Viable Counts of Bacteria—a New Method for Facultative Anaerobes

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SUMMARY

Large dilutions of bacterial suspensions were made at 45° in a buffered complex medium containing agar. The tubes of medium with the serial dilutions of bacteria were solidified by cooling; subsequent incubation at 37° permitted bacterial growth in discrete colonies. The density of colonial growth was enumerated by comparison with a set of prepared standards calibrated by the pour-plate technique. A large range ($\log_{10}$ 2·5 to 10·5) of colony counts was covered in only four test tubes without any prediction of the approximate count. The method has advantages of speed, economy of reagents and apparatus, as well as range and precision.

INTRODUCTION

There are many methods for the assessment of the number of viable bacteria in a population (Meynell & Meynell, 1965). In direct methods, a suspension containing a suitable number of bacteria is transferred to a solid nutrient medium which, under suitable conditions, permits each viable bacterium to grow to a colony. Direct count of the colonies and reference to the dilution of the original suspension yield the colony count. This type of method is generally satisfactory, but effort wasted in plating unsuitable dilutions can only be avoided to the extent that the order of magnitude of the result is predictable. The present paper describes a radically new approach which has advantages over other methods. Without loss of accuracy, it is more economical in the use of media and apparatus (especially graduated glassware), does not require a prediction of the colony count of the unknown, and does not demand the error-prone and tedious counting of thousands of colonies or mathematical processing of results. The method has only been applied to facultative anaerobes, cannot be used for aerobes, and would probably require modification for use with obligate anaerobes.

METHODS

Bacterial strain and media. *Escherichia coli* ATCC 11229 was grown on an orbital shaker (L.H. Engineering Co., Bells Hill, Stoke Poges, Bucks.) at 37° in a defined medium containing (g./l.): glucose, 12; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0·4; KH_2PO_4 , 5·4; $(\text{NH}_4)_2\text{SO}_4$, 1·2; NaOH to adjust to pH 7·2. Pour-plates for colony counts were made in nutrient agar (Oxoid Ltd., London). Colony counts by the method described in this paper were done in a medium containing (g./l.): tris (2-amino-2-hydroxymethyl-propane-1,3-diol; Sigma, London, Ltd.), 2·017; maleic acid (British Drug Houses lab. reagent), 1·93; peptone (Difco, Baird and Tatlock (London) Ltd.), 30; yeast extract (Difco), 50; agar (Oxoid ionagar no. 2), 6; adjusted to pH 7·2. This medium was sterilized at

108° (5 lb./sq.in. pressure of steam). This buffered complex medium (BCM) was normally stored at 4°, melted in boiling water and 5.9 ml. portions transferred into sterile 150 × 16 mm. rimless tubes which were then stored in water baths at 48° up to 18 hr. It was convenient to cap the test tubes with anodized aluminium caps (Oxoid) and to dispense the molten medium with an automatic syringe pipette (Froud and Sons Ltd., Dalston, London). Where undesigned, chemicals were British Drug Houses Ltd. Analar grade.

Apparatus. Transfers of 0.1 ml. volumes were done with special pipettes (H. J. Elliot Ltd., Pontypridd, Glamorgan) which had been made water-repellent by a silicone treatment. This consisted of immersion in a 1% (v/v) solution of SiCl_4 (M 441, Imperial Chemical Industries Ltd.) in carbon tetrachloride, followed by baking at 110° for 1 hr. Each pipette was fitted with a plastic teat which was controlled by the stainless steel lever carried in the brass pipette holder. The whole arrangement and the manner of operation are shown in Fig. 1. For assembly, the pipette is fitted with a teat and placed in the bottom part of the holder, the lever is located in its slot and the top part pushed home until the whole is firmly held together by friction. The dimensions and arrangements of the holder are not critical, but it should be reasonably light and fit conveniently to the hand. Above all, the lever should permit a high degree of sensitivity with an absolutely positive control.

Fig. 1. Arrangement and operation of pipette assembly.

To heat pipettes in boiling water it was found convenient to use a small instrument sterilizer (McCarthy's Hospital Equipment, Romford). After transfer to diluent, bacterial suspensions were mixed on a Vortex mixer (Scientific Industries International (U.K.), Ltd.). The growth in the tubes was compared visually under indirect illumination, and it was found convenient to house the set of standards in a viewing-box. The dimensions are not critical and depend on the number of standards

employed. A box which carried 24 standard tubes was 42 in. $\times$ 8 in. $\times$ 14 in. over-all and was illuminated from below by a 3 ft. 30 W fluorescent tube.

