CULTIVATION AND VISUALIZATION OF MYCOBACTERIA ON MOLECULAR FILTER MEMBRANES

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The molecular filter membrane (MF) was developed for the recovery of small numbers of bacterial contaminants in water and air (Goetz, 1953a,b; McKee et al., 1953). A number of pathogenic bacteria, including the tubercle bacillus, were grown on these filters by Orlando and Bolduan (1953). These membranes are worthy of further study in the cultivation of mycobacteria. The possible advantages of the membrane should include recovery of every tubercle bacillus in a large volume of liquid such as urine or spinal fluid, accelerated detection of growth, and the feasibility of washing residual antibiotics out of clinical inocula without loss of bacilli. The filters could be used also for morphological and biochemical studies of intact colonies.

Orlando and Bolduan (1953, personal communication) grew Mycobacterium tuberculosis on the filters over Lowenstein-Jensen egg medium, and we have repeated their work and extended it to include subcultures of a number of other mycobacterial species and strains. In this paper we shall stress visualization of growth with special reference to the use of a neutral red stain and its significance in the characterization of growth as well as to oblique lighting for early detection of colonies.

METHODS AND MATERIALS

Cultivation. Twelve ml of Lowenstein-Jensen egg medium were inpsized in screw capped, wide mouthed, 2 ounce jars having a 50 mm inside diameter. The lids were fitted with bibulous paper discs which were saturated, just before incubation of the cultures, with sterile distilled water.

The membrane used in this study was the Millipore Filter Disc, grid marked, of 47 mm diameter. Inoculum consisted of diluted cultures of the tested organisms grown in Dubos' Tween-albumin medium or homogenates of colonies grown on Lowenstein-Jensen medium and suspended in a 0.5 per cent solution of bovine albumin fraction V. The inocula were not standardized with respect to age or turbidity since qualitative rather than quantitative results were desired. Some of the membranes were inoculated by passage of the suspensions through the filters supported on the Pyrex filter holders which were obtained from the same source as were the filter discs (McKee et al., 1953). The inoculated discs were then placed, inoculated (grid) side up, on the surface of the egg medium. Others were placed on the medium, first, and a drop of inoculum spread on the surface with a wire loop. For our purposes either method of inoculation was satisfactory.

The caps with water saturated liners were placed loosely over the jars, and the cultures were incubated, inverted, in a large desiccator with water in its base. Maintenance of an atmosphere saturated with water vapor is essential for optimal growth on molecular filter membranes (McKee et al., 1953).

Visualization. Growth of tubercle bacilli was observed with the naked eye after a week of incubation, and saprophytes grew on the filters at a rate comparable to that on the medium alone. Very early observation of growth could be made by means of special lighting. The aperture of a standard microscope lamp was masked with cardboard in such a manner as to permit passage of a thin horizontal "sheet" of light. This was directed across the surface of the membrane culture at a very low angle to the surface, i.e., practically parallel to it, and the culture was examined with a stereoscopic dissecting microscope. Brief experimentation with the light will indicate optimal thickness and incident angle for the light beam.


2 Obtained from the Lovell Chemical Company, Watertown, 72, Mass.
Acid-fast staining of mycobacteria in situ was not satisfactory due to the tendency of colonies to be washed off the membrane. Impression smears made on slides could be subjected to acid-fast staining.

A differential stain in which physical washing was unnecessary was needed if intact colonies were to be observed in detail. The neutral red (cyto-chemical) test of Dubos and Middlebrook (1948) represents such a technique, in principle. The specific phenomenon observed in this test is elicited by a chemically induced alteration of the dye color, rather than by the physical washing out of unfixed dye. Its usefulness has been limited to recognition of virulent mammalian tubercle bacilli, which have been grown by routine methods (Richmond and Cummings, 1950; Morse et al., 1953). Modification of the technique to make it applicable to the staining of colonies has yielded a differential tool for visualization of tubercle bacillus colonies grown on molecular filter membranes.

The following reagents were employed: NR-6: A saturated solution of neutral red in 95 per cent ethanol, filtered and acidified with 1 per cent (v/v) of concentrated hydrochloric acid; Na₂CO₃ developer: A 1:10 dilution of saturated aqueous sodium carbonate solution; ethanol, 95 per cent.

A small segment of the filter, containing apparent growth, was excised and placed, growth side up, on paper pads saturated with the reagents indicated in the following sequence: (1) NR-6: 10 minutes (entire segment turns red); (2) Na₂CO₃ developer: 5 minutes (background turns amber); (3) 95 per cent ethanol: 1 minute (removes excess reagents).

The stained segment was dried and mounted on a slide in Permount mounting fluid* under a cover slide.

