Statistical methods in microbial disinfection assays
by Todd Alan DeVries

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Statistics
Montana State University

Abstract:
Disinfectant efficacy testing is performed by Federal regulatory agencies, disinfectant manufacturers, and consumer groups for the purposes of assessing the ability of a liquid chemical disinfectant to kill potentially harmful microorganisms, such as bacteria, spores and viruses, attached to inanimate surfaces. Carrier tests are microbial assays in which the disinfectant is applied to microbes that have been immobilized in a dried organic film on an impermeable surface such as glass. The goal of a carrier test is to provide a reproducible measure of disinfection efficacy. “Log Reduction” (“LR”), the reduction in numbers of viable microbes (log scale), is one conventional measure of efficacy.

The quantitative assay and the presence/absence assay are two microbial assays commonly used in disinfection efficacy testing. The quantitative assay is characterized by direct counts (quantitative data) of microbes surviving disinfection. The presence/absence assay is characterized by a series of presence/absence responses (qualitative data); a response of presence indicates one or more organisms have survived disinfection in a sample aliquot, while a response of absence indicates no organisms survived disinfection in a sample aliquot. Although the types of data collected from the quantitative assay and presence/absence assay are different, the goal of the two assays is the same - to estimate the “LR”.

Very little attention has been devoted to the statistical analysis of carrier tests. In this dissertation, statistical models are developed for data arising from the quantitative assay and the presence/absence assay. Mathematical parameters defining disinfection efficacy (i.e. “LR”) are presented, and various estimators and associated standard errors are derived for each parameter. Computer simulation and asymptotic efficiency results are used to document the small and large sample properties of the various estimators.

The simulation and theoretical results indicate that Method of Moments estimators are to be recommended over Maximum Likelihood Estimators for the quantitative assay. For the presence/absence assay, the form of the estimator to be recommended depends on the definition of “LR” used.

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APPROVAL

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Todd Alan DeVries

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ABSTRACT

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Very little attention has been devoted to the statistical analysis of carrier tests. In this dissertation, statistical models are developed for data arising from the quantitative assay and the presence/absence assay. Mathematical parameters defining disinfection efficacy (i.e. "LR") are presented, and various estimators and associated standard errors are derived for each parameter. Computer simulation and asymptotic efficiency results are used to document the small and large sample properties of the various estimators.

The simulation and theoretical results indicate that Method of Moments estimators are to be recommended over Maximum Likelihood Estimators for the quantitative assay. For the presence/absence assay, the form of the estimator to be recommended depends on the definition of "LR" used.

CHAPTER 1

Introduction

Various microbiological assays have been developed for assessing the ability of a liquid chemical disinfectant to kill potentially harmful microorganisms such as bacteria, spores and viruses. The disinfectants are intended for application on nonporous surfaces in hospitals, restaurants, the home, etc. In environments of potentially high microbial contamination, such as hospitals, a measure of a disinfectant's efficacy is necessary if the product is to be used with any degree of confidence.

Establishing an index of efficacy for a particular disinfectant is done via a standardized laboratory test. Federal regulatory agencies, disinfectant manufacturers and consumer groups routinely test for efficacy of a disinfectant, and as such, require reliable testing methods. Depending on the intended use of a disinfectant, a specific standardized laboratory test must be passed before the United States Environmental Protection Agency (EPA) or the United States Food and Drug Administration (FDA) will approve the disinfectant for public usage. The tests are defined by standardized protocols that clearly state the microbial assays, microbial strains, inoculum sizes, culture conditions, testing materials, etc. Standardized tests are designed to be inexpensive and reproducible laboratory approximations of real world conditions. A standardized test is said to be reproducible if the within- and between- laboratory variability of the test are "low".

