

Differential serologic response to *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* in lambs affected with chronic respiratory disease

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Abstract. An enzyme-linked immunoabsorbent assay (ELISA) was used to evaluate the levels of antibodies to *Mycoplasma ovipneumoniae* and *M. arginini* in lambs with chronic respiratory disease. Sera were obtained from lambs in several flocks at various stages of the clinical disease and tested with sodium dodecyl sulfate (SDS)-treated *M. ovipneumoniae* and *M. arginini* whole cells and a crude capsular extract of *M. ovipneumoniae* as the antigens. There were low levels of antibody to *M. ovipneumoniae* in flocks sampled at the early stages of infection, whereas increased levels of antibody were present in lambs from flocks that had apparently recovered from the clinical disease. Slowly rising titers of circulating antibodies to *M. ovipneumoniae* were confirmed by sequential bleeding of lambs during the course of the clinical disease. However, antibody levels of *M. arginini* were more likely to increase earlier in the disease process. There was significant cross-reactivity between the 2 SDS-treated antigens in both the ELISA test and western immunoblotting. In contrast, the crude capsular extract was specific for detecting antibodies to *M. ovipneumoniae*.

Mycoplasma ovipneumoniae (MO) is one of the most commonly isolated microorganisms from sheep with respiratory disease worldwide.⁴ Recently in this laboratory, this microorganism and *M. arginini* (MA) have been routinely recovered from young lambs with a respiratory disease that has been termed the “coughing syndrome.” The condition is associated with a severe paroxysmal cough, leading to rectal prolapses. This syndrome is widespread in the midwestern states of the USA, with morbidity and severity variable among flocks. The disease is chronic and persists for several weeks in most affected lambs. There is some evidence that the extended persistence of the MO organism in the respiratory tract of these lambs and the chronic nature of the disease may be due to failure of the immune system to generate protective immunity.¹⁰ Reasons for that failure are not known, but marked variation in the MO organisms isolated from sheep⁷ and expression of a polysaccharide capsule by the organisms are recognized.¹¹

Because of its simplicity the enzyme-linked immunosorbent assay (ELISA) has been extensively used in detecting antibodies to *Mycoplasma* species. However, concerns exist about its specificity because of cross-reactions among several *Mycoplasma* species.³ Several attempts have been made to overcome such specificity

problems by using different methods of antigen preparation.^{6,12} Solubilized whole cell antigens of MO have been used, and cross-reactivity problems were noted with these preparations.⁵ Consequently, a crude capsular material was extracted from MO for use as a diagnostic antigen, and the specificity of this reagent was confirmed in ELISA and western immunoblotting procedures. Humoral response to MO could be distinguished from humoral response to MA when measured with crude capsular material extracted from the MO organisms. Levels of antibodies to MO and MA antigens in the serum of naturally infected lambs were also measured to better understand the nature of the immune response against these organisms.

Materials and methods

Animals and sample collection. Lambs from 10 different sheep flocks around the state of Iowa were included in this study. The lambs were of various breeds, and most were approximately 10–12 wk of age at sampling. Blood samples were collected for serum, and nasal swabs were taken for bacterial isolation. The disease status, based on clinical signs including nasal discharge, cough, prolapses, and poor growth, was recorded for each flock at the time of sample collection by the investigators or reported by the owner or attending veterinarian. In 1 flock with a history of clinical disease blood samples were obtained sequentially, and nasal swabs were taken at 2–3-wk intervals starting at 3 wk of age and continuing over a 10-mo period. In addition, blood samples were taken from lambs in another flock with no history of clinical respiratory disease; these were free of mycoplasmas at the time of collection and provided reference negative serum samples.

Serum was harvested within 24 hr of collection and stored

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at -20°C until tested. Nasal swabs were processed immediately for mycoplasmas and bacterial isolation.

