JOURNAL OF BACTERIOLOGY, Nov. 1968, p. 1867-1869

Vol. 96, No. 5
Printed in U.S.A.

Application of a Fluorescent-Antibody Technique for the Detection of *Mycoplasma mycoides* Antigen and Antibody

W. N. MASIGA AND S. S. STONE[1]

East African Veterinary Research Organization, Muguga, P.O. Kabete, Kenya

Received for publication 13 August 1968

Serological and cultural methods for the identification of *Mycoplasma* often lack specificity and may require several days to establish the presence of *Mycoplasma*.

Fluorescent-antibody (FA) techniques are rapid and sensitive and have been used for the identification of *Mycoplasma* (3). However, the FA procedure has not been described for *M. mycoides*, the causative agent of contagious bovine pleuropneumonia (CBPP). We have described the use of FA to detect *M. mycoides* and its antibody in the sera of CBPP-vaccinated and infected cattle.

The virulent Gladysdale strain of *M. mycoides* was used to infect cattle by the endobronchial method (1) and to prepare standard slides. The CBPP T_1 culture vaccine was prepared and used as previously described (2). For the antibody-absorption experiments, *M. mycoides* was separated from the culture broth by centrifugation at ca. 26,000 $\times$ *g* in a continuous-flow centrifuge, washed three times with 20 volumes of saline, and lyophilized.

M. agalactiae and *M. capri* were obtained through the courtesy of E. P. Lindley, Near East Animal Health Institute, Khartoum, and *M. bovirhinis* was obtained from R. D. Leach, Central Public Health Laboratories, Colindale, London. The above *Mycoplasma* species were grown in tryptose broth containing penicillin, thallium acetate, sulfamethazine, and polymixin B as bacterial inhibitors.

Viable counts of all the mycoplasmas were made with 10-fold dilutions in the same broth, and the highest dilution giving turbidity after incubation for 48 hr at 37 C was considered the titer. Appropriate dilutions were then made to give 10^5 organisms per ml for preparing standard slides. Control slides were also prepared from noninoculated broth.

[1] Present address: U.S. Department of Agriculture, Agricultural Research Service, Animal Disease Laboratory, Plum Island, Greenport, Long Island, N.Y. 11944.

Sera were obtained from cattle naturally infected by contact with cattle that were endobronchially infected with the virulent Gladysdale strain of *M. mycoides*. Blood for serum was obtained approximately 3 weeks after the cattle had serologically positive reactions in complement-fixation test for CBPP. The serum was adjusted to *p*H 6.0 and dialyzed against 25 volumes of phosphate buffer (*p*H 6.0), ionic strength 0.01, for 24 hr at 5 C. The resulting euglobulin precipitate was separated by centrifugation and reconstituted in phosphate-buffered saline (PBS; *p*H 7.2) to one-half the original serum volume. Conjugation with fluorescein isothiocyanate was as described (6), and unreacted fluorescein isothiocyanate was removed with Sephadex G75. Conjugated globulin was lyophilized, reconstituted to the original serum volume, and dialyzed against PBS to remove the excess salts; it was then lyophilized in 1-ml samples and stored at 5 C.

Smears of mycoplasma containing 10^5 organisms per ml were fixed in methanol for 3 min. Various dilutions of conjugate were prepared, and two drops of each dilution was overlaid on the smears. The smears were then incubated in a moist chamber at 37 C for 30 min and washed in PBS for 10 min; cover slips were mounted by use of 10% glycerol in PBS.

To determine the presence of *M. mycoides* in the mucosa of CBPP-infected cattle, sterile cotton applicator sticks were inserted into the nostrils of the cattle. The swabs were incubated in the standard broth for 48 hr at 37 C. Smears from these cultures were stained with FA.

To determine the specificity of the antibody conjugate in the FA preparation, 5 mg of lyophilized *M. mycoides* was added to 1 ml of undiluted FA. The mixture was then incubated for 30 min at 37 C and refrigerated for 18 hr. The precipitate was separated by centrifugation, and the supernatant phase was tested for FA. The reaction was controlled by using untreated FA.

Standardized smears of *M. mycoides*, *M. capri*,

M. agalactiae, and *M. bovirhinis* were stained with FA diluted 1:40 to determine possible cross-reactions. Another series of slides was prepared with adjacent smears of *M. mycoides* and *M. bovirhinis*; a third series of standard *M. mycoides* slides was overlaid with immune CBPP serum for 30 min at 37 C, washed with PBS, and stained with FA.

The complement-fixation test, as described by Shifrine, Stone, and Davies (6), was used to determine CBPP antibody in cattle sera.

The inhibition method was essentially as described by Moller (5), but the reaction time was increased to 30 min, after which the fluorescent globulin was applied. The serum titer was considered to be the last dilution of serum in which the fluorescence was visually as bright as the normal serum control slide.

Fluorescent-stained slides were examined with a Zeiss fluorescence microscope with a BG 12 exciter filter, no. 41 barrier filter, and a bright-field condenser.

M. mycoides smears stained with homologous conjugate displayed typical yellow-green fluorescence which was observed with all dilutions of FA ranging to 1:640. A dilution of 1:40 was considered optimal for use in both the direct staining and inhibition of FA reaction.

The appearance of clumped FA-stained *M. mycoides* on the standard slides was similar to that of organisms stained by other direct methods and viewed with white light.

Slides prepared from cultures of *M. agalactiae*, *M. bovirhinis*, and *M. capri* were stained with FA but did not show fluorescence.

