THE COUNTING OF SURFACE COLONIES OF BACTERIA

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One of the several ways of performing colony counts is to spread a known volume of a bacterial suspension on the surface of an agar plate. This method has probably been used by many workers at various times, and Snyder (1947), comparing it with other methods, recommends it. It has not, as far as I am aware, been analysed statistically as Fisher, Thornton & Mackenzie (1922) analysed the pour-plate method or as Miles & Misra (1938) analysed their own method. In this paper the surface method is analysed and notes added on the effects of varying the culture medium and the care then necessary in the selection of the diluting fluid.

In contrast with the pour-plate and roll-tube methods the surface-counting method can be used with opaque media. The colonies of different bacterial species can be counted separately, and contamination by colonies of a different species to those sought is therefore of little importance. Moreover, nicer colonial differences can be distinguished than is usually possible in the case of deep colonies. Since melted media are not used when the plate is inoculated the method is cleaner and certainly quicker than the pour-plate and roll-tube methods. Further, both the latter methods are made much more difficult if media that cannot be remelted are used. Undoubtedly its main advantage over most other methods is that counting of the developed colonies is more certain and more comfortable and therefore, more accurate. In regard to the method of Miles & Misra these particular advantages do not apply, but, when the approximate count is known, it gives greater precision for the same expenditure of medium. Dropping pipettes are not needed and it can therefore be carried out with the apparatus found in any bacteriological laboratory.

The method has one serious disadvantage, namely, that if a colony, too small to be noticed, has already grown on the plate, it may be spread widely with the suspension and may prevent counting, though it does not necessarily do so. The frequency of such grave contamination with the complete loss of a plate depends on the medium used, the age of the plate and the care taken when pouring it. When blood or plain agar is used and a coliform suspension is being counted such a contamination may occur perhaps once or twice in a hundred plates.

EXPERIMENTAL METHOD

Of the suspension to be counted 0·1 ml. is slowly expressed from a delivery pipette fitted with a rubber teat on to the surface of an agar plate as this is rotated once with the other hand. In this way the fluid is made to form an almost complete circle two-thirds of the diameter of the plate. Self-made and calibrated capillary pipettes have usually been used but 1 ml. graduated serological pipettes can be, and have been occasionally, used. The fluid is spread with a nichrome wire, 10 cm. long, bent for the terminal 4 cm. to an angle of 150° with the holder. The plates should be dry to such a degree that the fluid is absorbed within 5 min. of spreading. In the laboratories in which this work has been done, this degree of dryness has been reached by a routine stock of prepared plates. In this way suspensions of broth cultures of coliform organisms and of faeces have been counted. Unless faecal suspensions contain less than 1 g. in 10 ml. the detritus interferes with colony growth and hence with counting. When there are fewer than 30 colonies on a plate counting is easy. Between this number and 200, marking the surface of the agar into irregular segments, avoiding individual colonies, makes counting easy and accurate. Up to 500 colonies may be counted, but counting so many colonies becomes tedious. If a large number of colonies is present a plate microscope is helpful though it is not necessary, at least for colonies of coliform organisms, if less than 100 are present.

The plates are counted after 24 hr. incubation. The increase in count of *Salmonella typhi* on blood agar plates was found to be less than 2% after a further 24 hr. incubation. On Wilson & Blair's medium the increase is about 6% but there is no increase in counts on Leifson's medium.

In Table 2 are included results with faecal suspensions and with cultures of coliform organisms at various ages in pure or in mixed culture, the rest of the results (with one exception) were obtained with a 24 hr. culture in tryptic digest broth of a strain of *Salm. typhi* recently isolated from a chronic carrier.

In most experiments the broth culture was serially diluted about one in 500,000 with quarter strength Ringer's solution. Of the final suspension 0·1 ml. contained sufficient organisms to form between 100 and 200 colonies.

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RESULTS

Internal consistency. The counts on replicate plates will not in general be the same. It is to be expected that they will be in a Poisson distribution. Table 1 gives the expected and observed distribution in two experiments with a specially dilute suspension.

Table 1. *Comparing the expected with the observed number of colonies developing on thirty plates in each experiment sown with* 0·1 *ml. of a suspension of* Salmonella typhi

	No. of colonies on plate				
	0	1	2	3	More than 3
Exp. 1					
Expected	17·6	9·4	2·5	0·4	0·1
Observed	17	10	3	0	0
Exp. 2					
Expected	10·3	11·0	5·9	2·1	0·7
Observed	9	14	5	1	1

table, a function of the total variability is compared with its expected value. Twice the variability exceeds the expected value and once falls short but never significantly. Finally, the results are pooled and, again, the expected variability is not significantly exceeded. The statistical tests of consistency are therefore satisfied, for the variability of counts does not significantly exceed or fall short of that which the nature of the method dictates. In consequence, the standard error of a count is equal to the square root of the number of colonies counted and the usual tests of significance of means, differences and sums may be applied.