Hyperimmune sera against MO and MA were raised in horses as described elsewhere.⁶

Preparation of mycoplasmal antigens. MO and MA organisms were grown in modified Friis broth medium for 3 days at 37°C as previously described.⁷ Cultures were harvested and washed 3 times in phosphate-buffered saline (PBS, pH 7.4) by centrifugation at $10,000 \times g$ at 4°C for 30 min. The protein concentration of washed mycoplasmas was determined with a commercial kit^a following the instructions of the manufacturer, and aliquots were stored at -70°C .

Sodium dodecyl sulfate (SDS)-treated MO and MA antigens were prepared by mixing the respective washed organisms with SDS (1 mg/mg of cell protein) at 37°C for 30 min.¹³ These preparations were dialyzed in 0.01 M Tris-NaCl buffer (pH 7.2) for 48 hr, and aliquots were stored at -20°C .

Crude capsular material of MO was prepared as previously described¹ with some modifications. Washed MO organisms were incubated in PBS at 56°C for 30 min and centrifuged as before. The supernatant containing the capsular material was filtered through a $0.22\text{-}\mu\text{m}$ membrane filter. The protein content of the preparation was determined as before, and aliquots were stored at -20°C . Removal of the capsule was indicated by an experiment demonstrating that the decapsulated cells could not be agglutinated by any of the lectins specific for the organism.

ELISA. Both SDS-treated MO and MA antigens were diluted in carbonate-bicarbonate buffer (pH 9.0) to $6\text{ }\mu\text{g}$ protein/ml. Crude capsular MO antigen was suspended in PBS to give a final concentration of $15\text{ }\mu\text{g}$ protein/ml. One hundred microliters of each antigen preparation was coated onto individual wells of 96-well flat-bottom microtiter plates.^b The plates were incubated overnight at 4°C . The coated wells were washed 9 times with an ELISA wash buffer (0.13 mM sodium chloride, 150 mM potassium phosphate monobasic, 320 mM sodium phosphate dibasic, 270 mM potassium chloride, and 0.05% Tween 20, pH 7.2) to remove unabsorbed antigens. Then $100\text{ }\mu\text{l}$ of ammonium chloride (0.1 M) was added to each well to block unreacted sites. After 1 hr of incubation at 37°C , the plates were again washed 9 times with ELISA wash buffer. One hundred microliters of each sheep test serum, diluted 1:800 in PBS containing 0.05% Tween 80 and 1% horse serum, was added in duplicate to individual wells except for 2 wells in each plate that served as background controls. Positive and negative control sera (determined in preliminary studies using convalescent and normal lamb sera, respectively) were included in each plate. The plates were incubated for 2 hr at room temperature and washed as before. Then $100\text{ }\mu\text{l}$ of rabbit anti-sheep IgG (H + L) conjugated to horseradish peroxidase,^c diluted 1:780 in PBS containing 1% horse serum, was added to each well. The plates were incubated and washed as before. The wells were developed with $100\text{ }\mu\text{l}$ /well of 1-component 2,2'-azino-di-3-ethyl-benzothiazoline sulfonate peroxidase substrate (ABTS)^c solution at room temperature for approximately 12 min, and the reaction was stopped by adding $100\text{ }\mu\text{l}$ of 1% SDS solution.

The optical density (OD) values were measured with an automatic microplate reader^d at 405 nm. The net OD values

of individual samples were determined by subtracting the OD value of background wells from that of each sample. The titer of each test serum was determined as previously described⁹ with some modifications. The mean OD value for each test serum (S) was divided by the mean OD of the positive control sera (P), and the resulting value was multiplied by 100; results were expressed as S/P ratios.

The reactivity of the different antigens against horse hyperimmune serum to each *Mycoplasma* was tested as described except that each serum was diluted at 1:200, 1:400, 1:800, and 1:1,600 with incorporation of normal rabbit serum instead of normal horse serum in the diluents. Peroxidase-labeled goat anti-horse IgG (γ)^c was used as the secondary antibody.

