Food Microbiology 60 (2016) 49–53

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Do bacterial cell numbers follow a theoretical Poisson distribution? Comparison of experimentally obtained numbers of single cells with random number generation via computer simulation

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ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form
16 May 2016
Accepted 19 May 2016
Available online 22 June 2016

Keywords:
Individual cell modelling
Single-cell
Poisson distribution

ABSTRACT

We investigated a bacterial sample preparation procedure for single-cell studies. In the present study, we examined whether single bacterial cells obtained via 10-fold dilution followed a theoretical Poisson distribution. Four serotypes of *Salmonella enterica*, three serotypes of enterohaemorrhagic *Escherichia coli* and one serotype of *Listeria monocytogenes* were used as sample bacteria. An inoculum of each serotype was prepared via a 10-fold dilution series to obtain bacterial cell counts with mean values of one or two. To determine whether the experimentally obtained bacterial cell counts follow a theoretical Poisson distribution, a likelihood ratio test between the experimentally obtained cell counts and Poisson distribution which parameter estimated by maximum likelihood estimation (MLE) was conducted. The bacterial cell counts of each serotype sufficiently followed a Poisson distribution. Furthermore, to examine the validity of the parameters of Poisson distribution from experimentally obtained bacterial cell counts, we compared these with the parameters of a Poisson distribution that were estimated using random number generation via computer simulation. The Poisson distribution parameters experimentally obtained from bacterial cell counts were within the range of the parameters estimated using a computer simulation. These results demonstrate that the bacterial cell counts of each serotype obtained via 10-fold dilution followed a Poisson distribution. The fact that the frequency of bacterial cell counts follows a Poisson distribution at low number would be applied to some single-cell studies with a few bacterial cells. In particular, the procedure presented in this study enables us to develop an inactivation model at the single-cell level that can estimate the variability of survival bacterial numbers during the bacterial death process.

1. Introduction

Predictive modelling that takes into account the individual heterogeneity and uncertainty of bacterial behaviour has recently received increasing attention. A definition of variability and uncertainty is described as follows (Begg et al., 2014). Variability is quantified by a distribution of frequencies of multiple instances of the quantity, derived from observed data. Examples of source of variability are individual cell heterogeneity, which cause different responses among cells in a same condition and number of bacterial cells caused by naturally occurring randomness. In contrast, uncertainty refers to the unknown, single, true value of some quantity. Uncertainty arises from multiple sources, such as statistical variation, approximation, subjectivity in measurement techniques, disagreement, variability and practical unpredictability. A deterministic approach results in limited predictions of bacterial behaviour at low bacterial numbers because this approach does not consider uncertainty and individual heterogeneity (Koutsoumanis and Lianou, 2013; Membré et al., 2006). The phenomenon of tailing described in bacterial death/inactivation kinetics has not yet been predicted well, which may represent the uncertainty and variability of surviving bacterial behaviour. It has been reported that in small populations, such as less than 100 cells, individual cell behaviours become distinct from population behaviour (Aspridou and Koutsoumanis, 2015). During bacterial inactivation or growth process, bacterial behavior would be difficult to predict by point

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http://dx.doi.org/10.1016/j.fm.2016.05.019
0740-0020/

estimation due to the variability and uncertainty. To estimate bacterial numbers in foods contaminated at low levels, the variability of bacterial behaviour should be combined into a predictive model. A probabilistic approach is indispensable for appropriately describing the uncertainty and variability of bacterial behaviour.

Salmonella enterica and enterohaemorrhagic *Escherichia coli* can cause foodborne illness following the ingestion of less than 100 cells (Hara-Kudo and Takatori, 2011). Thus, it is important to appropriately estimate the behaviour (growth and/or death) of small bacterial numbers, such as less than 100 cells, for risk assessment of foodborne illness. Because of variability and uncertainty of bacterial behavior, it is difficult to assess the frequency of bacterial survival in foods contaminated at low levels.

