SHORT ARTICLE

A SIMPLE AND SAFE VOLUMETRIC ALTERNATIVE TO THE METHOD OF MILES, MISRA AND IRWIN FOR COUNTING VIABLE BACTERIA

MARY P. E. SLACK AND D. B. WHELDON

*Department of Bacteriology and Regional Public Health Laboratory,
Radcliffe Infirmary, Oxford*

THE method of Miles, Misra and Irwin (1938) has for many years been the standard technique for the determination of viable bacterial counts by surface inoculation of solid media. However, this method requires precision and skill if accurate results are to be obtained. One problem is the difficulty of preparing pipettes that accurately and consistently deliver 50 drops per ml.

The method is also not without hazard to the operator. Johansson and Ferris (1946) have demonstrated aerosol formation when a liquid is dropped on to an agar surface, and the necessary agitation of dilutions during mixing also produces airborne bacteria.

We describe and evaluate a procedure in which a semi-automatic pipette with glass capillary tubes is used. This method does not require the preparation and calibration of accurate dropping pipettes but has the accuracy of the Miles, Misra and Irwin technique; it also has the advantages of being faster and of producing less bacterial contamination of the working surface.

MATERIALS AND METHODS

Bacteria. Counts were performed on 24 bacterial broth cultures, each containing approximately 10^8 organisms per ml. The cultures were all enterobacteria (table I). The organisms were identified by methods described by Cowan (1974). None of the strains tested was unduly mucoid.

Culture media. Peptone water (Oxoid Bacteriological Peptone 1%, with NaCl 0.5%) was used for the initial broth cultures and for subsequent dilutions. Counts were made on overnight broth cultures of the test organisms diluted approximately 1 in 10^8 . Blood agar plates (Oxoid Columbia Agar 2%, with defibrinated horse blood 5%) were used in the micropipette method and in the method of Miles, Misra and Irwin; plates were dried for 2 h at 37°C before use.

In the pour-plate method, Columbia Agar (Oxoid) was used as the medium. As a less nutritious medium was used for the pour-plate method than for the other methods, a comparison of the two media was made by estimating the number of viable bacteria in 12 of the cultures listed in table I by the method of Miles, Misra and Irwin. The two media were inoculated in parallel. There was no significant difference between the plate counts obtained with the two media ($t = 0.73$, $df = 11$, $P > 0.05$).

The micropipette. An Oxford micropipette [Boehringer Corporation (London) Ltd, Bell Lane, Lewes, East Sussex] delivering a fixed 25- μ l volume was equipped with a disposable plastic tip supplied by the same manufacturer. A short length (8 mm) of pliable plastic tubing (Standard Auto-Analyser pump tubing, Technicon Ltd, Basingstoke, Hants.) of internal diameter 1 mm was attached to the end of the plastic tip. This tubing formed a cuff into which a disposable, uncalibrated glass capillary tube (Bilbate Precision Glass, 16 Middlemarch, Daventry, Northants.) was inserted (figure). This adaptation ensured that fluid was drawn only into the sterile capillary tube.

Received 16 Nov. 1977; revised version accepted 13 Feb. 1978.

TABLE I
Organisms used in evaluating the counting methods

Isolate no.	Organism
1	<i>Acinetobacter anitratum</i>
2,6,7,9,10,15,17	<i>Escherichia coli</i>
3,4,14,18	<i>Enterobacter aerogenes</i>
5,20,21,23	<i>Enterobacter cloacae</i>
8,11	<i>Serratia liquefaciens</i>
12,13,16	<i>Serratia marcescens</i>
19,24	<i>Enterobacter agglomerans</i>
22	<i>Providencia stuartii</i>

TABLE II
Number of viable bacteria in 24 broth cultures, estimated by three methods

Culture number	Viable count (10^6 per ml) determined by the		
	micropipette method	method of Miles, Misra and Irwin	pour plate method
1	1.55	1.53	1.52
2	2.50	2.50	2.41
3	1.51	1.53	1.46
4	0.54	0.57	0.52
5	3.00	3.16	3.03
6	0.36	0.36	0.31
7	1.35	1.35	1.21
8	0.89	0.86	0.88
9	0.23	0.29	0.21
10	5.00	5.58	5.20
11	9.80	10.25	9.90
12	15.50	15.10	16.80
13	16.90	16.50	17.20
14	57.10	52.70	57.30
15	27.90	24.10	28.60
16	45.90	48.80	46.10
17	43.40	41.70	44.70
18	5.53	5.83	5.60
19	13.10	13.50	16.60
20	16.00	15.60	18.10
21	12.60	12.90	12.30
22	16.80	16.50	16.90
23	3.80	4.08	3.82
24	18.40	18.00	18.20

The following analysis of variance is made by the method of Armitage (1971).

