Immunoelectrophoretic Analysis of *Mycoplasma mycoides* var. *mycoides*

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Acrylamide gel electrophoresis was used to show the similarities and differences in the membrane proteins of two vaccine and two virulent strains of *Mycoplasma mycoides* var. *mycoides*. Immunoelectrophoretic (IEP) analysis was also used to partially characterize the associated antigens. Antibody spectra to the antigens of *M. mycoides* differ in rabbit, pig, and cattle sera. Rabbits produce better precipitating antibody against the anodic migrating protein mycoplasma antigens than cattle and pigs as seen in IEP. However, rabbit anti-*M. mycoides* serum did not show precipitating antibody against the heat-stable carbohydrate antigen. As judged by IEP, the major carbohydrate antigen extracted from the media, or boiled whole organism, is similar to that present in the sera-infected cattle and knee joints of calves. This carbohydrate antigen has a cathodic migration in IEP at pH 8.6. Periodate oxidation, classically used to destroy carbohydrate, also destroys most of the protein antigens. Heating the antigens to 56 C for 10 min destroys many of the noncarbohydrate antigens and 100 C eliminates all but the carbohydrate antigen. Extraction of *M. mycoides* with chloroform-methanol, phenol, ethanol, or ethanol-acetone reduced or eliminated most of the protein antigens. Some of the isolated antigenic fractions of *M. mycoides* were tested to determine their activity in the diagnostic complement fixation test for contagious bovine pleuropneumonia and their inhibitory effect in this test by using bovine anti-*M. mycoides* antisera having precipitating antibody and circulating antigen. The complement fixation antigen is not the galactan, cannot be extracted by chloroform-methanol, but is stable to boiling at 100 C and may be extracted by phenol and partially precipitated by ethanol-acetone.

Previous studies on the antigens associated with *Mycoplasma mycoides* var. *mycoides* hereafter called *M. mycoides*) have been limited to extracting antigens for diagnostic testing (29), comparison to bacteria (32) or viruses (22), or for comparative serological reactions to other mycoplasmas (18).

There has been a great resurgence of interest in mycoplasmas, and mycoplasmas are being implicated in many disease situations as they can be isolated virtually from all biological systems. However, of all the mycoplasmas, the first one isolated, *M. mycoides*, the causative agent of contagious bovine pleuropneumonia (CBPP), is still important today. This disease is an ever-present threat to the development of a cattle industry in many parts of the world (2).

For the comparative analysis of mycoplasmas, in particular *M. mycoides*, better methods of antigenic analyses are required. Serological techniques are foremost in the analytical methods used for identifying mycoplasmas. These include the metabolic and growth inhibition tests (1, 9), fluorescent antibody (20; M. F. Barile and D. B. Riggs, Bacteriol. Proc., p. 83, 1961), complement fixation (CF), agglutination, and agar gel diffusion precipitin (AGD) tests (34). Except for AGD, which represents the antigens as distinct entities in the form of precipitin bands, none of the aforementioned procedures characterize the antigens associated with mycoplasma.

One analytical method offering great resolution is the acrylamide gel electrophoresis (AGE) procedure (25). Mycoplasma membranes can be

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solubilized, and, after electrophoretic separation in the gel and staining, the bands depicting these cell proteins are usually unique for a particular species of Mycoplasma. As such, this procedure has been extensively used for taxonomic studies (23).

In contrast, immunoelectrophoresis (IEP), although also depending upon the electrophoretic migration of solubilized mycoplasma membranes, can depict only those that are antigenic in a given host in which the serum was prepared. The electrophoretically separated fractions are, therefore, identified as precipitin lines formed by the antibody in given serum.

It was the purpose of this study to examine primarily by IEP the spectrum of antigens associated with M. mycoides, compare these to antigens extracted by previously published procedures for mycoplasma, and to further relate these extracts to the CF test (CPT) which is at present the most reliable field test for detecting CBPP antibody in cattle.