Working at the single-cell level requires the preparation of single bacterial cells. However, obtaining single cells is difficult due to the statistical uncertainty associated with current experimental techniques (Hedges, 2002). In a previous study, a protocol for isolating single cells using two-fold dilutions was developed (Francois et al., 2003), and the individual lag phase of *Listeria monocytogenes* was evaluated (Francois et al., 2005, 2006). In contrast, there has been only one report on bacterial inactivation models at the single-cell level (Aspridou and Koutsoumanis, 2015). One of the reasons for the limited number of such studies is the difficulty involved in obtaining exactly one cell with high reproducibility. For single-cell-level bacterial inactivation studies, single bacterial cell samples must be stably and reproducibly prepared, and the probabilistic distribution of the cell numbers should be known. Obtaining stable single-cell populations following a mathematically described distribution would enable us to simulate the variability of bacterial cell numbers.

The Poisson distribution has been considered to provide a good fit for bacterial cell number when obtained under carefully controlled experimental conditions (El-Shaarawi et al., 1981). It is assumed that a bacterial cell number following Poisson distribution will be experimentally obtained via a dilution series. To evaluate the individual lag phase, a single-cell-level study of *Listeria monocytogenes* was performed previously; the study dealt with a few bacterial cell populations that followed a Poisson distribution (McKellar and Knight, 2000; Robinson et al., 2001). However, the bacterial cell numbers in the prepared inocula were not directly counted in these experiments, and the accuracy with which bacterial cell numbers followed the Poisson distribution remained unclear. Other studies conducted with low-level bacterial populations have not experimentally demonstrated whether bacterial cell counts follow a Poisson distribution. If a distribution of bacterial cell numbers is mathematically described, variability of bacterial cell numbers would be available.

A Poisson distribution applies to various phenomena with discrete properties whenever the probability of the phenomenon is constant in time or space. Experimental data have been estimated to fit a Poisson distribution in other fields of study, such as the distribution of seismic hazards (Wang and Chang, 2015). Thus, a Poisson distribution would fit to a random distribution in nature.

The objective of the present study was to develop a protocol to obtain single cells following Poisson distribution for use in various single-cell studies. We investigated whether single bacterial cells obtained via 10-fold dilution followed a theoretical Poisson distribution. An inoculum of each pathogenic microorganism was prepared in 10-fold dilution series to obtain bacterial cell counts with mean values of one or two. Furthermore, to examine the validity of the parameters of the Poisson distributions obtained from bacterial cell counts, we compared these parameters with those of a Poisson distribution, having used random number generation in a computer simulation. The fact that bacterial number distributions follow a Poisson distribution at low number would be applied to some single-cell studies. In particular, the procedure presented in this study would enable us to develop an inactivation model that simulates the variability of survival bacterial numbers in a stochastic inactivation process.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains were kindly provided by the Research Institute for Microbial Diseases (RIMD) of Osaka University, the Hokkaido Institute of Public Health (HIPH), and the Aomori Prefectural Research Laboratory of Public Health. Three strains of *Escherichia coli* O111 (RIMD 05092013, RIMD 05092017, and RIMD 05092026), two strains of *E. coli* O26:H11 (RIMD 05091996 and RIMD 05091997), four strains of *E. coli* O157:H7 (RIMD 0509939, RIMD 05091896, RIMD 05091897, and HIPH 12361), a strain of *Salmonella* Stanley (RIMD, 1981001), two strains of *S.* Typhimurium (RIMD, 1985007 and RIMD, 1985009), a strain of *S.* Chester (from Aomori), a strain of *S.* Oranienburg (from Aomori), and six strains of *Listeria monocytogenes* (ATCC, 19111, ATCC, 19117, ATCC19118, ATCC 13932, ATCC 15313, and ATCC 35152) were used. All strains were maintained at −80 °C in brain heart infusion broth (Merck, Darmstadt, Germany) containing 10% glycerol. A sterile metal loop was used to transfer the frozen bacterial cultures by scratching the surface of the frozen culture into 5 ml of tryptic soy broth (Merck, Darmstadt, Germany) in a sterile plastic tube. The cultures were incubated without agitation at 35 °C for 24 h and transferred using a loop inoculum at two successive 24-h intervals to obtain a more homogeneous and stable cell population. Grown cells were collected via centrifugation (1000 × *g*, 10 min at 25 °C), and the resulting pellet was washed with 0.1% peptone water twice and subsequently resuspended in 5 ml of 0.1% peptone water. To generate a single sample of each pathogen comprising every strain, equal volumes of the cell suspensions from multiple strains of each pathogen were combined to achieve an approximately equal population of each strain.