Colony count by pour plates. Serial dilutions for pour plates were made by transfer of 1 ml. portions in serological pipettes to 9 ml. sterile water in capped 150 $\times$ 16 mm. rimless tubes. A fresh pipette was used for each dilution and was rinsed three times in both suspension and diluent. Triplicate 1 ml. portions of appropriate dilutions were transferred to sterile, disposable, plastic Petri dishes (Oxoid), thoroughly mixed with about 10 ml. nutrient agar at 48°, allowed to set, and incubated at 37° for 24 hr. The plates were then set against a dark background and the discrete colonies counted. The count was obtained from this value by reference to the serial dilution used.

Colony count by tube dilution. Normally, four 0.1 ml. pipettes were used. Of these, one was in use, two were suspended so that the glass parts were in the sterilizer of boiling water while one was cooling after boiling. A single pipette was used to select the 0.1 ml. sample, transfer it to 5.9 ml. BCM and, after mixing, for successive transfers. The pipette was rinsed in the boiling water between transfers and four tubes of BCM were normally employed although, with *Escherichia coli*, a culture not showing visible turbidity could be assessed in three tubes. When the dilutions were completed the pipette was suspended in the boiling water and the tubes of BCM were placed in cold running water to solidify. With *E. coli* incubation at 37° overnight was usually sufficient to permit maximal colony development, but 40 hr was required for some strains of *Staphylococcus aureus* (unpublished results). Each set of tubes was then compared with the standards. The $\log_{10}$ count was computed by reference to the dilution (1/60, 1/60², 1/60³ or 1/60⁴) of the sample and the colony count of the standard with which it was matched.

The standard tubes were prepared so that each tube differed from its immediate neighbours by $\log_{10}$ of 0.1. Since the standards were used over a range much greater than can be directly counted, a method for their preparation was devised. An overnight culture of *Escherichia coli* was diluted 10^{-5} in distilled water and two further dilutions of $10^{-0.1}$ and $10^{-0.2}$ made from this by dilutions of 200/252 and 200/317 respectively. These primary standards were then further diluted by successive factors of $10^{-0.3}$ by adding 100 ml. to 100 ml. sterile distilled water. Each bacterial suspension was assessed for colony count by the pour-plate technique applied in triplicate and was also used to inoculate 24 tubes of BCM. The 'true' count of each member of the dilution series was calculated from *all* of the pour plates and, as this depended on 166,428 colonies selected by 66 independent processes, presumably the sampling error was minimal. There were 24 tubes available for the calibration series at each bacterial concentration. To select the 'best' tube at the most concentrated end of the series, each set of 24 tubes was first graded visually according to density. The middle three tubes (two as spares) from each set were selected and preserved by adding 1 ml. 40% (w/v) formaldehyde (May and Baker Ltd., Dagenham). After standing for 1 week the formaldehyde was changed and the tubes sealed by drawing out in a gas flame. At the lower end of the scale, tubes were selected which were shown by direct count to contain the 'correct' number of colonies 'randomly' distributed in the medium.

Some practice is required in matching an unknown tube to the set of standards on the basis of an over-all visual impression of colony size as well as density. It is the diminution in size of individual colonies, as their number increases, which allows the use of a set of standards covering more than a 100-fold range. Once an unknown tube

has been matched to a standard, the colony count of the culture from which the unknown has been derived is easily calculated from a knowledge of the number of 1/60 dilutions made and the known colony content of the standard. In practice it has proved convenient to construct a table from which the colony count of the unknown is read off directly by reference to the position of the standard in the calibration series and the number of dilutions made.

RESULTS

The validity of the calibration was tested by determination of colony counts with both the pour-plate and tube-dilution methods. Several suspensions of *Escherichia coli* 11229 were prepared by dilution of an overnight culture in water and were deliberately chosen to cover a very wide range of concentrations. The results are shown in Fig. 2. The reproducibility of pour-plate and tube-dilution methods was determined over a large range of bacterial concentrations. Both methods were used to make 10 replicate counts on each of several suspensions; a statistical analysis of the results is given in Table 1.

