TRANSMISSION OF LOW PASSAGE *EHRLICHIA CANIS* TO DOGS BY NATURALLY FED AND EXPERIMENTALLY INJECTED *RHIPICEPHALUS SANGUINEUS* TICKS

by

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(Under the Direction of Donald Champagne)

ABSTRACT

Experimental transmission of *Ehrlichia canis* was conducted using laboratory-reared *Rhipicephalus sanguineus* ticks that were fed as nymphs on dogs infected with low passage field isolates, or by experimental injection of replete nymphs with white blood cells isolated from infected dogs. After molting, 30 naturally infected and 70-150 injected ticks were applied to *E.canis* negative dogs. The ticks infected by feeding successfully transmitted *E.canis* to dogs demonstrated by the development of clinical signs typical of Ehrlichiosis (12 of 12 dogs) and by PCR (10 of 12 dogs). *E.canis* transmission to dogs was also demonstrated with the experimentally injected ticks through analysis of clinical signs (4 of 4 dogs) and PCR (3 of 4 dogs). This study accomplished successful transstadial and horizontal transmission of *E.canis* using low numbers of ticks infected with *E.canis* field isolates that were not laboratory cultured. This study also demonstrated for the first time, successful transmission of *E.canis* by ticks that were experimentally injected with the bacterium as engorged nymphs.

INDEX WORDS: *Ehrlichia canis*, Transstadial Transmission, *Rhipicephalus sanguineus*, Experimental Injection, Canine Moncytic Ehrlichiosis (CME)

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CHAPTER 1

INTRODUCTION

Ehrlichia canis is an obligate intracellular bacterium that causes Canine Monocytic Ehrlichiosis, an important veterinary disease. Canine Monocytic Ehrlichiosis (CME) is a tick-vectored disease that was first discovered in Africa and has since been reported throughout the world. In the United States it is reported nationally, with most reports coming from the southeastern and southwestern states. The brown dog tick, *Rhipicephalus sanguineus*, was the first arthropod to be described as a vector of *Ehrlichia canis* (1). The vector potential of *Rhipicephalus sanguineus* for *E. canis* was re-established in a more recent study in which high numbers of adult stage ticks were used to transmit *E. canis* to dogs (2). While the large number of ticks that were used for this study did not represent the natural environment, the study did reconfirm the transmission of the organism by *Rhipicephalus sanguineus* ticks. To date the American dog tick, *Dermacentor variabilis*, is the only other tick vector that has been shown to transmit this pathogen (3). It is reported that the tick only transmits this bacteria transstadially and that trans-ovarial transmission does not occur (11).

While it is known that *E. canis* is transmitted horizontally through blood feeding by an infected tick, very little detail has been reported about the process of transmission for this pathogen. In comparison, the transmission process has been extensively described for *Rickettsia rickettsi*, and this model has been used as the scenario for most Ehrlichial and Rickettsial diseases (8). If *E. canis* follows a similar transstadial transmission process, then it may be assumed that after a larvae or nymph stage tick acquires an infectious blood meal the Ehrlichiae will initially infect the midgut epithelium, then spread throughout the tick's body infecting most, if not all, tissues. Ultimately, the

salivary glands will become infected and the tick will transmit the bacteria through its saliva during blood feeding as a nymph or adult. The efficiency with which a tick can acquire and transmit *E. canis* has not been completely determined. This detail in the transmission process needs to be further explored so that a comprehensive model of *E. canis* transmission can be described.

Once the *Ehrlichia* enter the canine host, the organisms circulate throughout the body infecting mononuclear cells and tissues such as the spleen and lymph nodes. This process of Ehrlichial invasion of the canine host has been developed using needle inoculation of the bacterium. The delivery of *Ehrlichia* by a tick may or may not change the process in which invades the host animal. Needle inoculated *Ehrlichia* invade canine host cells as small spherical structures about 1-2 micrometers in diameter. These initial bodies develop into larger structures termed morula, that form membrane-bound inclusions within the cytoplasm of the cell. Ultimately the morula will lyse the host cells and dissociate into small granules that infect new host cells and tissue. Generally an 8-20 day incubation period occurs after introduction of the ehrlichial organisms into the host by needle inoculation (6). This is followed by an acute phase of the disease, followed by a sub-clinical phase, and in some cases a chronic phase of Ehrlichiosis will occur. Hematological analysis reveals thrombocytopenia, mild anemia, and mild leukopenia during the acute phase, which typically occurs between weeks 2 -5 post needle inoculation. Mild thrombocytopenia generally occurs in the sub-clinical phase, which can last from 2 to 3 months or up to years post inoculation. Some dogs will enter a chronic phase, where pancytopenia is typically observed, resulting in the severe form of clinical Ehrlichiosis (6). It is not known if the infected canine host is infectious to feeding ticks throughout the life of the infection or only during the acute and/or chronic phase of the disease.

Diagnosis of CME is achieved through several analytical methods. These tests include indirect immunofluorescence antibody test, polymerase chain reaction, western immunoblotting,

and visualization of the morula within the moncytes by microscopy. Often more than one test is needed to reach a definitive diagnosis of CME.

Previous studies have attempted to recreate the model of natural *E. canis* transmission in a reproducible laboratory setting. There are two problems that are routinely encountered with these studies. The first is the lack of successful tick transmission using a high passage, laboratory adapted *E. canis* isolate. Lab-adapted *E.canis* isolates that grow well in cell culture are routinely used to infect dogs by needle inoculation for Ehrlichiosis studies. These dogs are also used as feeding sources for producing infected ticks, which are then used for various transmission studies. Due to minimal success with these studies in the past, concerns have arisen in regards to the susceptibility of the tick vector to cell-cultured strains of *E. canis*. These concerns were discussed after negative results were yielded by a transmission study in which a cell culture isolate was used (12). Additionally, other attempts to use *E. canis* cell cultured isolates for transmission studies have yielded minimal positive results (3, 16). This problem of using lab adapted isolates, either from repeated passage through cell culture or vertebrate host species, for transmission studies has also been encountered with other rickettsial organisms, including *E. chaffiensis* and *E. platys* (12, 13, 14, 15). It has been speculated that during repeated passages in mammalian cell cultures (ie. DH82) and/or by repeated blood passages, the *Ehrlichia* may lose their infectivity in ticks (2). However, this hypothesis has not yet been tested. The second problem encountered in *E.canis* transmission studies is the use of biologically unrealistic numbers of ticks. Successful *E.canis* transmission is routinely completed using large numbers of ticks (100+) (1,2,3). The need for using such high numbers of ticks to complete *E. canis* transmission could be attributed to the use of cell culture isolates rather than low passage field isolates. However, allowing a high number of infected ticks to feed on one

dog at one time does not accurately reflect the natural route of *E.canis* transmission. Although, this method has served as a proof of concept to show that transmission can occur from infected ticks to dogs, it does not lead to an accurate concept of the efficiency with which ticks acquire or transmit the pathogen. The use of fewer ticks in a transmission study would be more ideal when trying to duplicate the natural conditions of *E.canis* transmission.

The use of a low-passage *E.canis* field isolate may improve the efficiency of transmission when studied in a controlled laboratory setting. The first objective of my research was to test this hypothesis by demonstrating that low passage *E.canis* field isolates can be transmitted more efficiently than previous transmission studies using cell cultured isolates. This research was conducted using two low-passage *E.canis* field isolates, designated as strains New Mexico and Arizona, in place of laboratory-adapted strains. Unlike previous studies that used unrealistically large numbers of ticks per dog to prove the concept of transmission, we decided to reduce the number of ticks per dog to a level closer to that of the natural environment. This research will identify the tick's efficiency of transmitting the low-passed bacteria, as well as identify a new lower limit to the number of ticks that can be used for successful transmission of the pathogen. It is reported in this paper that using a low-passed field isolate can improve transmission results and that *E. canis* can be successfully transmitted using numbers of ticks that are closer to what would be observed in the field.

Past studies have shown that it is possible to inoculate ticks with pathogens through experimental injection into the hemoceol. Several pathogens such as *Anaplasma marginale*, *Hepatozoon canis*, and *Theileria* have all been successfully introduced into the tick via percutanious inoculation (4,5,7). This form of tick infection is ideal for certain studies of tick-host transmission, because it avoids the complications associated with natural feeding on

parasitemic vertebrate hosts. Some of these complications include the issue of when to feed ticks on the parsitemic host, how long to allow feeding, monitoring the host for peak parasitemia, or determining how much infected material each tick received. Additionally, this technique would provide a humane and cost effective alternative to exposing a donor dogs to *E. cani*s for the sole purpose of infecting ticks. The second objective was to test the hypothesis that it may be possible to use this technique to inoculate ticks with *E. canis*. To do this, a method of experimentally inoculating ticks with *E. canis* was developed and then tested by allowing the infected ticks to feed on dogs. The validation of this model will offer a new technique to studying the transmission process of *E. canis* without requiring an infected vertebrate host for tick acquisition of the bacteria. This study found that it is possible to experimentally inoculate ticks with *E.canis* and these ticks can successfully transmit the pathogen to dogs.

CHAPTER 2

TRANSMISSION OF *EHRLICHIA CANIS* TO DOGS BY NATURALLY-FED *RHIPICEPHALUS SANGUINEUS* TICKS

The disease Canine Monocytic Ehrlichiosis is caused by the intracellular bacterium *Ehrlichia canis*. This important veterinary pathogen is transmitted to canine hosts through the blood feeding of infected ticks. Tick species such as *Rhipicephalus sanguineus* and *Dermacentor variabilis* have been described as vectors of the *E. canis* bacterium. Tick transmission studies have been performed with *E. canis*, but not without problems. The two issues that are routinely encountered are the lack of successful tick transmission using a high passage, laboratory adapted *E. canis* isolate and the use of biologically unrealistic numbers of ticks. The lack of consistent success with these studies has caused concerns in regards to the susceptibility of the tick vector to cell-cultured strains of *E. canis*. Additionally, the need to use high numbers of infected ticks to transmit the pathogen does not accurately reflect the natural route of *E.canis* transmission. It was hypothesized that the use of a low-passage *E.canis* field isolate may improve the efficiency of *Ehrlichia* transmission when studied in a controlled laboratory setting. This hypothesis was tested with two low passage *E.canis* isolates that were transmitted to dogs using numbers of ticks that were representative of the natural environment. The two *E.canis* isolates were obtained from chronically infected dogs identified in veterinary hospitals and were designated as New Mexico and Arizona. It was expected that by using these low-passage field isolates, *E.canis* transmission to dogs would be more efficient and subsequently fewer numbers of ticks would be needed to successfully transmit the bacteria.