2.2. Preparation of single cells

Single cells of each pathogenic bacterium were prepared via 10-fold dilutions of bacterial cultures. The initial cell count was assumed to be approximately 10^9 CFU/ml. The inoculum was further diluted (10-fold dilution series) to obtain a 10^3 CFU/ml solution. An aliquot of 10^4 CFU/ml solution was stored at 5 °C for 24 h. The bacterial cell number was determined via direct plating of 100 μl of inoculum of a 10^3 CFU/ml onto five tryptic soy agar (Merck, Darmstadt, Germany) plates. The colonies on the plates were counted after 24 h of incubation at 35 °C. $\bar{x}$ represents the mean value of five plate counts. An aliquot of the 10^4 CFU/ml solution stored from the previous day was diluted $\bar{x}/5$ fold and $\bar{x}/10$ fold to obtain 500 CFU/ml and 1000 CFU/ml, respectively. This procedure resulted in 1 CFU/2 μl and 2 CFU/2 μl, respectively. The cell density in each inoculum was determined by plating 2 μl of the inoculum onto TSA plates 96 times. The colonies were counted after a 24-h incubation at 35 °C.

2.3. Statistical analysis

2.3.1. Characteristics of bacterial counts

To determine whether bacterial cell counts (n = 96) follow a Poisson distribution after adjusting to 1 CFU/2 μl or 2 CFU/2 μl, a likelihood-ratio test of goodness of fit between the experimentally obtained bacterial cell counts (n = 96) and a Poisson distribution estimated by maximum likelihood estimation (MLE) was

conducted (Haas et al., 1999). If the *p*-value was smaller than 0.05, the hypothesis that bacterial cell counts followed a Poisson distribution was rejected. The λ parameters of the bacterial cell counts (n = 96) were estimated using the maximum-likelihood method. Furthermore, we confirmed that the experimentally obtained bacterial cell counts (n = 96) follow a binomial distribution and/or a negative binomial distribution by using a likelihood-ratio test of goodness of fit.

2.3.2. Parameter estimation from random number generation via computer simulation

To examine validity of the experimentally observed λ parameters of the Poisson distribution, we compared the experimentally obtained parameters with those obtained via random number generation in a computer simulation. A set of 96-number random generation for Poisson distribution (λ = 1 or λ = 2) was obtained as a random sampling set. The λ parameter was estimated for each random sampling set, and this procedure was repeated 1000 times. A Kolmogorov-Smirnov test was conducted to determine whether the distribution of the parameters obtained from the random sampling set (n = 1000) followed a Gaussian distribution. If the *p*-value was smaller than 0.05, the hypothesis that the random variable of the estimated parameter from the random sampling set followed a Gaussian distribution was rejected. If bacterial cells were appropriately prepared, parameter of Poisson distribution would be within the range of 95% confident interval of Gaussian distribution.

All statistical analyses were conducted with R statistical software (Version 3.1.2 for Mac OS X; http://ww.r-project.org). The goodness-of-fit test for a Poisson distribution was conducted using library (grid) and library (vcd).

