STUDIES ON TWO TICK-BORNE DISEASES OF DOGS: EHRLICHIOSIS AND HEPATOZOONOSIS

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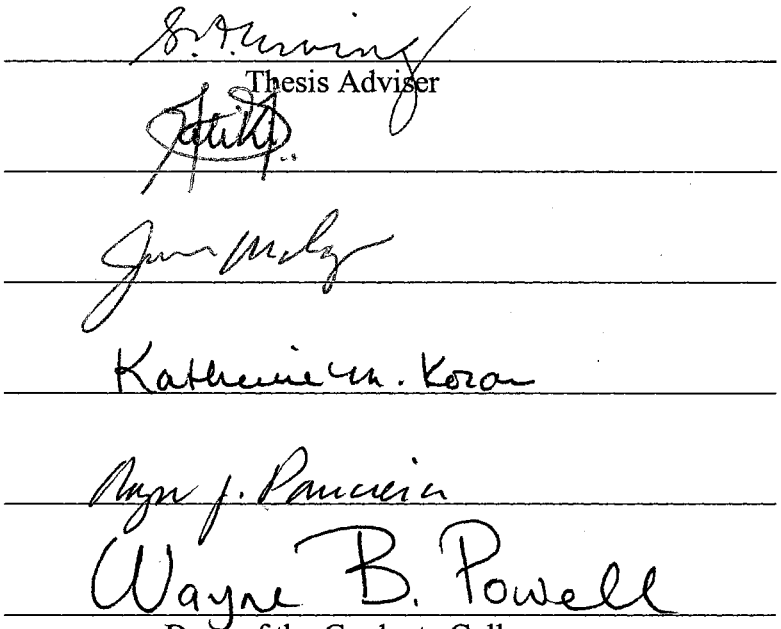

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

INTRODUCTION

Background and Significance

Ehrlichiosis:

The genus *Ehrlichia* is classified in the Order Rickettsiales. Organisms that belong to this genus are obligatory intracellular parasites that produce disease in a variety of vertebrate animals. The majority of species assigned to this genus are transmitted by ixodid ticks. *Ehrlichia canis* infects dogs and is designated as the type species of the genus. The organism was first discovered in dogs in Africa[1] and was assigned to the genus Rickettsia.[1] Subsequently it was reported in many parts of the world, including the United States.[2-5] The role of *Rhipicephalus sanguineus* (brown dog tick) as a vector for *E. canis* was first documented in 1935[1] and later the vector potential of this ixodid was demonstrated experimentally by Groves et al.[6], Lewis et al.[7] and Mathew et al.[8] This tick is believed to transmit *E. canis* transstadially under natural conditions, and there is one unconfirmed report of transovarial transmission.[9] *E. canis* has also been successfully transmitted experimentally by the American dog tick, *Dermacentor variabilis*.[10]

Dogs infected with *E. canis* exhibit fever, anorexia, thrombocytopenia, non-regenerative normocytic/normochromic anemia, leukopenia, petechiae on mucous membranes and epistaxis.[11,12] Confirmatory antemortem diagnosis of *E. canis* infection formerly required demonstration of morulae in peripheral blood smears. The first successful serologic test developed to detect *E. canis* infection, an indirect fluorescent antibody (IFA) assay, was described in 1972.[13] A polymerase chain reaction (PCR) assay was developed using species-specific primers to amplify *E. canis* DNA from infected animals in 1992.[14] Subsequently, the usefulness of PCR and a combination of PCR and

DNA hybridization, using a digoxigenin-labeled 287bp oligonucleotide probe, was demonstrated as useful in diagnosis of *E. canis* infections in acutely infected dogs.[15,16]

Hepatozoonosis:

American canine hepatozoonosis, caused by *Hepatozoon americanum*, is characterized by a chronic, often fatal, infection in dogs and was first reported from Texas and Louisiana.[17,18] More recently cases were reported from Oklahoma, Alabama and Georgia.[19,20] Morphologically, *H. americanum* is similar to *H. canis*, which produces a mild infection in dogs in the Old World.[17] Dogs infected with *H. americanum* exhibit muscle wasting, weakness, leukocytosis with a mature neutrophilia, periosteal bone proliferation and are non-responsive to treatment.[19] The vector potential of the brown dog tick, *Rhipicephalus sanguineus*, in transmission of *H. americanum* and *H. canis* has been experimentally demonstrated.[21,22] *Hepatozoon*-like oocysts were reported from the Gulf Coast tick, *Amblyomma maculatum*, collected from a dog with symptoms of *H. americanum* infection.[23]

In the Old World, *H. canis* infection is easily detected in dogs by demonstration of gametocytes in circulating neutrophils. In contrast, dogs with *H. americanum* infection characteristically present with a much lower parasitemia than do dogs with *H. canis* infections.[17] An IFA test that employs *H. canis* gamonts (recovered from buffy coat preparations) as antigen is used for serologic testing.[24] In contrast, *H. americanum* infections of dogs are currently diagnosed primarily by clinical signs, radiography and demonstration of asexual stages of the parasite in canine skeletal muscles.[25]

The genus *Hepatozoon* is classified in the Phylum Apicomplexa, Order Eucoccidiorida, and Family Haemogregarinidae. There are more than 300 species (based

on morphological analysis) assigned to this genus; they infect a wide variety of vertebrate species, including amphibians, reptiles, birds and mammals.[26] Nuclear small subunit ribosomal RNA (18S rRNA) gene sequence has been used extensively to determine phylogenetic relationships among various Apicomplexan parasites.[27-29] Restriction enzyme analysis of a 600bp fragment from the 18s rDNA of *H. mocassini*, a parasite of cottonmouth snakes (*Agkistrodon piscivorus*), revealed unique restriction sites when compared to other haemogregarine species known to infect snakes.[30]

The Research Problem

Ehrlichiosis:

Diagnosis of *E. canis* infections has always been difficult due to the low parasitemia associated with the infection.[12] Accurate detection of *E. canis* infection is very important in identifying carrier animals, e.g., prior to import and export of pets, in selecting donor dogs for blood transfusion, etc. Carrier animals can also serve as a source of infection for ticks. Serologic tests, using the IFA technique with cell-cultured *E. canis* as antigen, have been used for routine laboratory diagnosis. *Ehrlichia chaffeensis* and *E. ewingii* are closely related species that can infect dogs and both species cross react with *E. canis* serologically, making it difficult to differentiate the infections.[31,32] Experimental studies showed that dogs remained seropositive for many months after spontaneous loss of infection and after curative treatment.[33] PCR assay has been used successfully to amplify *E. canis* DNA from infected dogs during the acute phase of infection. More recently PCR combined with DNA hybridization has been used to increase the sensitivity of the PCR assay.[16] A test that can equal the best of existing ones in sensitivity and specificity, while

reducing the time and expense required for execution, would enhance the ability to manage canine ehrlichiosis.

Hepatozoonosis:

Identifying a vector for *H. americanum* will be important in understanding its transmission and epidemiology. Even though *R. sanguineus* is believed to be a vector for this agent, it is not unequivocally proved. Most of the cases of canine hepatozoonosis reported from Oklahoma are among rural, outdoor dogs. Since *R. sanguineus* is primarily an in-house tick, it seems likely that there may be other acarine vectors involved in transmission.

At present diagnosis of American canine hepatozoonosis (ACH) is made mostly in terminally ill dogs and is based upon clinical signs, radiography and demonstration of asexual stages of the parasite by skeletal muscle biopsy.[25] Multiple biopsies may be needed for confirmatory diagnosis. Developing a serologic test will be very useful for epidemiological investigations and for routine laboratory diagnosis of *H. americanum* infections. A serologic test may facilitate earlier detection of infection than is now possible which, in turn, can assist in proper management of the disease.

Classification of the genus *Hepatozoon* is based primarily upon morphological analyses of various stages of sporogony, merogony, and gamogony in definitive and intermediate hosts.[26] Unfortunately, morphological analyses do not always provide adequate information for understanding phylogenetic relationships. Various studies have shown that analyses of 18S rRNA gene sequence can illuminate phylogenetic relationships among various Apicomplexan species.[27-29] However, few molecular phylogenetic studies have been carried out on species of the genus *Hepatozoon*, and there

are no descriptions of the sporogonic phase of *H. americanum*. Determination of sporogonic developmental stages and of 18S rRNA gene sequence of *H. americanum* will help to understand its relationship to other *Hepatozoon* species as well as to other members of the phylum Apicomplexa.

Objectives

Ehrlichiosis:

I. To develop a technique for rapid detection of *Ehrlichia canis* in dogs with either acute or chronic infection.

Hepatozoonosis:

II. To identify the acarine vector(s) for *Hepatozoon americanum* and to produce experimental infections in dogs.

III. To describe the development of *H. americanum* in its acarine host vector, using light microscopy.

IV. To determine the phylogenetic relationship between *H. americanum* and some selected species of the Phylum Apicomplexa, based on 18S rRNA gene sequence.

V. To develop a serologic test for detection of *H. americanum* infection in dogs.

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CHAPTER II

EFFICACY OF A MODIFIED POLYMERASE CHAIN REACTION (PCR) ASSAY FOR DETECTION OF *EHRLICHIA CANIS* INFECTION

Abstract

Detection of *Ehrlichia canis* in acutely infected and convalescent dogs is important for effective treatment and control; however, accurate detection has been difficult to achieve, in part, because dogs that have been cured therapeutically often remain seropositive for extended periods. A new method, viz., polymerase chain reaction (PCR) assay using biotinylated, *E. canis*-specific primers (PCR-BP), was developed for detection of *E. canis*. Four dogs experimentally infected with *E. canis* by intravenous inoculation of whole blood from carrier dogs and two naturally infected convalescent carriers were used to compare the specificity and sensitivity of the new method with that of microscopy/blood smear evaluation, serologic test, and conventional PCR assay using *E. canis*-specific primers. In experimentally infected animals, infection was detected as early as 7 days post exposure using PCR-BP. Although the two naturally infected dogs were positive by serologic test and PCR-BP, both were negative by conventional PCR. Results suggest that the new method is a sensitive assay for detection of *E. canis* infection. In addition, results were obtained more rapidly than with other PCR-based assays.

Body

Ehrlichia species (Order Rickettsiales) are obligatory intracellular parasites that produce disease in a variety of vertebrate animals, and most of them are transmitted by ixodid ticks. *Ehrlichia canis*, the type species of the genus, was first discovered in dogs in Africa and was originally assigned to the genus *Rickettsia*.[4] Dogs infected with *E. canis* exhibit fever, anorexia, thrombocytopenia, non-regenerative normocytic/normochromic anemia, leukopenia, petechiae (especially on mucous membranes) and epistaxis.[5, 8] Carrier dogs can serve as a direct source of infection for other dogs through blood transfusion and indirectly via the bite of ticks.

Accurate and rapid methods to detect *E. canis* infection are needed to identify carrier animals, e.g., prior to import/export of pets, and selection of donor dogs for blood transfusion. Diagnosis of *E. canis* infections by evaluation of blood smears has always been difficult due to the low parasitemia associated with infection.[8] A serologic test, using the IFA technique with cell-cultured *E. canis* as antigen, has been developed[12] and used widely. One shortcoming of this test is that antibodies stimulated by *E. chaffeensis* and *E. ewingii*, closely related species that also infect dogs, both cross react with *E. canis* serologically, making it difficult to differentiate the individual infections.[2, 3, 13]

Experimental studies have demonstrated that dogs remain seropositive to *E. canis* for many months after spontaneous loss of infection and after curative treatment with tetracycline.[2, 7] Since dogs often remain seropositive for extended periods, it may be important to reconsider the usefulness of serologic assessment for differentiating true carriers from animals that are cured of ehrlichial infection.

Polymerase chain reaction (PCR) assay has been used successfully to amplify *E. canis* DNA from infected dogs during the acute phase of infection.[1, 9] More recently, PCR combined with DNA hybridization (PCR-CH)[10] and nested-PCR[14] have been used to improve sensitivity over that of the conventional PCR assay. Herein we report PCR assay using biotinylated primers (PCR-BP) for rapid detection of *E. canis* infections and compare efficacy of the new technique with three older methods, viz., blood smear evaluation, serologic test using the FIAX[a] system, and conventional PCR assay.

