DETECTION OF *ANAPLASMA MARGINALE* THEILER, 1909 IN TICK GUT HOMOGENATES USING A MICROFLUOROMETRIC IMMUNOASSAY

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PREFACE

This study is concerned with the detection of *Anaplasma marginale* organisms in gut homogenates of adult *Dermacentor andersoni* that were infected as nymphs. Various techniques are attempted using a micro-immunofluorescence method.

The author wishes to thank those who have encouraged and assisted her in this endeavor. This group of perservering individuals includes those on my committee: Dr. Carl Fox, Dr. John Homer, Dr. Kathy Kocan, and Dr. Sidney Ewing. It also includes other friends and supporters. Dr. Helen Jordan's encouragement often kept me from giving up when I was disheartened at my progress. Other friends, too numerous to name, also deserve my heartfelt thanks. Thanks, too, to Jim Hellwege for typing the manuscript and for the suggestions he made.

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CHAPTER I

INTRODUCTION

Anaplasma marginale Theiler, 1909 was observed by Smith and Kilborne as small coccoid bodies on the edge of many erythrocytes from animals suffering from Texas cattle fever. They interpreted these bodies to be a stage in the life cycle of *Babesia bigemina*, the causative agent of Texas cattle fever (Smith and Kilborne, 1893). About 10 years later in South Africa, Theiler differentiated between the two organisms and named the smaller organism *Anaplasma marginale*.

The name *Anaplasma marginale* is descriptive of the organism's morphology, as observed by light microscopy. *Anaplasma* is derived from Greek words meaning "without form" and refers to the appearance of the organism as a clump of chromatin material without cytoplasm. The organism also is most often located near the periphery of its host red blood cell, resulting in the specific name *marginale* (Theiler, 1910). The taxonomic classification of *A. marginale* has been considered uncertain for many years. The electron microscope has been especially useful in studying the organism. Ultrastructural studies have demonstrated a rickettsial-like morphology and a developmental cycle involving several stages (Kocan et al, 1984). In the ninth edition of *Bergey's Manual of Determinative Bacteriology* (Moulder, 1974), *Anaplasma* is classified in the Order Rickettsiales and Family Anaplasmataceae.

Different developmental stages have been described in bovine

erythrocytes (Ristic and Watrach, 1962; Ristic, 1980) and in the ticks *Dermacentor andersoni* Stiles and *Dermacentor variabilis* (Say) (Kocan et al, 1982; Kocan, Hair, et al 1984; Kocan, Yellin, et al, 1984). Ristic (1962) suggested and later research verified (Kocan et al, 1980; Francis et al, 1979) that the initial body of *A. marginale* enters the cell by invagination of the erythrocyte cytoplasmic membrane. A parasitophorous vacuole forms from the host cell membrane, and the initial body replicates by binary fission.

Kocan et al (1982) described colonies of *Anaplasma* in the epithelial cells of tick gut as rickettisal-like and demonstrated the existence of two distinct morphologic forms. However, these organisms exhibited more pleomorphism than other rickettsiae, and their reproductive characteristics appear to be more similar to chlamydiae.

CHAPTER II

THE NATURAL DISEASE

Cattle that become infected with A. marginale and display clinical signs are generally older than one year of age. Usually, calves are resistant to infection and are only mildly affected and usually become carriers. Susceptible animals that develop acute disease initially exhibit an elevated body temperature of 105° to 107° F. After a few days, the temperature drops to normal, and icterus and signs of anemia appear. Normally, there is no hemoglobinuria because the hemolysis that occurs is extravascular. Kreier and Ristic (1963) indicated that the anemia results from erythrophagocytosis initiated by parasitic damage to the erythrocytes and from "antierythrocytic antibody" (Schroeder and Ristic, 1965). Additional clinical signs include decreased milk production, loss of weight, a pounding jugular pulse, constipation, and abortion. Postmortem lesions include watery-appearing blood, an enlarged gall bladder, swollen and edematous lymph nodes, and a dark, soft, and enlarged spleen. Convalescent animals become carriers of the disease and maintain a healthy appearance but are capable of transmitting the organism to a vector (Bruner and Gillespi, 1973; Stiles, 1939).

