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Estimating the Antimicrobial Log Reduction: Part 2. Presence/Absence Assays

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Abstract. This is part 2 of a pair of papers on antimicrobial assays conducted to estimate the log reduction (LR), in the density of viable microbes, attributable to the germicide. Two alternative definitions of LR were defined in part 1, one based on the mean of the log-transformed densities; the other is based on the logarithm of the mean of densities. In this paper, we evaluate statistical methods for estimating LR from an antimicrobial assay in which the responses are presence/absence observations at each dilution in a series of dilutions. We provide a model for the presence/absence data, and, for each definition of LR, we derive the maximum likelihood estimator (mle). Using computer simulation methods, we compare the mle to several alternative estimators, including an estimator based on averaging the log-transformed most probable number (mpn) values. Standard error formulas for the estimators are also derived and evaluated using computer simulations. This investigation results in the following recommendations. If the parameter of interest is based on the mean of log-transformed densities, then the results favor use of the log-transformed mpn method. If, however, the parameter of interest is based on the logarithm of the mean of densities, then the results show that the mle should be used.

Key words: antimicrobial, serial dilution assay, disinfectant, log reduction, statistical models

1. Introduction

Various microbiological assays have been devised for assessing the ability of a liquid chemical disinfectant to kill potentially harmful microorganisms such as bacteria, spores, fungi, and viruses, that contaminate hard, nonporous surfaces in hospitals, restaurants, the home, etc. Each standard assay is conducted according to a protocol that clearly specifies the microbial species and strain, inoculum sizes, culture conditions, testing materials, and the carriers. The *carriers* are small objects constructed of glass, stainless steel, or other nonporous material, designed for ease of handling in the laboratory. They are inoculated with microorganisms to create laboratory surrogates for contaminated surfaces. Each assay utilizes multiple test carriers (exposed to the chemical) and multiple control carriers (exposed only to a non-germicidal liquid). The standardized carrier assays are designed to provide an inexpensive and reproducible laboratory assessment of each disinfectant's activity under conditions similar to real world applications. The goal of an antimicrobial assay is to provide a reproducible, quantitative measure of a chemical's disinfection

efficacy. One conventional measure of efficacy is the Log Reduction (LR), which is the reduction in numbers of viable microbes ($\log_{10}$ scale) caused by exposure to the chemical.

In a companion paper, we briefly review the importance of antimicrobial assays, discuss the main statistical issues that arise when estimating the LR, list the key steps involved in the typical assay, and then focus exclusively on quantitative carrier assays (DeVries and Hamilton, 1999). Because we will often refer to that paper, it will be convenient to use the shorthand reference DH:I. In this paper, we discuss presence/absence assays. The distinction between the two types of antimicrobial assays occurs at the final laboratory step (Step 7 in DH:I). After the microbes have been removed from each carrier into suspension via sonication, vortexing, or scraping, the final step is to determine the number of microbes per carrier. In a quantitative assay, the response data are viable cell counts at each dilution in a series of dilutions of the liquid suspension. In a presence/absence (PA) assay, the response data are quantal responses (presence/absence) at each dilution in the series of dilutions. The primary reason for using a PA assay is that, in some applications, it may be very expensive, perhaps impossible, to count viable microbes, although it is feasible to observe presence/absence. For example, some viruses, such as adenoviruses, do not readily form colonies or plaques, however, it is possible to determine whether one or more live viruses are present in a sample of the suspension.

For the PA assay, the suspension is serially diluted. At each dilution stage, a number of sample aliquots are each placed into a separate tube or well (henceforth called well). Following incubation, each well is examined for a presence/absence response. It is assumed that a well will exhibit a positive response (presence) if and only if one or more organisms in the sample is viable. Otherwise, the well will exhibit a negative response (absence), indicating that no organisms in the sample are viable. From the series of presence/absence responses and the associated dilution levels, it is possible to estimate the number of viable organisms in suspension.

In a PA assay using viruses, the wells in a microtiter plate are prepared with a lawn of living cells. An aliquot of virus suspension is pipetted into each well, then the plate is incubated. Each well is inspected by a virologist to see if the cells exhibit a cytopathic effect which is a characteristic sign of viral infection. It is assumed that the cytopathic effect will be absent in a well if and only if it contains no viable viruses.

Table 1. Numbers of positive wells in a PA assay. There were 4 wells at each dilution × carrier combination.

	Control carriers			Test carriers		
Dilution	Carrier 1	Carrier 2	Carrier 3	Carrier 1	Carrier 2	Carrier 3
10^{-1}	4	4	4	4	4	4
10^{-2}	4	4	4	4	4	4
10^{-3}	4	4	4	4	2	4
10^{-4}	4	4	4	0	1	4
10^{-5}	4	4	4	0	0	2
10^{-6}	4	3	3	0	0	0
10^{-7}	0	1	0	0	0	0
10^{-8}	0	0	0	0	0	0

Table 1 shows data collected for a PA assay in which there are four wells per dilution for each of three control and three test carriers. Notice that for control 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} through 10^{-6} dilutions. These data indicate that there are between 100–1000 times more viable organisms on control carriers than on test carriers, which loosely translates into a Log Reduction (LR) of 2.0–3.0. This example will be used later to illustrate calculations.

2. Statistical model

We present here a probability model for the observable data. Some notation is required. Let A denote the number of control carriers and B denote the number of test carriers. The data recorded for a PA antimicrobial assay are the number of wells and the number of positive wells at each dilution level for each carrier. For control carrier i, $i = 1, \ldots, A$, let D_i denote the number of dilution levels; let n_{ij} denote the number of wells at dilution j, $j = 1, \ldots, D_i$; let u_{ij} denote the fraction of the initial suspension volume contained in the sample aliquot that is placed in a well at dilution j; let the random variable $Y_{ijk} = 1$ if presence is observed in well k of dilution j, and $Y_{ijk} = 0$ otherwise; let $R_{ij} = \sum_{k=1}^{n_{ij}} Y_{ijk}$. Define the vectors $\vec{R}_i = (R_{i1}, R_{i2}, \ldots, R_{iD_i})$ and $\vec{u}_i = (u_{i1}, u_{i2}, \ldots, u_{iD_i})$.

