# NATURAL AND ARTIFICIAL INFECTION OF *DERMACENTOR ANDERSONI* STILES WITH THREE ISOLATES OF *ANAPLASMA MARGINALE* THEILER

By

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NATURAL AND ARTIFICIAL INFECTION OF DERMACENTOR ANDERSONI STILES WITH THREE ISOLATES OF ANAPLASMA MARGINALE THEILER

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

# INTRODUCTION

## Bovine Anaplasmosis

*Anaplasma marginale* Theiler is an intraerythrocytic parasite that infects many species of wild and domestic ruminants including cattle, water buffalo, deer, antelope, sheep and camels. The organism is currently classified in the Family Anaplasmataceae, Order Rickettsiales (1). In stained blood smears, *A. marginale* appears as a round inclusion body near the periphery of erythrocytes. A separate species, *A. caudatum*, was established more recently to distinguish the causative agent of clinically indistinguishable infections in which an appendage-like structure is associated with the marginally located inclusion. Tail-like inclusion appendages were seen attached to the inclusion membrane in several isolates when hemolyzed erythrocytes were stained (2-21). It has been posited that the organism enters erythrocytes by pinocytosis, forming an inclusion vacuole in which the organism divides by binary fission. Because there has been some controversy over the classification of the anaplasmal organism, all isolates employed in studies reported in this thesis are designated *A. marginale*.

In cattle, infection of erythrocytes results in the disease anaplasmosis, which is characterized by progressive anemia, weight loss, dehydration and sometimes abortion or temporary sterility. The

mortality rate in adult cattle is 50-60%, but young calves usually have mild symptoms or inapparent disease. Cattle that recover from anaplasmosis are resistant to further clinical episodes because of premunity; low levels of the organism persist in a carrier state which keeps the immune system stimulated. Anaplasmosis occurs throughout the world, including Canada, Southeast Asia, Latin America, Africa, and the United States. In the United States, the disease is endemic in Pacific Northwest, inter-mountain states, the Mississippi Valley, the Gulf Coast and the Atlantic Coast south of New Jersey (22, 23). It has been ranked by some as second in importance of diseases that affect the cattle industry in the United States and causes economic losses estimated at $100,000,000 yearly (22).

## *Anaplasma marginale* in Ticks

The anaplasmal organism can be transmitted from one vertebrate to another by ticks, biting flies or blood-contaminated surgical equipment (23, 24). To date, ixodid ticks are the only organisms proved to be biological vectors of *A. marginale* (23, 25, 26). Several species of ticks have been shown to transmit this parasite transstadially; transovarial transmission has been reported but not confirmed (25, 27-31). Colonies of *A. marginale* were described in midgut epithelial cells of the nymphal and adult *Dermacentor andersoni* Stiles that were exposed as nymphs to the parasite (32-40). The identity of the organisms within the colonies was confirmed with fluorescent antibody (FA) (32) and peroxidase-antiperoxidase (PAP) (33) techniques and electron microscopy (37,40). Ticks were proved to become infected from feeding on calves with parasitemias as well as on carrier animals

with inapparent infections (31).

Light and electron microscope studies of gut tissue taken from nymphal and adult ticks revealed several morphologically distinct colony types that appeared to represent a developmental sequence (39, 40). Three types of colonies were found in replete nymphal ticks that had fed on an infected calf and these colonies were most often found along the basal portion of the gut epithelial cells near the basement membrane. Colonies observed in replete nymphs were classified as nymphal type 1 (Ny1), transitional (TsN) and nymphal type 2 (Ny2) colonies. Colonies first appeared in nymphs 5 days post repletion and were classified as Ny1 colonies. These colonies were usually round and contained large separate organisms that were uniform in size and stained densely. Electron microscopy revealed that these organisms were reticulated, surrounded by a double membrane and moderately electron-dense. No background matrix was visible in Ny1 colonies and the organisms appeared to divide by binary fission. Transitional colonies were seen at 10 and 15 days post repletion and were round, oval, or irregular in shape. These colonies contained organisms that varied in size and staining density. At 10 days post repletion, colonies contained reticulated forms of A. marginale that were not as large as organisms within Ny1 colonies. At 15 days post repletion, reticulated organisms were observed that contained small particles within the cell membrane. These colonies appeared to have a dense matrix. Nymphal type 2 colonies contained rod-shaped reticulated organisms and had an electron dense matrix. No electron-dense forms of the anaplasmal organism were found in replete nymphal ticks.

The tail-like inclusion appendage was also found in the gut of

nymphal ticks at 5 and 10 days post repletion (41). The appendage was similar to that described in bovine erythrocytes infected with some isolates of A. marginale. The appendage observed in the lumen of the gut was associated with clusters of vesicular particles or with degenerating initial bodies. Frequently, appendages were seen attached to the luminal surface of midgut epithelial cell membranes by an electron-dense attachment complex. Small particles were often seen immediately across the cell membrane from the attachment complex-portion of the appendage. These particles appeared to be arising from the appendage. This suggests that the appendage may have been involved in the introduction of the organism into the midgut epithelial cells of the tick.

In the adult ticks, anaplasmal colonies were also observed in the basal portion of the gut epithelial cells near the basement membrane but occurred in 5 different morphologic types (36,40). Colony types 1 and 2 contained small electron dense forms of the anaplasmal organism. Type 3 colonies contained electron-dense forms, early reticulated forms and small particles that were often found outside the parasite's limiting membrane. Type 4 colonies contained many reticulated forms that often had small particles within the cell membranes. Type 5 colonies had fewer well-formed reticulated organisms than did type 4, and they often contained large pleomorphic reticulated forms. Incubated (2.5 days at 37°C) unfed adult ticks, that were naturally infected as nymphs, were found to contain increased numbers of colonies (38, 39) compared with unincubated controls.

Hemolymph has been suggested as a possible site of further A. marginale development. Hemolymph collected from infected ticks

produced anaplasmosis when injected into cattle (40). The presence of anaplasmal bodies in hemolymph was confirmed with fluorescent antibody studies.

Salivary glands have also been mentioned as possible developmental sites of the anaplasmal organism, but conclusive evidence of such a role has not been reported. Salivary glands have been proved to be the main route of transmission of several disease-causing agents in ixodid ticks, such as Theileria parva, Babesia canis and Babesia ovis (43-46).

## Artificial Infection of Ticks

Several methods have been developed to infect ticks artificially with infective organisms and have included simulation of the natural feeding processes of ticks or direct inoculation of organisms into the body cavity of the tick. Ticks have been infected with Theileria parva by feeding on rabbits that were inoculated with parasitemic blood during the final phase of tick feeding (47-49) and on isolated rabbit heads which were perfused with parasitemic bovine blood (50). A more widely documented method involved feeding ticks on a skin or membrane stretched over a container of blood, serum, or plasma (51-57). It has also been possible to feed ticks a parasitemic bloodmeal by placing a capillary tube directly over the hypostome and chelicerae of immobilized ticks (58-62). Placement of parasitic organisms directly into ticks has been accomplished by percutaneous inoculation (62-67) in which whole or lysed erythrocytes that contained the parasitic organisms were injected through the cuticle of the tick. This method has been used to infect D. andersoni with A. marginale (68).