In the series of slides comparing *M. mycoides* and *M. bovirhinis*, fluorescence could be seen only on the portion of the slide with *M. mycoides*.

Before absorption with *M. mycoides*, the titer of the FA with standard *M. mycoides* smears was

TABLE 1. *Comparative titers of complement fixation test and the inhibition of the FA reaction in the sera of contagious bovine pleuropneumonia-vaccinated cattle*

No. of animal	Days postvaccination											
	0		3		8		14		21		86	
	IFAR[a]	CFT[b]	IFAR	CFT	IFAR	CFT	IFAR	CFT	IFAR	CFT	IFAR	CFT
870	20[c]	0[c]	640	40	320	40	1,280	40	640	80	640	20
875	20	0	NT	0	320	40	640	40	320	40	320	20
926	NT[d]	0	640	40	640	20	320	40	320	40	320	20
929	20	0	1,280	40	1,280	160	1,280	80	320	80	320	20
974	0	0	640	0	1,280	160	1,280	160	1,280	160	320	20
980	0	0	640	0	320	20	320	20	320	40	320	20

[a] Inhibition of FA reaction.
[b] Complement-fixation test.
[c] Reciprocal dilution.
[d] Not tested.

TABLE 2. *Comparative titers of complement fixation test and inhibition of the FA reaction in the sera of cattle exposed to contagious bovine pleuropneumonia*[a]

No. of animal	Days postcontact									
	0		45		59		66		73	
	IFAR	CFT	IFAR	CFT	IFAR	CFT	IFAR	CFT	IFAR	CFT
B30	10[b]	0	NT	0	20[b]	0	80	20	160	20
908	0	0	80	0	1,280	>160	NT	>160	1,280	>160
948	20	0	640	40	NT	20	320	20	320	10
959	0	0	80	0	160	0	160	40	NT	>160
960	20	0	40	0	40	0	320	40	160	20
962	0	0	NT	0	80	0	320	40	640	40

[a] Abbreviations as in Table 1.
[b] Reciprocal dilution.

1:640. After absorption, fluorescence could not be detected in the undiluted FA or in dilutions to 1:640.

Fluorescence was not observed on slides on which the smears of *M. mycoides* were first overlaid with the immune CBPP serum and then stained with FA.

Titers obtained by the complement-fixation test and the inhibition of FA reaction with sera from vaccinated cattle were compared (Table 1). A similar comparison of inhibition of FA reaction and the complement-fixation test on the sera of cattle in contact with CBPP-infected cattle is shown in Table 2, which shows that the inhibition of FA reaction detected CBPP antibody before it could be determined by the complement-fixation test, and, in all cases, inhibition of FA reaction was more sensitive than overnight complement-fixation test.

Nasal swab cultures were positive by the direct FA test, indicating the presence of *M. mycoides* in 12 cattle tested. Two cattle later died of CBPP; the remaining 10 had typical clinical signs of CBPP.

Fluorescent antibody was used for the identification of *M. mycoides*. It has been particularly useful when contamination with *Mycoplasma* or other microorganisms is prevalent, such as in nasal swabs. This procedure had reduced the necessity of subculturing and using growth inhibition methods. The specificity of the FA staining for *M. mycoides* was confirmed by using four criteria: (i) fluorescence was not observed with *M. agalactiae*, *M. capri*, and *M. bovirhinis*; (ii) fluorescence could be eliminated by absorption of the antibody with the antigen, *M. mycoides*; (iii) fluorescence was not observed in noninoculated broth smears; and (iv) CBPP-immune sera inhibited the FA reaction.

The specificity of FA has been determined with only three species of *Mycoplasma*. It is probable, however, that others may not cross-react, since Leach (4) examined 22 strains of bovine *Mycoplasma* and no cross-reaction in growth inhibition or complement-fixation test with *M. mycoides* antiserum was observed.

We thank G. S. Colgrove for the assistance with the fluorescent microscopy; M. L. Burdin, Director of EAVRO, and J. R. Hudson for their review of the manuscript; and F. R. Gilbert and W. C. S. Read for their help in growing the *Mycoplasma* cultures.

This investigation was supported by the U.S. Agency for International Development under the terms of the STRC/AID Joint Project 16 for Research on Contagious Bovine Pleuropneumonia.

LITERATURE CITED

1. Brown, R. D. 1964. Endobronchial inoculation of cattle with various strains of *Mycoplasma mycoides* and the effects of stress. Res. Vet. Sci. **5**:393–404.
2. Brown, R. D., R. N. Gourlay, and A. K. Macleod. 1965. The production of *T* broth culture contagious bovine pleuropneumonia vaccine. Bull. Epiz. Diseases Africa **13**:149–155.
3. Clark, H. W., J. S. Bailey, R. C. Fowler, and T. McP. Brown. 1962. Identification of *Mycoplasmataceae* by the fluorescent antibody method. **85**:111–118.
4. Leach, R. H. 1967. Comparative studies of mycoplasma of bovine origin. Ann. N. Y. Acad. Sci., **143**:305–316.
5. Moller, G. 1961. Demonstration of mouse isoantigens at cellular level by the fluorescent body technique. J. Exptl. Med. **114**:415–434.
6. Shifrine, M., S. S. Stone, and G. Davies. 1968. Contagious bovine pleuropneumonia: Serologic response of cattle after single and double vaccination with *T* culture vaccine. Rev. Elevage Med. Vet. Pays Trop. **21**:49–58.
7. Wood, B. T., Thompson, S. H., and G. Goldstein. 1964. Preparation of fluorescent-isothiocyanate-labeled antibodies. J. Immunol. **9:5** 225–229.