A RAPID MEMBRANE FILTER METHOD FOR DIRECT COUNTS OF MICROORGANISMS FROM SMALL SAMPLES

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Several methods have been described by which membrane filters may be used in making total counts of the microorganisms in fluid samples (Ehrlich, 1955; Jannasch, 1953, 1958). When a sample is filtered the cells are well distributed on the filter surface of known area. The cells are stained and counted by direct microscopic observation, after the filter has been made transparent by application of immersion oil. The technique described here, using simple apparatus and few manipulations, is useful in situations which require the examination of many small samples at frequent intervals.

MATERIALS AND METHODS

Plain white, type HA 1.00 in MF filters (Millipore Filter Corporation, Watertown, Massachusetts) are divided into several sampling areas by stamping them with grease using a simple metal die. This die has a flat, polished surface, in the face of which are a series of circular indentations of known diameter. The face of the die is smeared with a thin film of a mixture consisting of 10 per cent paraffin in petroleum jelly, and warmed slightly. The greased die is then pressed firmly against the filter. In this way the filter is rendered impermeable except for a series of circular areas corresponding to the indentations in the face of the die. It is possible to divide each 1.00 in filter into 12 individual filtering areas, each 2.5 mm in diameter.

For filtration of samples, the prepared filter is placed on the fritted glass base of a pyrex hydrosol microanalysis filter holder (Millipore Filter Corporation, Watertown, Massachusetts). It is not necessary to use the upper portion of the filter holder. Small, accurately measured amounts of appropriately diluted samples are placed on each filtering area by use of a microliter pipette. Sample volumes of 50 to 100 μL are most convenient when filter areas of 2.5 to 3.0 mm diameter are used. Surface tension prevents the drops of sample from coalescing before filtration has been completed. The holder is placed in a filter flask which is attached to a laboratory vacuum line and connected with a mercury U-tube manometer. A vacuum of 5 to 10 mm is optimum for filtration, which is completed in a matter of seconds.

Since dilution of samples is usually necessary to obtain optimum numbers of cells on the filter surface for counting purposes, it is convenient to combine the dilution and fixation steps. Appropriate dilutions are therefore made in 0.85 per cent NaCl solution containing 1 per cent picric acid. (If picric acid alone is used, there is sometimes considerable lysis of the cells.) Media such as nutrient broth, which have a high content of organic material, may form a precipitate with picric acid. In such instances, initial dilutions are made in distilled water, and only the final dilution, which is to be filtered, is made in the fixative. If the number of cells in a sample is so small that dilution is unnecessary, a drop of the saline-picric acid solution is placed on each area after filtration and permitted to stand for about one minute before staining.

After filtration, a drop of the staining solution consisting of 0.1 per cent acid fuchsin (60 per cent dye content) in distilled water at pH 3.0 is placed on each filtering area and allowed to stand for 1 min. The filter is then subjected to full vacuum (50 to 60 mm of Hg) for 15 to 20 sec, removed, taped to a glass slide, and dried at 37 C for 15 to 20 min before observation. No washing is necessary, since excess dye is removed by the filtration. To prevent accumulation of debris which would interfere with counting the cells on the filter surface, all reagents must be filtered through an HA Millipore Filter before use. Microliter pipettes are rinsed with distilled water and dried with acetone after each use.

A drop of immersion oil is placed on the dried filter, and the cells enumerated by direct microscopic observation. The size of the field must of

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course be known at the magnification used. Rather than using an entire field, we have found it most convenient to employ a calibrated Whipple ocular, and to consider the grid area as the standard "field." Since the grid is divided into lesser portions, it is possible to count the cells in a fraction of a field when desirable. The count is then converted to cells per ml of the