Similar notation is adopted for test carriers. For test carrier s, $s = 1, \ldots, B$, let H_s denote the number of dilution levels; let w_{sm} denote the number of wells at dilution m, $m = 1, \ldots, H_s$; let v_{sm} denote the fraction of the initial suspension volume contained in the sample aliquot that is placed in a well at dilution m; let the random variable $Z_{smh} = 1$ if presence is observed in well h of dilution m and $Z_{smh} = 0$ otherwise; let $G_{sm} = \sum_{h=1}^{w_{sm}} Z_{smh}$. Define the vectors $\vec{G}_s = (G_{s1}, G_{s2}, \ldots, G_{sH_s})$ and $\vec{v}_s = (v_{s1}, v_{s2}, \ldots, v_{sH_s})$. As is conventional in statistics, the realized value of a random variable will be denoted by the corresponding lower case symbol.

We will derive a plausible statistical model for the observable counts in steps that employ intermediate, unobservable random variables. Define the *recoverable count* on a carrier as the number of viable organisms removed into suspension (step 7 in DH:I) if the carrier were not exposed to a disinfectant. The recoverable count is not directly observable because control carriers contain too many microbes to count and test carriers have been exposed to a disinfectant.

Assumption 1 (DH:I): The (unobservable) recoverable counts for the A control carriers and the B test carriers, denoted by $\theta_i^{(C)}$, $i = 1, \ldots, A$ and $\theta_s^{(T)}$, $s = 1, \ldots, B$, respectively, are independent and identically distributed (iid) as gamma (mean $= \alpha\beta$, variance $= \alpha\beta^2$) random variables.

Assumption 2 (DH:I): The survivals of individual microbes on a randomly chosen test carrier are independent Bernoulli trials with common probability P which is a random variable that follows a distribution with mean ϕ and variance ζ^2. Also P and $\theta_s^{(T)}$, $s = 1, \ldots, B$, are statistically independent.

See DH:I for discussion of Assumptions 1 and 2. The quantity P is the mean survival fraction for organisms on a randomly chosen test carrier, and ϕ is the mean of P across test carriers. As in DH:I, we will use the term *variable survival fraction* to indicate the case where P has a positive variance, and the term *constant survival fraction* to indicate that $P \equiv \phi$; i.e., $\zeta^2 = 0$.

To simplify the notation let $C_i \equiv \theta_i^{(C)}$, $i = 1, \ldots, A$, and let μ_C denote the expected recoverable count for control carriers. Define $X_i = \log(C_i)$ and $\mu_X = E(X_i)$. It can be shown (DeVries, 1997) that $\mu_X = [\Psi(\alpha) + \ln(\beta)]/\ln(10)$, where $\Psi(\cdot)$ is the derivative of the natural logarithm of the gamma function and ln is the natural logarithm. Similarly for test carriers, let T_s be a random variable denoting the number of organisms surviving the disinfection steps for test carrier s ($s = 1, \ldots, B$), and let μ_T denote the expected number of organisms surviving the disinfection steps for test carriers. Define $Y_s = \log(T_s)$ if $T_s > 0$; otherwise $Y_s = 0$ if $T_s = 0$. Define $\mu_Y = E(Y_s)$. Note that C_i, X_i, T_s, and Y_s are not directly observable, but can be estimated from the presence/absence serial dilution data.

Assumption 3 (similar to Assumption 4 in DH:I): $T_s|\theta_s^{(T)}, P$ are conditionally distributed independently as Poisson $(\theta_s^{(T)} \cdot P)$ random variables, $s = 1, \ldots, B$.

Technically, $T_s|\theta_s^{(T)}, P$ is distributed as a binomial random variable with $\theta_s^{(T)}$ trials and probability of "success" P. Because $\theta_s^{(T)}$ is large and P is relatively small, the Poisson approximation to the binomial is appropriate (Feller, 1968).

Assumptions 1–3 imply that marginally, T_s is distributed iid with mean $= \alpha\beta\phi$ and variance $\alpha\beta\phi[1 + \alpha\beta\phi[(\phi^2 + \zeta^2(\alpha + 1)/(\alpha\phi^2)]]$. For convenience, denote $[\phi^2 + \zeta^2(\alpha + 1)]/\phi^2$ as d; $d \geq 1$. If $d = 1$, then $\zeta^2 = 0$ and the distribution of P is degenerate at ϕ. If $d > 1$, then $\zeta^2 > 0$ and the survival fraction varies among carriers. Instead of attempting to model the distribution of P, we have chosen to model the distribution of T_s.

Assumption 4 (similar to DH:I Assumption 5): The marginal distribution of T_s can be reasonably approximated by a negative binomial distribution with parameters $(\rho = \alpha/d, p = 1/(\beta\phi d + 1), q = \beta\phi d/(\beta\phi d + 1))$, $s = 1, \ldots, B$, and the T_s are iid.

DeVries (1997) has shown that the marginal distribution of T_s is exactly negative binomial when $d = 1$. Assumption 4 implies that $E(T_s) = \alpha\beta\phi$, $\mathrm{Var}(T_s) = \alpha\beta\phi[1 + \alpha\beta\phi[(\phi^2 + \zeta^2(\alpha + 1)/(\alpha\phi^2)]]$, and $\mu_Y = \sum_{t=1}^{\infty} \log(t) \times \Gamma(t + \rho)/\Gamma(\rho)t!q^t p^\rho$.

Assumption 5 (identical to DH:I Assumption 6): The C_i and T_s are all statistically independent; $i = 1, \ldots, A$; $s = 1, \ldots, B$.

