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Enumeration of Mycoplasmas After Acridine Orange Staining

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Samples of liquid mycoplasma cultures were mixed with equal parts of a 0.01% solution of acridine orange and placed on agar plates. The number of fluorescing organisms per field was counted in an epifluorescence microscope at an ×1,000 magnification. When the number of fluorescing organisms per field was related to the number of colony-forming units per milliliter during the growth cycle, highly significant correlation was found in cultures with $\geq 10^6$ colony-forming units per ml during the exponential growth phase. The counts were weakly correlated during the stationary phase and not correlated during the death phase. This technique provides a mean to enumerate mycoplasmas in liquid cultures.

Acridine orange is a fluorochrome with affinity for nucleic acids. In ultraviolet light, stained ribonucleic acid and single-stranded deoxyribonucleic acid appear orange to red. Double-stranded deoxyribonucleic acid appears green (6). Mycoplasma colonies stain well with acridine orange (4), and mycoplasma organisms contaminating cell cultures may be visualized with this stain (1).

Mycoplasmas are commonly harvested from broth cultures at the end of the exponential growth phase. Enumeration of mycoplasmas in liquid cultures is retrospective and requires time-consuming plate counting or specialized biochemical techniques (5, 7). In an attempt to develop a rapid method for immediate enumeration of mycoplasmas in broth cultures, measured samples were stained with acridine orange, and fluorescing organisms were counted in an epifluorescence microscope.

MATERIALS AND METHODS

Cultures were prepared of *Mycoplasma bovis* strains 427 (obtained from H. L. Ruhnke, Veterinary Services Laboratory, University of Guelph, Guelph, Ont., Canada) and A42 and the following glucose-catabolizing strains: *Mycoplasma hyopneumoniae* (J), *Mycoplasma dispar* (462/2), *Mycoplasma canis* (PG14), *Mycoplasma edwardii* (PG24), *Mycoplasma molare* (H542), *Mycoplasma cynos* (H831), *Mycoplasma felis* (CO), *Mycoplasma neurolyticum* (type a), *Acholeplasma laidlawii* (PG8), *Mycoplasma* sp. strain HRC 689. Cultures were also prepared with the following arginine-catabolizing strains: *Mycoplasma gateae* (CS), *Mycoplasma spumans* (PG13), *Mycoplasma maculosum* (PG15), *Mycoplasma opalescens* (MH5408), and *Mycoplasma arthritidis* (158P10). *M. hyopneumoniae* (J) and *M. dispar* (462/2) were grown in the medium described by Friis (2). Hayflick's medium was used for the remaining strains (3). The medium for the glucose-catabolizing strains was supplemented with glucose (0.5%, wt/vol) and phenol red (0.06%) and adjusted to pH 7.8. Medium with arginine (1.0% wt/vol) and phenol red (0.06%) was adjusted to pH 7.3 and used for arginine-catabolizing strains.

One part of liquid culture was mixed with one part of 0.01% acridine orange (Sigma Chemical Co., St. Louis, Mo.) solution in phosphate-citric acid buffer (pH 3.0) and left at room temperature for 5 min.

The effects of acridine orange concentration and pH of the buffer on the staining intensity of mycoplasmas and background fluorescence were evaluated in preliminary experiments with cultures of *M. bovis* strain 427. The 0.01% concentration in phosphate-citric acid buffer (pH 3.0) gave optimal staining with the least disturbing background. Using a calibrated loop, a 0.01-ml sample was transferred to the center of an agar plate (1% Noble agar in normal saline) and covered by a clean cover slip. The preparations were examined at an ×1,000 magnification in a Zeiss Universal microscope equipped with an HBO mercury lamp and epifluorescence condenser III RS which had the BG-38 filter and the KP490 interference filter for excitation, the FT580 chromatic beam splitter mirror, and the LP520 barrier filter.

To quantitate the growth in liquid cultures, one colony of each strain was inoculated into 5 ml of medium. Each day over an 8-day period, the number of colony-forming units (CFU) per milliliter was determined by a standard plate counting technique, and each culture was observed for color change. For comparison, a 0.5-ml sample was stained, and the average number of fluorescing mycoplasmas (AO count) in five microscope fields was calculated. Growth curves of all 17 cultures were drawn on the basis of the $\log_{10}$ CFU per milliliter count and superimposed upon each other, with the stationary phase (the day when the CFU per milliliter was highest) being given the same arbitrary time value. The average and standard deviation were calculated in the stationary growth phase, the exponential growth phase (1 day before the highest CFU per milliliter count), and the death phase (1 day after the highest CFU per milliliter count). The average and

standard deviation were similarly calculated on the corresponding $\log_{10}$ AO counts. The coefficient of correlation (r value) between $\log_{10}$ CFU per milliliter and $\log_{10}$ AO counts was calculated for the three points on the growth curve representing the exponential growth phase, the stationary phase, and the death phase, respectively.

RESULTS AND DISCUSSION

The mycoplasmas appeared with green or orange-green fluorescence on the surface of the agar (Fig. 1). They were usually evenly dispersed, but a few strains had clumps which, however, could be dispersed if the stained culture was agitated for 30 s.

