Estimation of Microbial Concentration in Food Products from Qualitative, Microbiological Test Data with the MPN Technique

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Microbial concentration in samples of a food product lot has been generally assumed to follow the log-normal distribution in food sampling, but this distribution cannot accommodate the concentration of zero. In the present study, first, a probabilistic study with the most probable number (MPN) technique was done for a target microbe present at a low (or zero) concentration in food products. Namely, based on the number of target pathogen-positive samples in the total samples of a product found by a qualitative, microbiological examination, the concentration of the pathogen in the product was estimated by means of the MPN technique. The effects of the sample size and the total sample number of a product were then examined. Second, operating characteristic (OC) curves for the concentration of a target microbe in a product lot were generated on the assumption that the concentration of a target microbe could be expressed with the Poisson distribution. OC curves for Salmonella and Cronobacter sakazakii in powdered formulae for infants and young children were successfully generated. The present study suggested that the MPN technique and the Poisson distribution would be useful for qualitative microbiological test data analysis for a target microbe whose concentration in a lot is expected to be low.

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Introduction

Food safety is one of the major public health concerns worldwide. To ensure the safety of our daily food products, they are routinely examined in laboratories of food companies and regulatory agencies in many countries. Indeed, to prevent the occurrence of food poisoning by pathogenic bacteria such as Salmonella, pathogenic Escherichia coli, and Listeria monocytogenes, microbiological tests for food products are indispensable.

There are two types of microbiological tests for food, namely qualitative and quantitative. Qualitative tests are done just to detect target pathogens and the results are expressed as presence/absence, or positive/negative for the pathogens. In reality, most food products are now negative in qualitative microbiological tests. That is, the prevalence and the contamination level of pathogens in most of processed products are actually low. For example, Sagoo et al.1 reported that only 5 (0.13%) and 88 (2.3%) out of 3,852 bagged, prepared, ready-to-eat salad vegetable samples were positive for Salmonella and L. monocytogenes, respectively, in the UK. Shimojima et al.2 found that L. monocytogenes was isolated from 52 (1.7%) out of 2,980 ready-to-eat food samples in Tokyo. Gonzales-Barron et al.3 also reported that zero counts of total coliforms and Escherichia coli on beef carcasses sampled from Irish abattoirs were often observed in quantitative microbiological tests.

Nevertheless, microbiological testing of food products is still important for confirmation of the safety of food products worldwide. Moreover, microbiological tests of food samples with an appropriate sampling plan can provide reliable results. However, design of the sampling plan is critical. The International Commission on Microbiological Specifications for Foods (ICMSF)4 has provided urgently needed guidance on the use of sampling plans and microbiological criteria for foods in international trade.

The Codex Alimentarius Commission (CAC)5 suggested two types of sampling plan for microbiological tests, i.e., two-class and three-class attributes plans. Two-class attributes plans should be principally applied for microbes that present a severe hazard or a moderate

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direct health hazard with potentially extensive spread in food, such as pathogenic *E. coli*, *Salmonella*, and *L. monocytogenes*. Three-class attributes plans are for microbes with no or low direct health hazard, such as aerobic microorganisms, yeasts, and coliforms.

In two- and three-class attributes plans, the CAC suggested that when a total of *n* samples of a given lot is tested, there should be an acceptance number, *c*, which is the maximum number of nonconforming units allowed in the sample if the lot is to be accepted. Here 0 ≤ *c* < *n*. For example, when *c* = 2 in a qualitative microbiological test, lots with zero to two positive samples are accepted, but the lot with three (or more) positives is not accepted. The operating characteristic (OC) curve is very important in making a sampling plan*. The OC curve shows the probability of acceptance for a given lot in relation to the rate of non-conforming items or the concentration of a target in the lot*.

The concentration of a target microbe in a food product is often estimated with the most probable number (MPN) technique when the concentration of the target is low**. The MPN technique employs a combination of the Poisson and binomial distributions and is a maximum likelihood method*. With this technique, serial dilutions of a food sample are cultured in test tubes containing microbiological selective media and the concentration of a target microbe is estimated from the number of positive test tubes among the total test tubes at each dilution**. The MPN technique has been applied in complex models of microbial contamination in food. In the present study, it was considered that samples of an actual food lot would contain a low or zero concentration of target pathogen, as described above, so the pathogen concentration was estimated on the basis of presence/absence data with the MPN technique. The characteristics of the present MPN technique were then evaluated.

