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Simple Staining Procedure Permits Rapid Counting of Mycoplasma Colonies

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A staining procedure employing oil red O and Coomassie R-250 was developed to increase visualization of mycoplasma colonies. This procedure permits CFU determination of mycoplasmas without additional microscopy.

Mycoplasma colonies are small, ranging from 0.01 to 0.6 mm in diameter (3). Often, colonies cannot be seen without magnification, and microscopy is needed for recognition or for colony counting. For these reasons, quantitation of mycoplasma growth by CFU determination is cumbersome and time consuming.

There are numerous reports of attempts to facilitate quantitation of broth-grown or agar-grown organisms (1, 2, 4, 11, 12, 16, 17). For example, Low and Eaton (10) used a dilution of the Dienes stain as an aid in determining CFU. Also, hemolytic plaque assays employing guinea pig (13) or sheep (6) erythrocytes were used to assist in identification and quantitation of *Mycoplasma pneumoniae* colonies.

Del Giudice et al. (8) demonstrated that colonies of *M. lipophilum* are distinguished by their ability to stain with lipid-soluble dyes. Now, we report a simple procedure which employs the lipid stain oil red O and a protein dye for staining mycoplasma colonies of various species. This procedure allows rapid quantitation of CFU without the need for microscopy.

Four media formulations were used in growing mycoplasmas. SP4 medium (14) contained fetal bovine serum. Hayflick medium (9) included horse serum, and SSR_2 medium contained bovine serum fraction. In a modified SSR_2 formulation (5), phosphatidylcholine-cholesterol liposomes and albumin replaced the serum fraction. Agar medium for each formulation contained 1% (wt/vol) Noble agar (Difco Laboratories, Detroit, Mich.). Petri dishes (15 by 60 mm) containing 8 ml of agar medium were held at room temperature for 48 to 72 h to evaporate excess moisture.

Oil red O (C.I. 26125; Matheson, Coleman, and Bell, Cincinnati, Ohio) and a dye reagent containing Coomassie R-250 were used to stain the mycoplasma colonies. First, we prepared a saturated stock solution of oil red O (0.25 to 0.50%, wt/vol) in isopropanol. To prepare a working solution of oil red O, six parts of saturated stock solution were diluted with four parts of distilled water (7); after 10 min, the solution was filtered through Whatman no. 1 paper. The working solution of oil red O is stable for 2 h. The working solution of protein stain consisting of 0.25 g of R-250 dye (C.I. 42660; BioRad Laboratories, Richmond, Calif., lot 25527) was dissolved in a mixture of methanol, 45.4 ml; distilled water, 45.4 ml; and glacial acetic acid, 9.2 ml (15). Before use, this solution was filtered through Whatman no. 1 paper. The filtered working solution of protein stain is stable at least 1 month.

M. pneumoniae 65-2161, *M. gallisepticum* S6, *M. fermentans*, PG18, *M. pulmonis* Nelson 3, *M. hyorhinis* GDL, *M. arginini* G-230, and *M. arthritidis* PG27 were tested for their ability to accept the stains. Each petri dish was inoculated with a 0.1-ml suspension of organisms and incubated at 37°C for 7 to 10 days. Older mycoplasma colonies (4 to 6 weeks of incubation) stained as intensely as the younger colonies. To stain colonies, agar surfaces were flooded with 1.3 ml of the oil red O stain and rotated 3 or 4 times during a period of 10 to 15 min. The lipid stain was removed, and the agar was gently washed three times with 5-ml volumes of distilled water. The plates were inverted to drain excess water and then were counterstained for no more than 30 to 45 s with approximately 1 ml of protein stain. The stain was then decanted, and the agar surface was rinsed with distilled water. Excess dye washes were sterilized before being discarded.

The seven *Mycoplasma* species, each grown on all four media, were stained by the lipid-protein procedure. At first, colony centers were red, and their peripheries were dark blue. After 3 h, the central areas of each colony had changed to bluish black, which further differentiated the colonies from the light blue agar background. Figure 1A and B demonstrates this contrast for unstained and stained colonies of *M. fermentans*. With *M. arthritidis* (Fig. 1C), the colony centers remained sharply delineated after staining. Figure 1D shows a petri dish containing stained colonies which may be counted with a bacterial colony counter.

