Acridine Orange Staining for Diagnosis of *Mycoplasma bovis* Infection in Cow Milk

DONALD E. JASPER,† SOREN ROSENDAAL, AND DONALD A. BARNUM

Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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*Mycoplasma* organisms were readily recognized in samples of milk or udder secretions from cows with clinical *Mycoplasma bovis* mastitis when these samples were stained with 0.01% acridine orange at pH 3.0. Samples could be stored at −4°C for several days or subjected to repeated freezing and thawing without loss of staining or fluorescence properties. Use of this procedure in diagnostic laboratories on suspect samples from cows with clinical mastitis could hasten inauguration of control measures against this highly contagious disease by several days; however, definitive diagnosis still requires standard culture methods.

Acridine orange staining has proved to be a very helpful technique for the early detection of bacterial growth in blood cultures (4, 6, 7, 9) and in cerebrospinal fluid and other clinical specimens (5). Air-dried films, usually methanol fixed, were stained with 0.01 to 0.025% acridine orange at pH 3.5 to 4.0. Bacteria fluoresced orange against a pale-green or varicolored background.

When liquid culture samples of 13 *Mycoplasma* species and *Acholeplasma laidlawii* were mixed in equal parts with an 0.01% acridine orange solution (pH 3.0) and placed on agar plates under cover slips, all *Mycoplasma* species and *A. laidlawii* fluoresced a strong apple green against a pale-green background (8). When the number of fluorescing organisms per field was related to the number of CFU per milliliter during the exponential growth phase, a very significant correlation was found. The counts were weakly correlated during the stationary phase but were not correlated during the death phase, although organisms continued to fluoresce. *Mycoplasma* species in liquid preparations mounted on glass slides fluoresced a bright apple green, but active Brownian movement precluded accurate counts.

Mastitis caused by *Mycoplasma* infection, especially *Mycoplasma bovis* infection, is a serious problem in some dairy areas (3). *M. bovis* mastitis is highly contagious and often spreads rapidly at milking time. Diagnosis is routinely made by culturing milk or udder secretions, but the 2 to 5 days required for growth of *Mycoplasma* species imposes a serious delay in the inauguration of control procedures. This research was undertaken to determine whether staining of suspect samples with acridine orange could be useful for establishing an early tentative diagnosis of *M. bovis* infection of the udder.

**MATERIALS AND METHODS**

The presence of *M. bovis* infection in milk and udder secretions was determined by culturing on Hayflick agar medium (2). Cultures were transferred or stored frozen (−70°C) in Hayflick liquid medium as needed. The identification of species as *M. bovis* was done by immunofluorescence testing (1). Milk from uninfected cows was obtained for comparative purposes.

Acridine orange was prepared at a 0.01% concentration in phosphate-citrate buffer (pH 3.0) (8) and stored in a rubber-stoppered brown bottle at 4°C. One part of a sample was mixed with one part of the acridine orange solution; the mixture was allowed to stand for 2 to 3 min or more. Preparations for observation were made by using a 0.01-ml loop to transfer 0.01 or 0.02 ml of the mixture to a glass slide or to the surface of a Noble agar plate and covering the slide or agar with a clean cover slip. Both slide and agar preparations were examined under oil at a 1,000× magnification with a Zeiss Universal microscope equipped with an HBO mercury lamp and epifluorescence condenser III R equipped with the BG-38 filter and the KP 490 interference filter for excitation, the FT 580 chromatic beam splitter mirror, and the LP 520 barrier filter.

Observations of fresh normal milk and infected milk or udder secretion samples were made on both slide and agar preparations. Observations were made at intervals of normal and infected samples stored at 4°C before staining with acridine orange, of normal and *M. bovis*-infected samples after freezing and repeated thawing at −4 and −70°C, of sample-acridine orange mixtures stored at 4°C, and of stored final slide and agar preparations. Normal milk with added *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Escherichia coli*, common udder pathogens, was also examined by the acridine orange method.

**RESULTS**

Normal milk contained many small fat droplets and a protein precipitate both of which could be seen on the glass slide preparations. The latter tended to form tiny or larger filaments, with active Brownian movement, or aggregated into large clumps. The small particles somewhat resembled *Mycoplasma* organisms but usually fluoresced a very pale apple green and should not confuse an experienced observer. Occasionally, normal milk contained more brightly fluorescing particles, which could create uncertainties. Both the fat droplets and the proteinaceous material were not apparent when the same material was examined by the agar plate method.