Western immunoblotting. Washed MO or MA organisms were adjusted to a final concentration of 1 mg protein/ml with PBS. Crude capsular material of MO was adjusted to a final concentration of 4 mg protein/ml. Antigens were separated under reducing conditions using a 4% stacking gel and a 10% running gel⁸ and a vertical slab mini-gel apparatus.^a Prestained SDS-polyacrylamide gel electrophoresis standards (broad range)^a were included in each gel as molecular mass standards. The electrophoresed proteins along with the molecular mass standards were electrophoretically transferred onto $0.45\text{-}\mu\text{m}$ pore size nitrocellulose membranes^a at 4°C for 90 min at 100 V in transfer buffer consisting of 25 mM Tris, 192 mM glycine^a (pH 8.3), and 20% (v/v) methanol. The membranes containing the separated proteins were blocked overnight at 4°C with 1% gelatin in TBS (500 mM NaCl, 20 mM Tris, pH 7.5). The membranes were subsequently washed with gentle agitation 3 times (5 min each) in TTBS (TBS containing 0.05% Tween 20) and reacted for 1 hr at room temperature with the appropriate test lamb or horse sera diluted in diluent solution (TTBS containing 1% gelatin). The membranes were again washed as before and incubated at room temperature for 1 hr with gentle agitation in peroxidase-labeled anti-species IgG (L + H)^c antibody diluted in diluent solution. After washing, 1-component 3,3',5,5'-tetramethylbenzidine (TMB) membrane peroxidase substrate solution^c was used for color development. After 10–15 min of incubation at room temperature, the reaction was stopped by rinsing the membranes in deionized water.

Bacterial examination. Immediately after collection, nasal swabs were placed in tubes containing brain-heart infusion broth without antibiotics for transport to the laboratory. Ten-fold serial dilutions were made in modified Friis broth medium culture, and the mycoplasmas were isolated and identified as described previously.⁷ All the nasal swabs were also streaked onto 5% blood agar and McConkey's agar plates for isolation and identification of bacteria of pathogenic importance.

Statistical analysis. Most data were examined for trends. It became evident that there were large differences in reactivity between unaffected flocks and flocks late in the disease, so *t*-tests were run on ELISA S/P ratios, grouping all lambs in either early or late-affected flocks.

Results

The presence of both MO and MA organisms in lambs in the various flocks was indicated by the pres-

Table 1. Reactivity in ELISA of crude capsular material of *M. ovipneumoniae* (MO-CAP) and SDS-treated MO (MO-SDS) and *M. arginini* (MA-SDS) antigens against horse hyperimmune serum to each mycoplasma.

Hyper-immune serum	Dilutions	Optical density*		
		MO-CAP	MO-SDS	MA-SDS
Negative	1:200	0.157 ± 0.040	0.196 ± 0.050	0.135 ± 0.025
	1:400	0.090 ± 0.020	0.119 ± 0.054	0.111 ± 0.030
	1:800	0.054 ± 0.010	0.056 ± 0.033	0.079 ± 0.028
	1:1,600	0.041 ± 0.010	0.046 ± 0.015	0.075 ± 0.025
Anti-MO	1:200	1.693 ± 0.221	1.734 ± 0.200	0.497 ± 0.155
	1:400	1.535 ± 0.116	1.656 ± 0.162	0.266 ± 0.029
	1:800	1.400 ± 0.013	1.604 ± 0.135	0.165 ± 0.032
	1:1,600	1.177 ± 0.105	1.500 ± 0.104	0.100 ± 0.027
Anti-MA	1:200	0.189 ± 0.091	1.161 ± 0.163	1.735 ± 0.197
	1:400	0.173 ± 0.044	0.890 ± 0.164	1.590 ± 0.139
	1:800	0.096 ± 0.023	0.544 ± 0.165	1.579 ± 0.164
	1:1,600	0.062 ± 0.013	0.295 ± 0.074	1.493 ± 0.145

* Mean ± SD of 3 separate experiments.

ence of serum antibodies and isolation of the agents from nasal swabs. Other bacteria that were frequently isolated from the same flocks included *Neisseria ovis*, *Pasteurella multocida*, *P. haemolytica*, *Escherichia coli*, and *Klebsiella* sp.

