Rev. Inst. Med. trop. São Paulo
6(3):89-92, maio/junho, 1964

UDC 576.8.093.6

A METHOD FOR THE TITRATION OF NON-VIABLE OR SLOW-GROWING CULTURES OF BACTERIA

C. A. SALLES

SUMMARY

A titration method of non-viable or slow-growing cultures of bacteria is described. Indicator bacteria Y, titrated by the colony count method, are mixed with bacteria X. The mixture is atomized and the micro-drops are collected on a microscope slide. Both bacteria X and Y are counted in several micro-drops.

The titre of X can be obtained by use of a simple formula.

INTRODUCTION

Precise calculation of the number of bacterial cells in a given volume of culture is one of the most important and frequently used quantitative methods in micro-biology. Existing titration methods are perfectly adequate in most cases. However, where the culture has been processed so that the micro-organism is non-viable, or where it is of a type which develops slowly, the titration methods based on bacterial multiplication are difficult or impossible.

In other cases the indirect physical or chemical methods (nephelometry, dryweight measure, constituent cell dosage etc.) may not be applicable or may be unpractical, costly, lengthy or imprecise.

A method has been developed which may be applied in cases where other methods have proved unsatisfactory, based on the work reported by LURIA *et al.*[1] who developed a direct counting method for the titration of bacteriophage.

PRINCIPLE OF THE METHOD

An untitrated suspension of bacteria X is mixed with a titrated suspension of bacteria Y.

Bacteria Y is chosen so that it is easily distinguished from X either morphologically or by staining. The mixture is then atomized and the micro-drops are collected on a common microscope slide. The slides are fixed, stained and examined by light microscopy at 1,000 × magnification. With adequate dilutions, the number of X and Y bacteria in each drop are easily counted. After counting the bacteria in a certain number of micro-drops, the total number of X and Y bacteria is obtained and the following formula is applied:

$$T_x = T_y \cdot N_x/N_y$$

T_x is the titre of X which is to be discovered. T_y is the known titre of Y. N_x and N_y are the respective number of cells in the total number of micro-drops examined.

MATERIALS AND METHODS

Escherichia coli, strain "B" was used as indicator bacteria (Y), while *Staphylococcus* (diplococcus) *albus*, isolated in our laboratory was used as test bacteria (X). Both were kept in a simple broth with the following composition: Lab-lemco beef extract (Oxoid) 8 g; sodium chloride 5 g; distilled water qsp 1 litre, pH adjusted to 7.4 with 10% NaOH. For Plate counting, the above medium was added to 15 grams of agar and placed in Petri dishes.

National Institute of Health and Medical Research. P.O. Box 2848, Accra, Ghana.

Preparation of suspension for the micro-drop technique

1. *Medium* — The medium used for preparing the suspension is the maintenance medium enriched by the addition of tryptone, 5 g; soy peptone, 5 g; glucose 2 g. 20 ml of medium is placed in each 25 × 200 tube.

2. *Culture* — The tubes are inoculated with 0.3 ml of saturated culture (18 hrs) and shaken 300 times per minute in an incubator at 37°C, for 3½ hours.

3. *Suspensions* — After 3½ hours, the cultures are removed from the incubator and placed in an ice bath. After the samples are taken from each tube for titration by colony counter, 2.0 ml of 40% formalin and 0.3 ml of Indian Ink are added to each tube. We use "Pelikan Indian Ink for fountain pens" (Gunther Wagner, Germany). The ink serves to distinguish one drop from another by clearly marking the contour of each and making it possible to avoid gross errors in counting the number of bacteria contained in a single drop.

4. *Dilutions* — The dilutions of X are prepared at a constant volume (3 ml) and mixed with 3 ml of indicator bacteria "Y" suspension. Each dilution of X is prepared independently. The dilutions for the colony count method are successive dilutions as usual.

5. *Atomization* — A total volume of 6 ml (3 ml of X and 3 ml of Y suspension) is placed in an atomizer of the kind used for developing paper chromotograms. Microscope slides chosen for their regular shape and clear surface (of best quality glass and without marks or oxidation stains) are cleaned and passed over a Bunsen burner. The source of air for the atomizer may be a compressor, an ordinary rubber bulb or the experimenter's lungs. The best results are obtained with a compressor which produces the smallest and most uniform micro-drops.