In the 1980's, environmental microbiologists expressed concern that the standardized tests used to register disinfectants were not reproducible, and that manufacturer label claims regarding germicidal activity were overstated. For

example, Rutala and Cole (1987) reported a collaborative study that assessed the reproducibility of the Use-Dilution-Method (UDM). Eighteen laboratories which were familiar with the UDM protocol tested six EPA-registered disinfectants, each of which had previously passed the performance criterion for the UDM. The results of the study by organisms were as follows: for 20%, 34%, and 62% of the trials, the disinfectants failed when tested against *Salmonella choleraesuis, Staphylococcus aureus*, and *Pseudomonas aeruginosa*, respectively. These results call into question the manufacturers' product claims of germicidal activity for the six randomly chosen disinfectants used in the study. Also noted in the study was a high degree of variability in test results among the eighteen laboratories. Because of this inconsistency among laboratories, Rutala and Cole declared that the UDM was not reproducible, and therefore questioned the use of the UDM for registration purposes. They also hypothesized that other standardized tests were similarly not reproducible.

A government review of the statistical properties of standardized disinfectant tests concluded that up to 20% of the disinfectants on the market would not meet registration performance standards if they were retested; these disinfectants may be ineffective for their intended use (U.S. General Accounting Office 1990, as quoted in Bond et al. 1991). Bond et al. (1991) nicely summarize the necessity for reproducible standardized tests.

> "The importance of establishing consistently appropriate and reproducible laboratory test methods to evaluate and compare the germicidal capabilities of liquid chemical agents cannot be overstated. If these test methods are not appropriate and verifiable, or if the resulting data are not reflective of in-use conditions, then the overall effectiveness of many generic recommendations and guidelines for selection and use of liquid chemical germicides will be weakened."

Since 1990, the EPA has adopted a multidisciplinary approach involving microbiologists, virologists, statisticians and other scientists to develop new

standardized tests which will be more reliable and reproducible than the preceding tests. Prior to the new tests, little attention was given to statistical analysis of antimicrobial assays. The EPA realized that statistical thinking was necessary to achieve the goal of developing improved standardized tests. Some important statistical tasks include developing statistical models for microbial assay data, mathematically defining parameters measuring a disinfectant's efficacy, identifying sources of variability in the tests, and suggesting designs that will reduce variability to acceptable levels. The general purpose of this dissertation is to describe statistical methodology for two microbial assays commonly used in disinfection efficacy testing; the quantitative assay and the presence/absence assay. A description of both assays will first be given before defining the specific goals of this dissertation.

Description of the Quantitative Assay

Central to the quantitative assay (as well as the presence/absence assay) is the concept of "check" and "test" "carriers". The carriers are often small stainless steel or glass penicylinders, or some other nonporous medium, which are inoculated with microorganisms for testing purposes. Figure 1 shows the major steps common in all quantitative assays for test carriers.

Step 1: A suspension of microbes is prepared according to a targeted density of microbes per unit volume. Included in the suspension are culture medium, which acts as a food source for the microbes, and possibly added "organic soil", in addition to the microorganisms.

Step 2: A sterilized carrier is selected.

Step 3: The carrier is placed in the prepared inoculum.

Step 4: The inoculum is dried on the carrier, leaving the microorganisms embedded in a protective organic film that represents a realistic challenge to the disinfectant.

After drying, the targeted inoculum size ranges between 10^5 and 10^8 viable organisms depending on whether bacteria, spores, or viruses are the test organism of interest.

Step 5: The carrier is treated with a purported disinfectant for a specified amount of time.

Step 6: Following neutralization of the disinfectant, the carrier is sonicated and/or vortexed, thus removing organisms from the carrier into a well-mixed liquid suspension.

Step 7: The suspension is serially diluted, plated or filtered, incubated, and colony forming units (cfu) are counted. The series of dilution levels, or synonymously, the fraction of total volume sampled, are recorded with the cfu counts so that cfu counts can be converted into a per carrier estimate of the number of viable organisms surviving disinfection. If a disinfectant is effective in killing organisms, few cfu counts will be observed for the test carriers.

