Hydrophobic Grid-Membrane Filters: New Approach to Microbiological Enumeration

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Received for publication 11 April 1974

A square grid pattern, printed in hydrophobic material on conventional membrane filters, subdivided the surface into a plurality of areas. This separated colonies from one another and prevented lateral growth, spreading, and confluence. Very high colony-packing densities were achieved (2,500/in², ca. 1.61 × 10⁹/cm²) reducing the need to dilute samples. Recovery of organisms was better than on conventional filters, particularly at high inoculum levels. At the same time, visibility of normally pale or diffuse colonies was improved, since colonies grew upwards instead of sideways, and counting was facilitated because colonies grew in orderly arrays.

Enumeration of microorganisms by growing colonies on the surface of membrane filters is a well-established technique (1) with advantages over the plate count of speed, reproducibility, economy of media, and incubator space. At high colony concentrations, the apparent recovery of organisms by membrane filter and plate count methods decreases because of the increased probability of colonies overlapping. With spreading organisms the limit may occur below 300 colonies, and confluence may make an estimate of the number of microorganisms impossible. Obviously, any means of increasing the ability of membrane filters to separate colonies makes counting easier and more accurate and reduces the need to make several dilutions of samples of unknown history. The modification described below has this effect.

MATERIALS AND METHODS

Preparation of membrane filters. Cellulose ester membrane filters (HAWP 04700, 0.45-μm pore size, Millipore Corp.) were printed with a square grid pattern, using silicone grease or hydrocarbon wax, to subdivide the surface into an array of small, unconnected, growth areas. A zinc printing plate, prepared from a master drawing, was "inked" with molten paraffin wax (52 C; BDH Ltd., Poole, Dorset, England) using a roller. A filter, covered by protective parchment-paper disks, was laid on the surface of the plate, and the wax was transferred by applying gentle pressure from a clean rubber roller. An electric hot plate served as the wax reservoir, and the zinc plate was kept warm on a second hot plate between operations. With this apparatus, gridded filters of satisfactory quality could be prepared quickly and easily. The filters did not become contaminated, provided they were handled with sterile forceps.

Zinc plates containing 8, 25, and 50 lines per inch (2.54 cm) were used. The number of squares capable of being inoculated by filtration through a 1 3/8-inch (ca. 3.49-cm) diameter circle were thus approximately 93, 940, and 3,650, respectively. Only results using 50 line per inch filters are reported.

Bacterial cultures. Cultures of Escherichia coli, Serratia marcescens, Streptococcus faecalis, Proteus vulgaris, Salmonella typhimurium, and Klebsiella pneumoniae were grown overnight in Trypticase soy broth (BBL) and diluted in 0.1% peptone solution immediately before filtration. Specimens containing approximately 10, 50, 250, 1,250, 6,300, 31,300, and 156,000 colony-forming units (CFU) in 100 ml were filtered through each filter in a Gelman magnetic filter holder and then rinsed with 10 ml of sterile water. Filters were laid on Trypticase soy agar in the case of S. marcescens, P. vulgaris, S. typhimurium, and K. pneumoniae, M-Endo agar LES or M-FC agar in the case of E. coli, and M-Enterococcus agar (all BBL) in the case of S. faecalis. Incubation was at 20, 30, or 35 C for 26 or 48 h, depending on the organism. Comparison Millipore filters, of the type printed with conventional hydrophilic black squares, were always inoculated from the same suspensions and incubated together with "gridded" filters in cluster dishes (60 by 15 mm; type 1065, Falcon Plastics, Oxnard, Calif.) in polyethylene bags.

RESULTS

At high colony concentrations particularly, colony counts on membrane filters printed with hydrophobic squares were appreciably higher than those from untreated ones. Table 1 compares counts for six species of bacteria. On untreated filters, colony counts reached maxima of 350 to 6,400, depending on the species (a function of colony size), and at higher CFU
concentrations colonies became uncountable as a result of confluent growth.