6. *Atomizing technique* — Fig. 1 shows the apparatus used. A filter is employed in order to reduce the flow of micro-drops falling onto the slide, to increase the homogeneity of the mixture and to block the micro-drops travelling obliquely and producing blurred images which are difficult to count. A test tube rack was used as a filter. The rack was made from a folded sheet of aluminium with a surface of 20 × 14 cm containing 24 holes, each 1.5 cm in diameter. Three cm behind the first sheet is a second sheet exactly like the first and 4 cm behind the second is a third aluminium sheet with no holes in it.

Between the second and third sheets a vertical precipitation chamber is formed where the micro-drops fall vertically onto the slide and produce round images. Only a very short vaporizing action is necessary; approximately one second, though the time varies slightly according to the source of air.

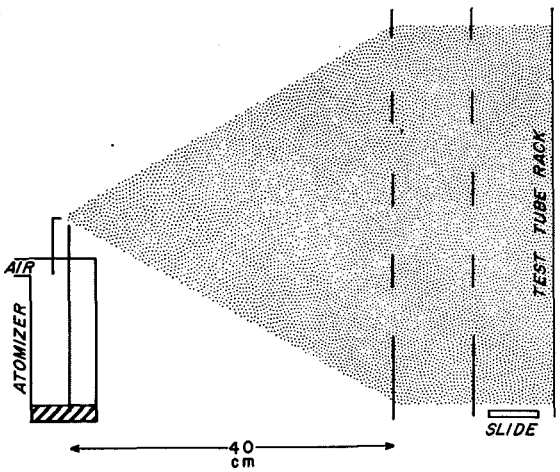

Fig. 1

7. *Preparation of the slide for counting* — After atomizing, the slide is removed, and dried at 37°C. After drying, the slide is fixed in the flame, and stained with a 0.5% of fuchsin solution. Figure 2 is a drawing of a micro-drop as seen at oil immersion magnification.

8. *Counting the bacteria in the micro-drops* — Once the microscope is focussed, the slide is moved horizontally from one end to the other and all the micro-drops which are smaller than or equal to the size of the microscope's field and which contain bacteria are counted.

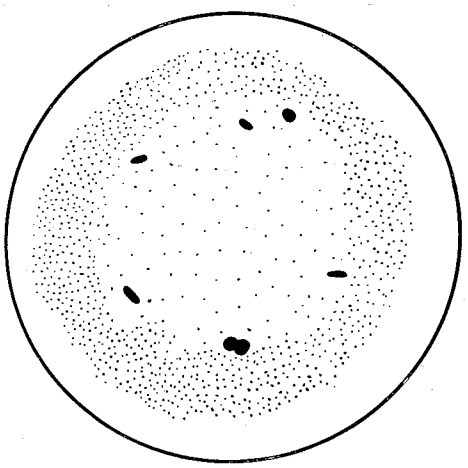

Fig. 2

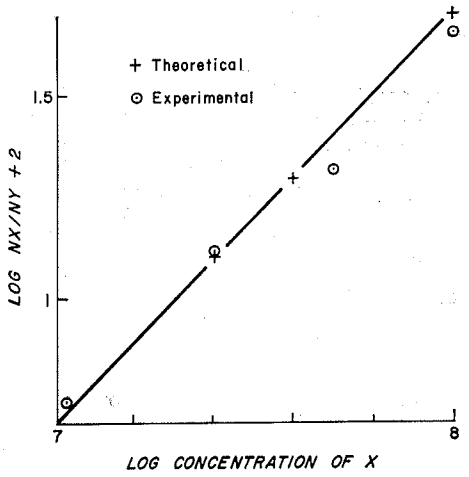

Fig. 3

TABLE I

Suspension dilution	Total drops counted	N_x	N_y	N_x/N_y experimental	N_x/N_y theoretical	Titre (by Colony counter method) (cells/ml)	Titre (method of micro-drop) (cells/ml)
1:1	45	214	484	0.450	0.500	1.0×10^8	9.0×10^7
1:2	83	80	390	0.205	0.225	5.0×10^7	4.2×10^7
1:4	78	54	402	0.135	0.112	2.5×10^7	2.7×10^7
1:10	20	14	253	0.055	0.050	1.0×10^7	1.1×10^7
1:100	30	4	435	0.009	0.005	1.0×10^7	1.8×10^6

RESULTS

The results of an experiment are shown in Table I. Both bacteria used in this experiment were previously titrated by the colony counter method. The titre was 4×10^8 for *E. coli* (Y) and 2×10^8 for *S.* (diplococcus) *albus* (X). The ratio T_x/T_y was 0.5.