In the absence of knowledge about the initial numbers of organisms exposed to disinfection, one cannot discern if low test carrier cfu counts are due to a strong disinfectant or to a weak disinfectant applied to carriers that contained few organisms. For this reason, a series of check carriers are run in addition to test carriers. Check carriers are treated in an identical manner as test carriers except an inert solution is applied to the carrier in lieu of an actual disinfectant. Check carrier cfu counts, along with the recorded dilution levels, provide an estimate of the number of viable organisms exposed to disinfection (on a per carrier basis). Thus in the quantitative assay, direct counts (quantitative data) are observed on both check and test carriers. Data from both check and test carriers are required in order to measure disinfectant efficacy. Because of inherent variability involved at each step in the test protocol, each assay requires multiple check and test carriers.

Figure 1: Schematic chart for test carriers in the quantitative assay

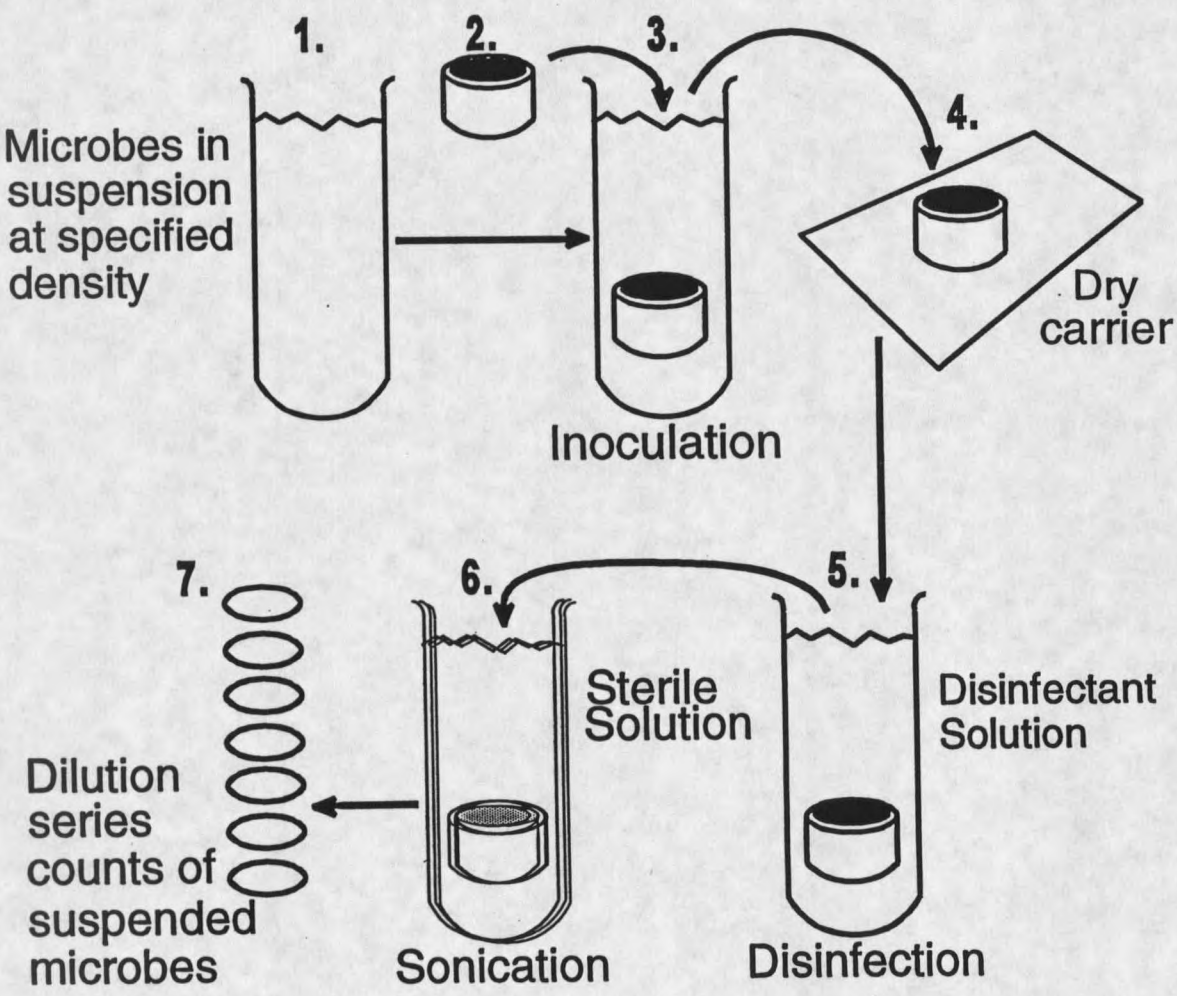

Table 1 gives an example of the type of data arising from a quantitative assay with three check carriers and ten test carriers. In this example, the check carriers contain approximately 10^8 organisms (check carrier counts divided by volume sampled for check carriers) and the test carriers contain approximately 1000 organisms (test carrier counts divided by volume sampled for test carriers) .

Table 1: Example data from the quantitative assay

Check Carriers			Test Carriers		
Carrier Number	Volume Sampled	Counts	Carrier Number	Volume Sampled	Counts
1	3×10^{-6}	189	1	0.33	267
2	3×10^{-6}	195	2	0.33	291
3	3×10^{-6}	219	3	0.33	99
			4	0.33	270
			5	0.33	318
			6	0.33	102
			7	0.33	297
			8	0.33	126
			9	0.33	222
			10	0.33	489

Description of the Presence/Absence Assay

The presence/absence assay (pa assay) is characterized by a series of presence/absence responses (qualitative data) for both check and test carriers. The pa assay can be thought of as a sequence of serial-dilution assays for test and check carriers. The serial-dilution assay (Loyer and Hamilton 1984) has proved valuable for determining the density of organisms in solutions in situations where colony-count methods are not effective (Eaton et al. 1992). For example, some viruses, such as the adenovirus, do not readily form colonies or plaques, hence the serial-dilution assay is commonly used in the field of virology.

