Application of the Most-Probable-Number Procedure to Suspensions of *Leptospira autumnalis* Akiyami A

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Various statistical tests are presented as evidence that the most-probable-number (MPN) procedure is a reliable method for estimating the density of washed-cell suspensions of *Leptospira autumnalis* Akiyami A.

Colonization of *Leptospira* was first reported in 1967 by Cox and Larson (2). There is only one known report of the use of this counting procedure for describing growth (4) and there are no reports of its application to survival studies. According to Bodily et al. (1), not all serotypes of *Leptospira* will colonize, and results between laboratories have not always been reproducible. My attempts at quantitative recovery of pathogenic serotypes on solid media produced, at best, erratic results.

Motivated by a desire to study survival of *Leptospira* at low cell concentrations, and recognizing that semisolid and liquid media have long been used successfully for maintenance and recovery of *Leptospira*, I undertook an investigation of the applicability of the most-probable-number (MPN) procedure.

Estimation of bacterial density by the MPN procedure is based on two assumptions. (i) The distribution of individual cells is assumed to be random in the suspension with complete independence. This precludes use of the method with any microorganism which tends to aggregate in any way. (ii) It is assumed that growth will ensue with the introduction of one or more cells into a tube of medium. Applicability of the procedure is, therefore, essentially dependent on the ability to recover a single cell.

MATERIALS AND METHODS

**Test organism.** The test strain of *L. autumnalis* Akiyami A was obtained from A. D. Alexander, Walter Reed Army Medical Center. The organism was maintained in Fletchers semisolid medium (Difco) containing 10% rabbit serum (Pel-Freez) with transfer every 5 weeks. Antigenic stability was verified periodically by the slide agglutination test with antiserum provided by the Center for Disease Control.

**Recovery medium.** Cell recovery was accomplished with Fletchers medium supplemented with (per liter): ZnSO₄·7H₂O, 0.2 mg; CaCl₂·2H₂O, 10 mg; MgCl₂·6H₂O, 10 mg; asparagine, 150 mg; and rabbit serum, 100 ml. The asparagine and serum were filter-sterilized and added aseptically. Amounts of 4 ml of the medium were placed in each sterile, plastic, disposable tube (12 by 75 mm, Falcon Plastics).

**Preparation of cells.** Cell cultures were grown at 30°C in a medium containing (per liter): Na₂HPO₄·1.0 g; KH₂PO₄·0.3 g; NaCl, 1.0 g; NH₄Cl, 0.25 g; thiamine, 0.005 g; and rabbit serum, 100 ml. After 86 hr of incubation, the cells were recovered by centrifugation for 30 min at 3,000 rev/min, washed twice in buffer (total phosphate, 5.33 mM; pH 7.6), and resuspended in buffer. Cell concentration was standardized by direct count with a Petroff-Hausser chamber and dark-field microscopy.

**Dilution blanks.** Dilutions for MPN determinations were made in 9.0-ml blanks containing *Leptospira* medium base EJM (Difco) with 1% rabbit serum. Tubes of recovery medium were inoculated with 0.1 ml of the appropriate dilutions.

**RESULTS**

A suspension of washed cells of *L. autumnalis* was standardized and then serially diluted to 1.0 cell per ml. Three series of 120 tubes each were inoculated with 0.1 ml of the last three decimal dilutions to give a theoretical MPN of 100 cells per ml. Table 1 compares the observed and theoretical ratios of negative tubes to total tubes for the three cell concentrations.

