IN VITRO AND IN VIVO STUDIES OF THEILERIA CERVI BETTENCOURT, 1907

By

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I would like to dedicate this thesis to my wife, Patience and my parents Herbert and Salome Wanyangu.

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CHAPTER I

INTRODUCTION

Protozoan hemoparasites belonging to the genus Theileria are known to infect a wide variety of domestic and wild ruminants in many parts of the world. In eastern Africa alone there are more than 40 species of wild Bovidae, the majority of which harbor Theileria parasites (Brocklesby and Vidler, 1966). The economic significance of theileriosis in cattle in eastern Africa results from death of half a million cattle annually from East Coast Fever (ECF) caused by Theileria parva parva (Miller et al., 1977).

Vectors for *Theileria* spp.

Various ticks have been incriminated as vectors for different species of Theileria. T. parva parva which infects domestic cattle (Bos indicus and Bos taurus) as well as African buffalo (Syncerus caffer), is transmitted mainly by the 3-host tick Rhipicephalus appendiculatus. Rhipicephalus zambiensis and other ixodid ticks have also been incriminated as vectors for this parasite (Koch, 1906; Gonder, 1910, 1911; Cowdry and Ham, 1932; Reinchow, 1937, 1938, 1940; Schein, et al., 1977 as cited in Mehlhorn and Schein, 1984). Theileria parva lawrencei is transmitted by

R. appendiculatus to cattle and buffalo; Theileria annulata is transmitted to cattle and the domestic water buffalo by both Hyalomma spp. and Amblyomma spp. Theileria velifera of cattle and buffalo is transmitted by Amblyomma spp. (Young, et al., 1977 a,b.; Warnecke, et al., 1979, 1980). Theileria taurotrogi of cattle and eland (Taurotragus oryx) is transmitted by several species of Rhipicephalus ticks including R. appendiculatus (Martin and Brocklesby, 1960).

Development of Theilerial Parasites

The life cycle of Theileria spp. in domestic cattle has been well documented (Hullinger, et al., 1964; Mugera and Munyua, 1972; Schein, et al., 1978; Young, et al., 1978 and Fawcett et al., 1982 a,b,c). The cycle is initiated when an infected tick attaches to a suitable animal host and begins to feed. As the tick feeds, the Theileria parasite develops along with salivary glands and transmission occurs after 3-5 days of feeding (Mehlhorn and Schein, 1984). Theileria sporozoites are released into the host along with tick saliva and enter lymphocytes. In vitro electron microscopy studies (Fawcett, et al. 1982 c) have shown that penetration of sporozoites into lymphocytes occurs rapidly and is completed within 10 minutes. The apical complex of sporozoites of Theileria spp. does not function in attachment to and penetration of lymphoid cells as it does with related protozoan parasites, e.g., Babesia spp. Actual entry of Theileria sporozoites into a host cell

involves recognition of specefic antigens and occurs when the membranes of the parasite and those of the host cell come into close apposition with each other. The overlapping of the membranes spreads laterally and the host cell membrane begins to invaginate resulting in endocytosis of the sporozoites. During the process of endocytosis, the sporozoite sheds off its surface antigen coat leaving it outside the host cell (Webster, et al., 1985). The invagination continues until the host cell membrane fuses over the parasite, forming an inclusion vacuole of host origin around the parasite. The host cell membrane surrounding the sporozoite disintegrates within 24 hours.

Schein, et al., (1978) observed that Theileria sporozoites within lymphoid cells, like those of Eimeria spp. and Plasmodium spp., reduce the two inner membranes of their pellicles and remain bound by a single membrane. The parasite grows rapidly and measures about 2 µm in diameter at 72 hours. As the parasite grows, its nucleus begins to divide by binary fission resulting in the production of a large schizont measuring 10-15 µm (Mehlhorn and Schein, 1984). The nuclei of the schizont measure 1.5-2 µm in diameter. The presence of schizonts in lymphocytes stimulates the latter to divide. As the cells divide, schizonts divide with them. Daughter cells that result from this division are, therefore, infected. Uninfected lymph node tissue is replaced by infected cells resulting in lymphadenopathy, the extent of which depends on the severity

of the infection and the susceptibility of the host animal. T. parva parva infection in Holstein-Fresian cattle, for example, produces severe lymphadenitis. Schizonts that have been observed in different species of theilerial organisms are morphologically similar (Mehlhorn and Schein, 1984) with variations occurring only in the number (13-50) of nuclei that they possess.

In bovids infected with T. annulata, spherical schizonts begin to form merozoites in 8-10 days after infection, but those of T. parva parva appear at 12-14 days after infection. Merozoite formation has not been readily observed in vitro. In vivo, merozoites penetrate and infect erythrocytes. Mehlhorn and Schein, (1984) observed that in cattle infected with T. annulata up to 90% of the erythrocytes are parasitized. Parasitemia of a similar magnitude can be observed in bovids with T. parva parva prior to death.

When a suitable uninfected tick feeds on an infected host, it ingests erythrocytes containing piroplasms. These piroplasms develop into slender spindle-shaped "microgamonts" in the gut of the tick in 1-4 days post repletion (Levine, 1985). Microgamonts then form 1-4 nuclei and several flagellum-like appendages. Fertilization has not been observed, but it is presumed to occur by the union of microgamonts and macrogametes. Resulting zygotes enter cells of the tick gut wall, elongate and leave to enter the hemolymph of the tick on days 14-17 post repletion. In the

hemolymph, zygotes transform into kinetes which are fully differentiated by day 17-20 post-repletion and enter salivary glands. Hazen-Karr, (1986) described the ultrastructure of sporogony for Theileria cervi in experimentally infected Amblyomma americanum. Complete development of sporozoites in the salivary glands is dependent on the tick feeding during the next blood meal.

Review of *Theileria cervi* in Deer

Schaffler (1962) reviewed the history of Theileria spp. in various species of deer. He cites the first case as being reported by Bettencourt, et al. in 1907 in fallow deer (Dama dama) in Portugal. The parasite in red blood cells appeared in various shapes including comma, oval, round and maltese crosses. No schizonts were observed in any of the lymphatic tissues. An attempt to transmit the parasite to goats failed. The organism was at that time referred to as Theileria cervus. Theileria spp. parasites reported from deer include T. cervus in 2 deer (Cervus aristotelis) from Indochina; "Piroplasma species" in spotted deer (Cervus axis); blood parasites in young deer (Cervus muntjac); Theileria dama in Japanese deer (Cervus sikka); blood parasites in spotted deer (Cervus hortulorum); Theileria spp. in European deer (Cervus elaphus). Although the parasites described by these various authors differed in their morphologic appearance, each felt that there was adequate resemblance to the parasites described by

Bettencourt, et al.(1907) (as cited in Schaffler, 1962) to warrant assignment to the genus Theileria.