When the concentration of a target microbe in a product is low, it can be considered to follow the Poisson distribution, which is a discrete distribution and thus gives probabilities for discrete cell concentrations such as 1, 2, and 6 cells/g. It can deal with the concentration of 0 cells/g for a target microbe in a sample. For example, when the mean of the Poisson distribution is 6 cell/g, the probability of 0 cell/g is 0.0025. A characteristic feature of this distribution is that the mean of the distribution is equal to the variance, meaning that the standard deviation is defined by the mean.

On the other hand, sampling plans for microbiological tests have generally been analyzed on the assumption that the distribution of a target microbe in a food sample is log-normal**. But, a log-normal distribution does not allow for complete absence of microbes in a sample**. That is, this distribution cannot give a probability at the concentration of 0 (cell/g), since the concentration of 0 in log (cell/g) is not 0, but 10^0=1 (cell/g). Negative numbers in log can only approach 0, such as 10^-3=0.0001, but never reach 0. In the present study, therefore, the OC curves for a two-class attributes sampling plan were generated on the assumption that the concentration of a target microbe in a product lot would follow the Poisson distribution.

** Materials and Methods

1. MPN technique

Let us assume that a target microbe is distributed in samples of a food product lot at a low concentration, *μ* (cells/g) following the Poisson distribution. The MPN technique can be used to estimate a low concentration of a microbe in a sample. In the MPN technique, serial dilutions of a test sample are cultured in 3 (or 5) test tubes that contain a medium selective for a target microbe. After incubation, the number of positive test tubes in which the target microbe has grown is counted. The Poisson distribution function with the mean *μ* is described by Equation (1).

\[
f(r) = \frac{(\mu \times a)^r \exp(-\mu \times a)}{r!}
\]

where *r* is the number of test tubes in which the target microbe exists and *a* is the aliquot or sample size of the sample (g or mL). *μ* is the concentration of the target microbe in a sample (cell/g or cell/mL) and exp is an exponential function. The probability that a test tube contains no cells, i.e., a negative test tube is obtained by substituting zero for *r*; namely it is exp(-μ×a). Thus, the probability that a test tube is positive (containing at least one viable cell) is 1−exp(-μ×a). The probability, *p*, of having a positive test tube in the *i*-th dilution is then expressed as follows.

\[
p_i = 1 - \exp(-\mu \times a)
\]

The number *i* is three (or five) in most routine tests. For example, 3 dilutions containing 1, 0.1 or 0.01 g are inoculated in test tubes with selective medium. The number of positive test tubes, *x*, among the total test tubes, *n*, in the *i*-th dilution would follow a binomial distribution. Therefore the probability, *p*, of having *x* in the *i*-th dilution of the series is expressed as follows.

\[
p_{x,i} = \prod_{j=1}^{m} \left[ \frac{n_i}{x_i} \right] \left[ 1 - \exp(-\mu \times a) \right]^{x_j} \left[ \exp(-\mu \times a) \right]^{n-x}
\]

where \( \prod \) means the combination, and *m* is the number of dilution series, which takes the value of 3 in most routine cases.

It is considered that the MPN technique can be applied to the results of a qualitative test for samples of a food product. Namely, when 20 samples of a product lot are tested, this is considered as a case with *m*=1 and *m*=20 in MPN, because the food samples correspond to a single series of non-diluted samples in the MPN dilution series. The probability *p* that *x* positive samples are ob-

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*FDA, 2010. Bacteriological analytical manual (BAM) Appendix 2: Most Probable Number from Serial Dilutions. Available at http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm*
served in the total of \( n \) samples of a given product, thus, can be simply expressed with Equation (4), which is derived from Equation (3).

\[
p = \binom{n}{x} [1 - \exp(-\mu \times a)]^x [\exp(-\mu \times a)]^{n-x} \tag{4}
\]

Therefore, when the number of positive samples \( x \) is obtained in the total of \( n \) samples with a qualitative test, the probability \( p \) at a given value for \( \mu \) of the sample is calculated using Equation (4). Among many values for \( \mu \), the value which has the highest probability (or likelihood) for a particular combination of \( x \) and \( n \) is the estimate of the cell concentration of a target microbe, following the maximum likelihood method in the MPN technique.\(^{30,31}\)

The probabilities for the values of \( \mu \) of 0, 1, 2, ⋯ cells/100 g with an increment of 1 cell/100 g were calculated for the estimation for \( \mu \). All calculations were done with spreadsheet software, Microsoft Excel, in the present study.\(^{30}\)

