Colony Counts on Strips of Agar in Tubes

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SUMMARY: For viable counting, 0.1 ml. volumes of bacterial suspension were incorporated in 1.5–2 ml. nutrient agar, which after mixing was allowed to solidify in an almost horizontal test-tube so as to produce a 3–4 in. agar strip. After incubation in the vertical position, the counts of the strips gave estimates of a bacterial population which did not differ significantly from that obtained from plates inoculated with 1 ml. quantities.

The study of bacterial populations requires a simple method for determining the viable count. The method most often used is the plate count, but with a large number of plates this method becomes rather cumbersome and expensive in equipment, medium and laboratory labour. Modifications of the plating method have been suggested from time to time with a view to reducing the amount of materials and labour involved without impairing the accuracy of the count (Esmarch, 1886; Miles & Misra, 1938). In an endeavour to find a simple method by which colony counts might be made, inoculation of small quantities of agar in test-tubes was tried.

EXPERIMENTAL

Preliminary trials were carried out to determine the most suitable quantity and concentration of agar to use in each tube, and the best methods of mixing and of forming the agar strips. In the technique finally adopted $6 \times \frac{1}{8}$ in. test-tubes were used, each containing 1.5–2 ml. of 2 % nutrient agar. The agar was melted and held at 45° for $\frac{1}{2}$ –1 hr. before it was inoculated. This allowed condensation water to run down into the agar, thus lessening the amount of moisture appearing at the foot of the tubes during incubation. Much moisture encouraged spreading growth, especially when the count was high.

A 1 ml. pipette graduated in 0.01 ml. was used to deliver 0.1 ml. of inoculum into each tube.

The agar and inoculum were mixed by rotating the tube five times, and the tube was then laid on the bench with the plugged end slightly raised so as to produce a layer of agar 3–4 in. long. When necessary the tubes were slightly tilted when being laid down to ensure that the agar flows evenly over the glass. The colonies that developed were counted with greater ease when the strip of agar was of good length. The agar solidified rapidly and when the tubes were cool they were set upright in racks and placed in the incubator.

Experiments were carried out to provide material for a statistical analysis, using a suspension of the spores of *Bacillus megatherium* which was stable and gave reproducible results.

Exp. 1. This experiment was designed to compare the counts obtained in tubes with those obtained by the standard plating method in which 1 ml.

quantities of spore suspension were mixed with approximately 10 ml. of nutrient agar in Petri dishes.

Six workers each inoculated 120 tubes and ten plates; each worker used one pipette for the inoculation of both tubes and plates. In order to detect any differences due to pipetting 0.1 ml. quantities the tube inoculations were divided into three groups:

(a) Forty tubes were inoculated with consecutive 0.1 ml. amounts.

(b) Forty tubes were inoculated with alternate 0.1 ml. amounts, i.e. 1st, 3rd, 5th, 7th and 9th 0.1 ml. amounts.

(c) Forty tubes were inoculated with the 1st and 6th amount of 0.1 ml. from each pipetteful. In (b) and (c) the unused quantities were discarded into a sterile tube.

Table 1. *Mean colony counts and standard errors obtained by plate and strip counts*

Worker ...	A	B	C	D	E	F	All workers
Mean count in 10 plates	302.6 ± 7.5	288.8 ± 4.7	295.5 ± 9.6	302.8 ± 8.7	296.4 ± 5.4	312.7 ± 5.2	299.8 ± 2.9
Mean count in 120 tubes (× 10)	312.0 ± 5.4	299.2 ± 5.7	298.2 ± 5.0	309.6 ± 6.7	296.7 ± 5.1	290.9 ± 4.8	301.1 ± 2.2

The mean count for all the plates (Table 1) (1 ml. inoculum) was 299.8 ± 2.9 , and that for tubes (× 10, since in this case the inoculum was 0.1 ml.) was 301.1 ± 2.2 . These counts do not differ significantly.