The three series of 120 tubes each represent 24 replicate MPN values with 5 tubes per
Table 1. Recovery of Leptospira autumnalis in a supplemented Fletchers medium at three cell concentrations

| Determination | No. of cells per tube |
|---------------|-----------------------|
|               | 10                    | 1.0                   | 0.1                   |
| No. of negative tubes observed, s | 0 | 48 | 100 |
| No. of positive tubes observed, r | 120 | 72 | 20 |
| Observed frequency, q = s/n | 0.000 | 0.400 | 0.833 |
| Theoretical frequency, q = s/n^* | 0.000045 | 0.367879 | 0.904837 |
| Expected error, e = \sqrt{pq/n} | 0.000612 | 0.044021 | 0.026877 |

*Halvorson and Ziegler (3); n = s + r = 120; p = 1 - q.

dilution and 12 replicate MPN values with 10 tubes per dilution. These MPN values and their 95% confidence intervals are presented in Table 2. Figure 1 shows the 24 replicate MPN values on log-probability paper. A reasonably straight line results, the consequence of a logarithmically normal distribution. The true cell concentration should lie at the 50% probability intersection. Figure 2 is a graphical representation of the 12 replicate MPN values with their 95% confidence intervals. Each confidence interval includes the true cell concentration of 100 per ml based on direct microscopic count and dilution.

MPN determinations were completed on a series of cell suspensions standardized by direct microscopic count and dilution. The results are compared in Table 3. MPN values are expressed as duplicate determinations with 5 tubes per dilution and one determination with 10 tubes per dilution.

To compare observed frequencies of MPN codes with theoretically expected frequencies, MPN values from two separate experiments with six different cell suspensions were chosen (Table 4). These MPN tests were completed over time and, therefore, represent different cell concentrations because death was proceeding. This constitutes a stricter test than selecting codes obtained on a single cell suspension.

**DISCUSSION**

Each of the statistical tests applied demonstrates that, with supplemented Fletchers medium used for cell recovery, the MPN procedure is a reliable technique for estimating the density of suspensions of *L. autumnalis*. The observed frequencies shown in Table 1 are within the theoretical frequency plus or minus the expected error for 10 and 1 cells per tube. This is not true for 0.1 cell per tube, but here the problem becomes one of an increasing expected error with a decreasing number of positive tubes. For reliable results, the percentage of positive tubes should be roughly between 35 and 85 (6), which is the case for one cell per tube.

The logarithms of MPN values are distributed normally. The mean of the log distribution of MPN values should equal the true cell concentration. The means of 24 five-tube MPN values (106) and 12 ten-tube MPN values (103) agree well with the true cell concentration of 100 per ml. Repeat MPN values from a

Table 2. Replicate MPN values on a standardized suspension of Leptospira autumnalis

| 5 tubes per dilution | 10 tubes per dilution |
|----------------------|-----------------------|
| MPN | 95% C.I.* | MPN | 95% C.I. |
| 130 | 35-300 | 171 | 76.7-381 |
| 220 | 57-700 | 125 | 56.1-279 |
| 79 | 25-190 | 58.9 | 26.4-131 |
| 46 | 16-120 | 58.9 | 26.4-131 |
| 110 | 31-250 | 125 | 56.1-279 |
| 33 | 11-93 | 102 | 45.8-227 |
| 140 | 37-340 | 102 | 45.8-227 |
| 70 | 23-170 | 74.2 | 33.3-165 |
| 79 | 25-190 | 74.2 | 33.3-165 |
| 63 | 21-150 | 87.2 | 39.1-194 |
| 130 | 35-300 | 133 | 59.7-296 |
| 240 | 68-750 | 133 | 59.7-296 |
| 94 | 28-220 | 133 | 59.7-296 |
| 170 | 43-490 | 133 | 59.7-296 |
| 110 | 31-250 | 133 | 59.7-296 |
| 110 | 31-250 | 109 | 48.9-243 |
| 110 | 31-250 | 197 | 88.4-439 |
| 130 | 35-300 | 109 | 48.9-243 |
| 350 | 120-1,000 | 197 | 88.4-439 |
| 79 | 25-190 | 79.2 | 35.5-176 |
| 79 | 25-190 | 79.2 | 35.5-176 |
| \( x \ | 106 | 31.2-262 | 103 | 46.3-230 |

*True cell concentration based on direct microscopic count and dilution = 100 per ml.
* Confidence interval.
FIG. 1. Bacterial density (MPN per ml) versus the probability of not exceeding the density. MPN values based on five tubes per dilution. True density, based on direct microscopic count and dilution, is 100 cells per ml.

normal distribution should plot as a straight line on log-probability paper, which is demonstrated in Fig. 1. The mean cell concentration determined graphically is 104, which compares well with the true concentration of 100 cells per ml based on direct microscopic count and dilution.