Blood from an infected animal can be inoculated into another animal to produce infection with A. marginale. The disease may be spread mechanically by blood-contaminated mouthparts of horseflies or

mosquitoes. Blood-contaminated instruments may also transmit the disease (Harwood and James, 1979). Several species of ticks have been shown to transmit *A. marginale* biologically. Studies have shown that developmental stages can occur in the tick, *Dermacentor andersoni*, beginning with infection of nymphal ticks and continuing through transmission of the organism to cattle by subsequently molted adults (Kocan, Hair, and Ewing, 1980).

Ticks as Transmitters of Disease

Ticks may serve as both vectors and reservoirs for a large number of disease-causing organisms. Factors which account for the success of ticks in the transmission of diseases include the fact that they are persistent blood suckers that attach firmly and do not dislodge easily while feeding. They are also slow feeders, which creates the opportunity for dispersion when hosts migrate while they are attached and allows time for ingestion of pathogens. Some ticks have a wide host range, giving them considerable opportunity to acquire pathogens and transmit them from one animal species to another. The extreme longevity of most tick species allows time for pathogens to undergo development or to survive adverse environmental conditions. Smith and Kilborne (1893) were first to demonstrate that ticks were involved in the transmission of protozoans. Ticks have since been shown to transmit viruses, rickettsiae, bacteria, and protozoans (Philip and Burgdorfer, 1961).

It is thought that rickettsiae were originally symbiotes of acarines and may have become secondary pathogens for vertebrates through the blood-feeding habits of their arthropod hosts (Philip, 1961). In many instances, the rickettsiae trannsmitted by ticks do not exhibit high

or long-lasting parasite levels in vertebrate hosts. In such cases, the ticks usually serve as the reservoirs of infection (Philip, 1961; Bertram, 1962).

Ticks become infected with disease agents by ingesting blood or tissue fluids from a vertebrate host. The blood from the mouth parts enters the digestive diverticula of the midgut which expands to allow consumption of large quantities of blood. Within the tick, the organisms may grow and multiply in cells of various organs. *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (tick-borne typhus), can be found in many tick tissues, including salivary glands, hemocytes, sperm, and ova. Transstadial transmission of some rickettsiae and protozoans also have been reported in ticks (Philip and Burgdorfer, 1961; Wolbach, 1919; Philip, 1959; Philip, 1963; Burgdorfer and Varma, 1967).

Anaplasma marginale in Ticks

Several species of ticks have been shown in experimental studies to transmit anaplasmosis (Ristic, 1968). Transstadial transmission of *Anaplasma* was first demonstrated by Anthony (1964). In later studies, nymphs of *Dermacentor andersoni* and *D. variabilis* were fed on an infected calf and then allowed to molt to adults. Inoculation of susceptible animals with tick gut homogenate from adults infected as nymphs resulted in patent anaplasmosis (Kocan, Teel, et al, 1980; Kocan, Hair, Ewing, and Stratton, 1980).

Anaplasma marginale organisms are acquired by ticks during the ingestion of a blood meal from infected cattle. Colonies of *A. marginale* have been observed in midgut epithelial cells of replete

nymphs throughout development to the adult stage. Two distinct morphologic types of colonies have been observed and categorized by light microscopy. When the colonies were examined with electron microscopy, the colonies were observed to comprise distinctive organisms of different types. Colonies of A. marginale have also been found in midgut epithelial cells of adult Dermacentor andersoni that were infected as nymphs. Light microscopy revealed five morphologically different colony types. Ultrastructural features of these colonies showed a high degree of pleomorphism in developmental stages (Kocan, Hair, and Ewing, 1980; Kocan, Ewing, Holbert, et al, 1982).

Several techniques have been used to demonstrate the A. marginale organisms in tick midgut epithelial cells. A ferritin-labeled antibody technique with the electron microscope demonstrated the organisms in infected adult ticks that had been fed as nymphs on Anaplasma-infected cattle (Kocan, Hsu, et al, 1980). The light microscope was used to demonstrate colonies of A. marginale in both frozen and plastic sections of tick gut which were stained with Mallory's stain (Oberst et al, 1981). Oberst et al also produced Anaplasma antibody which was conjugated with fluorescein isothiocyanate and were able to demonstrate colonies in tick gut epithelial cells with a direct fluorescent antibody technique. Immunocytochemical labeling of A. marginale using peroxidase-antiperoxidase labels allowed the organisms to be demonstrated within intact host cells with the electron microscope (Staats et al, 1982).