## Anaplasma marginale Isolates

Researchers in several geographic locations have reported Anaplasma marginale isolates with slightly different morphologic and antigenic properties (14-16, 21, 69-77). The various isolates included: 1) eight Mexican isolates (Michoacan 1, Michoacan 2, Michoacan 3, Tuxpan, Cuernavaca, Tulyehaulco, Ixtacalco, and Valle de Bravo) (14), 2) Venezuela (21), 3) Oregon (15, 16, 69-71), 4) Virginia (69), 5) Florida (15, 69-71), 6) California (12), 7) Illinois (72), 8) North Texas (69), 9) Colombia (69), 10) Pawhuska (20), 11) two Washington isolates (Washington - O, Washington - C) (73, 74), 12) South Idaho (75), 13) Louisiana (76), 14) Wyoming (72) and 15) Gulf Coast (72). All of the isolates except the Florida, Wyoming and Gulf Coast have been reported to have a tail-like inclusion appendage attached to the marginal bodies. The percentage of inclusion bodies with the appendage was found to vary with the isolate (14-16, 21, 69, 75).

Immunologic differences have been reported between the Virginia and Florida isolate (69), the Colombia and Oregon isolate (69), and the Florida and Oregon isolate (71). Monoclonal antibodies demonstrated common antigens, and at the same time, several antigenic differences between the Virginia, Florida, Washington - O, Washington - C, North Texas, and South Idaho isolates (75). It has been suggested that the Virginia and Florida isolates were similar enough immunologically to provide cross protection in cattle. However, when animals were rendered immunoincompetant they lost the cross protection. The commercially[1] produced anaplasmal vaccine provided an inadequate level of protection to field challenge from the Colombia isolate (69). It was also

demonstrated that calves that were carriers of the Florida isolate were susceptible to a challenge with the Oregon isolate, but calves that were carriers of the Oregon isolate were resistant to challenge with the Florida isolate (71). In conducting complement-fixation tests a greater reactivity of antibody has been observed when homologous antigens are used than when a heterologous antigen was employed (74).

## The Research Problem

With increased reports of different isolates of A. marginale there is a need to characterize them on the basis of infectivity for cattle and ticks, cross immunity, and severity of disease. The purpose of this study was to compare infectivity of three isolates of Anaplasma marginale [Virginia (VAM), Illinois (IAM) and Florida (FAM)] in known vector, ixodid ticks. Ticks were exposed to each isolate by natural infection through feeding of nymphs on a calf with anaplasmosis. Nymphal ticks were also exposed to each isolate artificially by percutaneous inoculation with bovine erythrocytes infected with A. marginale. Infection of ticks was tested by feeding exposed ticks on susceptible calves to test for transmission. In addition, gut was examined with light and electron microscopy for the presence of colonies in sections and salivary gland tissues from ticks exposed to each of the isolates were examined with fluorescent-labeled antibody technique and methyl green-pyronin stain as a further attempt to confirm infection with A. marginale.

## ENDNOTES

[1] Anaplaz vaccine, Fort Dodge Laboratories, Fort Dodge, Iowa.

# CHAPTER II

# MATERIALS AND METHODS

## General

### *Anaplasma marginale* Isolates

Three isolates of *Anaplasma marginale* were compared in this study: 1) the Virginia isolate (VAM), 2) the Illinois isolate (IAM) and 3) the Florida isolate (FAM). The VAM was obtained from Dr. K. M. Kocan at Oklahoma State University, who obtained it originally from the USDA Animal Parasitology Institute at Beltsville, Maryland. The IAM was obtained from Dr. Ron Smith at the University of Illinois. The FAM was obtained from Dr. Ken Kuttler at the USDA Hemoparasitic Disease Research Unit at Washington State University with the permission of Dr. Miodrag Ristic. Blood from cows infected with each isolate was inoculated intravenously (IV) into each of three complement-fixation (CF) negative calves. The studies were done in two trials for each isolate (six calves total).

### Experimental Calves

Twenty-four Holstein bull calves (2-3 months of age) that were negative to the anaplasmosis complement-fixation (CF) test were splenectomized for use in these studies. Six calves were used as controls on which uninfected *Dermacentor andersoni* nymphs were

allowed to feed. Two calves (six total) were injected IV with infected blood for each of the isolates (VAM, IAM, and FAM) and were used as donor calves to infect D. andersoni nymphs. These calves were monitored daily for the presence of A. marginale by Wright-stained blood smears and packed cell volume (PCV). When marginal bodies were detected in blood smears, the donor calves were used to infect large numbers of D. andersoni nymphs either by natural feeding or by artificial inoculation procedures. The remaining 12 calves were used for feeding the subsequently molted adult ticks to test for transmission. If transmission did not occur within 90 days after the ticks attached, the calves were challenged with infected blood stabilate.

Laboratory Propogation of Ticks

Dermacentor andersoni nymphs were reared at the Oklahoma State University, Medical Entomology Laboratory which is under the direction of Dr. Jakie Hair (78). Larvae were fed on rabbits and allowed to molt to the nymphal stage. Ticks were placed in a humidity chamber (90% to 98% relative humidity) at 25°C with a fourteen-hour photophase period until used for transmission studies. At 1 month post molting from the larval to nymphal stage the ticks were infected with the three isolates (VAM, IAM and FAM) by either natural feeding or by percutaneous inoculation.

Exposure of Nymphs by Natural Feeding

When each donor calf's parasitemia reached approximately 3%, uninfected D. andersoni nymphs were placed in an orthopedic

stockinette attached to the infected calf and allowed to feed until replete. After the nymphs became replete, they were placed in a humidity chamber and allowed to molt to the adult stage.

Exposure of Nymphs by Artificial Feeding

When each donor calf, infected with one of the three isolates (VAM, IAM and FAM) by IV inoculation of blood stabilate, reached a parasitemia of approximately 1%, uninfected (control) D. andersoni nymphs were placed into orthopedic stockinettes on an anaplasmosis complement-fixation negative calf and allowed to feed until replete. When replete, the nymphs were injected percutaneously with infected erythrocytes from the donor calves. Heparinized blood (50 ml) containing each of the isolates (VAM, IAM and FAM) was drawn from infected calves. In all three cases the erythrocytes were sedimented with a centrifuge at 2000 rpms for twenty minutes and washed with RPMI medium 1640, containing 25 mM HEPES buffer and L-glutamine[1]. The replete nymphs were placed dorsal side down on double-sided tape. The concentrated erythrocytes were inoculated through the cuticle using a 30 gauge needle attached to a tuberculin syringe. The erythrocytes were injected into the body cavity through the ventral posterior area of the cuticle of the newly replete control nymphs until the body cavity was fully distended. The inoculated nymphs were then carefully removed from the tape and immediately placed into a humidity chamber where they were allowed to molt to the adult stage.