3. Results

3.1. Experimentally obtained bacterial cell numbers

The experimentally obtained bacterial numbers (n = 96) targeting the mean values of one and two is shown as a histogram for each serotype (Fig. 1 and Fig. 2). The experimentally obtained cell counts followed a Poisson distribution regardless of the serotypes and the target cell number (λ = 1 and 2) (Figs. 1 and 2), representing by which all the 16 *p*-values were lager than 0.08. In addition, the relationship between the mean values and the variances of obtained bacterial cells is shown in Fig. 3. The mean values and variances of bacterial counts of each serotype are similar, which is a characteristic of Poisson distribution. These results indicated that the experimentally obtained bacterial cell numbers followed a Poisson distribution. All the 16 p-values of negative binomial distribution and binomial distribution were lager than 0.09 and 0.07, respectively. These two distributions would also be able to appropriately describe the obtained bacterial cell counts.

3.2. Validity of the parameters of the poisson distribution obtained from bacterial cell counts

We examined the validity of the Poisson distribution parameters obtained from the experiments mentioned above by comparing them with random numbers generated in a computer simulation. We assumed that an experiment developing a predictive model describing the uncertainty of bacterial survivors would be conducted using 96-well microplates in the future. As shown in Fig. 4, the distribution of the λ in the random sampling sets was described as normal, which was confirmed by the Kolmogorov-Smirnov test (Fig. 4, solid line). The experimentally obtained λ parameters of the Poisson distributions were within the 95% confidence interval of the normal distribution. After comparing these results with experimentally obtained parameters and parameters from the random sampling set, the validity of the experimentally obtained parameters was confirmed.

