THE RELATIVE ERRORS OF BACTERIOLOGICAL PLATE COUNTING METHODS

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It is generally conceded that the degree of precision ordinarily attained with dilution plate counting methods of enumerating viable bacteria is not so great as could be desired. The extent of variation encountered even under the best conditions is considerably larger than the maximum permitted, for example, of routine chemical analyses. Coefficients of variation consistently as high as 30 per cent of the mean have been reported (Mudge and Lawler, 1928; Ziegler and Halvorson, 1935), although considerably lower values have also been encountered (Jennison, 1937). Some of the principal sources of variation which may be identified at the present time are:

(1) The presence of clumps, consisting of various numbers of organisms, in the original suspension (Jennison, 1937).

(2) The error involved in preparing a dilute suspension (Jennison and Wadsworth, 1940).

(3) The error involved in measuring aliquots of this suspension into plates.

(4) The distribution of organisms in these aliquots (Fisher, Thornton, and Mackenzie, 1922; James and Sutherland, 1939; Sutherland and James, 1938; Wilson and Kullmann, 1931).

(5) Factors which influence the development of the inoculated cells into visible colonies (Harmsen and Verweel, 1936–1937; McNew, 1938).

The relative importance of these different sources of error depends considerably upon the conditions of experimentation; but it appears probable that if species of bacteria characterized by persistent or tenacious clumping are excluded, the contribution of the first two sources listed above may be reduced through careful technique to a small part of the total. It is not an uncommon practice to describe the variability of replicate plates as the total error of the plate count, and this is probably not often a serious misrepresentation. However, methods of estimating "dilution error" have been described (Jennison and Wadsworth, 1940) and are undoubtedly valuable for precise studies.

The sum of the variations due to the three remaining sources may be estimated from the observed variability of replicate plates. The chief purpose of the present study is an analysis of this sum, separating the variation related to the measurement of aliquots into plates from the sum of the remaining two factors, and estimating its relative importance. The data required for this purpose were obtained by comparing several measuring methods with respect to the volumes of suspension delivered and the plate counts obtained with each method. This problem does not appear to have been investigated specifically, although methods for increasing the precision of measurement have been devised and recommended for use (Miles, Misra, and Irwin, 1938; Wilson, 1922). Since the comparison was also designed to assist in the selection of appropriate bacterial counting methods for practical use, several modifications, which are not strictly essential to the principal objective, were included and assessed.

METHODS

Three methods of measuring aliquots of inoculum were employed, and these, together with other modifications of technique, provided five plating methods for comparison, as follows:

A. Prepared and dried plates were inoculated with 6 drops of a known dilution of organisms delivered by means of a single calibrated capillary pipette.[1] The drops were allowed to fall at well-separated points on the surface of the agar.

(1) The area covered by the individual drops was increased by repeatedly tilting the plate in all directions. This method is commonly used with *Brucella* species.

(2) The inoculum was distributed over the entire surface of the plate with a sterile, bent glass rod.

B. Prepared and dried plates were inoculated with 0.1-ml quantities of dilute suspension delivered by means of a single "exax" serological 1-ml pipette graduated in tenths and hundredths of a ml. The inoculum was distributed by means of a sterile, bent glass rod. This method has been used routinely with *Bacterium tularense* (Snyder, Engley, Penfield, and Creasy, 1946) and is a modification of the method of Anderson and Stuart (1935).

C. Nine-tenths ml of dilute suspension were delivered by means of a single "exax" 1-ml serological pipette into the following:

(1) Sterile, empty plates, which then received 15 ml melted agar medium held at 45 C. The medium and inoculum were mixed and allowed to solidify. This is the conventional poured plate, with the single exception that an attempt was made to increase accuracy by avoiding delivery of the final 0.1 ml of the inoculum.

(2) Culture tubes (16 by 150 mm) containing 2 ml of melted 3/2 strength agar medium held at 45 C. The medium and inoculum were mixed and allowed to solidify on the walls of the tube by rotating the tube in a horizontal position under the cold water tap. This is the rolled tube method (see Wilson, 1922) modified with respect to the size of the inoculum.