## Tissue Collection for Light and Electron Microscopy

Twenty pairs of naturally infected and twenty pairs of artificially inoculated unfed adult ticks from each isolate (VAM, IAM and FAM) were placed in an incubator for 2.5 days at 37°C (120 pairs total). Gut tissues were dissected from all of these ticks. Salivary glands were dissected from ten pairs of naturally infected and ten pairs of artificially inoculated adult ticks exposed to each isolate (VAM, IAM and FAM) that fed on a sheep for 8 days (120 pairs total). A sheep was used so that the ticks would go through the natural feeding process on a vertebrate host but without any risk of exposure to A. marginale. The tissues were fixed in 2% glutaraldehyde for 6-8 hours, post fixed in 2% osmium tetroxide overnight, alcohol dehydrated, embedded in resin[2] using propylene oxide as a transitional fluid, and thick sectioned with an ultramicrotome.[3] The 1μm sections were stained with Mallory's blue stain (79) and examined with a light microscope for the presence of A. marginale colonies. Thin sections were prepared using an ultramicrotome, stained with uranyl acetate and lead citrate (80) and examined with the electron microscope[4] to confirm presence of Anaplasma colonies.

## Determination of Colony Density

Colony density (No. of colonies/.1 $mm^2$ gut tissue examined) was determined for gut samples from ticks exposed to each isolate. The gut cross section area was measured with a calibrated ocular grid.

Collection of Salivary Glands for Light Microscopy

After the ticks were allowed to feed on a sheep for eight days salivary glands were dissected from naturally infected and from artificially inoculated adult ticks exposed to each isolate (VAM, IAM and FAM). Salivary glands from ticks (20 pairs per isolate, total of 120 pairs) were teased out onto 3x1 inch microscope slides and allowed to dry. Slides were stained with either fluorescent antibody or methyl green-pyronin technique.

Fluorescent Antibody Technique

Salivary glands placed on slides were fixed in acetone for ten minutes, air dried, and stored in a low temperature freezer at -74°C until stained. For staining, slides were allowed to reach room temperature, then fluorescein-labeled antibody was placed onto the slides which were then placed in an incubator at 37°C for 90 minutes. Slides were rinsed twice in phosphate buffer for a total of four minutes, allowed to air dry and examined with a light microscope for the prescence of FA staining. Fluorescent-labeled antibody technique was applied simultaneously to a slide containing salivary glands of ticks exposed to the anaplasmal isolate, a corresponding slide of non-exposed tick salivary glands as a negative control and an erythrocyte smear infected with A. marginale as a positive control. This was done on ticks exposed to each isolate (VAM, IAM and FAM) by both methods of exposure (natural feeding and percutaneous inoculation). The positive fluorescence was graded on a scale of +/- to ++++. A +/- represented fluorescence of an unusual appearance and ++++ was the brightest positive fluorescence as compared to the controls. A - indicated that

there was no noticeable difference between the exposed salivary gland and the negative controls.

Methyl Green-pyronin Stain Techniques

Salivary gland slides were fixed in Carnoy's fixative (64) for five minutes, rinsed in 70% alcohol for two minutes, placed into distilled water for two minutes, stained in methyl green-pyronin stain (64) for five minutes, and washed with distilled water. The slides were air dried and examined with a light microscope for the presence of A. marginale in stained cells.

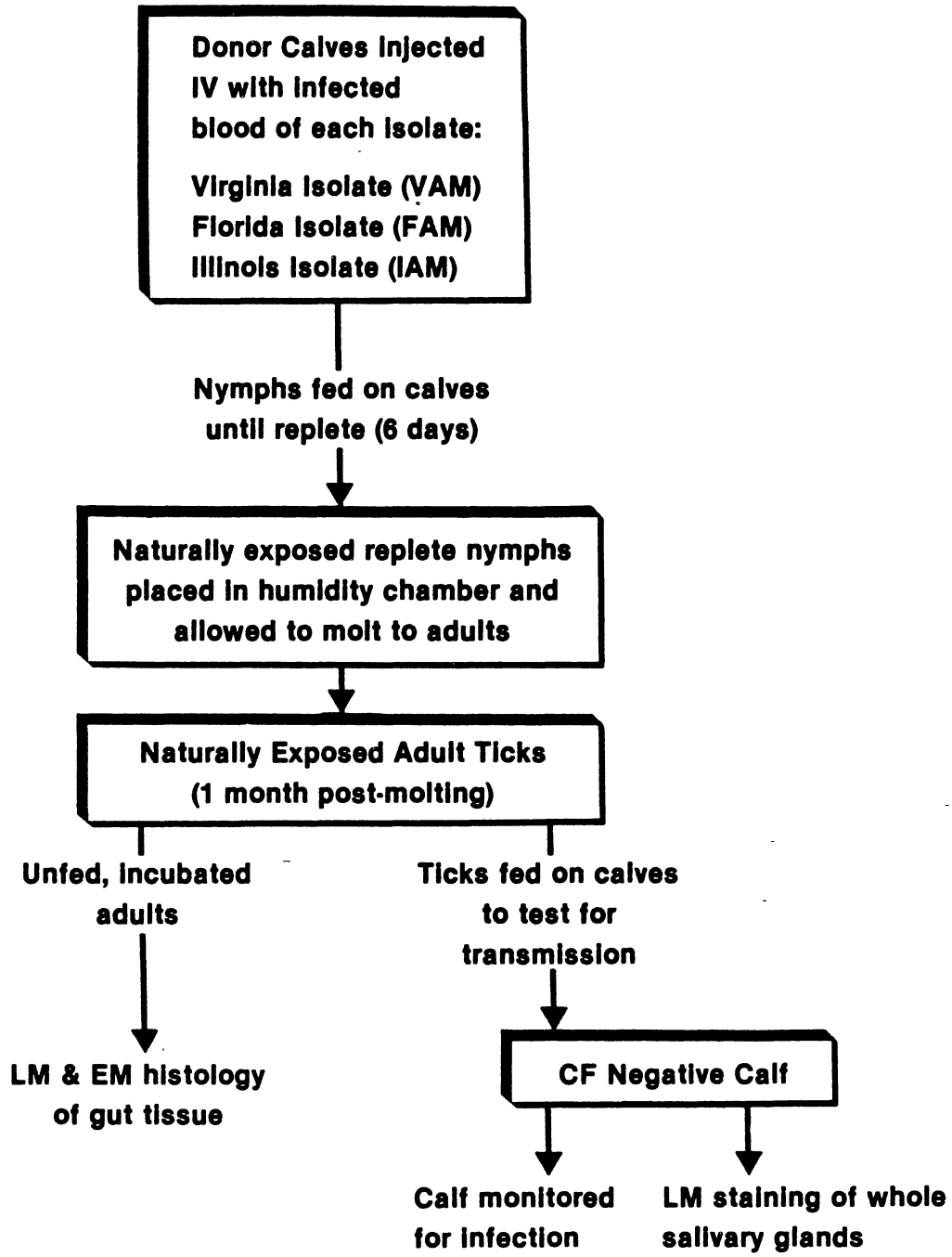
## Experimental Design

Comparison of Isolates by Natural Feeding

Six calves that were found negative by the anaplasmosis complement-fixation test were injected IV with the three isolates of A. marginale, the VAM, FAM and IAM isolates (two calves for each isolate) (Figure 1). The calves were monitored for infection in the aforementioned ways. When the calves reached a 3-5% parasitemia, approximately 1000 D. andersoni nymphs were placed in orthopedic stockinettes on each animal. The nymphs were allowed to feed to repletion and placed in a humidity chamber. At one month post-molting, 20 pairs of unfed exposed ticks were incubated for 2.5 days and gut samples were dissected and used for light and electron microscope studies. Twenty-five pairs of exposed ticks (from each of the six groups) were placed into orthopedic stockinettes on each of six calves, found to be negative by the anaplasmosis complement-fixation test, and

