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## An Antigenic and Serologic Comparison of Two Virulent Strains and an Attenuated Strain of *Anaplasma marginale*

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### SUMMARY

Soluble antigens of 3 *Anaplasma marginale* strains were compared by agar gel diffusion and immunoelectrophoretic techniques. Serum proteins from calves infected with each of the 3 *A. marginale* strains were separated electrophoretically and tested with rabbit anti-bovine serum in immunoelectrophoretic systems. There was no detectable difference between the soluble antigens of the 3 *A. marginale* strains. A *beta* globulin arc, which was not detectable in normal bovine serum, was present in serum of acutely affected calves, and a *gamma* globulin arc was lengthened in the latter serum as compared with that in serum of normal calves.

Various isolates of *A. marginale* in the United States were reported<sup>5</sup> to have antigenic differences. Since an isolate of United States origin was used in the production of a modified live anaplasma vaccine,<sup>14</sup> the present study was made

to determine the antigenic compositions of the attenuated organism and a strain from Colombia where the efficacy of the vaccine was being determined by field studies.<sup>7</sup> The antigenic composition of the attenuated organism was also compared with the Texas strain of *A. marginale*. Both the Texas and Colombian strains of *A. marginale* produced a more severe form of the disease than was produced by the attenuated *A. marginale*.

Various soluble antigens have been extracted from hemoparasites, and sensitive methods for detecting their presence and the presence of their antibodies have been described.<sup>1,11,12</sup> One soluble antigen,<sup>1</sup> the exact nature of which was not determined, was used in the present experiment. Since the use of only one

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TABLE 1—Identification of Experimental Calves

Animals	Strain of <i>A. marginale</i> in inoculum	Use of animals in the experiment
<b>EXPERIMENTAL CALVES</b>		
1 (splenectomized)	Attenuated (U.S. vaccinal strain)	Produce soluble antigen I*; donor for calves 4 through 7
2 (splenectomized)	Colombian	Produce soluble antigen II*; donor for calves 8 through 11
3 (splenectomized)	Texas	Produce soluble antigen III*; donor for calves 12 through 15
4, 5, 6, 7	Attenuated (U.S. vaccinal strain)	Examine seroimmunologic response
8, 9, 10, 11	Colombian	Examine seroimmunologic response
12, 13, 14, 15	Texas	Examine seroimmunologic response
16, 17, 18, 19 (Controls)	None (saline solution)	Examine seroimmunologic response
<b>OTHERS</b>		
38 (nonsplenectomized)	None	Produce normal soluble antigen (soluble antigen IV)
700 (splenectomized steer)	Colombian	Donor for calf 2
701 (splenectomized steer)	Texas	Donor for calf 3

\* Percentage parasitemia of blood from calves 1, 2, and 3 used for preparation of the 3 soluble antigens was 8, 15, and 12%, respectively.

antigen extraction process does not allow for reporting on complete antigenic characterization of *A. marginale*, the changes in the serum protein of animals infected by the 3 strains were determined to obtain additional information.

### Materials and Methods

Nineteen male Holstein-Friesian calves (No. 1-19) were obtained from a commercial dairy on the Bogota Savannah (plateau) of Colombia (Table 1). Each

TABLE 2—Development of Detectable Antibody to the Soluble Antigen of 3 Strains of *Anaplasma marginale*

Inoculum	Calf No.	Antibody first detected (days)
Soluble antigen I (attenuated vaccinal strain)	4	23
	5	23
	6	23
	7	23
Mean (± S.D.)		23.0 ± 0
Soluble antigen II (Colombian strain)	8	15
	9	36
	10	23
	11	29
Mean (± S.D.)		25.8 ± 15.4
Soluble antigen III (Texas strain)	12	23
	13	19
	14	29
	15	48
Mean (± S.D.)		29.8 ± 22.2

There is no significant difference in the number of days which elapsed prior to detection of the antibody in the 3 groups. ( $P < 0.05$ )  
S.D. = Standard deviation.

calf was placed in an individual stall and was fed milk and starter rations until approximately 3 months of age. Calves were free of anaplasmosis as determined by results of examinations of Giemsa-stained blood smears and of complement-fixation (CF) tests done each week for 2 months before they were inoculated. Calves 1, 2, and 3 were splenectomized 20 days before they were inoculated with the strains of *A. marginale*. Calf 1 was used for the production of soluble antigens from the attenuated *A. marginale* (soluble antigen I). Calf 2 was used for the production of soluble antigens from the Colombian strain of *A. marginale* (soluble antigen II). Calf 3 was used for the production of soluble antigens from the Texas strain of *A. marginale* (soluble antigen III). Soluble antigen IV was prepared from red blood cells (RBC) of a normal calf.