Theileria infections in North American white-tailed deer (Odocoileus virginianus) were first reported in 1961 from a splenectomized deer that was part of an anaplasmosis experiment (Kreier, et. al., 1962). The deer died 12 weeks after splenectomy with a piroplasm parasitemia of about 50% and severe anemia. The parasite was easily transmitted to other deer by intravenous inoculation of infected whole blood. Attempts to transmit it to a calf and a sheep via the same route failed. This parasite was called Theileria mutans because it could not be transmitted to cattle. This name was changed to T. cervi when Schaffler (1962) did a morphological comparison with the theilerial organism described by Battencourt, et al. in 1907 in fallow deer. Schaffler confirmed that the Theileria from deer could be transmitted by intravenous inoculations of infected whole blood as well as subcutaneous inoculations of similar blood. In all of Schaffler's studies the deer developed severe anemia and died.

In an attempt to determine the role of T. cervi infections in free-ranging deer populations in Texas, Robinson, et al. (1967) inoculated splenectomized and intact fawns subcutaneously with 5 ml. of infected whole blood. One of the splenectomized fawns died while the 4 that remained developed anemia but survived. All intact fawns developed a piroplasm parasitemia less than 4% and none of

them developed anemia. It was concluded from the results that T. cervi could cause severe disease or death in immunosuppressed or malnourished deer.

Kuttler, et al. (1967 a) showed a positive correlation between the distribution of the lone star tick, A. americanum, and the occurrence of T. cervi in deer from Texas. A prepatent period of 14-21 days was established for T. cervi infection using A. americanum ticks which had been infected as nymphs. This study, as well as the one by Barker, et al. (1973) demonstrated that T. cervi was transmitted transstadially by A. americanum.

Serological tests, including fluorescent antibody test (FAT), gel-diffusion test, capillary tube agglutination test (CA) and complement-fixation test (CFT) were used to detect deer Theileria by Schaffler (1962) and Kuttler, et al. (1967 a, b, c). The CA test was found to be sensitive although cross-reactivity was observed between T. cervi antigen and sera from animals infected with T. recondita, T. annulata, T. lawrencei and T. parva whereas CFT did not identify animals with low parasitemia. The CA test was preferred partly because of its sensitivity, but also because it was selective for Theileria spp. in deer and did not produce a reaction with other deer hemoparasites. Durham, et al. (1976) detected sporozoites in salivary secretions of field-collected A. americanum using an FA test. The results suggested that T. cervi overwinters in these ticks.

The tissue stages of T. cervi, if they exist, have neither been found nor clearly described. Schaffler (1962) mentioned that he found "structures resembling Koch bodies" on impression smears of bone marrow of an experimentally infected deer. Structures suspected to be schizonts were found in primitive cells of the erythropoetic system and in undifferentiated mesenchymal cells. Similar structures were not observed in smears of other organs. Kreier, et al., (1962), in reference to as yet unpublished work by Schaffler, reported that Schaffler infected deer with T. cervi by subcutaneous inoculation of lymph node homogenates.

Research Objectives

Since no proof exists for the presence of a schizogonous tissue stage for T. cervi in white-tailed deer, a major emphasis of this study was to attempt to find such a tissue stage. Most studies dealing with the life cycle of T. cervi in white-tailed deer have been initiated by the inoculation of infected whole blood containing piroplasms. Few studies dealing with the life cycle of this parasite in its deer host have involved tick-initiated infections.

The objectives of this study were:

1. To infect Amblyomma americanum with Theileria cervi by allowing the ticks to feed on naturally or experimentally infected deer, and to use these ticks as the source of infection for further studies.
2. To prepare a crude stabilate of T. cervi sporozoites and

test its infectivity at the time of its preparation; after 1 week, 1 month, 6 months, and 1 year of storage at -70° C.

3. To monitor deer for piroplasms and schizonts following exposure to infected A. americanum or a sporozoite stabilate by daily lymph node biopsies.
4. To attempt in vitro cultivation of T. cervi by exposure of deer lymphocytes and erythrocytes to sporozoites.

CHAPTER II

MATERIALS AND METHODS

General Procedures

Agent

Theileria cervi used in these studies was obtained from a white-tailed deer (*Odocoileus virginianus*) captured at Cookson Hills Game Management area in eastern Oklahoma.

Deer Facility

Deer were maintained at Camp Redlands, a 6 hectare research facility located 20 kilometers west of Stillwater, Oklahoma. The facility is comprised of two, two-hectare fenced pens, a barn with six 2X7-meter enclosed runs and four, 7X7-meter cement-floored holding pens. This facility is located at the far western edge of the known range of *Amblyomma americanum* which naturally transmit *T. cervi* (Hair & Bowman, 1986). Natural transmission of *T. cervi* has not been observed in the 10 years that the facility has been in operation.

Experimental Animals

Thirteen white-tailed deer (O virginianus), designated A-M for the purpose of this study, and ranging from 6 months to 2 years of age were used. Uninfected deer were either obtained from western Oklahoma, or were raised in captivity at Camp Redlands. Animals were fed a 1:1 mixture of corn and oats (1-2 pounds/head/day) as well as alfafa hay. Animals were determined to be uninfected when repeated examination of blood films did not demonstrate piroplasms. In the present study uninfected deer are referred to as `susceptible deer.' If T. cervi piroplasms were found on blood films an animal was considered infected.

Surgical Procedures

When splenectomy of deer was necessary, it was done under halothane anesthesia at the College of Veterinary Medicine Teaching Hospital. When surgical removal of prescapular lymph nodes was needed, deer were sedated using Rompoun^{R} (xylazine) prior to the operation.

Tick Maintenance

Amblyomma americanum were reared and maintained at the Oklahoma State University, Tick Laboratory. The laboratory occupies 1225 square meters, and provides facilities for rearing and maintaining tick stages. A humidity chamber maintained at 25°C and 90-98% relative humidity was used to retain ticks while they molted.

Molting ticks were subjected to a 14 hour photoperiod.

Infection of Nymphal Ticks

Amblyomma americanum nymphs were placed on an infected deer in a 0.6X1.2X1.0 m wooden box for 24 hours to facilitate nymphal attachment. Seams on the box were sealed using adhesive tape to prevent escape of nymphs. Holes present on the box for ventilation were also surrounded with adhesive tape to prevent escape of nymphs. After 24 hours, the deer was transferred to a 1.0X0.7X0.9 m. steel cage that was positioned over steel pans lined with newspapers. The edges of the pans were lined with adhesive tape to prevent escape of ticks. Replete nymphs were collected, counted and placed in paper cartons with porous lids. Labelled cartons were placed in a humidity chamber.

Exposure of Deer to Laboratory-Infected Adult *Amblyomma americanum*

Adult *A. americanum*, exposed to *T. cervi* as nymphs were placed in a wooden box with an uninfected deer for 24 hours. The deer was transferred to a steel cage positioned over metal pans as previously described for collection of replete ticks.