From the data of Table I, a correlation curve has been drawn (Fig. 3) showing the close relationship between the ratio N_x/N_y and the titre of the culture.

DISCUSSION

Two major limiting factors are involved in this method. First, the area of the microscopic field, which is the superior limit of the sample of drops, and second, the dilution factor which is increased when the size of the drop tends to zero.

Drops collected on the slide, and the resulting circular images, can be seen as a population of drops with different areas which are normally distributed. This population can be divided into groups according to the radii of the drop images, which ultimately means the volume of the drops. The drops came from a suspension of bacteria and can be seen as randomized sets of aliquots taken from the bacterial culture. Each group of drops has its own mean of bacterial cells. Since the initial concentrations of X and Y in the atomizer are kept constant, the mean bacteria/drop is a function of the volume of the drop, or in two-dimensional terms, of the radius of the drop. Assuming a normal distribution for the population of drops, then

$$m_x = (V_x - V_v)\ C + K \qquad (1)$$

in which m_x is the mean bacteria/drop of

the group Gx of drops with volume X; V_x is the volume of the drop, V_v is the mean volume of the population of drops, and C and K are constants where, $C = sm/sV$, i.e. the ratio between the two standard deviations, and K is the mean of the means. The distribution of bacteria per drop is assumed to be a Poisson's distribution. Within each group Gx, with a mean mx of bacteria per drop, the frequency of drops containing one or more bacterium will be

$$B_x + 1 - e^{-mx} \qquad (2)$$

or, if the whole population is considered,

$$B_x = (1 - e^{-mx})\ P_x \qquad (3)$$

in which P_x is the frequency of the group Gx in the whole population of drops and e is the base of natural logarithms.

Consider now, a new set of drops formed by all the drops containing bacteria. The fraction of the population of drops which is included in this new set will be:

$$B_i = \sum_{i=a}^{q} (1 - e^{-mi})\ P_i \qquad (4)$$

in which i corresponds to the different groups Ga, Gb, Gc ... Gq. This last group Gq is the upper limit of the populations, formed by drops with the biggest radius which can be seen in the field of the microscope.

Finally, the contribution given by each set of drops containing bacteria belonging to the different groups Ga, Gb ... Gq of the population, to this new set, will be:

$$F_x = (1 - e^{-mx})\ P_x / \sum_{i=a}^{q} (1 - e^{-mi})\ P_i \qquad (5)$$

F_x is a function of m and of q, each of them related to the two limiting factors mentioned above.

For practical reasons it would be interesting to evaluate how many drops must be examinated in order to find one drop which contains bacteria; this is given by

$$\sum_{i=a}^{q} R_i \qquad (6)$$

in which

$$R_x = P_x/B_i \qquad (7)$$

E.G.5 shows that when q tends to a then F_x tends to i, and the importance of the groups which contain small drops increases. In these groups the mean of bacteria/drop are the smallest of the whole population.

When the mean becomes small (m tends to zero), then $(1 - e^{-mi})\ P_i$ tends also to zero and B_i becomes increasingly smaller.

The practical consequence when m is small and q tends to a is that the number of drops which must be examined in order to find one drop containing bacteria becomes infinitely great.

The equations 5 and 6 indicate that improvements may be made either on the mean or on the limiting factor q. The mean is determined by the concentration of the bacteria in the suspension which is atomized. An increase in the mean may thus be obtained by concentrating this suspension. To increase the limiting factor q, large field optics may be used. In particular cases the observation of the bacteria may be carried out using high dry objectives, in which case the limiting factor q may be increased up to 3-4 times.

Concerning the precision of the method, measures made in five independent experiments have given a coefficient of variation (standard deviation/mean) of 0.15. Since the bacteria of the suspension in the atomizer are killed by formalin, there is no danger of aerial contamination. Inhalation of allergenic substances from the bacterial bodies or from constituents of the medium may however occur, and to prevent this a surgical mask is used during the few seconds of the atomization.

ACKNOWLEDGEMENTS

I would like to express my great debt to Dr. C. Bierwood and Dr. N. Rothstein by the valuable sugestions and criticisms offered during the course of the experiments and the revision of the manuscript.

REFERENCES

LURIA, S. E.; WILLIAMS, R. C. & BACKUS, R. C. — *Journal of Bacteriology* 61:179, 1951.

Recebido para publicação em 5 dezembro 1963.