DNA was extracted from 200 µl of whole blood collected from experimental dogs and 200 µl of IDE8 (*Ixodes dammini* embryonic) cells infected with *Ehrlichia canis* (as a positive control) using either QIAMP® blood kit or QIAMP® tissue kit[b] according to manufacturer's instructions. DNA was also extracted from 200 µl suspension of DH82 cells (ATCC No. CRL-10389) infected with *E. chaffeensis* and from dog platelets infected with *E. platys*. Recovered DNA was used subsequently for conventional PCR assay and for PCR-BP.

Ehrlichia canis-specific primers ECAN5 and ESPEC3[11] were synthesized at the Recombinant DNA/Protein Resource Facility, Oklahoma State University. The PCR assay was performed in 50 µl reaction volumes containing 5 µl of 10X PCR buffer, 1.25 units of Taq DNA polymerase[c], 20 *p*Moles of each primer, 10 µl (30 *n*g/µl) of DNA extracted from blood samples, and 0.2 mM of dCTP, dATP dGTP, and dTTP. DNA extracted from samples positive for *E. canis, E. chaffeensis* and *E. platys* and sterile distilled water (instead of template DNA) were used as controls in the PCR assay. Reactions were carried out in a thermal cycler[d] using an initial denaturing temperature of 94° C for 3 min for one cycle followed by denaturing at 94° C for 1 min, an annealing

temperature of 60° C for 1 min and an extension temperature of 72° C for 2 min. The amplified products were separated on a 1.5% agarose gel using 10μl of the PCR product, stained with ethidium bromide and visualized using ultraviolet illumination.

In the PCR-BP assay, one of the primers (ECAN5) was labeled with biotin; labeling was done at the Recombinant DNA/Protein Resource Facility, Oklahoma State University. PCR assay conditions were similar to those described above and similar controls were used. The resultant PCR product was purified using a Wizard® PCR purification system[c] and 5-10 μl of the purified PCR product was blotted onto a nylon membrane manually or by using a Bio-dot apparatus.[e] The labeled PCR product was detected by immunological reaction using 1:3,000 diluted anti-biotin antibody[f] conjugated with alkaline phosphatase and the substrates, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT).

Three methods were used to assess the sensitivity of PCR-BP. First, the template *E. canis* DNA extracted from infected IDE8 cells was diluted to 1:10 (3 *ng*/μl), 1:100 (300 *pg*/μl), 1:1,000 (30 *pg*/μl) 1:10,000 (3 *pg*/μl) and 1: 100,000 (300 *fg*/μl) and used (10 μl of template per reaction) in the PCR-BP assay. The PCR product was detected using both gel electrophoresis and immunological methods. Second, PCR product obtained using biotinylated primers was diluted to 800 *n*g, 160 *n*g, 32 *n*g, and 6.4 *n*g per 10 μl concentration and was employed for gel electrophoresis and immunological detection. Third, DNA extracted from four dogs experimentally infected with *E. canis* and two naturally infected dogs was used to test the new method.

For the third testing of the new method, infections were produced in experimental dogs by intravenous inoculation of either 10 ml whole blood (dog #s 3152, 3154 and

3307) from donor dogs that were *E. canis* carriers or 2 ml of infected IDE8 (50% infected) cells (dog # 3325). These 4 dogs were monitored daily by blood smear evaluation and weekly by serologic test (FIAX[a]). Conventional PCR assay using *E. canis*-specific primers, and the new PCR-BP assay were performed on samples from all four dogs daily from one day prior to inoculation of infectious blood or culture material until morulae were first observed in peripheral blood smears.

In addition to the experimentally infected dogs, samples (serum and DNA) from 2 naturally infected, privately-owned, dogs that were also referred to our laboratory for confirmatory diagnosis of *E. canis* infection were used to test the new method. The serologic test, conventional PCR, and PCR-BP assays were all performed on samples from these two naturally infected, convalescent carriers.

Biotinylated primers were very specific in amplifying *E. canis* DNA; no PCR product was obtained when *E. chaffeensis* and *E. platys* DNA were used as template. The PCR-BP assay detected parasite DNA in experimentally infected dogs as early as 7 days after exposure (Table 1). As little as 6.4 *n*g of PCR product was detected by PCR-BP assay when serially diluted PCR product was tested. The assay performed using diluted template DNA yielded a PCR product that was detectable by gel electrophoresis at 1:100 dilution but not at 1:1,000. However, using immunological detection, the PCR product was consistently visible at 1:1,000 dilution (sometimes even at 1:10,000 dilution) when 10 µl of purified PCR product was blotted on to the membrane (Fig 1).

The prepatent periods determined by blood smear evaluation, serologic test, PCR assay and PCR-BP assay for the experimentally infected dogs are presented in Table 1. Two naturally infected dogs were *E. canis* positive by serologic test. Although *E. canis*

DNA could not be detected when blood was tested by conventional PCR, both dogs were positive when tested with the PCR-BP assay.

The new method detected *E. canis* infection in experimentally infected animals sooner than did either conventional PCR assay, serologic test or blood smear evaluation. The new method appears to be as sensitive as subinoculation of whole blood into susceptible pups for detection of *E. canis* parasitemia. In a previous study[6] a series of susceptible pups were transfused with whole blood every successive 24 hours after an "incubating donor" was exposed to *E. canis*, demonstrated that, beginning at day 7 after exposure, all pups that received blood became infected. The first 6 pups exposed at 24-hour intervals after the incubating carrier was exposed, all failed to become infected. In the present study, the PCR-BP assay was almost equally sensitive, detecting infection at 7 days in 1 of 4 dogs and 8 days in the other 3.

Although we do not know precisely the amount of *E. canis* DNA used in our sensitivity assay there is at least a 10 - 100 fold increase in sensitivity in detecting *E. canis* DNA using the PCR-BP assay compared to conventional PCR assay. A previous study[14] showed that nested-PCR and PCR-CH are specific and about 100 times more sensitive than conventional PCR assay in detecting *E. canis* DNA. Our observations demonstrate that the PCR-BP assay is comparable in sensitivity and specificity to nested-PCR and PCR-CH.

Based upon our experiments, we recommend use of biotinylated primers in PCR assay to substitute for nested-PCR and PCR-CH because the biotin-tagged to the primer amplifies the PCR signal considerably. The detection step requires only about 1 - 1.5 hrs, making this method faster and, therefore, more cost effective than nested PCR or PCR-

CH assays. Another advantage of PCR-BP is that it can be converted to a quantitative assay using densitometry to measure the intensity of the spots on the nylon membrane.

Our observations are consistent with results of an earlier study[10] that used PCR-CH with respect to early detection of parasite DNA (7 days after exposure) in experimentally infected dogs. However, our technique did not detect *E. canis* DNA as early as 4 days as demonstrated by Wen et al.[14] using nested-PCR. In that study, dogs were experimentally exposed to 10^7 infected DH82 cells and the larger infective dose could account for the difference in time of detection. Even though we do not know precisely the infective dose in our experiments, other studies conducted in our laboratory have shown that shorter prepatent periods (as brief as 10 days; determined by blood smear evaluation) are experienced when dogs are exposed to heavily infected cell culture material. In contrast, dogs infected by transfusion of whole blood from carrier animals typically have prepatent periods of 14-20 days (unpublished data). Another explanation for why Wen et al.[14] detected *E. canis* DNA earlier by using the nested-PCR assay may lie in differences in the amount of whole blood from which DNA was extracted; we used 200 µl whereas those workers extracted DNA from 200 µl of buffy coat (from 4 - 5 ml of whole blood). Alternatively, nested-PCR may, in fact, be a more sensitive technique than is our PCR-BP assay.

The PCR-BP assay has proved useful in detecting *E. canis* infection in 4 dogs with acute infection and 2 with latent infections. The technique may prove useful for the detection of other types of ehrlichial infections and for differentiating true carriers from animals that have been cured but remain serologically indistinguishable from infected animals.

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Sources and Manufacturers

a. Bio-Whittaker, Walkersville, MD

b. Qiagen, Santa Clarita, CA

c. Promega Corp., Madison, WI

d. Perkin Elmer, Foster City, CA

e. BioRad, Hercules, CA

f. Sigma, St. Louis, MO

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Table 1. Prepatent periods (days) determined by different methods for detection of *Ehrlichia canis* in blood of experimentally infected dogs

	Dog Numbers			
Technique	3152	3154	3307	3325
Blood smear evaluation	15	14	13	15
Serologic test (IFA)	16	21	14	21
Conventional PCR	11	9	9	10
PCR using Biotinylated primers	8	7	8	8

Figure 1. Results of conventional PCR assay and the new PCR-BP assay in the detection of *E. canis* DNA. **1a.** Results of conventional PCR assay; A 359 bp *E. canis*-specific product is obtained at 1:1. 1:10 and 1:100 dilution of the template DNA by this method. **1b.** Results of PCR-BP assay; *E. canis* DNA was detected in up to 1:10,000 dilution of the template DNA when 10µl of the purified PCR product was used for the PCR-BP assay.

1a
1 Kb plus ladder
-VE
1:1
1:10
1:100
1:1000
1:10000
1b
1:1
1:10
1:100
1:1000
1:10000
-ve
10µl
5µl

CHAPTER III

EXPERIMENTAL TRANSMISSION OF *HEPATOZOON AMERICANUM* VINCENT-JOHNSON ET AL., 1997 TO DOGS BY THE GULF COAST TICK, *AMBLYOMMA MACULATUM* KOCH

Abstract

Experimental transmission of *Hepatozoon americanum* to dogs was attempted with four ixodid ticks, viz., *Rhipicephalus sanguineus, Amblyomma americanum, Amblyomma maculatum* and *Dermacentor variabilis.* Ticks that dogs ingested included some that were laboratory-reared and experimentally fed as nymphs on a dog with naturally occurring hepatozoonosis; other ticks were collected as replete or partially engorged larvae, nymphs and adults from dogs that had hepatozoonosis and natural infestations of ticks. Whole ticks used to expose susceptible dogs orally were partially dissected to help release oocysts. Among eight dogs exposed, only the three that were fed *A. maculatum* adults experimentally acquisiton-fed as nymphs became infected. Dogs developed elevated body temperature and other evidence of clinical disease starting 4 weeks after exposure. "Cysts" typical of *H. americanum* were found in skeletal muscle when samples were first examined 5 weeks after dogs ingested ticks, and parasites were also observed in peripheral blood smears at approximately the same time. Our study demonstrates that *A. maculatum* nymphs can acquire *H. americanum* by feeding on a parasitemic dog and that transstadial transmission of the protozoan occurs, with dogs acquiring infection when they ingest newly-molted adult ticks.

Key Words: *Hepatozoon americanum, Amblyomma maculatum*, Experimental transmission

Introduction

Canine hepatozoonosis is an emerging tick-borne disease of dogs in the United States. The disease was first reported from Texas by Craig et al. (1978) and subsequently has been reported from Louisiana, Alabama, Georgia and Oklahoma (Gossett et al., 1985; Macintire et al., 1997; Panciera et al., 1997). The causative agent was originally thought to be a particularly virulent strain of *H. canis* (Craig et al., 1978), the causative agent of canine hepatozoonosis in the Old World. The American form of the disease, however, is more virulent and otherwise dissimilar from that in the Old World (Vincent-Johnson et al.,1997a; Panciera et al., 1998). For example the asexual stages of the parasite seen in the USA occur predominantly in skeletal and cardiac muscle rather than in spleen and liver. This difference in location of asexual stages, clinical signs and structure of the gametocytes in the vertebrate host formed the basis for classification of the protozoan as a new species, *H. americanum* (Vincent-Johnson, et al., 1997b).