	S Sq.	DF	M Sq.	VR
Cultures	16799.09	23	730.40	773.33 ($P < 0.005$)
Methods	5.12	2	2.56	2.71 ($P > 0.05$)
Residual	43.45	46	0.94	1.00

Micropipette method. A Selectapette [Becton and Dickinson (UK) Ltd, York House, Empire Way, Wembley, Middlesex] was used to dispense 0.9 ml of broth into each of three glass tubes (6 cm $\times$ 1.5 cm). The adapted micropipette was used to take up 25 μ l of the initial broth culture and to deliver this volume on to the surface of a blood agar plate, the tip of the capillary just touching the surface of the agar. This was repeated five times to give six replicates on one plate. Four more 25- μ l amounts of the initial culture were then transferred

FIGURE.—The adapted micropipette.

to the first of the tubes containing 0.9 ml of broth, to give a 10-fold dilution of the original broth. The glass capillary was then discarded and replaced by a fresh, sterile one. The whole procedure was then repeated with the 10-fold dilution broth, and subsequently with serial 10-fold dilutions up to 1 in 1000. Six replicate drops on a blood agar plate were taken from each dilution.

After the drops had dried, the blood agar plates were incubated aerobically for 18 h at 37°C, and the number of countable colonies growing in each of the inoculated areas was recorded. The dilution that gave the largest number of discrete colonies was selected for the estimation of the number of viable bacteria in each initial broth culture.

Method of Miles, Misra and Irwin. The original technique of Miles, Misra and Irwin (1938) was used. Nominal 50-dropper pipettes were prepared with a Morse gauge (no. 57). Of the total number of pipettes prepared, a sample (20) was calibrated as follows: each pipette was used to dispense, in drops, 10-ml of 1% peptone water measured with a volumetric pipette, and the total number of drops was noted. The range of counts for the 20 pipettes was 465.5–490.5 drops per 10 ml and the individual counts were 471, 466, 487, 469, 489, 480, 467, 465, 471, 490, 467, 471, 469, 476, 465, 488, 468, 473, 487, and 471. Each count was increased by 0.5 to allow for any fraction of a drop retained in the pipette. The mean number of drops delivered was 47.5 per ml; this figure was used in the subsequent calculations of viable bacteria in the test cultures.

Ten-fold dilutions of the broth cultures were prepared (1 in 10, 1 in 100 and 1 in 1000) and six replicate drops of each dilution were dropped on to the surface of blood agar plates. After the drops had dried the plates were incubated for 18 h at 37°C. Colonies were counted and bacterial numbers estimated as described for the micropipette method.

Pour-plate method. Serial decimal dilutions were made with 1.0-ml volumetric pipettes from the broth cultures in screw-capped glass bottles containing 9 ml of peptone water. A 1-ml amount of each dilution was mixed with 18 ml of molten Columbia agar and poured into a Petri dish. After drying, the plates were incubated aerobically for 18 h at 37°C. Those bearing the largest countable numbers of colonies were selected for the estimations of viable count.

Comparison of the methods. The micropipette method, the method of Miles, Misra and Irwin, and the pour-plate method were used in parallel to estimate the numbers of viable bacteria in the 24 broth cultures. For the two surface methods, the calculations were based on the mean colony counts obtained from replicate drops.

Statistical analyses. (a) The six plate counts obtained for each broth by the micropipette method and the six obtained by the method of Miles, Misra and Irwin were tested for homogeneity (Armitage, 1971). (b) A two-way analysis of variance (Armitage, 1971) was applied to the viable counts obtained by the three methods, results being considered significantly different when $P = < 0.05$.

Detection of environmental contamination. The number of viable bacteria in an overnight broth culture of a pigmented strain of *Serratia marcescens* was determined by the micropipette method, and by the method of Miles, Misra and Irwin. In each method the degree of environmental contamination during the preparation and plating of the dilutions was assessed by carrying out these procedures over separate square culture plates (24 cm × 24 cm) containing Columbia agar.

After overnight incubation at 37°C the number of pigmented colonies of *S. marcescens* on each square plate was noted.

RESULTS

None of the sets of replicate counts obtained by the micropipette method or the method of Miles, Misra and Irwin suggested a significant degree of variation; it was therefore considered

valid to use the mean plate counts for the calculation of bacterial counts in the original broth cultures.