Mycoplasmas have Brownian movements in liquid preparations. Our technique facilitates counting because the liquid soaks into the agar, leaving the mycoplasmas immobile on the surface (Fig. 1). With acridine orange only elements containing nucleic acids will stain intensely. In dark-field or phase-contrast microscopy preparations, protein precipitates and other nonviable particles are difficult to distinguish from mycoplasmas.

The color of the cultures of the glucose- and arginine-catabolizing strains invariably changed during the stationary growth phase. Three points representing the exponential growth phase, the stationary phase, and the death phase from the superimposed growth curves of the 17 cultures are depicted in Fig. 2. The corresponding $\log_{10}$ AO counts are also shown. The $\log_{10}$ AO count axis is drawn so that the means and standard deviations of the $\log_{10}$ CFU per milliliter and $\log_{10}$ AO counts in the exponential growth phase coincide. During the exponential growth phase, one mycoplasma organism per field in the AO preparation corresponds to approximately 10^6 CFU per milliliter (Fig. 2). The r value in

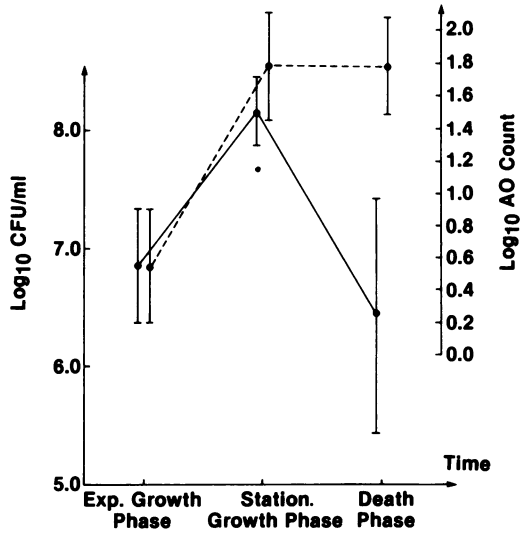

FIG. 2. *Average and standard deviation of the $\log_{10}$ CFU per milliliter count (——) and the $\log_{10}$ AO count (- - -) of 17 mycoplasma cultures during the exponential growth phase, the stationary growth phase, and the death phase.*

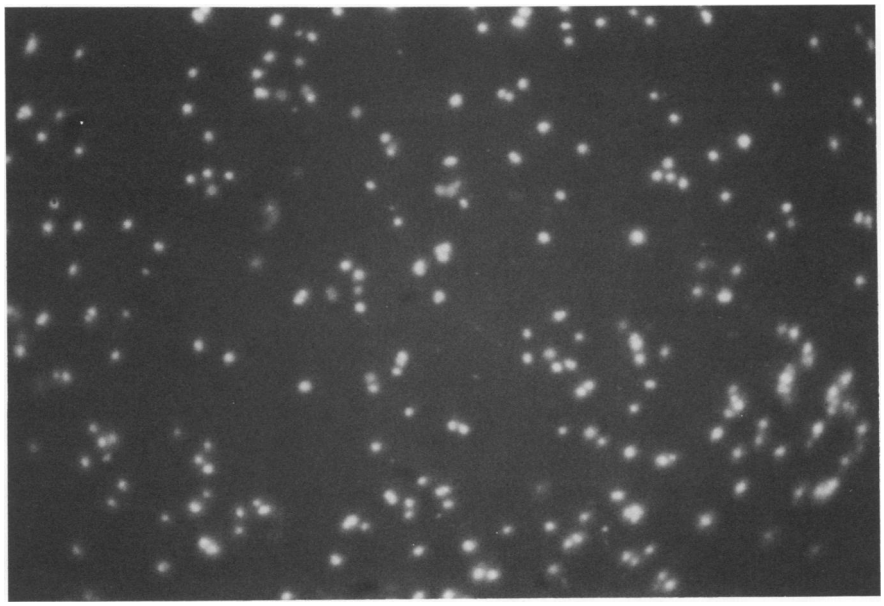

FIG. 1. *Mycoplasmas stained with acridine orange and viewed with an epifluorescence microscope.*

the exponential growth phase was 0.86, which is significant ($P \leq 0.001$). Thus, 74% ($r^2 = 0.74$) of the variation in CFU per milliliter can be predicted by variation in the AO count.

In the stationary phase, the r value was 0.46 ($P \leq 0.05$), and in the death phase, the r value was estimated to 0.02 (not significant). The decreasing correlation in the stationary phase and loss of correlation in the death phase is probably because both viable and nonviable mycoplasmas are counted in the acridine orange preparations, whereas the CFU per milliliter count represents viable mycoplasmas only.

The AO counting technique provides a simple and rapid method to enumerate mycoplasmas in liquid cultures. To follow growth, the AO count must be performed at least daily. In the exponential growth phase, the number of CFU per milliliter can be predicted with reasonable confidence. We have used the technique for several years as a guide to determine the appropriate time to harvest or store liquid cultures.

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