Hepatozoon canis is transmitted by the brown dog tick, *Rhipicephalus sanguineus,* and the same vector was suspected for the "Texas strain" of *H. canis* (presumably *H. americanum*) (Craig et al., 1978). Later, the potential of this ixodid to transmit the Texas isolate was demonstrated experimentally by Nordgren and Craig (1984). More recently, Vincent-Johnson et al. (1997b) reported three unsuccessful attempts to transmit *H. americanum* with *R. sanguineus* adults that had acquisition-fed as nymphs on dogs with naturally-occurring hepatozoonosis. These same workers reported finding *Hepatozoon*-like oocysts in four adult *Amblyomma maculatum* ticks collected from a dog naturally infected with *H. americanum* (Vincent-Johnson et al., 1997b). In the present

study we explored the potential of four ixodids, viz., *R. sanguineus, A. americanum, A. maculatum*, and *D. variabilis* to transmit *H. americanum*.

Materials and Methods

2.1 Experimental dogs

One mixed breed dog (#3048) naturally infected with *H. americanum* (diagnosed by characteristic parasite stages in skeletal muscle) and eight mixed breed pups (#s 2783, 2825, 3069, 3070, 3071, 3152, 3305 and 3306) raised by the Laboratory Animal Resources Unit (LAR) of Oklahoma State University (OSU) were used in this study. [Three other privately owned adult dogs (designated a, b, and c) with hepatozoonosis were the source of Awild@ ticks used in phase I of this four-part study.] All the dogs were housed in tick-free isolation facilities and cared for in accord with conventional laboratory animal practices. Dog #3048 was the source of infective blood meals for experimentally fed nymphal ticks. Six of the eight dogs that were fed ticks orally were conventionally preadapted to LAR facilities. Two others were adapted with the intent to enhance susceptibility: dog # 3152 was a chronic carrier of *Ehrlichia canis* at the time of exposure to ticks and dog #3305 was splenectomized. Dog #s 3152, 3305 and 3306 were determined to be *H. americanum*-naive by examination of skeletal muscle biopsy prior to being fed ticks.

2.2 Ticks

Replete or partially engorged larval, nymphal and adult ticks collected from naturally infested dogs that were also infected with *H. americanum* were used in phase I. In phases II, III, and IV, specific pathogen free ticks were purchased as nymphs from the tick rearing facility of the Oklahoma Agricultural Experimental Station. Adult ticks of

the previous generation fed on sheep; nymphs employed in this study had fed as larvae on rabbits. Four different species of laboratory-reared ticks, viz. *R. sanguineus, A. americanum, A. maculatum* and *D. variabilis,* were used in this study.

2.3 Acquisition-feeding of ticks

2.3.1 Phase I -AWild@ ticks collected from dogs with hepatozoonosis

Ticks used in this phase were partially or fully engorged larvae, nymphs and adults collected from privately owned naturally infested dogs that were confirmed by muscle biopsy to be infected with *H. americanum* (Fig 1). The adult ticks were identified as *R. sanguineus, D. variabilis*, and *A. americanum;* larval and nymphal ixodids were not identified to taxon.

2.3.2 Phases II, III and IV-Laboratory-reared nymphal ticks

Prior to exposure to the various species of nymphal ticks, the dog (#3048) was determined to have gametocytes circulating in the peripheral blood. At each exposure ticks were placed under a stockinet snugly fitted on the dog. The stockinet was removed 48 hours after release of ticks. While the ticks were feeding the dog was housed in a metabolism cage positioned over a water moat. As nymphs became replete they left the host and were collected from the refuse pan or the moat. Engorged nymphs were maintained in a humidity chamber at a relative humidity of 90% and 14:10 hours (light:dark) photoperiod to molt. Dog #3048 was exposed to nymphal *R. sanguineus, D. variabilis* and *A. americanum* simultaneously in phase II (Fig 2); to *R. sanguineus* and *A. americanum* simultaneously in phase III; and to *A. maculatum* alone in phase IV (Fig 3).

2.4 Attempted transmission trials

2.4.1 Phase I - Oral exposure to Awild@ ticks

Two pups (#s 2783 and 2825) were exposed orally to ticks collected from three privately owned naturally infested dogs that had naturally occurring hepatozoonosis (Fig 1). Dog #2783 was fed ticks twice (at 3 mo and 7 mo of age), first in April, 1996, from dog Aa@ and a second time in August, 1996, from dog Ab@, after being found negative from previous exposure by muscle biopsy; dog #2825 (6 mo) was exposed to ticks from dog Ac@ (Fig 1). Partially or fully engorged ticks were crushed, mixed with dog food, and fed to dogs orally. Both the dogs used in phase I were given prednisone (3mg/Kg) *per os* daily for a month after they ingested ticks. Dog #2783 was euthanatized 16 weeks after the second exposure, and dog #2825 was euthanatized 25 weeks after ingesting ticks; necropsy examinations were conducted on both dogs.

2.4.2 Phase II - Oral exposure to R. sanguineus, A. americanum *and* D. variabilis

Adult ticks *(R. sanguineus* and *A. americanum)* that were acquisition-fed as nymphs on dog #3048 were activation-fed for 4 days on a rabbit. Ticks were removed from the rabbit, crushed using a mortar and pestle, mixed with milk and fed to littermate pups (3 mo) through a stomach tube. Ticks were not examined for oocysts prior to being fed to dogs. Two dogs (#s 3070 and 3071) used in this phase (Fig 2) each received 60 adult *R. sanguineus* (20 M and 40 F); a third dog (#3069) received 40 adult *A. americanum* (20 M and 20 F). Dogs were euthanatized for necropsy examination 16 weeks after exposure. Although nymphal *D. variabilis* were acquisition-fed with the expectation that transmission studies with adults would be done in this phase, that portion of the study was abandoned after examination of dissected ticks failed to reveal oocysts.

2.4.3Phase III - R. sanguineus *and* A. americanum

After molting, 20 adult ticks each of *A. americanum* and *R. sanguineus* were dissected in an effort to detect *Hepatozoon* oocysts. If oocysts were not found, transmission to dogs were not attempted.

2.4.4 Phase IV - Oral exposure to A. maculatum

After molting, a number of adult ticks were dissected and examined by light microscopy for the presence of parasites. 100 adults (50 M and 50 F) were activation-fed on dog #3306 for 4 days after which ticks were removed, bisected, mixed into canned food and fed to three pups of 6 to 9 mo age (Fig 3). Dog #3152 ate 20 ticks, #3305 ate 50 ticks and dog #3306, the dog on which activation-feeding was done, ate 13 ticks.

2.5 Monitoring the dogs

Dogs that ingested adult, nymphal or larval ticks in phase I and those that were fed adult ticks in phase II were monitored daily by body temperature and physical examination. Skeletal muscle biopsies were taken at 8, 12 and 16 weeks for histopathologic examination in phase I and at 8weeks in phase II. In phase IV, dogs were monitored similary by physical examination and body temperature starting at 8 (dog #3152) and 26 days (dog #s 3305 and 3306) after ingesting ticks. Blood smears were made daily once there was a rise in body temperature. Skeletal muscle specimens were examined 5 (#3152) and 6 weeks (#s 3305 and 3306) after exposure.

All dogs in phases I , II and IV were monitored on a weekly basis by CBC and platelet count. Differential counts were made when the WBC count exceeded 15,000/μl.

Results

3.1 Phase I

Dogs exposed orally to ticks collected from dogs naturally infected with *H. americanum* did not show clinical evidence of infection during 32 weeks (#2783, two exposures) and 25 weeks (#2825) of monitoring (Fig. 1). Body temperatures remained within the reference range. Muscle biopsies taken from #2783 at 8, 12 and 16 weeks after the first ingestion of ticks and 6, 10 and 14 after the second exposure were negative for parasites. Histopathologic examination of muscle biopsies taken from #2825 at 8, 12, and 16 weeks post exposure did not show evidence of parasites. The CBC values and platelet counts remained within normal range throughout for both dogs (data not shown) and there were no detectable lesions specific for *H. americanum* infection at necropsy.

3.2 Phase II

Dogs #s 3069, 3070 and 3071 that ingested either *R. sanguineus* or *A. americanum* adults acquisition-fed as nymphs on dog #3048 showed no evidence of infection throughout 16 weeks of monitoring (Fig 2). The CBC values and platelet counts were within the reference range for all three dogs throughout the study (data not shown). No parasites were found in muscle biopsies taken 8 weeks post exposure nor were histologic lesions of hepatozoonosis observed at necropsy performed 16 weeks after exposure.

Dermacentor variabilis nymphs that were acquisition-fed in this phase of the experiment were found not to contain oocysts. As mentioned in materials and methods, the transmission experiments originally planned with dogs were not conducted.

3.3 Phase III

Following experience gained in phase II, we dissected a representative sample of *R. sanguineus* and *A. americanum* adults exposed as nymphs for this phase. No *H. americanum* oocysts were found in the hemocoel of either species. Accordingly, as stated in materials and methods, transmission studies planned with dogs in phase III were abandoned.

3.3 Phase IV

When 88 newly-molted adult *A. maculatum* that fed as nymphs on dog #3048 were dissected, *Hepatozoon*-like multisporocystic oocysts were found in the hemocoel (Fig 4). The number of oocysts per tick ranged from 4 to more than 150. Oocysts that were measured ranged from 300 to 1000μm in diameter (n=20). Sporocysts were spherical, measuring 20-30μm in diameter (n=20). Sporocysts typically contained 8-16 tightly packed sporozoites (Fig 5). Upon manual rupture of sporocysts, sporozoites were found to be banana-shaped (Fig 6) and measured 13-17μm in length and 4-7μm in width (n=20).

Each of the three dogs (#s 3152, 3305 and 3306) that ingested infected *A. maculatum* adults developed hepatozoonosis (Fig 3). The clinico-pathologic changes observed in these dogs are summarized in Table I. All dogs experienced waxing and waning elevations of body temperature (Fig 7) and neutrophilic leukocytosis (Fig 8) about 4 weeks after exposure. Myasthenia, bone pain and ocular discharge were observed 5 weeks after exposure. These signs continued throughout the period of observation [5 weeks (#3152) and 7 weeks (#s 3305 and 3306)].

Dog # 3152, euthanatized 5 weeks after exposure, was found to have "cysts" typical of *H. americanum* in skeletal muscle (Fig 9). Skeletal muscle biopsies taken at 6 weeks after exposure from dog #s 3305 and 3306 revealed numerous parasite stages such as Acysts@, meronts and granulomas as depicted by Panciera et al. (1998).

Discussion

Canine hepatozoonosis in the USA is caused by a haemogregarine originally designated as the "Texas strain" of *H. canis* (Craig et al., 1978). We assume that the recently described new species, *H. americanum* (Vincent-Johnson et al., 1997b), is more similar to the ATexas strain@ than to Old World *H. canis*. The disease produced in dogs by *H. americanum* has been well characterized as a chronic, progressive, often fatal condition accompanied by muscle weakness, wasting, leukocytosis and periosteal bone proliferation (Craig, et al., 1978; Macintire, et al., 1997; Panciera, et al., 1997; Vincent-Johnson et al., 1997a). Clinical recognition of the disease has been limited mostly to terminally ill animals. The acute stage of *H. americanum* infection has been little studied except for one instance (Nordgren and Craig, 1984).

Craig et al. (1978) suggested *R. sanguineus* as a vector for the "Texas strain" of *H. canis,* and later the vector potential of this ixodid was experimentally demonstrated (Nordgren and Craig, 1984). Similar results were reported with *H. canis* in the Old World; successful experimental transmission studies were conducted using *R. sanguineus* (McCully et al., 1975; Baneth et al., 1998). Adult *R. sanguineus* acquisition-fed as nymphs on a dog naturally infected with the "Texas strain" of *H. canis* (presumably *H. americanum*) transmitted the infection when fed to two splenectomized

dogs. These dogs were found to have muscle lesions 8 weeks post exposure and gamonts were found in peripheral blood smears 4 weeks after that (Nordgren and Craig, 1984).