Table 2. *Analysis of variance of strip counts of experiment recorded in Table 1*

Method of inoculation	Factors	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Consecutive 0.1 ml. amounts	Position of 0.1 ml. amounts in the pipette (<i>P</i>)	9	880.0	97.8	2.77*
	Interaction of workers and positions (<i>W</i> × <i>P</i>)	45	1,504.6	33.4	0.95
	Pipettefuls (<i>Pp</i>)	18	820.3	45.6	1.29
Alternate 0.1 ml. amounts	<i>P</i>	4	188.9	47.2	1.34
	<i>W</i> × <i>P</i>	20	855.8	42.8	1.21
	<i>Pp</i>	42	900.2	21.4	0.61
1st and 6th 0.1 ml. amounts	<i>P</i>	1	82.8	82.8	2.35
	<i>W</i> × <i>P</i>	5	348.0	69.6	1.97
	<i>Pp</i>	114	4,395.0	38.6	1.09
Combined results	Workers (<i>W</i>)	5	437.0	87.4	2.48†
	Methods (<i>M</i>)	2	137.1	68.5	1.94
	Interaction (<i>W</i> × <i>M</i>)	10	401.7	40.2	1.14
	Error	433	15,277.6	35.3	—
	Total	708	26,228.5	—	—

* Significant at the 1 % level.

† Significant at the 5 % level.

The results of the tube counts were subjected to an analysis of variance (Table 2) to ascertain the accuracy of the technique. The variance ratio calculated from the combined results of the six workers indicated a significant difference between workers at the 5 % level, which may have been due to inexperience with the method. There was no significant difference between the various pipettefuls measured or between the three methods of inoculation, but counts from consecutive 0.1 ml. inocula differed significantly at the 1 % level when the results for all workers were combined, although not when the results were examined separately for each worker. The discrepancy with the consecutive 0.1 ml. amounts appeared to be due mainly to the last 0.1 ml. delivered by each pipette, which in most cases gave a higher mean count than the remainder, as is shown in Table 3. This higher count may have been due to the slowness of

Table 3. *Mean counts of consecutive 0.1 ml. amounts delivered by 1 ml. pipettes*

Successive 0.1 ml. amount ...	1	2	3	4	5	6	7	8	9	10
Mean count	31.25	28.92	29.79	29.79	29.58	28.17	26.46	30.08	27.88	33.88

delivery of the pipette which was obvious with the last 0.1 ml. volume, or to the difficulty in seeing the graduation mark, which came within the test-tube. This source of error could be avoided by not using the last 0.1 ml. quantity. The seventh 0.1 ml. quantity appears to be somewhat smaller than the others, though the divergence is not so marked as with the tenth. A check of the pipettes by weighing the quantities delivered did not reveal any peculiarity of the seventh 0.1 ml. volume.

The value of χ^2 for tube counts, calculated by dividing the error sum of squares of the analysis of variance (Table 2) by the mean count for all tubes, was 507.4 with 433 degrees of freedom. This is significantly large at the 1 % level. Further examination revealed, however, that this high value was almost entirely due to the variable counts obtained by one worker, for which there may have been some special reason. When the results for this worker were omitted the value of χ^2 was 388.4, which with 363 degrees of freedom is not significant. There is thus no indication that the counts obtained from tubes deviated significantly from the expected Poisson distribution. A given number of tubes did not, however, give as reliable a result as the same number of plates, the coefficient of variation for plates being 7.3 % and for tubes 19.9 %. This was thought to be due to the number of colonies counted in plates having been larger than in tubes, so a second experiment was designed to show the influence of colony numbers.

Exp. 2. In this experiment two dilutions of spore suspension were used, one ten times as concentrated as the other. Two workers each inoculated twelve plates with 1 ml. quantities of the higher dilution and 120 tubes with each dilution, using consecutive 0.1 ml. amounts of inoculum. The number of colonies developing on a plate should therefore be the same as the number developing in a tube inoculated with the lower dilution and thus permit a direct comparison of the two methods.

Table 4. *Mean counts in plates and strips, and the observed and expected coefficients of variation (Exp. 2)*

Mean count			Coefficient of variation	
			Observed (%)	Expected (%)
12 plates	Worker 1	75.25 ± 2.46	11.3	11.5
12 plates	Worker 2	76.25 ± 2.37	10.8	11.4
Low dilution				
120 tubes	Worker 1	74.99 ± 0.89	13.0	11.6
120 tubes	Worker 2	72.08 ± 0.75	11.3	11.8
High dilution				
120 tubes	Worker 1	7.77 ± 0.24	33.3	35.9
120 tubes	Worker 2	7.62 ± 0.24	33.8	36.2

For the same mean count there was no significant difference between the plate method and the tube method (Table 4). The means given by the two workers were in good agreement. The observed coefficients of variation agree well with their expected values. In only one case, namely that of worker 1 with the 'low dilution' tubes, was the observed coefficient of variation greater than or appreciably different from expectation. An analysis of variance showed no significant difference between counts from different 0.1 ml. quantities or from different pipettefuls.