Calves 4 through 19, allotted to 4 groups of 4 animals each, were used to determine the serologic response to *A. marginale* infection. Calves 4 through 7 were infected with the attenuated vaccine *A. marginale*. Calves 8 through 11 were infected with the Colombian strain of *A. marginale*. Calves 12 through 15 were infected with the Texas strain of *A. marginale*. Calves 16 through 19 were used as control animals.

**SOLUBLE ANTIGENS OF *A. MARGINALE***  
*Inoculation of Calves and Collection of Samples.*—Calf 1 was inoculated subcutaneously with 17 ml. and intravenously

with 3 ml. of viable attenuated *A. marginale* in sheep blood. The inoculum had been hand-carried from Mexico\* to Colombia in a chest (solid carbon dioxide was used as refrigerant) and kept at  $-65^{\circ}\text{C}$ . for 6 months prior to use. After calf 1 was inoculated, paired blood samples were collected 3 times each week, with and without added anticoagulant (balanced oxy-late).<sup>15</sup> Giemsa-stained blood smears and specimens tested for packed cell volume (pcv) were prepared from blood samples which contained anticoagulant. Serum collected from the clotted samples was tested for CF activity. At the time calf 1 had maximal parasitemia (8%), 500 ml. of blood was collected in a flask containing 25 ml. of 12% sodium citrate solution. The blood was immediately centrifuged<sup>b</sup> in a 4 C. atmosphere at 480 g<sup>c</sup> for 10 minutes, and the plasma and buffy coat were removed. The RBC was washed 4 times by centrifugation at 480 g for 10 minutes in cold (4 C.) 0.85% sodium chloride (saline) solution. The RBC (100 ml.) was stored at  $-65^{\circ}\text{C}$ . in plastic containers.

Steer 700, which was a carrier of the Colombian strain of *A. marginale*, was the source of infective material for calf 2, and steer 701, a carrier of the Texas strain (College Station, Texas)<sup>d</sup> of *A. marginale*, was the source of infective material for calf 3. Both calves were inoculated intravenously. Flasks containing 12% sodium citrate solution were used for collection of blood from the 2 donor steers. The RBC was separated from plasma and buffy coat by centrifugation and washed 4 times in saline solution prior to use as inoculum in calves. Blood samples (oxylated) were also collected from the donor steers, and the parasitemia was determined by examining Giemsa-stained blood smears. Blood samples were taken from the calves 3 times per week during the prepatent period and 1 time each day after the onset of the patent period. Thin blood smears were stained by the Giemsa technique, and pcv was measured. Blood (500 ml.) for antigen production was collected from each calf soon after the level of infection had reached 8% of parasitized RBC; calf

2 had a parasitemia of 15% and calf 3 had a parasitemia of 12% at the time of blood collection. Blood was collected in flasks containing 25 ml. of citrate solution. The RBC was separated, washed as described in the previous paragraph, and stored at  $-65^{\circ}\text{C}$ .

Calf 38 was a normal calf from which blood (500 ml.) for antigen production was collected and processed as described in the preceding paragraph.

*Process for Extraction of Soluble Antigens of A. marginale.*—Frozen RBC from calves 1, 2, and 3 was thawed at ambient temperature. Lysed RBC from each of the calves (50 ml. from calf 1, 26 ml. from calf 2, and 34 ml. from calf 3) was used for antigen extraction. Barbital buffer (pH 7.3 to 7.4) was added to the lysed RBC (1 vol.:1 vol.), and the solutions were sonicated<sup>e</sup> at 4 C. for 60 seconds, using 5 ma. of current at 15-second intervals separated by 15-second rest intervals. The material was then centrifuged<sup>f</sup> at 100,000 g for 1 hour; the pellets (which were discarded) contained the particulate RBC stroma and parasites and the supernatant fluid, soluble antigen, and hemoglobin. The supernatant fluid was mixed with diethylaminoethyl (DEAE) cellulose to extract soluble *A. marginale* antigen by the method described by Amerault and Roby.<sup>1</sup>