Plating medium. It is to be expected that the medium on which the suspension is spread will affect the count, though this need not be an important factor with an organism of simple requirements. An *aerogenes*-like coliform organism was counted on sets of five plates containing: (1) phosphate buffer in distilled water solidified with agar; (2) Koser's citrate solidified with agar; (3) the same to which glucose was added; and (4) blood horse muscle digest agar. The counts were 156, 178, 167 and 169 respectively. The variance within sets exceeds that between them though, according to Fisher's z test, quite insignificantly.

But the results with the special media would be more pertinent. Three questions might be asked of these results: (1) Are counts within sets of the same batch and one age of all media consistent? (2) How do batch and age affect the count? (3) How do counts on different media compare? To try to answer these points a suspension of *Salm. typhi* was counted on blood agar, Leifson's medium, and Wilson & Blair's

Table 2. *The expected and observed numbers of sets of plates of the same* χ^2 *range and a function of the sum of* χ^2 *compared with its expected value*

No. of sets of five plates		No. of sets of six plates		No. of sets of three plates	
Expected	Observed	Expected	Observed	Expected	Observed
0·87	0	0·64	2	0·71	0
0·87	1	0·64	2	0·71	0
2·61	4	1·92	2	2·31	2
4·35	2	3·2	2	3·55	7
8·70	8	6·4	5	7·1	3
8·70	12	6·4	3	7·1	6
17·40	17	12·8	14	14·2	20
17·40	17	12·8	13	14·2	11
8·70	10	6·4	6	7·1	7
8·70	2	6·4	4	7·1	8
4·35	7	3·2	4	3·55	4
2·61	3	1·92	7	2·31	2
0·87	3	0·64	0	0·71	1
0·87	1	0·64	0	0·71	0
$\sqrt{(2 \times \text{total } \chi^2)}$	$=27{\cdot}26$		$=25{\cdot}95$		$=16{\cdot}68$
$\sqrt{(2 \times \text{degrees of freedom} - 1)}$	$=26{\cdot}36$		$=25{\cdot}27$		$=16{\cdot}87$

$\sqrt{(2 \times \text{total } \chi^2 \text{ of all columns})} = 41.16$
$\sqrt{(2 \times \text{degrees of freedom} - 1)} = 40{\cdot}24$

When, as is usual, only a few counts are made of each suspension such a comparison is not instructive. Instead, the observed distribution of variability within many sets of plates can be compared with the expected (Fisher, 1944).

In Table 2 such a comparison has been made for all the sets of this work except six.* In the same

* Six sets have been omitted from this table and the omission needs justification. All six sets were on special media for the isolation of the intestinal pathogens and were prepared on a single day. One set had no colonies, two had a normal degree of variability and the remaining three had individual plates with no colonies and others bearing 12, 15 or 31. The last will be referred to later.

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medium. Three sets of each medium, differing in age and batch, were used and each set consisted of five plates.

Question (1) is partly answered by the first pair of columns of Table 2 where sets of all media are included, but it is instructive to pick out those of this experiment, distinguish the media, and compare the observed with the expected variability. For the same suspension but with the two special media only plates of the same batch and age do so.

Can this undue variability be ascribed to age or batch exclusively? Old plates of Wilson & Blair's medium give larger counts but even allowing for this a significant amount of variability remains. Age is of relatively less importance for plates of Leifson's medium, but all the variability cannot reasonably be ascribed to batch differences (Table 5). Both the special media were made by adding the recommended solutions to the nutrient base. To seek a possible and simple cause for the excessive variance between batches the amounts of these solutions were varied. Table 6 shows that increasing the amount lowers the count but that this is most marked on Leifson's medium, a finding parallel to the greater batch variability of this medium in comparison with Wilson & Blair's medium as shown in Table 5.

Regarding question (3) it has been an invariable finding that more colonies of *Salm. typhi* have developed on blood plates than on either of the special blood agar and Leifson's medium variability exceeds the expected, and for Wilson & Blair's medium falls short, but in each case by an insignificant amount. Counts on all three media are consistent, therefore, for sets of the same batch and age (Table 3).

In regard to question (2), on blood agar the variance between sets of different age and batch does not significantly exceed that within sets. This is not true for Leifson's and Wilson & Blair's media (Table 4). In other words, any blood plate (of this experiment) gives substantially the same count when spread with

Table 3. *Comparing the expected with the observed variance of counts, on sets of plates of the same age and batch*

	Blood	Leifson's medium	Wilson & Blair's medium
Variance			
Expected	42·0	29·2	27·5
Observed	50·0	30·7	23·5
Probability of difference as great or greater:			
Less than	0·7	0·7	0·3
Greater than	0·5	0·5	0·2

Table 4. *Analysing the total variance into that between and that within sets of different age and batch*

	Blood	Leifson's medium	Wilson & Blair's medium
Variance between sets	109·1	161·9	162·3
Degrees of freedom	6	4	4
Variance within sets	50·0	30·7	23·5
Degrees of freedom	36	24	24
Fisher's z	0·39	0·83	0·97
Probability of this value being exceeded:			
Less than	—	0·01	0·001
Greater than	0·05	0·001	0·001

