Proficiency Test Specimens for Water Bacteriology

RONALD L. CADA
The University of Texas Health Science Center at Houston, School of Public Health, Houston, Texas 77025

Received for publication 15 October 1974

A procedure for the preparation and distribution of simulated water specimens for coliform density testing is described. Lyophilization of Escherichia coli in a cooked meat-glucose supporting substrate provides stable samples which may be distributed to participants in a proficiency testing program. Logarithmic conversion of the data allows statistical evaluation of the results for inter- and intralaboratory variation. Comparisons between the most probable number and membrane filter techniques indicate no significant difference in the accuracy of these techniques, although the membrane filter technique is more precise.

Performance evaluation is a tool used to determine whether a laboratory is providing useful and dependable service to the public (11). The elements of an acceptable evaluation are (i) personnel; (ii) maintenance of records, equipment, and supplies; (iii) internal quality control management; and (iv) external quality control, or proficiency testing.

Proficiency testing has been recommended by the U.S. Department of Health, Education, and Welfare and has been used extensively in the approval of laboratories performing analyses of milk samples (10). No comparable program has been developed in water bacteriology due to sample instability during distribution, and disagreement on whether the standardized procedures are equivalent in the measurement of bacterial numbers (5, 7, 8).

The development of a simulated sample is basic to the concept of external evaluation. Freeze-drying (lyophilization) is used extensively in the preservation of bacterial strains (6), and is used for sample preparation in at least one proficiency test survey (4).

This study was proposed with three objectives: (i) to demonstrate the suitability of lyophilized cell suspensions for proficiency test specimens, (ii) to assess the effects of distribution on the lyophilized specimens, and (iii) to compare results of analysis between the most probable number, membrane filter, and the standard plate count.

MATERIALS AND METHODS

The supporting substrate used in the simulated water samples was prepared according to the following formulation: (i) 1 g of cooked meat medium (Difco); (ii) 10 ml of distilled water; and (iii) 0.05 g of D-glucose.

The mixture was allowed to stand for 10 min, and was then homogenized for 3 min on a Lourdes tissue grinder (model 1A). A commercial blender will give equivalent results. The suspension was dispensed in 3-g aliquots into 1-oz. (about 29 ml) prescription bottles and autoclaved for 10 min at 121°C with slow exhaust.

A stock culture of Escherichia coli exhibiting all the characteristics of a fecal coliform was incubated in brain heart infusion broth (BBL) for 18 h at 35°C. Culture densities were measured at 600 nm with a Coleman (model 6A) spectrophotometer. Sterile brain heart infusion broth was used to set the 100% transmission, and to adjust the 18-h culture to the predetermined density.

The adjusted cultures were serially diluted in buffered dilution water (1). One milliliter of the diluted culture was added to each bottle of sterile substrate; the mixture was quick-frozen in a bath of dry ice-alcohol and dried according to the manufacturer's instructions (Virtis Freeze-Mobile, model 10-140 BA).

The samples were sent to the participants by airmail, but were not insulated against temperature variations. Before testing, the samples were stored at room temperature in the dark.

On day 7 after lyophilization, the participants reconstituted the samples as follows. Approximately 10 ml of sterile water was pipetted from a 99-ml dilution blank into the bottle containing the freeze-dried material. This was allowed to stand for 1 min, and swirled gently, and then the suspension was pipetted or poured back into the same dilution blank. The dilution blank, containing approximately 100 ml of water, was treated as a sample just received in the laboratory.

The participants analyzed the samples according to their routine procedures. If more than one technique for water analysis was used, they were asked to test the samples in parallel. As an aid in analysis, the laboratories were supplied with a simulated most probable number (MPN) per 100 ml, representing the results obtained from the "water source by previous
testing." This allowed the analysts to set the dilution range as they would with their routine water samples.

The results received were converted to logarithmic format because it is assumed that the logarithms of the bacterial counts are normally distributed (10). The conversion allowed statistical evaluation of the data for intralaboratory and interlaboratory procedural variation.

RESULTS

Preliminary measurements indicated that an 18-h culture of E. coli in brain heart infusion broth achieved 30 to 40% transmission. When the culture was diluted to the 50% transmission level, approximately 10⁴ viable organisms were consistently recovered. Serial dilution of these cultures allowed the inoculum to be standardized throughout the study. Concentration 1 corresponds to a standardized prelyophilization inoculum of 10⁴ coliforms/100 ml, and concentration 2 corresponds to 10⁴ coliforms/100 ml.

