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Computation of Most Probable Numbers

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A rapid computational method for maximum likelihood estimation of most-probable-number values, incorporating a modified Newton-Raphson method, is presented. The method offers a much greater reliability for the most-probable-number estimate of total viable bacteria, i.e., those capable of growth in laboratory media.

The most-probable-number (MPN) method is an important technique for microbiologists in the enumeration of viable bacteria in samples of food, water, and natural products. The method has undergone some evolution (3), but the principle itself is essentially unchanged since it was first developed. Some samples do not lend themselves to viable bacterial enumeration by any other procedures, so the MPN technique is convenient and necessary in some instances, and its use will continue.

Many uses for MPN have been described in the literature in the fields of food microbiology, water quality, and public health (3, 9). MPN methods have been treated statistically by a number of investigators (deMan [5, 6], Finney [7], Halvorson and Ziegler [8], Moran [12], and Taylor [15]), but the general computation has not changed significantly.

We report here a rapid computational method for maximum likelihood estimation of MPN values utilized in existing tables (1), incorporating a modified Newton-Raphson method, as discussed by Kalbfleisch and Prentice (10). Use of such an algorithm eliminates the need for tables, but also, more importantly, allows an investigator to select the number of dilutions and replicates per dilution according to individual need. The method presented produces d (the MPN), its standard error, and a 95% confidence interval. In contrast to the method discussed by deMan (5), it does not eliminate improbable values; deMan's method can yield confidence intervals that are not truly 95% intervals. Obtaining unlikely extremes is more often the product of bacteria not being randomly dispersed throughout the medium. Such values invalidate the use of MPN tables, but can be detected by using a test proposed by Moran (12) or, if the number of replicates per dilution is large, by using the method described here.

Finney (7) discusses a method for fitting MPN, based on weighted least squares. With large numbers of replicates per dilution, results should be equivalent to those based on maximum likelihood. However, as pointed out by Finney (7), this approach presents problems if the bacterial concentrations are low and the proportion of positives is small. Maximum likelihood estimates offer some advantages in this regard, but both approaches allow computation of confidence intervals.

Winter (16) suggests an alternative method for computing MPN values; this method, however, corresponds to neither of the above. The MPN resulting from the computations presented here corresponds to that presented by Koch (11) and Cochran (2). However, Koch (11) presents an algorithm which is dependent upon an HP41C calculator and does not compute confidence intervals. Cochran's method for formulating confidence limits involves the use of a table, whereas the method presented here does not.

Most of the methods used in the computation of interval estimates for MPN values are based on a large number of replicates. While the term "large" is vague, what it means is that the larger the number of replicates per dilution, the more robust the 95% confidence interval. Since it is not economically feasible, or even practical, in many cases to run hundreds of replicates per MPN, a simple simulation study is provided as an example of the ability to study small-sample properties where the methods are employed. This simulation study is preferable because with a simulation one is assured that the assumptions of the model are correct. Methods which cannot perform adequately when assumptions hold cannot be expected to perform at all when assumptions fail. In addition, since the true concentration is known, i.e., fixed in advance, problems of validation are minimized.

MATERIALS AND METHODS

Statistical model. Assuming there are k dilution levels ($k \geq 1$) with: v_i = volume in ith dilution ($i = 1$,

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. . ., k); n_i = number of replicates in ith dilution; and s_i = number of sterile tubes in ith dilution.

The assumption of bacteria randomly dispersed leads to the probability of observing s_i sterile tubes being:

$$P(s_i) = \binom{n_i}{s_i} \left(e^{-v_i d}\right)^{s_i} \left(1 - e^{-v_i d}\right)^{n_i - s_i} \quad (1)$$

Since the outcomes at each dilution are statistically independent, solving for d involves finding the d that maximizes $P(s_1) \times P(s_2) \times \ldots \times P(s_k)$. This leads to finding the d that solves:

$$\sum_{i=1}^{k} v_i s_i = \sum_{i=1}^{k} (n_i - s_i)/(e^{v_i d} - 1) \quad (2)$$

Computational method. The computational method used is described by Kalbfleisch and Prentice (10) and utilizes maximum-likelihood methods. First, an initial estimate for d is made. The computer can do this by using data for those dilution levels where the percentage of sterile samples, i.e., no growth, lies between 0 and 100, i.e., excluding all negative or all positive dilutions. Then, one can use the average of $-(1/v_i)$ log (s_i/n_i) for these concentrations. Alternatively, a reasonable guess can be specified by the user. As long as this guess is within an order of magnitude, and strictly greater than zero, the method works.