The next two assumptions are specific to PA assays. They are the final building blocks in the model we use for estimating LR.

Assumption 6: Assume the "single hit Poisson model" (Cyr *et al*., 1990); that is, $Y_{ijk}|C_i, u_{ij}$, $i = 1, \ldots, A$; $j = 1, \ldots, D_i$ are distributed as independent Bernoulli random variables, where $\Pr(Y_{ijk} = 1) = 1 - e^{-C_i \cdot u_{ij}}$ for $k = 1, \ldots, n_{ij}$; and $Z_{smh}|T_s, v_{sm}$, $s = 1, \ldots, B$; $m = 1, \ldots, H_s$ are distributed as independent Bernoulli random variables, where $\Pr(Z_{smh} = 1) = 1 - e^{-T_s \cdot v_{sm}}$ for $h = 1, \ldots, w_{sm}$.

Assumption 7: Assume independence among replicates. Thus $R_{ij}|C_i, u_{ij}$ are distributed independently as binomial $(n_{ij}, 1 - e^{-C_i \cdot u_{ij}})$ random variables; $i = 1, \ldots, A$; $j = 1, \ldots, D_i$, and $G_{sm}|T_s, v_{sm}$ are distributed independently as binomial $(w_{sm}, 1 - e^{-T_s \cdot v_{sm}})$ random variables; $s = 1, \ldots, B$; $m = 1, \ldots, H_s$.

The joint conditional probability mass function (pmf) of the vector of positive samples for control carriers, given the number of organisms surviving the simulated disinfection, is given by (1).

$$\Pr(\vec{R}_1, \ldots, \vec{R}_A|C_1, \ldots, C_A) = \prod_{i=1}^{A}\left[\prod_{j=1}^{D_i}\binom{n_{ij}}{R_{ij}}(1 - e^{-C_i \cdot u_{ij}})^{R_{ij}}(e^{-C_i \cdot u_{ij}})^{n_{ij}-R_{ij}}\right]. \tag{1}$$

The joint conditional pmf of the vector of positive samples for test carriers, given the number of organisms surviving the disinfection steps, is given by (2).

$$\Pr(\vec{G}_1, \ldots, \vec{G}_B|T_1, \ldots, T_B) = \prod_{s=1}^{B}\left[\prod_{m=1}^{H_s}\binom{W_{sm}}{G_{sm}}(1 - e^{-T_s \cdot v_{sm}})^{G_{sm}}(e^{-T_s \cdot v_{sm}})^{w_{sm}-G_{sm}}\right]. \tag{2}$$

Because the C_i and T_s are statistically independent, the joint conditional pmf of the vector of positive samples for control and test carriers, given the C_i and T_s, is just the product of the pmf's given by (3).

$$\begin{aligned}\Pr(\vec{R}_1, \ldots, \vec{G}_B|C_1, \ldots, T_B) &= \Pr(\vec{R}_1, \ldots, \vec{R}_A|C_1, \ldots, C_A)\\ &\quad \times \Pr(\vec{G}_1, \ldots, \vec{G}_B|T_1, \ldots, T_B).\end{aligned} \tag{3}$$

Equation (3) is of little practical use, because the C_i and T_s are never exactly observed, although they can be estimated. To produce a likelihood function of directly observable quantities, we integrate or sum out the unobservable C_i and T_s from the joint distribution of $\vec{R}_1, \ldots, \vec{G}_B$ and $C_1, \ldots, T_B$. The details of this derivation are presented in the Appendix.

Let $\vec{\tau}$ denote the vector parameter $(\phi, \zeta, \alpha, \beta)'$, and let $l(\vec{\tau}|\vec{r}_1, \ldots, \vec{r}_A)$ and $l(\vec{\tau}|\vec{g}_1, \ldots, \vec{g}_B)$ be the log likelihood functions of the observed data for control and test carriers, respectively. Then $l(\vec{\tau}|\vec{r}_1, \ldots, \vec{r}_A)$ and $l(\vec{\tau}|\vec{g}_1, \ldots, \vec{g}_B)$ are given in (4) and (5), respectively. The constants ξ, γ, and M in (4) and (5) are explained in the Appendix.

$$l(\vec{\tau}|\vec{r}_1, \ldots, \vec{r}_A) = \sum_{i=1}^{A}\ln\left[\prod_{j=1}^{D_i}\binom{n_{ij}}{r_{ij}}\left(\sum_{L_i=1}^{M_i}\gamma L_i\left(\frac{1}{1+\beta\xi_{L_i}}\right)^{\alpha}\right)\right]. \tag{4}$$

$$l(\vec{\tau}|\vec{g}_1, \ldots, \vec{g}_B) = \sum_{s=1}^{B}\ln\left[\prod_{m=1}^{H_s}\binom{w_{sm}}{g_{sm}}\left(\sum_{L_s=1}^{M_s}\gamma L_s\left(\frac{1}{1+\beta\phi d(1-e^{-\xi_{L_s}})}\right)^{\alpha/d}\right)\right]. \tag{5}$$

The log likelihood function of the observed control and test carrier data is given by (6).

$$l(\vec{\tau}|\vec{r}_1, \ldots, \vec{g}_B) = l(\vec{\tau}|\vec{r}_1, \ldots, \vec{r}_A) + l(\vec{\tau}|\vec{g}_1, \ldots, \vec{g}_B). \tag{6}$$

3. Log reduction

For the quantitative antimicrobial assay, DH:I presented two alternative definitions of LR, the *mean of the logs* and the *log of the means*. The same definitions of LR apply for the PA assay. The mean of logs and the log of the means parameters, denoted by Υ_1 and Υ_2, respectively, are defined in (7) and (8).

$$\Upsilon_1 = \mu_X - \mu_Y. \tag{7}$$

$$\Upsilon_2 = -\log\left(\frac{\mu_T}{\mu_C}\right) = -\log(\phi). \tag{8}$$

where $\phi = \mu_T/\mu_C$ is the probability of a microbe survives disinfection. Consult DH:I for reasons to prefer Υ_1 to Υ_2 and vice-versa.