The MPN is not a precise method of measurement; it is only an estimate of the true cell concentration. The 95% confidence interval expresses the imprecision of the method. This interval will include the true cell concentration 95% of the time. Each of the 95% confidence intervals for 12 replicate 10-tube MPN values includes the true concentration of 100 cells per ml. Two of the 24 confidence intervals for replicate five-tube MPN values do not include the true concentration (Table 2). It is expected that the confidence interval will not include the true concentration 5% of the time or 1.2 times in every 24 tests. In the case of MPN tests completed on 12 different cell suspensions, 2 of

milliliter based on direct microscopic count and dilution.

FIG. 2. Replicate MPN values with 95% confidence intervals on a standardized suspension of Leptospira autumnalis. MPN values based on 10 tubes per dilution; \( \bar{x} \) = true cell concentration per
Table 3. Comparison of MPN values with cell densities based on direct microscopic count and dilution

| Density | 5 tubes per dilution | 10 tubes per dilution |
|---------|----------------------|-----------------------|
|         | MPN | 95% C.I. | MPN | 95% C.I. |
| 126     | 130 | 35-300   | 130 | 58.3-290 |
| 107     | 33  | 11-93    | 220 | 39.1-194 |
| 114     | 70  | 23-170   | 220 | 56.1-279 |
| 182     | 170 | 43-490   | 350 | 102-508 |
| 106     | 350 | 120-1,000| 220 | 118-586 |
| 189     | 110 | 31-250   | 170 | 59.7-296 |
| 109     | 130 | 35-300   | 49  | 35.5-176 |
| 134     | 130 | 35-300   | 31-250 | 52.1-258 |
| 177     | 49  | 17-130   | 43-490 | 41.9-208 |
| 86.3    | 33  | 11-93    | 23-170 | 21.3-106 |
| 86.3    | 170 | 43-490   | 79  | 52.1-258 |
| 86.3    | 63  | 21-150   | 25-190 | 25.2-125 |
| 86.3    | 110 | 31-250   | 17-130 | 33.3-165 |
| 86.3    | 33  | 11-93    | 17-130 | 17.9-88.9 |
| 86.3    | 130 | 35-300   | 130 | 58.3-290 |
| 90.9    | 170 | 43-490   | 49  | 41.9-208 |
| 91.4    | 240 | 68-750   | 43-490 | 88.4-439 |
| 85.3    | 79  | 25-190   | 130 | 45.3-225 |

* Based on direct microscopic count and dilution.
* Confidence interval.

Table 4. Comparison of theoretical and observed MPN code frequencies for five-tube tests

| Group | Theoretical percentage of results | Observed percentage of results |
|-------|----------------------------------|--------------------------------|
| Class 1 codes: | 550, 551, 552, 553, 554, 500, 510, 520, 530, 540, 100, 200, 300, 400 | 67.5 72.5 |
| Class 2 codes: | 511, 521, 531, 541, 542, 110, 210, 310, 410, 420 | 23.6 20.9 |
| Class 3 codes: | 501, 010, 532, 320, 522, 220, 543, 430, 120, 533, 330, 502, 020, 544, 440, 301, 401, 341, 201, 441, 101, 311, 421, 211, 001 | 7.9 6.5 |

* Woodward (7).
* Based on 153 tests.

18 (11.1%) 10-tube MPN values have confidence intervals not including the true concentration and 4 of 36 (11.1%) 5-tube MPN values have confidence intervals not including the true concentration (Table 3). This frequency is slightly greater than the expected 5%. However, the true cell concentrations are based on 12 separate microscopic counts, a method which also has a significant error.

The comparison of observed and theoretical frequencies for five-tube MPN codes shows good agreement (Table 4). An unusually large number of improbable codes would indicate either that the basic assumptions for the MPN procedure do not hold or that something is wrong in the technique.

This procedure was subsequently used to describe exponential death rates for L. autumnalis in defined solutions (5).

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