

Agar Block Technique for Identification of Mycoplasmas by Use of Fluorescent Antibody¹

E. J. BAAS² AND D. E. JASPER

Department of Clinical Pathology, School of Veterinary Medicine, University of California, Davis, California 95616

Received for publication 19 November 1971

A procedure for staining mycoplasmata colonies directly on agar blocks for examination by fluorescent microscopy is described. Areas of the agar surface appropriate for staining were demarcated by use of Lucite cylinders. Direct fluorescent-antibody staining was superior to indirect staining. The technique was very useful for determining whether cultures were mixed and for identification of mycoplasmas in either pure or mixed cultures.

The need to consider mycoplasmas in the etiology of a variety of disease problems has required development and modification of identification techniques. Mycoplasmas can be isolated from a variety of body locations and conditions in the bovine, but species identification is often a problem. Many identification methods present technical difficulties which limit their application in diagnostic situations involving numerous samples.

Many methods fail to detect mixed species adequately in initial cultures. These cultures can be hard to purify by cloning when more than one species is present within a colony (4, 7).

A fluorescent-antibody (FA) technique has many potential advantages for characterization of mycoplasma isolates.

Several FA methods have been used for identification of mycoplasmas by Clark (2), Tully (14), Masiga and Stone (9), Dowdle et al. (5), and Ertel et al. (6). Each method has some limitations for expedient identification of mycoplasmas and mixed mycoplasmata cultures.

A satisfactory method for identifying mixed cultures appears to be that of staining colonies grown on an agar surface. Early methods involved transfer of colonies to slides by a hot-water fixation procedure involving colony transfer problems and heat destruction of the

antigens (2, 3, 8, 13).

As pointed out by Razin (12), direct staining of mycoplasma colonies on the agar is clearly the best method for distinguishing between mixed cultures of different serotypes. Del Guidice et al. (4) and Barile (1) stained entire plate cultures and examined them with incident ultraviolet fluorescent lighting equipment which is not available to most laboratories.

It was desirable, therefore, to devise a practical and reliable method for routine staining and examination of cultures obtained from disease conditions involving numerous and mixed cultures.

MATERIALS AND METHODS

Cultures. Mycoplasma serotypes represented nine available bovine mycoplasma serotypes (Table 1) as classified and furnished by Al Aubaidi and Fabricant (Cornell University, Ithaca, N.Y.). They were grown on a variety of pleuropneumonia-like organisms (PPLO)-agar media. Other observations were made on stock culture preparations and primary plate cultures from mycoplasmata-infected tissues and milk.

Antisera. Antisera were prepared in rabbits essentially by the methods of Morton and Roberts (10). The gamma globulin fraction was conjugated by the procedure of Nairn (11).

Staining mycoplasma colonies for FA examination: direct method. Culture plates were incubated at 37 C in a candle jar (with moisture added) until good colony growth was obtained (2 to 8 days). Petri plates (90 mm) containing 15 ml of 1.5% agar culture media were most satisfactory. Thicker plates were optically undesirable and thinner plates resulted in manipulative problems.

A Lucite cylinder (15 mm long by 13 mm in diameter) was pressed through the agar to circumscribe an area containing well developed, discrete, representative mycoplasmata colonies. The cylinder ends

¹ Based upon a thesis presented by E. J. Baas to the faculty of the Graduate School of the University of California at Davis, California, in partial fulfillment of the requirements for the Ph.D. degree.

² Present address: Veterinary Resources Branch, Division of Research Service, National Institutes of Health, Bethesda, Md. 20014.

were beveled slightly on the inside rim to incorporate an excess of agar pressure which sealed the edges and prevented leakage of antisera. With good colony distribution, 8 to 12 cylinders could be placed on a single plate if so desired. (Fig. 1)

Three drops of diluted conjugate were placed in each cylinder with a Pasteur pipette. The plates were placed in a shallow pan over moist paper towels and incubated for 30 min at 37 C with the covers slightly open.

