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Estimation method for serial dilution experiments

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ABSTRACT

Titration of microorganisms in infectious or environmental samples is a corner stone of quantitative microbiology. A simple method is presented to estimate the microbial counts obtained with the serial dilution technique for microorganisms that can grow on bacteriological media and develop into a colony. The number (concentration) of viable microbial organism is estimated from a single dilution plate (assay) without a need for replicate plates. Our method selects the best agar plate with which to estimate the microbial counts, and takes into account the colony size and plate area that both contribute to the likelihood of miscounting the number of colonies on a plate. The estimate of the optimal count given by our method can be used to narrow the search for the best (optimal) dilution plate and saves time. The required inputs are the plate size, the microbial colony size, and the serial dilution factors. The proposed approach shows relative accuracy within $\pm$ 0.1 $\log_{10}$ from data produced by computer simulations. The method maintains this accuracy even in the presence of dilution errors of up to 10% (for both the aliquot and diluent volumes), microbial counts between 10^4 and 10^{12} colony-forming units, dilution ratios from 2 to 100, and plate size to colony size ratios between 6.25 to 200.

1. Introduction

Quantitative estimation of the number of viable microorganisms in bacteriological samples has been a mainstay of the microbiological laboratory for more than one-hundred years, since Koch first described the technique (Koch, 1883). Serial dilution techniques are routinely used in hospitals, public health, virology, immunology, microbiology, pharmaceutical industry, and food protection (American Public Health, 2005; Hollinger, 1993; Taswell, 1984; Lin and Stephenson, 1998) for microorganisms that can grow on bacteriological media and develop into colonies. A list of bacteria that are viable but nonculturable (VBNC), the detection of such microorganisms, and the process of resuscitation of cells from VBNC state are addressed by Oliver (2005, 2010). In the work presented here it is assumed that the microorganisms are culturable.

The objective of the serial dilution method is to estimate the concentration (number of colonies, organisms, bacteria, or viruses) of an unknown sample by counting the number of colonies cultured from serial dilutions of the sample, and then back track the measured counts to the unknown concentration.

Given an unknown sample which contains n_0 colony forming units (CFUs), a series of J dilutions are made sequentially each with a dilution factor α. From each of the J dilutions a fraction α_p^{-1} is taken and spread (plated) on an agar plate (assay) where colonies are counted. Thus, in general there are two dilution factors: α and α_p. For example, $\alpha = 10$ indicates a 10-fold dilution, e.g., by diluting successively 0.1 ml of sample into 0.9 ml of media; and $\alpha_p = 1$ means that the entire volume (e.g., 1 ml) is spread (plated) on the agar plate. For an experiment with a larger dilution factor α_p, multiple plates may be spread at the same dilution stage. For example, $\alpha_p = 20$ represent a 5% plating of the 1 ml dilution, and thus up to 20 replicates could be created. At each dilution the true number of colonies is $n_j = n_0\alpha^{-j}\alpha_p^{-1}$ and the estimated number is $\hat{n}_j$. The estimated quantities are denoted with a "hat" (estimated quantities can be measured quantities, or quantities that are derived from measured or sampled quantities); symbols without a "hat" denote true quantities (also known as population values in statistics) that do not contain any sampling or measurement error. In this work both n_j and n_0 are "counts", i.e., number of colonies. Knowing the aliquot volume, one can easily convert counts to concentration (for example CFU/ml).

The importance of serial dilution and colony counting is reflected by the number of standard operating procedures and regulatory guidelines describing this methodology. In all of these guidelines the optimal number ($\hat{n}_j$) of colonies to be counted has been reported (Park and Williams, 2010; Wilson, 1922; Jennison and Wadsworth, 1940; Tomasiewicz et al., 1980; FDA, 2001; Goldman and Green, 2008) as 40–400, 200–400, 100–400, 25–250, 30–300. It is interesting to note that these

Abbreviations: TE, total error; RE, relative error; MPN, most probable number; TNTC, too numerous to count; VBNC, viable but nonculturable; CFU, colony forming unit.