MATERIALS AND METHODS

Ehrlichia canis Low Passage Isolates

Dr. Yasuko Rikihisa at the Ohio State University provided two low passage *E.canis* isolates for these studies. The material was obtained from chronically infected dogs that were naturally infected by tick transmission. The dogs had been identified at veterinary hospitals in New Mexico and Arizona and tested positive to *E. canis* by PCR and serology (IFA). The two isolates were designated as New Mexico and Arizona and were IV-passaged in dogs two times before 5ml of this infectious blood was obtained.

IV Inoculation of Dogs with *E. canis*

The blood from these two *E. canis* infected dogs was used as challenge inoculum. Five milliliters of challenge, containing approximately 10^7 cell/ml (total target administration > 10^5 organisms/dog) was delivered via intravenous injection into dogs 16604 (New Mexico isolate) and 53303 (Arizona isolate). Weekly blood samples and temperatures were taken to monitor the *E. canis* infection. In addition to fever, 16S rRNA nested PCR, CBC counts and clinical signs were used to monitor the infection. Once infected, these dogs were used to feed ticks and provide infectious material for laboratory inoculation of ticks (Chapter 3).

Hematological Analysis

Samples of blood from each dog were collected in EDTA vaccutainer tubes and blood samples were analyzed using the Hemavet ® system. The Hemavet system is a quantitative, automated multispecies hematology analyzer for in vitro diagnostic use in clinical laboratories. The following paramenters were monitored in the study: lymphocytes, monocytes, platelets,

hemoglobin and white blood cell counts (K/ul) as well as mean platelet volume (MPV). Canine control blood was used daily to validate the system prior to analysis of blood samples. The Hemavet system provided the following reference values for canine blood samples:

Parameter (unit)	Normal Range
Lymphocytes (K/μL)	1.0-4.8
Monocytes (K/μL)	0.2-2.0
Platelets (K/μL)	200-500
Hemoglobin (K/μL)	12.0-18.0
WBC (K/μL)	6.0-17.0
MPV (fL)	5.0-15.0

The platelet counts that were below the normal range of 200, indicated thrombocytopenia and were used as one of the primary indicators for *E. canis* infection in the dogs.

Preparation of Naturally Infected Nymphs

Approximately 1000 *Rhipicephalus sanguineus* nymphs were obtained from Oklahoma State University. The dogs infected with the two low passage *E. canis* isolates had an area of hair, 10cm in diameter, shaved from their sides. A 5 cm diameter plastic capsule was secured to the side of each dog using a Resin/Beeswax (4:1) mixture. The mixture was heated using a hot plate until the resin and beeswax were melted and combined. The mixture was set aside to cool and when a workable temperature was reached the capsule was attached to the side of the dog using the resin beeswax mixture. Care was taken not to apply the mixture to the dog until it had cooled enough to avoid causing burns. The cap to the capsule had a fine mesh material covering holes that had been cut into the top. Vet wrap was used to completely secure the capsules to the dogs.

E-collars were used to prevent the dogs from biting the capsules or vet wrap (figures 1,2). The feeding ticks were observed daily and allowed to blood feed until they detached from the dogs (5-6 days). Replete ticks were collected and housed in an incubation chamber (27°C and 95% humidity). Once the nymphs molted into adults, a random sample (5 to 10%) of the molted ticks were tested for *E. canis* infection by PCR. The remaining ticks were used to transmit *E. canis* to naive dogs.

Figure 1: Feeding capsule attached to IV inoculated dog.

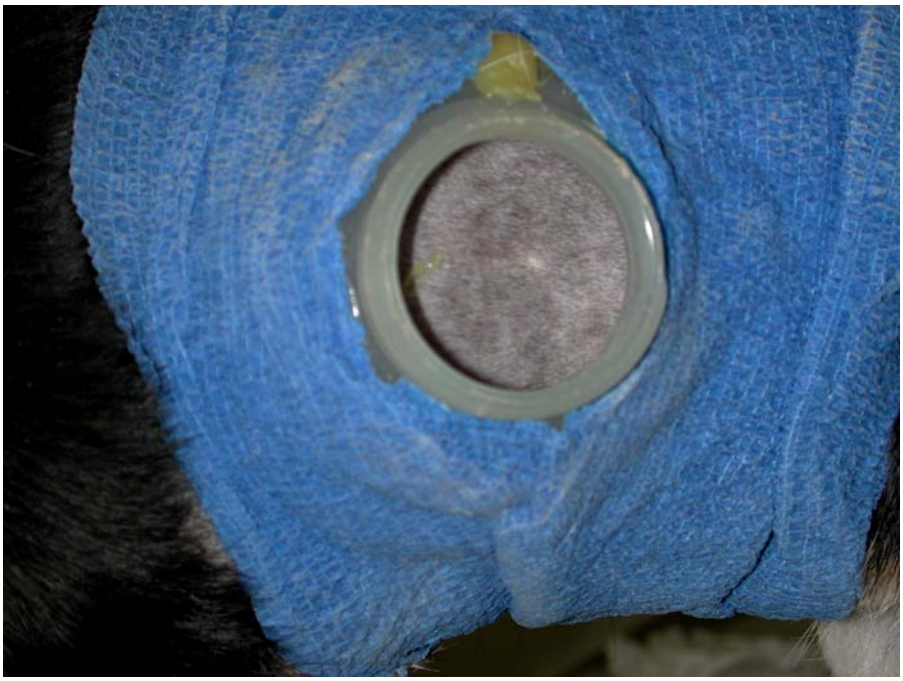

Figure 2: Close-up view of feeding capsule.

DNA Extraction from Ticks for E. canis Detection by PCR

DNA was purified from adult and nymph *Rhipicephalus sanguineus* ticks by using a modified method of the DNeasy Tissue Kit (Qiagen). Groups of 5-10 ticks were placed in 1.5-ml microcentrifuge tubes containing 180µl of Buffer ATL and 20µl of Proteinase K from the Qiagen kit and were crushed with disposable micropestles (Kontes Scientific Glassware/Instruments, Vineland, N.J.). The manufacturer's protocol for DNA purification from Gram-negative bacteria was followed to extract total DNA. Briefly, tissue lysis was carried out in the presence of Proteinase K for 10 min at 70°C. The lysed material was applied to a spin column containing a silica gel-based membrane and was washed twice. Purified DNA was eluted from the columns in 200 µl of elution buffer and was stored at 4°C until it was used as the template for PCR amplification. The PCR was carried out to detect *E. canis*16S rRNA gene regions.

16S Nested PCR

PCR amplifications were performed in two rounds in a Perkin-Elmer 9600 thermal cycler as described previously (19), with slight modifications. Reagents used were from Qiagen's *Taq* PCR Master Mix Kit, which was supplemented with 25mM $MgCl_2$ from Promega. Primary reactions used 5 µl of purified DNA (equivalent to 0.125-0.25 ticks) as the template in a total reaction volume of 25ul (containing 12.5ul master mix, 5ul template, 4.2ul H_2O, 1.3ul of 25mM MgCl, and 1.0ul of each primer pre-diluted to 5uM). The primers used for the first reaction were previously described (19) as follows:

ECC (5'-AGAACGAACGCTGGCGGCAAGCC-3')

ECB (5'-CGTATTACCGCGGCTGCTGGC- 3').

Cycling conditions involved an initial 10-min denaturation at 94°C, followed by 40 cycles, each consisting of a 30-s denaturation at 94°C, a 1-min annealing at 60°C, and a 1-min extension at 72°C. These 40 cycles were followed by a 7-min final extension at 72°C. Reaction products were maintained at 4°C until they were used as templates for nested reactions. Nested amplifications used 1µl of the primary PCR product as the template in a total volume of 25µl (containing 12.5ul master mix, 1ul template, 8.2ul H_2O, 1.3ul of 25mM MgCl, and 1.0ul of each primer pre-diluted to 5uM). The primers used were again previously described (19) as follows:

"canis" (5'-CAATTATTTATAGCCTCTGGCTATAGGA-3')

HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT3')

Nested cycling conditions were identical to those described for the primary amplification. Reaction products were subsequently maintained at 4°C until they were analyzed by 1.5 % agarose gel electrophoresis. Sybr gold DNA stain was used to illuminate PCR products. The expected size of an E.canis amplicon is approximately 400kD. Both positive (DNA extracted

from a chronically infected *E. canis* positive dog) and negative (no DNA) controls were extracted and PCR amplified in parallel with all specimens. In order to minimize the potential for contamination, DNA extractions, PCR setup, and agarose gel electrophoresis were performed in three separate rooms.

Feeding of Infected Adult Ticks on Dogs

Due to problems associated with using the capsule method of feeding the nymphal stage ticks, a new method using "ear bags" was employed for feeding of the adult ticks. The eight dogs used for these studies had a two-inch wide area of hair shaved from around the base of their ears (just above the ear canals). Each ear was placed in a fine mesh stocking that was partially taped to the ear. The adult ticks were inserted into the ear bags and tape was applied to secure the stockings. Once the ear bag was secured to the ear, both ears were secured on top of the dog's head using elastic tape (Figures 3,4). Great care was taken to ensure that the tape was applied as loosely as possible. E-collars were used to prevent the dogs from scratching the taped areas. The feeding ticks were observed daily and allowed to blood feed until they detached from the dogs (10-14 days). Detached ticks were collected and housed in an incubation chamber (27°C and 95% humidity). Random samples of the blood fed ticks from each dog were tested for *E. canis* infection by 16S rRNA nested PCR.