* A synthetic mounting fluid marketed by Fisher Scientific Co., New York, N. Y.
slip. The preparation was transparent and suitable for microscopic examination at all powers.

RESULTS

By means of the low angle incident illumination and observation through the dissecting microscope it was possible to detect growth of *Mycobacterium tuberculosis*, strain H37Rv, on the membrane in three or four days of incubation (figure 1). This type of observation alone, however, did not permit identification of these early colonies.

Thirty two stock strains* of various species of the genus *Mycobacterium* were grown on membrane filters and examined by the neutral red staining technique. The following organisms stained red against an amber background, i.e., were neutral red positive: *M. tuberculosis* var. *hominis*, 6 strains; *M. tuberculosis* var. *bovis*, 4 strains; *M. avium*, 2 strains; *M. tuberculosis* var. *hominis*, attenuated variants, 3 strains; BCG strain, 4 strains; *M. ulcerans*, 1 strain; *Mycobacterium* spp. (saprophyte), 1 strain.

The following organisms remained yellow and blended with the background, i.e., were neutral red negative: *M. tuberculosis* var. *hominis*, attenuated variant, 1 strain; *M. avium*, 1 strain; *M. phlei*, 3 strains; *M. smegmatis*, 2 strains; *M. butyricum*, 1 strain; *M. stercoris*, 1 strain; *Mycobacterium* spp. (saprophytes), 2 strains.

In no case was it necessary to incubate the cultures longer than 6 days to produce sufficient growth to be studied by this method. Certain of

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*Figure 2. Portion of cored microcolony of *Mycobacterium tuberculosis*, strain H37Rv, on the MF. Neutral red stain, oil immersion examination.*
the attenuated variants which are listed as positive gave weak coloration only.

On microscopic examination it was possible to observe cording of the colonies of virulent strains of \textit{M. tuberculosis}. Figure 2 is a photograph of a corded strain of \textit{M. tuberculosis} stained in this manner.

**DISCUSSION**

The use of the molecular filter membrane for subcultures of mycobacteria permits detection of early growth. The optical system described is simple to set up and permits observation of colonies of diameter as small as 10 micra without disturbing the growing culture.

By staining with neutral red and developing with alkali it is possible to visualize these colonies and observe, in detail, the colonial morphology of the neutral red positive mycobacteria. Hughes \textit{et al.} (1954) tested numerous nonacid-fast bacteria, yeasts, and molds and found them to be neutral red negative by the standard method as were four strains of the acid-fast fungus genus \textit{Nocardia}. We have found strains of various corynebacteria to be neutral red negative on molecular filter membranes. Although, as may be seen from our results, a positive neutral red reaction is not an assurance of virulence, we have not encountered any virulent mammalian tubercle bacilli which were neutral red negative. For this reason, in the search for tubercle bacilli on molecular filter cultures, the neutral red stain should not fail to visualize all colonies of these organisms.

On occasion one may encounter aborted growth or contamination on cultures for tubercle bacilli. By ordinary culture methods, tubercle bacillus colonies in such cultures will not be observed or recognized. In the former case, a few generations of bacteria may be produced when, for no obvious reason, multiplication ceases before the appearance of any grossly visible colonies. We have observed colonies of this apparent type by examination, under oil immersion lens, of neutral red stained membrane preparations. Rudimentary cords, characteristic of mammalian tubercle bacilli (Middlebrook \textit{et al.}, 1947), may be seen in colonies with as few as 20 bacilli. Cultures of the tubercle bacillus which were overgrown with yeast contaminants, resulting in aborted growth, have also been subjected to this stain, and it was possible to observe small colonies of the neutral red positive organisms through the yeast film.

Since the neutral red stain technique permits detailed observation of colonial morphology (i.e., cording), as well as the cytochemical reaction, this method may be useful as a combined virulence test. We have been investigating this possibility, and the results will be published at a later date.

Practical application of the molecular filter membrane to the cultivation of tubercle bacilli from clinical specimens is not yet feasible since many biological fluid materials tend to clog the pores of the membrane. Attempts to overcome this difficulty are under way in this laboratory at the present time.

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**SUMMARY**

A number of different mycobacteria have been grown on molecular filter membranes, over Lowenstein-Jensen egg medium, in an atmosphere saturated with water vapor. A method for detection of very early growth, using a simple optical arrangement, was described.

A colonial stain has been developed, based on the cytochemical reaction of tubercle bacilli with neutral red. The implications of this stain were discussed. By means of the technique described, it was possible to recognize microcolonies of virulent mammalian tubercle bacilli after as little as three days of incubation.

**REFERENCES**


Hughes, D. E., Moss, E. S., Hood, M., and Henson, M. 1954 Virulence of \textit{Mycobacterium