Figure 1. Experimental Design for Natural Infection of *Dermacentor andersoni* With Three Isolates of *Anaplasma marginale*.

## EXPERIMENTAL DESIGN FOR NATURAL INFECTION OF *Dermacentor andersoni* WITH THREE ISOLATES OF *Anaplasma marginale*



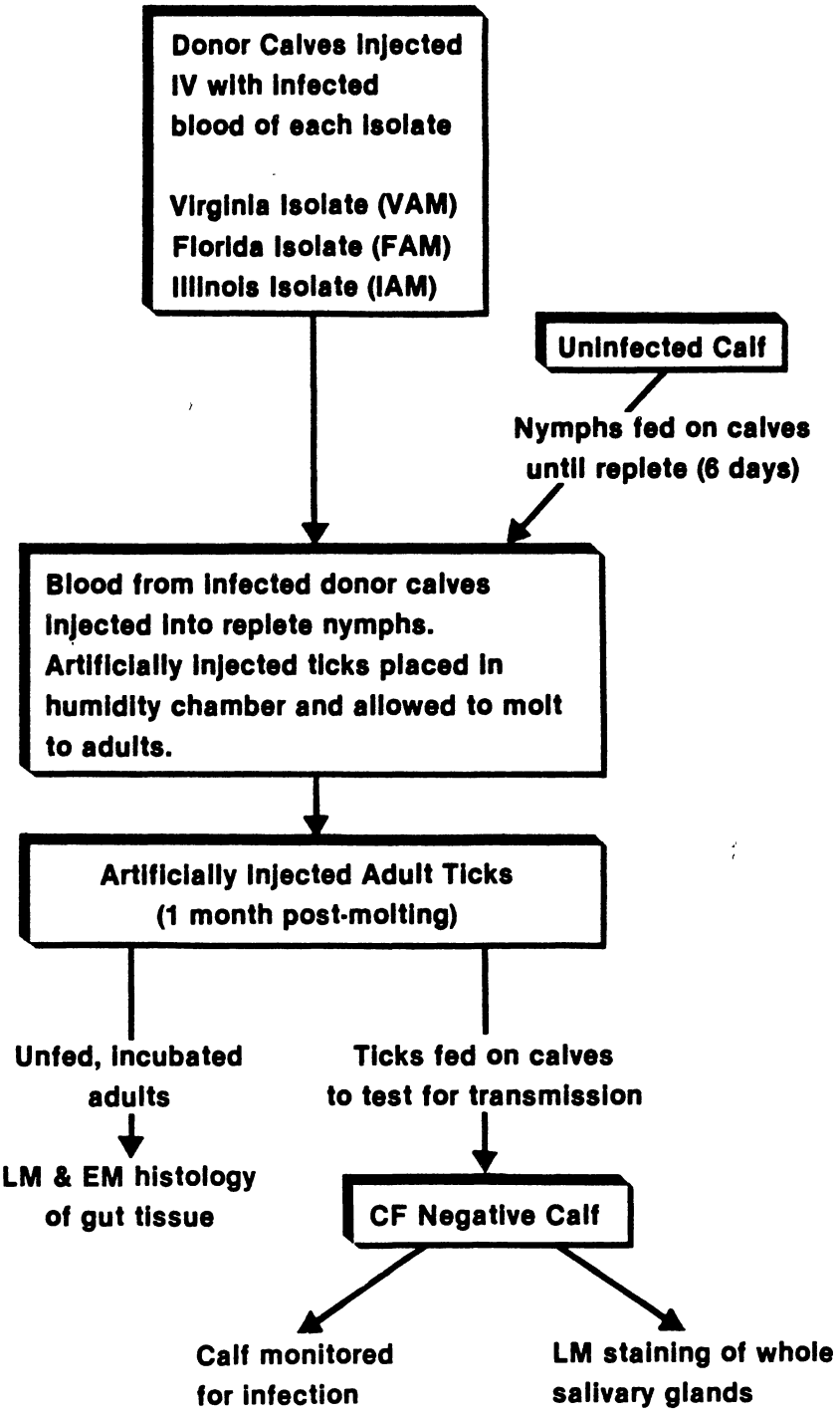
allowed to feed to repletion. These calves were then monitored for infection as described previously. Fifty pairs of ticks were allowed to feed for eight days on a sheep. The salivary glands were dissected from these ticks and used for the following studies: 1) ten pairs of ticks were used for morphologic studies by light and electron microscopy; 2) ten pairs were used for methyl green-pyronin studies; and 3) ten pairs were used for FA studies. The three isolates were compared in ticks in three ways: 1) animal transmission studies; 2) colony density in gut cells of unfed adult ticks; and 3) staining characteristics of the salivary glands on day 8 of feeding of adult ticks that had been exposed to the three isolates.

Comparison of Isolates by Artificial Infection

Uninfected D. andersoni nymphs (approximately 1,000) were placed into orthopedic stockinettes on each of six calves found to be negative by the anaplasmosis CF test (Figure 2). As the ticks became replete they were injected percutaneously (two trials of each of the three isolates VAM, IAM and FAM). At one month post molting, 20 pairs of each group were incubated for 2.5 days and gut samples were dissected for light and electron microscopy. Twenty-five pairs of each group were allowed to feed on calves, known to be negative by the anaplasmosis CF test, for nine days. These calves were then monitored for infection as stated previously. Fifty pairs were fed on a sheep for eight days and the salivary glands dissected for morphologic studies by light and electron microscopy, methyl green-pyronin staining and FA staining.

Figure 2. Experimental Design for Artificial Infection of *Dermacentor andersoni* With Three Isolates of *Anaplasma marginale*.

## EXPERIMENTAL DESIGN FOR ARTIFICIAL INFECTION OF *Dermacentor andersoni* WITH THREE ISOLATES OF *Anaplasma marginale*



## ENDNOTES

[1] Gibco Inc., Grand Island, NY.

[2] Dow Epoxy Resin (736), Polysciences Inc., Pauls Valley Industrial Park, Warrington, PA.

[3] Sorvall MT 5000, DuPont Instruments, Wilmington, Del.