Once an initial guess is specified, one computes:

$$d_{NEW} = d_{OLD} + U(d_{OLD})/I(d_{OLD})$$

where:

$$U(d_{OLD}) = \sum_{i=1}^{k} n_i v_i/(e^{v_i d_{OLD}} - 1) - \sum_{i=1}^{k} v_i s_i$$

and:

$$I(d_{OLD}) = \sum_{i=1}^{k} n_i v_i^2/(e^{v_i d_{OLD}} - 1)$$

where d_{OLD} is the initial guess during the first iteration.

The value of d_{NEW} is then called d_{OLD}, and the procedure is repeated until little or no change in the value of d occurs. If the initial guess is a good one, very few iterations are needed. One of the attractive features of the algorithm is that it provides the 95% confidence interval for d as described by Cochran (2) and Parnow (13), i.e., by noting that the standard error for d is approximately $1/\sqrt{I(d)}$ and the standard error for ln(d) (ln denotes the logarithm, base e, e = 2.71 . . .) is approximated by $1/[d\sqrt{I(d)}]$. When the number of replicates is large, a 95% confidence interval for d is $d \pm 1.96$ SE(d). However, as indicated by Cochran (2) and Finney (7), using ln(d) $\pm$ 1.96 SE[ln(d)] and taking anti-logs is a better approach.

Goodness-of-fit tests. As indicated earlier, the derivation of MPN assumes that bacteria are randomly dispersed throughout the sample. Obvious violations exist when replicates for more diluted media yield more positive values than the original, a result that may be caused by clumping, affinity of the bacteria for the surface or walls of the test tube, or heterogeneity in the medium itself.

Not rejecting a null hypothesis of randomness does not guarantee randomness, but rather is a rejection of situations in which randomness appears to be markedly violated. With very high bacterial concentrations, randomness becomes more difficult to achieve, because the MPN model assumes bacteria occupy an infinitesimal proportion of the space available, and achieving adequate mixing is difficult.

With 10-fold dilutions, violations may be obvious. With twofold dilutions they may be less so. Moran (12) suggests a test which rejects randomness if $T = \Sigma s_i(n_i - s_i)$ is large. This test is quite easy to perform with 2-fold dilutions, but less so in 5- and 10-fold series, because of computations associated with the standard error of T.

However, when $k \geq 2$ a simple χ^2 test can be derived by using the maximum likelihood estimate derived above. Let $E_i = n_i e^{-v_i d}$. Then:

$$\chi^2 = \sum_{i=1}^{k} (s_i - E_i)^2 \left[\frac{1}{E_i} + \frac{1}{n_i - E_i}\right] = \sum_{i=1}^{k} n_i (s_i - E_i)^2/E_i(n_i - E_i)$$

If χ^2 is greater than χ^2 table value with $k - 1$ degrees of freedom (e.g., k = 2, use value 3.841; k = 3, use value 5.991), randomness is rejected. However, as is true of most χ^2 goodness-of-fit tests, it is advisable that E_i and $n_i - E_i$ should be large (some suggest greater than 5). Although the number of replicates required is large, this test for randomness need not be done with every MPN performed, but only at the initial stages and perhaps repeated later on to assure validity of the method. This test is a straightforward application of methods described by Rao (14).

Simulation. A technique commonly used to test the validity of statistical methods based on large sample approximations, as in the case of standard errors and confidence intervals presented here, is that of a Monte Carlo simulation. One can, in effect, simulate hundreds of replicate samples from the same "population." In this study, 500 samples were generated (using the IMSL routine GGBIR) from each population defined, using values of n_i, v_i, and d commonly found in practice. Values of d were chosen according to the suggestion of Cochran (2), i.e., that one chooses volumes so that d falls between 1 and 2 (the volume of the original undiluted replicates being 1). Tests and estimates which do not meet such expectations, in fact, should not be employed in a laboratory.

For each sample generated, the MPN and its confidence interval were recorded, and the results were tallied. An illustration of the computational method is presented in Fig. 1. With 500 replicates, approximately 95% or 475 confidence intervals should contain d.