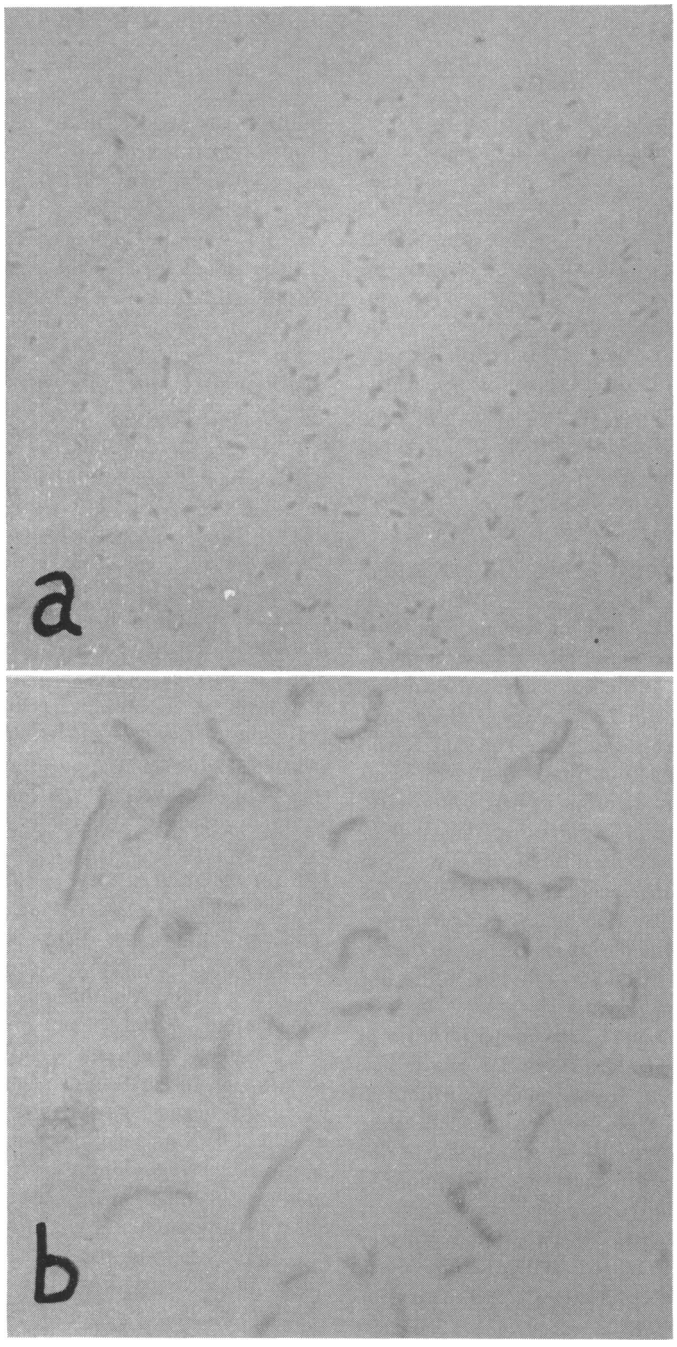

Figure 1. Appearance of bacterial cells on membrane filters prepared as described in text. (*a*) *Escherichia coli*, (*b*) *Bacillus megaterium*.

original culture by the formula:

$$\text{Cells/ml} = \frac{\text{conversion factor} \times \text{dilution factor} \times \text{cells counted}}{\text{number of fields counted}},$$

where the conversion factor is the filtering surface area divided by the area of the Whipple field at the magnification used.

RESULTS AND DISCUSSION

Cells fixed and stained on the membrane filter appear deep red on a clear to light pink background when viewed microscopically (figure 1). Cells may readily be distinguished from extraneous material, and the necessary contrast for ease in counting is obtained without treatment or counterstaining of the filters. Cells left unfixed or those fixed with 3 per cent formalin show much less contrast, appear smaller, and are not retained as well on the filter surface.

TABLE 1

Direct counts of Escherichia coli obtained on membrane filters

Filter No.	Dilution	Area No.	Counted Cells per Field			Mean Cells per Field	Calculated Cells per ml Original Culture
I	1:200	1	288	270	263	274	4.3×10^8
		2	210	282	247	246	
	1:800	1	69	66	60	65	4.1×10^8
		2	62	44	70	59	
		3	71	52	74	66	
	1:2000	1	20	25	30	25	4.3×10^8
		2	31	32	25	29	
II	1:200	1	270	225	269	255	4.3×10^8
		2	296	261	262	273	
	1:800	1	63	73	54	63	4.1×10^8
	1:2000	1	41	18	47	35	5.3×10^8
		2	27	37	28	31	
III	1:200	1	285	259	226	257	4.1×10^8
		2	235	243	232	237	
	1:800	1	51	77	73	67	4.2×10^8
		2	49	77	71	66	
		3	51	65	64	60	
	1:2000	1	23	29	47	33	5.3×10^8
		2	26	38	35	33	

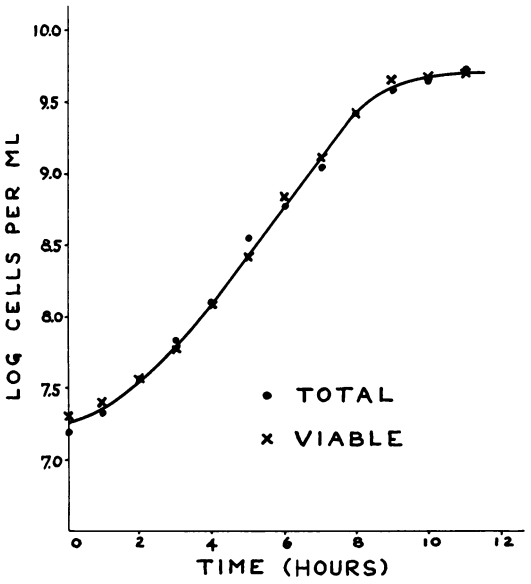

Figure 2. Growth curve of *Escherichia coli* in aerated synthetic medium at 37 C. *Total:* direct membrane filter count described in text. *Viable:* plate count in complete medium. All points are means from 3 determinations.