All participants performed the standard 5-5-5 multiple-tube fermentation (MPN) technique. These data are analyzed in Table 1, and indicate the number of bottles tested at each concentration, the means of the log values, and standard deviations. Also tabulated are the overall (total) means and standard deviations for each trial. Table 1 lists similar data for laboratories performing parallel testing by the standard membrane filter (MF) technique. Analysis of the samples by the standard plate count (SPC) technique was performed by laboratory 4 (Table 3).

Comparability of the means of the MPN and MF techniques was tested by the one-way analysis of variance (9). The variance observed due to technique differences (procedure variance) is divided by the error variance to give the variance ratio (F value). The procedure variance measures the variation of the laboratory means from the overall mean, while the error variance measures the pooled variation of the individual values about each laboratory's mean. In this study (Table 4), 3 of the 25 evaluations showed an F value indicating significant differences between the means of the two techniques. Two of these differences indicated a significantly higher MPN mean, and the other indicated a higher MF mean. Study of the sources of variance show that the error variance was very small in two of these significant differences, while in the other the procedure variance was large.

The SPC was used as the criterion for accuracy and precision. An analysis of variance tested the difference between the means of the SPC, MPN, and MF techniques (Table 5). One of the six comparisons showed a significant difference between the means.

It has been suggested that better estimates of the true density may be obtained by increasing the number of samples at each dilution (2). Table 6 shows two trials where the standard 5-5-5 MPN was compared with an estimate (3) by using 20 tubes at each dilution. Although the precision is higher when the sample size is in-

### Table 1. Logarithms of coliform density in simulated water samples as determined by the MPN technique

| Laboratory no. | Density during trial: | 1     | 2     | 3     | 4     | 5     | 6     |
|----------------|-----------------------|-------|-------|-------|-------|-------|-------|
|                | n*                    | i*    | SD    | n     | i     | SD    | n     | i     | SD    | n     | i     | SD    | n     | i     | SD    |
| Using conc 1⁴  |                       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 1              | 2.5192 (0.213)        | 3.4689 (0.866) | 3.5901 (0.212) | 3.5621 (0.450) | 3.5081 (0.168) | 3.4467 (0.091) |
| 2              | 2.4872 (0.038)        | 3.5475 (0.099) | 3.5970 (0.125) | 3.5441 (0.296) | 3.5467 (0.091) | 2.5301 (0.00)  |
| 3              | 2.4780 (0.372)        | 1.5342 |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 4              | 4.6293 (0.301)        | 4.5089 (0.139) | 2.5630 (0.379) | 9.5664 (0.180) | 4.823 (0.319)  | 5.4925 (0.364) |
| Total          | 10.4820 (0.313)       | 11.5108 (0.506) | 8.5859 (0.242) | 15.5611 (0.261) | 16.5054 (0.327) | 13.6488 (0.645) |
| Using conc 2⁵  |                       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 1              | 2.3402 (0.407)        | 3.7500 (0.128) | 3.5253 (0.164) | 3.5277 (0.321) | 3.0631 (0.075) | 3.1570 (0.231) |
| 2              | 2.3678 (0.019)        | 3.844 (0.301)  | 3.4970 (0.354) | 3.5265 (0.232) | 3.5160 (0.057) | 1.4301 (0.00)  |
| 3              | 2.3810 (0.421)        | 1.4114 |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 4              | 4.2867 (0.186)        | 4.2962 (0.458) | 4.5252 (0.227) | 3.4982 (0.212) | 3.3043 (0.371) | 4.3170 (0.218) |
| Total          | 10.3326 (0.475)       | 11.3522 (0.540) | 10.3515 (0.226) | 14.3508 (0.219) | 14.3283 (0.538) | 11.3465 (0.377) |