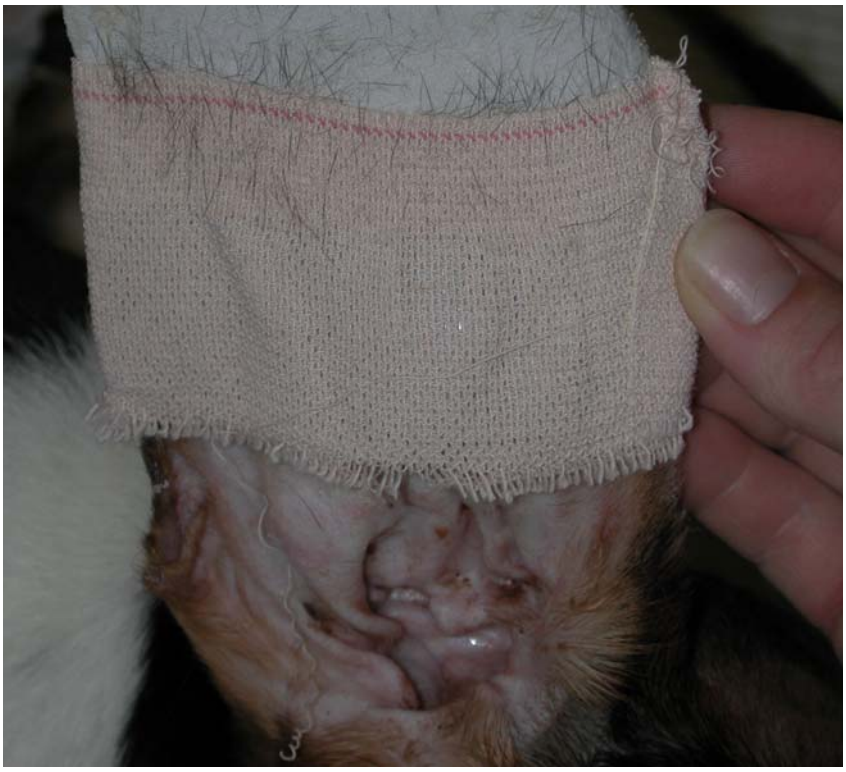

Figure 3: Application of adult ticks using the ear bag feeding method.

Figure 4: Ear bags with adult ticks are secured to the top of the head.

White Blood Cell Isolation

Approximately 3ml of blood was collected in 5ml vaccutainer tubes containing heparin. The blood was mixed 1:1 with sterile PBS and then layered over 3ml of Lymphocyte Separation Medium obtained from ICN Biochemicals Inc. in a 15ml conical tube. The tubes were spun at 1500 rpm for 25 minutes. After centrifugation the WBC layers were harvested and used for total DNA extraction.

DNA Purification from WBCs for 16S Nested PCR

White blood cells were harvested from dogs as described above. The WBC extractions were performed using the Qiagen Dneasy® Tissue Kit, following the manufacturer's protocol for Gram-negative bacteria. Briefly, tissue lysis was carried out in the presence of Proteinase K for 10 min at 70°C. The lysed material was applied to a spin column containing a silica gel-based membrane and was washed twice. Purified DNA was eluted from the columns in 200 µl of elution buffer and was stored at 4°C until it was used as the template for PCR amplification. The purified DNA was amplified by 16S nested PCR as described above.

Western Immunoblotting Analysis

Ehrlichia canis antigen was obtained from a cell-cultured isolate that has been adapted to grow well in canine macrophage cell lines. The antigen was obtained by harvesting *E. canis* infected cells from five 150cm^2 tissue culture flasks that had great than 90% of the cells heavily infected with *E.canis* (17). The cell suspension was centrifuge for 5 minutes at 1500 rpm and resuspended in a phosphate buffer solution at 1x10^6 cells per ml. The cell suspension was lightly sonicated 3 times for 15 seconds and confirmation of host cell lysing was shown by Diff Quik-stained preparations. The sonicated material was centrifuged at 10,000xg for 15 minutes and the

suspension from this centrifugation was used as Ehrlichia antigen for western immunoblot analysis. The antigen was treated with sodium dodecyl sulfate at a 1:1 concentration and run on a 10% polyacrylamide gel with 10ul of antigen per well. The separated antigens were blotted onto nitrocellulose membranes and reacted with primary antibodies at a 1:25 dilution. A peroxidase-conjugated affinipure rabbit anti-dog IgG produced by Jackson ImmunoResearch Laboritories, INC. was then incubated with the membrane at a 1:2,000 dilution. Lastly, TMB membrane peroxidase substrate by Kirkegaard and Perry Laboratories was added to the membrane for color development.

Sequence Analysis of PCR Products

Nested PCR products that resulted in positive bands for *E.canis* were submitted to Integrated Biotech Laboratories (IBL) at the University of Georgia for sequence analysis. Sequences were determined in both directions to resolve any ambiguities. The sequences generated from these PCR products were used in a BLAST search at the NCIB web site to identify homologous sequences to our generated sequences. Sequence alignments were done using the GCG software package.

Dog and Tick Follow-Up Testing

Dog

Weekly blood samples and temperatures were taken from the tick challenged dogs to monitor the *E. canis* infection. The infection was monitored by temperature, 16S nested PCR, CBC counts, and western blots. A dog was diagnosed as successfully infected after exhibiting multiple

symptoms including, high temperatures, low platelet counts, and PCR positive. Dogs that exhibited the acute stage of CME for multiple weeks were removed from the study for treatment.

Tick

Adult ticks that were collected after 10-14 days of blood feeding were housed in the tick incubation chamber. The adult ticks were checked 3 weeks post blood meal for presence of *E. canis*. Pools of 5-10 ticks were prepared to assess the presence of *E. canis* post blood meal. The ticks were tested by 16S-nested PCR.

RESULTS

IV Challenge

Both New Mexico and Arizona *E. canis* strains induced thrombocytopenia in dogs within 14 days post IV-challenge (Table 1). The thrombocytopenic state was identified at day 14, with white blood cell counts and hemoglobin levels declining in both challenge groups. These CBC counts never recovered to a normal level for the remainder of this study. An elevated temperature of >104°F was recorded on day 14 for both challenge groups as well (Table 2). Additionally, both dogs tested positive by Day 14 and remained positive by 16S nested PCR throughout the study. In addition to fever, the clinical signs in each group included icterus and paleness of mucous membranes indicative of anemia, as well as ocular signs such as congestion and mucous discharge. Serological analysis by western blot of the sera taken at day 21 indicated a strong antibody response against *E.canis* antigen (Figure 5).

Table 1: Hematological results of intravenous inoculated dogs. High or low counts are listed in bold.

WBC (K/ul)		Day Post IV-Challenge					
Group	ID	7	14	21	28	35	42
NM Strain	16604	**5.26**	**4.58**	**4.34**	**5.38**		
AZ Strain	53303	16.02	8.18	9.24	17.02	15.30	
Control	16602	11.28	8.64	10.60	9.04	8.24	10.98
Monocytes (K/ul)		Day Post IV-Challenge					
Group	ID	7	14	21	28	35	42
NM Strain	16604	0.83	0.25	0.24	0.30		
AZ Strain	53303	**2.08**	1.58	0.48	0.79	**3.15**	
Control	16602	1.23	1.25	1.90	0.38	0.69	1.07
Platelet (K/ul)		Day Post IV-Challenge					
Group	ID	7	14	21	28	35	42
NM Strain	16604	336.00	**41.00**	**35.00**	**41.00**		
AZ Strain	53303	555.00	**165.00**	**50.00**	**54.00**	**70.00**	
Control	16602	585.00	503.00	561.00	551.00	455.00	288.00
Lymphocytes (K/ul)		Day Post IV-Challenge					
Group	ID	7	14	21	28	35	42
NM Strain	16604	3.06	1.15	**0.77**	**0.61**		
AZ Strain	53303	**6.93**	3.26	2.21	2.89	**7.39**	
Control	16602	5.39	1.82	6.75	3.15	2.73	3.97
Hemoglobin (K/ul)		Day Post IV-Challenge					
Group	ID	7	14	21	28	35	42
NM Strain	16604	12.3	**10.9**	**7.1**	**6.5**		
AZ Strain	53303	13.1	11.9	**7.5**	**7.6**	**8.2**	
Control	16602	11.8	11.4	11	13.2	12.8	14.5
MPV (fL)		Day Post IV-Challenge					
Group	ID	7	14	21	28	35	42
NM Strain	16604	12.00	**18.20**	**18.80**	**18.50**		
AZ Strain	53303	13.70	**17.30**	**17.10**	**18.90**	**16.60**	
Control	16602	9.90	10.00	9.90	10.10	10.60	11.70

Table 2: Temperature results for intravenous inoculated dogs. High temperatures listed in bold.

Temperature °F		Day Post IV-Challenge											
Group	ID	0	4	7	11	14	18	21	25	28	32	34	35
AZ Strain	53303	103.2	101.8	101.6	103.4	**104.5**	**104.3**	102.2	103.9	102.6	102.9	NT	103.6
Control	16602	103.1	103.0	101.6	103.2	101.8	102.3	101.6	103.7	102.9	102.2	NT	102.4
NM Strain	16604	102.9	103.6	102.4	102.8	**104.7**	103.2	102.4	101.6	102.9	102.8	NT	102.4
NT = No Temperature available for this day													

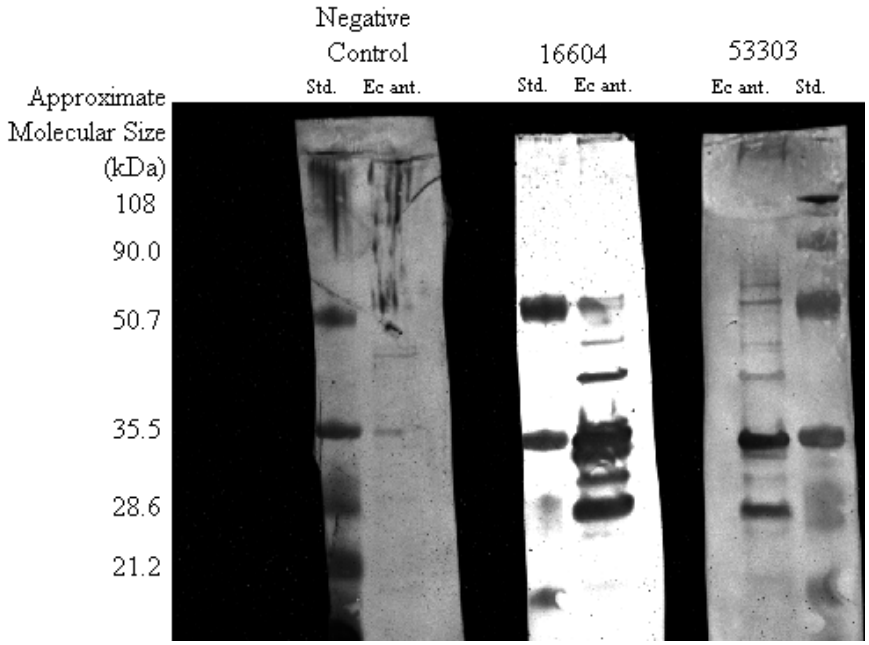

Figure 5: Western blot analysis of IV inoculated dogs. Dog 16604 was inoculated with the Arizona isolate and 53303 with the New Mexico isolate. Low range standard is noted as Std. and *E.canis* antigen is noted as Ec ant.