The results in Table 1 highlight the reactivity of SDS-treated MO or MA antigens, or the crude capsular material of MO to horse hyperimmune sera against each organism. At various serum dilutions, there was substantial cross-reaction of anti-MA serum with SDS-treated MO antigen, less cross-reaction of anti-MO serum with SDS-treated MA antigen, and no cross-reaction of the anti-MA serum with crude capsular material of MO. Several convalescent sheep sera reactive to MA antigens were tested for their reactivity against the SDS-treated or crude capsular MO antigens. Although these sera reacted with SDS-treated MO, there was no detectable reactivity with capsular MO antigen (Table 2).

Reactivity of the same antigens with each of the horse hyperimmune sera or the convalescent sheep

sera was also evaluated with immunoblotting. Each antigen preparation reacted with the homologous hyperimmune serum (Fig. 1). Although several bands developed for SDS-treated MO antigen when reacted with anti-MA sera, no reactivity could be seen when the crude capsular material of MO was reacted with the same anti-MA sera. A few bands were present when SDS-treated MA antigen was reacted with anti-MO hyperimmune serum (Figs. 1, 2).

The results of serologic testing of lambs from the 10 flocks are provided in Tables 3–5. Clinical disease was not evident in three flocks (early affected flocks HD, MY, and ES1) at the time of blood collection, whereas flocks KP, SN, ES2, and AB were at midstage (based on the percentage of lambs affected and the severity of clinical signs) of the disease. Only a small percentage of lambs were still coughing in flocks HR, MR, and RB, considered to be in late stages of the disease. There was extensive variability in titers among lambs in each flock. Appreciable levels of antibodies to MA were present in lambs without clinical disease, and high levels of antibody to MA were present in lambs at the midstage of the disease process. Low levels of antibody to MO were present in lambs in the early affected flocks; however, lambs had developed high levels of antibody to MO late in the disease process when most lambs had apparently recovered. Significant differences ($t = 10.1$, $P > 0.0005$) were found in antibodies to crude capsular material of MO when comparing lambs from early and late-affected flocks.

A similar pattern of antibody production to MO and MA was observed in lambs from a flock with endemic disease, when blood samples were collected sequentially prior to, during, and after the disease process (Fig. 3). Many of the lambs in the flock developed a

Table 2. Reactivity in ELISA of various convalescent (S4-352–S5-560) and negative (C1–C3) sheep sera with crude capsular material of *M. ovipneumoniae* (MO-CAP) and SDS-treated MO (MO-SDS) and *M. arginini* (MA-SDS) antigens.

Serum no.	Optical density*		
	MO-CAP	MO-SDS	MA-SDS
S4-352	0.084 ± 0.013	0.382 ± 0.033	0.755 ± 0.014
S4-359	0.213 ± 0.008	0.571 ± 0.010	1.190 ± 0.030
S5-485	0.174 ± 0.001	0.348 ± 0.005	0.769 ± 0.009
S5-560	0.191 ± 0.007	0.390 ± 0.018	0.559 ± 0.043
C1	0.117 ± 0.005	0.164 ± 0.018	0.168 ± 0.013
C2	0.087 ± 0.012	0.112 ± 0.000	0.109 ± 0.016
C2	0.156 ± 0.014	0.123 ± 0.003	0.115 ± 0.006

* Mean ± SD in duplicate wells.