Preparation of a *Theileria cervi* Sporozoite Stabilate

Forty pairs of infected adult A. americanum were fed on sheep for 6 days to allow development of T. cervi sporozoites in tick salivary glands. The ticks were removed and crushed in a hand-held glass pestle and mortar in 30 ml. of cold (4°C) RPMI Medium 1640[R1] with 25 mM HEPES buffer and L-glutamine. The tick suspension was filtered through 2 layers of cheesecloth and the supernatant was centrifuged at 1000 X g for 5 minutes, passed through a 5 µm filter and resuspended in RPMI medium to establish a final volume of 8 ml. Thus, when well mixed, 1 ml of the resulting suspension contained sporozoites equivalent to those maintained by 10 ticks. Approximately 3 ml. of the stabilate was inoculated into deer soon after its preparation while the rest was mixed with 10% glycerin in a 1:1 ratio and stored in plastic vials at -70° C.

Monitoring of Deer for Parasites

Deer were bled either by jugular venipuncture or by pricking an ear vein and thin blood smears were made and stained with Diff Quik[R2]. Buffy-coat smears were made

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[1]Gibco Laboratories Life Technologies Inc., Grand Island, New York 14072 U.S.A.

[2]Diff Quik Solutions consist of a fixative containing
(Footnote Continued)

following centrifugation of whole blood in micro-capillary tubes. Lymph node aspirates were obtained after a prescapular lymph node was fixed in position between the index finger and the thumb. A 20-guage biopsy needle was inserted through the skin into the lymph node and the plug inside the needle removed. A 3 ml. syringe was carefully attached to the needle and the plunger pulled to aspirate lymph. Lymph smears were prepared, stained and examined using a light microscope (LM) at 1000X (oil immersion).

Collection of Tissues for Electron Microscopy (EM)

Prescapular lymph nodes were surgically removed, cut into approximately 1 mm^3 pieces and immediately placed in 2% glutaraldehyde (pH 7.4) in 0.2 M sodium cacodylate buffer and fixed at $4^{\circ}C$ for 24-48 hours. The ratio of tissue to glutaraldehyde was at least 1:10. Liver, spleen, lymph node, bone marrow and lung were collected at necropsy and processed in a manner similar to that described for biopsy material.

Processing of Tissue for Electron Microscopy

(Footnote Continued)
1.8 mg/L triaryl methane dye, 100 PDC in methanol; Solution 1 which contains 1 gm/L Xanthene dye 100 PDC and Solution 2 which contains 1.25 gm/L thiazine dye mixture, 100 PDC (0.625 gm/L azure A and 0.625 gm/L methylene blue).

Tissues collected for EM were washed 3 times in 0.2 M sodium cacodylate buffer and post-fixed in a 1:1 solution of 2% osmium tetroxide and 0.27 M cacodylate buffer for 1 hour. Tissues were washed twice in buffered wash (10 minutes each), dehydrated in a graded series of ethanol and infiltrated with Dow Epoxy Resin (DER 736) using propylene oxide as the transitional solvent. The vials were capped for 24 hours, uncapped for approximately 12 hours after which tissues were then embedded in 100% DER and placed in a vacuum oven at 60° C and 5-8 mm Hg for 48-96 hours. Thick sections (1 µm) were prepared using glass knives and a Sorvall MT 5000 ultramicrotome and stained with Mallory's stain at 60°C (Richardson, et al., 1960). Fine sections (gold reflective) were cut with a Diatome diamond knife and collected on 200 mesh copper grids. The sections were stained with 3% uranyl acetate and with lead citrate (Venable and Coggeshall, 1965). Sections were examined and photographed using a JEOL 100CX transmission-scanning EM at 100 KV.

Experimental Design

Infection of Ticks and Transmission of *Theileria cervi* to Deer

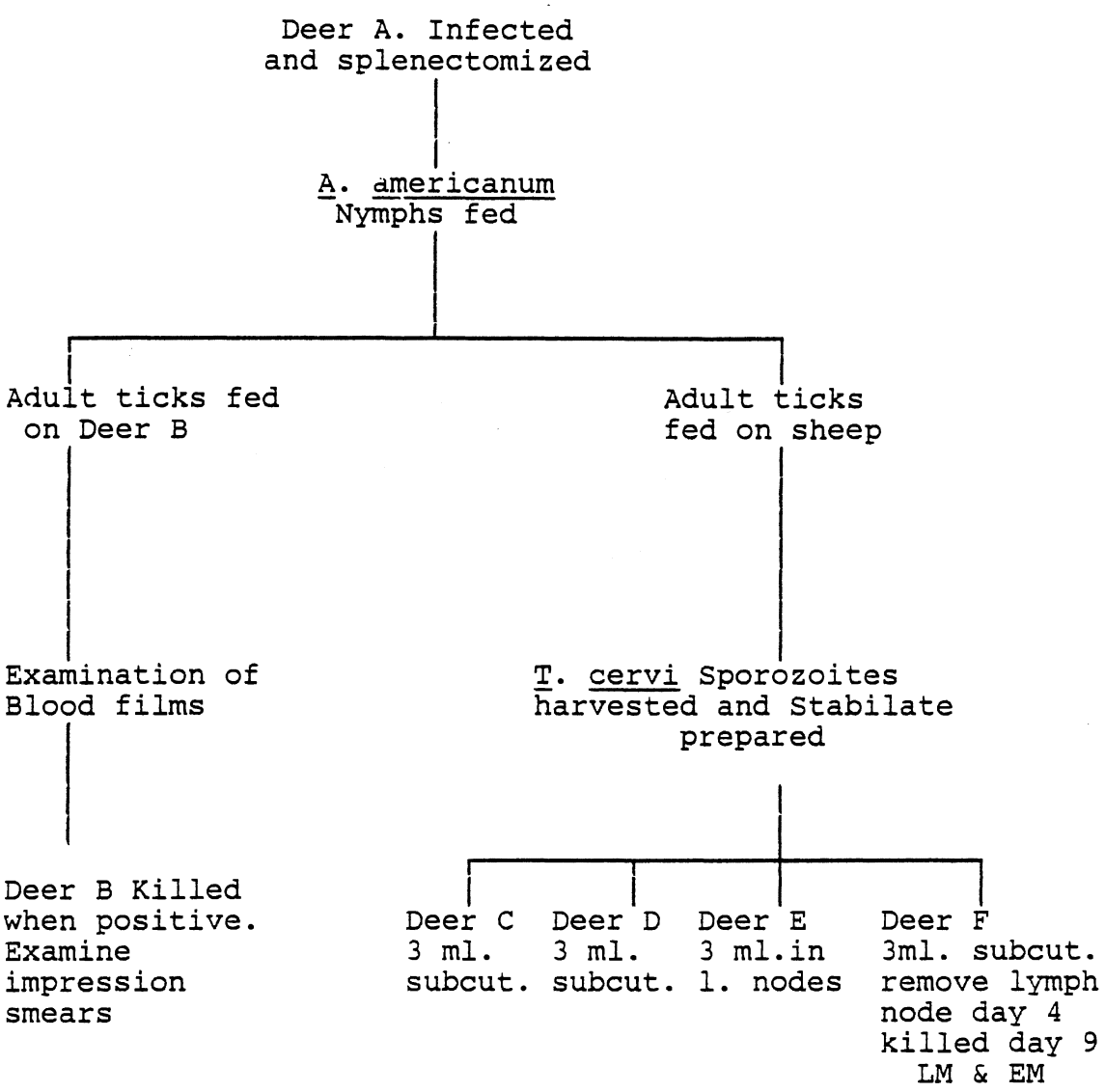
A white-tailed deer (deer A), with a T. cervi piroplasm parasitemia of 1% was splenectomized to increase its parasitemia. After splenectomy, the deer was monitored daily by blood film examination. When the