Purpose of the Study

Microfluorometric tests using an FIAX 100™ fluorescent immunoassay

system (MA Bioproducts, Walkersville, Maryland) have been used to detect serum antibodies against such organisms as Toxoplasma gondii (Walls and Barnhart, 1978; Gordon et al, 1981; Hyde et al, 1980), Entamoeba histolytica (Taylor and Rez, 1978), Candida albicans (Estes et al), and Anaplasma marginale (Fox et al, 1982; Logan et al, 1985). In this system, antigen is adsorbed to a cellulose nitrate disk affixed to a plastic handle (StiQ^{TM} Sampler). The antigen-coated StiQs are then incubated in test serum, and the amount of bound antibody is detected using fluorescein isothiocyanate (FITC) which is conjugated to antiserum against specific classes of immunoglobulin from the animal species to be tested (indirect) or to antiserum against the organisms being tested (direct). Both the indirect and direct methods of measuring antibody binding are very sensitive; on the other hand, the specificity depends on the quality of the antigens used. In either case, the amount of fluorescence read as fluorescent signal units is directly related to the amount of antigen on the StiQs or antibody in the test serum.

The purpose of the present study was to determine if FIAX serology could be adapted to demonstrate A. marginale in homogenates of experimentally infected ticks. This would represent a unique approach for an infectious agent, although similar techniques have been used for detection of drugs and globulins.

CHAPTER III

MATERIALS AND METHODS

Ticks for experiments were propagated and maintained at the Oklahoma Agricultural Experiment Station, Entomology Tick Laboratory (Patrick and Hair, 1975). Larval *Dermacentor andersoni* were fed on rabbits and allowed to molt to the nymphal stage. Some nymphs were placed in muslin cells that were glued to the sides of *Anaplasma marginale*-infected calves. Infected calves were inoculated with a Virginia isolate of *A. marginale* which had been used successfully in other tick transmission studies (Kocan, Hair, Ewing, and Stratton, 1980). Tick feeding was timed so that the rapid engorgement stage (between days 6 and 10 after attachment) coincided with periods of highest parasitemia levels in the calves. After fully engorged ticks detached, they were maintained in a chamber with 90 to 98% relative humidity at 20° C with a 14-hour photoperiod. After molting to the adult stage, the ticks were used to prepare gut homogenates by the methods of Kocan, Hsu, et al (1980).

Control ticks were treated in a similar manner, with the exception that the nymphs were fed on noninfected calves without complement fixation (CF) titers to *Anaplasma* as determined by the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Oklahoma.

Preparation of Seeded and Infected Gut Homogenates

After molting to the adult stage, infected ticks were incubated for an additional two days at 37° C to facilitate the maximum development of *A. marginale* colonies (Kocan, Hair, Ewing, and Stratton, 1980). All ticks were immersed in 0.1% aqueous solution of merthiolate to decontaminate their body surfaces. They were then washed several times in sterile phosphate-buffered saline (PBS). Batches of 50 ticks were dissected, and the guts were removed and pooled in RPMI 1640 medium. The tissues were macerated in a glass tissue homogenizer and centrifuged for 15 minutes at 3,500 g. The sediment was collected and frozen at -70° C until the time of testing.

Some of the gut homogenate from control ticks was seeded with CF antigen obtained from the National Veterinary Services Laboratory, Ames, Iowa. One-ml portions of homogenate were seeded with 25 ul of CF antigen. The seeded and nonseeded control hemogenates were used as controls in the various experiments.

Preparation of Antisera

Bovine anti-*Anaplasma* sera were obtained from cattle previously infected with *Anaplasma*. High-titered sera (CF tested) were tagged with fluorescein isothiocyanate (FITC) for direct immunofluorescent assays (Oberst et al, 1981).

Rabbit anti-*Anaplasma* (CF antigen) serum was produced in a New Zealand white rabbit. Two ml of CF antigen was mixed with 6.0 ml PBS (pH 7.3). On day one, the rabbit was given an intramuscular injection in the subscapular region with 1.0 ml of the antigen preparation. On

alternate days, an additional 1.0 ml of the antigen mixture was injected intramuscularly for a total of four injections. In an attempt to elicit an anamnestic response, the rabbit was injected with 2.0 ml of the antigen preparation one week after the fourth injection. Eight days later, the rabbit was bled from a marginal ear vein. The serum was harvested and incubated at room temperature for 10 minutes with washed bovine erythrocyte stroma prepared from freshly lysed bovine RBC's. After centrifugation, the adsorbed serum was removed and frozen in 5.0-ml aliquots.