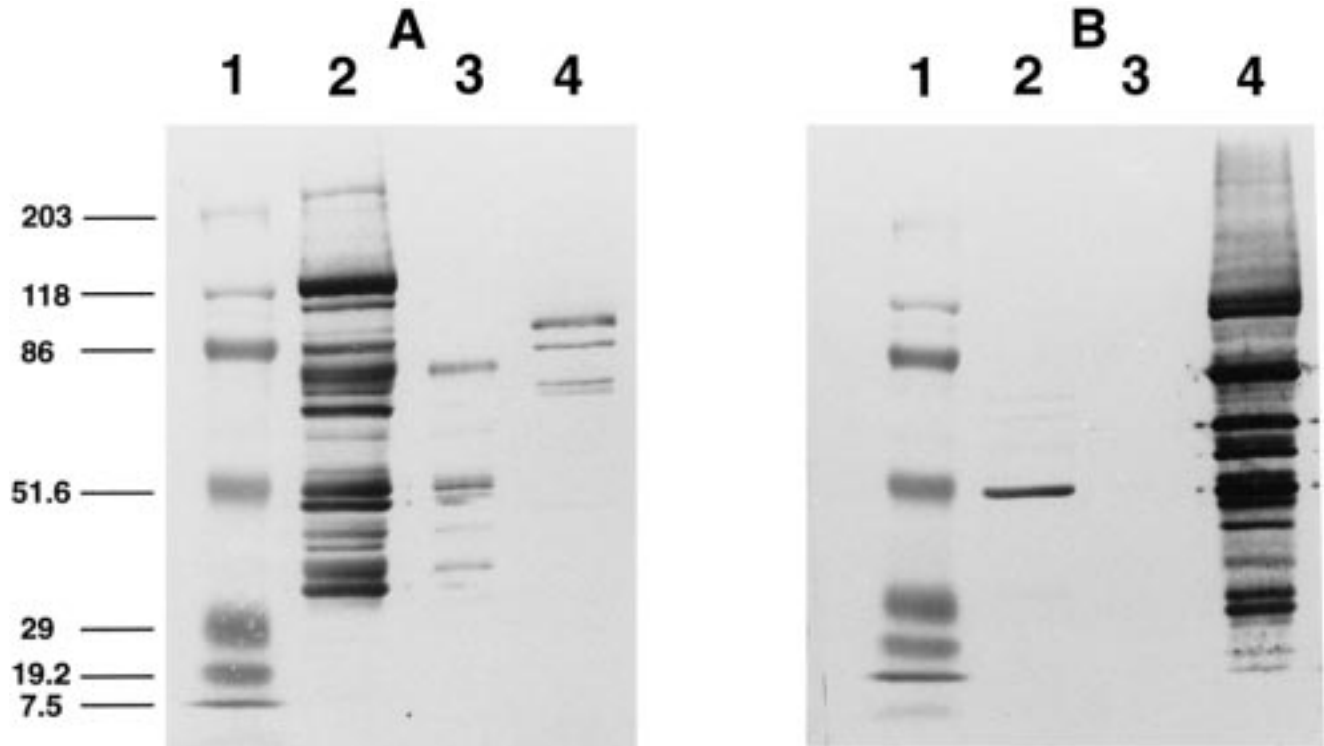


Figure 1. Immunoblots of SDS-treated *Mycoplasma ovipneumoniae* (MO) whole cells (lane 2), crude capsular material prepared from the same MO organisms (lane 3), and SDS-treated *M. arginini* (MA) whole cells (lane 4) developed with horse hyperimmune serum prepared against MO (A) and horse hyperimmune serum prepared against MA (B). Lane 1: molecular mass standards in kD. Peroxidase-conjugated rabbit anti-horse IgG whole molecule was used as a secondary antibody. Multiple bands developed to SDS-treated MO whole cell antigens when reacted with anti-MA serum, but no bands could be seen when the crude capsular material from MO was reacted with the same anti-MA serum. Bands were present when the SDS-treated MA antigen was reacted with anti-MO serum.

paroxysmal cough at 10–12 weeks of age. At that time, the mean antibody levels to both MO and MA were relatively low. As the disease progressed, more lambs seroconverted, and the mean antibody levels to crude capsular material of MO rose dramatically late in the disease process when most lambs had apparently recovered, at approximately 25–27 weeks of age. In contrast, the mean antibody levels to MA increased early during the disease process and rapidly declined during the recovery phase. Both MO and MA were routinely isolated from nasal swabs taken from some of the lambs in the flock throughout the duration of the experimentation. Other bacteria that were frequently isolated from the same samples included *N. ovis*, *Pasteurella* spp; and *E. coli*.