4. Estimators of log reduction

We apply two general strategies for deriving estimators of LR. The first strategy is to use the joint distribution of the control and test carrier observations to estimate the important parameters α and β that occur in both the control and test carrier components of the log likelihood function. Equation (6) is the log likelihood function for the observable control and test carrier. The maximum likelihood estimator (mle) is found by maximizing (6) with respect to $\vec{\tau}$, then the log reduction estimate is calculated. If the model is correct, the mle is asymptotically $(A, B \to \infty)$ fully efficient and may also have good small sample efficiency. The mle procedure requires a rather complicated computer program.

Let $\hat{\Upsilon}_{1,\text{mle}}$ and $\hat{\Upsilon}_{2,\text{mle}}$ denote the maximum likelihood estimators of Υ_1 and Υ_2, respectively. Let $\hat{\vec{\tau}}$ denote the value of $\vec{\tau}$ that maximizes (6). The mle estimator of Υ_1 is given in (9). It is based on (7) after substituting the components of $\hat{\vec{\tau}}$ into the formulas for μ_X and μ_Y and letting $\hat{d} = [\hat{\phi}^2 + \hat{\zeta}^2(\hat{\alpha} + 1)]/\hat{\phi}^2$.

$$\hat{\Upsilon}_{1,\text{mle}} = \frac{\Psi(\hat{\alpha}) + \ln(\hat{\beta})}{\ln(10)} - \sum_{t=1}^{\infty} \log(t) \times \frac{\Gamma(t + \hat{\rho})}{\Gamma(\hat{\rho})t!}(\hat{q}^t)(\hat{p}^{\hat{\rho}}), \tag{9}$$

where $\hat{\rho} = \hat{\alpha}/d$, $\hat{p} = 1/(\hat{\beta}\hat{\phi}d + 1)$, and $\hat{q} = 1 - \hat{p}$. Notice that $\hat{\Upsilon}_{1,\text{mle}}$ as given in (9) is not of closed form, however, DeVries (1997) provides guidelines for approximating (9).

The mle estimator of Υ_2 is given by (10), where $\hat{\phi}$ is the first element in the vector $\hat{\vec{\tau}}$.

$$\hat{\Upsilon}_{2,\text{mle}} = -\log(\hat{\phi}). \tag{10}$$

The second strategy involves estimating μ_C or μ_X from the control carrier data and μ_T or μ_Y from the test carrier data. The following four steps lead to estimates of LR. First, estimate C_i (and hence X_i) from the ith control carrier data using a standard technique for serial dilution assays. Second, estimate T_s (and hence Y_s) from the sth test carrier data. Third, estimate μ_C, μ_X, μ_T, and μ_Y as follows:

$$\hat{\mu}_C = \frac{1}{A}\sum_{i=1}^{A} \hat{C}_i; \qquad \hat{\mu}_X = \frac{1}{A}\sum_{i=1}^{A} \hat{X}_i; \qquad \hat{\mu}_T = \frac{1}{B}\sum_{s=1}^{B} \hat{T}_s; \qquad \text{and} \qquad \hat{\mu}_Y = \frac{1}{B}\sum_{s=1}^{B} \hat{Y}_s.$$

Fourth, estimate Υ_1 and Υ_2 using (11) and (12), respectively.

$$\hat{\Upsilon}_1 = \hat{\mu}_X - \hat{\mu}_Y. \tag{11}$$

$$\hat{\Upsilon}_2 = -\log\left(\frac{\hat{\mu}_T}{\hat{\mu}_C}\right) = \log(\hat{\mu}_C) - \log(\hat{\mu}_T). \tag{12}$$

We have investigated four standard techniques for accomplishing the first and second steps, the most probable number (mpn) technique (Cochran, 1950), the assay-wise jackknife (jack) method (Does, Strijbosch, and Albers, 1988), the modified score function (msf) (Mehrabi and Matthews, 1995), and the Spearman-Kärber (sk) method (Johnson and Brown, 1961). The mpn and sk methods are commonly used by virologists. All of these methods are easier to calculate than the mle.

Although the mpn is consistent (Cochran, 1950), it is well known that for small replicate sizes, the mpn has a significant positive bias (Mehrabi and Matthews, 1995). Because the replicate sizes will likely be small when the PA assay is used in practice, we sought less biased alternatives to the mpn estimator, such as the assay-wise jackknife and modified score function estimators. It should be noted that the bias of the mpn is mostly a function of the number of wells tested for presence or absence at each dilution stage. Thus if the number of wells per dilution is the same for both test and control carriers, the bias induced by the mpn approach for estimating LR will, for the most part, cancel out when performing the subtractions of equations (11) or (12).

Technical problems are faced in the computation of the msf when all replicate wells at every dilution stage are negative for a test carrier (e.g., $g_{sm} = 0$ for $j = 1, \ldots, H_s$), and in the computation of the mpn or jack when all replicate wells are positive for a control carrier (e.g., $r_{ij} = n_{ij}$ for $j = 1, \ldots, D$). We followed the rules of Mehrabi and Matthews (1995) and Cyr *et al.* (1990) for computing the msf, mpn, and jack estimates in the presence of such extreme observations. Specifically, if all wells at every dilution stage are negative for a test carrier, then the msf estimator does not exist. However, in these cases it is natural to set msf estimate equal to the mpn estimate of zero. If all replicate wells are positive for a control carrier then the mpn estimate is infinite. To overcome this obstacle we act as if one well at the most dilute dilution were in fact negative. This same rule was adopted in the computation of the jack estimate whenever necessary.