In order to provide a valid basis for comparison, a single bacterial species was counted with all five methods. *Shigella dysenteriae*, strain 14-4, was selected for this purpose because its rather simple nutritional requirements and its relative indifference to oxidation-reduction potential could be expected to permit abundant growth in all cases.

Final dilutions for plating were prepared from 24-hour stock cultures in tubes of nutrient broth, using as the diluent 0.2 per cent Difco gelatin in 1.0 per cent disodium phosphate ($12H_2O$) adjusted to pH 6.8. Whenever the same dilution

[1] We are indebted to M/Sgt. D. E. Drukenmiller, Jr., for a supply of these pipettes, which were prepared essentially according to the directions of Donald (1915).

could be used for several plating methods, all samples were taken from a single bottle of that dilution.

Tryptone agar of the following composition was used in all plates:

Difco tryptone	2.0 per cent
Sodium chloride	0.5 per cent
Glucose	0.5 per cent
Difco agar	2.0 per cent
Adjusted to pH 7.0 to 7.2	

In the case of method C2 (rolled tubes), all ingredients were originally present in concentrations 1.5 times those indicated, but the addition of the inoculum reduced them to approximately those listed.

All surface-inoculated plates were dried before being used by storage at 34 C for 3 to 5 days. This is not recommended as a method for producing uniformly dried plates, but it is believed that the extent of drying is a relatively unimportant factor in the case of *S. dysenteriae*.

Plates and tubes were incubated at 34 C for 24 hours after inoculation. Plates were counted with the aid of a Quebec colony counter, whereas rolled tubes were counted over oblique illumination, each colony being marked with a wax pencil. Counting rolled tubes was found to be extremely tedious.

The volumes of fluid delivered by means of the three pipetting methods were determined gravimetrically. Approximately 60 replicates were delivered into weighed sample bottles by each method, the exact technique used in plating being followed as nearly as possible. The bottles were closed immediately after the addition and weighed to the nearest one-tenth of a milligram. However, in order to reduce the total number of weighings required, distilled water was substituted in these measurements for gelatin-phosphate diluent, so that the bottles might be merely dried, rather than cleaned and reweighed, before being used again. This method appeared to be valid, since the difference in weight of the dry sample bottles before and after completion of all measurements averaged only +0.7 mg, and in only one case (apparently an error in the initial weighing) did it exceed 2.0 mg, or 2 per cent, of the smallest volume measured.

The substitution of distilled water necessitated a correction for the volume of gelatin-phosphate diluent delivered by the capillary-dropping pipette method (A), since the volumes of the drops formed are influenced by the surface tension of the fluid delivered. The correction factor required was determined by counting the number of drops of distilled water and diluent delivered by the particular capillary pipette used throughout. The pipette was mounted vertically in a fixed position, and the drop rate was controlled by means of a capillary air inlet. A constant volume was assured by horizontal markings on the pipette, and the portion of the last drop delivered was estimated to the nearest one-tenth of a drop. The results were quite constant with water, averaging for 11 determinations $102.05 \pm 0{\cdot}05$ drops. They were equally constant for any one sample of gelatin-phosphate diluent, but different samples differed appreciably: the average of 5 determinations on each of 6 samples was 105.56 ± 0.18 drops. The difference between water and diluent is highly significant. On the basis of these averages the ratio of volume of drops of diluent to volume of an equivalent number of drops of water is 0.96675. This is considerably less than the theoretical ratio calculated from surface tension measurements and probably may be accounted for by the small diameter of the pipette tip.

STATISTICAL METHODS

In order to compare the magnitude of plate counts obtained by different methods of plating, the observed mean counts were adjusted according to the dilution and volume of suspension inoculated. In all cases the adjusted mean count is that expected with 0.1 ml of the 10^{-5} dilution.

Standard deviations,

$$s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{N - 1}}$$

(x = counts of individual plates, $\bar{x}$ = mean count, and N = number of plates), were derived from the unbiased estimate of the second central moment and are thus independent of the particular distribution function with which the counts may conform.

In order to determine the significance of differences between adjusted mean counts, standard errors,

$$s_{\bar{x}} = \frac{s}{\sqrt{N}},$$

were adjusted to conform with the adjustment of the means:

$$\text{adjusted } s_{\bar{x}} = \frac{s_{\bar{x}}(\text{adjusted } \bar{x})}{\bar{x}}.$$

This adjustment is dependent upon the assumption that a strict proportionality exists between the standard error and the mean, and may be only approximately correct. Consequently, most confidence may be placed in tests of significance of differences between means when the required adjustment is minimal.