The main distinction between the pa and quantitative assays comes after the organisms are removed from a carrier into suspension via sonication. For the pa assay, the suspension is serially diluted. At each dilution stage, a number of sample aliquots are placed into tubes or wells and, following incubation, examined for a presence/absence response. It is assumed that a tube or well will exhibit a positive response (presence) if and only if one or more organisms in the sample survived disinfection. Otherwise it will exhibit a negative response (absence), indicating that no organisms in the sample survived disinfection. From the series of presence/absence responses and the associated dilution levels, an estimate of the number of viable organisms in suspension can be determined. Figure 2 shows the steps common to serial-dilution assays. A check carrier is treated in the same manner as a test carrier except that an inert solution is used in place of the disinfectant. The type of data recorded for a check or test carrier is then the number of wells exhibiting a positive response at each dilution stage. There is evidence to believe a disinfectant is effective if wells are negative at low dilutions for test carriers, but wells are positive at high dilutions for check carriers.

Table 2 gives an example of the data collected for a pa assay with four wells per dilution for each of three check and three test carriers. These data indicate that there are about 1000 times more viable organisms on check carriers than on test carriers. Notice that for check carriers, high dilutions of 10^{-7} or 10^{-8} are required to show absence of viable organisms in all four wells, whereas for test carriers, absence in all four wells occur at 10^{-4} or 10^{-5} dilutions.

Common Measures of Disinfectant Efficacy

As one can see from Tables 1 and 2, there are distinct differences in the types of data collected in the quantitative and presence/absence assays. The goal of each