2. OC curve for two-class attribute sampling plan

Let us assume that a target microbe is distributed in samples of a food product lot at a low concentration, \( \mu \) (cell/g), following the Poisson distribution. The probability of acceptance \( p \) that \( x \) positive samples are observed in the total of \( n \) samples of a given product is then expressed by Equation (4). The values of \( p \) are calculated at various cell concentrations of the target microbe in a sample, to generate an OC curve. The probability of acceptance with the acceptance number \( c \) in a two-class attribute sampling plan \( p_c \) is the sum of the probabilities at the value for \( x^c \). Here \( x=0, 1, 2, ⋯ c \). Namely, the probability of acceptance \( p_c \) is expressed by the following Equation (5).

\[
p_c = \sum_{x=0}^{\infty} \binom{n}{x} [1 - \exp(-\mu \times a)]^x [\exp(-\mu \times a)]^{n-x} \tag{5}
\]

Calculations for generating OC curves were done with Microsoft Excel in the present study.

Results and Discussion

1. Probability distribution curves for positive sample numbers at various values of cell concentration

Let us consider that \( x \) (≥0) samples in the total of \( n \) samples of a food product are positive in a qualitative microbiological test for a target microbe. The probability distribution curves for \( x \) with various values for the concentration of a target microbe \( \mu \) are generated with the present MPN technique. Examples for the case of \( n=10 \) with aliquots of 10 and 25 g per sample are shown in Fig. 1A, B. Each curve for \( \mu \) in Fig. 1A, B has a peak. The curve that has the highest probability at a given value for \( x \) can give the best estimate for \( \mu \), following the maximum likelihood method. For example, when 4 samples are positive in the total samples with aliquot sizes of 10 and 25 g/sample, the estimates for \( \mu \) are 5 and 2 cells/100 g, respectively, as shown in Fig. 1A, B.

2. Probabilities for cell concentration at various positive sample numbers.

The probabilities for \( \mu \) at various values of \( x \) can also be obtained with the present MPN technique. Examples of probability curves for \( x \) with aliquots of 10 and 25 g per sample with \( n=10 \) are shown in Fig. 2A, B. Each curve for \( x \) in Fig. 2A, B has a peak. The curve of \( x=4 \) has a peak at \( \mu=5 \) cells/100 g in a 10-g aliquot (Fig. 2A) and at \( \mu=2 \) cells/100 g in a 25-g aliquot (Fig. 2B). These values for \( \mu \) are the same as estimated in Fig. 1A, B. These results, thus, show that when 4 samples in a total of 10 samples of a given lot are positive in the microbiological test with a 10-g aliquot, the microbial concentration of the lot is estimated to be 5 cells/100 g with the present MPN technique.

3. Estimation of cell concentration of a product with a qualitative test

From the results shown in Figs. 1 and 2, the cell concentration of a target microbe at various combinations of \( x \) (ranging from 1 to 20) and \( n \) (5, 10, and 20) with aliquots of 10 and 25 g can be estimated with the MPN technique (Table 1). The cell concentration estimated at a given \( x \) in \( n \) for each sample aliquot is obtained from this MPN table. For example, when the microbiological
test result is \( x = 3 \) in \( n = 10 \) with a 10-g aliquot, the estimated concentration is 4 cells/100 g (Table 1).

When none of the samples tested is positive, or \( x = 0 \) with any value of \( n \), the cell concentration is estimated to be 0 cell/100 g (Table 1). When all samples are positive, or \( n = x \), the concentration cannot be stochastically limited to a single value (Table 1); this is because the possibilities for multiple cell concentrations are high and very similar to each other, even for concentrations of \( >100 \) cells/100 g.

### 4. Effect of the aliquot size of sample on the probability of the number of positive samples

The probability for \( x \) changes with the aliquot size of a sample, as shown in Fig. 1 A and B. As the aliquot size becomes larger, the peak of the probability curve for \( x \) changes to a higher value (Fig. 3A, B). Here \( n = 20 \). For example, when the cell concentration is low such as 1 cell/100 g, the number of positive samples with a 5-g aliquot is mostly 0 or 1 in the total of 20 samples (Fig. 3A). When the cell concentration is 5 cells/100 g, the number of positive samples with a 50-g aliquot is mostly

### Table 1. Cell concentration estimated from the number of positive samples with the MPN technique

<table>
<thead>
<tr>
<th>Aliquot (g)</th>
<th>10</th>
<th>20</th>
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<td>16</td>
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<td>8</td>
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<td>9</td>
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<td>10</td>
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The unit of cell concentration is cells/100 g. 
\( n \) is the total number of samples. 
\( x \) is the number of positive samples. 
ND: Not determined.
5. Effect of the total number of samples on the probability of the number of positive samples

As the total number of samples, \( n \), increases, the peak of the probability curve for \( n \) changes to a higher value of \( x \) and becomes lower, when the values for the aliquot size and \( \mu \) are fixed (Fig. 4). The positions of the peaks of the curves are 1, 2, 5, and 8 positive samples with the total numbers of 5, 10, 20, and 30 (Fig. 4).

