ELSEVIER

Antiviral Research 41 (1999) 145–152

Antiviral Research

One-hit models for virus inactivation studies

Michael Kundi *

Institute of Environmental Hygiene, University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria

Received 7 October 1997; accepted 30 December 1998

Abstract

All biologicals whose production involves materials of human or animal origin are at risk of viral contamination. Testing the capacity of the production processes to remove or inactivate viruses is an essential step in establishing the safety of biological products. The one-hit model which is essentially based on the assumption that the assay will show a positive reaction if and only if there is at least one infectious particle in a small sample drawn from the material, is often used as a basis for the estimation of the number of infectious particles per unit volume, or equivalently, to estimate the ID_{50} (the dose which results in 50% positive reactions). Due to the availability of computers it is no longer necessary to use inadequate and biased methods like Spearman–Kärber to estimate the ID_{50}. Depending on the details of the experiment the average bias of Spearman–Kärber ID_{50} estimates is 10–30%. Maximum likelihood estimation procedures of the parameters, the computation of ID_{50}, reduction factors, and their confidence limits are presented. Furthermore, hints for the design of the experiments are given. The incorporation of kinetics models is also discussed. Although the method represents the state of the art in the biostatistical literature, the problem of random variations of doses has not been addressed appropriately. Based on 36 000 simulated experiments it is shown that the parameters of the model are robust with respect to random variation of doses. Designs using 10-fold dilution series, however, are generally less appropriate and also more affected by dose variability.

Keywords: Virus inactivation; One-hit model; Dose variability; Kinetics models

1. Introduction

During vaccine production or the processing of sera it is essential that the 'virus reduction' (virus removal or virus inactivation) capacity is assessed by adequate methods, to guarantee predefined safety standards (Brown, 1993). While the development of sensitive and specific assays for virus detection as a basis for the assessment of such production processes is mainly a microbiological problem, the design and evaluation of inactivation experiments is within the scope of biostatistics.

* Tel.: +43-1-404-90-64, ext. 726; fax: +43-1-402-05-10.

0166-3542/99/$ - see front matter
PII: S0166-3542(99)00008-X

For the following discussion it is irrelevant whether the virus is added during the production process to study virus removal or whether the virus is already present and the inactivation capacity is assessed.

To test for the presence of infectious particles, samples from the material being processed are drawn at several time points or production steps. The aim of this procedure is to assess the reduction capacity, to analyse the inactivation kinetics, to establish the time necessary to reach a given limit value of infectious particles or for the routine control of inactivation or removal. There are several possibilities to measure the concentration of the infectious agent (e.g. antigen ELISA, Plaquetest). In cell culture experiments the sample is diluted in several steps and each dilution is inoculated into several wells. It will be assumed that one infectious particle is sufficient to cause a positive reaction. However, since a single particle is sufficient to cause the effect, the number of infectious particles per unit volume can only be inferred from the dose response curve. It is the same situation which is known as the 'most probable number' (MPN) problem in several fields of microbiology (Hoskins, 1933; Cochran, 1950; Koch, 1982; Russek and Colwell, 1983).

In virus inactivation studies, however, typically, a 50% infectious dose (ID_{50}) is calculated, which is the dose (dilution) estimated to result in 50% of positive wells. The model appropriate for this type of assay is referred to as one-hit model. It is based on the assumption of a Poisson distribution of the number of infectious particles within samples, and it makes use of the fact that a single infectious particle is sufficient to cause a positive reaction. Furthermore, if a reasonable assumption exists about the inactivation kinetics, this kinetics model can directly be incorporated into the one-hit model and the total number of parameters be further reduced and hence the precision of the estimates enhanced.

The main assumption that a single infectious particle is sufficient to cause a positive reaction has to be tested in advance for the assay in question. The presence of defective interfering (DI) particles in the tissue culture as well as in the tested medium might lead to a violation of this assumption, either due to a failure of virus assembly and therefore to less positive reactions, or due to an interaction of DI particles in the tested volume with the genome of the cells leading to a positive reaction without the presence of an intact infectious virus, or to titre fluctuations in coinfected cultures (Alemany et al., 1998; Lancaster et al., 1998).

In the following sections the model and appropriate estimation procedures, tests of the model and estimations of confidence limits are presented and a sample application is shown. It will be further demonstrated, that the maximum likelihood (ML) estimates are robust with respect to random variation of doses.