4. Discussion

In the present study, an inoculum preparation procedure to

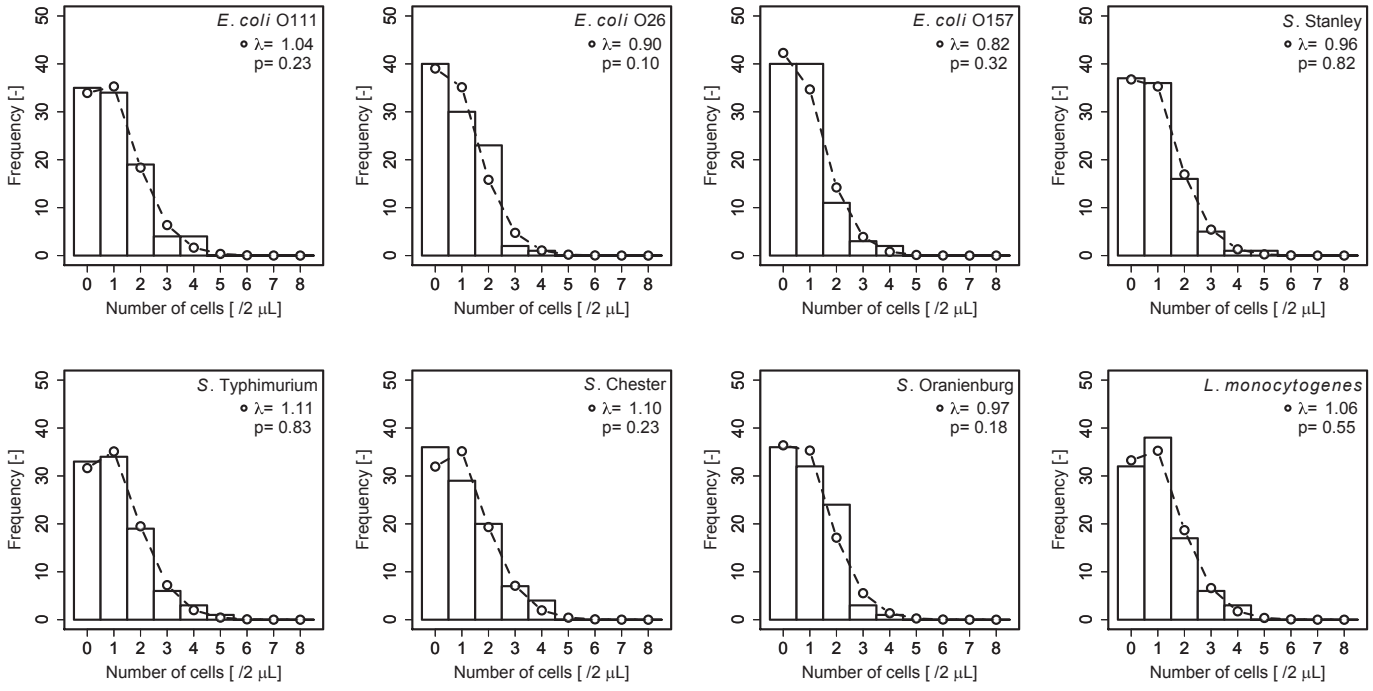

Fig. 1. Distribution of bacterial cell counts obtained from the experiment (histogram) and estimated using maximum likelihood (○). The target cell number, λ parameter, was 1 CFU/2 μl. A *p*-value was estimated using a likelihood-ratio test. If the *p*-value was smaller than 0.05, the hypothesis that the bacterial cell counts followed a Poisson distribution was rejected. Parameters of each strain were estimated using the maximum likelihood.