The relative error of the different plating methods was compared on the basis of the coefficient of variation,

$$V = \frac{s}{\bar{x}}.$$

This definition of the coefficient of variation is that of Cramér (1946), and it differs from the usual percentage representation by a factor of 1/100. The standard deviation of the coefficient of variation,

$$s_V = \sqrt{\frac{s^2}{2N\bar{x}^2}\left(1 + 2\frac{s^2}{\bar{x}^2}\right)},$$

is also due to Cramér (1946).

Tests of significance of differences between the adjusted mean counts of different plating methods, and between coefficients of variation of different methods, were based on Student's ratio,

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(s_{\bar{x}_1})^2 + (s_{\bar{x}_2})^2}}$$

or

$$t = \frac{V_1 - V_2}{\sqrt{(s_{V_1})^2 + (s_{V_2})^2}}.$$

Values of t exceeding the 5 per cent level of the t-distribution (1.960) for an infinite number of degrees of freedom are considered significant, those exceeding the 1 per cent level (2.576) highly significant.

TABLE 1

Analysis of gravimetric measurements of water volumes delivered by three pipetting methods

STATISTICS	METHODS OF PIPETTING*		
	A	B	C
Number of replicates	60	60	59
Mean weights of water (grams)	0.1801	0.1025	0.9087
Calculated volumes of diluent (ml)†	0.1741	0.1025	0.9087
Standard deviations	0.0036	0.0028	0.0090
Standard errors	0.0005	0.0004	0.0012
Coefficients of variation	0.02001	0.02727	0.00989
Standard deviations of coefficients of variation	0.00183	0.00249	0.00090

* Methods of pipetting:

A. Six drops delivered with capillary pipette.

B. One-tenth ml delivered with 1-ml serological pipette.

C. Nine-tenths ml delivered with 1-ml serological pipette.

† Calculations based on the ratio of drop volumes of distilled water and gelatin-phosphate diluent.

EXPERIMENTAL RESULTS

The results of gravimetric measurements of volumes delivered by the three pipetting methods are shown in table 1. The weights may be converted directly into volumes without appreciable error, since the density of water at 25 C is 0.9970 grams per ml (Hodgman and Holmes, 1940). It may be noted that the capillary pipette used delivers in 6 drops 0.180 ml of distilled water or 0.174 ml of gelatin-phosphate diluent. The serological pipettes delivered slightly in excess of the volumes intended, and presumably the volumes were the same in the case of both water and diluent.

The variability of even the most inaccurate method (B) was small, the coefficient of variation being less than 3 per cent of the mean. The coefficients of variation necessarily included errors of weighing, but these were apparently small. The variabilities of the three pipetting methods, however, differed appreciably. The order of increasing error was C, A, B in the ratio of 1:2:2.7. It may be shown that the difference between any two of these coefficients of variation was statistically significant and that method C was more accurate to a highly significant degree than either of the other two. It may be concluded, therefore, that of the methods tested most precise delivery was obtained when 0.9 ml were delivered from a 1-ml pipette, and the least precise when only 0.1 ml was delivered from the same type of pipette. When it is necessary to use small inocula of the order of 0.1 to 0.2 ml, the capillary pipette provides more accurate delivery than do 1-ml pipettes.

In order to compare the variabilities of the three pipetting methods with the total error of the plate count, of which they are a part, two plating experiments were conducted using the five plating methods described. The two experiments were essentially repetitive. In experiment 1 plates were inoculated by methods

TABLE 2

Analysis of plate counts (experiment 1)

STATISTICS	PLATING METHODS*			
	A1	A2	B	C1
Number of replicates	66	64	67	49
Mean counts (colonies per plate)	293.14	308.86	178.34	153.37
Adjusted mean counts†	168.36	177.39	173.91	168.77
Standard deviations	21.949	22.928	13.999	11.727
Standard errors	2.7017	2.8660	1.7104	1.6753
Adjusted standard errors‡	1.5517	1.6460	1.6679	1.8435
Coefficients of variation	0.07488	0.07423	0.07850	0.07646
Standard deviations of coefficients of variation	0.00655	0.00660	0.00683	0.00777

* For plating methods, see text.