**MATERIALS AND METHODS**

**M. mycoides.** The M. mycoides representative strain PG-1 and the T-1 and KH24 vaccine strains, as well as the virulent strains Gladysdale (Australia) and Archers Post (AP) (Kenya, Africa), were grown in tryptose phosphate broth media as cited (32). At the late logarithmic growth stage, M. mycoides was concentrated by centrifugation at 15,000 × g for 60 min. The pellet was washed five times with phosphate-buffered saline (pH 7.3) and finally suspended in 5 vol of 0.85% saline and lyophilized. For AGE, the percentage of NaCl was calculated from the dry weight, and all subsequent weights of M. mycoides were adjusted to compensate for the NaCl. No adjustment for NaCl which ranged from 40 to 60% was made in the M. mycoides preparations used for pig and rabbit inoculation. The NaCl is equivalent to 2.4 to 3.5 times isotonicity, and the hypertonicity had no apparent affect on the precipitin bands.

**Antisera.** Antisera were prepared in cattle by using the endobronchial intubation method of Brown (5) with the Gladysdale strain of M. mycoides. Blood for serum was collected 3 weeks after the first appearance of complement-fixing antibody.

**Pig antiserum.** Antiserum was prepared in pigs inoculated with T-1 strain of M. mycoides grown in media containing pig sera (32) and in rabbits inoculated with T-1 cultures grown with a rabbit serum supplement.

Adult New Zealand white rabbits were inoculated weekly with a 50 mg/ml suspension of M. mycoides mixed with an equal volume of Freund complete adjuvant. The inoculation regime consisted of 0.1 ml in the hind leg plantar pad and 1 ml intramuscularly (i.m.) weekly for 2 weeks, and 1 ml i.m. for 2 additional weeks. Rabbits were bled for serum at 7, 9, and 11 days after the last inoculation.

Rabbits were also immunized by the same inoculation regime by using purified M. mycoides galactan (16) at a concentration of 1, 100, and 1,000 μg/ml in an equal volume of complete Freund adjuvant.

All pig and rabbit sera were tested for the presence of precipitin M. mycoides antibody by IEP, using sonically disrupted M. mycoides (50 mg/ml) as antigen. Strong sera, as judged by the number and intensity of precipitin lines, were pooled, and the weaker reacting sera were discarded. In all instances, the precipitin reaction was controlled using the culture media undiluted, at a 5× concentration, and with preinoculation sera.

**AGDP.** The AGDP procedure of Stone and Shifrine (32) was modified to use three equidistant wells, 5-mm in diameter, surrounding a fourth central well of the same diameter, with 3 mm between the edges of the central and outside wells.

**AGE.** The AGE procedure as described (25) was followed. The gel system contained 7% acrylamide and 35% acetic acid in 5 M urea. Gels were prepared in glass tubes (6 by 100 mm). Mycoplasma membranes were dissolved in phenol-acetic acid-water, 2:1:0.5 (wt/vol/vol), and electrophoresis was carried out at room temperature for 2 h at a constant current of 5 mA per tube. The gels were stained with 1% amido black, and in some cases the intensity of dye binding was traced by using a spectrophotometer equipped with a scanner.

**IEP.** The micro procedure of IEP (27) was employed by using barbital-acetate buffer (pH 8.6), ionic strength 0.025, and a constant current of 2 mA/cm for 90 min. For IEP analysis, 30 to 50 mg/ml of freeze-dried M. mycoides was disrupted ultrasonically at 0 to 3°C using five 30-s bursts of energy. A 0.1% tincture of bromophenol blue was used as an indicator for electrophoretic migration, and a minimum dye migration of 3 cm was used on each run. Precipitin patterns were developed with the appropriate antiserum for 18 h in a moist chamber and then photographed.

**Antigen from cattle.** Serum and pleural fluid were obtained at necropsy from a cow that had an acute CBPP infection. Joint fluid was aspirated from the swollen knee of the calf that had been experimentally infected with virulent M. mycoides.

**Extractions.** Except for the preparation of galactan, all extractions and heat treatments were made by using 30 mg of lyophilized Gladysdale strain of M. mycoides. Resulting extracts were adjusted to isotonicity and the same starting volumes prior to testing.

**Galactan preparation.** Galactan F from both the virulent Gladysdale and the T-1 vaccine strains was extracted as outlined (16, 21).

**Lipid extraction.** M. mycoides was extracted by the lipid extraction method of Razin et al. (24) by using a 2:1 chloroform-methanol mixture. The lipid phase was reduced to dryness under a stream of nitrogen and then resuspended in saline and homogenized by sonic treatment.

**Hot phenol extraction.** The hot phenol extraction procedure used was as described by Buttery and Plackett (7). Both the phenol and aqueous phases were extensively dialyzed against saline in the cold to remove all traces of phenol.