Let $\hat{C}_{i,\text{mpn}}$ denote the mpn estimate of the number of organisms surviving the simulated disinfection steps for control carrier i. Define $\hat{X}_{i,\text{mpn}} = \log(\hat{C}_{i,\text{mpn}})$. Similarly for test

carriers, denote as $\hat{T}_{s,\text{mpn}}$ the mpn estimate of the number of organisms surviving the simulated disinfection steps for test carrier s. Define

$$\hat{Y}_{s,\text{mpn}} = \begin{cases} \log(\hat{T}_{s,\text{mpn}}), & \text{if } \hat{T}_{s,\text{mpn}} > 0 \\ 0, & \text{if } \hat{T}_{s,\text{mpn}} = 0. \end{cases}$$

Denote the mpn estimates of μ_C and μ_X as $\hat{\mu}_{C,\text{mpn}}$ and $\hat{\mu}_{X,\text{mpn}}$, respectively. Then $\hat{\mu}_{C,\text{mpn}} = 1/A \sum_{i=1}^{A} \hat{C}_{i,\text{mpn}}$ and $\hat{\mu}_{X,\text{mpn}} = 1/A \sum_{i=1}^{A} \hat{X}_{i,\text{mpn}}$. Similarly, $\hat{\mu}_{T,\text{mpn}} = 1/B \sum_{s=1}^{B} \hat{T}_{s,\text{mpn}}$ and $\hat{\mu}_{Y,\text{mpn}} = 1/B \sum_{s=1}^{B} \hat{Y}_{s,\text{mpn}}$. The mpn estimators of Υ_1 and Υ_2 are given by (13) and (14), respectively.

$$\hat{\Upsilon}_{1,\text{mpn}} = \hat{\mu}_{X,\text{mpn}} - \hat{\mu}_{Y,\text{mpn}}. \tag{13}$$

$$\hat{\Upsilon}_{2,\text{mpn}} = \log(\hat{\mu}_{C,\text{mpn}}) - \log(\hat{\mu}_{T,\text{mpn}}). \tag{14}$$

The jack, msf, and sk estimators of μ_C, μ_X, μ_T, μ_Y, Υ_1, and Υ_2 are calculated similarly and the estimates will be denoted by the subscript notation, jack, msf, or sk.

5. Standard error estimators

DeVries (1997) suggests formulas for $\text{SE}(\hat{\Upsilon}_{1,\text{mle}})$, and $\text{SE}(\hat{\Upsilon}_{2,\text{mle}})$ based on the asymptotic variances of the mles. The details are quite complicated and, due to limitations of space, will not be presented here.

Let $S^2_{X,\text{mpn}}$ and $S^2_{Y,\text{mpn}}$ denote the sample variance for the log mpn estimates of the number of organisms surviving disinfection for the control and test carriers, respectively. Thus,

$$S^2_{X,\text{mpn}} = \frac{1}{A-1} \sum_{i=1}^{A} (\hat{X}_{i,\text{mpn}} - \hat{\mu}_{X,\text{mpn}})^2 \quad \text{and} \quad S^2_{Y,\text{mpn}} = \frac{1}{B-1} \sum_{s=1}^{B} (\hat{Y}_{s,\text{mpn}} - \hat{\mu}_{Y,\text{mpn}})^2.$$

Let $\text{CV}_{C,\text{mpn}}$ and $\text{CV}_{T,\text{mpn}}$ denote the sample coefficients of variation for the mpn estimates of organisms surviving disinfection for the control and test carriers, respectively; i.e.

$$\text{CV}_{C,\text{mpn}} = \frac{\sqrt{S^2_{C,\text{mpn}}}}{\hat{\mu}_{C,\text{mpn}}} \quad \text{and} \quad \text{CV}_{T,\text{mpn}} = \frac{\sqrt{S^2_{T,\text{mpn}}}}{\hat{\mu}_{T,\text{mpn}}}.$$

Equations (15) and (16) give the standard errors for the estimators of Υ_1 and Υ_2 using the mpn approach. The delta method was used to derive standard error formulas for estimators of Υ_2.

$$\mathrm{SE}(\hat{\Upsilon}_{1,\mathrm{mpn}}) = \sqrt{\frac{S^2_{X,\mathrm{mpn}}}{A} + \frac{S^2_{Y,\mathrm{mpn}}}{B}}. \tag{15}$$

$$\mathrm{SE}(\hat{\Upsilon}_{2,\mathrm{mpn}}) = \frac{1}{\ln(10)} \times \sqrt{\frac{\mathrm{CV}^2_{X,\mathrm{mpn}}}{A} + \frac{\mathrm{CV}^2_{Y,\mathrm{mpn}}}{B}}. \tag{16}$$

The standard error formulas for the jack, msf, and sk approaches are derived in an identical manner as those using the mpn approach.

6. Example

Table 1 is an example data set with $A = 3$ control carriers and $B = 3$ test carriers. Ten-fold dilutions were performed between 10^{-1} and 10^{-8}, inclusive. Four wells were tested at each dilution stage. Table 2 shows the associated estimates of Υ_1 and Υ_2, and accompanying standard errors, when calculated by each of the methods described in the preceding sections. Notice the difference of roughly 0.50 logs between the estimates of Υ_1 and Υ_2. This difference is due in part to the variability among test carriers.

7. Simulation study design

We conducted 16 computer simulation experiments, denotes as cases 1–16, to assess the statistical properties of the various estimators of Υ_1 and Υ_2, and of the associated standard errors. Each of the 16 experiments consisted of 1000 simulated PA assay data sets. Table 3 shows the parameter settings for each of the cases. The settings make up a 2^4 factorial layout where B, ϕ, d, and the coefficient of variation for control carriers ($\mathrm{CV}_{\mathrm{control}}$) were each set at two levels. The number of test carriers B was either 3 or 10. The mean survival

Table 2. Estimates of the log reduction parameters Υ_1 and Υ_2 and associated standard errors for the data of Table 1.