† Values expected with 0.1 ml of 10^{-5} dilution.

‡ Adjusted to conform with adjusted mean counts.

A1, A2, and B from the 10^{-5} dilution, and plates and tubes by methods C1 and C2, respectively, from the 10^{-6} dilution, of a single culture. In this experiment the rolled tube counts were low and extremely erratic, presumably because of having been held in the water bath at 45 C for 10 to 20 minutes after inoculation, and therefore they were not included in the analysis. In experiment 2, which employed a second culture, the dilutions used with each method were so chosen that approximately equal mean counts could be expected with all methods. These dilutions were 0.6×10^{-5} in the case of methods A1 and A2, 1.0×10^{-5} with method B, and 0.11×10^{-5} with methods C1 and C2. Furthermore, in this experiment rolled tubes were rolled and cooled immediately after inoculation, a procedure requiring the attention of two operators.

The analyses of these two experiments are shown in tables 2 and 3. Since rather similar values were obtained for the adjusted mean counts of the various methods, as well as for their variabilities as indicated by coefficients of variation (which may legitimately be compared), it is necessary to resort to statistical tests to determine the significance of the differences between them. The significances of the differences between all possible combinations of adjusted mean counts

TABLE 3

Analysis of plate counts (experiment 2)

STATISTICS	PLATING METHODS*				
	A1	A2	B	C1	C2
Number of replicates	56	56	56	56	56
Mean counts (colonies per plate)	152.91	161.36	158.30	159.05	167.48
Adjusted mean counts†	146.37	154.46	154.37	159.11	167.54
Standard deviations	12.807	13.568	11.513	14.157	12.338
Standard errors	1.7115	1.8132	1.5386	1.8919	1.6488
Adjusted standard errors‡	1.6382	1.7356	1.5003	1.8926	1.6494
Coefficients of variation	0.08376	0.08409	0.07273	0.08901	0.07367
Standard deviations of coefficients of variation	0.00797	0.00800	0.00691	0.00848	0.00700

* For plating methods, see text.

† Values expected with 0.1 ml of 10^{-5} dilution.

‡ Adjusted to conform with adjusted mean counts.

TABLE 4

Significance of differences between mean counts of various plating methods

EXPERIMENT	METHODS* COMPARED	DIFFERENCES BETWEEN ADJUSTED MEANS	STANDARD ERRORS OF DIFFERENCES	STUDENT'S† RATIO
1	A1, A2	−9.03	2.262	3.992(++)
	A1, B	−5.56	2.278	2.440(+)
	A1, C1	−0.41	2.410	0.171
	A2, B	+3.47	2.343	1.482
	A2, C1	+8.62	2.471	3.487(++)
	B, C1	+5.14	2.486	2.070(+)
2	A1, A2	−8.09	2.387	3.389(++)
	A1, B	−8.00	2.221	3.602(++)
	A1, C1	−12.74	2.503	5.091(++)
	A1, C2	−21.18	2.325	9.110(++)
	A2, B	+0.09	2.294	0.038
	A2, C1	−4.66	2.568	1.813
	A2, C2	−13.09	2.394	5.467(++)
	B, C1	−4.74	2.415	1.964(+)
	B, C2	−13.18	2.230	5.910(++)
	C1, C2	−8.43	2.510	3.359(++)

* For plating methods, see text.

† Ratios exceeding the 5% level of the *t*-distribution (1.960) are considered significant (+), those exceeding the 1% level (2.576), highly significant (++).

within each of the two experiments are shown in table 4. Since these experiments were not designed primarily for the comparison of mean counts, probably only a few of the tabulated comparisons are valid. The significance of the difference between means of plates inoculated from different dilutions, or even from different dilution bottles, should be questioned because the error involved in the preparation of dilutions was not determined. Methods A1, A2, and B in experiment 1 are not subject to this criticism. It may be seen that the adjusted mean counts of methods A2 and B were not significantly different, whereas both were significantly greater than that of method A1. The higher counts are undoubtedly related to the more efficient spreading of the inoculum which was obtained in methods A2 and B by the use of a bent glass rod. It is suggested that the opportunity for the formation of congruous colonies is decreased by this procedure. The only comparisons of mean counts within experiment 2 which may not be questioned are those between methods A1 and A2 and between methods C1 and C2, each pair of which was inoculated from a single dilution bottle. Again, method A2 gave a significantly higher count than method A1, confirming the results of the first experiment. In addition, the adjusted mean of method C2 (rolled tubes) was significantly higher than that of method C1. This confirms a similar observation of Wilson (1922), and the magnitude of our difference (5 per cent) is approximately the same as his.