**Ethanol extraction.** Ethanol extractions were
made as described (11). This resulted in two fractions: “A,” obtained by precipitating an aqueous extract of boiled M. mycoides with ethanol, and “B,” an alcoholic extract of unboiled M. mycoides from which the active fraction was precipitated with acetone. The extract residue was also resuspended in saline and homogenized by sonic treatment.

**Effect of heat.** Standard suspensions of M. mycoides were each heated for 15 min at 56°C and 100°C in a water bath. The suspension was clarified by centrifugation at about 1,000 × g for 15 min. Serum from CBPP-infected cattle and joint fluid from the knee of an infected calf were heated at 100°C and clarified in the same way, but at about 5,000 × g.

**CFT.** To determine the CFT potential of the various extracts of M. mycoides, two bovine CBPP antisera were used in a micro (0.025-ml vol) CFT. The first serum was one that contained precipitating antibody as determined by the AGDP. The second serum was taken from a CBPP-infected cow that, because of the severity of the infection, had circulating serum galactan. Except for the galactan, each of the extracts was adjusted to the same dilution factor as that of the standard M. mycoides antigen (30) and used as a CF antigen. The galactan F was used at an initial concentration of 0.1 mg/ml. To determine if any of the extracts of M. mycoides may have had a haptenic effect, each of the extracts was added to the test mixture of serum and complement and allowed to react for 30 min at 37°C; then, 2 U of the standard M. mycoides antigen was added and the test was incubated for another 30 min. Sensitized red blood cells (RBC) were then added followed by incubation for 30 min at 37°C. The micro trays were then transferred to a tray of crushed ice, and the unreacted RBC were allowed to settle. The serum end point was established by comparison of the wells containing unlysed RBC with known RBC controls containing 25 and 50% of the RBC used in the test. The highest dilution of serum having 50% or less hemolysis was considered to be the end point. Enhancement or inhibition from the prior addition of the M. mycoides extract was determined by comparing these titers to that obtained with the standard antigen. If the titer of the serum-extract-standard antigen mixture was higher than that of the serum-standard antigen mixture, the enhancement was plus; if lower, it was minus. No change in the titer was considered to be zero.

**Periodate oxidation.** A standard suspension of M. mycoides was disrupted by sonic treatment before the potassium periodate was added (17). Oxidation was allowed to proceed in the dark for 10 days at room temperature after which the suspension, the pH, and the isotonicity were adjusted for IEP.

**RESULTS**

The AGE patterns of the two vaccine and two virulent M. mycoides strains were compared to the reference PG-1 strain in Fig. 1. One band in the KH12 vaccine strain appeared to be different from the others. However, when the weight of the membranes was adjusted to compensate for the NaCl, this difference disappeared. Densitometer tracings of the two vaccine and two virulent strains are shown in Fig. 2. Qualitatively, there is a close similarity between the two vaccine strains, T-1 and KH12, and similarity between the two virulent strains, Gladysdale and Archers Post. However, by this procedure there is a difference in the amplitude of some peaks in the avirulent and virulent strains, indicating that some components are in higher concentrations in the avirulent strains.

Precipitin bands were not obtained when using bovine, pig, and rabbit anti-M. mycoides sera and the media either at its standard dilution or in the 5× concentrated form, nor were there any visible precipitin bands in the AGDP or IEP tests when using preinoculation sera.

Precipitin patterns formed by M. mycoides and bovine, porcine, and rabbit sera in double diffusions and by IEP are shown in Fig. 3. The advantage of separating the antigens electrophoretically before precipitation with anti-serum is evident. Further, it appears that each of the three animal species—bovine, porcine, and rabbit—produces its own spectrum of antibody. Notable is the absence of precipitin lines on the anode side of the plate with the bovine antisera and the reduced number of precipitin lines formed by pig antisera as compared to that of rabbit antisera. However, with the rabbit antisera, there is no precipitate band against the major antigen galactan migrating toward the negative pole (Fig. 3).

Rabbits injected with 1, 100, and 1,000 μg of galactan in complete Freund adjuvant by using the same inoculation regime did not have detectable antibody by either IEP or double diffusion in agar.

There is apparently no difference in galactan extracted from either virulent or avirulent strains of mycoides (Fig. 4). Further evidence that the galactan is the major antigen associated with mycoides is illustrated in Fig. 5.