Figure 2: Schematic diagram for a serial-dilution assay

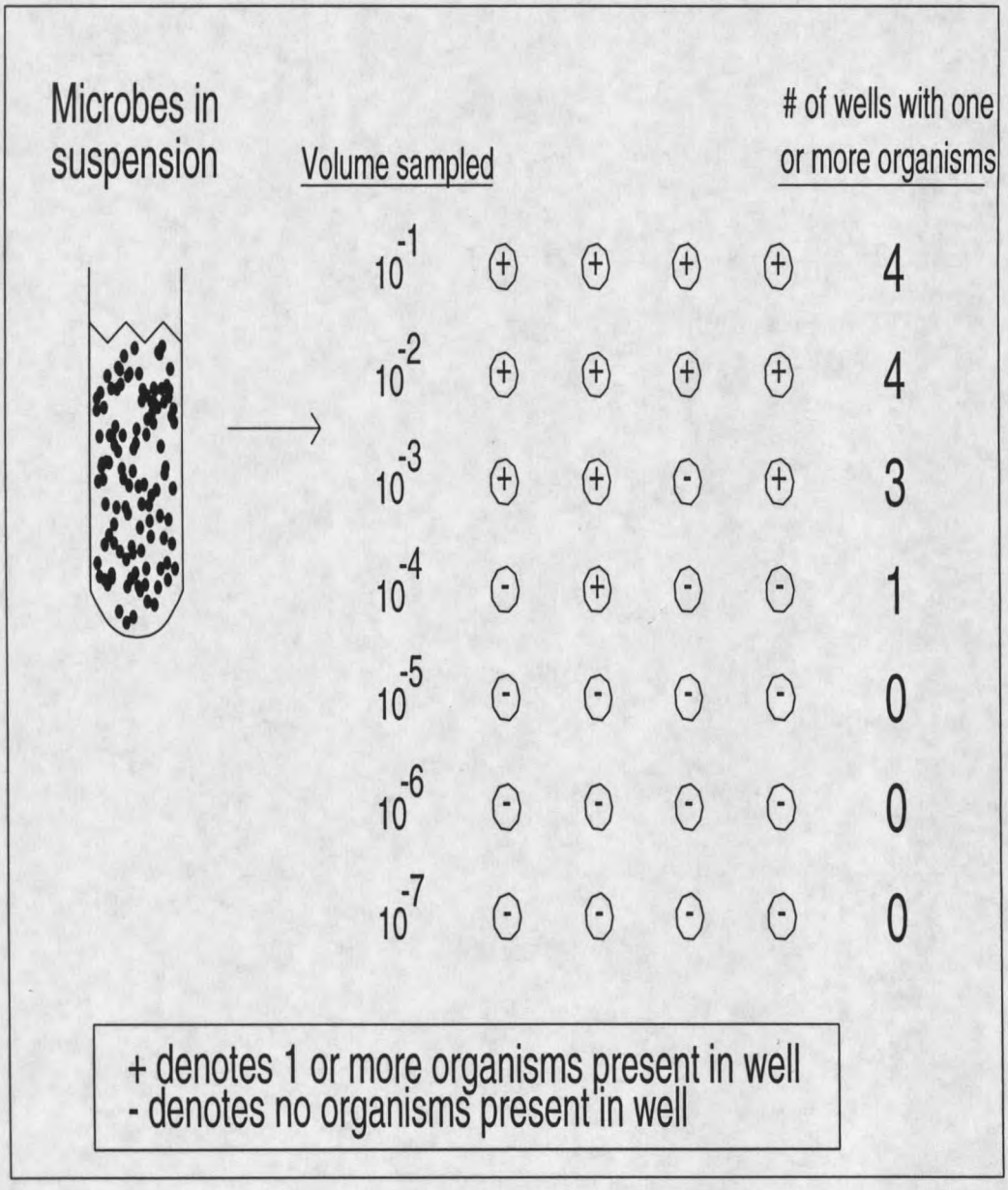

Table 2: Example data from the presence/absence assay

	Check Carriers			Test Carriers		
	Carrier 1	Carrier 2	Carrier 3	Carrier 1	Carrier 2	Carrier 3
Dilution	Number of wells positive			Number of wells positive		
10^{-1}	4	4	4	4	4	4
10^{-2}	4	4	4	4	4	4
10^{-3}	4	4	4	4	3	4
10^{-4}	4	4	4	0	1	4
10^{-5}	4	4	4	0	0	0
10^{-6}	4	3	3	0	0	0
10^{-7}	0	1	0	0	0	0
10^{-8}	0	0	0	0	0	0