Table. 5. *Apportioning the variance between sets to different causes*

	Leifson's medium			Wilson & Blair's medium		
	'Batch'	'Age'	'Within sets'	'Remainder'	'Age'	'Within sets'
Variance	784	288	34·2	124	201	23·5
Degrees of freedom	1	1	16	2	2	24
Fisher's z	1·56	1·06	—	0·83	1·07	—
Probability of this value being exceeded:						
Less than	0·001	0·05	—	0·05	0·01	—
Greater than	—	0·01	—	0·01	0·001	—

Table 6. *Showing the colony counts on media of different strengths*

	Amount of solutions added to nutrient base expressed as a factor of the recommended amount		
	1·4	1·0	0·7
Leifson's medium	236	431	529
Wilson & Blair's medium	603	652	710
Blood agar plates	—	827	—

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media whether suspensions of faeces from carriers of the organism or dilutions of pure broth cultures were plated. Since the special media show considerable batch differences in the number of colonies becoming visible no significance attaches to the ratio of colonies on the various media actually observed in these experiments. However, it may be noted that for every four colonies developing on blood plates about three have done so on Wilson & Blair's medium and rather less than this on Leifson's medium.

Comparison with other methods of counting. Throughout the working day a suspension of *Salm. typhi* was counted by the spread-plate and roll-tube methods, five of each being sown on each occasion. The same batch of nutrient agar was used for all plates and tubes. Since the count declined by less than one-fifth and the methods were used alternately the order of sowing seems immaterial. Both plates and tubes were counted after 24 hr. incubation at 37° C. There were 3400 colonies counted by the former method and 3244 by the latter. In another experiment, 1584 and 1538 colonies respectively were counted. In a third, on five blood agar plates there were 827 colonies and in nutrient agar in roll tubes 679. Snyder (1947) got different results, his roll-tube counts exceeding the spread plates but he spread his plates in some experiments by tilting and in others with a glass spreader. However, he did not get a considerable excess.

Though some fluid, and presumably a number of organisms, is removed by the spreading wire, the count is on this account only slightly reduced or not at all. The fact that, in this work, the spread-plate count was the larger need not be disturbing. G. S. Wilson (1922) found, and T. L. Snyder (1947) has confirmed, that the roll-tube count exceeds that of the pour plate and in a similar way the spread plate may provide even better conditions for the development of visible colonies than the roll tube.

Diluting fluid. Some erratic results on the special media have been noted above (see footnote, p. 427), and they have also been found on a few other occasions. This observation suggested that although colonies were still able to develop on blood, on the special media they did so erratically or not at all. It is easy to show that exposure to certain batches of quarter strength Ringer's solution reduces the count on the special media more rapidly than on blood agar (Table 7).

In 1¾ hr. the count fell to less than one-fifth on Leifson's medium. In 15 min., the time sometimes taken in performing a dilution, it will fall by about 20% and therefore significantly. Other batches of diluent have not been so damaging. Raising the temperature accelerates the damaging effect. After 10 min. in diluting fluid at 47° C. organisms produced no colonies on Leifson's and Wilson & Blair's media though 53 developed on blood agar plates. A sample of the same suspension kept for the same time at room temperature gave counts of 103, 128, and 167 respectively on the three media.

Table 7. *Comparing the decline in colony count on two different media sown at intervals with a suspension of* Salmonella typhi

Time of day ...	10.45 a.m.	12.30 p.m.	2.15 p.m.	4.00 p.m.	5.45 p.m.
Count on blood agar	522	500	204	65	26
Count on Leifson's medium	277	48	17	8	4

DISCUSSION

The colony-counting method described gives consistent results on the same plating medium. Using blood agar plates, age and batch differences have little influence on the counts. This is good, though not a sufficient, reason to recommend it. Its superiority over other methods can only be judged by experience and depends to some extent on ease in recognition and comfort in counting which cannot be readily communicated in a paper. Irregularity in the volume of fluid removed by the wire which spreads the suspension does not seem to add appreciably to the variability of the counts, nor, if carried out in this way, are they lower than, for instance, roll-tube counts.

With suspensions which have been prepared for less than 5 min., counts of enteric and food poisoning organisms are only slightly lower on the special media used for the isolation of intestinal pathogens than on blood agar. In consequence, the method may be used for rough enumerations of these organisms in faeces. The deformation of colonies by detritus and consequent inability to recognize them sets, however, a limit on the use of the method, and also indicates that direct plating of faeces is not likely to yield positive results with fewer than 100 pathogens in 1 g. It is necessary to use the same batch of the special medium or an undue variability will be introduced into the counts. Moreover, the suspension should be fresh; if it has stood at room temperature for up to 2 hr., the count on the special medium may be as little as one-tenth that on blood agar plates.

SUMMARY

A colony count performed by spreading a known volume of suspension on the surface of a plate possesses certain advantages over other methods, of which the chief is ease of counting.

The distribution of counts of several samples of the same suspension on medium of the same age and batch is as expected on statistical grounds.

Different batches and ages of blood plates may give substantially the same count but this is not true for Leifson's and Wilson & Blair's media.

A diluting fluid, which is satisfactory if blood agar is the plating medium, may not necessarily be so if special media are to be used.

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