We failed three times in phase I to transmit *H. americanum* to susceptible pups by feeding them assorted ticks we collected from tick-infested dogs that had naturally occurring hepatozoonosis. In that phase we speciated the adult ticks (but not larvae and nymphs), and we pooled species and stages when they were fed to dogs (Fig 1). We also failed to demonstrate oocysts in any of the few adult Awild@ ticks that we dissected. It is possible, of course, that some of the Awild@ ticks were infected but failed to be infectious for dogs that ingested them. While we cannot account for the failure, we speculate that none of the three species of Awild@ticks (*R. sanguineus, A. americanum* and *D. variabilis*) collected from dogs infected with hepatozoonosis were infected with *H. americanum.*

In phase II we failed to transmit *H. americanum* with laboratory-reared *R. sanguineus* adults acquisition-fed as nymphs on a known carrier (Fig 2). Likewise, in Phase III, failure to see oocysts in adult *R. sanguineus* signaled poor compatability between this tick and *H. americanum.* Dog #3048, that served as donor host for all experimentally exposed nymphal ticks in these experiments, was parasitemic before and during all the acquisition feedings by laboratory-reared nymphal ticks.

We do not know why our attempts to transmit the infection by *R. sanguineus* failed. Our results are similar to those reported by Vincent-Johnson et al. (1997b) but differ from those of Nordgren and Craig (1984). It is possible that the ATexas strain@ of *H. canis* is not identical to *H. americanum* or that our experimental conditions were

unlike those followed in the study by Nordgren and Craig (1984). It may be that *R. sanguineus* is a marginal host for *H. americanum*.

Most of the 25 naturally-occurring cases of hepatozoonosis seen in Oklahoma dogs occurred in rural, outdoor dogs. (Nineteen of these cases were reported by Panciera et al. In press; the others are of more recent occurrence.) This fact caused us to question whether *R. sanguineus*, which is commonly an in-house tick, is the sole vector. Tick vectors of canine hepatozoonosis are known to differ in various parts of the world. For example, *H. canis* oocysts were reported from *Haemaphysalis* sp. in Japan (Murata et al., 1995) and from *Rhipicephalus* sp. in Israel (Baneth et al., 1998), South Africa (McCully et al., 1975) and India (Christophers, 1912). Vincent-Johnson et al. (1997b) reported finding *Hepatozoon*-like oocysts in four adult Gulf Coast ticks, *A. maculatum*, recovered from a dog naturally infected with *H. americanum*. These authors found that A... oocysts and sporocysts were similar to *H. canis* but differed in size, shape of the sporocysts and number of sporocysts per oocysts.@ To our knowledge that is the only report, other than the present one, implicating *A. maculatum* as a possible host for *H. americanum*. Moreover, all the cases of naturally-occurring hepatozoonosis observed in Oklahoma dogs have come from geographic parts of the state where the Gulf Coast tick is known to occur (Semtner and Hair, 1973).

Dog # 3071 that ingested laboratory-reared adult *A. americanum* acquisition-fed as nymphs on dog #3048 also failed to develop infection (Fig 2). Moreover, adult *D. variabilis* that acquisition-fed as nymphs on dog #3048 in Phase II and *A. americanum* acquisition-fed as nymphs in Phase III did not have demonstrable oocysts in them.

These findings suggest that these ixodids (*R. sanguineus*, *A. americanum* and *D. variabilis*) are unlikely to be prime vectors of *H. americanum*.

Laboratory-reared adult *A. maculatum* acquisition-fed as nymphs on dog # 3048 were found to have *Hepatozoon* oocysts (Fig 4) in the hemocoel. When ticks from this cohort were ingested by three dogs (#s 3152, 3305 and 3306), all showed clinical evidence of infection, viz., elevated body temperature, leukocytosis, myasthenia and bone pain and had parasites in skeletal muscle when first sampled at 5 weeks (#3152) and 6 weeks (#s 3305 and 3306) (Table I). Nordgren and Craig (1984) reported parasites in skeletal muscle 8 weeks after exposure to infected adult *R. sanguineus*. Our results indicate that it takes as little as 5 weeks for the parasites to encyst in striated muscles (Fig. 9). We do not know whether there is a site of asexual multiplication between the time that sporozoites are ingested and when they are seen in striated muscle. Further studies that include earlier sampling will be needed to examine this possibility.

We performed activation feeding on dog #3306 to cover the possibility that *H. americanum* must undergo development in its tick vector similar to that required by *Theileria cervi* (Hazen-Karr et al., 1987) and *Anaplasma marginale* (Kocan et al., 1985). We did not see any changes in oocyst structure during or after the activation feeding. We speculate that such a feeding period is likely unnecessary for the transmission of *H. americanum* by *A. maculatum*. Although dog #3306 was exposed by tick bite as well as by ingestion, dog #s 3152 and 3305 became infected following exposure only via ingestion of infected ticks. It seems probable that *H. americanum,* like other *Hepatozoon* spp. for which life cycles are known, is transmitted by ingestion of the infected vector, not by the vector=s mouth parts.

Nordgren and Craig (1984) achieved experimental transmission of the "Texas strain" of *H. canis* in two splenectomized dogs. We chose to use one splenectomized dog (#3305), one intact (#3306) and a third (#3152) that was assumed to be immuno-compromised owing to *E. canis* infection. [Larsen et al. (1994) reported that *E. phagocytophila* infections can cause immunosuppression in sheep.] We fed 50 ticks to dog #3305, 20 ticks to dog #3152 and 13 ticks to dog #3306. We do not know the number of sporocysts or sporozoites ingested in any of these exposures, and we made no attempt to determine the minimum infective dose. Successful transmission of *H. americanum* to dog #3306 demonstrates that dogs need not be splenectomized or otherwise immuno-compromised for infection to occur.

Although we did not enumerate the oocysts in the ticks we fed to dogs, we did dissect numerous ticks from the same cohort for other studies. Among 88 ticks dissected, almost 97% (85 ticks) were found to contain oocysts; the number of oocysts recovered ranged from 4 to >150. This infection rate suggests that *A. maculatum* is easily infected with *H. americanum* and is well adapted as a suitable definitive host of *H. americanum.* Oocysts were multisporocystic and each sporocyst appeared to contain 8 or 16 sporozoites (Figs 5 and 6).

Most of the cases of canine hepatozoonosis reported from the USA are from areas where *A. maculatum* is known to occur. The Gulf Coast tick was formerly thought to feed on dogs only rarely. Larvae and nymphs feed mostly on birds, rodents and other small mammals; adults typically parasitize larger mammals (Bishopp and Hixson, 1936; Krull, 1969). There is likely a natural cycle in the wild that involves *A. maculatum* and some vertebrate host for *H. americanum*; dogs are probably accidentally inserted into that cycle

when they ingest ticks while grooming. We have made no attempt to determine whether larval *A. maculatum* will acquire infection nor have we attempted to produce infection by feeding either flat or replete nymphs to dogs.

Obviously, extensive studies are needed to determine the Anatural@ endemic cycle of this parasite. Once the Ausual@ vertebrate host(s) is (are) identified, development of the protozoan in these animals should be compared with that observed in naturally- and experimentally-infected dogs. The course of development of the parasite in *A. maculatum* should be compared with that of *H. canis* in its vectors and with other acarine-transmitted *Hepatozoon* spp.

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Table I

Summary of observations made on dogs that ingested either 'wild' ticks taken from dogs with hepatozoonosis or laboratory reared adult ticks that had been experimentally fed as nymphs on a dog with *Hepatozoon americanum* infection.

Dog No.	Leukocytosis	Fever	Muscle weakness	Cysts in muscle	Parasite in blood
2783[a, e]	-	-	-	-	-
2825[a, e]	-	-	-	-	-
3069[b]	-	-	-	-	-
3070[c]	-	-	-	-	-
3071[c]	-	-	-	-	-
3152[d, f]	+(4 wk)	+(27 d)	+(33 d)	+(5 wk)	+(34 d)
3305[d, g]	+(4 wk)	+(28 d)	+(36 d)	+(6 wk)	+(43 d)
3306[d]	+(5 wk)	+(29 d)	+(44 d)	+(6 wk)	+(47 d)

[a] Dogs exposed to 'wild' ticks.

[b] Dogs exposed to laboratory-reared *Amblyomma americanum.*

[c] Dogs exposed to laboratory-reared *Rhipicephalus sanguineus.*

[d] Dogs exposed to laboratory-reared *Amblyomma maculatum.*

[e] Dogs were given prednisone orally for a month after ingesting ticks.

[f] Dog was a convalescent carrier of *Ehrlichia canis* when ticks were ingested.

[g] Dog was splenectomized prior to being fed ticks.

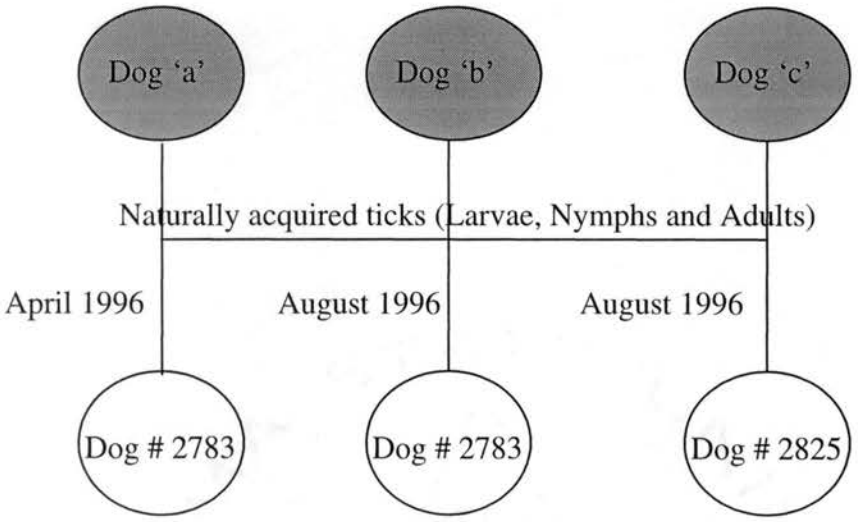
- indicates changes not observed following ingestion of ticks.

+ indicates deviation from 'normal'; numbers in parenthesis specify the time elapsed (d=days; wk= weeks) from ingestion of ticks until changes were first observed.

Fig 1. Experimental design and results, phase I; shaded circles represent three privately owned dogs with naturally occurring hepatozoonosis; nonshaded circles represent animals that remained uninfected after ingesting Awild@ ticks that had fed on the naturally infected dogs. Dog # 2783, exposed twice, was determined by muscle biopsy to be uninfected prior to second exposure.

Fig 2. Experimental design and results, phase II; shaded circle represents a dog naturally infected with *Hepatozoon americanum* that served as a donor-host for laboratory-reared nymphal ticks; nonshaded circles represent dogs that ingested those ticks as newly molted adults and remained uninfected.