2. Materials and methods

2.1. The one-hit model

The following three assumptions are necessary and sufficient for the one-hit model:

- The number of infectious particles in small volumes of the bulk suspension has a Poisson distribution. (If the organisms are distributed at random within the bulk suspension and the sample volumes are small compared to the total volume, this assumption is justified.)
- The nature of the assay is such that a positive reaction will occur in every sample if and only if the sample contains at least one infectious particle.
- The samples are drawn randomly and independently from the bulk suspension and are diluted without systematic or random error (the doses are determined with negligible error—however, the influence of random variations of doses will be tested later).

As a single particle is sufficient to cause a positive reaction, its probability based on the assumption of an underlying Poisson distribution for volume (dose) i at time or production step t is,

$$P_{i,t} = 1 - \exp(-\mu d_{i,t})$$

where $d_{i,t}$ denotes the tested volume and μ the infectivity (virus concentration), which could be interpreted as the mean number of infectious particles per unit dose.

In the context of inactivation experiments, μ is a function of t and one or more parameters θ. The most simple case is a first order inactivation kinetics, where the number of infectious particles at time t obeys the simple differential equation:

$$d\mu = -\mu\theta\ dt$$

where θ is related to the inactivation half-time by $t_{1/2} = \ln 2/\theta$. The solution of this differential equation is $\mu = \mu_0 \exp(-\theta t)$. μ_0 being the virus concentration at the start of the inactivation. An estimate of the total time (t_c) necessary to remove all infectious particles from the bulk follows immediately: $t_c = 1\,d(\mu_0 V)t_{1/2}$ (where V denotes the bulk volume).

It is sometimes claimed that the inactivation is not a first order reaction and has a fast initial phase followed by a slower one. However, unless the rate constants are very different there is no practical way to experimentally differentiate this assertion from simple first order kinetics. If there is doubt whether or not biphasic kinetics are valid, samples at the time point when all infectious particles will have been removed (under the assumption of first order kinetics) should be drawn and tested.

According to the experimental procedure, at each time point or production step t and each dose, $n_{i,t}$ replicates are tested. Let $x_{i,t}$ be the number of positive responses at dose $d_{i,t}$. The total likelihood is thus (with the constant term omitted)

$$\ln L = \sum_{i,t} \ln L_{i,t} = \sum_{i,t} x_{i,t} \ln(e^{\mu d_{i,t}} - 1) - \sum_{i,t} n_{i,t} d_{i,t} \mu$$

Let $S(\theta) = \partial \ln L/\partial\theta$ be the score function. The solution of $S(\theta) = 0$ yields the ML estimates of the parameters θ.

The score function is

$$S(\theta) = \sum_{i,t} \left[\frac{x_{i,t} d_{i,t}}{1 - e^{-\mu d_{i,t}}} - n_{i,t} d_{i,t} \right] \frac{\partial \mu}{\partial \theta}$$

In the general case $\mu(\theta) = \theta_a$ for $a \in T$, T being the set of time points or production steps, the score for θ_a is

$$S(\theta_a) = \sum_{i} \left[\frac{x_{i,a} d_{i,a}}{1 - e^{-\theta_a d_{i,a}}} - n_{i,a} d_{i,a} \right]; \quad a \in T$$

which is a set of $N(T)$ independent score functions. However, since θ_a is always positive, a better approach is to use the transformation $\theta_a^* = \ln(\theta_a)$. For this case the score function and its derivative have to be adjusted accordingly.

If $\mu(\theta) = \mu_0 \exp(-\theta t)$ then the score function is given by

$$S(\theta) = -\sum_{i,t} \left[\frac{x_{i,t} d_{i,t}}{1 - e^{-\mu(\theta) d_{i,t}}} - n_{i,t} d_{i,t} \right] \mu(\theta) t$$

From an estimate $\hat{\mu}$ the ID_{50} is computed:

$$ID_{50} = \frac{\ln 2}{\hat{\mu}}$$

The titer is obtained as the inverse of the ID_{50}.

The estimator's $\hat{\theta}$ asymptotic variance is

$$\mathrm{Var}(\hat{\theta}) = 1/I(\hat{\theta})$$

where $I(\theta) = E\{-\partial^2 \ln L/\partial\theta^2\}$.

