0.09	0.03
E	8.13		4.43		0.24	1.88	1.79
F	7.59		5.45	6.06		0.74	
G	7.61		6.42		2.05	0.10	0.55
H	7.82		4.08			0.06	0.02
I	8.15		3.95			-0.01	-0.01
J	-0.11	6.21	3.28			-0.06	0.02
K	7.18		2.12	2.67		0.09	-0.24
L	7.46		2.32		0.77	0.04	-0.16
M	8.16		5.06			-0.70	-0.06
N	7.34		5.76	5.93		-0.01	0.17
O	7.30	8.21	3.43			-0.05	-0.10
Mean	7.150	7.510	3.843	4.988	0.937	0.127	0.167
Std Dev	2.031	0.892	1.362	1.581	0.777	0.561	0.502

Table A2. Analysis of Variance Summary for the Data of Table A1, After Grouping the Disinfectants According to Mean LR (averaged across laboratories).

		Estimated Standard Deviation (Standard Error of Estimated SD)			**Sources of Variability** Percentage of Total		
Disinfectant Group (No. formulations)	**Mean LR**	**Lab-to-Lab**	**Repeat-ability**	**Reproduc-ibility**	**Lab-to-Lab**	**Day-to-Day**	**Assay**
G_I (7 formulations)	< 1.40	0.5471 (0.0601)	0.1935 (0.0303)	0.5803 (0.0566)	89%	8%	3%
G_{II} (2 formulations)	3.97 & 6.11	1.0740 (0.2594)	0.7976 (0.1904)	1.3378 (0.1931)	65%	31%	4%
G_{III} (4 formulations)	7.41 to 7.85	0.4813 (0.1651)	0.8947 (0.1071)	1.0159 (0.0857)	23%	75%	2%

Figure A1. Log Reduction (LR) values for the Unedited Data of Table A1. Each point is for a single laboratory. the two lines of dots at a single concentration show the scatter for the two blind assessments. Concentrations a and b were tested by all laboratories, but the blind duplicates were tested at only 3 or 4 laboratories. Note the one outlier (unusually small) LR for concentration a of Chlorine dioxide.



Appendix B. STATISTICAL NOTATION AND MODEL

The Model

The data for each of three formulation groups (G_I, G_{II}, and G_{III}) were analyzed separately. Preliminary analysis indicated that disinfectants within a group had similar LR, repeatability, and reproducibility. We used a mixed effects linear statistical model for each formulation group. One important advantage of analyzing a group of disinfectants, instead of individual disinfectants, was increased degrees of freedom for estimating variance components.

Let μ denote the true, unknown overall mean LR for the group; L_k denote the random effect due to laboratory *k*, β_s denote the fixed effect for formulation *s* in the group, $(L\beta)_{ks}$ denote the random effect for the interaction of laboratory *k* with formulation *s*, and E_{ksm} denote the random, within laboratory *k* effect for replicate *m* of formulation *s*. Let Y_{ksm} denote the observed Log Reduction (LR) for replicate *m* of formulation *s* in laboratory *k*. The mixed effects linear statistical model is:

$$Y_{ksm} = \mu + L_k + \beta_s + (L\beta)_{ks} + E_{ksm} \text{ ; where}$$

k = 1,2,3,...,15 for laboratories, s=1, ..., S for formulations, and m = 1,...,M for duplicates. The number of formulations S=7, 2, and 4 for G_I, G_{II}, and G_{III}, respectively. For a value of *k* corresponding to a lab that analyzed masked duplicates of the formulation, M=2; for other values of *k*, M=1. Let σ_L^2 denote the variance of L, $\sigma^2_{(L\beta)}$ denote the interaction variance, σ_E^2 denote the variance of E (the repeatability variance), and σ_Y^2 denote the variance of Y (the reproducibility variance, also called total variance). Let $\sigma^2_{L\text{-to-}L}$ denote the lab-to-lab variance defined by $\sigma^2_{L\text{-to-}L} = \sigma_L^2 + \sigma^2_{(L\beta)}$. Then $\sigma_Y^2 = \sigma^2_{L\text{-to-}L} + \sigma_E^2$.

Relationship between the model and Tables 2 and A2

The variances are in LR units squared. It is conventional to take the square root of the variance to arrive at a measure of variability in LR units. The square root of the variance is called the standard deviation. The three standard deviation columns in Table 2 (and Table A2) show, for each formulation group, estimates of the standard deviations $\sigma_{L\text{-to-}L}$, σ_E, and σ_Y. These standard deviations are the quantities used in AOAC official guidelines for analyzing collaborative studies (Helrich 1990).

We derived a formula that uses observed carrier-to-carrier variability to estimate the inherent assay variance; that is the smallest variance possible given inherent statistical variability associated with inoculating carriers, drying carriers, performing a dilution series, counting colonies, etc. We partitioned σ_E^2 into two components, σ_A^2 , which denotes the assay variance, and σ_D^2, which denotes the day-to-day variance. Thus we essentially divided the reproducibility variance into three components, $\sigma_Y^2 = \sigma^2_{L\text{-to-}L} + \sigma_D^2 + \sigma_A^2$. We used the collaborative data to estimate these variances for each formulation group. The last 3 columns of Table 2 (and Table A2) show what percentage of σ_Y^2 is attributable to each of $\sigma^2_{L\text{-to-}L}$, σ_D^2, and σ_A^2.