Phase I



Phase II

(*Rhipicephlus sanguineus, Amblyomma americanum* and *Dermacentor variabilis*)

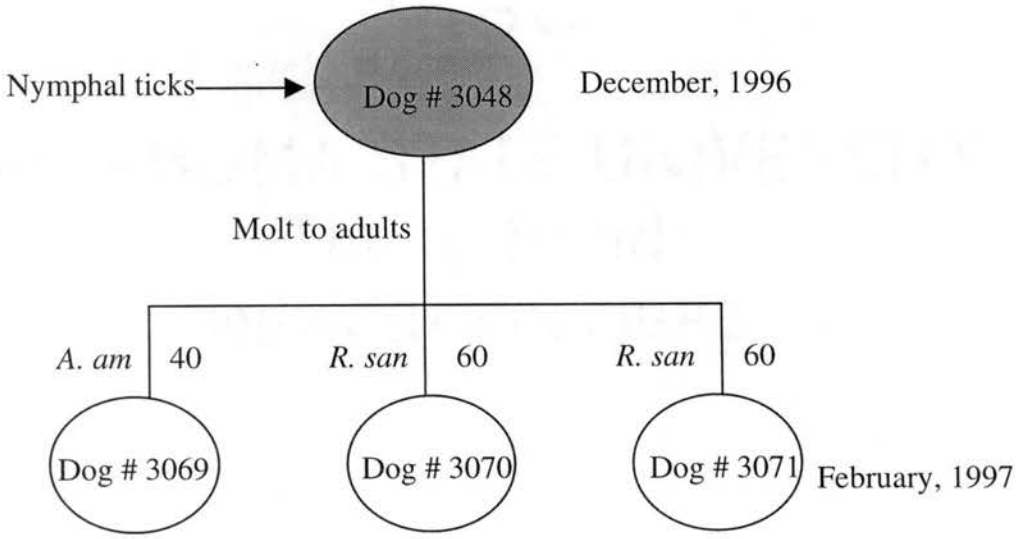
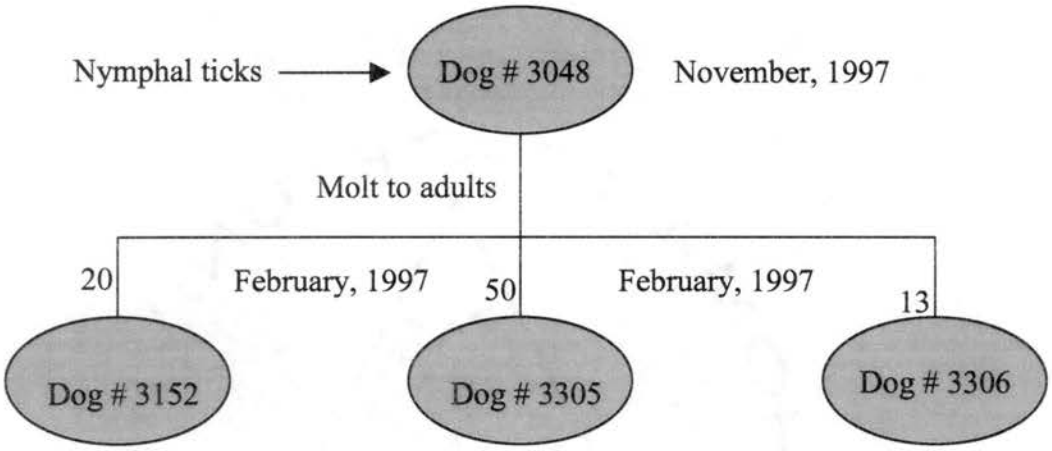


Fig 3. Experimental design and results, phase IV; shaded circles represent dogs infected with *Hepatozoon americanum;* dog #3048 had naturally occurring hepatozoonosis; and the other three developed disease after ingesting laboratory-reared adult *Amblyomma maculatum* that had fed as nymphs dog # 3048.

Fig 4. Unstained oocyst of *Hepatozoon americanum* mounted in saline; recovered from an adult *A. maculatum* experimentally infected as nymph. Notice oocyst wall (solid arrows), margin of air bubble in mounting fluid (open arrow) and another artifact (arrow head) on the surface of the oocyst. Bar = 200μm

Phase IV

(*Amblyomma maculatum*)



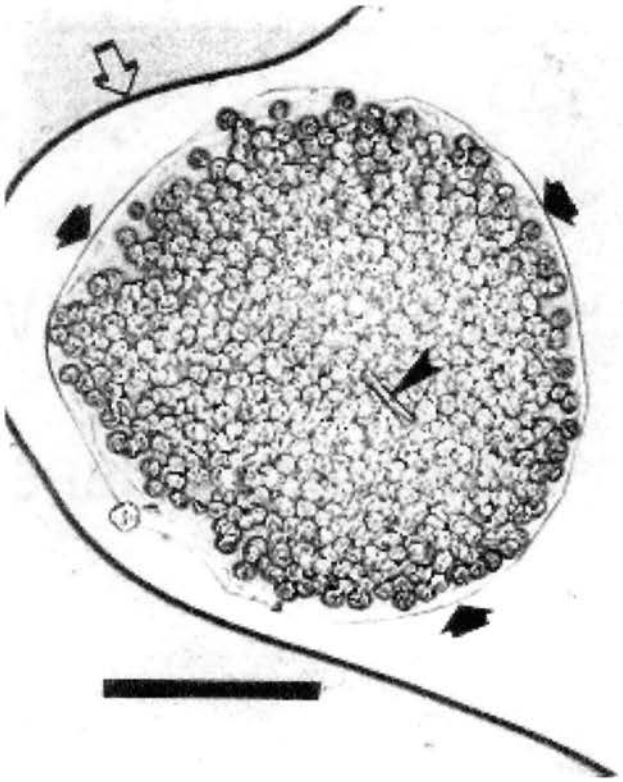


Fig 5. Unstained *Hepatozoon americanum* sporocysts containing tightly packed sporozoites. Bar =25μm

Fig 6. *Hepatozoon americanum* sporozoites mounted in saline; sporocyst was ruptured manually by applying pressure on the cover slip; arrow indicates nucleus of sporozoite and arrow head, the wall of the ruptured sporocyst. Bar =25μm

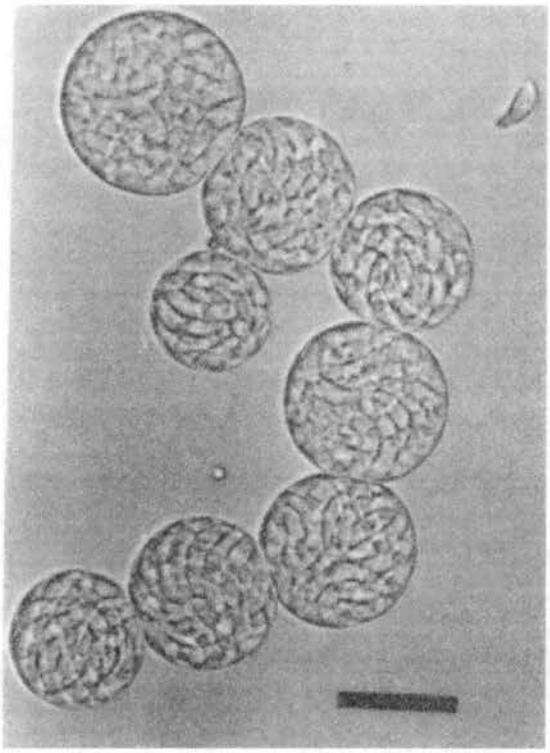


Figure 5

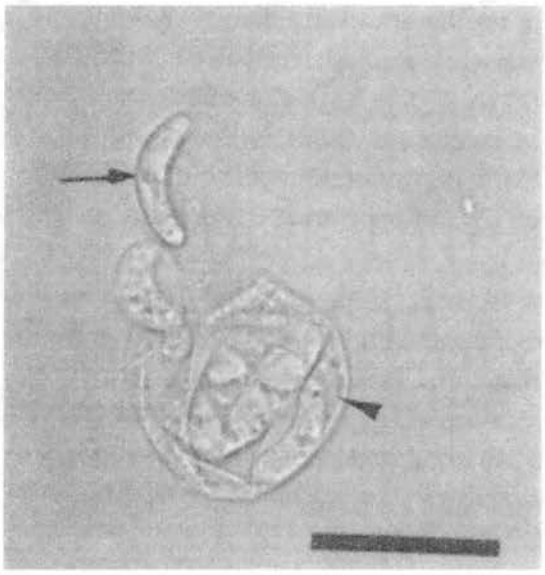
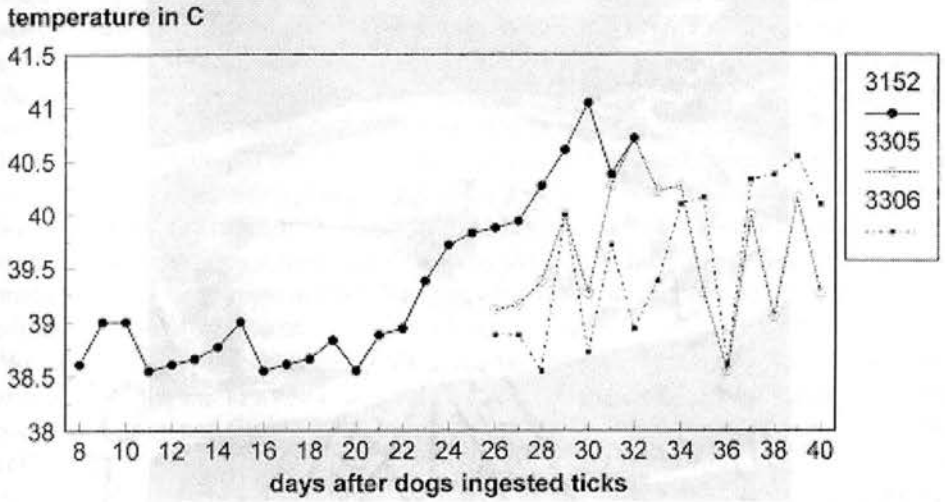


Figure 6

Fig 7. Rectal temperature changes of three dogs experimentally infected with *Hepatozoon americanum*, starting 8 (#3152) and 26d (#s 3305 and 3306) after exposure; dog #3152 was euthanatized 35d after ingesting infected ticks.

Fig 8. WBC count (number per microliter) of three dogs experimentally infected with *Hepatozoon americanum*; dog #3152 was euthanatized 35d after ingesting infected ticks.

Body temperature



WBC count

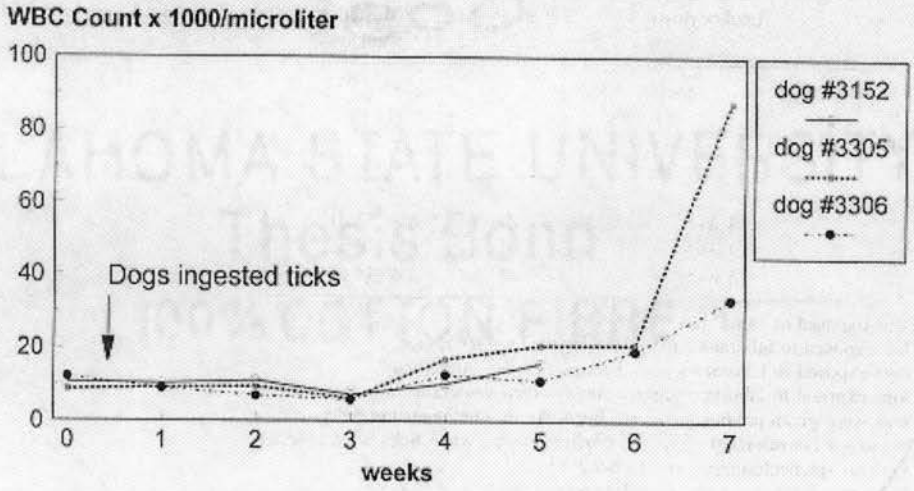
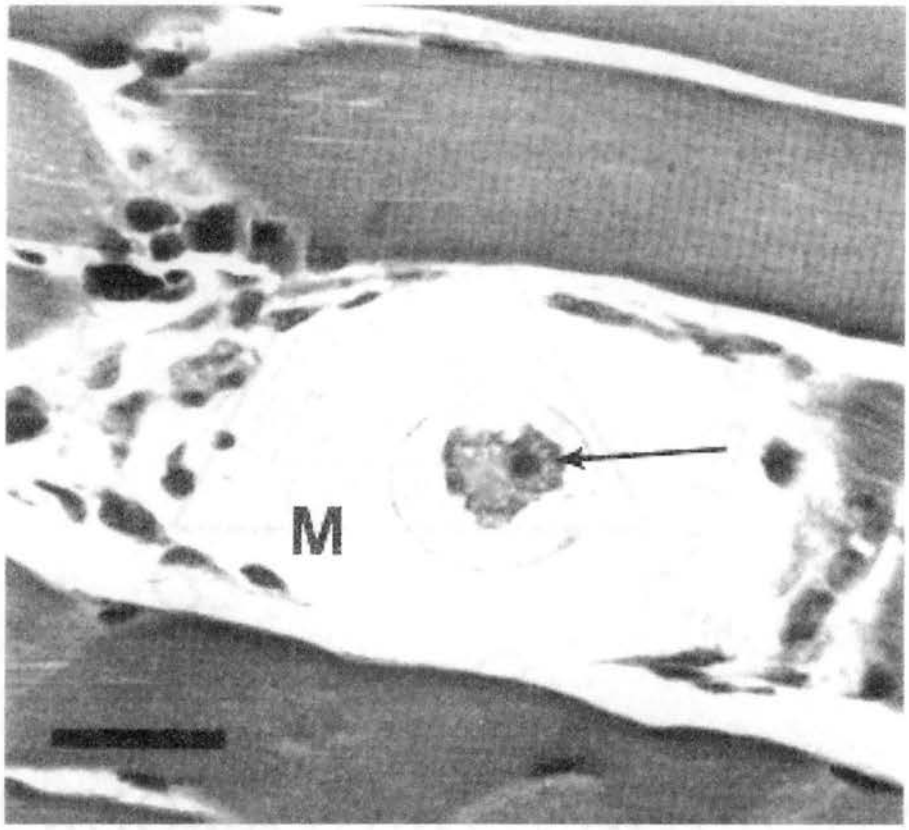