*Immunodiffusion Technique.*—The medium for immunodiffusion was prepared by dissolving 1 Gm. of noble agar<sup>g</sup> per 100 ml. of borate buffer solution. The buffer contained 9 Gm. of boric acid and 2 Gm. of NaOH per liter of distilled water and was adjusted to a pH of 8.4. Thiomer-sal<sup>h</sup> (1:10,000 final concentration) was added. Portions (4 ml.) of the warmed (93 C.) medium were pipetted onto the microscope slides which had been coated with a thin layer of 0.1% borate buffered agar containing 0.05% glycerol. Patterns were cut into the agar, using a cork borer with outside diameter of 6 mm. The central well was surrounded by 4 circumferential wells whose perimeters were spaced 2 mm. from the perimeter of the central well. Soluble antigens I, II, and

\* Diamond Laboratories, Mexico City, Mexico.

<sup>b</sup> Sorvall RC2-B Refrigerated Centrifuge, Ivan Sorvall, Inc., Norwalk, Conn.

<sup>c</sup> Sorvall Relative Centrifugal Force Chart, Ivan Sorvall, Inc., Norwalk, Conn.

<sup>d</sup> Department of Veterinary Microbiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas.

<sup>e</sup> Branson Sonifier, Model 8-15, Branson Instruments, Inc., Danbury, Conn.

<sup>f</sup> Beckman L-2 Ultracentrifuge, Spinco Division, Beckman Scientific Instruments, Palo Alto, Calif.

<sup>g</sup> Special Agar-Noble, Difco Laboratories, Detroit, Mich.

<sup>h</sup> Merthiolate, Eli Lilly Company, Inc., Indianapolis, Ind.

III, as well as normal (noninfected) ABC material (soluble antigen IV), were compared in circumferential wells surrounding a central well containing 1 of the 4 reference serums derived from serums of the experimental calves.

*Immuno-electrophoretic Technique.*—The micromethod introduced by Sheidegger<sup>1</sup> and outlined in an operating manual<sup>1</sup> was used with some modifications. The medium was prepared as prescribed for immunodiffusion technique. Holes were cut in the agar, using a cork borer with an outside diameter of 6 mm., and these were 2 mm. distant from troughs which were 2 mm. wide. Agar was removed and approximately 60  $\lambda$  of soluble antigen solution was placed in the well. Electrophoretic separations<sup>1</sup> were made at ambient temperature with a current of 350 v. and approximately 10 ma. per tray for 1 hour. Troughs were filled with approximately 150  $\lambda$  of a reference serum. Soluble antigens I, II, and III were tested with homologous and heterologous reference serums.

#### SEROLOGIC EXAMINATIONS

*Inoculation of Calves and Collection of Samples.*—Blood was collected in sodium citrate solution (0.5 ml./10 ml. of blood) from calves 1, 2, and 3 which had been used for antigen production. Calves 4 through 7 were inoculated intravenously each with 5 ml. of blood from calf 2; and calves 12 through 15, with 5 ml. of blood from calf 3. Calves (No. 16 through 19) in the control group were each inoculated intravenously with 5 ml. of saline solution. Approximately 20 ml. of blood was collected from each inoculated calf on the day of inoculation and twice each week thereafter, and serum was prepared. Serum was stored at  $-20^{\circ}\text{C}$ . Blood samples (oxylated) were also collected twice each week for examination for parasitemia and determination of pcv. Blood smears were stained by the Giemsa technique.

*Preparation of Alum-Precipitated Antigen and Rabbit Inoculation.*—The CF test<sup>2</sup> was performed on serums which had been prepared (2 times per week) from calves 4 through 19 after they were inoculated.

<sup>1</sup> Gelman Equipment for Immuno-electrophoresis and Immunodiffusion, Gelman Instrument Company, Ann Arbor, Mich.

<sup>2</sup> Deluxe Electrophoresis Chamber, Gelman Instrument Company, Ann Arbor, Mich.

Serums separated from blood which had been drawn from each calf within one week after the CF titer became maximal were used to prepare an aluminum potassium sulfate (alum-precipitated) antigen<sup>3</sup> for rabbit inoculation.<sup>3</sup> Serum from which protein was precipitated for rabbit inoculation was collected from control calves (No. 16 through 19) 34 days after they were inoculated with saline solution, this being the average time between the day of inoculation and the development of maximum CF titers in the exposed calves.