Tick Acquisition Feeding On Parasitemic Dogs

On day 21 post IV-challenge, approximately 500 unfed *Rhipicephalus sanguineus* nymphs were applied to each dog. The nymphs were allowed to feed on the dogs until they detached and then collected (Figure 6). All feeding ticks had detached from the dogs by day 6. The naturally fed nymphs molted to adults at an average of 92% survival rate (Table 3). A pool of 5 molted ticks from each naturally fed group were tested for *E. canis* infection using the 16S nested PCR. The New Mexico group tested positive while the Arizona group tested negative. A decision was made not to sacrifice further molted ticks for PCR analysis and to move forward with the transmission study using the remaining ticks.

Figure 6: Nymphs feeding on intravenous inoculated dog.

Table 3: Molting results of naturally infected nymphs

E. canis Strains	AZ Dog	NM Dog
Days Post IV-Challenge	21	21
Total Days Blood Fed	6	6
Total Ticks Blood Fed	120	266
Number that Molted	111	243
% Molted	93%	91%

Infected Adult Ticks Feeding on Dogs

Eight SPF beagles were used to study transmission of E.canis by the adult ticks previously infected by feeding on IV challenged dogs in the acute stage of CME. The ticks were applied to the dogs in the following order: Dogs-85302, 53804, 53703, and 53802 received Arizona naturally fed ticks at 29 ticks per dog; Dogs-53805, 85306, 53702, and 53803 received New Mexico naturally fed ticks at 44 and 88 ticks per dog. The ticks were collected from the dogs after feeding for 10-14 days (Figure 9). All but one dog had full engorgement of more than 50% of the applied ticks (Table 8). The dog to have less than 50% was 53703 (45%). Once the fed

ticks were counted, they were housed in an incubation chamber for 3 weeks before testing for *E. canis* infection by 16S nested PCR (Table 9). All tick groups had pools of either 5 or 10 ticks test positive for *E. canis* infection. See Figure 10 and 11 for PCR results from the pools of 5 and 10 ticks. Further analysis of the groups of ticks was not made after obtaining positive PCR results from the pools of ticks.

Both groups of tick challenged dogs showed clinical signs and became PCR positive (16S nested) during the study. An increase in white blood cells, mean platelet volume, monocytes and lymphocytes was observed between days 42 and 49, while a decrease in platelet counts was observed between days 42 and 49 (Tables 13,14,15,17,18). Results from temperature monitoring revealed decreases between days 35 and 38 and increases in average temperatures between days 42 and 63 (Tables 11, 12). Hemoglobin analysis of the groups during the 91 day study did not reveal any changes (Table 16). The first dog became PCR positive on day 28 (dog 85306) and all dogs, except 85302, became PCR positive at least once during the study (Table 10). None of the PCR positive dogs maintained positive results for more than three consecutive weeks. An analysis was made comparing temperature and platelet counts with PCR positive days (Figures 12,13,14). Dogs 85306, 85304, and 53804 were used for the comparison. No correlation could be found between each of the variables.

The serological analysis by western blot of the eight dogs indicated an antibody response against *E.canis* antigen by all dogs at day 91 (Figure 15,16). However, only dogs 85306, 85302, and 53806 developed strong antibody responses as compared to the IV challenged dogs. The other five dogs had mild antibody responses against the *E.canis* antigen.

For the final confirmation of PCR positive products, nested PCR products were submitted to IBL for sequence analysis. PCR products analyzed were from the IV challenged dogs (New Mexico and Arizona), tick challenged dogs (53804 and 85306), and the pools of infected ticks that had previously fed on dogs (85302 and 85306). To resolve any ambiguities, PCR products were sequenced in both forward and reverse directions. The sequence of the Arizona isolate is given in Figure 8, and the New Mexico isolate is given in Figure 7. Sequences from ticks fed on IV-infected dogs, and from dogs infected by these ticks, did not differ from the initial sequences obtained from IV-infected dogs. When the sequences were submitted to a nucleotide BLAST all the sequences aligned with *Ehrlichia canis* 16S ribosomal RNA gene (Table 6, 7). The Arizona isolate matched previously sequenced isolates from dogs from China and Venezuela (22), and a Venezuelan human isolate (23), with 100% identity (Table 5, 6). The New Mexico isolate differed from the Arizona isolate in having a 3 nucleotide insertion in position 379-381, a T in place of A at position 430, and CT in place of AG in position 553-554 (Table 4). Despite these differences the New Mexico isolate is clearly closest to a number of *Ehrlichia canis* sequences (Table 7).

New Mexico sequence

```
  1  TGCGTAGGAA TCTACCTAGT AGTACGGAAT AGCCATTAGA AATGGTGGGT
 51  AATACTGTAT AATCCCCGAG GGGGAAAGAT TTATCGCTAT TAGATGAGCC
101  TACGTTAGAT TAGCTAGTTG GTGAGGTAAT GGCTTACCAA GGCTATGATC
151  TATAGCTGGT CTGAGAGGAC GATCAGCCAC ACTGGAACTG AGATACGGTC
201  CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGGACAA TGGGCGAAAG
251  CCTGATCCAG CTATGCCGCG TGAGTGAAGA AGGCCTTCGG GTTGTAAAAC
301  TCTTTCAATA GGGAAGATAA TGACGGTACC TATAAAAGAA GTCCCGGCAA
351  ACTCTGTGCC AGCAGCCGCG GTAATACGAC AAGCTAGAAC GAACGCTGGC
401  GGCAAGCCTA ACACATGCAA GTCGAACGGT CAATTATTTA TAGCCTCTGG
451  CTATAGGAAA TTGTTAGTGG CAGACGGGTG AGTAATGCGT AGGAATCTAC
501  CTAGTAGTAC GGAATAGCCA TTAGAAATGG TGGGTAATAC TGTATAATCC
551  CCGCTGGGGA AAGATTTATC GCTATTAGAT GAGCCTACGT TAGATTAGCT
601  AGTTGGTGAG GTAATGGCTT ACCAA
```

Figure 7: 16S rRNA sequence of *Ehrlichia canis* New Mexico strain.

Arizona sequence

```
   1  ACCTAGTAGT ACGGAATAGC CATTAGAAAT GGTGGGTAAT ACTGTATAAT
  51  CCCCGAGGGG GAAAGATTTA TCGCTATTAG ATGAGCCTAC GTTAGATTAG
 101  CTAGTTGGTG AGGTAATGGC TTACCAAGGC TATGATCTAT AGCTGGTCTG
 151  AGAGGACGAT CAGCCACACT GGAACTGAGA TACGGTCCAG ACTCCTACGG
 201  GAGGCAGCAG TGGGGAATAT TGGACAATGG GCGAAAGCCT GATCCAGCTA
 251  TGCCGCGTGA GTGAAGAAGG CCTTCGGGTT GTAAAACTCT TTCAATAGGG
 301  AAGATAATGA CGGTACCTAT AAAAGAAGTC CCGGCAAACT CTGTGCCAGC
 351  AGCCGCGGTA ATACGAGCTA GAACGAACGC TGGCGGCAAG CCTAACACAT
 401  GCAAGTCGAA CGGACAATTA TTTATAGCCT CTGGCTATAG GAAATTGTTA
 451  GTGGCAGACG GGTGAGTAAT GCGTAGGAAT CTACCTAGTA GTACGGAATA
 501  GCCATTAGAA ATGGTGGGTA ATACTGTATA ATCCCCGAGG GGGAAAGATT
 551  TATCGCTATT AGATGAGCCT ACGTTAGATT AGCTAGTTGG TGAGGTAATG
 601  GCTTACCAAG GCTATGATCT ATAGCTGGTC TGAGAGGACG ATCAGCCACA
 651  CTGGAACTGA GATACGGTCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT
 701  ATTGGACAAT GGGCGAAAGC CTGATCCAGC TATGCCGCGT GAGTGAAGAA
 751  GGCCTTCGGG T
```

Figure 8: 16S rRNA sequence of *Ehrlichia canis* Arizona strain.

Table 4: Alignment of the Arizona and New Mexico sequences.

```
                 .         .         .         .         .
AZ     1 ACCTAGTAGTACGGAATAGCCATTAGAAATGGTGGGTAATACTGTATAAT 50
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM    14 ACCTAGTAGTACGGAATAGCCATTAGAAATGGTGGGTAATACTGTATAAT 63
                 .         .         .         .         .
AZ    51 CCCCGAGGGGGAAAGATTTATCGCTATTAGATGAGCCTACGTTAGATTAG 100
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM    64 CCCCGAGGGGGAAAGATTTATCGCTATTAGATGAGCCTACGTTAGATTAG 113
                 .         .         .         .         .
AZ   101 CTAGTTGGTGAGGTAATGGCTTACCAAGGCTATGATCTATAGCTGGTCTG 150
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM   114 CTAGTTGGTGAGGTAATGGCTTACCAAGGCTATGATCTATAGCTGGTCTG 163
                 .         .         .         .         .
AZ   151 AGAGGACGATCAGCCACACTGGAACTGAGATACGGTCCAGACTCCTACGG 200
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM   164 AGAGGACGATCAGCCACACTGGAACTGAGATACGGTCCAGACTCCTACGG 213
                 .         .         .         .         .
AZ   201 GAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCTA 250
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM   214 GAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCTA 263
                 .         .         .         .         .
AZ   251 TGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAACTCTTTCAATAGGG 300
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM   264 TGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAACTCTTTCAATAGGG 313
                 .         .         .         .         .
AZ   301 AAGATAATGACGGTACCTATAAAAGAAGTCCCGGCAAACTCTGTGCCAGC 350
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM   314 AAGATAATGACGGTACCTATAAAAGAAGTCCCGGCAAACTCTGTGCCAGC 363
                 .         .         .         .         .
AZ   351 AGCCGCGGTAATACG...AGCTAGAACGAACGCTGGCGGCAAGCCTAACA 397
         ||||||||||||||||   ||||||||||||||||||||||||||||||||
NM   364 AGCCGCGGTAATACGACAAGCTAGAACGAACGCTGGCGGCAAGCCTAACA 413
                 .         .         .         .         .
AZ   398 CATGCAAGTCGAACGGACAATTATTTATAGCCTCTGGCTATAGGAAATTG 447
         ||||||||||||||||| ||||||||||||||||||||||||||||||||
NM   414 CATGCAAGTCGAACGGTCAATTATTTATAGCCTCTGGCTATAGGAAATTG 463
                 .         .         .         .         .
AZ   448 TTAGTGGCAGACGGGTGAGTAATGCGTAGGAATCTACCTAGTAGTACGGA 497
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM   464 TTAGTGGCAGACGGGTGAGTAATGCGTAGGAATCTACCTAGTAGTACGGA 513
                 .         .         .         .         .
AZ   498 ATAGCCATTAGAAATGGTGGGTAATACTGTATAATCCCCGAGGGGGAAAG 547
         ||||||||||||||||||||||||||||||||||||||||  ||||||||
NM   514 ATAGCCATTAGAAATGGTGGGTAATACTGTATAATCCCCGCTGGGGAAAG 563
                 .         .         .         .         .
AZ   548 ATTTATCGCTATTAGATGAGCCTACGTTAGATTAGCTAGTTGGTGAGGTA 597
         ||||||||||||||||||||||||||||||||||||||||||||||||||
NM   564 ATTTATCGCTATTAGATGAGCCTACGTTAGATTAGCTAGTTGGTGAGGTA 613
                 .
AZ   598 ATGGCTTACCAA 609
         ||||||||||||
NM   614 ATGGCTTACCAA 625
```