Table 1 summarizes the IEP analysis of the extracts of M. mycoides by using phenol, chloroform-methanol, ethanol, and ethanol-acetone as extractants and bovine, pig, and rabbit anti-M. mycoides sera to develop the precipitin.
was visible with both the phenolic and aqueous phases. The aqueous phase had a more intense reaction.

With the chloroform-methanol extract, a precipitin band was not observed with any of the three antisera. In contrast, the solubilized residue formed a strong precipitin band on the cathode side of the origin with the bovine and pig antisera and the anode side with the pig and rabbit sera.

Extraction of boiled *M. mycoides* by ethanol (fraction A) produced an antigen that formed precipitin bands on the cathode side of the origin only with the bovine and pig antisera. The alcohol-acetone extract of *M. mycoides* (fraction B) showed precipitin bands on the cathode side with all three antisera, and the solubilized, extracted residue developed a similar reaction with the two antisera.

**Fig. 1.** Acrylamide gel electrophoretic patterns of *Mycoplasma mycoides* var. mycoides cell proteins. Left to right: PG-I, representative strain for *M. mycoides*; T-I, avirulent vaccine strain; AP, Archers Post, Kenya, East Africa, virulent strain; KH,J, avirulent vaccine strain; Glad, Gladysdale, Australia, virulent strain.

**Fig. 3.** Double diffusion precipitin (left) and immunoelectrophoretic (right) tests of *Mycoplasma mycoides* var. mycoides (MM). Precipitin lines were developed with bovine (B), pig (P), and rabbit (R) anti-*M. mycoides* sera.

**Fig. 2.** Densitometer tracings of acrylamide gel electrophoretic patterns of *Mycoplasma mycoides* var. mycoides cell proteins. Top right: Archers Post virulent strain. Top left: KH,J vaccine strain. Bottom right: Gladysdale virulent strain. Bottom left, T-I vaccine strain.

**Fig. 4.** Immunoelectrophoretic and double-diffusion precipitin patterns of galactan F extracted from vaccine (GF vac) and virulent (GF vir) strains of *Mycoplasma mycoides* var. mycoides. Precipitin lines were developed with pig anti-*M. mycoides* serum.
Before heating (MM), after heating (56 and 100 °C), and compared to galactan F (GF). Precipitin lines were developed with bovine, pig, and rabbit anti-M. mycoides sera.

**Table 1. Immunoelectrophoretic analysis of M. mycoides extracted with phenol, chloroform-methanol, and ethanol-acetone, using bovine, pig, and rabbit anti-M. mycoides sera to develop precipitin lines**

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Fraction tested</th>
<th>Precipitin band</th>
<th>Anti-M. mycoides serum used to develop precipitin lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anode side</td>
<td>Bovine</td>
</tr>
<tr>
<td>Phenol</td>
<td>Phenol phase</td>
<td>Cathode side</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anode side</td>
<td>Yes-weak</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cathode side</td>
<td>None</td>
</tr>
<tr>
<td>Chloroform-methanol</td>
<td>Lipid phase</td>
<td>Anode side</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cathode side</td>
<td>Yes-weak</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anode side</td>
<td>Yes-strong</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cathode side</td>
<td>None</td>
</tr>
<tr>
<td>Alcohol-acetone</td>
<td>Solubilized alcohol</td>
<td>Anode side</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>precipitate (A)</td>
<td>Cathode side</td>
<td>Yes-weak</td>
</tr>
<tr>
<td></td>
<td>Solubilized acetone</td>
<td>Anode side</td>
<td>Yes-strong</td>
</tr>
<tr>
<td></td>
<td>precipitate (B)</td>
<td>Cathode side</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Solubilized extracted</td>
<td>Anode side</td>
<td>Yes-strong</td>
</tr>
<tr>
<td></td>
<td>residue</td>
<td>Cathode side</td>
<td>None</td>
</tr>
</tbody>
</table>

*Precipitin band formation, area relative to point of application of antigen extract.

Figures 6 and 7 show galactan present in the knee joint fluid of a calf and in the serum and pleural fluid of a cow killed in extremis after artificial infection with virulent M. mycoides. The galactan is heat stable (Fig. 6) and does not form a precipitin line with the rabbit anti-M. mycoides serum (Fig. 7). Periodate oxidation (Fig. 8) eliminates the galactan precipitin lines with the bovine and rabbit antisera; and with the rabbit and pig antisera, the antigens migrating toward the positive pole are eliminated. The galactan normally migrating toward the negative pole was sharply reduced and appeared as two lines when the precipitin pattern was developed with pig antiserum.