From

$$\frac{\partial^2 \ln L}{\partial \theta^2} = \sum_{i,t} \left\{ \left[\frac{x_{i,t} d_{i,t}}{1 - e^{-\mu d_{i,t}}} - n_{i,t} d_{i,t} \right] \frac{\partial^2 \mu}{\partial \theta^2} - \frac{x_{i,t} d_{i,t}^2}{2(\cosh(\mu d_{i,t}) - 1)} \left(\frac{\partial \mu}{\partial \theta} \right)^2 \right\}$$

the following is found

$$I(\hat{\theta}_a) = \sum_i \frac{n_{i,a} d_{i,a}^2}{e^{\hat{\theta}_a d_{i,a}} - 1}; \quad a \in T$$

for the general case and

$$I(\hat{\theta}) = \sum_{i,t} \frac{n_{i,t}}{e^{\mu(\hat{\theta}) d_{i,t}} - 1} d_{i,t}^2 t^2 \mu(\hat{\theta})$$

for the case of first order inactivation kinetics.

As start value for the estimation algorithm the least squares estimate of the parameter could be computed (e.g.$(1/k)\Sigma \ln(n/x)/d$ for the general case; k…number of doses).

2.2. Design considerations

Consider an assay applying serial dilutions with a dilution factor f, starting from a volume V. Then the doses $d_{i,t}$ are given by $d_{i,t} = V/f^{i-1}$. Hence the standard error of $\hat{\theta}_a$ in the general case is (if there is a constant number of replicates $n_{i,t} = n$) indirectly proportional to volume V and

the square root of n. The design of the experiment should be adjusted in such a way that the expected number of doses with at least 1 and at most $n-1$ reactions exceeds a predefined value $k_{\min}$ (≥ 1). From the definition of the model it follows immediately that the minimum dilution step (leading to just one negative reaction) is given by

$$\text{dilution step}_{\min} = \frac{\ln\left(\frac{V\theta}{\ln(n)}\right)}{\ln(f)}$$

and the maximum dilution step (leading to just one positive reaction) by

$$\text{dilution step}_{\max} = \frac{\ln\left(\frac{V\theta}{\ln(n)-\ln(n-1)}\right)}{\ln(f)}.$$

Hence, the number of steps between the maximum and minimum (including the limits) is independent of the sample volume and the parameter θ:

$$k_{\text{pos}} = \frac{\ln\left(\frac{\ln(n)}{\ln(n)-\ln(n-1)}\right)}{\ln(f)} + 1.$$

If $k_{\text{pos}} < k_{\min}$, either by increasing n or reducing f, the appropriate number of dilutions could be determined. The predilution is found by computing the minimum dilution step.

The expected maximum number of doses with less than 100% and more then 0% reactions (k_{pos}) as a function of the number of observations per dose shows that with less than 20 observations per dose 10-fold dilution series result in less than three informative doses. Therefore, 2-fold or half-log dilutions are more appropriate.

If no positive reaction is found at all tested doses, no point estimate of the number of infectious particles from the general model is possible. However, as a conservative approach it can be assumed that there is just one particle in the volume at the highest dose. The total volume at that dose is nV. The estimated concentration of infectious particles is then $1/nV$. The ML estimate differs somewhat from this figure:

$$\hat{\theta} = -\frac{1}{V}\ln\left[1-\frac{1}{n}\frac{f^k - f^{k-1}}{f^k - 1}\right].$$

The standard error for this estimate is approximately equal to e, the base of the natural logarithm.

2.3. Reduction factor

The results of inactivation experiments are often expressed as reduction factors or rather as base 10 logarithm of the reduction factor. It is given by the expression

$$\log_{10}\frac{V_b}{V_a}\frac{\text{ID}_{50}^{(a)}}{\text{ID}_{50}^{(b)}}$$

where V_b and $\text{ID}_{50}^{(b)}$ is the volume of the starting material and the ID_{50} at the beginning of the inactivation, and V_a and $\text{ID}_{50}^{(a)}$ is the volume and ID_{50} after the inactivation (step).

2.4. Tests of the model

Let $S_{i,t}(\hat{\theta}) = \partial \ln L_{i,t}(\hat{\theta})/\partial\hat{\theta}$ be the score function for dose level i at time point t at the location of the maximum of the total score $S(\theta)$, and $I_{i,t}(\hat{\theta})$ be Fisher's information with respect to the parameter θ at location $\hat{\theta}$. Then $z_{i,t}^2(\hat{\theta}) = S_{i,t}^2(\hat{\theta})/I_{i,t}(\hat{\theta})$ is the square of an asymptotically normally distributed variate with expected value 0 and unit variance. Hence the sum

$$\chi_{kN-p}^2 = \sum_{i,t} z_{i,t}^2(\hat{\theta})$$

is an approximate χ^2 statistic with $kN-p$ degrees of freedom (where k is the number of doses per time point and N is the number of time points tested and p is the number of parameters estimated). This statistic (Rao, 1973) tests the homogeneity of the estimates over doses (and time points, if the model includes inactivation kinetics).