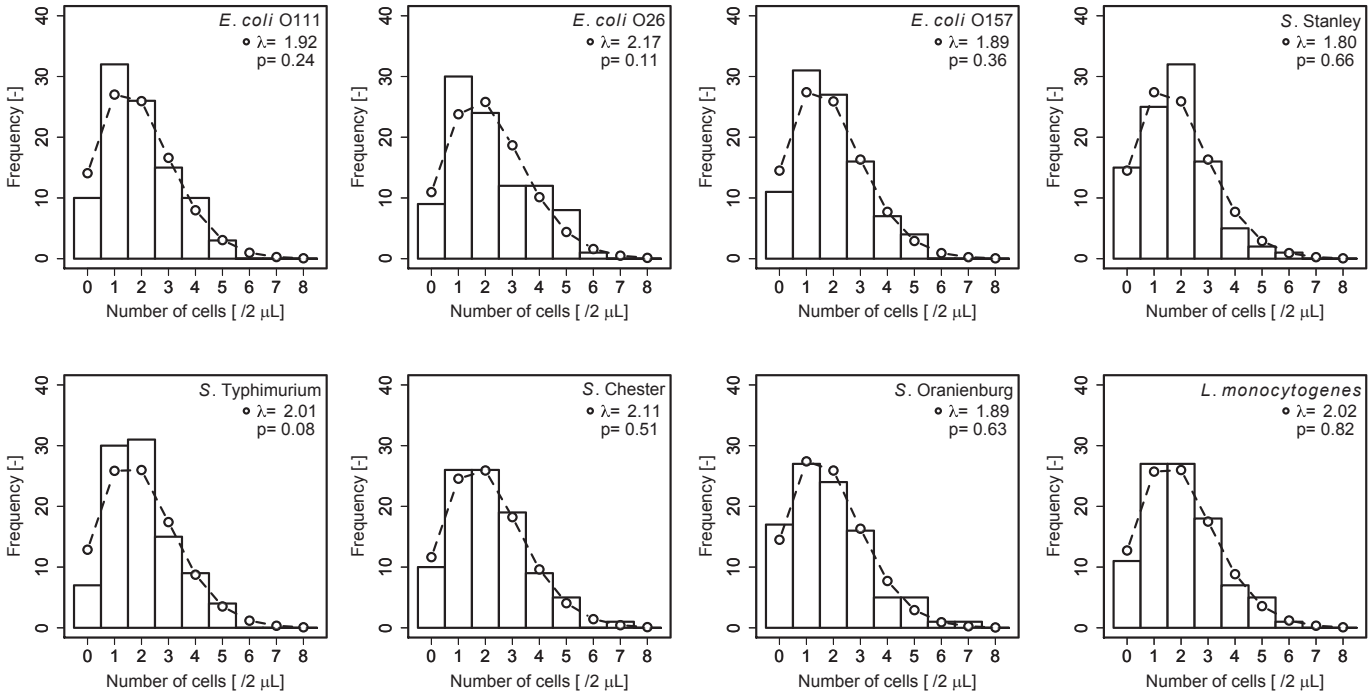

Fig. 2. Distribution of bacterial cell counts obtained from the experiment (histogram) and estimated using the maximum likelihood (○). The target cell number, λ parameter, was 2 CFU/2 μl. The *p*-value was estimated using the likelihood-ratio test. If the *p*-value was smaller than 0.05, the hypothesis that the bacterial cell counts followed a Poisson distribution was rejected. The λ parameters of each strain were estimated using maximum likelihood.

isolate single bacterial cells following a Poisson distribution via 10-fold dilution was proposed. As a matter of fact, some of dilution results in ca. 10-fold or 20-fold dilution during preparing for bacterial sample. We demonstrated that the experimentally obtained bacterial cell counts (λ = 1 or 2) in a 10-fold dilution series can be assumed to followed a Poisson distribution (Figs. 1–3). The validity of the experimentally obtained Poisson distributions has been verified through a comparison of the observed means with those obtained from randomly generated Poisson distributions (λ = 1 or 2) (Fig. 4). These results indicated that experimentally obtained bacterial cell numbers accurately followed the Poisson distribution.

The result of the present study illustrated that bacterial cell counts followed not only Poisson distribution but also negative binomial and binomial distributions. As sampling numbers of negative binomial distribution sufficiently increase (i.e., n = 96), the relationship between the mean and variance value of negative binomial distribution will be similar to a Poisson distribution (Feller, 1970) (Fig. 3). In addition, since the mean values of the targeting bacterial cell numbers were very small such as one or two, a binomial distribution would be very similar to a Poisson distribution (Feller, 1970) (Fig. 3). Considering similarity of the negative binomial distribution and binomial distribution to Poisson distribution, bacterial cell counts, in particular such as single cell level, would be reasonably described as Poisson distribution.

In a previous study, it has been reported that bacterial cell number was successfully described as a negative binomial distribution rather than Poisson distribution in water environment, because bacterial cell number was over-dispersed (Haas and Heller, 1988). In contrast, the procedure in the present study was conducted with carefully experiment to obtain bacterial cell numbers following natural distribution. Considering not only likelihood-ratio test (Figs. 1 and 2) but relationship between the mean and variance of bacterial cell numbers (Fig. 3), we judged bacterial cell number obtained in our experiment followed Poisson distribution.

Previous studies on single-cell growth developed a protocol for

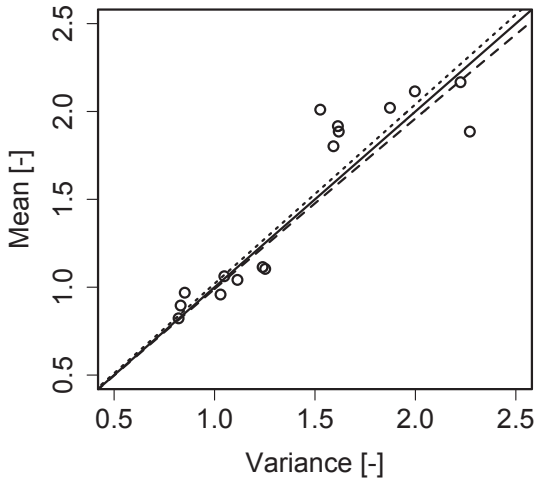

Fig. 3. The relationship between the mean values and variance values of observed bacterial cell counts of each bacterial serotype. The solid, dashed and dotted lines show the relationship between the mean values and variance values of Poisson distribution, negative binomial distribution (n = 96) and binomial distribution (size = mean × 50, probability = 0.02), respectively.

isolating a single cell via 2-fold dilution (Francois et al., 2003), and the variability of the bacterial lag phase among single cells was investigated using this protocol (Francois et al., 2006, 2005). McKellar and Knight (2000) and Robinson et al. (2001) performed single-cell studies by isolating a few cell samples. In those studies, they assumed that the number of bacteria followed a Poisson distribution. However, the exact number of bacteria was not accurately determined. In contrast, the results of the present study clearly demonstrated that bacterial cell number follow a Poisson distribution.