Fig 9. Mucopolysaccharide Acyst@ characteristic of *Hepatozoon americanum* infection, in skeletal muscle; Acyst@ contains a host (dog) cell; arrow indicates host cell nucleus containing enlarged nucleolus; M designates mucopolysaccharide Acyst@ material. The parasite is not visible in this specimen. Bar = 25μm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CHAPTER IV

SPOROGONIC DEVELOPMENT OF *HEPATOZOON AMERICANUM* (APICOMPLEXA) IN ITS DEFINITIVE HOST, *AMBLYOMMA MACULATUM* (ACARINA)

Abstract

Light microscopic observations of the sporogonic development of *Hepatozoon americanum* are described in its acarine host, *Amblyomma maculatum*. Laboratory-reared nymphal ticks were fed on 2 dogs infected with *H. americanum*. Nymphal ticks were sampled daily, starting 3 days after being placed on a parasitemic dog, until 18 days after infestation (PI), and then every 3 or 4 days until replete nymphs molted. Ticks were examined as unstained wet mounts and hematoxylin-eosin stained paraffin sections. Gametes were found within the gut cells of nymphs 4 and 6 days PI. Although differentiation of gamonts into gametes was not detected, syngamy and sporogony were observed. Sporogony appears to occur wholly within tick gut cells, followed by release of mature oocysts into the hemocoel. The earliest evidence of sporoblast formation was observed 23 days PI and of sporozoite formation, 10 days later. Mature oocysts were first found 42 days PI in newly molted adult ticks. Most adult ticks (>98%) that were dissected contained mature oocysts. Oocysts were multisporocystic and sporocysts contained a variable number of sporozoites. Oocysts in various stages of development were often seen within the same tick, and the number of mature oocysts ranged from 4 to 573.

Introduction

Canine hepatozoonosis is an emerging tick-borne disease of dogs in the USA. The disease was first reported from dogs in Texas (Craig et al., 1978) and subsequently from Louisiana (Gosset et al., 1985), Alabama, Georgia (Macintire, et al., 1997), and Oklahoma (Panciera et al., 1997). The etiologic agent was originally suspected to be a particularly virulent strain of *Hepatozoon canis* (Craig et al., 1978), which causes canine hepatozoonosis in the Old World (Africa, Asia and Europe). Later, the causative organism was classified as a new species, *H. americanum*, based on the asexual stages of the parasite in dogs and severity of the disease it produced (Vincent-Johnson et al., 1997). Recently, experimental transmission studies demonstrated that the Gulf Coast tick, *Amblyomma maculatum,* is an excellent definitive host for *H. americanum* (Mathew et al., 1998).

The genus *Hepatozoon* was erected by Miller (1908) and is classified in the Hepatozoidae, created by Wenyon (1926). The life cycle of the members of Hepatozoidae is characterized by merogony occurring in vertebrate host tissues, resulting in production of merozoites; gamonts that arise from merozoites are found within circulating blood cells from where they are ingested by a blood-sucking, invertebrate, definitive host. Syngamy and sporogony then occur in the invertebrate host, leading to production of sporozoites that are infective for vertebrate hosts upon ingestion of the definitive host (Wenyon, 1926). Based on the structure of gamonts and on life-cycle patterns in both vertebrate and invertebrate hosts, more than 300 species are currently assigned to the genus *Hepatozoon* (Smith, 1996).

In studies on the development of *H. canis* in its definitive host, *Rhipicephalus sanguineus,* gametes were shown to fuse in the tick gut lumen with subsequent development occurring in pouches or diverticula attached to the outer gut wall (Wenyon, 1911; Christophers, 1912). Studies on the sporogonic development of other *Hepatozoon* spp. indicate that the gametes may fuse in the gut lumen, hemocoel, or within a host cell, with subsequent development of the zygote occurring in the hemocoel of the arthropod host (Miller, 1908; Hoogstraal, 1961; Smith, 1996).

Histopathologic examination of tissues from dogs infected with *H. americanum* revealed merogony to occur primarily in skeletal muscles, and gamonts are found within circulating leukocytes (Craig et al., 1978; Panciera et al., 1997; Vincent-Johnson et al., 1997). Syngamy and the sporogonic phase of development of *H. americanum* in its definitive host have not been described. Herein, we report light microscopic observations on the development of *H. americanum* in its invertebrate host, *A. maculatum*. Comparisons are made with other *Hepatozoon* species transmitted by arthropods, with special attention given to *H. canis* in *R. sanguineus*.

Materials and Methods

Experimental animals

A dog (# 3048) naturally infected with *Hepatozoon americanum,* another dog (# 3306) experimentally infected with this parasite and a laboratory-reared sheep were used in this study. Dogs and sheep were cared for by the Laboratory Animal Resources Unit (LAR) of Oklahoma State University (OSU).

Laboratory-reared nymphal ticks (*Amblyomma maculatum*) were purchased from the Tick Rearing Laboratory, Oklahoma Agricultural Experiment Station, Oklahoma State University. These nymphal ticks were allowed to feed on either dog # 3048 or dog #

3306 as described by Mathew et al. (1998). Dogs were determined to be parasitemic during tick feeding by evaluation of blood smears. Dog #3048 was a chronic carrier (infected for more than 2 years) of *H. americanum* and dog #3306 was in the acute phase of infection at the time of exposure to ticks. Replete nymphs were kept in a humidity chamber [relative humidity 90%; photoperiod of 14:10 (light:dark)] until they molted to the adult stage. Uninfected control nymphal ticks were allowed to feed to repletion on a sheep. Control ticks were maintained under conditions similar to those of ticks that fed on infected dogs.

Examination of developmental stages

Amblyomma maculatum nymphs that acquired infection on dog #3048 were dissected and examined as wet mounts (ticks were dissected in phosphate buffered saline, placed on a glass slide, and examined immediately by light microscopy) only after they had molted to become adults and oocysts were already fully mature. In contrast, nymphs exposed by feeding on dog #3306 were examined daily beginning 3 days post-infestation (PI) at which time the nymphs were partially replete through 18 days PI and then every 3 or 4 days until the engorged nymphs molted to the adult stage (molting occurred 42-45 days after unfed nymphs were placed on the host). Of 5 ticks collected from dog #3306 on each sampling day, 2 were examined as wet mounts and 3 were bisected, fixed in phosphate buffered formalin, and embedded in paraffin. Sections stained with hematoxylin-eosin were examined by light microscopy (USNPC No. 88505 is a hematoxylin-eosin stained section of a nymphal tick, 34 days PI, containing developing oocysts). Uninfected control nymphal ticks obtained at days 4, 10, 18, and 43 after being placed on a sheep were processed and examined in similar manner. Dimensions of

developmental stages of the parasite were determined at 6, 10, 15, 20, 27, and 33 days PI, using a calibrated ocular micrometer. Measurements of mature oocysts and sporocysts were made by using CLEMEX Vision Lite® image analysis software (Clemex Corporation, Dublin, Ohio, USA) and those of sporozoites using a calibrated ocular micrometer.

The number of oocysts per infected tick was determined by counting those from 120 adult *A. maculatum* (96 that fed on dog #3048 and 24 that fed on #3306). The number of sporocysts per oocyst was estimated by counting those from 20 oocysts of various sizes; each oocyst was ruptured separately and the sporocysts from individual oocysts were suspended in 200 µl of distilled water. Sporocysts were then counted in 20 µl aliquots of the suspension using a compound microscope at 100X magnification.

Sporocysts from mechanically ruptured oocysts were treated in different ways to determine the optimum conditions for excystation of sporozoites. In preliminary trials, digestive juice from both the duodenum and jejunum of a dog [as well as various components of digestive juice such as pepsin (1, 5, and 10%), trypsin (1, 5, and 10%), diluted hydrochloric acid (1 and 5%), bile, and pancreatic extract (concentration not determined)] were tried, and bile was found most satisfactory. Sporocysts from mechanically ruptured oocysts were treated with either undiluted or diluted (50% and 10%, diluted with distilled water) bile. Observations were made by placing sporocysts in a drop of bile solution on a glass slide with a coverslip. Excystation was monitored through a compound microscope at 400x magnification, and the released sporozoites from 15 sporocysts were counted individually.

Results

Among the 400 ticks that fed as nymphs on the infected dogs (#s 3048 and 3306) and that were dissected as adults, all but 4 were infected with *H. americanum.* The number of oocysts within individual ticks was estimated from 120 of the 400 ticks and found to range from 4 to 573. Examination of replete or partially replete nymphal ticks that fed on dog # 3306 (acutely infected) revealed syngamy and sporogonic development of *H. americanum* in cells located in the wall of the gut (Fig. 1a). Developmental stages of *H. americanum* were not observed in uninfected control ticks that engorged on sheep (Fig. 1b). Sporogony was observed in a single type of cell, although specific identity of this cell type was not determined. *Hepatozoon americanum* development was not observed in any other tick tissue, including salivary glands. Oocysts were found at various stages of development within a given tick (Fig. 2).

Fertilization of gametes

Microgametes and macrogametes (Fig. 3) were observed in gut cells of ticks dissected 4 days and 6 days PI. Fusion of 2 gamete nuclei, interpreted as syngamy (Fig. 4), was observed in cells of ticks 7 days PI. Differentiation of gamonts to gametes was not observed in this study, nor was evidence of exflagellation of microgametes and ookinete formation detected in any of the ticks examined.

Development of oocysts

Early oocysts (zygotes) were first demonstrable within a host cell at 6 days PI; some sections with zygotes also contained gametes. The zygote was a round structure with a large, diffuse nucleus containing a dark-staining nucleolus (Fig. 5). Zygotes increased in size as they developed to become mature oocysts. Dimensions of developing oocysts at

6, 10, 15, 20, 27, and 33 days PI are presented in Table I. At 10 days PI, the zygote was found in a large parasitophorous vacuole within the host cell, displacing the host cell nucleus (Fig. 6); at this time, parasites were first visible in wet mount preparations. Several irregularly arranged nucleoli were visible within the nucleus and dark-staining granules appeared in the cytoplasm of the parasite between 13-17 days PI (Fig. 7). The nucleolus of the zygote/uninucleate oocyst began to divide by 13-15 days PI and the parasite cytoplasm became highly vacuolated (Fig. 8). By 18 days PI, multiple nucleoli were found radially arranged within the parasite nucleus. The dark nucleoli disappeared prior to first nuclear division, the parasite nucleus became spindle shaped and positioned towards the periphery of the zygote (Fig. 9). Nuclear division was first observed 20 days PI (Fig. 10a). In wet mount preparations at this stage, developing oocysts appeared to be in pouches attached on the hemocoel side of the gut (Fig. 10b).