Fig. 2. Comparison of viable count of suspension of *Escherichia coli* by pour-plate and tube-dilution methods.

DISCUSSION

All colony-counting procedures are subject to the errors inherent in sampling a population. This inescapable inaccuracy in sampling is frequently expressed as standard error which is the square root of the number sampled. Clearly, sampling

Table 1. *Colony counts of Escherichia coli by pour-plate and tube methods*

Overnight cultures were diluted in sterile water and ten replicate counts made of each by both counting methods.

Plate count		Tube count	
Mean (log ₁₀)	Standard deviation	Mean (log ₁₀)	Standard deviation
8.480	± 0.157	8.402	± 0.059
8.371	± 0.056	8.392	± 0.053
8.023	± 0.107	8.035	± 0.154
7.845	± 0.077	7.817	± 0.022
7.747	± 0.098	7.755	± 0.041
7.715	± 0.162	7.662	± 0.056
4.487	± 0.071	4.455	± 0.069
3.685	± 0.034	3.632	± 0.089
3.444	± 0.045	3.510	± 0.032
2.926	± 0.087	2.983	± 0.022

error, as a percentage of the number sampled, decreases as the number sampled increases. Thus a sample of 50 must have a standard error (S.E.) of about 14% while 100 counts have 10% S.E., 200 counts about 7% S.E., 400 counts about 5% S.E. and 800 counts about 3% S.E. In addition, the actual errors are increased by imperfections in technique so that the

$$\text{total error} = \sqrt{[(\text{technical error})^2 + (\text{sampling error})^2]}$$

(Jennison & Wadsworth, 1940). The data presented here show that the tube-dilution method was somewhat more reliable than the pour-plate technique. However, the count of the 'unknown' suspensions was known sufficiently well to enable the pour plates to be prepared with a final colony count of between 100 and 400. In laboratory practice it is not always possible to do this and the precision of pour plates decreases considerably when fewer colonies are counted. In the tube-dilution method, however, the lowest count which is assessed is usually 600, being 1/60 of the maximum concentration included in the calibration series. Of course the tubes at the higher end of the calibration series do not contain the stipulated density of colonies because of coincidence. The method of calibration allows for this, as is shown by the excellent correlation between tube-dilution and pour-plate methods in Results. In addition, the number of dilutions used is less in tube counts and consequently the technical error should also be less.

As compared with the pour-plate technique, the combination of larger sampling numbers and fewer dilutions increases the accuracy of the tube-dilution method. However, its real advantages lie in convenience. The technique has four main advantages: (i) it is economical in use of reagents and apparatus; (ii) it is rapid and easy in operation because it relies on one sampling procedure and four transfers only; (iii) it is automatic in use in that it does not demand a decision as to which dilutions to plate and, in work on activity of antibacterials where the count can vary over a wide range, this may well be the greatest single advantage; (iv) the assessment of a count is simple, since the considerable effort of counting individual colonies is stored in the calibration series and can be used again and again.

There are two main disadvantages in the method as described in this paper. First, the lower end of the range is restricted by the size (0.1 ml.) of the sample taken to the

first tube. Obviously any count of less than 10/ml. is going to give less than 10^2 colonies in the tube used for matching, with consequent increase in sampling error. This factor was introduced deliberately, as the method is currently being used to assess the effect of synthetic antibacterial compounds on culture viability, and a large dilution is required to extinguish the effect of drug carried over to the counting medium. However, when there is no such obligation to dilute out toxic material, it is quite feasible to increase the sample to 1 ml. (decreasing BCM to 5 ml.) which improves the range tenfold.

The second disadvantage is imposed by the type of vessel used for the culture of the counting medium. This limits aerobiosis and prevents the method's application to strict aerobes. Nor has any effort been made to count strict anaerobes. Even with facultative anaerobes only a small number of species has been counted. These include some of the enteric bacteria (species of *Escherichia*, *Salmonella*, *Shigella*) and many strains of *Staphylococcus aureus* (unpublished results). There is probably little restriction on its use for facultative anaerobes, although some modifications in the medium might have to be made to accommodate the nutritional requirements of particular species.

The development of this method was begun when the author was working at Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire. The technical assistance of Mrs Glenda Crook is gratefully acknowledged. The correlation of the method with the standard plate count was verified in Glasgow by Mrs F. Mahmood.

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