CF activity of the various fractions of M. mycoides are shown in Table 2. The serum with precipitating antibody had a higher CF titer with the same antigen than the serum having circulating antigen. The latter serum had a lower CF titer with all of the M. mycoides fractions tested. Galactan F did not fix complement nor did it have enhancing or inhibiting activity when added to the sera before adding the standard CF antigen. The phenol extract of M. mycoides was CF positive with both sera and exhibited enhancing activity as well. In contrast, the aqueous fraction in this extraction had only a minimal CF activity with the precipitating serum and no enhancement or inhibition of CF activity.
After heating the *M. mycoides* to 100 C, the CF antigen was retained in the precipitate fraction, and the supernatant fraction had minimal CF activity with the precipitating serum. Both fractions had CF-enhancing properties with each of the sera.

The lipid extract of the *M. mycoides* had trace CF activity with the serum-containing precipitating antibody and inhibited the CF reaction with the galactan-containing serum. However, the aqueous suspension of the extracted residue had CF and enhancing activity with both test sera.

The alcoholic extract A fraction of boiled *M. mycoides* did not have CF activity with either sera but enhanced the CF titer of the precipitating serum and depressed the CF titer of the serum containing circulating galactan. In contrast, the B fraction, an alcoholic extract of whole *M. mycoides* in which the antigen was precipitated with acetone, had CF and enhancing activity with both sera. The residue after this extraction had both CF and enhancing properties.

**DISCUSSION**

It is important to observe that the AGE patterns of the four major strains of *M. mycoides* analyzed are qualitatively similar to the PG-1 reference strain. However, the densitometer tracings of the AGE patterns (Fig. 2) show a quantitative difference between the two vaccine strains, T-1 and KH,J, and the two virulent strains, Archers Post (Kenya) (8), and Gladysdale (Australia) (33). Although these two strains were isolated at least 10 years apart in different continents, the similarity between them is significant because it points to a possible stabilization of the virulent strains.

There are few reports describing IEP using mycoplasma (4, 12, 15, 26). This may be because, due to electrophoresis, a good precipitating antiserum is required and a much higher concentration of mycoplasma antigen is needed as it must be spread over a larger area than in the direct double-diffusion procedure. The superiority of the IEP procedure over the double-diffusion precipitin test is well illustrated in Fig. 3. The multiplicity of antigens associated with *M. mycoides* is lost in the latter test because of overlapping precipitin lines. Of importance is the finding that rabbits do not readily produce antibody against the major
galactan F antigen. The lack of precipitating antibody against the galactan does not appear to be due to immune paralysis as the injection of rabbits with 1, 100, and 1,000 μg also failed to produce precipitating antibody. This is not entirely without precedent as Barber and Eylan (3) have reported that the polysaccharide moiety in Pasteurella pestis is a weak immunogen in rabbits. This poor antibody response with the M. mycoides galactan may also be due to the similarity of structure between the galactan and the pneumogalactan of rabbit lung (29). In contrast to the bovine, the rabbit appears to develop excellent antibody against the antigens migrating toward the anode (Fig. 3). These antigens can be considered to be proteins as they are apparently very heat labile (Fig. 5). Heating to 56 C is not only sufficient to inactive M. mycoides, but this temperature also appears to destroy some of the antigens; this may partly be the reason why heat-inactivated M. mycoides has not been very efficacious as a vaccine (28). Other mycoplasmas, namely M. salivarium, M. orale type I, and M. hyorhinis, have antigens that are sensitive to temperatures of 50 to 60 C. (15).

The data in Table 1 indicate that extraction of M. mycoides with organic solvents results in the destruction of the protein-like antigens and further demonstrates that the stable antigen is the carbohydrate that has cathodic migration at pH 8.6.

