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# Histochemical Observations on *Mycoplasma* After Staining with Acridine Orange

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ABSTRACT

JASPER, D. E. (University of California, Davis), AND N. C. JAIN. Histochemical observations on *Mycoplasma* after staining with acridine orange. Appl. Microbiol. **14**:720-723. 1966.—*Mycoplasma* colonies and *Mycoplasma* cells in preparations from infected milk and lymph nodes were observed for their fluorescent qualities after treatment with acridine orange. *Mycoplasma* colonies fluoresced brilliant red or red-orange. When treated after exposure to ribonuclease, the colonies fluoresced lime-green. There was no fluorescence when both ribonucleic acid and deoxyribonucleic acid were destroyed by perchloric acid. Detection of *Mycoplasma* in smears of mastitic milk or smears of infected lymph nodes was not definitive because of the large amount of nonspecific ribonucleic acid-rich material present during inflammatory reactions.

Acridine orange is unique in that it will fluoresce bright red under ultraviolet light when combined with ribonucleic acid (RNA) at an appropriate *p*H, and will fluoresce bright lime-green under the same circumstances when combined with deoxyribonucleic acid (DNA). As a consequence, acridine orange has been used extensively to demonstrate the presence of these nucleic acids in organisms, cells, and tissues. No reference to its application to *Mycoplasma* colonies has been found, although mycoplasmas have been reported (Hers et al., *unpublished data*) to fluoresce lime-green in frozen sections of infected chick embryo when stained by the acridine orange method at *p*H 3.8 or 5.4 (4).

The purpose of the present study was to determine the staining characteristics of *Mycoplasma*, to demonstrate histochemically the DNA and RNA components of *Mycoplasma*, and to explore the possibility that staining characteristics might be specific enough to be of value for identification or location of *Mycoplasma* in biological fluids, cells, or tissues.

## MATERIALS AND METHODS

Twelve isolates associated with one outbreak of bovine mastitis (3) and one strain of *Mycoplasma gallisepticum* (obtained from H. E. Adler, Department of Avian Medicine, School of Veterinary Medicine, University of California, Davis) were used. *Mycoplasma* colonies growing on PPLO Agar (Difco) containing 15% horse serum were transferred to a glass cover slip by pressing the cover slip gently over an appropriate area of the plate, and then carefully lifting the cover slip.

Smears of milk from normal cows, from cows with non-*Mycoplasma* mastitis, and from cows with *Mycoplasma* mastitis were prepared either by spreading with a loop, or by sliding a small drop between two cover slips, as for making a blood smear. Tissue imprints were made from the cut surfaces of normal mammary tissue and normal supramammary lymph nodes, from the same tissues of cows affected with acute non-*Mycoplasma* mastitis, and from cows affected with severe *Mycoplasma* mastitis.

Air-dried slides were fixed by passing them quickly through a low flame followed by immersion for 1 min in ether-alcohol. Staining with acridine orange then proceeded according to the methods of Armstrong (1) at *p*H 3.8 or of von Bertalanffy (7, 8) at *p*H 3.8 and 6.0. Use of Carnoy's fixative did not improve the results.

RNA was destroyed in preparations from five different bovine isolates by incubation in 1% ribonuclease (Nutritional Biochemicals Corp., Cleveland Ohio) at 37 C for 2 hr (2, 7). Both DNA and RNA were destroyed in other preparations from the same five bovine isolates by treatment with 10% perchloric acid at 60 C for 30 min (2). Suitable control slides were incubated in distilled water. Treated preparations were then stained with acridine orange at *p*H 6.0 (7, 8).

After observation of fluorescence, colony preparations were decolorized in 50% ethyl alcohol and restained with Wright-Leishman stain to verify the presence or absence of *Mycoplasma* on the slide.

## RESULTS

The results of staining *Mycoplasma* colonies with acridine orange are shown in Table 1. *My-*

*coplasma* colonies normally fluoresced a brilliant fiery-red or orange-red, indicative of high RNA content (Fig. 1). Intensity of staining was greater at *p*H 6.0 than at *p*H 3.8. Occasionally, thin edges of a colony or thin layers of *Mycoplasma* cells which had washed across the slide tended to stain green, especially when stained at *p*H 3.8.