*Testing (Screening) of Serum to Detect Antibody Against the Soluble Antigens.*—Serum separated from blood which was collected 2 times per week from the exposed calves (No. 4 through 15) was tested in immunodiffusion systems with homologous antigen preparations (soluble antigen I, II, or III). The initial visible reaction of antigen with antibody and degree of opacity of subsequent reactions were recorded. Serums from the calves that seemed to have high concentrations of antibody, as evidenced by production of strong precipitin lines, were retained as reference serums. Serums from control calves (No. 16 through 19) were also tested with the soluble *A. marginale* antigens for the presence of antibody.

*Serologic Response in Intact Calves.*—Microscope slides were prepared as described in the paragraph *Immunodiffusion Technique*. Tris-barbital-sodium buffer<sup>4</sup> was used in the concentration of 1:1,800 (pH 8.8) for agar preparation. Patterns were cut in the agar, using an immuno-electrophoresis punch<sup>1</sup> with a well diameter of 1.5 mm. and trough widths of either 1.0 or 2.0 mm. Electrophoresis separations were made at ambient temperature, using a current of 450 v. and 9 ma. per tray of 6 slides.

Serum samples of the 12 infected calves (No. 4 through 15) during the acute phase of disease (as determined by CF test results) were separated electrophoretically and then tested against serum from rabbits inoculated with the respective alum-precipitated antigen, as well as against serum from rabbits inoculated with alum-precipitated antigen derived from normal

<sup>4</sup> Gelman High Resolution Buffer, Gelman Instrument Company, Ann Arbor, Mich.

<sup>1</sup> Immuno-electrophoresis Punch Set, Gelman Instrument Company, Ann Arbor, Mich.

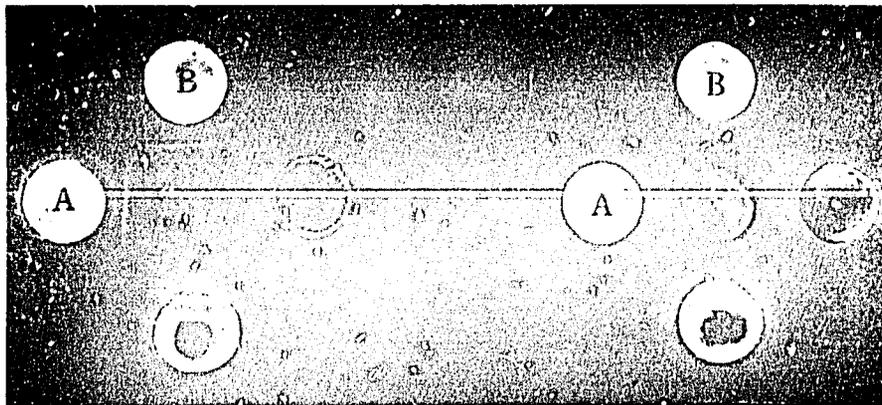


Fig. 1—Left: Centrally located reference serum from calf 4 infected with attenuated *A. marginale*. A, soluble antigen III; B, soluble antigen II; C, soluble antigen I; and D, soluble antigen IV.

Right: Reactants are same as those on left with respect to letters. Reference serum in center from calf 8 infected with the Colombian strain of *A. marginale*. After 72 hours of incubation.

bovine serum. Serums from control calves (16 through 19) were also tested against the respective hyperimmune serum from rabbits. Test serums (5  $\lambda$ ) were placed in the wells and hyperimmune serum (250  $\lambda$ ) was used in the troughs.

In another set of test patterns, serum from each infected calf and normal serum from control calves were placed in separate wells. After electrophoresis, this material was tested against serum from rabbits inoculated with the respective alum-precipitated antigen derived from serums of calves during the acute phase of infection.

## Results

### SOLUBLE ANTIGENS OF *A. MARGINALE*

*Immunodiffusion.*—Antigens I, II, and III formed one line of precipitation when tested with any of the 3 reference serums representing the various strains of *A. marginale* (Fig. 1). In each series of patterns, the formation of identity lines was evident. There was no evidence of precipitation produced by soluble antigen IV.