	Method				
	mle	mpn	jack	msf	sk
Estimate of Υ_1	2.52	2.50	2.40	2.54	2.50
Standard error	0.6014	0.5607	0.5687	0.5246	0.5528
Estimate of Υ_2	1.96	1.89	1.91	1.99	1.89
Standard error	0.4778	0.4119	0.4395	0.4019	0.4104

fraction, ϕ, was either 10^{-5} or $10^{-3.5}$, representing an active or moderately active disinfectant, respectively. The value of d was set either to 1.0 (constant survival fraction among test carriers) or to a value greater than 1.0 (variable survival fraction). In Table 3, the odd numbered cases have $d = 1.0$ and the even numbered cases have variable survival fractions. The $CV_{control}$ was either 0.10 or 0.40. We believe that these values for B, ϕ, d, and $CV_{control}$ cover the range of conditions likely to be encountered in real-world applications of the PA assay. Note that $\alpha = CV^{-2}$ and $\beta = \mu \cdot CV^2$ and that Υ_1 and Υ_2 are completely specified by α, β, ϕ, and d.

For all 16 cases, the mean (μ) recoverable count was fixed at 10^7, implying $\alpha\beta = 10^7$. Other assay characteristics were held constant across the 16 cases. The number of control carriers was $A = 3$, $n_{ij} = w_{sm} = 4$ for all i and j, and for all s and m, u_{ij} and v_{sm} were constant and were the same for both control and test carriers; the dilutions were

$$\begin{aligned}\vec{u}_i &= \vec{v}_s \\ &= (5 \times 10^{-2}, 5 \times 10^{-3}, 5 \times 10^{-4}, 5 \times 10^{-5}, 5 \times 10^{-6}, 5 \times 10^{-7}, 5 \times 10^{-8}, \\ &\quad 5 \times 10^{-9}),\end{aligned}$$

for all $i = 1, \ldots, A$ and $s = 1, \ldots, B$.

8. Simulation study results

In the interest of space, results of the simulations for cases 2, 6, 10, and 14, only, are presented in Tables 4 and 5. Cases 2, 6, 10, and 14 exhibit the variability among test

Table 3. Parameter settings for each of the 16 cases investigated in the simulation study.

Case	A	B	ϕ	d	CV control	CV test	Υ_1	Υ_2
1	3	3	$10^{-3.5}$	1.0	0.10	0.102	3.500	3.5
2	3	3	$10^{-3.5}$	56.22	0.10	0.750	3.631	3.5
3	3	3	$10^{-3.5}$	1.0	0.40	0.400	3.500	3.5
4	3	3	$10^{-3.5}$	3.51	0.40	0.750	3.598	3.5
5	3	10	$10^{-3.5}$	1.0	0.10	0.102	3.500	3.5
6	3	10	$10^{-3.5}$	56.22	0.10	0.750	3.631	3.5
7	3	10	$10^{-3.5}$	1.0	0.40	0.400	3.500	3.5
8	3	10	$10^{-3.5}$	3.51	0.40	0.750	3.598	3.5
9	3	3	10^{-5}	1.0	0.10	0.141	5.002	5.0
10	3	3	10^{-5}	24.0	0.10	0.500	5.055	5.0
11	3	3	10^{-5}	1.0	0.40	0.412	5.003	5.0
12	3	3	10^{-5}	1.50	0.40	0.500	5.021	5.0
13	3	10	10^{-5}	1.0	0.10	0.141	5.002	5.0
14	3	10	10^{-5}	24.0	0.10	0.500	5.055	5.0
15	3	10	10^{-5}	1.0	0.40	0.412	5.003	5.0
16	3	10	10^{-5}	1.50	0.40	0.500	5.021	5.0

Table 4. Results of the simulation study for estimators of Υ_1. Cases 2, 6, 10, 14 are defined in Table 3.

1. Case	2. Method	3. Bias $\hat{\Upsilon}_1$	4. Mean SE($\hat{\Upsilon}_1$)	5. SD(Υ_1)	6 $\widehat{\text{MSE}}$ $\hat{\Upsilon}_1$
2	mle	0.0049	0.2760	0.2941	0.0865
	mpn	− 0.0062	0.2808	0.2959	0.0876
	jack	0.0041	0.2782	0.2980	0.0888
	msf	− 0.0249	0.2859	0.2998	0.0905
	sk	− 0.0147	0.2910	0.3016	0.0912
6	mle	0.0024	0.2141	0.2249	0.0506
	mpn	− 0.0148	0.1961	0.2247	0.0507
	jack	0.0005	0.1854	0.2134	0.0456
	msf	− 0.0340	0.2008	0.2313	0.0546
	sk	− 0.0238	0.2046	0.2330	0.0549
10	mle	0.0156	0.2508	0.2665	0.0712
	mpn	0.0089	0.2267	0.2597	0.0675
	jack	0.0095	0.2023	0.2418	0.0586
	msf	− 0.0037	0.2306	0.2638	0.0696
	sk	0.0025	0.2360	0.2703	0.0731
14	mle	0.0209	0.1976	0.1902	0.0366
	mpn	0.0039	0.1737	0.1837	0.0338
	jack	0.0082	0.1520	0.1636	0.0268
	msf	− 0.0094	0.1786	0.1889	0.0358
	sk	− 0.0036	0.1822	0.1932	0.0373

carriers that we believe will be typical of real PA assays. The interested reader is referred to DeVries (1997) for a complete tabulation of the simulation study results.

8.1 Comparison of estimators of Υ_1

Table 4 shows the empirical bias and mean squared error (MSE) values, along with a comparison of the mean SE to the observed standard deviation (SD) of the estimators of Υ_1. As can be seen from column 3 Table 4, each estimator is essentially unbiased. No estimator is clearly superior to the others when comparisons of MSE are made (column 6 of Table 4). In general, the sk estimator had the largest MSE for the cases considered in Table 4. The jackknife estimator had the lowest MSE for cases 6, 10, and 14.