On the other hand, it would appear legitimate to compare the variabilities of any two plating methods within either experiment, or even those of different experiments, since the coefficient of variation is generally considered independent of the original culture, of the dilution used, and of the mean count. Actually, however, independence between the coefficient of variation and the mean count follows from the assumption that the standard deviation is strictly proportional to the mean. Since this may be only approximately correct, most confidence may be placed in comparisons between the variabilities of the plating methods of the second experiment because the unadjusted means are nearly equal in this case. This consideration is relatively unimportant, however, because it may be shown that no two of the coefficients of variation obtained in both experiments differed significantly. The 36 possible combinations may be covered by a single test, using the greatest difference observed (that between methods B and C1, experiment 2) and the smallest observed standard deviation of the coefficient of variation (method A1, experiment 1). Student's ratio then becomes

$$t = \frac{0.01628}{\sqrt{2(0.00655)^2}} = 1.757,$$

which is nonsignificant and at least as great as that of any pair of observed values. It may be concluded, therefore, that the variability or error of any one of the five plating methods tested was not significantly greater than that of any of the others.

It is now necessary to reconcile this conclusion with the finding that the errors of the three pipetting methods used did differ significantly. This may be done most conveniently by considering the components of error involved. The coefficients of variation of the plate counts will be regarded for this purpose as the total errors (T), consisting in part of variabilities due to the pipetting of aliquots into plates, which are represented by the coefficients of variation (P) of the appropriate pipetting methods as determined gravimetrically. The remaining variabilities (R) which complete the total are probably attributable to the distribution of organisms in the aliquots and to factors influencing the development of the organisms inoculated. Since the addition theorem is defined for terms of the same order as the variance (s^2), the relation between these components may be expressed by the equation,

$$T^2 = P^2 + R^2,$$

and R may be determined from the equation,

$$R = \sqrt{T^2 - P^2}.$$

TABLE 5

Analysis of error involved in various plating methods

EXPERIMENT	PLATING METHOD*	COEFFICIENTS OF VARIATION			$T - R$	$\frac{100\ (T - R)}{T}$
		Total† (T)	Pipetting‡ (P)	Remainder§ (R)		
1	A1	0.07488	0.02001	0.07216	0.00272	3.63
	A2	0.07423	0.02001	0.07148	0.00275	3.70
	B	0.07850	0.02727	0.07361	0.00489	6.23
	C1	0.07646	0.00989	0.07515	0.00131	1.71
2	A1	0.08376	0.02001	0.08134	0.00242	2.89
	A2	0.08409	0.02001	0.08168	0.00241	2.87
	B	0.07273	0.02727	0.06742	0.00531	7.30
	C1	0.08901	0.00989	0.08846	0.00055	0.62
	C2	0.07367	0.00989	0.07300	0.00067	0.91

* For plating methods, see text.

† Coefficients of variation taken from tables 2 and 3.

‡ Coefficients of variation taken from appropriate entries in table 1.

§ $R = \sqrt{T^2 - P^2}$. Additional preliminary analysis of this remainder variation (R), subtracting the variance due to a hypothetical Poisson distribution of organisms in aliquots of suspension, shows that residual variation is small and, in some cases, negative. There is at this time no plausible explanation for these negative residuals, but they suggest that there is something in the method which tends to keep the counts at an artificially even level, which would somewhat impair their use as measures of variation of bacterial populations.

The values of these statistics are given in table 5 for all the plating methods of experiments 1 and 2. It will be seen that the remainder (R) constitutes almost the total error (T). The final column of this table lists the percentage contributions of the pipetting errors to the total errors when reduced to terms of the same order as the actual measurements. Taking both experiments into account, this amounted to only 2.9 to 3.7 per cent in the case of capillary pipetting methods, to only 6.2 to 7.3 per cent in the case of 0.1-ml aliquots delivered with a 1-ml pipette, and to only 0.6 to 1.7 per cent in the case of 0.9-ml aliquots. This would appear to explain sufficiently the failure of the more precise methods of measurement to influence significantly the total error of the plate count.