Table 5: Alignment of Arizona strain sequence with Venezuelan isolate AF162860.

```
            .         .         .         .         .
370 AGAACGAACGCTGGCGGCAAGCCTAACACATGCAAGTCGAACGGACAATT 419
    |||||||||||||||||||||||||||||||||||||||||||||||||||
  4 agaacgaacgctggcggcaagcctaacacatgcaagtcgaacggacaatt 53
            .         .         .         .         .
420 ATTTATAGCCTCTGGCTATAGGAAATTGTTAGTGGCAGACGGGTGAGTAA 469
    |||||||||||||||||||||||||||||||||||||||||||||||||||
 54 atttatagcctctggctataggaaattgttagtggcagacgggtgagtaa 103
            .         .         .         .         .
470 TGCGTAGGAATCTACCTAGTAGTACGGAATAGCCATTAGAAATGGTGGGT 519
    |||||||||||||||||||||||||||||||||||||||||||||||||||
104 tgcgtaggaatctacctagtagtacggaatagccattagaaatggtgggt 153
            .         .         .         .         .
520 AATACTGTATAATCCCCGAGGGGGAAAGATTTATCGCTATTAGATGAGCC 569
    |||||||||||||||||||||||||||||||||||||||||||||||||||
154 aatactgtataatccccgaggggaaagatttatcgctattagatgagcc 203
            .         .         .         .         .
570 TACGTTAGATTAGCTAGTTGGTGAGGTAATGGCTTACCAAGGCTATGATC 619
    |||||||||||||||||||||||||||||||||||||||||||||||||||
204 tacgttagattagctagttggtgaggtaatggcttaccaaggctatgatc 253
            .         .         .         .         .
620 TATAGCTGGTCTGAGAGGACGATCAGCCACACTGGAACTGAGATACGGTC 669
    |||||||||||||||||||||||||||||||||||||||||||||||||||
254 tatagctggtctgagaggacgatcagccacactggaactgagatacggtc 303
            .         .         .         .         .
670 CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAG 719
    |||||||||||||||||||||||||||||||||||||||||||||||||||
304 cagactcctacgggaggcagcagtggggaatattggacaatgggcgaaag 353
            .         .         .         .
720 CCTGATCCAGCTATGCCGCGTGAGTGAAGAAGGCCTTCGGGT 761
    |||||||||||||||||||||||||||||||||||||||||
354 cctgatccagctatgccgcgtgagtgaagaaggccttcgggt 395
```

Table 6: Comparison of *E. canis* AZ strain with other rickettsial 16S RNA sequences.

Accession	Species	Source	E value	% Identity	Matches	Region
AF162860	*E. canis*	China	0.0	100	392/392	4-395
AF373613	*E. canis*	Venezuela	0.0	100	390/390	1-390
AF373612	*E. canis*	Venezuela	0.0	100	390/390	1-390
AF318946	*E. ovina*		0.0	99	388/389	1-389
M73226	*E. canis*		0.0	99	391/392	4-395
M73221	*E. canis*		0.0	99	391/392	4-395
U54805	*E*. sp	South Africa	0.0	99	389/390	1-390
U26740	*E. canis*	Israel	0.0	99	373/374	1-374
AF536827	*E. canis*	Japan	0.0	100	369/369	1-369
AY394465	*E. canis*	Spain	0.0	99	364/365	106-470
AF416764	*E. chaffeensis*	Arkansas	0.0	98	359/365	118-482
U23503	*E. chaffeensis*		0.0	98	359/365	118-482
U86665	*E. chaffeensis*		0.0	98	359/365	118-482
AY309970	*E*. sp	Japan	1e –94	96	377/390	1-390
AY309969	*E*. sp	Japan	1e –94	96	376/390	1-390
X62432	*Cowdria ruminatium*		9e –90	95	374/390	2-391

Table 7: Comparison of *E. canis* NM strain with other rickettsial 16S RNA sequences.

Accession	Species	Source	E value	% Identity	Matches	Region
AY394465	*E. canis*	Spain	0	99	377/378	93-470
AF536827	*E. canis*	Japan	0	99	377/378	78-455
AF318946	*E. ovina*		0	99	377/378	98-475
AF373613	*E. canis*	Venezuela	0	99	377/378	99-476
AF373612	*E. canis*	Venezuela	0	99	377/378	99-476
AF162860	*E. canis*	China	0	99	377/378	104-481
U26740	*E. canis*	Israel	0	99	376/378	83-460
M73226	*E. canis*		0	99	376/378	104-481
M73221	*E. canis*		0	99	376/378	104-481
U54805	*E*. sp	South Africa	0	99	376/378	99-476
AF416764	*E. chaffeensis*	Arkansas	0	98	372/378	105-482
U23503	*E. chaffeensis*		0	98	372/378	105-482
U86665	*E. chaffeensis*		0	98	372/378	105-482
AY309970	*E*. sp	Japan	0	97	369/378	98--475
AY309969	*E*. sp	Japan	0	97	369/378	99-476
X62432	*Cowdria ruminatium*		0	96	365/378	98-475

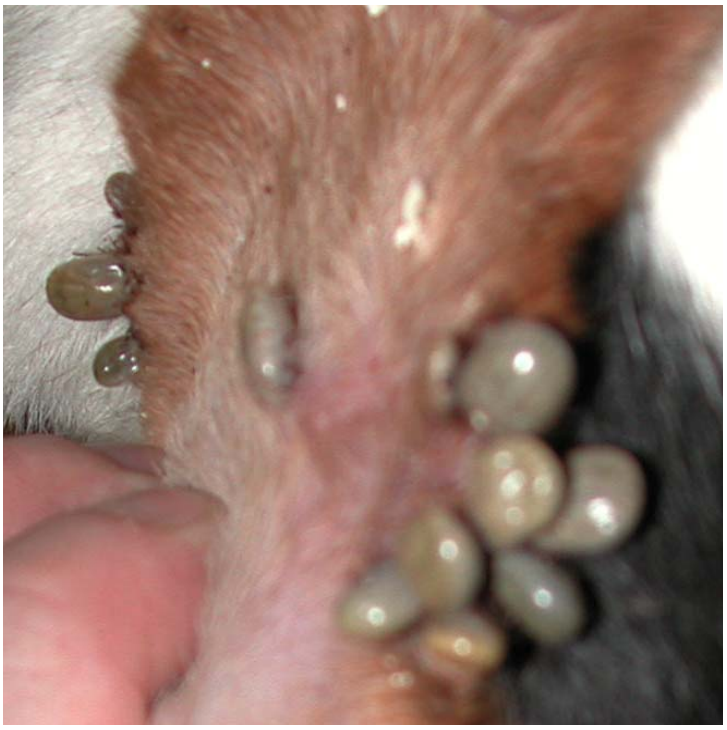

Figure 9: Infected adult ticks feeding on naive dog.

Table 8: Feeding success of adult ticks applied to each naïve dog.

Dog ID	Type of Tick Applied	Number of ticks	Fully Engorged	Partially Fed
85302	AZ Naturally Fed	29 Adults	19	10
53804	AZ Naturally Fed	29 Adults	16	13
53703	AZ Naturally Fed	29 Adults	13	16
53806	AZ Naturally Fed	29 Adults	21	8
53805	NM Naturally Fed	88 Adults	57	31
85306	NM Naturally Fed	88 Adults	47	41
53702	NM Naturally Fed	44 Adults	29	15
53803	NM Naturally Fed	44 Adults	27	17

Table 9: Post challenge PCR screen of adult ticks.

	Post Challenge PCR	16S Nested PCR	
Dog ID	Type of Tick Applied	5 Tick Pool	10 Tick Pool
85302	AZ Naturally Fed	Positive	ND
53804	AZ Naturally Fed	Negative	Negative
53703	AZ Naturally Fed	Negative	Positive
53806	AZ Naturally Fed	Positive	ND
53805	NM Naturally Fed	Negative	Negative
85306	NM Naturally Fed	Positive	ND
53702	NM Naturally Fed	Negative	Negative
53803	NM Naturally Fed	Negative	Negative
Tick Control	Uninfected Control Ticks	Negative	Negative
	Positive Control	Positive	Positive
	No DNA Control	Negative	Negative

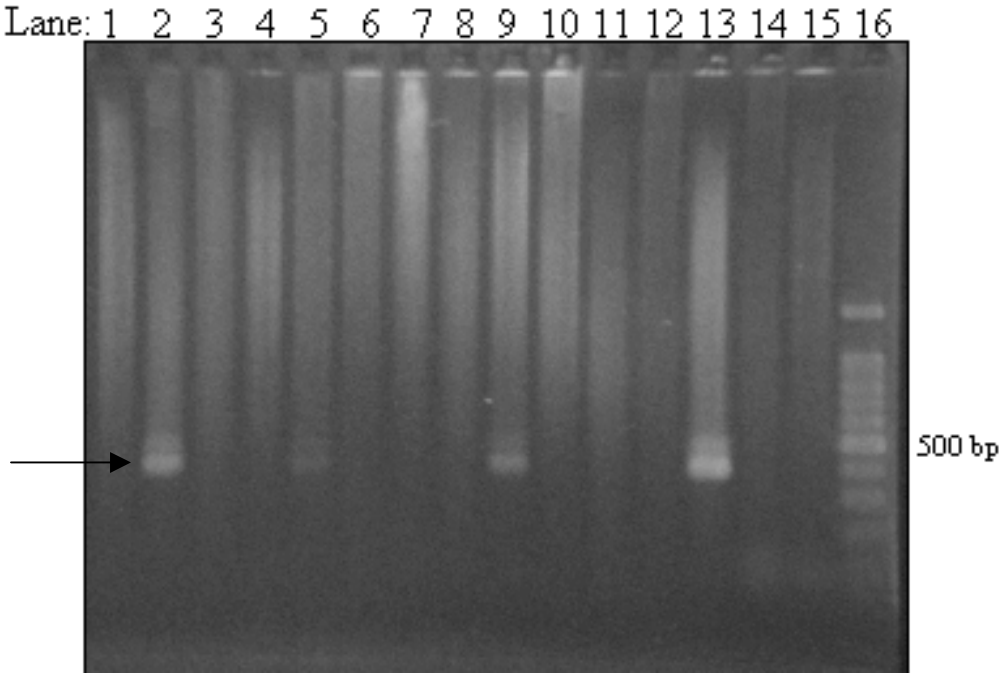

Figure 10: Five tick pools of adult ticks post transmission feeding. Lane: 1 (53804), 2 (85302), 3 (53703), 4 (53806), 5 (53805), 6 (53702), 7 (53803), 8 (53705), 9 (85306), 10 (85305), 11 (85304), 12 (53802), 13 (Positive control), 14 (negative tick control), 15 (no DNA control), and 16 (100 bp ladder). The arrow indicates an *E. canis* positive band. Lanes 2, 5, 9, and 13 are considered positive for *E. canis*.