This experiment indicates that one tube inoculated with a 0.1 ml. quantity can replace one plate inoculated with a 1 ml. quantity, provided the resulting mean colony counts lie at the same level.

DISCUSSION

The inoculation of agar in tubes in the manner described above is simple, and enables many replicate counts to be made with little expenditure of time, labour or materials. The method has advantages over roll-tube methods in that it permits easier counting and decreases the amount of manipulation. It also compares favourably with surface-counting methods (Snyder, 1947; Crone, 1948) where plates must be prepared beforehand and dried, and where considerable amounts of medium are required. There are, however, several points which require consideration.

The concentration of agar used. The accuracy of the count is diminished when the agar slips within the tubes during incubation. This can be avoided by using a suitable concentration of agar. With the several media and incubation temperatures that have been used, 2% agar gave a sufficiently large margin of safety without inhibiting bacterial growth. This concentration might have to be altered to suit special conditions.

The glassware. Since the quantity of agar in each test-tube is small, the tubes must be perfectly clean; any trace of an antibacterial substance might diminish the count or delay the appearance of colonies.

The inoculation. It is generally accepted that the sampling error is apt to become very large when volumes less than 1 ml. are measured, but our results

suggest that this need not be so. With normal care, the error introduced by the measurement of 0.1 ml. from a 1 ml. pipette is cancelled by other errors that are inherent in any plating method. Snyder (1947) found that 0.1 ml. delivered from a capillary dropping pipette was more accurate than 0.1 ml. from a 1 ml. pipette, but concluded that there was no appreciable contribution from the pipetting error unless the total error of the method was very small. One method recommended by Snyder was studied in more detail and analysed statistically by Crone (1948). Counts obtained by spreading 0.1 ml. inocula, measured by a capillary pipette or a 1 ml. graduated pipette, over the surface of previously prepared plates, were shown to conform within the limits of experimental error to a Poisson distribution, and the total count from all the plates agreed well with that obtained from a corresponding number of roll-tube cultures. No large error therefore appears to have been introduced in the measurement of 0.1 ml. inocula. Miles & Misra (1938) used a capillary pipette for the seeding of 0.02 ml. drops on agar plates. Greater accuracy of measurement of the inoculum could undoubtedly be achieved by the use of calibrated dropping pipettes, but for the inoculation of tubes the 1 ml. pipette is easier to handle, and provided that the last 0.1 ml. quantity is not used, there seems little disadvantage in the use of the 1 ml. pipette. Pipettes with good points and a suitable rate of delivery were used in our experiments and were checked by weighing the successive 0.1 ml. amounts delivered. Statistical analysis showed that there was no great error introduced by measuring consecutive 0.1 ml. amounts, and that the results obtained conformed to the expected Poisson distribution.

Counting of the colonies. Colonies developing within the agar can be counted in the lateral illumination box used for counts of Petri dishes. The lower groove should be slightly notched close to the glass in order to support the base of the tube; the opposite groove should be elongated to accommodate the upper part of the tube. In this way the tube is held in position and can be rotated slightly if required. A thin film of agar (a very small fraction of the total) is produced on the sides of the tube during mixing, but any colonies that develop on this are readily detected.

As with the plate count, the coefficient of variation (Table 4) rises rapidly as the mean count decreases, e.g. with a mean count of 72.08 the coefficient of variation was 11.8 % and with a mean count of 7.62 it was 33.8 %. This indicates that the number of colonies should be as large as possible without introducing any error due to overcrowding. It is suggested that the number of colonies counted in a tube should lie between 30 and 300. The maximum will depend on the type of colony that is being counted. Counts of 250–300 have been made quite readily and as many as 400 with small streptococcal colonies. The surface-seeded drop count recommended by Miles & Misra appears to have a more limited range, the maximum count quoted being 100. Fivefold instead of tenfold dilutions may be used if required to obtain a convenient count, but in all our work this was unnecessary.

Application of the method. The agar-strip method has now been used extensively in studying the growth of pure cultures of various bacteria and has

proved satisfactory. With *Bacillus subtilis* the spreading growths did not interfere with counting to a greater degree than they did in the usual plating method. The suitability of the method for the examination of mixed populations remains to be tested. Fungi that produce rapidly spreading growths and bacteria which form mucoid colonies that tend to flow, may give rise to greater difficulties in tubes than they do in Petri dishes.

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