USE OF POROUS CELLULOSE ESTER MEMBRANES IN THE PRIMARY ISOLATION AND SIZE DETERMINATION OF PLEUROPNEUMONIA-LIKE ORGANISMS

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Received for publication 6 August 1962

ABSTRACT

MOROWITZ, HAROLD J. (Yale University, New Haven, Conn.), MARK E. TOURTELLOTTE, AND MARY E. POLLACK. Use of porous cellulose ester membranes in the primary isolation and size determination of pleuropneumonia-like organisms. *J. Bacteriol.* **85**:134-136. 1963.—Millipore filters were used in the primary isolation and sizing of a number of strains of pleuropneumonia-like organisms (PPLO). Maximal pore diameters were checked by filtration of viruses of known sizes. All the PPLO strains studied produced cells capable of passing through a 0.22- μ filter. A number of strains produced forms which could be titered after filtration through a 0.1- μ filter. A number of primary isolations were made from filtrates.

One of the characteristic features of the pleuropneumonia-like organisms (PPLO) is their ability to pass through filters which do not permit the passage of bacterial cells (Klieneberger-Nobel, 1961). Such filters can, therefore, be used in the primary isolation of PPLO and the freeing of PPLO cultures from bacterial contamination. In addition, the availability of commercial filters of uniform quality provides an extremely simple method of size determination. In this paper, we shall report on the use of Millipore filters (Millipore Filter Corp., Bedford, Mass.) in the isolation and characterization of a number of strains of PPLO. We shall also discuss the use of these filters in the purification of very small forms of PPLO.

MATERIALS AND METHODS

Medium. Cells were grown in beef heart infusion (BHI) prepared in the following way. Fresh ground defatted beef heart (400 g) was infused in 1,000 ml of tap water at 4 C for 16 hr. The

suspension was then boiled for 20 min. Filtration was carried out through a wire screen, cheesecloth, and Whatman no. 1 filter paper (twice). The infusion was then diluted with 1,000 ml of distilled water and 20 g of Tryptose (Difco); 10 g of NaCl and 200,000 units of penicillin were added. The medium was adjusted to pH 8 with NaOH, sterilized by Seitz filtration, and supplemented with 1% Difco PPLO serum fraction.

Filtration apparatus. All filtrations, unless otherwise noted, were carried out with a 10-ml hypodermic syringe and a Swinney hypodermic adapter which holds a 13 mm diameter filter. The following is a list of filters and pore sizes (in μ) used with the manufacturer's specifications: AA, 0.80 ± 0.05 ; DA, 0.65 ± 0.03 ; HA, 0.45 ± 0.02 ; PH, 0.30 ± 0.02 ; GS, 0.22 ± 0.02 ; VC, 0.10 ± 0.008 ; VM, 0.05 ± 0.003 . Further calibrations were carried out and will be described later. Pore sizes were determined by the manufacturer by the Skau-Ruska mercury-intrusion method (Honold and Skau, 1954).

Filter calibration. To check the pore sizes, and in particular to determine the maximal pore sizes, a series of filtrations was performed with viruses of known size, including vaccinia virus, influenza virus, and T₂ coliphage. The procedure was to take 10 ml of virus suspension and filter it serially, starting with the largest pore size filter (AA) and continuing in sequence down through the smallest pore size filter used (VM). After each filtration, a 1.0-ml sample was taken for assay. The assays used were pock counting on the chorioallantois of 21-day-old chick embryos for the vaccinia and testing for positive hemagglutination of chicken erythrocytes in the case of influenza. Plaque formation was used for the phage assay.

Size determination of PPLO. The technique here employed was similar to that described in

the serial filtration of viruses for the calibration of filters. A 10-ml suspension of cells was serially filtered through a sequence of filters of decreasing pore size. After each filtration, a 1.0-ml sample was taken and assayed by the end point dilution method (Cleverdon and Morowitz, 1961). In some experiments, the filtrates were examined in an electron microscope. This was accomplished by centrifuging the cells, resuspending in distilled water, fixing with osmium tetroxide vapor, and drying on Formvar films.

Primary isolations. Primary isolations were done on nine rats showing symptoms of a respiratory ailment and four chickens having chronic respiratory disease. In the case of the rats, the area around the nares was swabbed with sterile cotton swabs; in the chickens, the trachea was swabbed. The swabs were immediately placed in beef heart infusion, and the medium was then filtered through an $0.45\text{-}\mu$ filter (HA). Cultures were incubated at 37 C for a period of 1 week, and were examined daily for evidence of growth.