Performance of the standard error formula can be determined by comparing the mean of the 1000 standard errors (column 4) with the standard deviation of the point estimates (column 5). The standard error formulas tend to underestimate the true standard deviation, although the bias is not excessive.

Overall, it is our opinion that there is little practice difference among the various estimators of Υ_1 explored in this simulation study. All estimators have little bias and have standard error formulas that appear to work reasonably well. The sk estimator has the advantage of being calculable in closed form. However, the sk estimator appears to be the

Table 5. Results of the simulation study for estimators of Υ_2. Cases 2, 6, 10, 14 are defined in Table 3.

1. Case	2. Method	3. Bias $\hat{\Upsilon}_2$	4. Mean SE($\hat{\Upsilon}_2$)	5. SD($\hat{\Upsilon}_2$)	6 $\widehat{\text{MSE}}$ $\hat{\Upsilon}_2$
2	mle	0.0629	0.2770	0.2934	0.0900
	mpn	0.0397	0.2384	0.3046	0.0943
	jack	0.0218	0.2348	0.3157	0.1002
	msf	0.0272	0.2423	0.3084	0.0959
	sk	0.0339	0.2454	0.3093	0.0968
6	mle	0.0319	0.2171	0.2283	0.0532
	mpn	− 0.0271	0.1837	0.2419	0.0592
	jack	− 0.0413	0.1718	0.2323	0.0557
	msf	− 0.0390	0.1864	0.2551	0.0666
	sk	− 0.0337	0.1902	0.2489	0.0631
10	mle	0.0372	0.2536	0.2671	0.0727
	mpn	0.0291	0.2046	0.2790	0.0787
	jack	0.0211	0.1776	0.2651	0.0707
	msf	0.0218	0.2133	0.3003	0.0907
	sk	0.0256	0.2133	0.2896	0.0845
14	mle	0.0348	0.2032	0.1966	0.0398
	mpn	− 0.0104	0.1693	0.2121	0.0451
	jack	− 0.0125	0.1502	0.2021	0.0410
	msf	− 0.0235	0.1813	0.2316	0.0542
	sk	− 0.0194	0.1759	0.2194	0.0485

most variable of the estimators considered in this paper. The mpn estimator appears to have reasonably good statistical properties in comparison with the other estimators, and has the advantage of being the easiest estimator to compute aside from sk. Because there is a precedent for using the most probable number estimator to obtain estimates of the density of viruses in suspension (Myers, McQuay, and Hollinger, 1994; Payment, Trudel, and Plante, 1985), virologists may prefer $\hat{\Upsilon}_{1,\text{mpn}}$ over other estimators of Υ_1.

8.2 Comparison of estimators of Υ_2

Table 5 provides the simulation results for estimators of Υ_2. The bias of each estimator appears to be negligible (column 3 of Table 5). Comparisons of MSEs indicate that mle is the best estimator of Υ_2. The mle had the smallest MSE, except for case 2 where mle had the second smallest MSE. The msf method had the largest MSE for cases 6, 10, and 14 which was somewhat surprising to us. Mehrabi and Matthews (1995) showed that the msf method had a superior MSE, relative to mpn and jack, when estimating the density of organisms in suspension via a serial- dilution experiment. We have reached that same conclusion in unpublished work not presented here. The poor performance of the msf method in this simulation study leads us to believe that the msf estimator is not as well suited to the log scale as are the other estimators.

The performance of the standard error formulas can be determined by comparing the mean of the 1000 standard errors (column 4) with the standard deviation of the point estimates (column 5) in Table 5. It is clear that the standard error formulas for mpn, jack, msf and sk underestimate the true standard deviation by 20%–30%. Such bias limits the usefulness of these method.

Clearly, mle is the superior estimator of Υ_2 when MSE and performance of the standard error formula are considered. The mle is difficult to calculate, however. Among the more easily computable methods, no estimator stands out as being superior on the basis of MSE and standard error comparisons.

9. Discussion

We believe that most microbiologists will follow the *Standard Methods for the Examination of Water and Wastewater* (Eaton *et al.*, 1995) and choose the geometric mean over the arithmetic mean as a measure of the typical density of viable organisms per carrier; i.e., they will choose the parameter Υ_1 as the measure of Log Reduction. DeVries (1997) showed that Υ_1 is more easily estimated than Υ_2 for the cases considered in this paper, particularly for the mpn, jack, msf, and sk methods. This result is opposite what he found for the quantitative assay as defined in DH:I. We suspect that when estimating the mean density of organisms from multiple serial-dilution experiments, it is more efficient to work on the log scale. The investigation of Garthright (1993) supports this argument. He showed that the log of the mpn from a serial-dilution experiment is less biased and more normally distributed than the mpn. Although Υ_1 is more easily estimated than Υ_2 for the cases considered in this paper, a microbiologist might well decide that Υ_2 is the more appropriate LR for the application at hand.

Should Υ_1 be chosen as the parameter of interest we believe that the mpn method is the method of choice. In terms of MSE and performance of the standard error formula, the mpn method performed as well as the other estimators considered in this paper. The mpn is already in common use.

If Υ_2 is determined to be the parameter of interest, then we recommend the mle. The mle of Υ_2 produced small MSEs and reliable standard errors in the simulation study. The mpn, jack, msf, and sk estimators of Υ_2 had standard error formulas which seriously underestimated the true standard deviation. Improving the standard error formulas for the mpn, jack, msf, and sk estimators of Υ_2 would be a significant contribution.