TABLE 1. *Fluorescent qualities of Mycoplasma colonies after staining with acridine orange by various procedures*

| Method of staining colonies | Fluorescence | | | |
|---|---|---|---|---|
| | Red | Green | Yellow-Green | Negative |
| A. Armstrong, *p*H 3.8 | | | 2 | 0 |
| B. Bertalanffy, *p*H 6.0 | 20[a] | | | 0 |
| C. Bertalanffy, *p*H 3.8 | | | | 0 |
| D. Bertalanffy, *p*H 6.0 after ribonuclease | 5 | 5 | | 0 |
| E. Bertalanffy, *p*H 6.0 after perchloric acid | | | | 5 |
| F. Bertalanffy, *p*H 6.0 after incubation in water | 3[b] | | | 0 |

[a] Includes 3 preparations from one strain of *M. gallisepticum* and 17 preparations from among 12 bovine isolates, 5 of which were repeated in conjunction with methods C, D, E, and F.

[b] Two control slides could not be read as the colonies had wasted away.

*Mycoplasma* colonies treated with ribonuclease fluoresced a bright lime-green, indicative of significant DNA content. When both DNA and RNA were destroyed, there was no fluorescence. Presence of organisms on such slides was later proved by use of Wright-Leishman stain. Good colony stains were obtained with this stain either before or after use of acridine orange. Colonies on control preparations incubated in water fluoresced red.

Smears of normal milk contained no fluorescent material suggestive of *Mycoplasma* cells. Imprints of normal supramammary lymph nodes contained occasional large mononuclear cells with red-staining cytoplasm.

Smears of milk and imprints of supramammary lymph nodes from severe *Mycoplasma* mastitis revealed many red-staining intracellular and extracellular particles (Fig. 2). Many young cells with abundant red cytoplasm were present in both types of preparations. The cytoplasm of mature neutrophils was generally dark.

Milk smears and supramammary lymph nodes from acute non-*Mycoplasma* mastitis also contained red-staining intracellular and extracellular particulate material in addition to large young

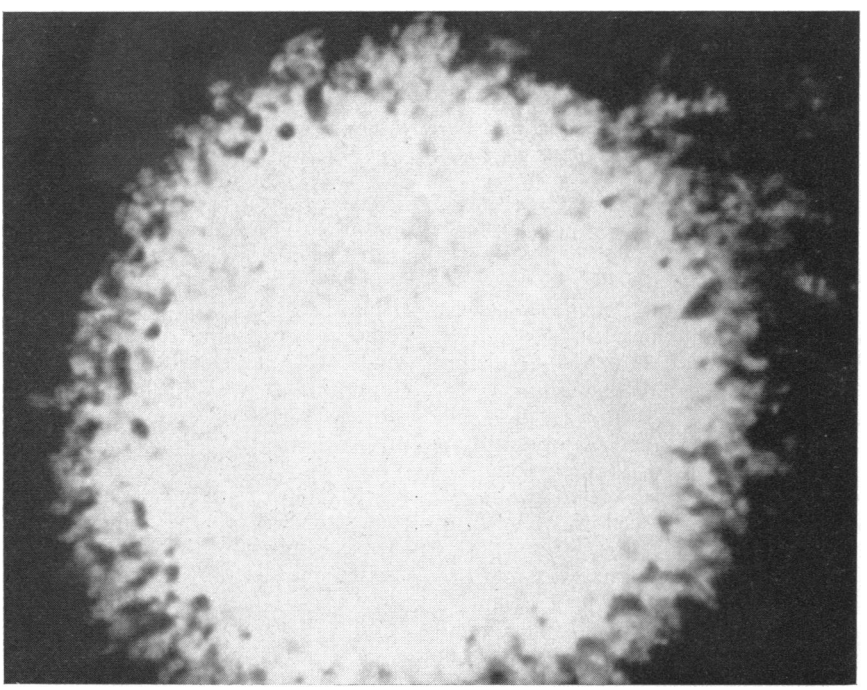

FIG. 1. *Mycoplasma colony fluorescing bright red after staining with acridine orange. Note fine details of fimbriated border.* × *450*