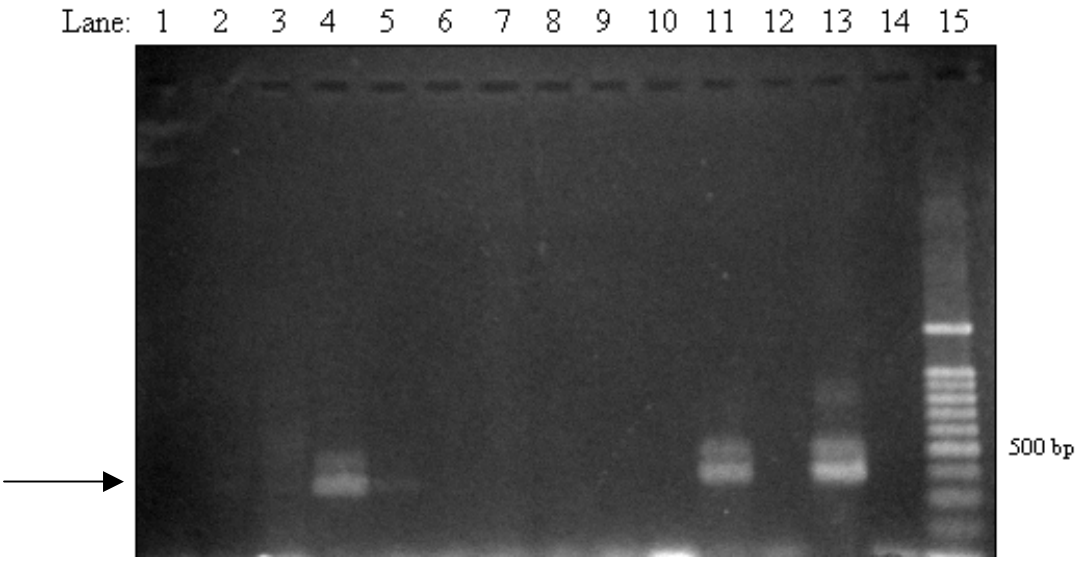

Figure 11: 10 tick pools of adult ticks post transmission feeding. Lane: 1 (empty), 2 (2nd group of nymphs fed on 85306), 3 (85304), 4 (85305), 5 (53705), 6 (53703), 7 (53805), 8 (53702), 9 (53803), 10 (53804), 11 (53802), 12 (negative tick contral), 13 (Positive control), 14 (no DNA control), 15 (100 bp ladder). The arrow indicates an *E. canis* positive band. Lanes 2, 3, 4, 5, 11, and 13 are considered positive for *E. canis*.

Table 10: PCR data for tick challenged dogs. PCR positive days are noted in bold.

		Days Post Tick Challenge												
ID	Type of Tick	–3	7	14	28	35	42	49	56	63	70	77	84	91
85302	AZ Blood Fed	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg
53804	AZ Blood Fed	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	**Pos**	Neg	**Pos**	**Pos**	Neg
53703	AZ Blood Fed	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg
53806	AZ Blood Fed	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**
53805	NM Blood Fed	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg
85306	NM Blood Fed	Neg	Neg	Neg	**Pos**	Neg	Neg							
53702	NM Blood Fed	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg
53803	NM Blood Fed	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	**Pos**
	Positive Control	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**
	No DNA Control	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Table 11: Temperatures for tick challenged dogs °F. High temperatures are noted in bold.

		Days Post Tick Challenge															
Type of Tick	ID	-3	1	2	3	4	7	10	14	17	22	24	28	31	35	38	42
NM Blood Fed	53702	101.7	102.8	102.8	102.8	102.7	103.0	103.2	102.7	102.6	102.0	102.9	101.9	101.5	102.7	102.2	103.1
	53803	103.3	101.8	102.6	102.5	102.5	101.7	102.4	102.7	101.4	102.0	102.4	101.8	102.4	102.4	101.5	102.0
	53805	102.8	103.0	103.2	102.8	102.9	102.6	102.7	103.1	102.5	101.5	102.3	101.8	102.7	102.3	102.1	102.3
	85306	102.4	101.8	103.2	102.2	102.6	102.5	102.8	102.7	103.2	**105.0**	**104.4**	103.8	103.6	103.8	103.6	103.4
Average		102.6	102.4	103.0	102.6	102.7	102.5	102.8	102.8	102.4	102.6	103.0	102.3	102.6	102.8	102.4	102.7
AZ Blood Fed	53703	102.7	102.1	101.8	102.3	102.5	102.0	101.1	102.9	102.4	101.8	102.8	101.1	101.6	101.9	102.1	102.8
	53804	102.8	102.4	102.8	102.3	102.5	102.3	103.3	103.5	103.5	103.2	102.3	102.3	102.2	102.9	102.4	102.7
	53806	102.5	102.9	102.6	103.0	102.0	103.4	102.3	102.8	101.8	101.9	102.7	102.1	101.9	102.2	101.3	102.1
	85302	102.6	101.8	101.9	101.9	101.8	102.2	102.5	102.3	103.1	101.0	102.0	101.2	102.0	103.2	102.6	103.1
Average		102.7	102.3	102.3	102.4	102.2	102.5	102.3	102.9	102.7	102.0	102.5	101.7	101.9	102.6	102.1	102.7

Table 12: Temperatures continued for tick challenged dogs °F. High temperatures are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	45	49	52	56	59	63	66	70	73	77	80	84	91
NM Blood Fed	53702	102.5	103.0	102.6	102.4	102.9	103.0	102.6	102.2	102.8	103.3	101.5	102.3	102.9
	53803	102.6	102.2	102.3	102.3	102.8	103.4	102.7	101.4	101.9	102.4	101.7	102.5	102.4
	53805	102.3	103.3	102.8	**105.1**	102.6	103.1	103.5	103.1	102.7	102.5	102.3	102.7	102.9
	85306													
Average		102.5	102.8	102.6	103.3	102.8	103.2	102.9	102.2	102.5	102.7	101.8	102.5	102.7
AZ Blood Fed	53703	102.3	102.4	102.3	101.4	103.0	102.4	102.2	102.9	102.0	102.5	100.6	102.6	102.7
	53804	102.4	102.2	102.6	102.3	102.5	101.9	103.3	102.4	103.3	102.2	102.2	102.7	102.0
	53806	102.7	102.3	100.8	102.6	101.4	101.7	101.8	102.4	102.4	102.5	101.5	102.1	102.8
	85302	101.5	102.2	102.4	102.3	102.9	102.9	102.8	102.9	102.8	102.2	102.2	102.9	101.4
Average		102.2	102.3	102.0	102.2	102.5	102.2	102.5	102.7	102.6	102.4	101.6	102.6	102.2

Table 13: White blood cell counts (K/ul) for tick challenged dogs. High and low counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
	53703	11.66	10.66	9.52	10.70	9.74	15.76	10.10	8.62	9.76	8.38	8.74	10.54	11.60
AZ Naturally Fed	53804	**18.68**	12.08	10.78	12.34	9.68	13.88	14.98	11.40	13.14	10.96	12.42	14.24	14.30
	53806	**18.40**	12.44	11.36	12.32	**17.56**	**17.30**	14.48	12.04	13.34	15.26	12.52	14.64	12.66
	85302	14.80	12.48	14.78	9.66	9.84	17.14	12.92	12.80	9.66	9.86	12.84	10.82	10.26
AZ Naturally Fed Avg		15.89	11.92	11.61	11.26	11.71	16.02	13.12	11.22	11.48	11.12	11.63	12.56	12.21
	53702	14.38	9.38	9.46	9.52	9.50	15.96	12.84	8.82	9.06	9.32	9.60	9.60	12.56
NM Naturally Fed	53803	13.16	13.04	9.62	13.94	10.06	15.54	11.68	9.84	11.84	11.22	15.52	14.54	13.02
	53805	15.50	14.94	14.84	14.16	13.76	15.10	**19.14**	15.44	12.88	11.10	12.28	15.50	13.86
	85306	16.38	12.06	9.36	7.34	**5.46**	**4.38**							
NM Naturally Fed Avg		14.86	12.36	10.82	11.24	9.70	12.75	14.55	11.37	11.26	10.55	12.47	13.21	13.15

Table 14: Lymphocyte counts (K/ul) for tick challenged dogs. High and low counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Naturally Fed	53703	4.73	3.58	3.67	3.49	**5.68**	**6.87**	4.61	2.57	2.65	2.60	3.43	1.87	3.90
	53804	**6.67**	4.68	3.93	4.33	3.65	4.25	4.21	3.01	2.89	2.54	2.69	3.22	3.65
	53806	**7.64**	**5.16**	4.07	**5.29**	**7.70**	**6.46**	3.79	3.62	3.94	5.26	2.73	4.02	4.07
	85302	**5.19**	4.93	5.58	4.02	**5.12**	**6.49**	**5.80**	4.17	2.26	2.75	2.61	2.82	2.91
AZ Naturally Fed Avg		6.06	4.59	4.31	4.28	5.54	6.02	4.60	3.34	2.94	3.29	2.87	2.98	3.63
NM Naturally Fed	53702	**5.64**	4.15	4.54	**5.49**	**5.20**	**6.83**	3.38	3.10	3.27	3.82	3.59	4.11	4.66
	53803	4.78	4.56	3.92	**5.85**	4.71	**5.87**	3.51	2.57	2.88	3.61	3.69	3.74	3.88
	53805	**6.53**	**5.56**	**5.51**	**5.37**	**5.44**	**5.66**	3.23	3.86	3.29	3.10	3.73	3.98	4.45
	85306	4.81	2.51	1.52	1.10	**0.80**	**0.67**							
NM Naturally Fed Avg		5.44	4.20	3.87	4.45	4.04	4.76	3.37	3.18	3.15	3.51	3.67	3.94	4.33