There was no significant difference between the estimates of viable bacteria obtained by the three methods ($P > 0.05$, $df = 2$) (table II). There was no evidence of any interaction between the row and column effects when the residuals were examined, from which we infer that none of the methods used produced an aberrant count for any particular organism.

The degree of environmental contamination was considerable when the method of Miles, Misra and Irwin was used; the square culture plates over which the dilution and plating procedures were carried out yielded 16 and 11 pigmented colonies of *S. marcescens* respectively. When the micropipette method was used, no pigmented colonies were found on the square plates.

DISCUSSION

The method of Miles, Misra and Irwin has for many years been the standard surface-inoculation method for estimating the number of viable bacteria in fluids. However, when dropping pipettes are used it is necessary to calibrate them accurately; even then, variation between pipettes leads to errors. Modifications of the method of Miles, Misra and Irwin have been described in which a volumetric pipette is used in place of a dropping pipette for measuring the diluent. However, if the dilutions are made by an inaccurately standardised dropping pipette, any error is magnified geometrically with each dilution, whereas when similar dropping pipettes are used for both measurement of diluent and dilution any error increases arithmetically with each dilution. The micropipette method described here uses calibrated volumetric equipment for both the distribution of volumes of diluent and for the diluting procedure; the risk of producing an increasing error in successive dilutions is thus minimised. In a comparative evaluation of mechanical handpipettes (McCormack and Robinson, 1975) it was found by a rigorous testing procedure that the levels of accuracy and precision of such instruments were acceptable and there was a high degree of mechanical reliability.

The use of semi-automatic pipettes for estimating numbers of viable bacteria has been considered recently by Bousfield, Smith and Trueman (1973) and by Watson, Duerden and Moyes (1977). The latter authors found that there was no significant difference between semi-automatic pipettes and dropping pipettes in respect of the counts or the reproducibility obtained, and they stressed the need for careful preparation and regular calibration of batches of dropping pipettes.

In the present study, the estimates of viable bacterial numbers given by the three test methods did not differ significantly. Counting by the micropipette method produced less environmental contamination than did counting by the method of Miles, Misra and Irwin. A common cause of aerosol formation in the latter method was found to be the placing of a drop, during the dilution procedure, on to the inner wall of the glass tube, necessitating the flushing of the tube walls with diluent to ensure an accurate dilution. The dropping of the dilutions on to agar surfaces was also found to give rise to contamination of the working area. Both these hazards seemed to be reduced when the micropipette method was used.

The new method was rapid and easy to perform; we have found it particularly useful where the exact timing of a count is of importance, as in the determination of bacterial growth curves (Wheldon and Slack, 1977). Other purposes for which we have found the new method useful include the testing of selective media and the measurement of inocula for estimating minimal inhibitory concentrations of antimicrobial agents.

SUMMARY

A rapid and simple method for counting viable bacteria is described. The technique involves the use of a semi-automatic micropipette and disposable glass-capillary tubes. Viable counts obtained by this method were not significantly different from those obtained by the Miles, Misra and Irwin method or by a pour-plate method. The micropipette procedure is less hazardous than techniques involving the use of calibrated dropping pipettes.

We wish to thank Dr R. T. Mayon-White for advice on the statistical analysis of the data.

REFERENCES

- ARMITAGE, P. 1971. Statistical methods in medical research, *Oxford*, pp. 214 and 217.
- BOUSFIELD, I. J., SMITH, G. L. AND TRUEMAN, R. W. 1973. The use of semi-automatic pipettes in the viable counting of bacteria. *J. appl. Bact.*, **36**, 297.
- COWAN, S. T. 1974. Cowan and Steel's Manual for the identification of medical bacteria, 2nd ed., *Cambridge*.
- JOHANSSON, K. R. AND FERRIS, D. H. 1946. Photography of airborne particles during bacteriological plating operations. *J. infect. Dis.*, **78**, 238.
- MCCORMACK, J. J. AND ROBINSON, R. 1975. Comparative evaluation of mechanical hand-pipettes. D.H.S.S. Report, abstracted in *Hospital Equipment Information Bull.*, no. 63 (March, 1976).
- MILES, A. A., MISRA, S. S. AND IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *J. Hyg., Camb.*, **38**, 732.
- WATSON, P. G., DUERDEN, B. I. AND MOYES, A. 1977. Modified method for surface viable counts. *Lab. Equip. Dig.*, **15** (5), 69.
- WHELDON, D. B. AND SLACK, M. 1977. Multiplication of contaminant bacteria in urine and interpretation of delayed culture. *J. clin. Path.*, **30**, 615.