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references do not specify the area in which the colonies grow, nor the diameter of the particular organism assayed. The result is that titration and counting colonies is done within a range that may be inadequate, and may introduce considerable error. In our work these parameters are addressed.

The main challenge in serial dilution experiments is the estimation of the undiluted microorganisms counts n_0 from the measured $\hat{n}_j$. There are two competing processes (Tomasiewicz et al., 1980) that affect the accuracy of the estimation: sampling errors and counting errors. Sampling errors are caused by the statistical fluctuations of the population. For example, if one sample on an average of 100 colonies, the fluctuations in the number of the population are expected to be $\pm\sqrt{100}$ when the sampling process is governed by a Poisson probability (Poisson and Binomial distributions are often used in statistical analysis to describe the dilution process (Hedges, 2002; Myers et al., 1994)) where the standard deviation equals square-root of the mean; the relative error (ratio of the standard deviation to the mean) is $\sqrt{100}/100 = 0.1$. Thus, the larger the sample size is, the smaller the relative sampling error; hence, one would like to use a dilution plate with the largest number $\hat{n}_j$ (i.e., the least diluted sample, $j \to 1$). However, as the number of colonies increases, counting error is introduced due to the high probability of two (or more) colonies to merge (due to overcrowding) and become indistinguishable, and be erroneously counted as one colony. An optimum ("a sweet spot") between these two processes (sampling and counting error) needs to be found for using the optimal dilution $\hat{n}_j$ (i.e., the optimal jth plate) with which to estimate n_0. Cells can grow into colonies in various ways. Wilson (1922) states that when two cells are placed very close together only one colony will develop, and when two cells are situated at a distance from each other both cells may grow and then fuse into one colony. Either way, the end result is the appearance of one colony which causes counting error.

Estimation of bacterial densities from the most probable number (MPN) method (Cochran, 1950) requires multiple replicates of the jth dilution plate, and analyzes the frequency of plates with zero colonies instead of using counts directly. MPN is often used to measure microbes in milk, water and food (Blodgett, 2010). The MPN method (Cochran, 1950) "is of low precision, as is to be expected from a method that does not use direct counts. Large number of samples [replicate agar plates] must be taken at each dilution if a really precise result is wanted". In our work we seek a method where the counts from a single plate are used to estimate bacterial concentrations.

A simple method to estimate the number of colonies n_0 in the unknown sample from the counted number of colonies $\hat{n}_j$ at the jth assay is presented. Our method is easy to implement. The method selects the optimal count (i.e., a best single plate) with which n_0 is estimated. There are only a few inputs needed: the incubation plate size, the microbial colony size, and the dilution factors (α and α_p). The dilution error (although present in the serial dilution experiment) is not an input. The relative accuracy of our method is within $\pm$ 0.1 $\log_{10}$ (i.e., within 100% error) which is much better than the common requirement of $\pm$ 0.5 $\log_{10}$ (i.e., within 500% error) that is often regarded as accepted accuracy in many biological experiments.