Discussion

ELISA and western immunoblotting performed in the present study indicated significant cross-reactivity between the SDS-treated antigens of MO and MA when horse hyperimmune sera of each specificity or convalescent sheep sera reactive to MA antigens were tested against these antigens. Cross-reactivity has previously been demonstrated between MO and other spe-

cies of mycoplasmas, including *M. hyopneumoniae*, *M. dispar*, and *M. flocculare* by immunoblotting.¹⁵ Cross-reactivity has also been observed with ELISA reactions of MO whole cell antigen solubilized in carbonate–bicarbonate buffer with hyperimmune sera of a variety of *Mycoplasma* species.^{5,15} Such cross-reactions would certainly interfere with the specificity of any serologic assay but in particular the ELISA. This cross-reactivity is particularly important when evaluating antibodies to MO and MA because both organisms frequently coexist in sheep.

Cross-reactions in ELISA between *M. hyopneumoniae* and a nonpathogenic *M. flocculare* in pigs is widely recognized and is a limiting factor for ELISA use in the serodiagnosis of mycoplasmal pneumonia of swine.³ Several attempts have been made to overcome this specificity problem by using different methods of antigen preparation. Several workers have used purified Tween 20 extracts of *M. hyopneumoniae* and found improved specificity.^{6,12} These improved ELISA antigens have been described as suitable for the practical serodiagnosis of mycoplasmal pneumonia of swine¹⁶ and are available in a commercial *M. hyopneumoniae* ELISA test kit.