*Immunelectrophoresis.*—All soluble

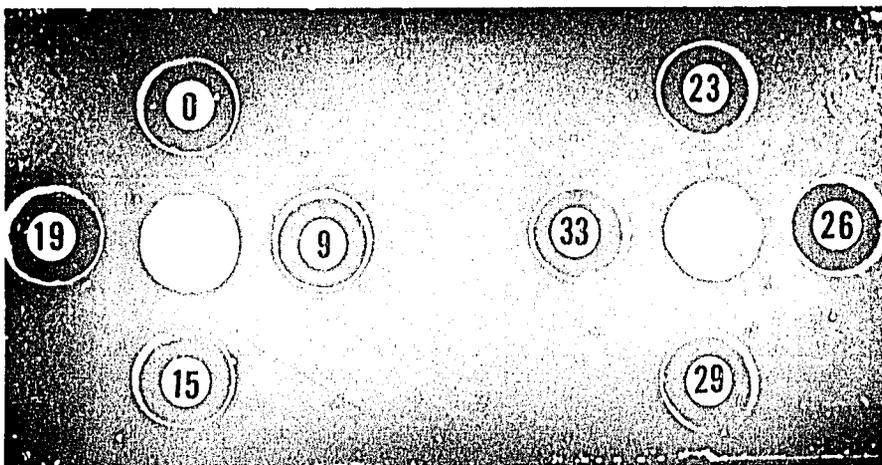


Fig. 2—Immunodiffusion reaction of centrally located soluble antigen II with serum samples from a calf inoculated with the Colombian strain of *A. marginale*. Moving clockwise from the top of patterns located at the left and continuing on the right the serum samples were obtained on the day of inoculation and at postinoculation days 9, 15, 19, 23, 26, 29, and 33. Arrow points to first visible line of precipitation. After 48 hours of incubation.

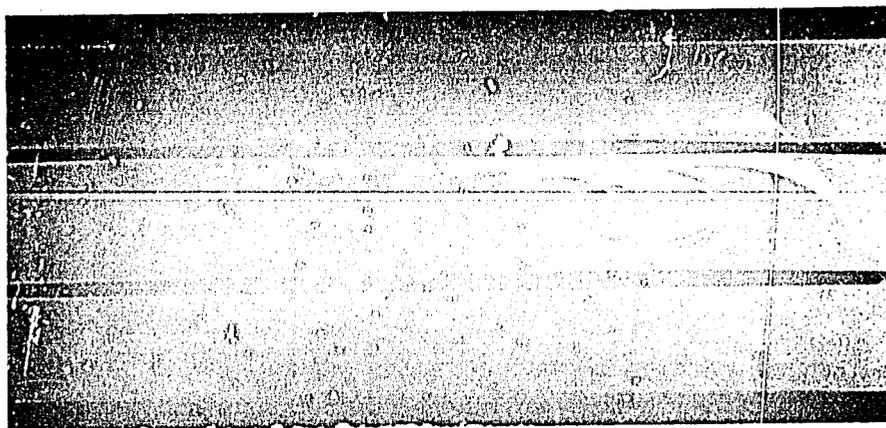


Fig. 3—Immunoelectrophoresis of serum (in well) from calf 4 during acute phase of infection with attenuated *A. marginale*. Top trough (1 mm. wide) contains hyperimmune serum from rabbit injected with alum-precipitated antigen prepared from the same serum which was exposed here to electrophoretic separation. Bottom trough contains hyperimmune serum from a rabbit injected with alum-precipitated antigen prepared from normal bovine serum. Arrows point to gamma globulin (left) and beta globulin (right) which appeared in serum from calf during acute phase, but which were shorter and indistinct, respectively, in normal bovine serum. After 48 hours of incubation.

antigens migrated approximately the same distance toward the anode and formed one arc of precipitation when tested with homologous reference serum.

#### SEROLOGIC RESPONSE

*Serum Testing (Screening) to Detect Antibody Against the Soluble Antigens.*—Antibody formed against the soluble antigen derived from the Texas strain appeared at an average of 29.8 days after inoculation (av. of 4 calves in group). Antibodies against the Colombian and the attenuated vaccinal strains appeared at an average of 25.8 days and 23.0 days, respectively, after calves 4 through 15 were inoculated with blood from carrier calves (Table 2). Concentration of antibody fluctuated as indicated by the density of precipitin lines (Fig. 2).

*Serologic Response in Intact Calves.*—The proteins in serums prepared from calves during the acute phase of induced disease, that were precipitated by hyperimmune serum from rabbits inoculated with alum-precipitated antigens prepared from serum of calves with acute infection or normal bovine serum, are shown (Fig. 3). Also, serum proteins of

animals acutely infected with *A. marginale* and normal serum proteins are shown (Fig. 4). A beta globulin<sup>3,8,9</sup> not present in normal bovine serum was demonstrated in most serum samples prepared during acute phases of infection in calves 4 through 15. A longer gamma globulin arc appeared in serums from acutely affected calves, as compared to that occurring in normal bovine serum (Table 3). These protein arcs were demonstrated in serum samples from calves which were infected with each of the 3 *A. marginale* strains. Other protein components varied inconsistently in serum samples from the infected calves and normal bovine serum.