piroplasm parasitemia reached 10%, about 1000 laboratory reared, uninfected A. americanum nymphs were allowed to feed to repletion on deer A (Figure 1). After molting, the exposed adults were fed on susceptible deer B. Deer B was monitored for piroplasms and schizonts daily beginning day 3 following tick attachment. When piroplasms were first observed, deer B was killed and the spleen, lungs, prescapular lymph nodes and bone marrow were processed for LM and EM.

Evaluation of *Theileria cervi* Sporozoite Stabilate

Immediately following its preparation, the T. cervi sporozoite stabilate was inoculated subcutaneously into susceptible deer C to test its infectivity. The deer was monitored daily by blood film examination for presence of piroplasms. At 1 week, 1 month, 6 months and 1 year following its preparation, the T. cervi sporozoite stabilate was tested for viability by subcutaneous inoculation of deer J,K,L and M respectively. Deer were monitored by blood film examination for presence of piroplasms (see `Monitoring of Deer for Parasites,' `General Procedures').

Figure 1. Flow chart summarizing *In Vivo* experimental design. Deer A was infected with *Theileria cervi* and splenectomized. *A. americanum* nymphs were fed on this deer. Nymphs molted and resulting adults were divided into 2 groups. One group was fed on deer B which was monitored daily for piroplasms by blood film examination. Deer B was killed when it became positive and impression smears were examined for schizonts by LM. The remaining ticks were fed for 6 days on sheep and a stabilate was made from them (see text). The stabilate was inoculated into deer C, D, E and F. All deer were monitored for piroplasms. Deer F was monitored for schizonts by surgical removal of a prescapular lymph node on day 4, and after the deer was killed on day 9.


Deer A. Infected and splenectomized
A. americanum Nymphs fed
Adult ticks fed on Deer B
Adult ticks fed on sheep
Examination of Blood films
T. cervi Sporozoites harvested and Stabilate prepared
Deer B Killed when positive. Examine impression smears
Deer C 3 ml. subcut.
Deer D 3 ml. subcut.
Deer E 3 ml.in l. nodes
Deer F 3ml. subcut. remove lymph node day 4 killed day 9 LM & EM

Examination of Deer for Schizogonous Tissue Stages of *Theileria cervi*

Three susceptible deer (D, E & F) were each inoculated with 3 ml. of the sporozoite stabilate. All the stabilate was injected subcutaneously in deer D and F; deer E was injected with 1.5 ml. of the stabilate directly into the right and left prescapular lymph nodes.

Deer were monitored daily for piroplasms and schizonts by blood film and buffy-coat examinations until piroplasms were seen in peripheral erythrocytes. A prescapular lymph node was surgically removed from deer F on day 4 post-inoculation and examined by LM and EM. When piroplasms were first observed deer F was killed, the remaining prescapular lymph node removed and processed for LM and EM. Impression smears of the lungs, spleen, liver and brain, as well as aorta scrapings were made, stained with Diff Quik and examined with LM.

In Vitro Studies

In Vitro Cultivation of *Theileria cervi* in Deer Lymphocytes and Erythrocytes

Eighty ml. of blood from an uninfected deer (deer G) was drawn asceptically by jugular venipuncture into 3 ml. of sodium citrate. The mononuclear-cell enriched fraction from this blood was isolated using a modification of the method described by Stagg, et al. (1976) .

Three ml. of Histopaque-1119[R3] was added to each of twenty, 15 ml. conical centrifuge tubes and carefully overlayered with 3 ml. of Ficcoll-Paque[R4]. Blood from deer G was mixed with an equal volume of isotonic phosphate buffered saline (PBS) and 6 ml. of this mixture was carefully layered onto the Ficcoll-Paque[R] in each of the 20 tubes. The tubes were centrifuged at 700 X g for 30 minutes at room temperature. Three distinct opaque layers resulted; a mononuclear cells/platelets layer, a granulocyte layer and a red blood cell layer. The mononuclear-cell/platelets layer was harvested from each tube and pooled into 4 tubes. Eight ml. isotonic PBS was added to each of the 4 tubes and the tubes were centrifuged at 200 X g for 10 minutes. The pellet of mononuclear cells was resuspended in 10 ml. of PBS before re-centrifuging at 200 X g for 10 minutes. This procedure was repeated 2 more times to remove the majority of the platelets. After the final washing, the pellets of cells were pooled and resuspended in 20 ml. of complete growth medium [RPMI 1640[R] supplemented with 20% fetal calf serum (FCS) that contained 100 ug/ml. streptomycin sulfate, 100 IU/ml. penicillin G and 25 ug/ml. amphotericin B (Sigma Cell Culture Reagents)]. Theileria cervi sporozoite stabilate was rapidly thawed and 5 ml. were added to the