of the two assays is the same: to measure how effective at killing microorganisms a disinfectant is; that is, to produce a quantitative summary of a disinfectant's efficacy. An example of a quantitative summary would be an estimate of the proportion of organisms surviving disinfection. Equivalent measures of efficacy are the percentage of organisms killed and the log reduction. For example, suppose it was known that N_1 of N_0 organisms would survive disinfection. Then $\frac{N_1}{N_0}$, $100 \times \left(1 - \frac{N_1}{N_0}\right)$ %, and $\log_{10}(N_0) - \log_{10}(N_1)$ are the survival fraction, the percentage killed, and the log reduction, respectively. Smaller survival fractions, or equivalently larger percentages killed and larger log reductions, indicate more active disinfectants. Table 3 gives numerical values for the survival fraction, percentage of organisms killed, and the log reduction assuming $N_0 = 1,000,000$ and $N_1 = 1$.

Table 3: Example of equivalent quantitative measures of disinfection efficacy

N_1	N_0	Survival fraction	Percentage killed	Log reduction
1	10^6	0.000001	99.9999%	6.0

Specific Goals of this Dissertation

The goals of this dissertation are to present statistical models for data arising from the quantitative assay and the presence/absence assay, to discuss alternative ways to quantify disinfection efficacy, to present methods I have derived for estimating these quantities and associated standard errors, and to compare alternative estimation methods. The comparisons are based on both theory and computer simulation. Separate chapters will be devoted to the quantitative assay and the presence/absence assay.

CHAPTER 2

Quantitative Assay

The goal of a quantitative assay is to estimate the Log Reduction (LR) for a disinfectant. Before defining LR it will be helpful if certain notation is listed.

Notation

A = Number of check carriers.

B = Number of test carriers.

D = Number of dilution stages for check carriers.

E = Number of dilution stages for test carriers. D does not have to equal E.

F = Number of samples per dilution for check or test carriers.

i = Subscript for check carriers; i = 1,...,A.

j = Subscript for dilution stage of a check carrier; j = 1,...,D.

k = Subscript for sample at a dilution stage for a check carrier; k = 1,...,F.

s = Subscript for test carriers; s = 1,...,B.

m = Subscript for dilution stage of a test carrier; m = 1,...,E.

n = Subscript for sample at a dilution stage for a test carrier; n = 1,...,F.

C_{ijk} = Random variable denoting the microbial count for check carrier i at dilution stage j and sample k.

c_{ijk} = Observed microbial count for check carrier i at dilution stage j and sample k.

$C_i = \sum_{j=1}^{D} \sum_{k=1}^{F} C_{ijk}$ = Random variable denoting the sum of microbial counts across dilutions and samples for check carrier i.

$c_i = \sum_{j=1}^{D} \sum_{k=1}^{F} c_{ijk}$ = Observed sum of microbial counts across dilutions and samples for check carrier i.

T_{smn} = Random variable denoting the microbial count for test carrier s at dilution stage m and sample n.

t_{smn} = Observed microbial count for test carrier s at dilution stage m and sample n.

$T_s = \sum_{m=1}^{E} \sum_{n=1}^{F} T_{smn}$ = Random variable denoting the sum of microbial counts across dilutions and samples for test carrier s.

$t_s = \sum_{m=1}^{E} \sum_{n=1}^{F} t_{smn}$ = Observed sum of microbial counts across dilutions and samples for test carrier s.

u_{ijk} = Fraction of total volume sampled for check carrier i at dilution j and sample k.

$u_i = \sum_{j=1}^{D} \sum_{k=1}^{F} u_{ijk}$ = Fraction of total volume sampled for check carrier i.

Notice that the total volume sampled may vary among check carriers.

v_{smn} = Fraction of total volume sampled for test carrier s at dilution m and sample n.

$v_s = \sum_{m=1}^{E} \sum_{n=1}^{F} v_{smn}$ = Fraction of total volume sampled for test carrier s.

Notice that the total volume sampled may vary among test carriers.