DISCUSSION

Experimental results have been presented which indicate that, within the limits of precision ordinarily employed in pipetting aliquots of inoculum into individual plates, the accuracy of measurement provides a negligible contribution to the total error, and that even so crude a method as that of measuring 0.1-ml quantities with a 1-ml pipette does not significantly increase the plating error. Since a conclusion of this sort naturally leads to the recommendation of changes in the technique of plate counting, it would be well to subject the experimental evidence to careful scrutiny.

In the first place, the statistical analysis appears to be valid throughout. Essentially identical experiments, each employing numbers of replicates generally considered sufficient to determine the pertinent statistics, were mutually confirmatory. Estimates of error were independent of the form of distribution function which may be involved, and in at least one experiment the possible influence of unequal means was minimal. Tests of significance were necessarily based on a normal distribution function, but it is known that even with markedly skewed distributions the statistics concerned are approximately normally distributed.

Also, it might be objected that the relative familiarity of the operators with the different techniques introduced a bias in favor of a particular method. It is especially probable that the coefficient of variation reported for the capillary-dropping pipette method is greater than that which might be obtained by an experienced operator. However, this issue cannot be considered critical for the present study. Since the most inaccurate pipetting method did not appreciably affect the total plating error, further improvement of the relatively precise capillary-dropping method would, at most, have no greater effect.

The specific coefficients of variation reported apply only under the experimental conditions described and with the particular organism used. The principal conclusion which is drawn from them, however, can be affected only by conditions or a bacterial species which leads to a *reduction* of the total error. The extent to which the total error must be reduced in order that the pipetting error may exert an appreciable effect depends upon the magnitude of the pipetting error. The limiting value (T_p) of the total error corresponding to various percentage contributions (p) of the pipetting error (P) is found by solution of the equations,

$$p = \frac{100(T - R)}{T}$$

and

$$R = \sqrt{T^2 - P^2},$$

which is

$$T_p = \frac{P}{\sqrt{\frac{p}{100}\left(2 - \frac{p}{100}\right)}}.$$

Values of T_p corresponding to various p are shown in table 6 for the three pipetting methods used. It may be seen that an appreciable contribution (for example, 20 per cent of the total error) from the pipetting error is not attained until the total error is reduced to 0.016 to 0.045, depending on the pipetting method used. It is questionable whether or not such low values for the total error are ever attained, in any real sense, with bacteriological plate counts. Although coefficients of variation as low as 0.01 have been reported[2] for individual counts derived from small numbers of plates, such occurrences are related to the characteristic fluctuation of the statistic with small samples and cannot be regarded as accurate estimates. Determinations based on large samples, or averages of numerous small samples, are rarely lower than our reported values.

TABLE 6

Limiting values of total error (T_p) corresponding to various percentage contributions of pipetting error*

PIPETTING ERROR PERCENTAGE OF TOTAL (p)†	METHODS OF PIPETTING‡		
	A	B	C
1	0.1419	0.1934	0.0701
5	0.0641	0.0874	0.0317
10	0.0459	0.0625	0.0227
20	0.0334	0.0454	0.0165
33	0.0270	0.0368	0.0133
50	0.0231	0.0315	0.0114

* $T_p = \dfrac{P}{\sqrt{\dfrac{p}{100}\left(2 - \dfrac{p}{100}\right)}}$.

† $p = \dfrac{100(T - R)}{T}$.