To test the kinetics model, the likelihood ratio statistic which is asymptotically χ^2 distributed with $N-p$ degrees of freedom could be used: Let L_{k} be the likelihood of the kinetics model with p parameters, at the location of the maximum and L_{g} be the likelihood of the general model with N parameters, at the location of the maximum then $-2\ln(L_{\text{k}}/L_{\text{g}})$ is the appropriate statistic.

Table 1
Results of an inactivation experiment[a]

log dose (mm/well)	Hours after start of inactivation							
	0.25	1	2	4	8	12	15	20
−1.0							16	16
−1.5							16	14
−2.0				16	16	16	16	7
−2.5				16	16	16	12	3
−3.0				16	16	15	7	1
−3.5				16	16	7	3	0
−4.0	16	16	16	16	11	3	0	0
−4.5	16	16	16	16	10	3	0	0
−5.0	16	16	15	13	2	0		
−5.5	16	12	12	3	0	0		
−6.0	10	7	5					
−6.5	3	2	1					
−7.0	1	0	0					
−7.5	0	0	0					
$-\log ID_{50}$	6.15	5.83	5.69	5.29	4.37	3.54	2.85	1.95
95% CI	5.96–6.33	5.65–6.01	5.51–5.86	5.09–5.49	4.19–4.55	3.36–3.72	2.67–3.03	1.77–2.13
Kinetics	6.05	5.89	5.68	5.26	4.41	3.56	2.93	1.87
Model	5.95–6.15	5.80–5.98	5.60–5.77	5.18–5.33	4.34–4.47	3.49–3.64	2.83–3.02	1.73–2.00

[a] Samples are drawn 0.25–20 h after start of inactivation. Number of positive wells (out of 16) for eight doses, estimate of $\log_{10} ID_{50}$ and 95% confidence limits from general model and from first order kinetics model.

2.5. Confidence intervals

As $\hat{\theta}$ is asymptotically normal, approximate confidence limits are given by:

$$\theta_{u,l} = \hat{\theta} \pm \frac{z_{\alpha/2}}{\sqrt{I(\hat{\theta})}}$$

where $z_{\alpha/2}$ is the $1-\alpha/2$ percentile of the standard normal distribution. If the estimation was not done based on the logarithmic transformation, then the confidence limits should be computed using the following formula:

$$\log \theta_{u,l} = \log \hat{\theta} \pm \frac{z_{\alpha/2}}{\sqrt{\hat{\theta} \cdot I(\hat{\theta})}}$$

and taking antilogs (Cochran, 1950; Finney, 1978).

An alternative method (Gart, 1991) involves the iterative solution of

$$z(\theta) = \frac{S(\theta)}{\sqrt{I(\theta)}} = \pm z_{\alpha/2}$$

which accounts for a possible variation in variances at the limits θ_l, θ_u.

The confidence intervals for reduction factors are computed either directly by the score method or by observing that the standard error of the difference between the estimates of the log-concentration of infectious particles is given by

$$\sqrt{\frac{1}{I(\hat{\theta}_b)} + \frac{1}{I(\hat{\theta}_a)}}$$

(with indexes b and a denoting before and after the inactivation step).

3. Results and discussion

3.1. Sample application

In an inactivation experiment a pool of sera is spiked with 6 $\log_{10}$ infectious units of HAV/ml. At eight time points during inactivation (0.25–20 h

Table 2
Results of simulated experiments with 2-fold dilution series[a]