Table 15: Monocyte counts (K/ul) for tick challenged dogs. High and low counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Naturally Fed	53703	1.05	0.65	0.92	0.29	1.52	**3.41**	0.98	0.49	0.92	0.35	0.77	0.63	0.64
	53804	1.17	0.66	0.65	0.77	0.69	0.60	0.65	0.55	0.64	0.51	0.60	0.88	0.79
	53806	0.90	0.55	0.63	0.81	1.60	0.68	0.74	0.65	0.78	0.85	0.77	0.99	0.46
	85302	1.23	0.87	0.71	0.71	1.26	**3.45**	**2.13**	1.51	0.63	0.66	0.99	0.83	0.65
AZ Naturally Fed Average		1.09	0.68	0.73	0.65	1.27	2.04	1.13	0.80	0.74	0.59	0.78	0.83	0.64
NM Naturally Fed	53702	1.12	0.74	0.54	1.28	1.42	1.50	0.90	0.45	0.59	0.58	0.53	0.77	0.66
	53803	1.25	0.76	0.57	1.13	1.09	**2.07**	0.74	0.60	0.78	0.55	0.87	1.04	0.59
	53805	0.97	0.75	0.76	0.86	1.01	1.64	0.91	0.91	0.61	0.42	0.79	1.07	0.66
	85306	1.09	0.82	0.43	0.38	0.30	**0.14**							
NM Naturally Fed Average		1.11	0.77	0.58	0.91	0.96	1.34	0.85	0.65	0.66	0.52	0.73	0.96	0.64

Table 16: Hemaglobin counts (K/ul) for tick challenged dogs. Low counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Naturally Fed	53703	14.6	14.8	15.2	14.5	14.0	13.7	**11.6**	13.8	14.3	14.2	15.0	15.5	14.6
	53804	14.5	13.9	14.4	13.2	16.2	14.3	12.5	14.1	13.9	13.9	14.1	14.6	14.7
	53806	14.0	14.3	14.5	14.2	14.1	15.4	12.2	13.9	14.8	13.4	14.1	14.4	13.4
	85302	15.5	15.4	15.7	14.5	15.4	15.8	14.6	15.4	16.1	16.0	16.6	17.1	16.6
AZ Naturally Fed Average		14.6	14.6	14.9	14.1	14.9	14.8	12.7	14.3	14.7	14.4	14.9	15.4	14.8
NM Naturally Fed	53702	13.9	13.1	14.5	12.7	13.2	13.8	15.0	13.4	14.1	13.9	14.6	14.9	14.5
	53803	12.5	12.9	13.0	14.2	13.8	14.6	13.4	13.6	13.5	14.3	14.2	15.2	14.0
	53805	13.2	13.0	13.5	14.0	14.4	14.5	13.8	15.3	13.8	14.5	14.1	14.6	12.9
	85306	14.5	14.1	13.6	**11.8**	**10.4**	**8.9**							
NM Naturally Fed Average		13.5	13.3	13.6	13.2	12.9	12.9	14.1	14.1	13.8	14.2	14.3	14.9	13.8

Table 17: Mean Platelet Volume (fL) for tick challenged dogs. High counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Naturally Fed	53703	14.4	11.5	10.9	12.6	11.7	**18.7**	12.9	13.2	12.2	12.7	13.6	13.8	11.3
	53804	**17.6**	14.9	14.4	14.5	14.6	**17.3**	**16.6**	**16.0**	**15.9**	14.5	14.9	**16.2**	**15.8**
	53806	**16.5**	13.3	13.9	13.9	**18.3**	**20.5**	**15.6**	**15.9**	14.9	13.2	13.9	14.4	14.3
	85302	**15.4**	13.2	**15.2**	**15.8**	14.1	**18.7**	**16.6**	**17.4**	**16.1**	**15.9**	**16.1**	**15.0**	14.9
AZ Naturally Fed Average		15.9	13.2	13.6	14.2	14.6	18.8	15.4	15.6	14.7	14.0	14.6	14.8	14.0
NM Naturally Fed	53702	13.4	11.5	12.4	12.1	11.7	**18.5**	13.7	12.8	11.8	12.0	12.4	11.7	14.2
	53803	**19.4**	**17.5**	**15.6**	**18.2**	**16.3**	**19.4**	**17.8**	**16.9**	**16.1**	**15.8**	**17.0**	**16.3**	**15.7**
	53805	13.9	11.8	11.7	12.0	12.4	**17.0**	14.3	12.4	12.5	12.7	13.9	12.1	12.6
	85306	14.2	12.2	**21.5**	**21.1**	**18.8**	**19.9**							
NM Naturally Fed Average		15.2	13.2	15.3	15.8	14.8	18.7	15.2	14.0	13.4	13.5	14.4	13.3	14.1

Table 18: Platelet counts (K/ul) for tick challenged dogs. Low counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Naturally Fed	53703	335. 0	365.0	329.0	266.00	288.0	**126.0**	299.0	319.0	296.0	287.0	326.0	237.0	297.0
	53804	**91.0**	304.0	305.0	259.00	363.0	**72.0**	236.0	227.0	225.0	238.0	229.0	256.0	218.0
	53806	285.0	384.0	284.0	307.00	**148.0**	**112.0**	343.0	295.0	291.0	**126.0**	307.0	325.0	281.0
	85302	259.0	267.0	**196.0**	269.00	292.0	**118.0**	**181.0**	**145.0**	263.0	244.0	324.0	270.0	258.0
AZ Naturally Fed Avg		242.5	330.0	278.0	275.25	272.7	107.0	264.7	246.5	268.7	223.7	296.5	272.0	263.5
NM Naturally Fed	53702	340.0	386.0	445.0	333.00	399.0	**125.0**	297.0	374.0	371.0	331.0	325.0	381.0	260.0
	53803	202.0	225.0	261.0	**111.00**	262.0	**93.0**	252.0	238.0	248.0	212.0	**195.0**	265.0	238.0
	53805	383.0	305.0	412.0	358.00	338.0	**137.0**	365.0	329.0	354.0	329.0	**193.0**	302.0	316.0
	85306	348.0	255.0	**76.0**	**63.00**	**35.0**	**47.0**							
NM Naturally Fed Avg		318.2	292.7	298.5	216.2	258.5	100.5	304.6	313.6	324.3	290.6	237.6	316.0	271.3

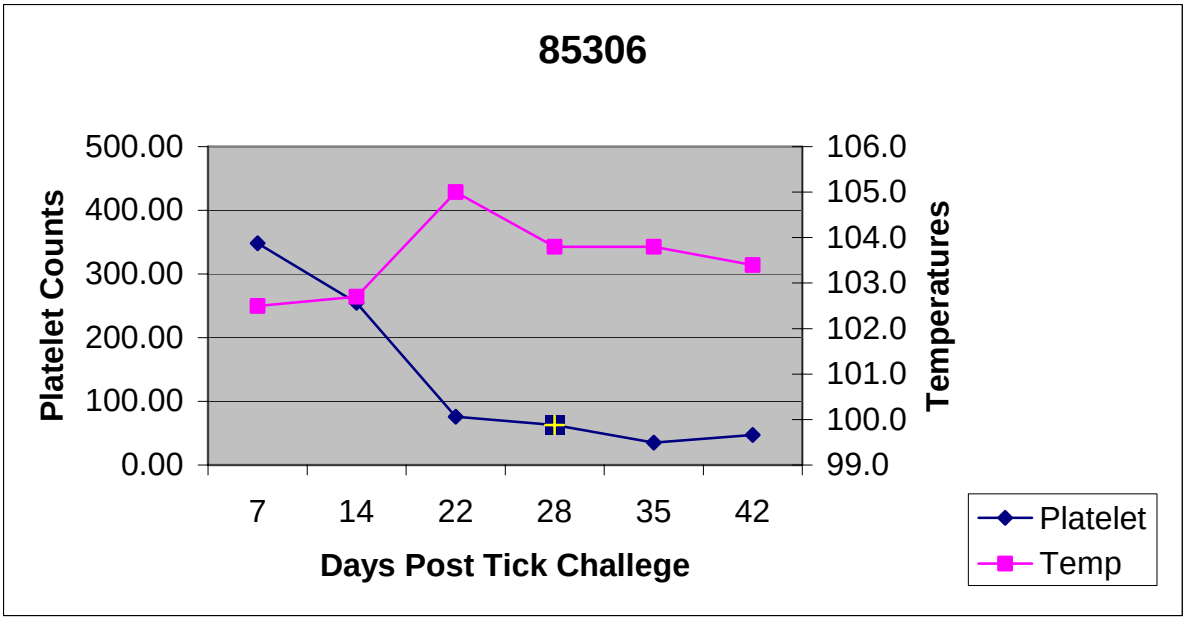

Figure 12: Dog 85306 platelet count and temperature comparison with PCR positive time points. PCR positive days are denoted by (+).