6. OC curves based on the Poisson distribution

OC curves for the concentration of a target microbe in a given product lot in the two-class attribute plan are generated on the assumption that the concentration of the target microbe in the lot follows the Poisson distribution in the present study. Several examples of OC curves at various values for the positive sample number, \( c \), with aliquot sizes of 10 and 25 g are shown in Fig. 5 A, B. With a smaller value for \( c \), lower probabilities of acceptance are observed, which corresponds to a more stringent sampling plan (Fig. 5A, B). Also, with a larger sample size, lower probabilities of acceptance are also observed (Fig. 5A, B). For example, the probability of acceptance at \( c=3 \) and \( \mu=4 \) cells/100 g with a 10-g aliquot (0.569) is higher than that with a 25-g aliquot (0.0345) (Fig. 5A, B). The OC curves at \( c=0 \) for various numbers of \( n \) are also generated on the assumption of the Poisson distribution (Fig. 6); as the number of \( n \) increases, OC curves with steeper declines are generated.

7. OC curves for Salmonella and Cronobacter sakazakii in powdered formulae for infants and young children

The CAC\textsuperscript{*4} has provided the guidelines for Salmonella and C. sakazakii in powdered formulae for infants and young children in a two-class attribute sampling plan. Namely, \( n=30 \) and \( c=0 \) with an aliquot size of 10 g for C. sakazakii and \( n=60 \) and \( c=0 \) with an aliquot size of 25 g.

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for *Salmonella*. With those values, OC curves for the two pathogens in the two-class plan can be generated on the assumption that the concentrations of the pathogens in a product lot follow a Poisson distribution (Fig. 7). Since the microbiological criteria of the guideline for *Salmonella* are more stringent than for *C. sakazakii*, the probabilities of acceptance estimated for *Salmonella* are lower than those for *C. sakazakii* (Fig. 7). Here the unit of the cell concentration for the OC curves is cells/1,000 g for easier visualization of lower cell concentrations.

The sum of the probabilities for the number of positives at a given μ is just one in Figs. 1, 3, and 4. Thus, the curves in these figures can be called probability mass function curves. On the other hand, the sum of the probabilities for μ at a given x in Fig. 2 is not equal to one, so that this probability curve is not a probability mass function curve. Namely, the sum depends on the value of x. For example, the sum of the probabilities for μ at x=0, 2, and 7 are 1.58, 1.25, and 3.33, respectively, with n=10 and a 10-g aliquot.

Our finding indicate that the Poisson distribution can be used to make OC curves for a two-class attribute sampling plan where the concentration of a target microbe in a lot is low. In the cases of *C. sakazakii* and *Salmonella* in powdered formulae, the CAC\(^*_5\)* assumed that the mean concentration of *C. sakazakii* is one colony forming unit (CFU) in 340 g (if the standard deviation is 0.8 and the probability of detection is 95%) or one CFU in 100 g (if the assumed standard deviation is 0.5 and the probability of detection is 99%) and one CFU in 526 g for *Salmonella* (if the standard deviation is 0.8 and the probability of detection is 95%) with the log-normal distribution.

One key advantage of the Poisson distribution in estimating the cell concentration and making OC curves in the present study is that there is no need to confirm the value of the standard deviation of the distribution, as described in the Materials and methods section. Namely, the Poisson distribution gives the probability at a positive sample number with the mean, as shown in Equation (1). Another advantage is that it can give the probability at the concentration of 0 cell/g of a target microbe in a lot, as described in the Introduction section.

Several probability distributions have been proposed for microbial concentration in food or water, including the log-normal distribution, the negative binomial distribution, and mixed distributions such as the Poisson-gamma and Poisson-lognormal distributions. It is very hard to say which distribution is superior to the others especially at low concentrations of a target microbe. Among them, the negative binomial distribution has been officially adapted in the sampling plans for aflatoxin analysis in peanuts and corn and is considered to be a distribution suitable for microbes in frozen food. The Poisson distribution is a special case of the negative binomial distribution. Consideration of stochastic features of those distributions and comparison of analytical results with actual microbial data might give us hints as to which distribution is most appropriate.

### References


\(^*_5\)FAO. Sampling plans for aflatoxin analysis in peanuts and corn. (http://www.fao.org/3/a-t0838e.pdf)