Fluorescein-conjugated rabbit antibovine immunoglobulin G and fluorescein-conjugated goat antirabbit immunoglobulin G (Cooper Biomedical, Cochranville, Pennsylvania) were rehydrated with 2.0 ml distilled water and diluted 1:200 with PBS (pH 7.3) containing Tween 20 (0.15%).

Serology

The equipment necessary for the FIAX serology was an FIAX 100* fluorometer, a microdiluter, and a horizontal shaker. Before taking fluorescence readings, measured as fluorescent signal (FS) units, both the gain control and baseline adjustment on the fluorometer were adjusted to zero. Pregain FS values were measured from all the StiQs. The highest fluorescing StiQ was again inserted into the fluorometer, antigen side up, and the gain control was set to a reading of 160. The FS units registered were then recorded for all specimens. Nonspecific fluorescence of each test was determined by measuring the fluorescence from the backside of the StiQ, and it was subtracted from the measurement obtained from the front or antigen-coated side to obtain the adjusted FS value for each sample. Depending on the conjugates used,

the technique produced either a direct or indirect measurement of bound antibody.

Detection of *Anaplasma* in Tick Homogenates Using FITC-Conjugated Bovine Anti-*Anaplasma* Antisera

Uninfected tick gut homogenates (1.0-ml aliquots) were seeded with 25 ul of CF antigen in order to determine if the antigen could be detected in the homogenates. The FIAX values were compared for homogenate with and without CF antigen. The unseeded and seeded homogenates were serially diluted to determine the effects of concentration. Twenty-five ul of each of the homogenate dilutions, with and without CF antigen, were evenly distributed over the StiQ surface and allowed to air dry for 3 to 24 hours.

The homogenate-coated StiQs were placed in a tube with 1.0 ml of a 1:200 dilution of fluorescein-tagged antiserum diluted in Tween 20 buffer at pH 7.2. StiQs in sera were placed on a horizontal shaker and incubated with the fluorescein-tagged bovine serum for 20 minutes. They were then transferred to 1 ml PBS-Tween 20 (0.15%) wash solution and agitated for five minutes. Fluorescence (FS) was then measured using the fluorometer. All samples were tested using six replicates of each homogenate.

Trapping of *Anaplasma* Organisms from Tick Homogenates Using Anti-*Anaplasma* Serum

Whole serum obtained from an *Anaplasma*-infected cow with a CF titer of 80 was coated in 25-ul volumes on StiQ samplers and allowed to air dry. The StiQs were reacted with 1-ml dilutions (1:2, 1:4, 1:8, 1:16,

and 1:32) of noninfected tick gut homogenate (as negative controls) and the CF antigen-seeded homogenate. After a 30-minute incubation, the StiQs were washed in 1 ml of PBS-Tween 20 solution for 10 minutes. The StiQs were then transferred to 1 ml of fluorescein-conjugated bovine anti-*Anaplasma* serum (1:100 dilution) for 20 minutes to allow for the binding of antibody. The StiQs were then transferred to 1 ml PBS-Tween 20 solution and washed 10 minutes. Fluorescence (FS) was read using the FIAX fluorometer. All samples were tested using six replicates of each treatment group.

Detection of *Anaplasma* in Tick Homogenates by Using Bovine Antiserum and Rabbit Antibovine Conjugate

Dilutions (1:2, 1:4, 1:8, 1:16, and 1:32) of infected, positive control, and negative control tick gut homogenates were made. Twenty-five ul of each dilution of homogenate were spotted on StiQs and allowed to air dry. The spotted StiQs were incubated for 10 minutes with 1 ml of bovine antiserum (1:100 dilution) that had an initial CF titer of 80 to *Anaplasma*. After a 10-minute wash, the StiQs were transferred to 0.5 ml of a 1:200 dilution of FITC-conjugated rabbit antibovine IgG for 20 minutes. After a second 10-minute wash, fluorescence was measured using the fluorometer. Six replicates of each dilution of each homogenate were evaluated.