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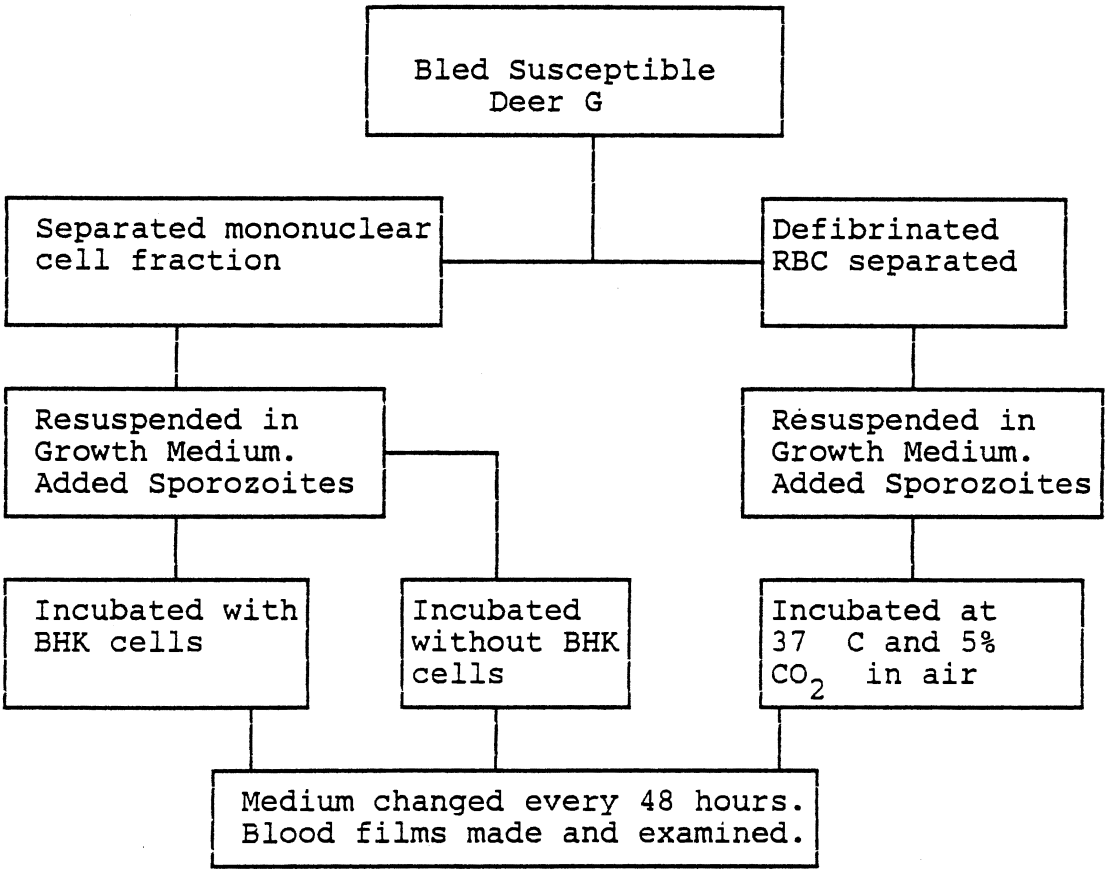
[3]Sigma Diagnostics, St. Louis, MO 63178 U.S.A.

[4]Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, NJ 08854 U.S.A.

cell suspension. The resulting solution was split in 2 equal volumes. One part was put in a 25 ml. plastic T flask with a pre-formed Baby Hamster Kidney (BHK) monolayer and the remaining portion was put in a similar flask without the BHK monolayer. The flasks were incubated at 37° C in air with 5% CO_2. Culture solutions were changed every 48 hours for 12 days by centrifuging the solution containing the cells, discarding the supernatant, taking an aliquot containing the cells and making a thin smear. Smears were stained with Diff Quik and examined with LM. The pellet was then resuspended in fresh growth medium and returned to the culture flasks. Cultures were monitored for 14 days.

Red blood cell cultures were established by defibrinating with glass beads 20 ml. of the blood drawn earlier from deer G (Figure 2). An equal volume of isotonic PBS was added to the defibrinated cells and the mixture centrifuged in 15 ml. conical centrifuge tubes at 500 X g for 10 minutes. The supernatant and buffy coat were discarded leaving red blood cells. The latter were washed 3 times, each time discarding the plateletes that remained suspended in PBS. At each washing any buffy coat layer that appeared was also discarded. The resulting erythrocytes were mixed with twice their volume of RPMI 1640 medium with 20% FCS. Antibiotics were added to achieve the concentrations mentioned above. Freshly-thawed T. cervi sporozoite stabilate (5 ml.) was added and the cultures were gently mixed. The solution was then split into 10 ml.

Figure 2. Flow chart summarizing first *in vitro* experimental design. Deer G was bled and mononuclear cells and erythrocytes separated from the blood. Both groups of cells were exposed to *T. cervi* sporozoites and incubated at 37°C with 5% CO_2 in air. Some of the mononuclear-cell flasks were incubated with BHK feeder-layer but some were not. All cultures were monitored every 48 hours for schizonts and piroplasms.


Bled Susceptible Deer G
Separated mononuclear cell fraction
Defibrinated RBC separated
Resuspended in Growth Medium. Added Sporozoites
Resuspended in Growth Medium. Added Sporozoites
Incubated with BHK cells
Incubated without BHK cells
Incubated at 37 C and 5% CO_2 in air
Medium changed every 48 hours. Blood films made and examined.

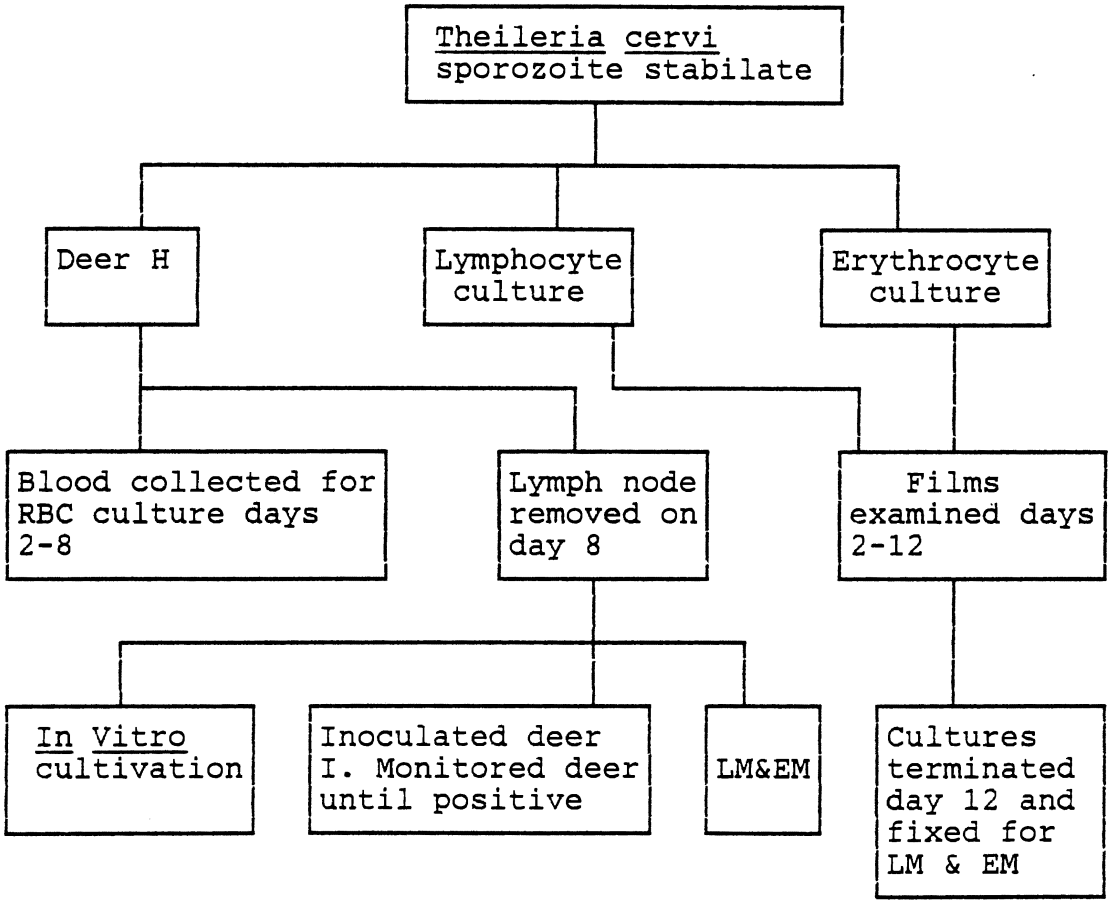
portions and incubated at 37° C in 25 ml. plastic T flasks and gassed with 5% CO_2 in air. Growth medium was changed and blood films made every 48 hours for 12 days by gently tilting the flask and asceptically drawing out the old medium and replacing it with new medium.