No of doses	n per dose	Variation of doses (%)	True ID_{50}	Sample ID_{50}	Expected S.D.	Sample S.D.	Variance ratio
3	5	0	100	106.3	0.462	0.514	1.238
		50		103.8		0.572	1.533
	10	0		101.2	0.327	0.339	1.075
		50		99.6		0.402	1.511
	16	0		101.8	0.258	0.260	1.016
		50		99.3		0.342	1.757
	5	0	50	49.9	0.377	0.412	1.194
		50		50.2		0.462	1.502
	10	0		49.9	0.266	0.270	1.030
		50		49.1		0.316	1.411
	16	0		50.4	0.211	0.213	1.019
		50		50.2		0.300	2.022
5	5	0	100	108.7	0.434	0.487	1.259
		50		99.6		0.499	1.322
	10	0		102.3	0.307	0.316	1.059
		50		99.8		0.370	1.453
	16	0		101.3	0.243	0.240	0.975
		50		100.9		0.312	1.649
	5	0	25	24.7	0.310	0.324	1.092
		50		25.0		0.347	1.253
	10	0		24.9	0.219	0.224	1.046
		50		24.4		0.276	1.588
	16	0		24.9	0.173	0.176	1.035
		50		25.3		0.236	1.861
8	5	0	100	108.7	0.426	0.459	1.161
		50		104.4		0.479	1.264
	10	0		99.5	0.302	0.319	1.116
		50		99.5		0.354	1.374
	16	0		101.2	0.238	0.255	1.148
		50		99.1		0.289	1.474
	5	0	8.84	8.58	0.294	0.297	1.021
		50		8.83		0.346	1.385
	10	0		8.76	0.208	0.206	0.981
		50		9.05		0.261	1.575
	16	0		8.75	0.164	0.163	0.988
		50		9.19		0.220	1.800

[a] Five hundred experiments per combination of number of doses, observations per dose, dose variation and ID_{50}. Sample geometric means of ID_{50}, expected asymptotic and observed S.D. of $\ln(\theta)$, ratio of observed to expected asymptotic variance of $\ln(\theta)$ are shown.

after start of inactivation) samples of 1 ml are drawn and serially diluted in 0.5 $\log_{10}$ steps. Starting with 10^{-4} ml at the first three time points, with 10^{-2} ml at the next three, and with 10^{-1} ml at the last two time points the diluted samples are inoculated each in 16 wells of a 8×16 microtiter plate containing the appropriate cell cultures. The number of positive wells as well as the estimates of the ID_{50} from the general model and from the kinetics model are shown in Table 1.

The χ^2 with 56 degrees of freedom (df) for the general model was 20.303 which indicates an excellent fit of the model. The likelihood ratio statistic for the kinetics model amounts to 3.293 with 7

Table 3
Results of simulated experiments with 10-fold dilution series[a]

No of doses	*n* per dose	Variation of doses (%)	True ID_{50}	Sample ID_{50}	Expected S.D.	Sample S.D.	Variance ratio
3	5	0	100	102.5	0.599	0.641	1.145
		50		98.3		0.912	2.318
	10	0		100.2	0.423	0.421	0.991
		50		99.8		0.914	4.669
	16	0		101.0	0.335	0.365	1.187
		50		101.2		0.937	7.823
	5	0	10	9.8	0.580	0.562	0.939
		50		11.0		0.986	2.890
	10	0		10.0	0.410	0.374	0.832
		50		10.9		0.936	5.212
	16	0		9.9	0.324	0.330	1.037
		50		11.3		0.866	7.144
5	5	0	100	104.5	0.598	0.633	1.120
		50		90.8		0.647	1.171
	10	0		110.3	0.423	0.460	1.183
		50		92.3		0.449	1.127
	16	0		103.7	0.335	0.362	1.168
		50		93.5		0.344	1.054
	5	0	1	0.96	0.577	0.549	0.905
		50		0.99		0.969	2.820
	10	0		0.97	0.408	0.390	0.914
		50		1.07		0.918	5.063
	16	0		1.00	0.322	0.306	0.903
		50		1.08		0.871	7.317
8	5	0	100	106.6	0.598	0.650	1.181
		50		82.7		0.916	2.346
	10	0		106.1	0.423	0.457	1.167
		50		87.3		0.922	4.751
	16	0		105.2	0.335	0.366	1.194
		50		96.5		0.938	7.840
	5	0	0.032	0.031	0.492	0.532	1.169
		50		0.033		0.965	3.847
	10	0		0.031	0.348	0.384	1.218
		50		0.034		0.868	6.221
	16	0		0.031	0.275	0.290	1.112
		50		0.035		0.815	8.783

[a] Five hundred experiments per combination of number of doses, observations per dose, dose variation and ID_{50}. Sample geometric means of ID_{50}, expected asymptotic and observed S.D. of $\ln(\theta)$, ratio of observed to expected asymptotic variance of $\ln(\theta)$ are shown.

df, hence the kinetics model shows no significant deviation. The time necessary to remove all virus particles from 1 l serum was estimated to be about 61.25 h. Hence, the virus removal is too slow, and appropriate measures have to be taken to increase the rate of virus inactivation.