Sporocyst formation

Sporocyst formation was first observed at 23 days PI; several nuclei were arranged along the periphery of the central cytoplasmic mass and there were infoldings of the oocyst around each nucleus (Fig. 11). Wet mount preparations examined at 23 days PI showed infoldings along the outer margin of the developing oocyst within the newly-visible oocyst wall. Sporocysts, each containing a nucleus, were first observed to form 27 days PI. Individual sporocysts appear to form by a process of budding that left a residual body (Figs. 12a and 12b). Newly formed sporocysts, initially irregular in shape (Fig 13), became spherical by 30 days PI (Fig. 14a) when sporozoites were not yet apparent within the sporocysts (Fig. 14b).

Sporozoite formation

Sporozoite formation was observed first at 33 days PI. In both wet mount preparations and paraffin sections, sporozoites appeared as small bodies arranged along the periphery of the sporocysts, mostly at 1 pole (Fig. 15). Sporozoites appeared to form by endopolygeny from the sporoblasts, leaving a residual body. Fully formed sporozoites were first observed in sporocysts at 42 days PI in newly molted adult ticks (Fig. 16).

Mature oocysts

Mature oocysts were found free in the hemocoel of adult ticks at 42 days PI; other, nearly mature, oocysts were still within host cells. Ticks that fed on the chronically infected dog (#3048) contained fewer oocysts (4-175, mean = 33 +/- 27, N= 100) than did those that fed on the acutely infected dog (#3306) (263-573, mean = 441+/- 107, N= 24). Oocysts recovered from ticks that contained fewer oocysts were greater in size (175-495μm, mean = 294 +/- 43, N = 100) than those from ticks containing larger numbers of oocysts (145-405μm, mean = 225 +/-37, N = 100). Oocysts were intact in the hemocoel, and neither sporocysts nor sporozoites were ever found free in the gut or hemocoel of the ticks. Oocysts were multisporocystic (Fig. 17) and the number of sporocysts per oocyst ranged from 260 to 1,040 (mean = 656 +/- 176, N=20). Sporocysts were spherical (Fig. 18) and had a thick wall and measured 18 – 39 μm (mean = 27.8 +/- 4.8 μm, N=100) in diameter.

Undiluted digestive juice from upper small intestine of dogs stimulated release of sporozoites from sporocysts. Sporozoites were not released when sporocysts were treated separately with pepsin, trypsin, hydrochloric acid or pancreatic extract. However, bile (undiluted and diluted) collected from the gall bladder of a dog consistently stimulated

sporozoites to leave the thick-walled sporocysts. The number of sporozoites/sporocyst (N=15) was variable, ranging from 10 to 26. Sporozoites were banana-shaped and nearly uniform in size (mean = 15 +/- 1.2 x 5 μm) with a centrally located nucleus (Fig. 19).

Discussion

Syngamy and sporogonic development of *Hepatozoon americanum* in its definitive host, *Amblyomma maculatum,* is reported for the first time. Identification of the invertebrate definitive host, successful experimental infections (Mathew et al., 1998), and consistently high infection rates of the ticks made this study possible. This is the first report of a *Hepatozoon* species in which syngamy and sporogonic development occurs entirely within the gut cells of the arthropod host. Smith and Desser (1997) reported that syngamy and sporogonic development of many *Hepatozoon* spp. occurs within the hemocoel of the definitive host. Syngamy and the entire sporogonic development of *H. americanum*, in contrast, appear to occur within gut cells of the tick host (Figs. 6, 7, 8, 9, 11, 14a).

In the present study, gametes were observed within unidentified gut cells of nymphal ticks 4 days and 6 days after they were placed on a dog infected with *H. americanum*. In contrast, macrogametes and microgametes of *H. perniciosum* (Miller, 1908) and of *H. catesbianae* (Desser et al., 1995) were found free in the gut lumen and hemocoel of their respective arthropod vectors (the mite, *Lelaps echidninus*, and the mosquito, *Culex territans*) 24 hr after ingestion of an infected blood meal. *Hepatozoon americanum* gametes were not found free in the lumen of the tick gut, but we did not look until the third day after ticks were placed on the dog and therefore cannot make definite

conclusions on this matter. Christophers (1912) observed vermicules (probably gamonts) of *H. canis* free within the gut of replete nymphal *R. sanguineus*.

We did not observe exflagellation of microgametes or ookinete formation in this study, a finding similar to that of Smith and Desser (1997) who studied *H. sipedon* in its mosquito definitive host, *Culex pipiens*. Ticks collected 7 days PI, revealed what is interpreted to be syngamy; the process appeared very similar to syngamy observed by Miller (1908) for *H. perniciosum* in its mite host, *L. echidninus*, and by Redington and Jachowski (1971) for *H. griseisciuri* in its mite vector, *Haemogamasus reidi*.

Allison and Desser (1981) observed in heavily infected mites that sporogonic development of *H. lygosomarum* occurred equally well in both sexes and that oocysts were found at various stages of development in a given mite. Similarly, in our study, sporogonic development occurred equally well in male and female ticks, and parasites were seen at various stages of development within the same tick.

Early oocysts of *H. americanum* observed within gut cells at 6 days PI appeared similar to those of other *Hepatozoon* spp. (Miller, 1908; Furman, 1966; Redington and Jachowski, 1971; Allison and Desser, 1981). By 10 days PI, considerable increase in the size of the parasite resulted in displacement of the host cell nucleus. Distortion of infected tick cells resembled that seen in the host cells of dogs that contain meronts of *H. americanum* (Panciera et al., 1998). By 13 days PI, there were several dark-staining granules in the parasite cytoplasm. These dark-staining granules appeared similar to the wall-forming bodies observed in the zygotes of many coccidia, which eventually contribute to the formation of the rigid oocyst wall (Hammond, 1973). We do not know the significance of the granules found in the zygotes of *H. americanum*, but their

disappearance prior to sporocyst formation suggests that they may have a role in formation of the sporocyst wall. Prior to the first nuclear division of the zygote, the nucleus became spindle-shaped and positioned towards the periphery of the cell (Fig. 9); this process appeared to be similar to meiosis described by Canning and Anwar (1968) in the zygotes of *Eimeria tenella* and *E. maxima*.

Sporocyst formation of *H. americanum* appeared to be identical to that described for *H. canis* by Wenyon (1911). After several nuclear divisions, daughter nuclei were arranged along the periphery of the developing oocyst. A similar process has been observed for other *Hepatozoon* species as well (Miller, 1908; Christophers, 1912; Hoogstraal, 1961; Redington and Jachowski, 1971; Allison and Desser, 1981). Sporocysts were eventually formed by a process that resembled budding, leaving a residual body. This process was very similar to that observed for *H. perniciosum* (Miller, 1908), *H. canis* (Wenyon, 1911), and *H. lygosomarum* (Allison and Desser, 1981).

The early process of sporozoite formation in the development of *H. americanum* appears to differ from that in *H. perniciosum* (Miller, 1908) and *H. canis* (Wenyon 1911). In those instances, after division of the sporocyst nucleus, daughter nuclei are arranged at 2 poles (bipolar) along the periphery of the sporocyst, whereas for *H. americanum* they were predominantly unipolar. The sporozoites of *H. americanum* grow out from the cytoplasmic mass of the sporocyst leaving a residual body similar to that observed for *H. perniciosum* (Miller, 1908) and *H. canis* (Wenyon, 1911; Christophers, 1912). This process of sporozoite formation in *H. americanum* resembles endopolygeny described by Hammond (1973) in the life cycle of certain coccidia.

Wenyon (1911) and Christophers (1912) reported finding mature *H. canis* oocysts in pouches formed by the membranes of the tick gut, appearing as if attached by a delicate pedicle to the outer gut wall; those authors interpreted the pouch to communicate with the gut lumen. Our observations of wet mount preparations from infected ticks also showed oocysts that appeared to be in pouches protruding into the hemocoel from the outer gut wall. However, examination of stained paraffin sections of companion ticks revealed that the pouches were, in fact, enlarged parasitophorous vacuoles within host gut cells and there was no connection to the gut lumen.

In newly molted adult ticks, mature oocysts of *H. americanum* were found free in the hemocoel. It appears that once oocysts are fully mature they are released from the host cell and are then free in the hemocoel. The precise time when oocysts are released from the host cell into the hemocoel could not be determined, but mature oocysts were first found free in the hemocoel 42 days PI. In heavily infected ticks that contained large numbers of mature oocysts free in the hemocoel, additional oocysts, still attached to the gut wall, were often observed as late as 5 mo after ticks molted.

Oocysts in the hemocoel were intact and free sporocysts and sporozoites were never observed in the hemocoel of any of the more than 400 ticks examined. Wenyon (1911) reported dissolution of the oocyst wall and liberation of sporocysts within the hemocoel of *R. sanguineus* during *H. canis* development, but this phenomenon apparently does not occur in *H. americanum*. Perhaps *H. canis* sporocysts are routinely released into the hemocoel of *R. sanguineus*; alternatively, forms observed by Wenyon (1911) might have been the result of oocysts rupturing during the dissection of ticks.

Mature oocysts of *H. americanum* were spherical (Fig. 17) and varied considerably in size. Smith (1996) suggested that the dimensions of oocysts and sporocysts, number of sporocysts per oocyst, and number of sporozoites per sporocyst can be influenced by a number of factors, e.g., infection load of the arthropod vector, species of the vector, and the temperature at which vectors live while parasites develop. Our observations support Smith's conclusion; oocysts were larger in ticks with fewer oocysts. Our results also demonstrate that nymphal ticks that fed on an acutely infected dog had larger numbers of oocysts than did those that fed on a chronically infected dog. We speculate that this difference may be the result of increased numbers of circulating gamonts during acute infection; however, the number of dogs on which ticks engorged is too small to draw conclusions.

As is typical for *Hepatozoon* species generally, the number of sporocysts per oocyst of *H. americanum* is variable, but among 20 oocysts for which counts were made, it was always >200 (260-1,040; 656 +/- 176). *Hepatozoon canis* oocysts that develop in *R. sanguineus* typically contain <150 sporocysts (Christophers, 1911; Wenyon, 1912). Sporocysts of *H. americanum* are spherical and in this way are similar to those of *H. lygosomarum* (Allison and Desser, 1981). They differ, however, from *H. perniciosum* (Miller, 1908), *H. canis* (Wenyon, 1911), *H. balfouri* (Furman, 1966), and *H. griseisciuri* (Redington and Jachowski, 1971), all of which have elliptical sporocysts.

The number of sporozoites per sporocyst in *H. americanum* ranged from 10-26. Our observations are consistent with others in that the number of sporozoites per sporocyst are often variable for *Hepatozoon* spp. For example, Miller (1908) reported 16-24 for *H. perniciosum*, Christophers (1912) 12-20 for *H. canis*, Allison and Desser (1981) 4-6 for

H. lygosomarum, and Furman (1966) 4-8 for *H. balfouri*. The sporozoites of *H. americanum* are banana-shaped with a centrally located nucleus similar to that of other *Hepatozoon* spp. (Wenyon, 1911; Christophers, 1912; Furman, 1966; Redington and Jachowski, 1971; Allison and Desser, 1981; Desser et al. 1995; Smith and Desser, 1997).

Like all other *Hepatozoon* spp. for which life cycle information is available, *H. americanum* infection of vertebrate hosts under natural conditions likely occurs only through ingestion of the infected definitive host. In this, our observations are consistent with those of Christophers (1912) who concluded that *H. canis* could be transmitted only by ingestion of infected ticks that contained mature oocysts because free sporocysts and sporozoites were not detected in salivary glands of infected ticks.

Sporozoites of *H. americanum* were easily liberated from the sporocysts when treated with bile collected from the gall bladder or with digestive juice from either the duodenum or the jejunum of a dog. Miller (1908) made similar observations, finding that sporozoites were liberated from sporocysts of *H. perniciosum* when treated with digestive juice from the small intestine of its vertebrate host, the white rat. These observations suggest that bile is essential for release of sporozoites from the sporocysts under natural conditions.

Further studies are needed to reveal the gametogenesis, and fertilization processes of *H. americanum.* Likewise, ultrastructural studies will be required to detail further the sporogonic developmental cycle. It may be important, as well, to learn whether or not sporogonic development will occur in larval ticks that ingest gametocytes. Such information could, in turn, help to better understand the epidemiology of American canine hepatozoonosis.