After incubation, the cylinders were examined for antisera leakage, and the agar plate was inverted over a beaker of disinfectant to remove surplus antiserum. The cylinders were removed with forceps and placed in the disinfectant. The agar blocks were then picked up with a narrow spatula and placed colony surface up on a cover glass. The cover glass was then held over the beaker of disinfectant with forceps, and the block was washed with 10 ml of phosphate-buffered saline (PBS) at pH 7.2. The cover glass and agar block were allowed to drain and dry before being mounted on glass slides. If a plug was dropped, the colony surface was identified by the smooth cylinder cut edges.

In an alternate method of washing, all cylinders were removed and the entire plate with agar blocks remaining in place was then washed with PBS. This did not appear to cause cross staining of negative serotypes.

A drop of buffered glycerol (9 parts glycerol to 1 part PBS, pH 7.2) for each preparation was placed on an identified slide, and the colony surface of the agar blocks was placed in the glycerol. A drop of PBS was applied to the top of the agar, and a clean cover glass was pressed firmly on top to assure good colony-glycerol contact, to smooth out uneven agar surfaces, to retard drying, and to prevent condensation of fluid on the objective lens. Two to four preparations could be placed on one slide by using sepa-

rate small cover glasses or placed in close proximity under one cover glass.

Staining mycoplasma colonies for FA examination: indirect method. The indirect procedure was performed similarly with some variation. After incubation for 30 min with unconjugated antiserum, the excess antisera was removed. Each cylinder plug was washed three times by filling the cylinder with PBS and allowed to stand 5 min before removal of the PBS. Three drops of a 1:20 dilution of fluorescein-conjugated goat antirabbit globulin (Difco) was placed in each cylinder and allowed to incubate for 1 hr. This was then washed away, as in the direct method, very gently with 5 ml of PBS. The remaining procedures were as for the direct method.

Controls. Controls for each trial consisted of using a known culture stained with homologous antiserum, heterologous antiserum, and one preparation without antiserum to enable observations relative to autofluorescence.

Microscopy. The agar block colonies were examined with a standard RA Zeiss microscope with the $\times 10$. Neofluor objective and the 1.2 to 1.4 numerical aperture oil immersion dark-field ultracondenser. They were illuminated with a fluorescent illuminator no. 11 and HBO 200 w/4 mercury vapor super pressure lamp. The light was filtered through a BG 12 (3 mm thickness) exciter filter and a 50/41 barrier filter. Even lighting of the entire field in this system was facilitated by focusing the condenser. Unstained colonies could be visualized by changing the lighting system to dark field by movement of the mirror in the light tunnel of this microscope system. Unstained colonies of some serotypes were also visible by an autofluorescence which differed in character and intensity from specific immunofluorescence. (Fig. 4).

Photomicrography. Fluorescent photographs were taken by using a Reichart Photoautomatic GAE

TABLE 1. Antigenic relationship of various bovine mycoplasmas as studied by the direct fluorescent-antibody technique

Mycoplasma serotype ^a	Dilution of conjugated antisera giving positive immunofluorescence (reciprocal ratio)								
	B	C	D	E	F	H	I	K	M
B. (<i>M. bovisgenitalium</i>)	320								
C. (<i>M. laidlawii</i>)		160							
D. (<i>M. bovirhinis</i>)			320	320					
E. (Unnamed)			320	320					
F. (<i>M. bovimastritis</i>)	20				160		20		
H. (Unnamed arginine utilizer)						20 160			
I. (Unnamed arginine utilizer)							160		
K. (Unnamed glucose utilizer)								640	10
M. (Unnamed glucose utilizer)								80	320

^a All mycoplasma colonies were grown on E medium agar plates and stained with conjugated antiserum produced in rabbits.

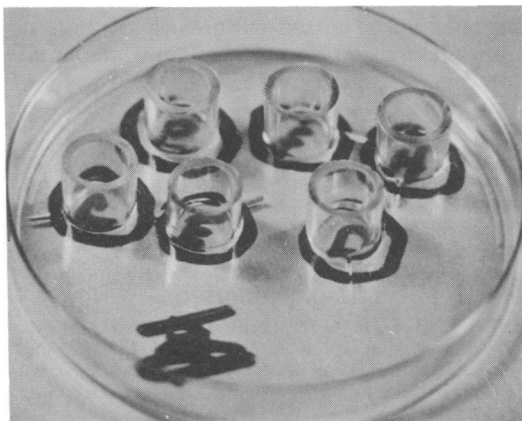


FIG. 1. Lucite cylinders pressed into agar media prior to staining colonies with conjugate. Conjugate and serotype designation can be seen on bottom of plate.

camera attached to the microscope. All dark-field (1 to 2 sec) and fluorescent exposures (8 to 15 sec) were made on Kodak high-speed Ektochrome EH 135 (ASA 160, DIN 23). All FA photographs were taken at $\times 100$ magnification.