Detection of *Anaplasma* in Tick Homogenates Using Erythrocyte-Adsorbed Rabbit Anti-CF Antigen and Antirabbit IgG Conjugate

Twenty-five ul of 1:2, 1:4, 1:8, 1:16, and 1:32 dilutions of tick gut homogenates (positive and negative controls) was placed on StiQs and allowed to air dry. The spotted StiQs were placed in 1.0 ml of rabbit anti-*Anaplasma* serum (1:100 dilution) and incubated for 30 minutes, after which the StiQs were washed for 10 minutes in PBS-Tween 20 (0.15%) and reacted for 20 minutes with 0.5 ml of FITC-conjugated antirabbit IgG diluted 1:200. The StiQs were then washed again in PBS-Tween 20 (0.15%) solution for 10 minutes, and the amount of fluorescence was measured. Six replicate samples of each homogenate dilution were evaluated.

Detection of *Anaplasma* in Experimentally Infected Ticks

Batches of ticks that were fed on *Anaplasma*-infected calves were tested for *Anaplasma* organisms. Twenty-five ul of homogenated tick gut from eight batches of ticks was diluted 1:2 (dilution determined in previous titrations) in PBS, spotted on StiQ samplers in 25-ul volumes, and allowed to dry. The spotted StiQs were placed in 1.0 ml of rabbit anti-*Anaplasma* serum at a 1:100 dilution. After reacting for 30 minutes, the StiQs were washed for 10 minutes and transferred to 0.5 ml of the FITC-conjugated antirabbit IgG diluted 1:200 for 20 minutes. The StiQs were washed in PBS-Tween 20 (0.15%) solution for 10 minutes, and the amount of fluorescence was measured. Twelve replicate samples of each homogenate were evaluated.

CHAPTER IV

RESULTS

Preliminary titrations using *Anaplasma*-seeded (CF antigen) gut homogenates indicated that a 1:200 dilution of the fluorescein isothiocyanate (FITC)-conjugated bovine anti-*Anaplasma* serum was optimal for detection of the *Anaplasma* in CF antigen. Dilutions of 1:200 of the unconjugated bovine and rabbit anti-*Anaplasma* sera also proved to be the optimal working concentrations when used in the indirect assays. The 1:2 and 1:4 (12.5 and 6.25 ul of CF antigen per ml of homogenate) dilutions of the seeded gut homogenate contained the optimally detectable amount of antigen.

When the CF antigen-seeded homogenate was reacted with FITC-conjugated bovine anti-*Anaplasma* serum, elevated FS values were noted in the 1:2 and 1:4 dilutions. FS values at the 1:8 dilution were similar to those of the positive and negative control tick gut homogenates at the 1:2 dilution (Table I); thus, the test was not sensitive enough to detect a difference in the positive and negative control tick gut homogenates.

Small increases in fluorescence levels were detected in the CF antigen-seeded homogenates (positive controls) at the 1:2 and 1:4 dilutions when the StiQs were spotted with bovine anti-*Anaplasma* antibody and reacted first with the homogenate and then with the FITC-conjugated bovine anti-*Anaplasma* serum (Table II). However, the negative control

TABLE I

FIAX VALUES[a] FROM StiQS SPOTTED WITH TICK GUT HOMOGENATES AND REACTED WITH FITC-CONJUGATED BOVINE ANTI-*ANAPLASMA* SERUM

	Tick Gut Homogenates		
Serum Dilutions	Infected[b]	Noninfected[c]	Seeded with CF Antigen[d]
1:2	38.0	42.0	89.0
1:4	16.0	18.0	55.5
1:8	6.5	8.0	38.0
1:16	6.5	6.5	17.5
1:32	2.0	2.0	5.5

[a]Mean of six replicates.

[b]Ticks fed on *Anaplasma*-infected calf.

[c]Ticks fed on *Anaplasma* CF-negative calf.

[d]One ml of homogenate with 25 ul CF antigen added before diluting.

TABLE II

FIAX VALUES[a] FROM StiQS SPOTTED WITH *ANAPLASMA*-SPECIFIC ANTISERUM AND REACTED WITH TICK GUT HOMOGENATES

	Tick Gut Homogenates		
Serum Dilutions	Infected[b]	Noninfected[c]	Seeded with CF Antigen[d]
1:2	17.0	12.0	20.5
1:4	10.5	16.0	20.5
1:8	3.5	2.5	14.0
1:16	1.0	1.0	12.5
1:32	4.0	1.0	10.0

[a]Mean of six replicates.