3.2. Simulation study

The one-hit model assumes that the doses $d_{i,t}$ are free from errors. However, in practical applications the doses will vary randomly from sample to sample. It is also possible that due to dilution

errors systematic deviations from the projected doses occur. In the latter case only direct experimental variations, applying different dilution factors, are suitable to detect this source of error. Random variation of doses might lead to underestimation of the variance of the estimator and to an increase of the number of invalid tests and also to biased estimates of the ID_{50}. Hence, the question arises whether the ML estimate of the ID_{50} based on the model presented is robust with respect to random variations of doses.

The simulation was done based on the assumption of a log-normal distribution of doses within dilution steps. For each choice of the ID_{50}, sample size, number of doses and dilution factor, 500 experiments were generated and evaluated. A total of 36 000 of such simulated experiments were carried out. Each design was evaluated with two choices of ID_{50} values: one at the first dose and one at the geometric mean of doses. The simulation was done under the assumption of error-free doses (0% variation of doses) and under the assumption of a log-normal distribution of doses with a standard deviation amounting to 1/2 of the logarithm of the dilution factor (50% variation of doses). The results of the simulations are presented in Tables 2 and 3.

With 2-fold dilution series the estimates are apparently not biased by random dose variations. As expected, the variance of the estimates based on randomly varying doses is somewhat greater than those based on error-free doses. The increase in precision by increasing the number of doses and/or the number of observations per dose is less pronounced if doses vary randomly, hence the variance ratio is greater the higher the number of doses and observations per dose. However, the simulation was based on rather a worst case scenario, since a 50% standard deviation means a 30% overlap of neighboring doses.

Due to doses with zero reactions, 10-fold dilution series result in considerably higher standard errors of estimates. This is especially true for higher numbers of doses. For the ID_{50} at the first dose the estimates based on data from randomly varying doses are slightly biased, and increasingly so as the number of doses increase. In this case the variance ratio is also considerably higher.

The observed mean variance in the case of error-free doses is almost equal to the asymptotic variance of the estimate for all designs tested.

From the results of the simulation study it is concluded that

1. designs with 10-fold dilution series are less appropriate due to the higher number of non-informative doses (with 0 or 100% positive reactions) and because of the greater influence of dose variability;
2. the width of the 95% confidence interval is less than one log-step for designs with 2-fold or half-log dilutions if the number of observations per dose is ten or more and at least five doses are tested (even if there is random variation of doses); and
3. experiments should be designed in such a way that the projected ID_{50} is as close as possible to the (geometric) mean dose.

References

Alemany, R., Dai, Y., Lou, Y.C., Sethi, E., Prokopenko, E., Josephs, S.F., Zhang, W.W., 1998. Complementation of helper-dependent adenoviral vectors: size effects and titer fluctuations. J. Virol. Methods 68, 147–159.

Brown, F., 1993. Virological safety aspects of plasma derivatives. Dev. Biol. Stand. 81, 103.

Cochran, W.G., 1950. Estimation of bacterial densities by means of the 'most probable number'. Biometrics 5, 105–116.

Finney, D.J., 1978. Statistical Method in Biological Assay, 3rd edition. Macmillan, New York.

Gart, J.J., 1991. An application of score methodology: confidence intervals and tests of fit for one-hit curves. In: Rao, C.R., Chakraborty, R. (Eds.), Handbook of Statistics, vol. 8. Elsevier, Amsterdam, pp. 395–406.

Hoskins, J.K., 1933. The most probable numbers of B. coli in water analysis. J. Am. Water Works Assoc. 25, 867–877.

Koch, A.L., 1982. Estimation of the most probable number with a programmable pocket calculator. Appl. Environ. Microbiol. 43, 488–490.

Lancaster, M.U., Hodgetts, S.I., Mackenzie, J.S., Urosevic, N., 1998. Characterization of defective viral RNA produced during persistent infection of Vero cells with Murray Valley encephalitis virus. J. Virol. 72, 2474–2482.

Rao, C.R., 1973. Linear Statistical Inference and Its Applications, 2nd edition. Wiley, New York.

Russek, E., Colwell, R.R., 1983. Computation of most probable numbers. Appl. Environ. Microbiol. 45, 1646–1650.