A duplicate study was conducted using Hank's balanced salt solution (without calcium and magnesium) instead of RPMI medium 1640. Cultures were maintained for 14 days at which time a fungal contamination was observed and the experiment discontinued.

Several vials of stabilate were thawed and pooled. An aliquot from this pool was used to initiate red blood cell and mononuclear cell cultures as described previously. Cultures were set up in duplicate using either RPMI containing 20% FCS (v/v) as described before, or containing 20% (v/v) deer serum. Three ml. of stabilate from the pool were inoculated into a susceptible deer (deer H) to test the viability of the sporozoites (Figure 3).

Beginning 2 days after deer H was inoculated with the T. cervi sporozoite stabilate and continuing daily until day 8, blood was drawn, defibrinated and divided into two portions; one portion was incubated using 20% FCS in RPMI while the rest was incubated using 20% deer serum. A prescapular lymph node was surgically removed on day 8 from deer H and divided into two portions. One portion was suspended in 6 ml. of RPMI medium, ground using a hand-held glass pestle and mortar, and inoculated subcutaneously into

Figure 3. Flow chart summarizing second *in vitro* experimental design. *Theileria cervi* sporozoite stabilate was inoculated into deer H and the deer monitored daily for schizonts and piroplasms. Deer H was bled daily starting day 2 post inoculation and the blood was put in culture. A lymph node was surgically removed from deer H and divided into 3 portions; one was inoculated into deer I, another put in culture and the third processed for LM and EM. Lymphocyte and RBC cultures were exposed to sporozoites and monitored daily for 12 days after which cultures were terminated and processed for LM & EM.


Theileria cervi sporozoite stabilate
Deer H
Lymphocyte culture
Erythrocyte culture
Blood collected for RBC culture days 2-8
Lymph node removed on day 8
Films examined days 2-12
In Vitro cultivation
Inoculated deer I. Monitored deer until positive
LM&EM
Cultures terminated day 12 and fixed for LM & EM

deer I. The remaining portion was divided in half and one part was placed in culture as described earlier for mononuclear cells, using RPMI medium. The other part was processed for EM as described under `General Procedures' after impression smears had been made for light microscopy. All cultures were monitored for T. cervi every 48 hours. Monitoring of cultures was done by carefully discarding old growth medium, making thin smears from cells at the bottom of the culture flasks, and adding fresh growth medium to the cells. All cultures were terminated on day 12 post inoculation of deer H, and samples were fixed from each culture for EM. Deer I was monitored for parasites until piroplasms were detected in erythrocytes.

CHAPTER III

RESULTS

Infection of Ticks and Transmission of *Theileria cervi* to Deer

Amblyomma americanum that fed as nymphs on deer A transmitted T. cervi as adults to deer B with a prepatent period of 11 days post-attachment. The piroplasm parasitemia was 1% at first detection and increased to 3.6% by day 13. On day 13 post-attachment deer B was euthanatized. Schizonts were not observed in any of the lymphocyte preparations nor in any of the organ impression smears prepared at necropsy.

Evaluation of *Theileria cervi* Sporozoite Stabilate

Electron microscopic evaluation of the T. cervi sporozoite stabilate revealed numerous T. cervi sporozoites and occasional cell fragments and granules (Figure 4). Freshly-prepared stabilate caused infection when inoculated into deer C with a prepatent period of 8 days post inoculation. The stabilate proved viable when it was tested in deer J at 1 week, K at 1 month, L at 6 months and M at 1 year after freezing.

Examination of Deer for Schizogonous Tissue Stages of *Theileria cervi*

Piroplasms were first observed in deer D and E on day 8 post inoculation following exposure to the sporozoite stabilate. Schizonts were not observed in smears prepared from lymph node aspirates. The prescapular lymph node that was surgically removed from deer F on day 4 post inoculation was hemorrhagic and slightly enlarged. Neither LM nor EM evaluation of this lymph node revealed schizogonous tissue stages in lymphocytes. Deer F developed a piroplasm parasitemia on day 9 post inoculation. When the deer was killed and impression smears of various organs examined by LM, no schizonts were observed.

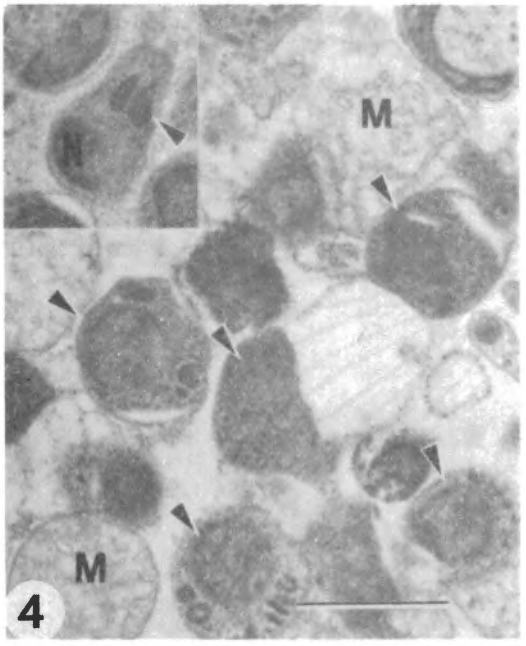
Electron microscopic studies of the lymph node removed from deer F on day 9 revealed lymphocytes containing structures that were compatible morphologically with early developmental stages of apicomplexa parasites (Figure 5). These stages were similar to structures described as early stages of Theileria parva parva schizonts in lymphocytes (Stagg, et al. 1984; Schein, et al. 1978). The suspect schizonts contained structures that looked like endoplasmic reticulum and mitochondria as well as developing rhoptries.

