**Mycobacterium microti** (Vole Bacillus): a Method for Viable Counts Within 21 Days of Culture

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A method is described for counting viable units of *Mycobacterium microti* (vole bacillus) suspensions after 21 days of culture on an oleic-albumin agar medium containing 5% defibrinated horse blood, with a tight seal to maintain humidity. This method is important because vole bacillus is an alternative to BCG in the prevention of tuberculosis.

**Mycobacterium microti**, causal agent of vole tuberculosis (9), has been used as an alternative to BCG for vaccination to prevent tuberculosis (6, 8). Counts of viable bacterial units in vaccine suspensions of this organism have generally been found difficult to make. By using various media (egg and semisynthetic), 4 to 8 weeks of incubation have been required to reveal colonies, whereas such media produce good growth of *M. tuberculosis* in 2 to 3 weeks (5). A method is now described for viable counts of vole bacillus suspensions obtainable within 21 days.

**Bacterium.** *M. microti* strain OV 254, originally isolated by A. Q. Wells, was used and maintained on slopes of glycerol-free Herrold egg medium (4) at 37 C.

**Media.** The following solid media, which satisfactorily support growth of *M. tuberculosis*, were tested: (i) 7H-10 (7), protected from heat and light during preparation; (ii) 7H-11 (1), similarly protected; and (iii) oleic-albumin agar, modified from that of Dubos and Middlebrook (2), and made up of agar base and Tween-albumin mixture. Agar base consisted of: KH$_2$PO$_4$, 1.0 g; Na$_2$HPO$_4$·12H$_2$O, 6.3 g; l-asparagine, 2.0 g; Casitone (Difco), 0.5 g; ferric ammonium citrate, 0.05 g; MgSO$_4$·7H$_2$O, 0.01 g; CaCl$_2$, 0.0005 g; ZnSO$_4$, 7H$_2$O, 0.0001 g; CuSO$_4$·5H$_2$O, 0.0001 g; agar (Difco), 15.0 g; and distilled water, 900 ml. The KH$_2$PO$_4$, Na$_2$HPO$_4$, and asparagine were dissolved in 100 ml of distilled water. The other ingredients except agar were dissolved in the remaining 800 ml of water. The pH was adjusted to 6.8 with concentrated HCl, the agar was added and dissolved, and the whole solution was autoclaved at 115 C for 25 min. Tween-albumin mixture consisted of polyethylene sorbitan monolaurate (Tween 80 [1.2 ml]) dissolved with stirring in 98.8 ml of 0.05 N NaOH. The albumin solution was made by adding glucose (50 g) to 0.85% NaCl (900 ml) in a 5-liter flask. A 50-g amount of bovine albumin fraction V (Armour) was added to the flask, which was placed at 4 C until the albumin was dissolved; the pH was then adjusted to 7.0 with concentrated NaOH and the volume was made up to 1 liter with 0.85% NaCl. After the addition of 52.2 ml of Tween 80 solution to the flask, the whole volume was sterilized by passing through a Seitz filter. To make the final oleic-albumin agar, the agar base was melted in a steamer, cooled, and then placed in a water bath at 56 C. A portion (32.0 ml) of Tween-albumin mixture was added to 280 ml of agar base and mixed thoroughly. The mixture was poured in 20- to 25-ml portions into plastic petri dishes (90 by 15 mm). The plates were stored at 4 C; before use they were inverted and dried overnight at 37 C.

In a fourth medium, blood-oleic-albumin agar, 5% defibrinated horse blood, hemolyzed by freezing and thawing, was added together with the Tween-albumin mixture.

**Viable counts.** Suspensions of vole bacillus were made by homogenizing 14- to 21-day-old cultures in a solution of 0.1% albumin in 0.85% NaCl, and adjusting the suspension visually to a concentration of 0.2 mg (weight)/ml by comparison with a standard. Suspensions were diluted serially in 0.1% albumin-saline, and drops were inoculated onto plates of the solid media at dilutions of 10$^{-1}$ to 10$^{-4}$, with a Pasteur pipette that delivered a known drop volume. The plates were individually sealed with Parafilm (Lindsay and Williams, supplied...
by Gallenkamp Ltd., London) and enclosed in a polyethylene envelope, inverted, and incubated at 37 C for 2 to 6 weeks, after which colonies were counted by use of a dissecting microscope.

**Total counts.** Total counts of bacterial suspensions were made by the method of Hart and Rees (3).

Table 1 shows the viable counts for the four media used. In the case of the blood-oleic-albumin agar, total counts are given for the suspensions used for plating.

The media without blood gave colonies too small to count even after prolonged culture. The blood-oleic-albumin agar showed discrete colonies of countable size at 21 days of culture; the counts were not increased after 28 days. The percentage of viability was of the order usually found in mycobacterial suspensions prepared by homogenization of surface cultures.

The slow development of colonies previously reported for media like those used for counting viable tubercle bacilli was confirmed. Long periods of culture carry the risk of dehydration of media on plates, even those sealed in the commonly used polyethylene envelopes. These difficulties have been overcome by incorporating 5% hemolyzed horse blood in an oleic-albumin agar base, and by using a tight seal of Parafilm around the petri dishes enclosed in polyethylene envelopes. Under these conditions, colonies are easily visible under 12.5× magnification and can be counted after 21 days of incubation.

Vole bacillus vaccines have been used in Czechoslovakia since 1953 as an alternative to BCG in the prevention of tuberculosis (8). Moreover, in its most recent report, the Medical Research Council's Tuberculosis Vaccines Clinical Trials Committee states, on the basis of its 15-year clinical trial, that "since at least one substrain of vole bacillus vaccine has been shown to confer substantial protection against tuberculosis without the complication of lupus, it would seem to be worth considering the reintroduction of the vole bacillus as an immunising agent against tuberculosis" (6).

It therefore seems advantageous to have an improved method of counting viable units of vole bacillus suspensions.

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| Culture medium                               | Incubation time (days) | No. of bacterial units/ml in original suspension | Mean* viable (range) | Total | No. viable (%) |
|----------------------------------------------|------------------------|-------------------------------------------------|----------------------|-------|----------------|
| 7H-10                                        | 21                     | Colonies too small to count                      | —                    | —     | —              |
| 7H-11                                        | 21                     | Colonies too small to count                      | —                    | —     | —              |
| Oleic-albumin agar                           | 35                     | Colonies too small to count                      | —                    | —     | —              |
| Oleic-albumin agar + 5% defibrinated horse blood | 21                     | $4.5 \times 10^7$ (2.8-6.6)                      | $7.0 \times 10^7$    | 64    |                |
|                                             | 21                     | $2.9 \times 10^7$ (2.6-3.4)                      | —                    | —     | —              |
|                                             | 21                     | $3.5 \times 10^7$ (3.3-3.6)                      | $4.0 \times 10^7$    | 87    |                |

* For three plates.