$X_i = \frac{C_i}{u_i}$ = Density for check carrier i; i = 1,...,A.

$$X_i^* = \begin{cases} \log(X_i), & \text{if } X_i > 0 \\ \log(\frac{1}{u_i}), & \text{if } X_i = 0 \end{cases}$$

$\overline{X} = \frac{1}{A} \sum_{i=1}^{A} X_i$ = Average check carrier density.

$\overline{X^*} = \frac{1}{A} \sum_{i=1}^{A} X_i^*$ = Average log check carrier density.

$Y_s = \frac{T_s}{v_s}$ = Density for test carrier s; s = 1,...,B.

$$Y_s^* = \begin{cases} \log(Y_s), & \text{if } Y_s > 0 \\ \log(\frac{1}{v_s}), & \text{if } Y_s = 0 \end{cases}$$

$\overline{Y} = \frac{1}{B} \sum_{s=1}^{B} Y_s$ = Average test carrier density.

$\overline{Y^*} = \frac{1}{B} \sum_{s=1}^{B} Y_s^*$ = Average log test carrier density.

P_s is the survival fraction for a randomly chosen microbe exposed to disinfection for test carrier s; s = 1,...,B. For notational convenience, the subscript will be dropped

from P for the remainder of this chapter. P is a random variable that follows a distribution with mean ϕ and variance ζ^2.

θ_{Ci} = Unobservable inoculum size for check carrier i; i = 1,...,A.

θ_{Ts} = Unobservable inoculum size for test carrier s; s = 1,...,B.

"Zero-adjusted" refers to the standard approach of adding 1 to zero counts before taking logs.

$\log(\cdot)$ is the base 10 logarithm.

$\ln(\cdot)$ is the natural logarithm.

$\hat{}$ is a symbol used to indicate "an estimator of".

Log Reduction (LR)

In my literature search of LR calculations for the quantitative assay, I have been able to find examples for the case of 1 check carrier and 1 test carrier, only. LR is defined (Robison et al. 1988) as follows:

LR = log(number of organisms per carrier before exposure to disinfection) –
log(number of organisms per carrier after exposure to disinfection).

Denote as c the check carrier count and its associated volume sampled as u. Denote as t the test carrier count and its associated volume sampled as v. Notice that $\frac{t}{v}$ is the estimated number of microbes per carrier surviving disinfection, while $\frac{c}{u}$ is the estimated number of microbes per carrier exposed to disinfection. The standard estimate of LR is: $LR = \log(\frac{c}{u}) - \log(\frac{t}{v})$. This quantity is sometimes called the microbicidal effect (ME) (Bloomfield et al. 1994) or the sterility assurance level (SAL) (Oxborrow and Berube 1991).

The new quantitative assays require multiple check and test carriers. Microbiologists calculate LR in a number of different ways when analyzing a multiple check and test carrier assay. To my knowledge, no published paper defines

and compares the various estimators of LR. In the following sections of this chapter two different conceptualizations of LR for multiple carrier assays will be discussed, and an intuitive estimator will be presented for each definition of LR. The main purposes of this chapter are to present a method of moments (MOM) and maximum likelihood estimator (MLE) and associated standard errors for the two conceptualizations of LR. The standard error is important because it indicates inherent variability in the quantitative assay. Knowledge of the inherent variability will be useful as a benchmark for within- and between- laboratory variances that will be observed in forthcoming multi-laboratory collaborative studies of disinfectants on various microorganisms. Large sample asymptotic results will be derived for purposes of evaluating the estimators' efficiencies. Knowledge of the theoretical efficiencies of the estimators will be useful in providing recommendations as to which estimators should be used. Specifically, efficiency calculations will give guidelines as to whether the more easily calculated MOM estimators of LR can be justified in their use over the more laboriously calculated MLE of LR. Finally, results from a computer simulation study will be given for purposes of determining the small sample properties of the various estimators.