* *Number of bottles analyzed.
   * Mean value of bottles analyzed.
   * Standard deviation.
   * Preliminary inoculum of 10⁴ coliforms per 100 ml.
   * Preliminary inoculum of 10⁴ coliforms per 100 ml.
TABLE 2. Logarithms of coliform density in simulated water samples as determined by the MF technique

| Density during trial: | Laboratory no. | 2 | 3 | 4 | 5 | 6 |
|-----------------------|----------------|---|---|---|---|---|
|                       | n | SD | n | SD | n | SD | n | SD | n | SD |
| Using concn 1*         | 2 | 3 | 5.342 | 0.035 | 3 | 5.760 | 0.068 | 3 | 5.671 | 0.042 | 3 | 5.249 | 0.228 | 3 | 5.848 | 0.151 |
|                       | 3 | 3 | 5.052 | 0.068 | 3 | 5.177 | 0.769 | 3 | 5.035 | 0.114 | 3 | 4.065 | 1.098 |
|                       | 4 | 4 | 5.074 | 0.089 | 3 | 5.474 | 0.100 | 9 | 5.553 | 0.057 | 7 | 4.828 | 0.430 | 5 | 4.650 | 0.064 |
| Total                 | 10 | 5.148 | 0.143 | 9 | 5.470 | 0.464 | 12 | 5.582 | 0.074 | 13 | 4.973 | 0.228 | 11 | 4.817 | 0.866 |
| Using concn 2*         | 2 | 3 | 3.715 | 0.473 | 3 | 3.752 | 0.304 | 2 | 3.539 | 0.088 | 3 | 3.107 | 0.640 | 3 | 3.502 | 0.174 |
|                       | 3 | 3 | 3.434 | 0.553 | 3 | 3.210 | 0.374 | 3 | 3.176 | 0.108 |
|                       | 4 | 3 | 3.286 | 0.331 | 8 | 3.490 | 0.100 | 8 | 3.523 | 0.132 | 6 | 2.985 | 0.453 | 4 | 3.037 | 0.132 |
| Total                 | 9 | 3.478 | 0.442 | 14 | 3.486 | 0.274 | 11 | 3.523 | 0.131 | 9 | 3.025 | 0.484 | 10 | 3.218 | 0.239 |

- Number of bottles analyzed.
- Mean value of bottles analyzed.
- Standard deviation.
- Prelyophilization inoculum of $10^5$ coliforms per 100 ml.
- Prelyophilization inoculum of $10^4$ coliforms per 100 ml.

TABLE 3. Logarithms of coliform density in simulated water samples as determined by the SPC technique

| Density during trial: | 3 | 4 | 5 | 6 |
|-----------------------|---|---|---|---|
|                       | n | SD | n | SD | n | SD | n | SD |
| 1*                    | 9 | 3.557 | 0.056 | 7 | 5.058 | 0.163 | 5 | 5.228 | 0.136 |
| 2*                    | 9 | 3.527 | 0.117 | 6 | 3.386 | 0.354 | 4 | 3.236 | 0.188 |

- Laboratory 4.
- Number of bottles analyzed.
- Mean value of bottles analyzed.
- Standard deviation.
- Prelyophilization inoculum of $10^5$ coliforms per 100 ml.
- Prelyophilization inoculum of $10^4$ coliforms per 100 ml.

TABLE 4. Comparison of MPN and MF techniques by one-way analysis of variance

| Concn     | Laboratory 2 | Laboratory 3 | Laboratory 4 |
|-----------|--------------|--------------|--------------|
|           | Trial | Procedure variance | Error variance | F* | Procedure variance | Error variance | F | Procedure variance | Error variance | F |
| 1*        | 2     | 0.027 | 0.0055 | 4.86 | 0.063 | 0.0033 | 18.96c | 0.0005 | 0.012 | 0.04 |
|           | 3     | 0.056 | 0.010 | 6.58 | 0.056 | 0.0017 | 3.13 |
|           | 4     | 0.079 | 0.045 | 1.77 | 0.021 | 0.0087 | 2.37 | 0.0001 | 0.056 | 0.001 |
|           | 5     | 0.071 | 0.030 | 2.35 | 0.003 | 1.164 | 0.002 | 0.189 | 0.058 | 2.77 |
|           | 6     | 0.359 | 0.015 | 23.77c | 0.003 | 0.002 | 11.9c | 0.040 | 0.033 | 1.23 |
| 2*        | 2     | 0.025 | 0.157 | 0.16 | 0.347 | 0.306 | 1.14 | 0.180 | 0.170 | 1.06 |
|           | 3     | 0.098 | 0.109 | 0.90 | 0.003 | 0.023 | 0.12 |
|           | 4     | 0.0002 | 0.031 | 0.01 | 0.002 | 0.033 | 0.06 |
|           | 5     | 0.0045 | 0.309 | 0.01 | 0.304 | 0.172 | 1.77 |
|           | 6     | 0.479 | 0.030 | 15.9 | 0.106 | 0.009 | 11.9c | 0.040 | 0.033 | 1.23 |