[b]Ticks fed on *Anaplasma*-infected calf.

[c]Ticks fed on *Anaplasma* CF-negative calf.

[d]One ml of homogenate with 25 ul CF antigen added before diluting.

had greater fluorescence than the infected homogenates at the 1:4 dilution.

When the StiQs were spotted with tick gut homogenate, reacted with bovine anti-*Anaplasma* serum, and then reacted with FITC-conjugated anti-bovine IgG, FS values were very high in the seeded homogenate at all dilutions (Table III). The 1:2 and 1:4 dilutions of the infected homogenate had higher FS values than did homogenates from the noninfected ticks examined at those dilutions. The FS values at the 1:2 dilution in the naturally infected ticks were similar to the FS values in the 1:32 dilution of the tick gut seeded with the CF antigen.

Results from a similar experiment, in which homogenates on StiQs were reacted with anti-*Anaplasma* serum of rabbit origin, are shown in Table IV. Again, apparent differences in antibody binding were noted between the infected and negative control tick gut homogenates. FS values for the positive control homogenate at the 1:8 dilution were similar to those for the homogenates from naturally infected ticks at a 1:2 dilution.

The FS values for batches of 50 ticks which were fed either on calves infected with *Anaplasma* (five groups) or on noninfected calves (two groups) are compared in Table V. In all instances, higher FIAX values were detected in the ticks fed on infected calves than in those fed on *Anaplasma*-negative animals.

TABLE III

FIAX VALUES[a] FROM StiQS SPOTTED WITH TICK GUT HOMOGENATES AND REACTED WITH BOVINE ANTI-*ANAPLASMA* SERUM AND FITC-CONJUGATED ANTIBOVINE IgG

	Tick Gut Homogenates		
Serum Dilutions	Infected[b]	Noninfected[c]	Seeded with CF Antigen[d]
1:2	73.0	39.5	125.0
1:4	44.0	38.0	146.0
1:8	33.5	20.5	146.0
1:16	20.0	9.0	108.0
1:32	8.0	3.5	85.0

[a]Mean of six replicates.

[b]Ticks fed on *Anaplasma*-infected calf.

[c]Ticks fed on *Anaplasma* CF-negative calf.

[d]One ml of homogenate with 25 ul CF antigen added before diluting.

TABLE IV

FIAX VALUES[a] FROM StiQS SPOTTED WITH TICK GUT HOMOGENATES SEEDED WITH *ANAPLASMA* ANTIGEN AND REACTED WITH RABBIT ANTI-*ANAPLASMA* SERUM AND FITC-CONJUGATED ANTIRABBIT IgG

	Tick Gut Homogenates		
Serum Dilutions	Infected[b]	Noninfected[c]	Seeded with CF Antigen[d]
1:2	58.5	37.5	120.0
1:4	22.0	10.0	99.0
1:8	8.0	5.5	52.5
1:16	1.0	3.0	25.5
1:32	1.0	1.0	10.0

[a]Mean of six replicates.

[b]Ticks fed on *Anaplasma*-infected calf.

[c]Ticks fed on *Anaplasma* CF-negative calf.

[d]One ml of homogenate with 25 ul CF antigen added before diluting.

TABLE V

FIAX VALUES FROM StiQS SPOTTED WITH TICK GUT HOMOGENATES FED ON INFECTED CALVES AND REACTED WITH RABBIT ANTI-*ANAPLASMA* SERUM AND FITC-CONJUGATED ANTIRABBIT IgG

Tick Homogenates[a]	Mean (N=12)	Range	Standard Error
Infected A[b]	56.2	53–74	4.9
Infected B	50.5	45–60	4.4
Infected C	40.5	36–53	3.7
Infected D	39.4	35–45	3.1
Infected E	89.8	73–108	7.9
Noninfected A[c]	26.7	16–47	3.6
Noninfected B	29.4	23–46	3.0

[a]1:2 dilutions of homogenates.

[b]Ticks fed on different *Anaplasma*-infected calves.

[c]Ticks fed on noninfected CF-negative calves.