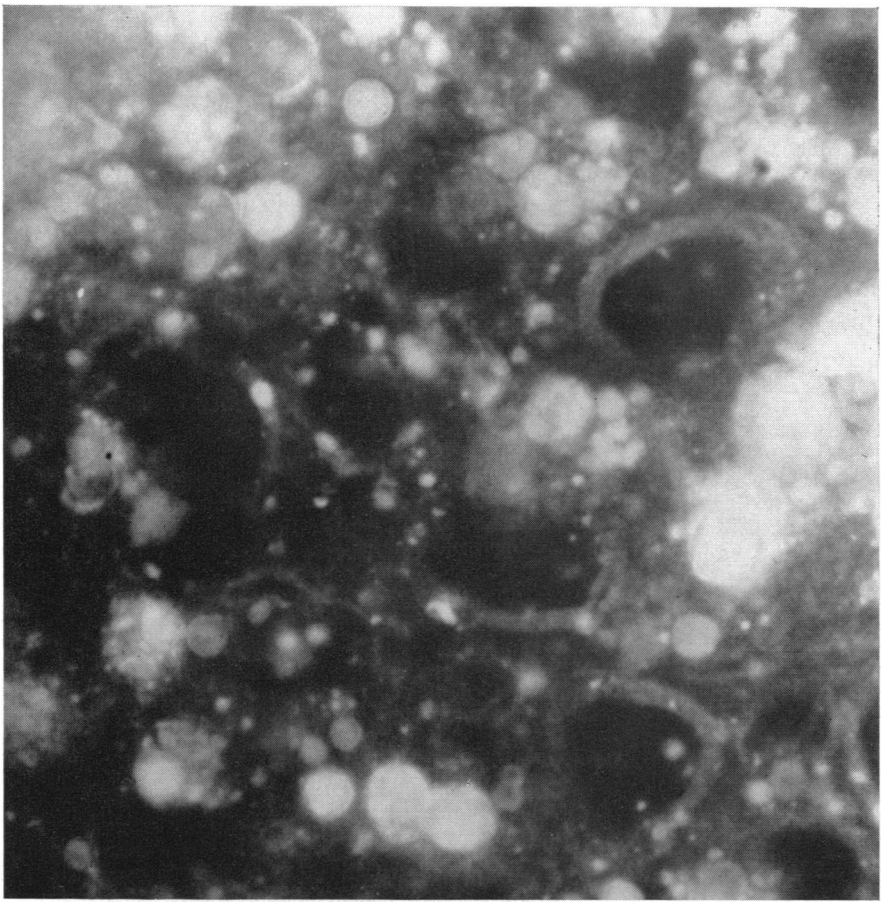

FIG. 2. *Milk smear from Mycoplasma mastitis. Intracellular and extracellular particles fluoresced bright red. Cytoplasm of large cells fluoresced red; nuclei fluoresced green.* × *280.*

cells with abundant red cytoplasm. The cytoplasm of mature neutrophils was generally dark.

## DISCUSSION

The brilliant red staining of colonies reflects a significant RNA content, which has been shown to be about double that of DNA (5, 6), although the DNA present fluoresced a strong lime-green after RNA was destroyed. Very thin or single cell layers of organisms were sometimes observed to fluoresce green instead of red. This characteristic may be related to the reported green staining of *Mycoplasma* in thin frozen sections of infected chick embryo (4).

Although the amount of intracellular and extracellular red-staining material appeared to be more abundant in material from the *Mycoplasma*-infected animals than in material from acute non-*Mycoplasma* mastitis, it was not possible to differentiate the *Mycoplasma*-infected material from noninfected material on this basis. Young RNA-rich cells and RNA-containing granular debris appeared to be common factors in inflammatory exudate and tissues whether *Mycoplasma* cells were present or not. The value of acridine orange staining for study of *Mycoplasma* in cells and tissues may, therefore, be limited to situations in which RNA and DNA from other sources is either lacking or well-defined in location.

Some problems were encountered from the washing away of *Mycoplasma* colonies during processing. The fixation method outlined was better than other variations tried, but washing still occurred occasionally. Additional procedures, such as incubation in ribonuclease or in water, increased this hazard.

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