The accuracy of these counts is totally dependent on a uniform distribution of cells over the filter area. Our data are in agreement with the report of Jannasch (1953) that effective distribution of cells is possible only if the depth of the added sample exceeds by many times the diameter of the filtering surface. We have found, as a rule of thumb, that the volume filtered should not be less than twice the cube of the diameter of the filtering surface. Thus, using a filtering surface with a diameter of 3 mm, the volume of sample should be no less than 50 μL. Although volumes of 25 μL will sometimes yield reasonably uniform distribution over this area, results are not generally reliable with this smaller volume.

In order to test the significance of possible variations between results obtained on different filters, in different areas on a single filter, or in different fields within a single area, a culture of *Escherichia coli* was diluted in the saline-picric acid fixative to 3 concentrations, and each concentration then filtered through a number of areas on each of 3 filters. Three fields were counted in each filtering area. The results of these experiments are shown in table 1. It can be seen that there is no significant difference between

filters or between areas on a single filter. The variation between fields in a single area is a function of cell distribution, and it is apparent that any effect of variable distribution remains negligible if the total number of cells counted per area reflects the average of several fields within the area. However, table 1 shows that a significant error is introduced when the average number of cells per field falls below 50. This could be anticipated, since the number of foreign particles mistakenly counted as cells will remain relatively constant for all fields. When too many fields are counted, the number of foreign particles enumerated will be multiplied into significance and the calculated cells per ml be too high.

Counts are most reliable when a total of 150 to 250 cells is counted, regardless of the concentration of cells on the filter surface. Accuracy is further enhanced by counting only a fraction of each field, if necessary, so that no more than 75 to 100 cells are counted in any single field. Thus the total of cells counted is representative of at least 3 fields in a given filter area. Although the upper limit of effective cell concentration appears to be dictated only by the difficulty encountered in distinguishing individual cells, it is convenient to hold the number of cells per field below 500. If counts are made at 1000× magnification, the sample to be filtered should, therefore, be diluted so that there will be between 5000 and 50,000 cells per square mm of exposed filter surface.

For a comparison of this technique with counts obtained by other procedures, an aerated culture of *E. coli* in synthetic medium (Davis and Mingioli, 1950) was sampled at intervals and direct counts made as described herein. Samples were also diluted in distilled water and plated in complete medium (Lederberg, 1950). Plates containing 30 to 300 colonies were counted after 24 hr at 37 C. Results are shown in figure 2. It is apparent that the membrane filter count is quite comparable to the viable count. Figure 2 illustrates also that the direct count is uniformly accurate regardless of the physiological age of the cells, a point which is substantiated by other experiments not shown here.

These counts seem fully as accurate as those obtained by other methods, and they offer a significant saving in time and materials. The dried filter may be preserved for future reference if desired. When it is not convenient to perform the entire procedure immediately after sampling, cells suspended in the diluent-fixative solution may be stored for at least 24 hr before filtration. There is some tendency toward clumping of the cells after storage for several hr in picric acid, but this is readily overcome by vigorous shaking.

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SUMMARY

A technique is described for direct counting of microorganisms on membrane filter surfaces. The method requires no elaborate apparatus or complex manipulations. The validity of data obtained by this method is discussed, and the results are compared with those obtained by plate counts.

REFERENCES

DAVIS, B. D. AND MINGIOLI, E. S. 1950 Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol., **60**, 17–28.

EHRLICH, R. 1955 Technique for microscopic count of microorganisms directly on membrane filters. J. Bacteriol., **70**, 265–268.

JANNASCH, H. W. 1953 Zur Methodik der quantitativen Untersuchung von Bacterienkulturen in flüssigen Medien. Arch. Mikrobiol., **18**, 425–430.

JANNASCH, H. W. 1958 Studies on planktonic bacteria by means of a direct membrane filter method. J. Gen. Microbiol., **18**, 609–620.

LEDERBERG, J. 1950 Isolation and characterization of biochemical mutants of bacteria. In *Methods in medical research*, pp. 5–22. Yearbook Publishers, Chicago.