To demonstrate the usefulness, or lack thereof, of confidence intervals, the average length of the confidence interval was recorded for each population. Confidence intervals which are wide are not very informative, but indicate that the value obtained for

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        REAL N(10),S(10),V(10)
*PROGRAM TO COMPUTE MPN FOR NDIL DILUTIONS(LE 10),
* ARBITRARY NO PER DILUTION, N-FOLD DILUTIONS
* N IS THE ARRAY HOLDING NO OF REPS PER DILUTION
* S IS THE NUMBER OF STERILE TUBES PER DILUTION
* V IS THE VOLUME OF ORIGINAL LIQUID IN DILUTION
    5 READ (5,6,END=3200) NDIL,NFOLD,IOPT
    6 FORMAT (I2,I3,I1)
      IF (NDIL .GT. 10) STOP
      READ (5,20) CONV,DNEW, (N(I),I=1,NDIL),(S(I),I=1,NDIL)
   20 FORMAT (2F10.4,10F5.0/10F5.0)
      WRITE (6,25) CONV,NFOLD,IOPT
   25 FORMAT ( ' CONVERGENCE,CRITERIA=',F10.4,2X,I5,'-FOLD DILUTION'
     1  , ' OPTION CODE=',I1 )
      DO 40 I=1,NDIL
 40   V(I)=1./NFOLD**(I-1)
      CALL MLE(N,V,S,NDIL,DNEW,IOPT,CONV)
      GO TO 5
 3200 STOP
      END
      SUBROUTINE MLE(N,V,S,NDIL,DNEW,IOPT,CONV)
      REAL N(NDIL),V(NDIL),S(NDIL)
* ON FIRST CALL DNEW HAS USER INITIAL VALUE IF IOPT=0
* U = ESTIMATED SCORE FUNCTION
* FISHER = INFORMATION VALUE
*IOPT=0 USE LEAST SQUARE EST, IOPT=0 USE USER GIVEN VALUE
*CONV=HOW CLOSE DOLD AND DNEW NEED TO BE TO STOP ITERATION(E.G. CONV=.001)
*N(I)=NUMBER OF TUBES IN ITH DILUTION
*V(I)= VOLUME OF ORIGINAL SAMPLE IN ITH DILUTION
*E.G.    DILUTION  I          1             2             3
*                         ---------     ---------     -----------
*      N(I)                   5             5             5          5 PER DIL
*     V(I)                    1.0           .5            .25        2-FOLD
      IF (IOPT .EQ. 0) THEN
                          DOLD = DNEW
                        ELSE
                          DOLD = 0.
                          JJ = 0
                          DO 10 I=1,NDIL
                          IF((ABS(N(I)-S(I))   .LT. .1)   .OR.
    1                     (S(I) .LT. .1)) GO TO 10
                          DOLD = DOLD + ALOG(N(I)/S(I))/V(I)
                          JJ = JJ + 1
   10                     CONTINUE
                          IF(JJ .EQ. 0) THEN
                                          DOLD = AMAX0(DNEW,1.5)
                                        ELSE
                                          DOLD = DOLD/JJ
                                        ENDIF
                        ENDIF
      DO 100 I=1,100
* UP TO 100 ROUNDS OF ITERATION FOR THE LIKELIHOOD ESTIMATE
      U=0.
      FISHER=0.
* ALGORITHM IS MODIFIED NEWTON-RAPHSON AS DESCRIBED IN
* KALBFLEISH AND PRENTICE SURVIVAL ANALYSIS TEXT
      DO 50 J=1,NDIL
      EVD=EXP(V(J)*DOLD)-1.
      U=U-S(J)*V(J)+(N(J)-S(J))*V(J)/EVD
 50   FISHER=FISHER+N(J)*V(J)**2/EVD
      DNEW=DOLD+U/FISHER
      IF (ABS(DNEW-DOLD) .LT. CONV) GO TO 200
 100  DOLD = DNEW
*WHEN SIMULATION WAS DONE--NO FAILURES WERE NOTED
      WRITE (6,120) DNEW
  120 FORMAT (' FAILURE TO CONVERGE',F10.5)
      RETURN
C COMPUTE CONF. INTERVAL AROUND LN(D),I(D)=FISHER INFO
 200  SE=EXP(1.96/(SQRT(FISHER)*DNEW))
      CLLOW=DNEW/SE
      CLHIGH=DNEW*SE
       WRITE(6,210) DNEW,CLLOW,CLHIGH
 210   FORMAT(' MPN=',F10.5,' 95% CONF.LIMITS (',F10.5,',',F10.5,')')
      RETURN
      END
```

FIG. 1. Fortran program illustrating MPN algorithm.

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TABLE 1. Number of times the true value of MPN was included in the 95% confidence interval ($k = 3$, $n_1 = n_2 = n_3 = n$)[a]

d	2-fold dilution		10-fold dilution	
	$n = 5$	$n = 10$	$n = 5$	$n = 10$
1.00	482	469	467	476
1.25	466	472	489	463
1.50	469	478	473	464
1.75	478	467	482	471
2.00	478	479	473	474
3.00	480	476	486	477

[a] Each set of parameters was replicated 500 times.

the MPN is highly variable and that if one repeated the test with another sample from the same volume the results might not be duplicated. Using MPN values, with large standard errors, in subsequent analyses may obscure relationships with other factors when the MPN values are themselves very imprecise.

RESULTS AND DISCUSSION