RESULTS

It was initially proposed that a low magnification ($\times 100$) be used with the Zeiss annular phase ring, as the low-power phase ring condenser is recommended for fluorescent use without oil on the condenser. However, the fluorescence was often weak, and much greening of the agar occurred when the colony surface of the agar block was upright.

The ultra dark-field condenser which requires oil on the condenser at all magnifications was then used with the colony surface of the agar block inverted in buffered glycerol. This system focuses the ultraviolet light at the plane of the colonies on the slides for best results and not within the agar block.

Other fluorescent microscopes and the selection of proper filters were found to influence the contrast of specific green immunofluorescence and the dull yellow autofluorescence.

The direct method was more satisfactory than the indirect method. Typical morphology of discrete colonies was preserved. The central areas stained a solid apple green, whereas the peripheral areas fluoresced less intensely (Fig. 2). Specific staining was obtained at dilutions of 1:40 to 1:80 for all serotypes studied. Specific staining was detected consistently at higher dilutions but could be confused with cross-staining heterologous antisera at low dilutions (Table 1). Atypical colonies could also

be identified as specific serotypes.

A dull yellow to yellowish green autofluorescence which varied in intensity was common with some strains of mycoplasmas when unstained or stained with heterologous antisera (Fig. 4). This was usually confined to the central portion of colonies but at times involved the entire colony. Care needed to be exercised so that autofluorescence not be confused with minor cross-reactions or poor staining from dilute antiserum. Autofluorescence tended to fade much more quickly upon exposure to ultraviolet light and caused difficulty in obtaining a good image. These characteristics were helpful in distinguishing



FIG. 2. Mixed culture containing serotype F colonies (*M. bovimastitidis*) stained directly with homologous antiserum. The large unstained colonies showing some autofluorescence stained with serotype C antiserum (*M. laidlawii*). The smaller and more distinct fluorescent colonies are showing some fluorescent peripheral area development. Note the positive fluorescent center within a large colony at the lower center indicating a mixture of serotypes within a single colony. $\times 100$.

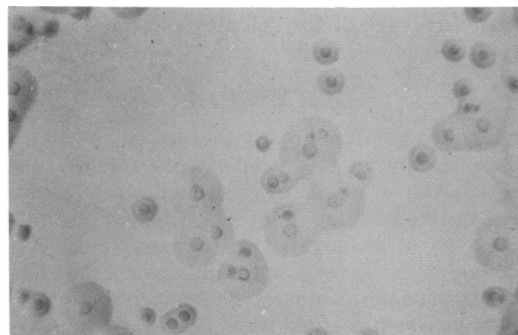


FIG. 3. Unstained preparation of a mixed culture showing small colonies with darker centers within large colonies. The smaller, darker colonies were identified as serotype F (*M. bovimastitidis*) and the large colonies were identified as serotype C (*M. laidlawii*). $\times 25$.

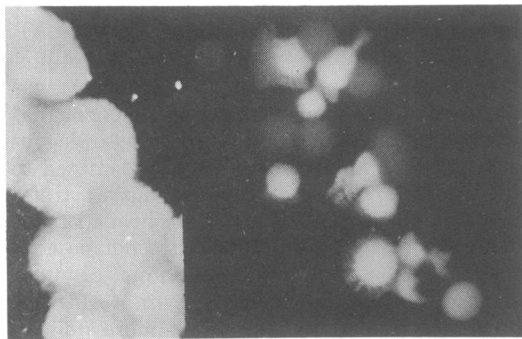


FIG. 4. Mixed culture from field material with positive direct staining to serotype F antisera (right). The large unstained colonies stained with conjugated antiserum to serotype C. Coalesced growth of the colonies does not reveal a mixed culture when examined by dark-field illumination (left). Note the inhibitory effect on the small colony growth by large colonies. This may account for the small atypical appearance of colonies within another serotype as seen in Fig. 2. $\times 100$.

positive fluorescence.