At a magnification of ×100, faintly stained liposomes were observed in SSR_2LIP medium. Since such staining artifacts may occur, this procedure would probably not be dependable for identifying mycoplasma colonies appearing on primary isolation.

The lipid-protein staining procedure provides an inexpensive means of readily visualizing mycoplasma colonies and allows quantitation of colonies without the need for a microscope.

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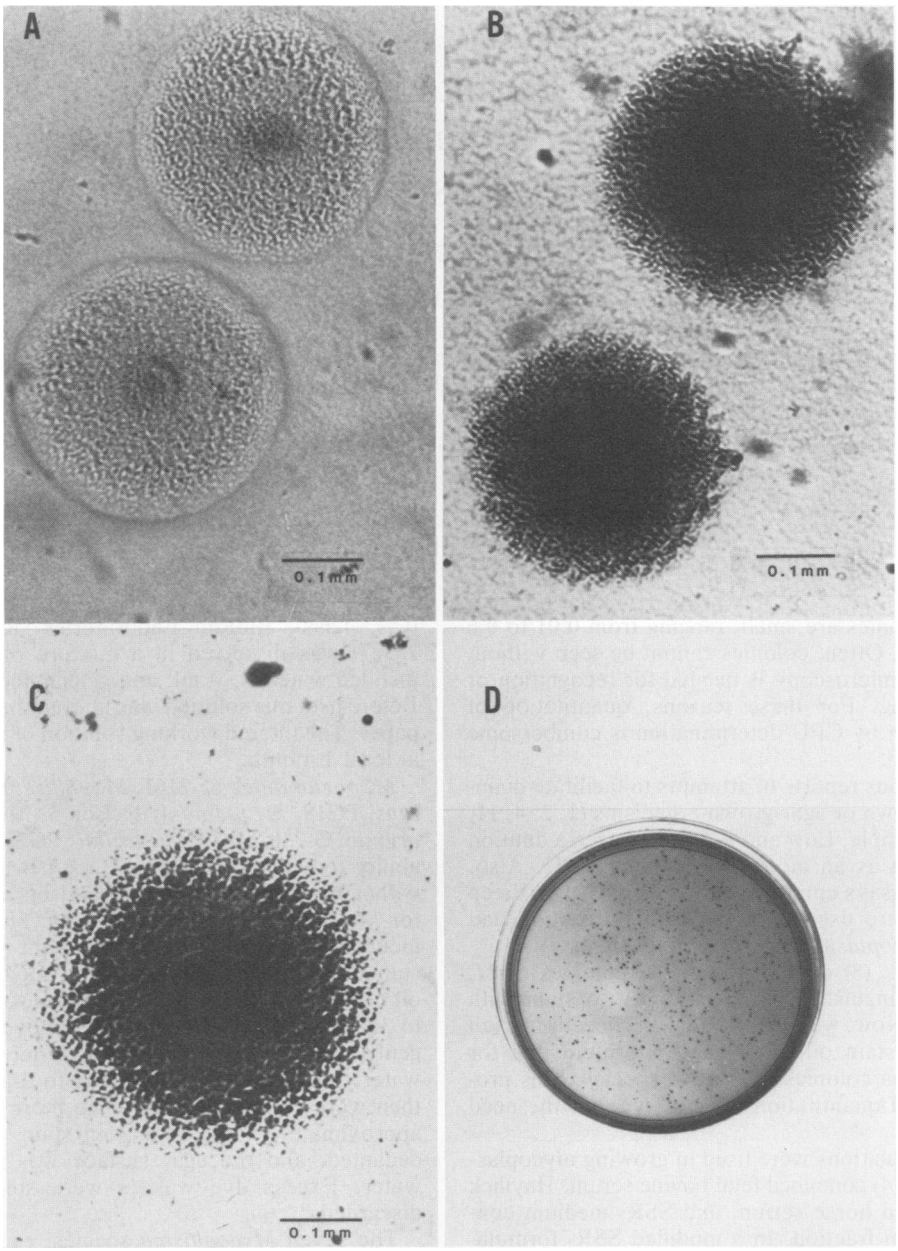

FIG. 1. Stained and unstained mycoplasma colonies grown on SP4 medium. (A) Unstained *M. fermentans*; (B) stained *M. fermentans*; (C) stained *M. arthritidis*; (D) entire agar surface containing stained *M. hyorhinis* colonies

John Kocka was responsible for all photography.

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