A total of 9,000 MPN dilution series were generated and evaluated in less than 38 s on a Univac 1100/82. The results of the simulation are presented in Tables 1 and 2. In spite of the fact that confidence intervals for MPN values are based on large-sample theory, the intervals one obtains do contain the true MPN value about 95% of the time, i.e., about 475 of 500 cases. This result indicates the use of log MPN in subsequent analyses such as an analysis of variance. If the MPN values vary considerably, a weighted analysis of variance (weights inversely proportional to their variance) may be better employed. However, it should be noted that the MPN method does slightly overestimate the bacterial concentrations present. Using 10-fold rather than 2-fold (i.e., serological) dilutions yields higher standard errors, as might be expected. Using a larger number of replicates per dilution obviously will reduce variability as well as bias.

The use of either 5 or 10 replicates per dilution yields an average confidence interval of about 1.2 to 2.3 on a natural log scale, or about 0.52 to 1.00 on a log base 10 scale, suggesting that one can estimate densities within an order of magnitude. However, less than five replicates per dilution is inadequate. In addition, an average confidence interval length of 1.00 on a $\log_{10}$ scale suggests that many of the intervals were in

TABLE 2. Sample statistics provided for the example given in the text

No. of replications per dilution	Dilution	True d	Sample avg MPN	True ln(d)	Sample avg (ln MPN)	Confidence interval avg length[a]
5	2-fold	1.00	1.0597	0	−0.0333	1.6728
		1.25	1.3319	0.2231	0.1914	1.5729
		1.50	1.5959	0.4055	0.3874	1.4973
		1.75	1.8684	0.5596	0.5447	1.4574
		2.00	2.2252	0.6931	0.7289	1.4227
		3.00	3.2539	1.0986	1.1188	1.4157
	10-fold	1.00	1.2445	0	0.0211	2.2524
		1.25	1.4629	0.2231	0.2072	2.1478
		1.50	1.8676	0.4055	0.5888	2.0709
		1.75	2.1228	0.5596	0.5902	2.0452
		2.00	2.5747	0.6931	0.7911	2.0469
		3.00	3.7970	1.0986	1.2010	2.0779
10	2-fold	1.00	1.0636	0	0.0131	1.1499
		1.25	1.3041	0.2231	0.2252	1.0827
		1.50	1.5541	0.4055	0.4066	1.0382
		1.75	1.8171	0.5596	0.5587	1.0129
		2.00	2.1021	0.6931	0.7108	0.9925
		3.00	3.1531	1.0986	1.1174	0.9880
	10-fold	1.00	1.0882	0	−0.0005	1.5385
		1.25	1.4052	0.2231	0.2600	1.4568
		1.50	1.6933	0.4055	0.4460	1.4251
		1.75	1.9257	0.5596	0.5827	1.4060
		2.00	2.2502	0.6931	0.7389	1.4059
		3.00	3.3947	1.0986	1.1497	1.4569

[a] Average length on natural-log scale, before anti-logs are taken. To convert to $\log_{10}$ multiply by 0.43429. Each line represents the average of 500 samples.

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fact larger than 1.0 in width. For comparing two sites or treatments, typically one will need to analyze more MPN values per treatment or site than direct counts, because of the larger standard error associated with the MPN values. If one is collecting environmental data, where changes of less than an order of magnitude in bacterial counts may be microbiologically significant, MPN values would probably not be a reasonable approach, unless the investigator is willing to increase greatly the number of replicates per dilution and the number of dilution concentrations.

Finally, the results of the simulation presented here suggest some strategies that can be developed for choosing sample sizes. Using the same number of replicates per dilution is a simple procedure but, under most circumstances, not optimal. If one selects three dilutions, as is often the case at present, with the intention that the highest concentration will yield almost all positive reactions and the lowest concentration will yield uniformly negative reactions, it is logical to replicate the middle concentration more than the upper and lower. After all, whenever the result is all or nothing, the variability observed is quite small. It is only when the results vary over a wide range, e.g., any value between 0 and 100%, that larger samples are required. This approach can be followed after a number of similar samples have been analyzed to assure that the assumptions made regarding the highest and lowest concentrations are, in fact, valid.

MPN values will continue to be used in microbiology, because alternative methods for estimating bacterial numbers have not yet been developed for those conditions where direct viable counts by epifluorescent microscopy (4) are too tedious or a problem because of large amounts of particulate matter present in the sample, or where a rapid estimate of viable bacteria, i.e., those bacteria capable of growth on selective media, is needed. The approach offered here provides a much greater reliability for the MPN estimate.

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