- Procedure variance/error variance.
- Prelyophilization inoculum of $10^5$ coliforms per 100 ml.
- Significant at 0.05 level.
- Prelyophilization inoculum of $10^4$ coliforms per 100 ml.
TABLE 5. Comparison of MPN, MF, and SPC techniques by the one-way analysis of variance*  

| Trial | Procedure variance | Error variance | F* | Procedure variance | Error variance | F |
|-------|--------------------|----------------|----|--------------------|----------------|----|
| 4     | 0.036              | 0.013          | 2.79 | 0.0015             | 0.026          | 0.06 |
| 5     | 0.126              | 0.049          | 2.59 | 0.268              | 0.156          | 1.72 |
| 6     | 0.419              | 0.052          | 8.09*| 0.042              | 0.034          | 1.25 |

* Laboratory 4.  
* Prelyophilization inoculum of 10⁴ coliforms per 100 per ml.  
* Prelyophilization inoculum of 10⁴ coliforms per 100 ml.  
* Procedure variance/error variance.  
* Significant at 0.05 level.

The effect of mailing the samples without temperature control was evaluated by sending a set of samples to the Texas State Health Department (160 miles, about 257.44 km), with instructions to return unopened. These “pigeon” samples were tested in parallel with samples stored at 23 to 25°C (Table 7). The samples in trial 2 spent 4 days in the mail system; those in trial 6, spent 6 days. Comparison of the means by analysis of variance shows that one of the laboratory-stored means is significantly higher than that of the pigeon. However, samples at the higher concentration, mailed in the same container, showed no significant difference between the means.

TABLE 6. Comparison of 5-5-5 and 20-20-20 multiple tube techniques: logarithms of coliform density and analysis of variance*  

| Concen | 5-5-5 | 20-20-20* | Procedure variance | Error variance | F |
|--------|-------|-----------|--------------------|----------------|----|
| n      | $\bar{x}$ | SD | n      | $\bar{x}$ | SD |
| Trial 3 |       |     |       |     |     |
| 1*     | 2     | 5.630 | 0.379 | 2 | 5.416 | 0.262 | 0.046 | 0.106 | 0.43 |
| 2*     | 4     | 3.522 | 0.227 | 4 | 3.528 | 0.147 | 0.00007 | 0.029 | 0.002 |
| Trial 4 |       |     |       |     |     |
| 1*     | 9     | 5.664 | 0.180 | 3 | 5.558 | 0.134 | 0.025 | 0.0296 | 0.86 |
| 2*     | 9     | 3.498 | 0.212 | 3 | 3.454 | 0.218 | 0.004 | 0.039 | 0.11 |

* Laboratory 4. n, Number of bottles analyzed; $\bar{x}$, mean value of bottles analyzed; SD, standard deviation.  
* Log $\lambda = x \log a - k$, where $\lambda = $ numbers of organisms; $x = $ total number of fertile tubes per number of tubes at each dilution; $a = $ dilution factor; $k = $ table value cubed.  
* Prelyophilization inoculum of 10⁴ coliforms per 100 ml.  
* Prelyophilization inoculum of 10⁴ coliforms per 100 ml.

TABLE 7. Comparison of pigeon samples and samples stored at room temperature: logarithms of coliform density as determined by the MPN technique  

| Concen | Laboratory stored | Pigeon | Procedure variance | Error variance | F* |
|--------|-------------------|--------|--------------------|----------------|----|
| n*     | $\bar{x}$ | SE | n | $\bar{x}$ | SE |
| Lab 1, trial 2 |       |     |       |     |     |
| 1*     | 3     | 4.689 | 0.866 | 3 | 5.116 | 0.427 | 0.274 | 0.466 | 0.59 |
| 2*     | 3     | 3.750 | 0.128 | 2 | 2.682 | 0.230 | 1.369 | 0.029 | 47.7 |
| Lab 4, trial 6 |       |     |       |     |     |
| 1*     | 5     | 4.925 | 0.364 | 3 | 5.191 | 0.067 | 0.133 | 0.090 | 1.48 |
| 2*     | 4     | 3.179 | 0.218 | 3 | 3.124 | 0.232 | 0.005 | 0.050 | 0.10 |