For preparative filtration, larger filters were used. Between 100 and 1,000 ml were serially filtered down through the $0.22\text{-}\mu$ filter. Electron micrographs were taken of the initial culture and of the final filtrate.

RESULTS

The virus calibration data and one set of determinations on *Mycoplasma laidlawii* B are shown in Table 1. This table shows that the smallest viable units of *M. laidlawii* B are smaller than vaccinia (0.22 by $0.26\text{ }\mu$) and larger than influenza (0.08 by $0.10\text{ }\mu$). This accords with electron micrographs (Fig. 1) of particles from the $0.22\text{-}\mu$ filtrate, which show particles between 0.1 and $0.2\text{ }\mu$. Table 2 shows

TABLE 1. Virus filtration, as a function of pore size, and comparison with *Mycoplasma laidlawii*

Organism	Filter pore diam (μ)							
	Control	0.80	0.65	0.45	0.30	0.22	0.10	0.05
Vaccinia....	1	1	1	0.3	0.02	0	0	0
T ₂ phage....	1	—	—	—	—	0.5	1.8×10^{-4}	0
Influenza....	1	1	1	1	1	1	0.08	0
<i>M. laidlawii</i>	1	1	1	1	1	0.01	10^{-8}	0

FIG. 1. *Mycoplasma laidlawii* B filtered through a $0.22\text{-}\mu$ filter. $\times 17,400$.

TABLE 2. Fraction of viable titer through various size filters

PPLO strain	Filter pore diam (μ)			
	0.3	0.22	0.10	0.05
H39(1)*.....	1.0	0.6	10^{-5}	0
H39(2).....	1.0	1.0	10^{-4}	0
H39(3).....	1.0	1.0	5×10^{-5}	0
<i>Mycoplasma gallinarum</i>				
(1).....	0.5	0.03	0	0
<i>M. gallinarum</i> (2).....	0.1	0.03	6×10^{-5}	0
<i>M. gallinarum</i> (3).....	1.0	0.6	10^{-5}	0
<i>M. laidlawii</i> B(1).....	1	0.6	0	0
<i>M. laidlawii</i> B(2).....	0.6	0.01	10^{-6}	0
<i>M. gallisepticum</i> (1)....	0.01	10^{-4}	0	0
<i>M. gallisepticum</i> (2)....	0.05	10^{-3}	0	0

* Each line represents an independent experiment.

the results of experiments on four strains of PPLO. The sizes of the smallest particles can be ordered. The smallest strain appears to be H39.

From both groups of animals in the primary

isolation study, rats and chickens, PPLO were isolated by use of the filtration procedure. In the experiment with rats, seven of nine cultures gave positive isolates; in the experiment with chickens, three of four cultures gave positive isolates. That the growth in broth was PPLO was confirmed by subculturing on agar; colonies formed were typical PPLO colonies. Further confirmation that the organisms isolated from the chickens were PPLO (*M. gallisepticum*; Edward and Kanarek, 1960) was demonstrated serologically. The organisms were agglutinated by antiserum prepared with an authentic strain of *M. gallisepticum*.

Agar plates (BHI plus serum fraction and penicillin) inoculated with material before filtration were grossly contaminated with bacteria.

DISCUSSION

In our limited study on primary isolation, we have found porous cellulose ester membranes (average pore size 0.45μ) to be of use in excluding bacteria in the initial cultures, at the same time permitting the passage of PPLO.

The use of these membranes in determining the smallest size units enabled us to establish the relative sizes. These sizes were in general agreement with electron micrographs of these preparations.

The most significant question raised by the data in Table 2 is whether there are strains of PPLO which produce viable subunits smaller than 0.1μ in diameter. Such units would represent the smallest autonomous clone-forming units yet described. On the basis of the virus

filtrations, one would assume that the titer after the filtration does indeed represent units smaller than the pore diameter. The viruses are, however, rigid, while the PPLO appear to be considerably more flexible so that the possibility exists of cell shape being distorted. This squeezing might allow larger cells to pass the filter pore. Various theoretical considerations, which will be discussed elsewhere, suggest that such squeezing will not be a major factor and that cultures of many strains of PPLO do give rise to extremely small reproductive units.

ACKNOWLEDGMENTS

The authors wish to acknowledge the help of John F. McCrea, who carried out the vacinia and influenza assays, and Phyllis Kahn, who carried out the phage assays.

This work was supported by grant AM-05226-02 from the U.S. Public Health Service.

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