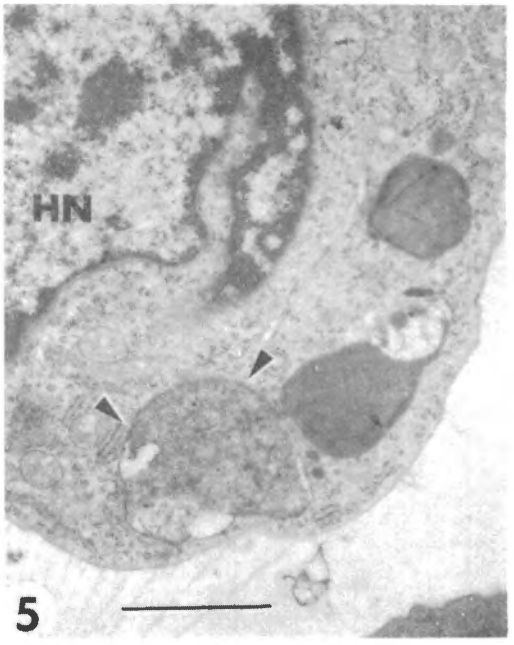
Neither developing nor mature schizonts were observed. Non-cell associated merozoite-like stages were observed, however, in the preparations (Figure 6). Structures suspected to be merozoites appeared singly and contained

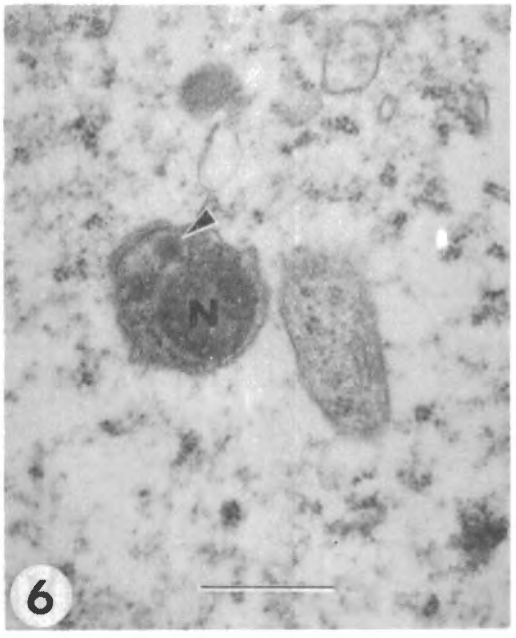
Figure 4. Electron micrograph of Theileria cervi sporozoite stabilate. Note sporozoites (arrows) and host cell mitochondria (M). Insert: Isolated sporozoite with nucleus (N), and rhoptries (arrows). Bar = 500 nm.

Figure 5. Electron micrograph of deer lymphocyte containing membrane-bound, electron-dense structure comparable to developing Theileria organism (arrows). HN = host nucleus. Bar = 500 nm.

Figure 6. Electron micrograph of merozoite-like structure free in lymph node tissue eight days after exposure of deer to the frozen stabilate. N = nucleus, arrows = rhoptries. Bar = 500 nm


M
M
4


HN
5


N
6

structures resembling nuclei, mitochondria and rhoptries. Two merozoite-like stages bound by one membrane were also observed.

In Vitro Studies

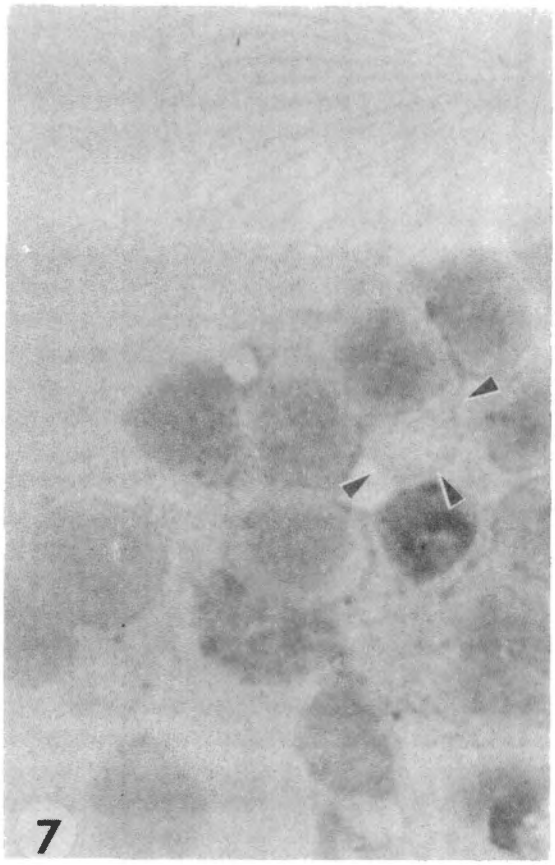
In Vitro Cultivation of *Theileria cervi* in Deer Lymphocytes and Erythrocytes

The mononuclear-cell fraction isolated from whole blood contained numerous platelets. Shizogonous tissue stages were not seen with LM or EM in cultures of mononuclear cells exposed to T. cervi sporozoites grown with or without BHK feeder-layer. Piroplasms were not observed over the 12-day culture period in red blood cells that were exposed to T. cervi sporozoites. Piroplasms were not seen with LM or EM in erythrocyte cultures that were set up using blood that was drawn from deer H for the first 7 days following infection. Blood drawn on day 8 post-infection contained piroplasms at the time of collection and continued to show them for 4 days in culture.

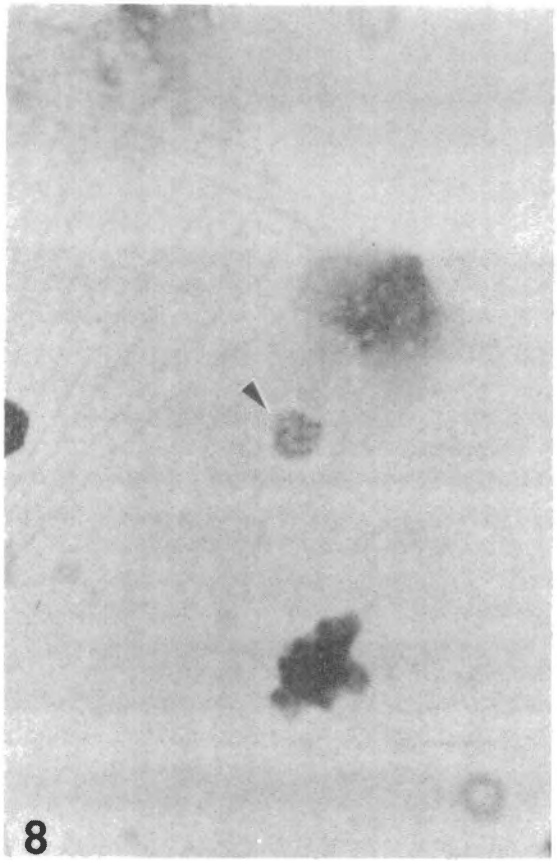
Light microscopic evaluation of impression smears made from the prescapular lymph node that was surgically removed from deer H on day 8 revealed one lymphocyte that contained a structure that resembled a schizont of Theileria parva (Figure 7). Films made after this lymph node had been in culture for 2 days also revealed structures that looked like schizonts of T. parva parva (Figure 8). These structures

Figure 7. Photomicrograph showing Theileria-like microschizont in lymph node lymphocyte (arrows). Taken eight days after exposure of deer to sporozoites. X 2000.