* Number of bottles analyzed.  
* Mean value of bottles analyzed.  
* Standard error of the mean.  
* Procedure variance/error variance.  
* Prelyophilization inoculum of 10⁴ coliforms per 100 ml.  
* Prelyophilization inoculum of 10⁴ coliforms per 100 ml.  
* Significant at 0.05 level.
DISCUSSION

The quantitative nature of results in water bacteriology makes it imperative that a proposed program for proficiency testing meet certain criteria. These criteria include: (i) adequate and appropriate specimens; (ii) specimen preparation and distribution procedures that can be used by testing agencies with minimum investment in personnel and equipment; (iii) specimens that possess maximum, definable stability throughout the preparation and distribution system; and (iv) valid data analysis of performance measurements. This study proposed to identify and measure some of the variables found in the preparation, distribution, and evaluation of the specimens.

The well-known instability of water samples (1) makes them inappropriate for use in a split-sample program measuring interlaboratory variation over wide geographical areas. Lyophilized specimens promise to provide more stable samples. Results of this study indicate satisfactory recovery of coliforms may be obtained over the 7-day time span from preparation to analysis. Additionally, the distribution of samples without strict temperature control promises to hold down the costs of applying such a program.

No attempt is made to address the controversy between the relative accuracy and precision of the MPN and MF techniques. This study shows that only three of the 25 comparisons made between these two techniques show any significant difference in recovery (Table 4). Two of these differences are due to a low level of variance within the laboratory, while the other shows an increased variance due to technique differences (laboratory 2). Further, Table 5 indicates little difference in accuracy between the MPN, MF, and SPC techniques.

Increasing the sample size of the MPN technique does not significantly alter the accuracy of the standard 5-5-5 technique (Table 6). Although increased precision (S.D.) is observed with larger samples, the magnitude of increase does not make it practical for use with routine water samples.

No test specimens were distributed at densities approaching the range of coliforms expected in potable water testing. Samples tested in the School of Public Health laboratory indicated there would be no major problems in preparation of such samples. However, the particle size of the supporting substrate interferes with the filtration technique as would a turbid stream sample. Further research will be necessary to determine whether this system would be appropriate for potable water evaluation by membrane filtration.

Mixed microbial flora can be used for the preparation of simulated water samples. Research concerned with mixed enteric bacteria (R. Cada, Health Lab. Sci., in press) demonstrates the feasibility of this technique. Fecal and nonfecal bacteria may be added to the substrate in desired proportions to assess the differential competencies of the analysis techniques.

ACKNOWLEDGMENTS

I acknowledge the assistance of the following laboratory directors and their staffs: C. D. McGuire, Colorado Department of Health; C. E. Sweet, Texas State Department of Health, and R. D. Wende, Houston City Health Department. The advice and assistance of Bartholomew P. Hsi, The University of Texas School of Public Health, is appreciated.

LITERATURE CITED

1. American Public Health Association. 1971. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, Washington, D. C.
2. Cochran, W. G. 1950. Estimation of bacterial densities by means of the "most probable number." Biometrics 6:105-116.
3. Fisher, B. A., and F. Yates. 1963. Statistical tables for biological, agricultural, and medical research, 6th ed. Oliver and Boyd, London.
4. Gavan, T. L. 1974. A summary of the bacteriology portion of the 1972 basic, comprehensive, and special, College of American Pathologists (CAP) quality evaluation program. Am. J. Clin. Pathol. 61:971-979.
5. Geldreich, E. E., H. L. Jeter, and J. A. Winter. 1967. Technical considerations in applying the membrane filter procedure. Health Lab. Sci. 4:115-135.
6. Greaves, R. I. N. 1962. Recent advances in freeze-drying. J. Pharm. Pharmacol. 14:621-640.
7. Kabler, P. 1954. A committee report: water examinations by membrane filter and most probable number procedures. Am. J. Public Health 44:379-396.
8. McCarthy, J. A., H. A. Thomas, and J. E. Delany. 1968. Evaluation of the reliability of coliform density tests. Am. J. Public Health 48:1628-1635.
9. Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods, 6th ed. The Iowa State University Press, Ames, Iowa.
10. U. S. Department of Health, Education and Welfare. 1965. Evaluation of milk laboratories recommended by the United States Public Health Service. Public Health Service Publication No. 999-FP-3. Washington, D.C.
11. U. S. Department of Health, Education, and Welfare. 1968. Laboratory performance evaluation—characteristics of an acceptable program. National Center for Disease Control, Atlanta, Ga.