‡ Methods of pipetting:

A. Six drops delivered with capillary pipette ($P = 0.02001$).

B. One-tenth ml delivered with a 1-ml serological pipette ($P = 0.02727$).

C. Nine-tenths ml delivered with a 1-ml serological pipette ($P = 0.00989$).

Since, then, there is every reason to believe that the conclusion derived from the experimental results is valid for general application, it is clear that efforts to improve the accuracy of bacteriological plate counting methods should at present be directed elsewhere than toward increased precision of measuring aliquots into plates. Two plausible sources of the remaining variability were indicated in the introduction. One of these, the distribution of organisms in the aliquots, has received considerable attention (Fisher, Thornton, and Mackenzie, 1922; James and Sutherland, 1939; Sutherland and James, 1938; Wilson and Kullmann, 1931) and is presumably susceptible to precise mathematical definition. If this is the case, the contribution of this particular source of variability may be reduced

[2] This figure is calculated from a value given by Jennison (1937) for the standard deviation of the mean (standard error) of 5 plates, expressed as percentage of the mean.

to whatever extent desired by sufficiently increasing the number of plates inoculated. It may be assumed that the standard error of replicate plates is approximately inversely proportional to the square root of the number of plates from which it is determined. Therefore, whatever the error obtained with the conventional usage of 3 plates, for example, it may be halved by the use of 12 plates or reduced by $\frac{2}{3}$ or $\frac{3}{4}$ by the use of 27 or 48 plates, respectively. Investigators are discouraged from making full use of this relationship by the inconvenience and expense involved in the preparation, inoculation, and counting of large numbers of plates. The present study provides some encouragement in this direction, in that it justifies the use of a plating method (B) with which large numbers of plates (9 to 18) may be as rapidly and conveniently inoculated as could small numbers (3 to 5) with standard methods. In addition, preparation of the plates is facilitated because they may be prepared on a large scale at convenient intervals and stored.

The other source of variability indicated in the introduction is more difficult to assess, but it has been shown (Harmsen and Verweel, 1936–1937) that in certain cases, at least, conditions that might be expected to favor the development of the inoculated bacteria also tend to bring the plate counts into better agreement with the Poisson distribution function; hence this type of study might be used to assess the suitability of various media. It would appear that this possibility merits more extensive investigation.

In view of all these considerations it is possible to make recommendations with respect to that part of the dilution plate count procedure subsequent to the preparation of dilutions. When the growth requirements of the bacterial species permit, prepared and dried plates should be inoculated with 0.1-ml aliquots delivered by means of 1-ml serological pipettes, and the inoculum should be distributed over the surface with a sterile glass rod. This method is quite as precise as any of those studied and has the advantage that one source of variability may conveniently be decreased by approximately 42 or 59 per cent by increasing the number of plates inoculated from 3 to 9 or 18. Using method B of the present study for an example, a standard error of 4.37 per cent of the mean count is provided by 3 plates, is reduced to 2.52 per cent with 9 plates, and to 1.78 per cent with 18 plates. These figures, like all the data presented, are exclusive of any error contributed by the process of preparing dilutions. In the case of an organism apparently characterized by a larger variability, as for example *Bacterium tularense* (Snyder, Engley, Penfield, and Creasy, 1946), the reduction of the standard error would be more impressive.

It is recognized that frequently practical limitations with respect to the number of plates inoculated are set by considerations of economy instead of manipulative convenience, so that the foregoing recommendations may well be referred principally to studies in which precision is of especial importance, rather than to routine practice. Even in the latter case, however, use of the surface plating technique with the usual number of 3 to 5 plates may be recommended on the basis of saving time and effort.

The rolled tube method may be recommended in cases where strict economy of medium and of space for incubation is important. There is suggestive evidence that this method will give a higher, and therefore a presumably more accurate, count. In our opinion, however, this method is not sufficiently convenient for large-scale studies.

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SUMMARY

Five bacteriological plate counting methods were compared with respect to the magnitude and reliability of the counts obtained with *Shigella dysenteriae*.

The coefficients of variation of the five plating methods were not significantly different, even though the coefficients of variation of the three methods of measuring aliquots of inoculum into plates did differ significantly. This phenomenon was referred to the relative magnitudes of the plating errors (coefficients of variation = 0.0727 to 0.0890) and of the pipetting errors (coefficients of variation = 0.0099 to 0.0273). It was demonstrated that no significant contribution to the total error can be expected from the pipetting methods used unless the total error is considerably less than that observed in this study.

Efficient spreading of the inoculum in the case of surface plating methods significantly increased the counts obtained. The rolled tube method gave a significantly higher count than the poured plate method.

As a result of these findings it is possible to recommend more extensive use of surface plate counting methods, which are conducive to greater replication of plates.

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