CHAPTER V

DISCUSSION

Anaplasma marginale is an important disease-producing organism of cattle worldwide, with several endemic areas in the United States. Studies with electron microscopy have shown the organisms in midgut epithelial cells of ticks (Kocan, Teel, et al, 1980; Kocan, Hsu, et al, 1980). Serologic tests are available for diagnosis of the acute form of bovine anaplasmosis. Although serologic tests are currently available to detect the organisms in ticks, there is no method to quantitate infections. Current research directed toward growth of *A. marginale* in cell culture systems would be aided by a serologic method to quantitate the organisms easily. Fluorometric methods have been used for measuring antibody to *Anaplasma* in cattle (Fox et al, 1982). The sensitivity of these tests and the flexibility of the FIAX serological system indicated that it might be possible to detect and to rapidly quantitate *Anaplasma* organisms in ticks.

Tick gut homogenates were serially diluted to determine the optimal working concentration. A decrease in FS units was measured in all of the homogenates upon dilution, including the negative controls. The background fluorescence observed in homogenates from control ticks probably represents a nonspecific binding of the fluorescein conjugates to components within the tick tissues, including intact bovine IgG and red cell stroma acquired when the ticks fed on calves.

When the tick gut homogenates were reacted with the FITC-conjugated bovine anti-*Anaplasma* serum, the seeded homogenates had higher FIAX values, indicating that the binding of antibody to the CF antigen was discernible. The finding also indicated that some of the background fluorescence might result from bovine IgG in the tick gut contents since the conjugated antisera would be expected to bind to it. Although the differences in FIAX values were not great, the CF antigen was detected when mixed with homogenated tick gut.

Anti-*Anaplasma* antibody was reacted first with the antigen and then with the FITC-conjugated bovine anti-*Anaplasma* serum (Table II). This antibody-antigen-antibody complex did not detect the *Anaplasma* antigen in the homogenates. Whole serum was used on the StiQs without attempting to purify the immunoglobulin specific for the *Anaplasma* organisms. Use of a purified immunoglobulin or of a monoclonal antibody might make this technique more feasible.

Detection of *Anaplasma* was possible by use of an indirect method. Specifically, this was done by first reacting the tick gut homogenates on a StiQ with sera containing antibodies against *Anaplasma* and then with FITC-conjugated anti-immunoglobulin as a measure of bound antibody. In this way, it was possible to detect *Anaplasma*-containing material irrespective of whether the bovine or the rabbit source of immunoglobulin was used (Tables III, IV, and V). The conjugated rabbit immunoglobulin was used to compare antigen content of different batches of ticks (Table V). It was possible to differentiate the homogenates prepared from groups of ticks that had fed on *Anaplasma*-infected calves from those of ticks that fed on negative control animals because of the higher fluorescent values. Unfortunately, there was considerable

variability within the tests as indicated by the high standard errors. For example, FIAX values from duplicate samples of one homogenate run simultaneously (Table V) ranged from 73 to 108. A cause for this unexpected variability could be that the *Anaplasma* antigen was not evenly distributed within the homogenate. This explanation seems unlikely because attempts were made to keep the homogenate well mixed while spotting the StiQs. Nevertheless, a better explanation for the results is not obvious.

The FIAX methods used were satisfactory for detecting the presence of *Anaplasma* in tick gut homogenates. These methods were not sensitive enough, however, to quantitate accurately the amount of antigen in these homogenates.

CHAPTER VI

CONCLUSIONS

A microimmunofluorescence system (FIAX) was employed to detect the presence of *Anaplasma marginale* antigen in groups of naturally infected ticks. The most useful approach was indirect and was done by placing the tick gut homogenate on a cellulose nitrate StiQ sampler and reacting it with an anti-*Anaplasma* serum made in a rabbit. The bound antibody was measured by using an antirabbit immunoglobulin G serum that was fluorescein conjugated. With this method, it was possible to determine the presence or absence of *Anaplasma* antigens in six groups of ticks. Results of attempts to correlate the amount of fluorescence with the absolute amount of antigen in the tick gut homogenates were unsatisfactory. Use of monoclonal antibodies to specific antigenic components of *Anaplasma* might provide the added sensitivity and specificity to facilitate a workable assay using the FIAX system. Producing an antiserum against the tick stages of the organism would also increase the usefulness of the test.

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