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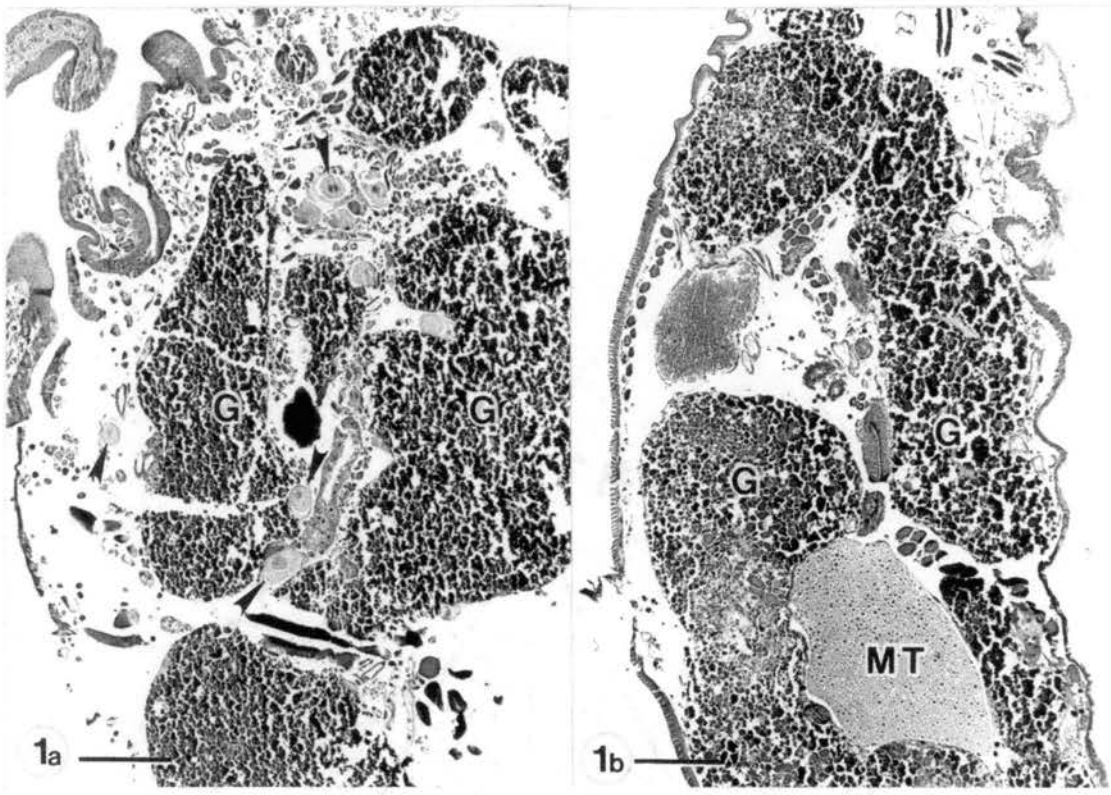
Table I. Dimensions of *Hepatozoon americanum* oocysts (in micrometers, μm) in unidentified tick gut cells, as determined from hematoxylin and eosin-stained paraffin sections.

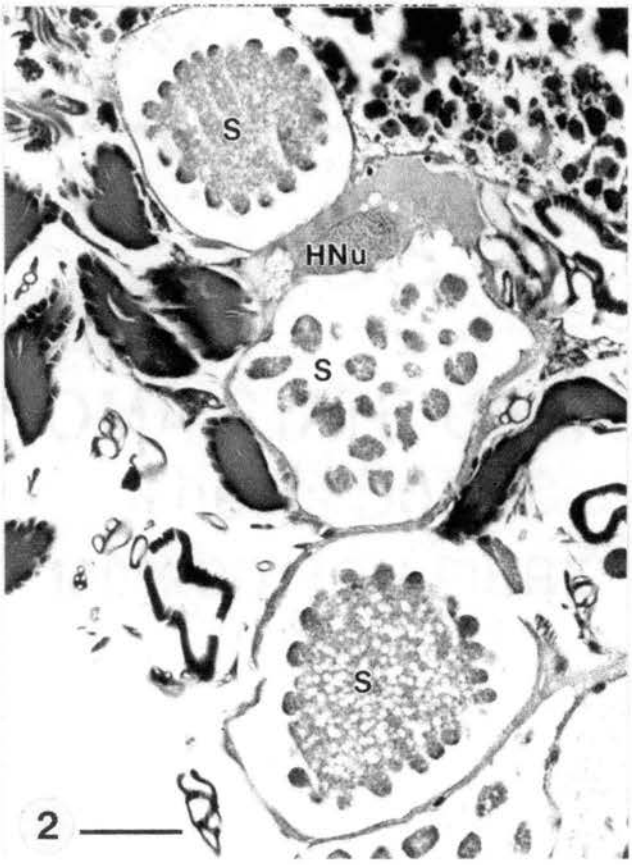
Time (days) after tick infestation of dogs	Number measured	Range (μm)	Mean (μm)
6	10	10-37.5	19.0
10	10	32.5-53.75	47.0
15	10	60-85	74.5
20	10	65-105	82.0
27	10	125-195	153.5
33	10	205-290	244.0

SPOROGONY - PLATE I

FIGURES 1a and 1b. Photomicrographs of hematoxylin and eosin stained paraffin sections of nymphal *Amblyomma maculatum* 18 days PI. **1a.** Developing oocysts (arrows) closely associated with tick gut (G). **1b.** An uninfected control; notice gut (G), Malpighian tubule (MT) and absence of oocysts. Scale bars: 1a and 1b = 250μm.

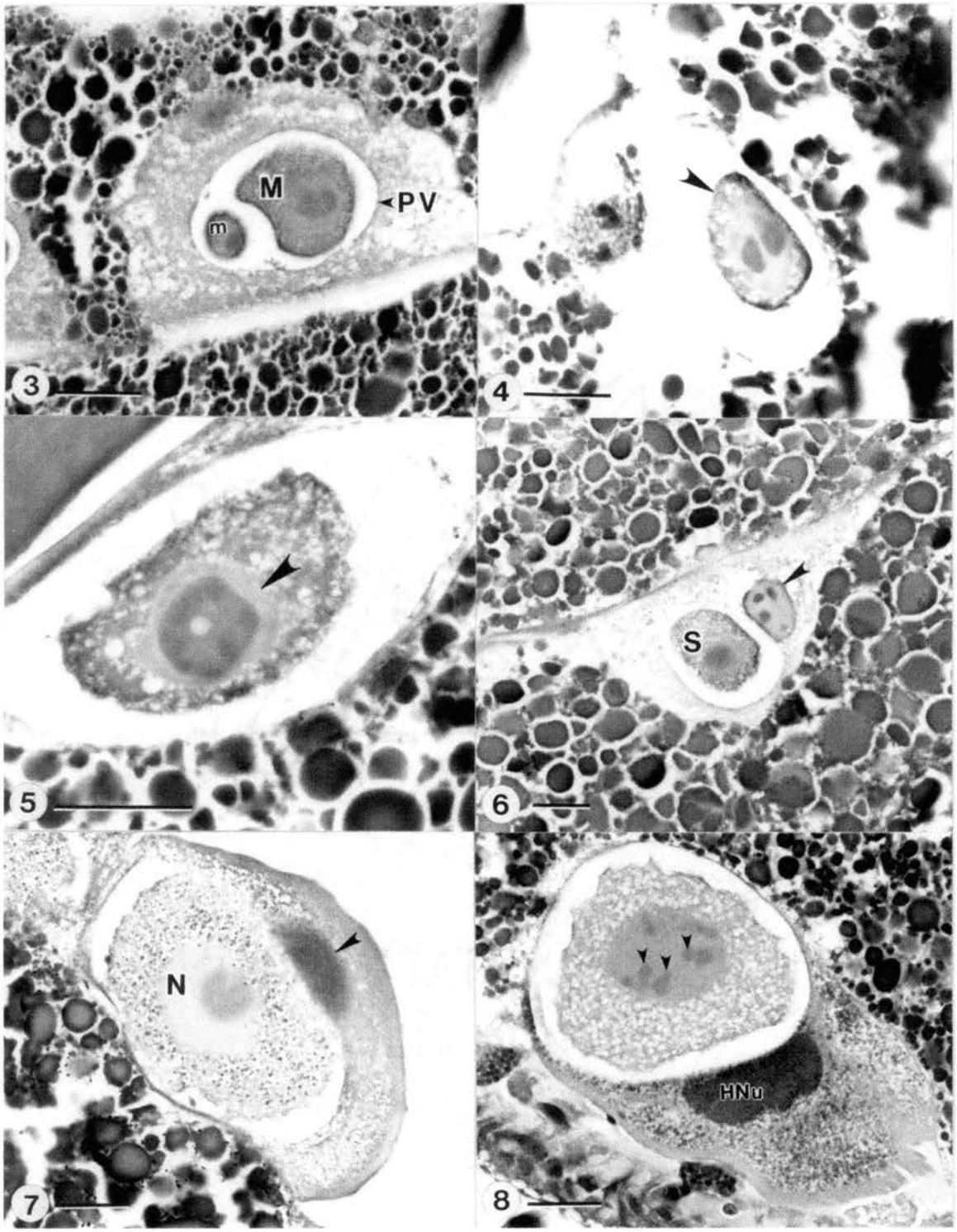
FIGURE 2. Photomicrograph of hematoxylin and eosin stained paraffin section of a nymphal *Amblyomma maculatum* 27 days PI; notice 3 developing oocysts (S) at various stages of development and host cell nucleus (HNu). Scale bar = 50μm.


G
G
1a
G
G
MT
1b


S
HNu
S
S
2

SPOROGONY - PLATE II

FIGURES 3-8. Photomicrographs of developmental stages of *Hepatozoon americanum* within gut cells of nymphal *Amblyomma maculatum* from 6 to 15 days PI in hematoxylin and eosin stained paraffin sections. **3.** Macrogamete (M) and microgamete (m) within a parasitophorous vacuole (PV), 6 days PI. **4.** Binucleate structure (arrow) interpreted to be syngamy, 7 days PI. **5.** An early oocyst (zygote) with a large, diffuse nucleus (arrow) containing a dense nucleolus, 9 days PI. **6.** Zygote (S) with vacuolated cytoplasm and a large, diffuse nucleus containing a dense nucleolus, 10 days PI. The host cell nucleus (arrow) containing multiple nucleoli is displaced by the developing parasite. **7.** Zygote with a dark staining nucleolus within the pale nucleus (N), 13 days PI. The cytoplasm of the parasite is vacuolated and contains dark staining granules; notice the displaced host cell nucleus (arrow). **8.** Zygote/uninuclear oocyst with several darkly stained irregularly arranged nucleoli (arrows) within the nucleus,15 days PI. Scale bars: 3-8 = 25μm.


M
m
PV
3
4
5
S
6
N
7
HNu
8

SPOROGONY - PLATE III

FIGURES 9-12b. Photomicrographs of developmental stages of *Hepatozoon americanum* within gut cells of nymphal *Amblyomma maculatum* from 20 to 27 days PI in hematoxylin and eosin stained paraffin sections and in unstained wet whole mounts. **9.** Zygote with spindle shaped nucleus (arrow) positioned towards the periphery prior to the first nuclear division; notice compressed host cell nucleus (HNu), 20 days PI. **10a.** Zygote with 2 nuclei (arrows) arranged along the periphery, 20 days PI. **10b.** Wet mount of a zygote in a parasitophorus vacuole; notice that the pouch like structure protrudes into the hemocoel and the developing oocyst has a distinct wall (arrow), 20 days PI. **11.** Developing oocyst with several peripherally-arranged nuclei (arrows) within a host cell in which the nucleus (HNu) is displaced, 23 days PI. **12a.** Section through a developing oocyst in which sporocysts (arrows) are forming, 27 days PI. **12b.** Wet mount of immature oocyst containing developing sporocysts (arrows) and a residual body (RB), 27 days PI. Scale bars: 9-12b = 25μm.