Secretions from infected glands were usually very abnormal, with serum separation, far less fat, and less precipitating protein or other interfering material, and usually contained very large numbers of *M. bovis*. These organisms fluoresced a bright apple green. On the slide preparations, they appeared as small bent rods or short branching filaments 1 to 3 μm in length, each with intense Brownian movement when free in a liquid environment. At times they
were caught up in clumps of precipitated protein, without Brownian movement but brightly fluorescent. On the agar preparations, the Mycoplasma organisms appeared to be gathered into small round masses ca. 1 μm in diameter and fluoresced a bright apple green, as described previously (8). These forms could be mistaken for individual staphylococci or streptococci but did not appear in clumps or chains.

The intensity of fluorescence in samples stored at 4°C before staining stayed strong for 4 to 5 days. By 8 days intensity began to diminish and color began to fade to a yellowish white. By 12 days fluorescence intensity and color quality were poor although still acceptable in some samples. A few bacteria fluorescing apple green appeared in the stored normal milk.

Samples stored at −4 and −70°C were examined after being frozen and thawed 1, 2, 4, and 8 times. The fluorescence intensity of M. bovis remained good to excellent throughout; the appearance of the negative samples did not change. By day 8 of observation there was a decreased number of Mycoplasma organisms, some irregularity in morphology and fluorescence intensity, and some increase in background debris.

Positive samples mixed with acridine orange and stored at 4°C changed very little for 4 days, but by 7 days of age the color quality and fluorescence intensity were diminished. By 14 days only an unsatisfactory weak yellowish-white fluorescence remained. Negative samples were unchanged.

Prepared slides stored at either 4°C or room temperature remained of good quality until drying or other deterioration spoiled them. This might occur as early as 1 day or as late as 7 days or more. On slides which did not dry, the fluorescence deteriorated to yellowish-white by 5 to 9 days. These preparations were no longer acceptable. Preparations on agar were less susceptible to drying than were slide preparations. However, the reduced intensity of fluorescence and quality of color were apparent by 4 days and continued thereafter. Although yellowish-white points of low staining intensity were still evident after 11 and 15 days of storage, these preparations had become unsatisfactory. Milk samples allowed to dry before staining or preparations which dried before observation were always unsatisfactory.

Bacteria, whether added to the milk or appearing naturally, usually fluoresced apple green, in contrast to the bright-orange fluorescence reported in air-dried preparations (4, 6, 7, 9). Occasionally, bacteria were orange or mixed in color. Mycoplasma organisms were easily distinguished from bacteria in the same sample on the basis of size and morphology, except when a single coccal form or a few coccal forms, which might be interpreted as either cocci or Mycoplasma organisms, were present on the agar plate preparations.

**DISCUSSION**

M. bovis populations, as other Mycoplasma species populations, tend to be very high (10^6 to 10^8) in abnormal udder secretions from infected cows with clinical mastitis, and they appear in typical fashion and in very large numbers in acridine orange slide and agar preparations. A tentative diagnosis of Mycoplasma infection under these circumstances is justified, especially in cows or herds whose clinical history suggests Mycoplasma infection. Confirmation by culturing is still required, and culturing is necessary for species identification.

Examination by the acridine orange method, especially with slides, is less useful in early or late stages of the infection, when the milk appears to be normal and only small numbers of organisms are present. Precipitated protein particles with Brownian movement occasionally fluoresce brightly enough to resemble Mycoplasma organisms in slide preparations, and fat droplets can mask them. Although the latter problems can be solved by using the agar plate method, a tentative diagnosis with samples showing small numbers of typically appearing bodies or none at all by acridine orange staining is unreliable and should always await examination by culture methods. Confidence in the tentative diagnosis can be greatly enhanced when 5 to 50 or more typical forms are present in every microscopic field. Milk or udder secretion samples can be stored long enough at 4°C to allow for normal shipment and handling procedures before examination. M. bovis was also very resistant to freezing and thawing at −4 and −70°C, thus permitting flexibility in sample handling. The persistence of fluorescence in mixtures of acridine orange and sample or in final slide or agar preparations also allows flexibility in reading times and an opportunity for repeat readings by the same observer or other observers.

Bacteria which may be present are usually easily distinguished by their larger size and distinct morphology. An exception might be when coccal forms of staphylococci are present and the agar plate technique is being used. Culturing for both bacteria and Mycoplasma species should always be done when Mycoplasma infection is suspected; sometimes both infections are present together.

Acridine orange examination of clinical samples from cows suspected of having Mycoplasma mastitis can provide an early tentative diagnosis which must be confirmed later by culturing. In a first diagnosis in a herd, control measures could be initiated at least 2 to 3 days earlier than otherwise. This could be a major benefit in early outbreak situations. In herds with an ongoing mastitis problem caused by Mycoplasma infections, early tentative identification of infected cows facilitates early segregation and reduction of the spread of infection among cows.

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**LITERATURE CITED**