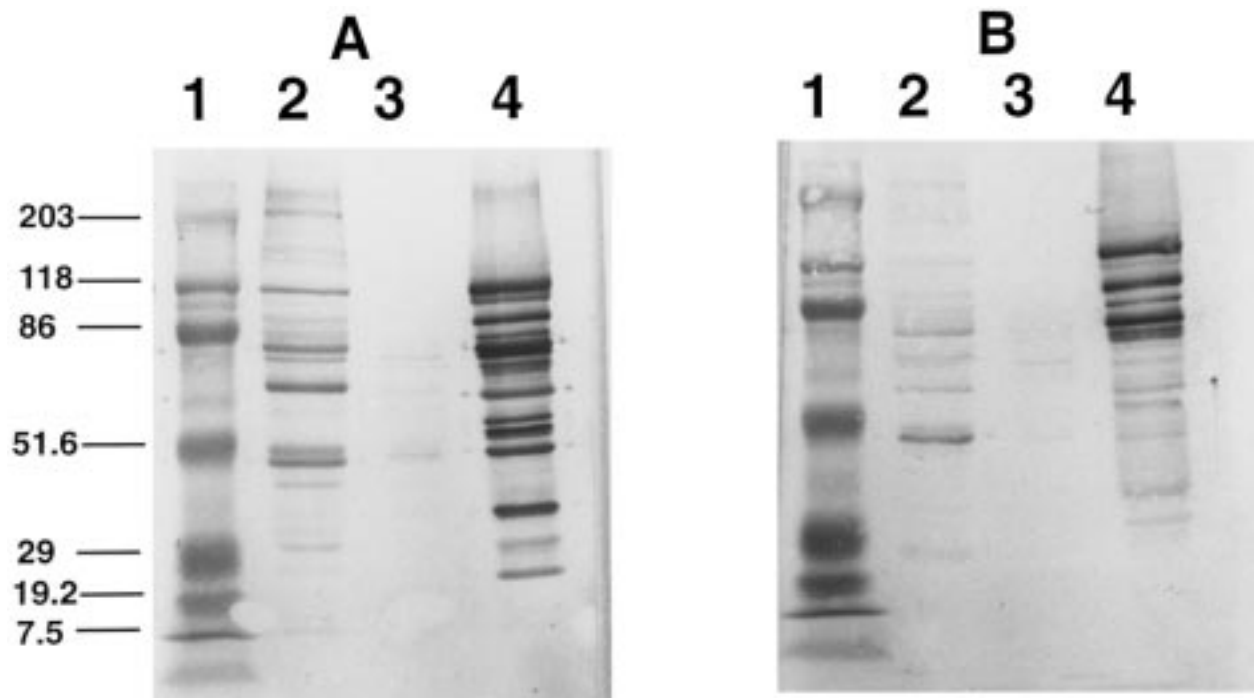


Figure 2. Immunoblots of SDS-treated *Mycoplasma ovipneumoniae* (MO) whole cells (lane 2), crude capsular material prepared from the same MO (lane 3), and SDS-treated *M. arginini* (MA) whole cells (lane 4) developed with 2 separate convalescent lamb sera reactive to MA antigen (A, B), which were positive by ELISA for both SDS-treated mycoplasma whole cell antigens but negative for MO crude capsular material. Lane 1: molecular mass standards in kD. Peroxidase-conjugated rabbit anti-sheep IgG whole molecule was used as a secondary antibody. Multiple cross-reactive bands developed between the 2 SDS-treated mycoplasma antigens. However, bands were absent or very weakly stained in the crude capsular material from MO.

A more specific ELISA antigen was needed to assist in the detection of mycoplasmal pneumonia of sheep. The crude capsular material of MO provided specificity in detecting MO antibodies. Presence of a specific capsule is a property of MO not shared by MA, and thus the antigen could be expected to provide specificity for MO.¹¹ The crude capsular material described here should be suitable as a reliable ELISA antigen in detecting MO-specific antibodies. Storage at $-20\text{ }^{\circ}\text{C}$

for at least 2 years did not affect the activity and the specificity of the antigen.

The results of this experiment indicated that lambs readily produce antibodies of IgG isotype in response to MA and MO, but the response to crude capsular material of MO was delayed. This slow development of circulating IgG antibodies to MO antigens could be confirmed by sequential collection of blood samples from lambs during the course of the disease. Other

Table 3. Antibody levels to SDS-treated *M. ovipneumoniae* in lambs from flocks sampled at different stages of disease.

Flock	No. tested	Titer*		Disease stage
		$\bar{x} \pm \text{SD}$	Range	
HD	12	19.496 \pm 4.196	11.411–27.500	early
MY	12	19.390 \pm 5.395	10.847–27.258	early
ES1	8	28.757 \pm 16.414	13.989–58.199	early
KP	20	35.284 \pm 9.717	25.021–58.285	middle
SN	8	27.932 \pm 10.783	14.552–48.266	middle
ES2	8	46.142 \pm 22.146	16.612–84.239	middle
AB	6	42.478 \pm 15.430	25.638–69.282	middle
HR	15	48.874 \pm 25.241	18.606–105.913	late
MR	10	35.284 \pm 9.717	25.021–58.285	late
RB	6	70.380 \pm 20.930	38.783–97.872	late

* Expressed in S/P ratios.

Table 4. Antibody levels to crude capsular material of *M. ovipneumoniae* in lambs from flocks sampled at different stages of disease.

Flock	No. tested	Titer*		Disease stage
		$\bar{x} \pm \text{SD}$	Range	
HD	12	11.573 \pm 4.715	5.508–20.469	early
MY	12	18.296 \pm 10.181	4.946–38.910	early
ES1	8	18.493 \pm 12.375	10.086–40.393	early
KP	20	25.510 \pm 13.752	6.453–57.342	middle
SN	8	26.777 \pm 12.456	13.433–51.102	middle
ES2	8	48.012 \pm 24.404	17.088–83.737	middle
AB	6	36.547 \pm 16.862	18.378–62.312	middle
HR	15	56.686 \pm 16.139	29.562–90.069	late
MR	10	50.874 \pm 21.556	33.696–107.325	late
RB	6	62.246 \pm 28.201	21.930–98.993	late

* Expressed in S/P ratios.

Table 5. Antibody levels to SDS-treated *M. arginini* in lambs from flocks sampled at different stages of disease.