[4] Philips EM-200, Philips Electronic Instruments, Mount Vernon, NY.

# CHAPTER III

# RESULTS

## Animal Transmission Studies

### Natural Exposure

Anaplasmosis developed in only those calves fed on by ticks that were naturally exposed as nymphs by feeding on a calf that had the VAM. The prepatent period of the disease was an average of 37 days and the average peak parisitemia was 31.5% (Table I). Calves fed on by ticks naturally exposed to the IAM and FAM did not develop anaplasmosis and were found to be susceptible to A. marginale upon challenge.

### Artificial Exposure

One of two calves fed on by ticks artificially exposed to VAM by percutaneous inoculation developed anaplasmosis. The prepatent period for the disease was 42 days post attachment. The calf had a peak parasitemia of 23.0% (Table I). The remaining calves fed on by artificially exposed ticks (2-IAM and 2-FAM) did not develop anaplasmosis and proved to be susceptible to A. marginale upon challenge.

TABLE I

TRANSMISSION OF *ANAPLASMA MARGINALE* BY ADULT *DERMACENTOR ANDERSONI* THAT WERE EXPOSED TO THREE ISOLATES AS NYMPHS EITHER BY FEEDING OR BY PERCUTANEOUS INOCULATION

a)EXPOSURE BY FEEDING

| Isolate | Calf No. | Prepatent Period[a] Day | Average | Peak Parasitemia % | Average | Donor No. | Calf[b] % Parasitemia |
|---|---|---|---|---|---|---|---|
| VAM | B-159 | 38 | 37 | 21.2 | 31.6 | B-143 | 41.1 |
| | B-162 | 36 | | 42.0 | | B-139 | 65.1 |
| IAM | B-100[c] | -- | -- | ---- | ---- | B-60 | 56.2 |
| | B-140[c] | -- | | ---- | | B-148 | 41.9 |
| FAM | B-107[c] | -- | -- | ---- | ---- | B-65 | 30.6 |
| | B-138[c] | -- | | ---- | | B-165 | 27.6 |

b)EXPOSURE BY PERCUTANEOUS INOCULATION

| Isolate | Calf No. | Prepatent Period[a] Day | Average | Peak Parasitemia % | Average | Donor No. | Calf[b] % Parasitemia |
|---|---|---|---|---|---|---|---|
| VAM | B-153 | -- | 42 | ---- | 23.0 | B-143 | 41.1 |
| | B-167 | 42 | | 23.0 | | B-139 | 56.1 |
| IAM | B-104[c] | -- | -- | ---- | ---- | B-60 | 56.2 |
| | B-136[c] | -- | | ---- | | B-148 | 41.9 |
| FAM | B-115[c] | -- | -- | ---- | ---- | B-65 | 28.0 |
| | B-133[c] | -- | | ---- | | B-165 | 23.8 |

[a] Prepatent period determined from day 1 of feeding to the appearance of a 1% parasitemia in the peripheral blood smear.

[b] Donor calf was used to infect nymphs with *A. marginale* isolates to use in transmission study.

[c] Calf was subsequently challenged and found to be susceptible to *A. marginale*.

## Colony Density Studies

### Natural Exposure

Colonies (Figures 3 and 4) were observed in thick sections of gut tissue of D. andersoni naturally exposed to the VAM. There was an average density of 1.7 for males and 1.4 for females (Table II). Colonies were not seen in gut samples from ticks naturally exposed to either the IAM or FAM isolates.

### Artificial Exposure

No colonies were seen in the gut samples from ticks artificially exposed to any of the three isolates (VAM, IAM and FAM).

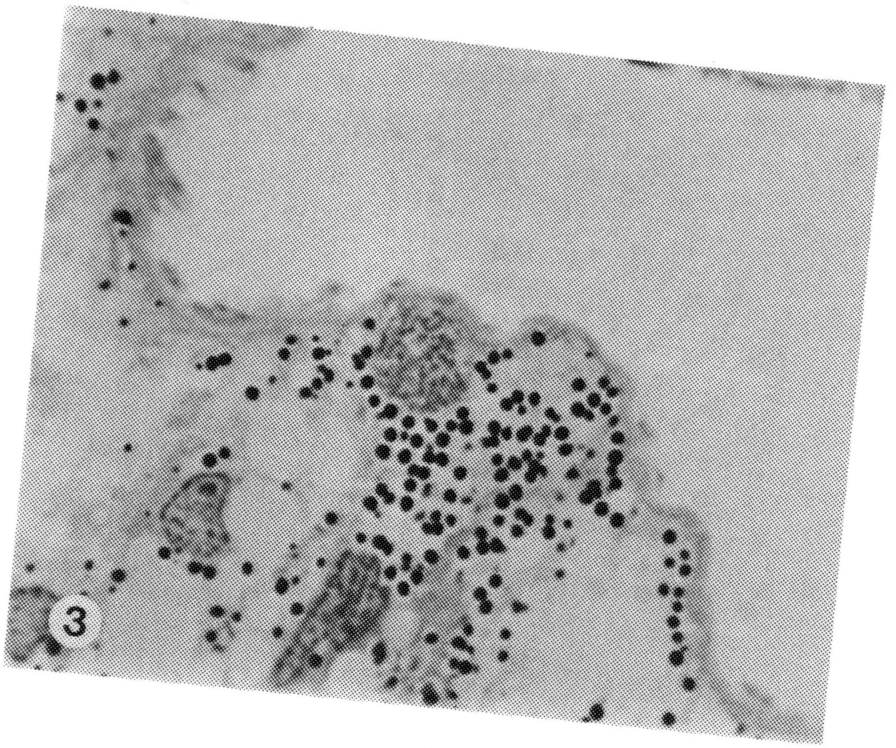
## Fluorescent-labeled Antibody (FA) Studies

### Natural Exposure

The salivary glands of female and male ticks naturally exposed to the VAM consistently had low numbers of a focal fluorescence within acini (Figure 5). The female salivary glands consistently had a +++ fluorescence and males consistently had a ++ (Table III). Salivary glands of male ticks naturally exposed to the IAM and FAM isolates had a grainy fluorescence that appeared to cover the entire acini, except for the nuclei. This fluorescence was seen in acini scattered throughout the salivary gland. Also, these acini were swollen in comparison to the non fluorescing acini on the same slide and the controls. This unusual pattern of fluorescence was assigned a +/- rating. The salivary glands from female ticks naturally exposed to the IAM and FAM isolates had no

Figure 3. Light Photomicrograph of an Anaplasma marginale Colony in Tick Gut Epithelial Cells.

Figure 4. Electron Photomicrgraph of an Anaplasma marginale Colony in Tick Gut Epithelial Cells.