Well separated discrete colonies of typical fried egg appearance where central and peripheral portions could be observed were especially helpful for detection of and identification in mixed cultures.

Older or dehydrated agar plates increased conjugate absorption and background agar fluorescence. Optimum dilutions of conjugated antisera gave good specific fluorescence as each greater dilution decreased the amount of background agar fluorescence.

The indirect method was helpful for testing purity of strains for which no specific conjugate was available and for testing antiserum being produced. It was less satisfactory in that the additional washing and staining appeared to cause breakage of the colonies and more background agar greening.

DISCUSSION

Lucite cylinders greatly facilitated the fluorescent staining of mycoplasma colonies on preselected areas of agar surface when using transmitted ultraviolet light and small amounts of conjugated antiserum. This FA technique was very specific when used for identification purposes. The morphology of colonies was preserved which facilitated purifi-

cation of colonies. This method is very useful for detection of mixed cultures (Figs. 2, 3, 4) and unclassified serotypes found in small numbers.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Training Grant ITTGM-1406 in Laboratory Animal Biology and Medicine and by Agricultural Experiment Station project no. 1064.

The skilled technical assistance of Jon Dellinger is gratefully acknowledged.

LITERATURE CITED

1. Barile, M. F., R. A. Del Guidice, T. R. Carske, H. M. Yamashiroya, and J. A. Verna. 1970. Isolation and rapid identification of *Mycoplasma* species from canine tissues by plate immunofluorescence. *Proc. Soc. Exp. Biol. Med.* **134**:146-148.
2. Clark, H. W., J. S. Bailey, R. C. Fowler, and T. M. Brown. 1963. Identification of mycoplasmataceae by the fluorescent antibody method. *J. Bacteriol.* **85**:111-118.
3. Corstvet, R. E., and W. W. Sadler. 1964. The diagnosis of certain avian diseases with the fluorescent antibody technique. *Poultry Sci.* **43**:1280.
4. Del Guidice, R. A., N. F. Robillard, and T. R. Carske. 1967. Immunofluorescence of *mycoplasma* on agar by use of incident illumination. *J. Bacteriol.* **93**:1205-1209.
5. Dowdle, W. R., and R. G. Robinson. 1964. An indirect hemagglutination test for diagnosis of *Mycoplasma pneumoniae* infections. *Proc. Soc. Exp. Biol. Med.* **116**:947-950.
6. Ertel, P. Y., I. J. Ertel, N. L. Somerson, and J. D. Pollack. 1970. Immunofluorescence of mycoplasma colonies grown on coverslips. *Proc. Soc. Exp. Biol. Med.* **134**:441-446.
7. Fabricant, J., and E. A. Freundt. 1967. Importance of extension and standardization of laboratory tests for the identification and classification of mycoplasmas. *Ann. N. Y. Acad. Sci.* **143**:50-58.
8. Lind, K. 1970. Preparation of antigen for the indirect fluorescent antibody test in diagnosis of *Mycoplasma pneumoniae* infection. *Acta Pathol. Microbiol. Scand.* **78**:149-152.
9. Masiga, W. N., and S. S. Stone. 1968. Application of a fluorescent antibody technique for the detection of *Mycoplasma mycoides* antigen and antibody. *J. Bacteriol.* **96**:1867-1869.
10. Morton, H. E., and R. J. Roberts. 1967. Production of anti-mycoplasma (PPLO) antibodies in rabbits. *Proc. Soc. Exp. Biol. Med.* **125**:538.
11. Nairn, R. C. 1969. Appendix, p. 303 to 305. In R. C. Nairn (ed.), *Fluorescent protein tracing*. The Williams & Wilkins Co., Baltimore.
12. Razin, S. 1969. Structure and function in mycoplasma. *Amer. Rev. Microbiol.* **23**:317-349.
13. Stewart, S. 1967. The use of fluorescent antibody techniques for the identification of cultures of mycoplasma. *Immunology* **13**:513-516.
14. Tully, J. G. 1965. Biochemical, morphological, and serological characterization of mycoplasma of murine origin. *J. Infect. Dis.* **115**:171-185.