2. Material and methods

The measured (counted) number of colonies $\hat{n}_j$ (corresponding each to one organism) is related to the true number of colonies n_j by $n_j = \hat{n}_j + \delta_j$ where δ_j is a bias that accounts for uncounted colonies due to the merging (overcrowding) of nearby colonies, and thus, $n_j \geq \hat{n}_j$. The challenge is to obtain an estimate of δ_j from a single measurement $\hat{n}_j$ of the jth Petri dish. The challenge is met in an ad-hoc manner. The following assumptions are made: (*i*) The true n_j (when no colonies are miscounted) is described by a Poisson probability for which the variance equals the mean (Forbes et al., 2011), (ii) The probability density function for $\hat{n}_j$ (with the effect of merging of nearby colonies) is a displaced version (by δ_j) of the Poisson distribution for n_j, see Fig. 1. Hence the variance of the probability density function for n_j is the same as that for $\hat{n}_j$, and therefore the variance $Var(\hat{n}_j) = Var(n_j) = E\left[\left(n_j - \mu_j\right)^2\right]$. $E(\cdot)$ is expectation operator (i.e., an average process), and μ_j is the mean of n_j. There is not enough data to compute a variance with the expectation operator (because only one plate with one value of $\hat{n}_j$ for a dilution $\alpha^{-j}\alpha_p^{-1}$ is available). Therefore, we define a measure of "spread" given by $V_n = (n_j - \mu_j)^2$, that is computed from a single value of n_j. The "spread" can be solved for μ_j by $\mu_j = n_j - \sqrt{V_j}$. The distance $\delta_j < \mu$ (see Fig. 1), and thus there must be a constant $c < 1$ such that $\delta_j = c\sqrt{V_n}$. Hence $\mu_j = cn_j - c\sqrt{V_n}$. Because we do not want to modify the individual n_j values (with c), we instead construct δ_j with a constant $k > 1$ where $\delta_j = n_j - k\sqrt{V_n}$, and k only modifies the spread $\sqrt{V_n}$. Our notion of "spread" is a substitute for the notion of variance (that we cannot compute) and is weak; hence, no harm is done in adjusting it with a fudge factor k. The factor k is a function of the geometry of the serial dilution experiment. At this point it suffices to state that k exists (the numerical value of k is addressed later). With the displaced Poisson assumption for n_j and for $\hat{n}_j$, we set $V_n = V_{\hat{n}} = \hat{n}_j$, leading to $\delta_j = n_j - k\sqrt{n_j}$. We don't have access to n_j, and therefore we replace the unknown population value n_j with the measured $\hat{n}_j$. This is inspired by the principle that is often used in signal processing of replacing unknown population parameters with maximum-likelihood estimates (as is done, for example, in the generalized likelihood ratio test (Scharf and Friedlander, 1994)).

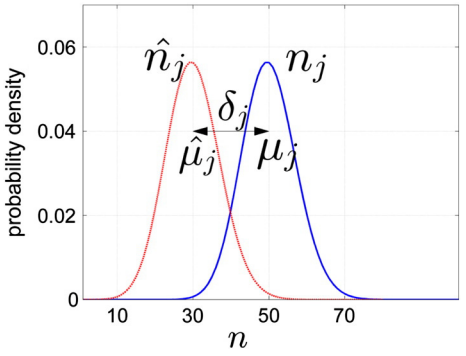

Fig. 1. The concept of δ_j is given with Poisson and a shifted-Poisson probability density functions for the true n_j, and for the counted (measured) $\hat{n}_j$, respectively. $\hat{n}_j$ is the observed (counted) number of colonies on plate j of the serial dilution process, for which the true number of colonies is n_j with a mean μ_j. Due to uncounted colonies in the measuring process (i.e., merging of colonies that are counted as one colony due to overcrowding), $\hat{n}_j < n_j$.

With an undetermined k (for the moment) a model-estimate of the true δ by $\hat{\delta}$ is given by

$$\left\{\begin{array}{l} \hat{\delta}_j = \hat{n}_j - k\sqrt{\hat{n}_j} \\ \hat{\delta}_j > 0 \quad for \quad 1 < k < \sqrt{\hat{n}_j} \\ \hat{\delta}_j = 0 \quad for \quad k \geq \sqrt{\hat{n}_j} \end{array}\right\}. \tag{1}$$

The inequalities in Eq. (1) are necessary to ensure $\hat{\delta}$ to be a non-negative quantity less than the value of μ. Eq. (1) implies that the counting error is negligible $\left(\hat{\delta} \to 0\right)$ when $\hat{n}_j < k^2$. Given $\hat{\delta}_j$ in Eq. (1) we proceed to estimate n_0 by

$$\hat{n}_0 = \left(\hat{n}_j + \hat{\delta}_j\right)\alpha^j\alpha_p. \tag{2}$$

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