3

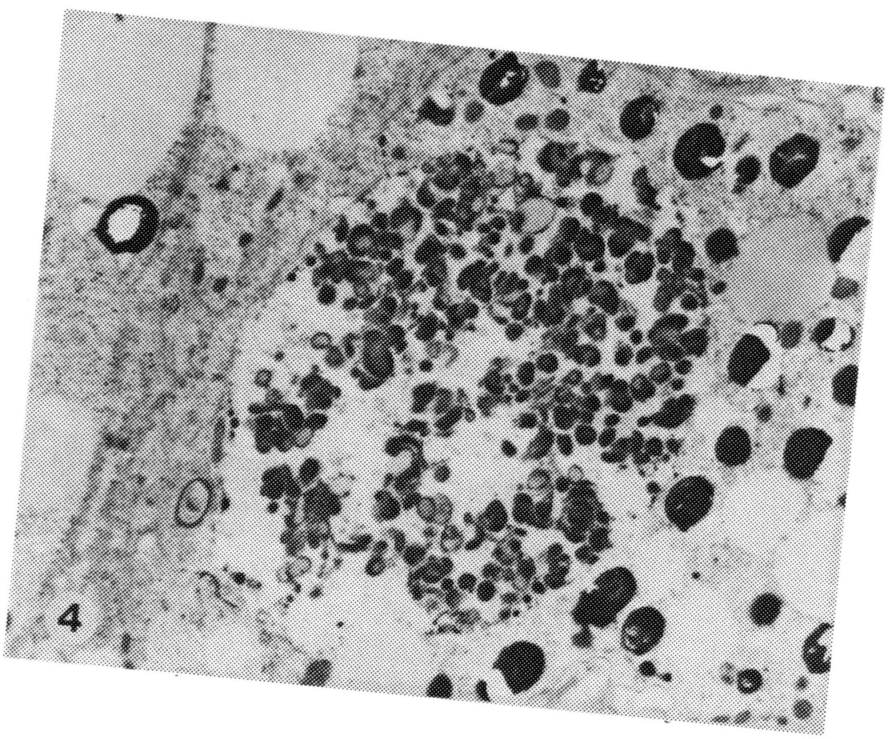

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TABLE II

DENSITY OF COLONIES IN GUT TISSUE OF ADULT MALE AND FEMALE *D. ANDERSONI* THAT WERE EXPOSED TO THREE ISOLATES OF *A. MARGINALE* AS NYMPHS EITHER BY FEEDING OR BY PERCUTANEOUS INOCULATION

a)EXPOSURE BY FEEDING

| Isolate | Sex of Ticks | Colony Density[a] Trial 1 | Trial 2 | Average |
|---|---|---|---|---|
| VAM | Male | 1.7 | 1.8 | 1.75 |
| | Female | 1.1 | 1.7 | 1.40 |
| IAM | Male | 0 | 0 | 0 |
| | Female | 0 | 0 | 0 |
| FAM | Male | 0 | 0 | 0 |
| | Female | 0 | 0 | 0 |

b)EXPOSURE BY PERCUTANEOUS INOCULATION

| Isolate | Sex of Ticks | Colony Density[a] Trial 1 | Trial 2 | Average |
|---|---|---|---|---|
| VAM | Male | 0 | 0 | 0 |
| | Female | 0 | 0 | 0 |
| IAM | Male | 0 | 0 | 0 |
| | Female | 0 | 0 | 0 |
| FAM | Male | 0 | 0 | 0 |
| | Female | 0 | 0 | 0 |

[a]No. colonies per .1 $mm^2$ gut tissue examined.

Figure 5. Light Photomicrgraphs of Fluorescence of Anaplasma marginale in Tick Salivary Glands Stained With Fluorescent-labeled Antibody.

a) Positive result of a female tick exposed naturally to the Virginia isolate (+++).
b) Corresponding negative control.
c) Positive result of a male tick exposed naturally to the Virginia isolate (++).
d) Corresponding negative control.
e) Characteristic fluorescence of male ticks exposed to the Illinois and Florida isolates naturally and artificially (+/-).
f) Corresponding negative control.

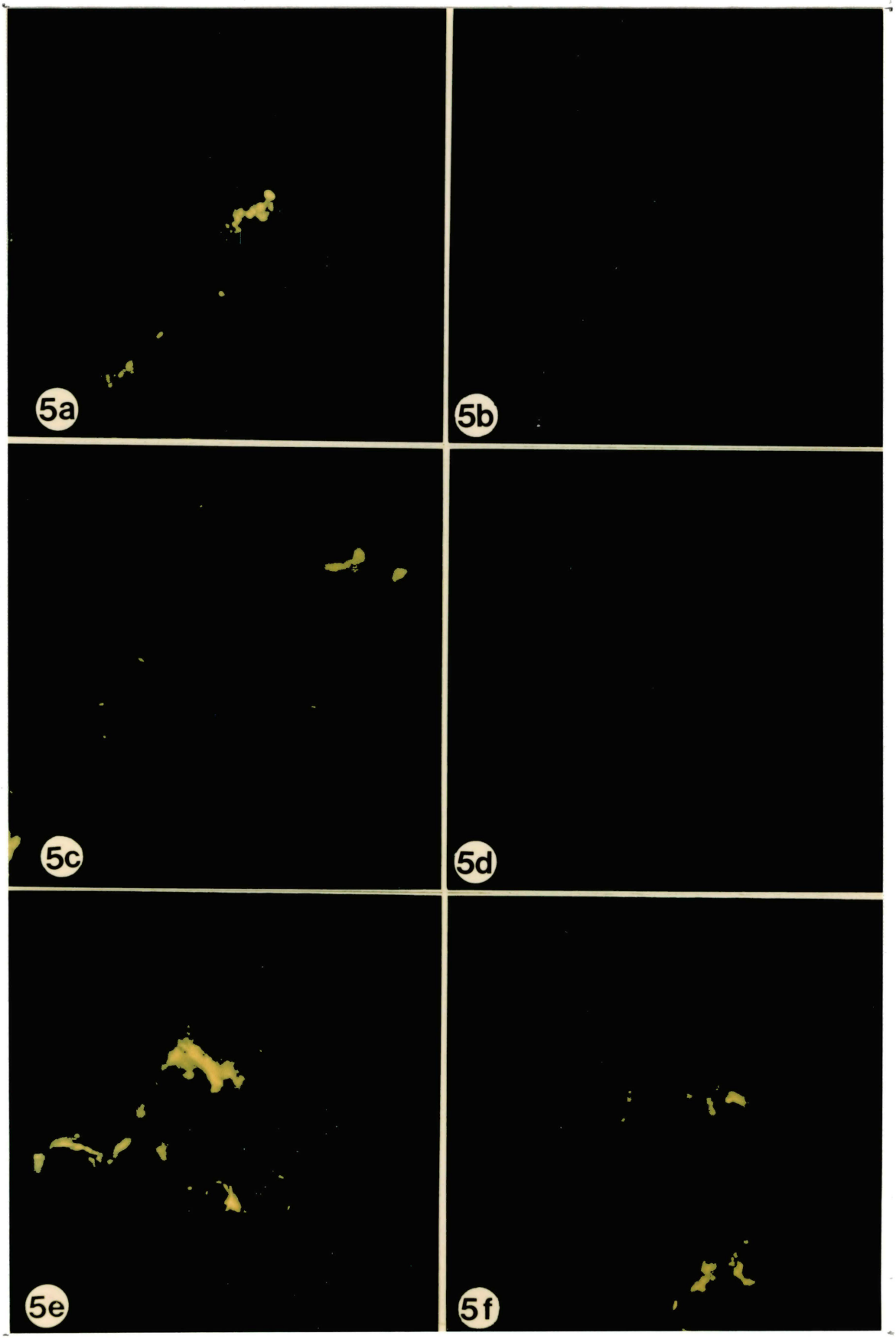

5a
5b
5c
5d
5e
5f

TABLE III

DIRECT FLUORESCENT ANTIBODY STAINING[a] OF SALIVARY GLANDS FROM ADULT MALE AND FEMALE D. ANDERSONI THAT WERE EXPOSED TO THREE ISOLATES OF A. MARGINALE AS NYMPHS EITHER BY FEEDING OR BY PERCUTANEOUS INOCULATION