There has been only one study on bacterial inactivation at

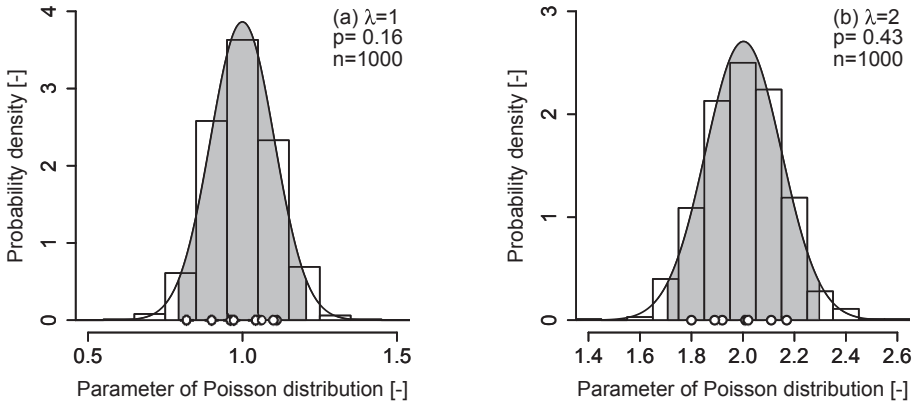

Fig. 4. Distribution of parameters obtained from random number generation (histogram) and the parameter from the experiment (○). The parameters obtained from random number generation followed a normal distribution (solid line). The grey area represents a 95% confidence interval of normal distribution. (a) Distribution with the λ parameter being equal to 1. (b) Distribution with the λ parameter being equal to 2.

single-cell level (Aspridou and Koutsoumanis, 2015) due to technical difficulties for monitoring bacterial cell death. Bacterial inactivation studies at single-cell level would be possible to express probability distribution of time to inactivation of each cell. In addition, changes in survival bacterial numbers along with its probability distribution could be estimated by initially Poisson distributed bacterial numbers. Thus, the conventional concept of time to death and/or *D*-value might be described as a probability distribution.

Although variability of initial bacterial cell numbers would be described as a Poisson distribution, it would be unclear whether variability of survival bacterial cell numbers during inactivation process would be followed a Poisson distribution. Even though initial bacterial cell numbers follow a Poisson distribution, survival bacterial numbers may not follow a Poisson distribution during inactivation process. The distribution of bacterial cell numbers during inactivation process should be clarified through experiments and be estimated via computer simulation in a future study.

To achieve a high probability of obtaining single cells for studying bacterial inactivation processes, it would be more efficient to employ the Poisson distribution with the λ parameter being equal to 2. When the Poisson distribution parameter λ was one, the rate of zero counts was approximately 37%. In contrast, when the parameter λ was two, the zero-count rate was reduced by approximately 14% without significantly changing the one-cell-count rate. Therefore, a Poisson distribution ($\lambda = 2$) for bacterial cell counts would be useful for single-cell studies. Stochastic inactivation approaches would be conducted with specific initial distributions to estimate the variability of number of survivors. This approach would enable us to assess bacterial survivors quantitatively. In the future, single-cell approaches to bacterial inactivation processes such as heat, desiccation, and pH will be applied to estimate stochastically changing distributions of surviving bacteria.

Acknowledgements

This study was supported by Grants-in-Aid from the Food Safety Commission, Japan (No. 1406). The authors sincerely thank the Research Institute for Microbial Diseases (RIMD) of Osaka University, the Hokkaido Institute of Public Health (HIPH), and Aomori Prefectural Research Laboratory of Public Health for kindly providing the bacterial cultures.

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