Figure 8. Photomicrograph showing Theileria-like microshizont free in lymph node (arrows). X 2000



7



8

were, however, free from lymphocytes and none were observed inside lymphocytes.

CHAPTER IV

DISCUSSION

Infection of Ticks and Transmission of *Theileria cervi* to Deer

Amblyomma americanum nymphs were allowed to feed on deer A when the Theileria cervi piroplasm parasitemia was rising and before the deer developed anemia. When adult ticks that developed from these nymphs were fed on deer B, the deer became positive with T. cervi piroplasms on day 11 post-attachment. The present findings confirmed the reports of Kuttler, et al. (1967 a) and Barker, et al. (1973) viz. that T. cervi is transmitted transstadially to white-tailed deer by A. americanum.

Evaluation of *Theileria cervi* Sporozoite Stabilate

Initiating T. cervi infections in deer using a sporozoite stabilate was unique since no previous reports of preparation or use of such stabilate exist. The stabilate was shown by EM to contain sporozoites and proved to be viable when it was inoculated into deer. The prepatent period of T. cervi was reduced from about 14-21 days using A. americanum (Kuttler, et al., 1967 a), to 8-9 days by

inoculation of the sporozoite stabilate. The difference in the prepatent periods could be due to the time taken by a tick to attach and feed before mature sporozoites were transmitted to the vertebrate host. In bovids infected with T. parva the time needed for Rhipicephalus spp. to feed before transmitting the parasite has been determined to range from 3-5 days (Mehlhorn ans Schein, 1984). Amblyomma americanum males and females that had fed for 3 and 6 days respectively were found to contain mature T. cervi sporozoites in their salivary glands (Hazen-Karr, 1986). Although the time required by ticks to pre-feed before transmitting T. cervi has not been determined, based on the findings of Hazen-Karr, A. americanum probably take 3-6 days of feeding to transmit T. cervi to deer.

The sporozoite stabilate provided a way of initiating infections that more closely approximates the natural mode of transmission of the parasite than does infection by blood transfusion.

Examination of Deer for Schizogonous Tissue Stages of *Theileria cervi*

The pre-scapular lymph node that was surgically removed from deer F on day 4 post-inoculation was hemorrhagic and slightly enlarged. Schizogony in pathogenic Theileria spp., e.g., T. parva parva (Levine, 1985), results in lymphadenitis manifested grossly by enlargement of pre-scapular lymph nodes. Lymphadenitis observed in deer F

may have, however, been due to repeated needle biopsies taken from this lymph node rather than by T. cervi. No lymphadenitis was observed in other deer with T. cervi. When impression smears from deer D, E and F were examined at necropsy, schizonts were not observed. This may have been because schizonts were present in very low numbers. EM studies done on the lymph node taken from deer F on day 9 revealed structures that were compatible morphologically with early developmental stages of apicomplexa parasites. Non-cell associated merozoite-like stages were observed with EM of the lymph node. These merozoite-like stages possessed structures that resembled rhoptries and micronemes which are observed in merozoites of other Theileria spp. We were unable to demonstrate mature T. cervi schizonts or merozoites inside deer lymphocytes, but based on what has been observed in other Theileria spp., it is likely that schizogony takes place in lymphocytes in lymph nodes. Therefore, the structures believed to be non-cell associated merozoites that were observed may have developed in lymphocytes but had already left them. Apicomplexans are intracellular parasites; schizogony takes place within host cells. The fact that the parasite stages observed were few in number suggests that schizogonous development occurs on a small scale and is of short duration.

When lymph node removed from deer H was inoculated into deer I, the latter deer became infected with T. cervi. Transmission of T. cervi from one deer to another by lymph

node inoculation supports the suggestion that the lymph node probably contained actual merozoites. The possibility that the lymph node also contained piroplasm-infected erythrocytes cannot, however, be eliminated. Deer H had a piroplasm parasitemia less than 1% when the lymph node was removed.

In Vitro Cultivation of *Theileria cervi* in Deer Lymphocytes and Erythrocytes

Erythrocyte cultures remained healthy throughout the study period and piroplasms were not observed in red blood cell cultures exposed to T. cervi sporozoites. These findings suggest that T. cervi sporozoites, like those of other members of this genus, do not penetrate erythrocytes directly.

Light microscopic evaluation of the lymph node surgically removed from deer H and grown in vitro for 2 days revealed structures resembling macroschizonts of Theileria spp. These macroschizont-like stages appeared membrane bound, non-cell associated and were few in number. Non-cell associated macroschizonts have been observed in other Theileria infections (Adams, et al. 1971). The culture material examined in the present study was, however, less than optimal and contained many lymphocytes with fragmenting nuclei. The possibility that these microschizont-like stages were artifacts resulting from the culture procedures cannot be eliminated.

No schizogonous tissue stages were seen with LM or EM in cultures of mononuclear cells exposed to T. cervi sporozoites. The routine procedure of harvesting lymphocytes from bovine blood described by Stagg, et al. (1976), did not prove directly applicable to work with deer blood. The poor yield of lymphocytes and the abnormal appearance of these cells in culture may have played a role in the inability to infect the cells with sporozoites. Successful in vitro cultivation of T. cervi has not been accomplished in deer lymphocytes. Further understanding of the development of this parasite will likely depend on the perfection of culture procedures.

CHAPTER V

SUMMARY AND CONCLUSIONS

A study of the life cycle of Theileria cervi was undertaken in white-tailed deer (Odocoileus virginianus). The objectives of the study were: to initiate infections using sporozoites rather than infected blood; to prepare a sporozoite stabilate and to attempt to find schizogonous tissue stages of T. cervi. Adult Amblyomma americanum that molted from exposed nymphs transmitted T. cervi transstadially to deer with a prepatent period of 11 days.

A T. cervi sporozoite stabilate was prepared from infected adult A. americanum that had pre-fed on sheep for 6 days. The ticks were crushed in a hand-held pestle and mortar and the stabilate passed through a 5µm filter and inoculated into deer. Exposed deer became infected with T. cervi with a prepatent period of 8 days. After a year of storage at -70° C, the sporozoites still proved to be viable.

Schizogonous tissue stages were not observed with LM in lymph node aspirates taken daily from infected deer. Prescapular lymph node impression smears made at necropsy from a deer that was killed on day 9 post inoculation revealed one lymphocyte that had what resembled a schizont

by LM. Impression smears of other organs revealed no schizonts. EM studies of a prescapular lymph node collected on day 9 post-inoculation revealed what appeared to be early stages of development of an apicomplexan parasite in lymphocytes. Non-cell associated merozoite-like stages were also observed in the lymph node.

No schizonts or piroplasms were observed in *in vitro* cell cultures that were exposed to *T. cervi* sporozoites. LM of films made from a lymph node that was surgically removed from a deer on day 8 post-inoculation and grown in an *in vitro* culture system revealed stages that resemled non-cell associated macroschizonts.

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