If it is assumed that useful counts are still obtained at up to 90% of the maximal recorded colony count, then the treated filters remained countable at CFU concentrations 16 to 38 times higher than on untreated ones (data obtained graphically). At very high CFU concentrations, the count on treated filters reached a plateau of 3,650 (for 50 lines per inch prints). High concentrations of colonies were easier to count on treated filters because of the orderliness of their locations (Fig. 1). The time required to count at first increased with the number of colonies, then decreased at high concentrations when it was sufficient to count the number of unoccupied squares and subtract from 3,650.

The hydrophobic lines generally were excellent barriers to the lateral spread of colonies, but did not retard colony formation in any of the six species. Reduction in the net area available for filtration increased the time required to filter a given volume of sample. For the 50 lines per inch filters the filtration time increased by about one-third.

**DISCUSSION**

It is possible, at least in principle, to extend the maximal attainable colony density even further by printing still finer squares. At 100 lines per inch, for example, one can expect to separate up to 14,600 colonies on the same area, increasing the CFU concentration at which the plateau is reached to as much as 160 times the normal value. Reducing the need to make dilutions may be particularly valuable in busy laboratories. Currently, we are investigating the printing process to reproduce the finest patterns without completely obliterating the pores of the membrane.

![Fig. 1. Colonies of P. vulgaris on gridded membrane filter. (Stained with triphenyltetrazolium chloride.)](image)

The ability of hydrophobic squared filters to resolve high colony concentrations may prove to be particularly valuable when completely automated microbiological instruments are produced, since the potentially serious situation in which confluent growth from a highly contaminated sample is recorded as a low value can be avoided. An instrument can easily give warning if the number of positive squares exceeds a certain proportion of the maximum.

Improved visibility of pale, translucent, or spreading colonies (e.g., *P. vulgaris*) results from the lateral growth restraint, and consequently thicker, bulging, colony appearance. It is important that the squares are smaller than the minimal normal colony size if this effect is to be taken advantage of. This condition did not apply to *S. faecalis*, which produced a high

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**Table 1. Comparison of colony counts on normal and hydrophobic grid membrane filters at various inoculum levels for six species of bacteria**

| Inoculum (approx) | Colony counts of bacteria |
|-------------------|--------------------------|
|                   | *S. marcescens* | *S. typhimurium* | *E. coli* | *P. vulgaris* | *K. pneumoniae* | *S. faecalis* |
|                   | Normal | HG | Normal | HG | Normal | HG | Normal | HG | Normal | HG | Normal | HG |
| 10^4              | 42     | 30 | 6      | 11 | 14    | 9   | 39     | 46 | 39     | 46 | 21     | 12 |
| 5 x 10^4          | 104    | 81 | 16     | 57 | 144   | 12  | 121     | 112 | 99     | 112 | 24     | 14 |
| 2.5 x 10^4        | 479    | 454| 54     | 111| 200   | 144 | 177     | 220 | 200    | 220 | 280    | 177 |
| 1.2 x 10^4        | 443    | 1,650| 292  | 380| 604   | 380 | 966     | 966 | 966    | 966 | 1,400  | 1,400 |
| 6 x 10^4          | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 |
| 3 x 10^4          | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 |
| 1.5 x 10^4        | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 | UNC 3,650 |

* Abbreviations: HG, hydrophobic grid; UNC, impossible to count or estimate.
  * Estimated count.
proportion of microcolonies. The apparent recovery of this organism by treated filters was low because a square was recorded as one, even if it contained several microcolonies. For this organism, at least, very much higher packing densities could be tolerated.

Reduced filtration rate could be a disadvantage in some situations. However, for suspensions relatively free from clogging debris, such as mineral or potable waters, an increase in filtration time from, for example, 15 to 24 s, may be quite acceptable if a dilution step is thereby eliminated. The effect on filtration rate depends on the proportion of total area covered by wax and therefore on the width of the line barriers. It is likely that, as the number of lines per inch is increased, the proportion of total area available for filtration will decrease, since the lines probably need to be at least 0.005 inches (ca. 0.127 cm) wide to separate colonies reliably.

We are currently investigating the possibility of preparing barriers raised above the surface of the filter to provide additional, mechanical, restraint to the lateral growth of colonies.

LITERATURE CITED

1. American Public Health Association. 1971. Standard methods for the examination of water and wastewater, 13th ed., p. 678-688. American Public Health Association, Inc., New York.