It also appears that galactan may also be the major circulating antigen in serum from severely infected cattle (Fig. 7) as well as from swollen knee joints (Fig. 6), and it appears that the galactan from virulent strains of mycoplasma is antigenically similar to that from avirulent strains (Fig. 4). The galactan is not only found in the growth media from which it is easily isolated, but it is also a major constituent of the organism and has been postulated to

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**TABLE 2.** Complement-fixing (CF) activity of various fractions of M. mycoides var. mycoides by using bovine serum having precipitating antibody and serum with circulating galactan F

<table>
<thead>
<tr>
<th>Determination*</th>
<th>Titer of M. mycoides var. mycoides fractions*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAg</td>
</tr>
<tr>
<td>PPTG serum</td>
<td>1:160</td>
</tr>
<tr>
<td>Enhancement of CF with PPTG serum</td>
<td>0</td>
</tr>
<tr>
<td>Ag serum</td>
<td>1:40</td>
</tr>
<tr>
<td>Enhancement of CF with Ag serum</td>
<td>0</td>
</tr>
</tbody>
</table>

* PPTG serum, Serum containing precipitating antibody as determined by agar gel diffusion precipitin test. Ag serum, Serum containing circulating galactan antigen.

* Enhancement of CF: +, higher titer; −, lower titer; 0, no change after addition of fraction prior to addition of standard CF antigen. Abbreviations: SAg, whole M. mycoides (standard CF antigen); F, galactan F; Phen, phenol extract; Aq, aqueous phase after phenol extraction; 100S, boiled whole M. mycoides supernatant phase; 100P, boiled whole M. mycoides precipitate phase; Lip, lipid extract; Res, lipid residue; A, alcohol “A” fraction; B, alcohol-acetone “B” fraction; BR, residue after extraction of “B” fraction.
affect virulence (14). It would also appear that the carbohydrate linkages are important in the other antigens associated with *M. mycoides*. Periodate oxidation (Fig. 8) destroys all galactan antigens as detected by bovine antiserum. The precipitin bands developed with the pig and rabbit sera are markedly reduced. Although periodate oxidation may affect tyrosine, the primary reaction is with carbohydrate; and because of the apparent carbohydrate composition of these antigens, it would be difficult to obtain a carbohydrate-free protein antigen that might increase the specificity of a CBPP diagnostic test. It has been shown that a large number of cattle from a CBPP-free area already have significant galactan antibody, as demonstrated by the indirect hemagglutination test (31).

The CF activity of the various fractions extracted from *M. mycoides* indicates that, except for acetone precipitate of the alcoholic and phenolic extract of *M. mycoides*, all of the procedures resulted in a decrease of CF titer. Buttery (6) used periodate oxidation for as little as 24 h and found it sufficient to destroy the CF activity of *M. mycoides*, indicating that the carbohydrate moiety is an essential part of the CF antigen. This is in agreement with our more prolonged periodate oxidation in which protein antigens are also destroyed. Using lecthin, he was also able to recover some CF activity in the aqueous phase after chloroform-methanol extraction of *M. mycoides*.

The galactan does not appear to fix complement nor possess inhibiting activity with either of the tests sera. However, the presence of circulating serum galactan-like antigen does reduce the CF titer, possibly because some antibody may be complexed with this antigen. Antigen-antibody complexes may have pathological importance in some phases of CBPP infection which causes infarcts, particularly in the alveolar capillaries, and which produces necrotic areas to promote further growth of *M. mycoides*.

The reduced CF titers of the galactan-containing serum support previous reports that the combination of serum CF and serum antigen AGDP tests are more effective in identifying CBPP-diseased cattle than with either test alone (30).

It is also apparent that the CF antigen resides primarily in the fractions that could be expected to be rich in protein; i.e., the phenol phase, the residues after lipid extractions, and the acetone precipitate of ethanol extract from whole mycoplasma.

The lipid extract of *M. mycoides* in our experiments did not have CF activity in contrast to the lipid reported by Yoshida (35), who used acetone-ether extraction. However, our CF data on the lipid extraction does agree with that of Marmion et al. (19).

The CF inhibiting effect of the alcoholic extract of boiled *M. mycoides* is probably due to univalent haptenic-binding antibody sites but not to the formation of complexes large enough to fix complement. In contrast, the enhancing CF effect of many of the extracts may be due to multivalent antigenic determinant groups reacting with sufficient antibody sites to fix additional complement.

These experiments illustrate in part the complexity of the antigens associated with *M. mycoides*. They also indicate that serological tests based upon extracts of a *Mycoplasma* must be carefully analyzed to determine the physicochemical, as well as antigenic, nature of the extracts. Such knowledge will help avoid cross-reactions with closely related antigens which would normally be expected to reside with carbohydrates as was found with *M. mycoides* and *M. pneumoniae* (19).

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**LITERATURE CITED**


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