Two Simple Estimators of LR

One approach microbiologists have used to calculate LR is: (i) transform the check and test carrier densities to the log scale and (ii) subtract the average log test carrier density from the average log check carrier density. In notation, this estimator is $\overline{X^*}$ - $\overline{Y^*}$. For obvious reasons I have decided to refer to this approach as the "average of logs". I denote the average of logs parameter as Υ_1. Specifically, letting $E(\cdot)$ denote the expectation operator,

$$\Upsilon_1 = \frac{1}{A} \times E\left(\sum_{i=1}^{A} X_i^*\right) - \frac{1}{B} \times E\left(\sum_{s=1}^{B} Y_s^*\right). \qquad (2.1)$$

By the Law of Large Numbers, the average of logs estimator is consistent for Υ_1. Notice, for the single check and test carrier situation, the average of logs estimate is $\log(\frac{c}{u}) - \log(\frac{t}{v})$.

An alternative estimator of LR is as the negative logarithm of the survival fraction estimator, $\frac{\overline{Y}}{\overline{X}}$; i.e. $LR = -\log\left(\frac{\overline{Y}}{\overline{X}}\right)$. This estimator can also be written as $\log\left(\overline{X}\right) - \log\left(\overline{Y}\right)$. Consequently, I refer to it as the log of averages approach. Notice, for the single check and test carrier situation, the survival fraction estimate is $\frac{t}{v} \div \frac{c}{u}$ and the log of averages estimate is simply $\log\left(\frac{c}{u}\right) - \log\left(\frac{t}{v}\right)$. Denote the log of averages parameter as Υ_2. Specifically,

$$\Upsilon_2 = -\log(\phi), \tag{2.2}$$

where ϕ is the population mean of the distribution of survival fractions, P. By the Law of Large Numbers, the log of averages estimator is consistent for Υ_2.

It should be emphasized that the average of logs and log of averages estimators are not estimating equivalent parameters. From Jensen's inequality it is known that $\log\left(\overline{X}\right) \geq \overline{X^*}$ and $\log\left(\overline{Y}\right) \geq \overline{Y^*}$, (assuming none of the test or check carrier densities are zero). However, no definitive statements can be made in terms of comparing the average of logs estimator to the log of averages estimator. For the single check and test carrier situation, however, the average of logs method and log of averages method yield identical results.

Arguments Favoring the Average of Logs Method

There are reasons to prefer the average of logs method to the log of averages method and vice versa. The average of logs estimator of LR is the log of the geometric mean of test carriers subtracted from the log of the geometric mean for check carriers. Because counts of microorganisms often exhibit positive skew, the preferred statistic for summarizing microbiological data is the geometric mean (Eaton et al. 1995).

For this reason, the average of logs method may be the method of choice by microbiologists. The assumption of microbial counts originating from distributions having positive skew appears to be reasonable for one particular application of the quantitative assay with which I am familiar. Figure 3 gives the Empirical Distribution Function (EDF) of the sample skewness coefficients from 195 check and test carrier data sets. Figure 4 gives the EDF of the sample skewness coefficients for the log transformed data. The data came from a multi-laboratory collaborative study (Springthorpe 1997) involving 15 laboratories each testing various disinfectant formulations. As seen from Figure 3, positive skew was evident in roughly 80% of the test carrier data sets. Positive skew does not seem to be as prevalent for the check carrier data sets. However, each check carrier data set consisted of only 3 observations (as opposed to 10 test carrier observations). Because the check carrier procedure should be constant from assay to assay within a laboratory, I felt it was appropriate to pool check carrier observations within laboratories. Results not shown here indicate that positive skew for the pooled check carrier data was evident in 12 of 15 laboratories. Thus it seems reasonable to believe that both check and test carrier data originate from distributions having positive skew.

From Figure 4 it is evident that the log test carrier counts are less skewed. There is some theoretical justification that the log transformation of microbiological data will induce less skew. For reasons to be presented later, I believe it is reasonable to assume negative binomial distributions for check and test carrier counts. The negative binomial distribution has positive skew and the log of a (zero-adjusted) negative binomial random variable has a more symmetric distribution. Some motivation for this statement can be provided by the gamma distribution. The log of a gamma has a more normal, and hence symmetric, distribution than the original gamma (Kotz, Johnson and Read 1985). Because the

Figure 3: EDF for skewness coefficient of 195 check and test carrier data sets