Flock	No. tested	Titer*		Disease stage
		$\bar{x} \pm SD$	Range	
HD	12	26.100 \pm 3.462	22.050–32.264	early
MY	12	32.299 \pm 12.521	11.153–50.379	early
ES1	8	21.515 \pm 12.709	12.054–50.8260	early
KP	20	53.529 \pm 27.910	14.731–113.342	middle
SN	8	40.813 \pm 13.470	18.204–62.611	middle
ES2	8	45.223 \pm 9.0603	26.809–56.472	middle
AB	6	38.772 \pm 13.980	25.647–59.2772	middle
HR	15	44.952 \pm 24.468	20.109–96.468	late
MR	10	35.786 \pm 17.518	12.720–64.292	late
RB	6	35.044 \pm 13.136	20.530–59.138	late

* Expressed in S/P ratios.

workers have reported that serum IgG responses to sonicated MO antigen peaked at 41 days after experimental inoculation of lambs.¹⁴ These results are comparable to responses of lambs to SDS-treated MO antigen under field conditions, as reported here. Antibody responses to the capsule of *M. dispar* were required for phagocytosis of this mycoplasma by bovine alveolar macrophages,² and a similar effect could explain why late IgG responses to crude capsular antigen of MO correlate with recovery from clinical signs.

No measurements of local immune responses were made in this study, and other immune response components could play a role in the late abatement of clinical symptoms. Nevertheless, there is an apparent fail-

ure to generate a systemic immune response to certain MO antigens until late in the clinical disease. When levels of specific antibodies to the organism rise in the serum, lambs seem to recover from the clinical disease. This finding corroborates our previous observations that infection with the microorganism has manipulative effects upon the respiratory system of lambs, leading to development of aberrant immune responses such as immediate hypersensitivity and ciliary autoantibodies during early stages of the disease process.¹¹ This hypothesis is further supported by reports of temporary abatement of clinical signs in lambs treated with antihistamines early during the clinical disease process (M. L. Kaeberle, personal observations) and demonstration of respiratory allergy in association with mycoplasmal infection in humans.¹⁷

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Sources and manufacturers

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- Immulon 1, Dynatech Laboratories, Alexandria, VA.
- Kirkegaard & Perry Laboratories, Gaithersburg, MD.
- Molecular Devices, Menlo Park, CA.

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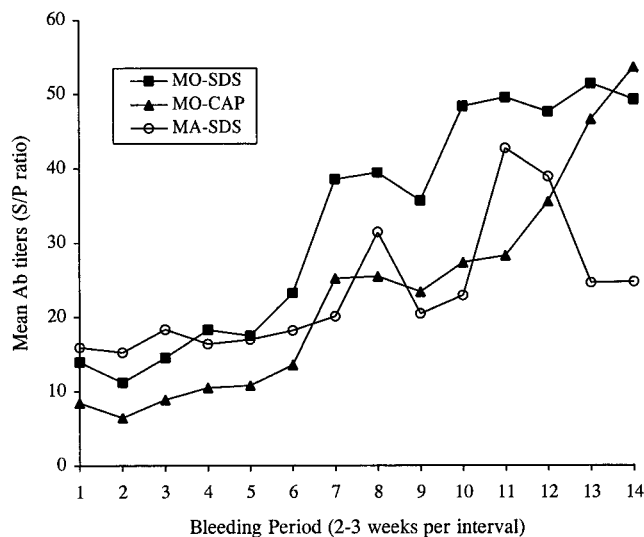


Figure 3. Antibody levels to *M. ovipneumoniae* (MO) and *M. arginini* (MA) in sera of lambs tested in a flock with clinical disease. Blood samples were collected from 3-week-old lambs sequentially over a 10-month period ($n = 16$ –20 lambs at each sampling time). Values represent mean titers expressed in S/P ratios. MO-SDS = SDS-treated MO whole cells; MO-CAP = MO crude capsular material; MA-SDS = SDS-treated MA whole cells.

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