The validity of the mle depends on the validity of the model we derived, whereas the mpn depends mainly on the components of the model pertaining to Assumptions 6 and 7. Although the statistical model that led to the likelihood function (6) is quite flexible, and we expect it to be practically correct for most assays, the mle approach is not as widely applicable as is the mpn approach.

Based on our experience with presence/absence antimicrobial assays, we believe that real experimental conditions will usually be contained within the range of conditions used in the simulation study. Nevertheless, our conclusions are circumscribed by the accuracy and scope of the simulation study on which they are based.

Appendix

Let the distribution of the number of organisms originally in suspension in a container be denoted by $g(\theta)$, where θ is a non-negative random variable with moment generating function $m_\theta(t)$. Consider a serial-dilution experiment. Let $\vec{R}$ and $\vec{u}$ denote vectors of the number of wells responding positive and the volumes sampled, respectively. Let n_{ij} denote the number of wells at dilution j.

Theorem A1: The marginal distribution of $\vec{R}$, denoted by $f(\vec{R})$, is

$$f(\vec{R}) = \left[\prod_j \binom{n_j}{R_j}\right]\left(\sum_{L=1}^{M} \gamma_L \times m_\theta(-\xi_L)\right),$$

where M, γ_L, and ξ_L are constants which are calculable.

Proof: For convenience, assume θ is a continuous random variable, although the proof works equally well for θ discrete. The conditional distribution of $\vec{R}$ given θ is,

$$f(\vec{R}|\theta) = \prod_{j=1} \binom{n_j}{R_j}(1 - e^{-\theta \cdot u_j})^{R_j}(e^{-\theta \cdot u_j})^{n_j - R_j}.$$

The marginal distribution of $\vec{R}$ is obtained by integrating out θ; i.e.

$$f(\vec{R}) = \int_0^\infty f(\vec{R}|\theta) \times g(\theta)\, d_\theta.$$

Thus,

$$f(\vec{R}) = \left[\prod_j \binom{n_j}{R_j}\right] \int_0^\infty \prod_j (1 - e^{-\theta \cdot u_j})^{R_j}(e^{-\theta \cdot u_j})^{n_j - R_j} \times g(\theta)\, d_\theta.$$

Note that

$$(1 - t)^= \sum_{k=0}^{p} \binom{p}{k}(-1)^k(t)^k,$$

hence

$$(1 - e^{-\theta \cdot u_j})^{R_j} = \sum_{k=0}^{R_j} \binom{R_j}{k}(-1)^k(e^{-\theta \cdot u_j \cdot k}).$$

So,

$$f(\vec{R}) = \left[\prod_j \binom{n_j}{R_j}\right] \int_0^\infty \prod_j \left[\sum_{k=0}^{R_j} \binom{R_j}{k} (-1)^k (e^{-\theta \cdot u_j k})\right] (e^{-\theta \cdot u_j})^{n_j - R_j} \times g(\theta)\, d_\theta$$
$$= \left[\prod_j \binom{n_j}{R_j}\right] \int_0^\infty \prod_j \left[\sum_{k=0}^{R_j} \binom{R_j}{k} (-1)^k e^{-\theta \cdot [u_j \cdot (k + n_j - R_j)]}\right] \times g(\theta)\, d_\theta.$$

Let $M = \prod_j (R_j + 1)$. Notice there are M terms involved in $f(\vec{R})$. Realizing that $(a \cdot e^c) \times (b \cdot e^d) = ab \times e^{c+d}$ for all constants a, b, c, and d, $f(\vec{R})$ can be written as

$$f(\vec{R}) = \left[\prod_j \binom{n_j}{R_j}\right] \int_0^\infty \left[\sum_{L=1}^{M} \gamma_L \times e^{-\theta \xi_L}\right] g(\theta)\, d_\theta,$$

where γ_L and ξ_L are appropriate constants. Notice that the γ_L terms are formed from the products of the coefficients from the binomial expansions of the $(1 - e^{-\theta \cdot u_j})^{R_j}$ terms. The ξ_L terms are formed from the products of the appropriate exponential terms. Thus,

$$f(\vec{R}) = \left[\prod_j \binom{n_j}{R_j}\right] \sum_{L=1}^{M} \gamma_L \int_0^\infty e^{-\theta \xi_L} \times g(\theta)\, d_\theta$$

because integration is a linear operator. Notice that $\int_0^\infty e^{-\theta \xi_L} \times g(\theta)\, d_\theta$ is the moment generating function of θ. Therefore,

$$f(\vec{R}) = \left[\prod_j \binom{n_j}{R_j}\right] \left(\sum_{L=1}^{M} \gamma_L \times m_\theta(-\xi_L)\right)$$

as claimed.

Applications of Theorem A1

Application 1: Let θ be distributed as gamma (mean $= \alpha\beta$, variance $= \alpha\beta^2$). The moment generating function for the gamma distribution parameterized in this manner is $m_\theta(t) = (1/(1 - \beta t))^\alpha$. Then for appropriate constants M, γ_L, and ξ_L, the marginal distribution of $\vec{R}$ is, $f(\vec{R}) = [\prod_j \binom{n_j}{R_j}](\sum_{L=1}^{M} \gamma_L \times (1/(1 + \beta\xi_L))^\alpha)$. Equation (4) was derived using the theorem.

Application 2: Let θ be distributed as negative binomial (mean $= \rho q/p$, variance $= \rho q/p^2$). The moment generating function for the negative binomial distribution parameterized in this manner is $m_\theta(t) = (p/(1 - qe^t))^\rho$. Then for appropriate constants M, γ_L,

and ξ_L, the marginal distribution of $\vec{R}$ is, $f(\vec{R}) = [\prod_j \binom{n_j}{R_j}](\sum_{L=1}^{M} \gamma_L \times (p/(1 - qe^{-\xi_L}))^{\rho})$. Equation (5) was derived using the theorem with $\rho = \alpha/d$, $p = 1/(1 + \beta\phi d)$, $q = 1 - p$.

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