a)EXPOSURE BY FEEDING

| Isolate | Sex of Ticks | Fluorescence Trial 1 | Trial 2 |
|---|---|---|---|
| VAM | Male | ++ | ++ |
| | Female | +++ | +++ |
| IAM | Male | +/- | +/- |
| | Female | - | - |
| FAM | Male | +/- | +/- |
| | Female | - | - |

b)EXPOSURE BY PERCUTANEOUS INOCULATION

| Isolate | Sex of Ticks | Fluorescence Trial 1 | Trial 2 |
|---|---|---|---|
| VAM | Male | -- | -- |
| | Female | -- | -- |
| IAM | Male | +/- | +/- |
| | Female | -- | -- |
| FAM | Male | +/- | +/- |
| | Female | -- | -- |

[a]FA staining of tissues graded from 1+ to 4+.

more fluorescence than the negative controls.

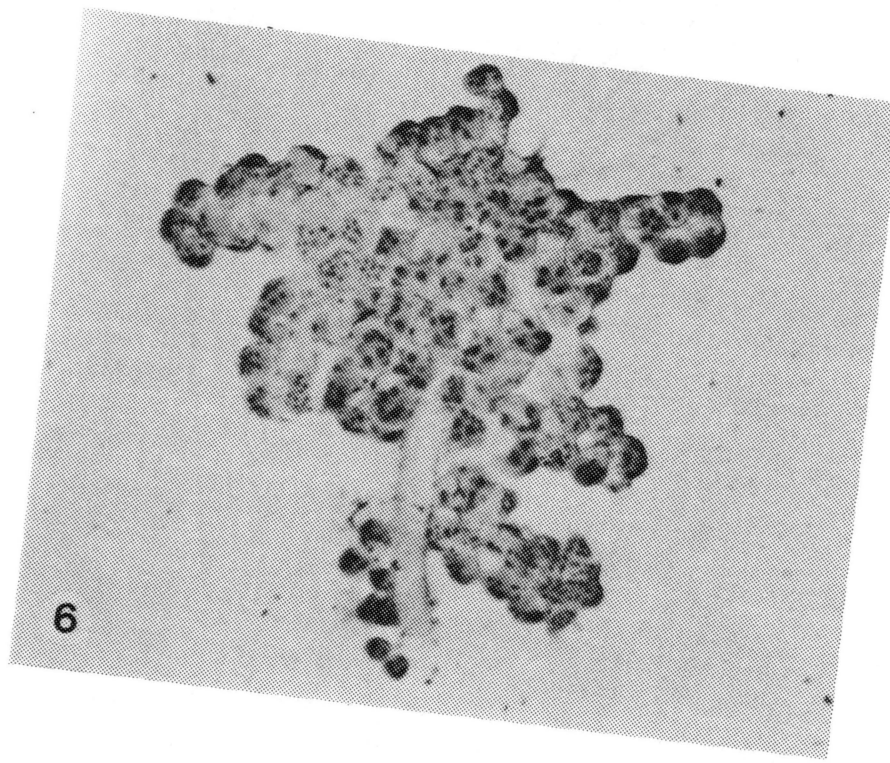
Artificial Exposure

Salivary glands from male and female D. andersoni that were artificially exposed to the VAM isolate had no fluorescence different from the associated negative controls. Salivary glands of male ticks that were artificially exposed to the IAM and FAM isolates had a grainy fluorescence similar to that of glands from ticks naturally exposed to these same isolates. Salivary glands from female ticks exposed artificially to these isolates had no noticeable fluorescence when compared to the negative controls.

## Methyl Green-pyronin Studies

No differences were apparent in methyl green-pyronin-stained salivary glands collected from ticks (naturally or artificially exposed to the three isolates of A. marginale) when compared with controls (Figure 6).

Figure 6. Light Photomicrograph of a Tick Salivary Gland Stained With Methyl Green-pyronin.


6

# CHAPTER IV

## DISCUSSION

Many isolates of Anaplasma marginale have been reported from various geographic areas of the United States (14-16, 21, 69-77). Isolates were found to differ in their morphology within bovine erythrocytes, antigenic determinants and infectivity for ticks (69-77). In the present study, three isolates were chosen for comparative studies in the invertebrate host, ixodid ticks: the Virginia (VAM), Illinois (IAM) and Florida (FAM). These isolates were selected because of their history of experimental use, reported antigenic differences and known morphologic differences of marginal bodies within erythrocytes. A tail-like appendage has been demonstrated in the VAM and IAM isolates but not the FAM (69-72). The appendage has been hypothesized to be important for infection of ticks. Previous attempts to infect ticks with FAM (without the appendage) have been unsuccessful (41, 81).

In the present studies, the only experimental groups from which ticks became infected with A. marginale were those exposed naturally and those exposed artificially to the VAM. IAM and FAM failed to infect ticks by either route of exposure, even though parasitemias of donor calves were high. Failure of ticks to become infected by natural exposure to FAM was not surprising because previous attempts at tick transmission were unsuccessful (41,81). It was hoped that artificial exposure of ticks would bypass any gut barrier that might exist and

allow for development of the organism in other tissues. However, this attempt apparently failed and infection of ticks was not demonstrable. It was surprising that the IAM, which does have an inclusion appendage, was not infective for ticks. Studies done in Illinois with the same isolate and Dermacentor variablis produced similar negative results (72). These data suggest that factors other than the presence of the inclusion appendage may be involved in determining infectivity of A. marginale for ticks.

Infectivity of isolates for ticks may correspond with the usual method of transmission in the geographic locations from which they were obtained. The mechanism of transmission of A. marginale has been thought to vary in geographic regions within the United States. A recent survey in Texas suggested that in some areas of the state, transmission of A. marginale was mechanical by mosquitos or horseflies, but in other areas tick transmission occurred (82). In areas of the western United States anaplasmosis has been shown to be tick transmitted (27, 84-87). It is possible that given isolates are adapted to different modes of transmission. This conjecture is supported by findings in the present study in that the Illinois isolate was recently obtained from the field and it appears incapable of infecting ticks. However, additional studies would be required to determine to what extent various isolates are preferentially adapted to mechanical or to biologocial transmission.