#### Discussion

*Soluble Antigens.*—The method of soluble antigen extraction used in the present experiment had been used to extract antigenic material from RBC parasitized by a strain of *A. marginale* of United States origin. There was no report, however, on the use of this method to extract soluble antigens from a Colombian strain or the attenuated organism. Reportedly,<sup>5,6</sup> antigenic differences may exist between the various strains of *A.*

*marginale*; thus the comparative experiment was undertaken. The work of Amerault and Roby<sup>1</sup> involved applying the extraction method to blood with 80% of RBC parasitized. The attenuated *A. marginale* strain characteristically produces a low percentage of parasitized RBC, and it was not known whether adequately potent antigen for testing by agar gel diffusion technique could be prepared from such blood. Calf 1 developed a maximal parasitemia involving 8% of RBC. Since blood was collected from calves 2 and 3 when their parasitemia was 15 and 12%, respectively, initial volumes of RBC used in the process for producing soluble antigens were reduced proportionally to equilibrate the amount of *A. marginale* protein subjected to extraction.

Soluble antigens I, II, and III were apparently identical in all characteristics that were tested. Adjacent lines of precipitation had continuity at the point where 2 lines met. Therefore, the antigens were considered identical. Further evidence of sameness was gained by immunoelectrophoretic migration of the various antigens to the same mobility zone and cross reactivity of all antigens with heterologous serum.

TABLE 3—Additional Beta and Lengthened Gamma Arcs Seen in Serum Prepared from Calves with Induced Acute Infections of *Anaplasma marginale*

Inoculum	Calf No.	Lengthened Gamma	Additional Beta
Soluble antigen I (attenuated vaccinal strain <sup>1</sup> )	4	Present	Present
	5	Present	Present
	6	Present	Present
	7	Present	Absent
Soluble ant. gen II (Colombian strain <sup>1</sup> )	8	Present	Present
	9	Present	Present
	10	Present	Absent
	11	Present	Present
Soluble antigen III (Texas strain)	12	Present	Present
	13	Present	Present
	14	Present	Present
	15	Present	Present

*Serologic Response.*—The serologic responses in the calf groups each infected with a given *A. marginale* strain were similar. By immunoelectrophoretic technique, 2 arcs of precipitation were usually identified in serums of calves during the acute phase of induced disease. The *beta* arc appeared only in serums of these calves and the *gamma* arc extended further toward the cathode portion.

The additional, or more extensive, protein components in serum of calves during the acute phase may be manifestations of protein present in smaller concentrations in normal bovine serums, or



Fig. 4—Immunoelectrophoresis of serum from calf 13 during acute phase of infection with the Texas strain of *A. marginale* (top well) and serum from calf 16, a normal control (bottom well). Trough (2 mm. wide) contains hyperimmune serum from rabbit injected with alum-precipitated antigen prepared from serum used in the top well. Arrows point to *gamma* globulin (left) and *beta* globulin (right) components, which appeared in serum from calf 16 during acute phase of infection, but which were shorter or indistinct, respectively, in normal bovine serum. After 72 hours of incubation.

of normal proteins which have been altered antigenically and electrophoretically during the immune response, or of specific proteins formed during disease. The long *gamma* arc may have been caused by partial retention of the antigen by the gel or by the presence of the same antigenic group on molecules of different electrophoretic mobilities.<sup>2</sup> However, Murphy *et al.*,<sup>8</sup> using immunoelectrophoresis technique, demonstrated that the *gamma* arcs are lengthened in serum collected during the course of anaplasmosis and shifted toward the trough, indicating increased concentration. Also, lengthening may have indicated synthesis of an electrophoretically slower subpopulation of the protein.

All arcs, however, should be labeled with caution, since they can be identified with absolute certainty only by the use of absorption of specific serum components with specific immune serum or simultaneous electrophoresis of known serum components.

Results of the present experiment that pertain to the efficacy of the attenuated agent in a program of anaplasmosis control in Colombia can be evaluated only in conjunction with field studies. Recent work in this area has indicated that the attenuated agent does not protect calves against field challenge exposure in Colombia.<sup>7</sup>

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