Colony density studies supported the tick transmission studies. Colonies were seen only in ticks that were naturally exposed to the VAM, and not in ticks exposed to IAM and FAM. Even though the artificially exposed VAM ticks transmitted A. marginale, colonies were not seen

in gut cells from these ticks. This indicates that either stages were present in too low numbers to be detected or that development of the organism proceeded in other tick tissues. The prepatent periods of anaplasmosis that developed in calves that were fed on by naturally exposed ticks were not remarkably different from that of those artificially exposed.

Anaplasma marginale has been shown to have a complex developmental cycle in its invertebrate host. Most of this developmental cycle has been documented and occurs in gut cells of nymphal and adult ticks (40). It is believed that the organism undergoes additional development in another tick tissue and is then transmitted to the vertebrate host. The organism was found to be transmitted after 6-7 days of feeding - a feeding time longer than that required for transmission of other tick-borne parasites (83). It is not known which tissue the final stage develops in but the gut is not a likely place because colonies were found to be present in very low numbers or were absent during the time of transmission (68). Salivary glands have been suggested as the most likely location because most tick transmitted organisms pass to the vertebrate host via these glands (43-46). Furthermore, injection of homogenates of salivary gland prepared from infective ticks produced anaplasmosis in susceptible calves (68). Colonies of rickettsial organisms have been found in a few salivary glands of ticks exposed to VAM but these were not demonstrated consistently enough for experimental manipulation to confirm their identity (68).

Another objective of these studies, in addition to comparison of the isolates in ticks, was to confirm infection of salivary glands with

A. marginale using fluorescent antibody (FA) techniques. Fluorescent-labeled antibody studies of salivary glands collected on day eight of feeding confirmed infection with the organism. Small amounts of focal fluorescence in each gland corresponded with the size, location and distribution of A. marginale colonies demonstrated with electron microscopy in salivary glands of previous studies (68). The absence of fluorescence in the salivary glands of IAM- and FAM-exposed ticks supported the conclusion that these ticks did not become infected with these isolates of the parasite. The origin of the grainy fluorescence seen in salivary glands of male ticks exposed naturally and artificially to the IAM and FAM is not known. However, the fluorescence was unusual and different from that seen in the controls and therefore rated +/-.

Methyl green-pyronin did not prove to be a useful stain in demonstrating the presence of A. marginale in infected salivary glands. It is a good diagnostic stain for Theileria parva in salivary glands of ticks (64). Methyl green-pyronin is an RNA stain and it was hoped that it would be able to stain A. marginale as other RNA stains such as acridine orange have been shown to do (15).

During the tick transmission portion of these studies, trace amounts of tetracyclines were suspected to be present in the calf feed because calves concurrently inoculated with blood stabilate for other experiments developed only low parasitemias of A. marginale without attendant clinical disease. Levels of the drug were apparently not high enough to prevent infection but did affect the parasite so that clinical response of these calves was minimal. The feeding procedure was altered at this time to minimize the risk of tetracycline being fed to experimental animals. The calves from the present study that became

infected when fed upon by ticks exposed to the VAM did not appear to be affected by tetracyclines because the prepatent periods and parasitemia levels were within expected ranges. Infections did not develop in susceptible calves when fed upon by ticks exposed to the FAM and IAM. Although infection could have been inhibited by the tetracyclines in the feed, evidence suggests that these ticks simply had not become infected with A. marginale; the organisms were not found in gut tissues with light and electron microscopy or in the salivary glands by FA techniques as was found in ticks exposed to the VAM and examined by similar techniques. The animal transmission studies are being repeated before these data will be published but it was not feasible to repeat them in time for inclusion in this thesis.

# CHAPTER V

## SUMMARY

Anaplasma marginale is a rickettsia-like organism that is an intraerythrocytic parasite of many species of wild and a few domestic ruminants. It produces anaplasmosis which has been ranked by some as second in importance among diseases that affect the cattle industry in the United States. The organism can be transmitted by blood-contaminated surgical equipment, biting flies and several species of ticks. The anaplasmal organism undergoes a complex developmental cycle in ixodid ticks but the cycle is not fully understood. The route of transmission to the vertebrate host is believed to be via the salivary glands, but this conjecture has not been confirmed. Several isolates of A. marginale have been documented from various geographic regions. These isolates appear to differ in their morphology and antigenic composition.

In the present study, three isolates of Anaplasma marginale, the Virginia (VAM), Illinois (IAM) and Florida (FAM), were compared in Dermacentor andersoni. These isolates were selected in part because a tail-like appendage has been demonstrated in the VAM and IAM but not the FAM. It was hypothesized that the appendage is important as a mechanism for infection of ticks. Ticks were exposed as nymphs to the isolates naturally by feeding on an infected animal or artificially by percutaneous inoculation administered immediately after the nymphs had

fed to repletion on a calf found to be negative for anaplasmosis by the complement-fixation test (CF). Infectivity of ticks was tested by animal transmission, colony density studies in sections of gut tissues and by fluorescent-labeled antibody (FA) and methyl green-pyronin staining of whole mount slides of salivary glands. Presence of colonies in gut cells was confirmed with light and electron microscopy.

The only ticks that became infected with A. marginale in this study were in experimental groups that were exposed naturally and artificially to the VAM. The Illinois and Florida isolates failed to infect ticks by either route of exposure, even though parasitemias of donor calves on which the clean ticks fed were high. The fact that the IAM did not produce infection in ticks even though it possesses an inclusion appendage, suggests that factors in addition to the presence of this appendage may be involved in infection of ticks with A. marginale.

Colony density studies supported transmission studies. Colonies were seen only in ticks that were naturally exposed to the VAM, and not in ticks exposed to the IAM and FAM. The absence of colonies in the gut of ticks that were artificially exposed to the VAM but that did transmit the organism to calves cannot be explained on the basis of data collected. It suggests that A. marginale colonies were present in numbers too low to be detected or that the organism underwent development in different tick tissue. Prepatent periods of anaplasmosis that developed in calves fed on by naturally and artificially exposed ticks were not remarkably different irrespective of route of tick exposure.

The FA technique was used to confirm the presence of A.

*marginale* in salivary glands collected from ticks, on day eight of feeding, exposed naturally to the VAM. Small amounts of focal fluorescence in each gland corresponded with the size, location and distribution of *A. marginale* colonies demonstrated with electron microscopy in salivary glands of previous studies (68). No fluorescence was seen in the salivary glands of ticks exposed to IAM and FAM. The absence of fluorescence supported the conclusion that the salivary glands were not infected with the anaplasmal organism.

Whole mounted salivary gland slides that were stained with methyl green-pyronin showed no noticeable difference between the glands of ticks exposed to the VAM, IAM and FAM and those of the control ticks. It was not known whether the organism was absent or was present in such low numbers as to escape notice.

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# VITA

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