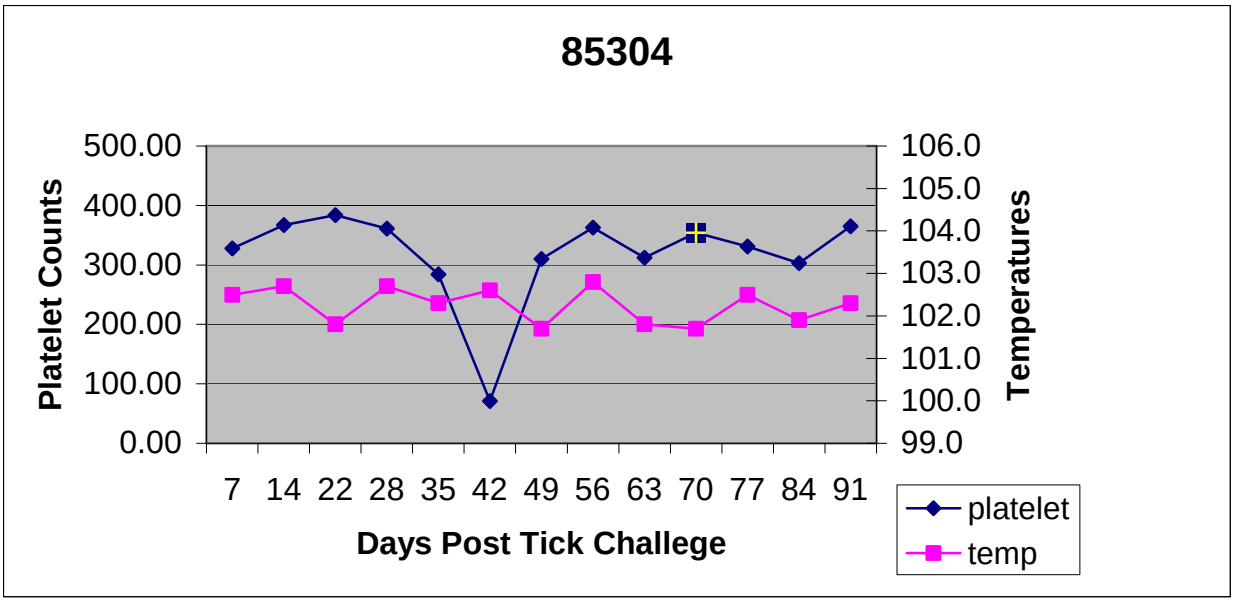

Figure 13: Dog 85304 platelet count and temperature comparison with PCR positive time points. PCR positive days are denoted by (+).

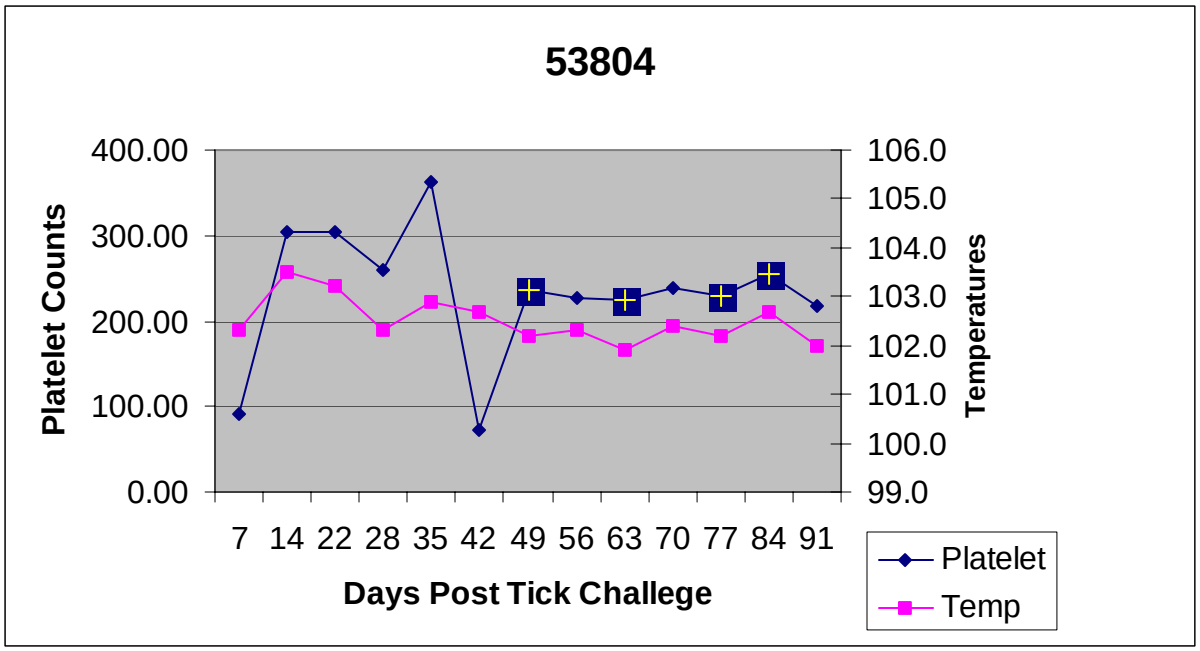

Figure 14: Dog 53804 platelet count and temperature comparison with PCR positive time points. PCR positive days are denoted by (+).

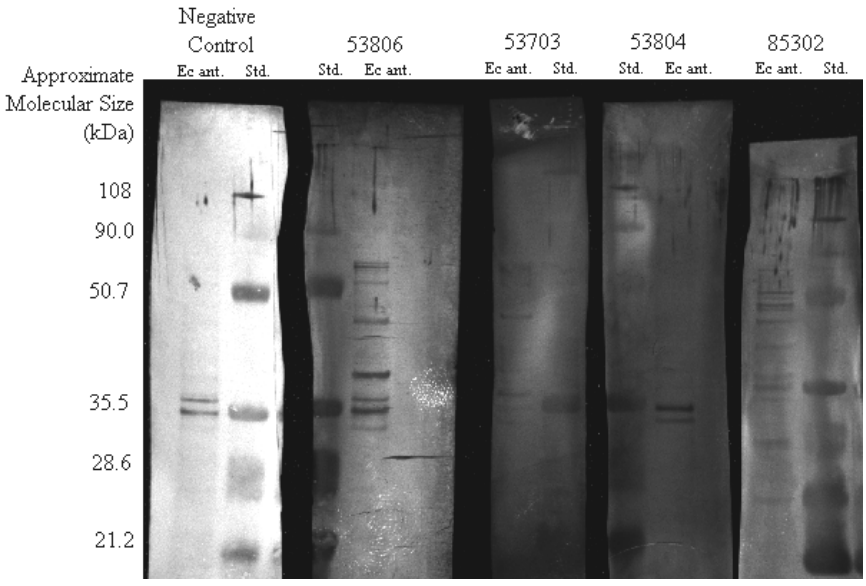

Figure 15: Western blot analysis of tick challenged dogs with the Arizona isolate. Low range standard is noted as Std. and *E.canis* antigen is noted as Ec ant.

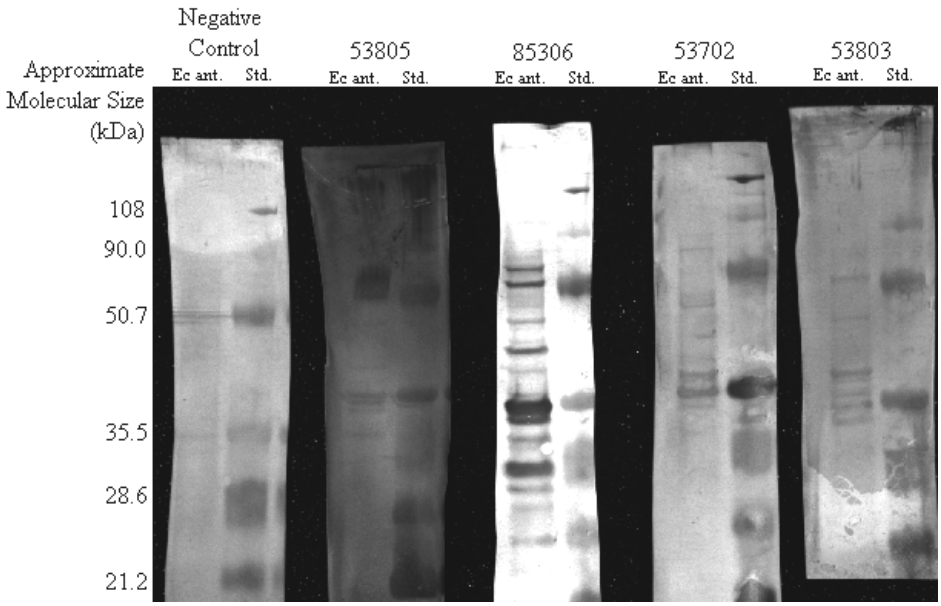

Figure 16: Western blot analysis of tick challenged dogs with the New Mexico isolate. Low range standard is noted as Std. and *E.canis* antigen is noted as Ec ant.

Second Transmission Study

In order to confirm efficient transmission from dog to tick and from tick to dog, we fed approximately 1000 nymphal ticks on dog 85306. This dog was chosen as it was exhibiting signs of CME for multiple weeks (PCR positive, low platelet counts, etc.). The ticks were attached to dog 85306 at day 42 post New Mexico tick challenge and allowed to feed for 6 days. After molting, a group of ten ticks were pooled and tested positive by PCR prior to feeing on naïve dogs (Figure 11). The remaining ticks were divided into 4 groups of 30 ticks and each group was fed on a naïve dog. The ticks were allowed to feed for 10-14 days on each dog. Based on results from the first transmission study, the dogs were monitored for Ehrlichial infection by platelet counts, serology, and PCR only. This decision was made based on the lack of changes in the other parameters from the previous transmission study. The tick challenged dogs showed clinical signs and 3 of 4 became PCR positive (16S nested) during the 77-day

study. Unfortunately CBC data was not available for days 0 and 7 and this data was collected starting on day 14. All dogs experienced a decrease in platelet counts on multiple days (Table 19). The first dogs became PCR positive on day 14 (350102 and 350302) and day 47 for dog 310105 (Table 20). Dog 350305 did not become PCR positive during this study. Only dog 350102 maintained PCR positive results for more than one consecutive week. Dogs 350302 and 310105 had only one day each when they tested PCR positive for *E.canis*. The serological analysis by western blot of the 4 dogs indicated a strong antibody recognition response against *E.canis* antigen by all dogs at day 48 (Figure 17).

Table 19: Platelet counts (K/ul) for second tick challenged dogs. Low counts are noted in bold

Platelet Counts (K/uL)		Days Post Tick Challenge									
Type of Tick	ID	14	22	29	34	41	48	55	62	70	77
2nd Tick-Dog Transmission	350102	**67**	**79**	**74**	**57**	**78**	**99**				
	310105	223	**98**	**115**	292	**154**	**136**				
	350302	205	207	213	279	230	260	**117**	**171**	352	276
	350305	335	262	**174**	303	311	219	**193**	207	228	**159**

Table 20: PCR data for second tick challenged dogs. PCR positive days are noted in bold.

16S Nested PCR		Days Post Challenge										
Type of Tick	ID	-3	14	21	28	33	40	47	54	61	69	76
2nd Tick-Dog Transmission	350102	N	**P**	**P**	**P**	**P**	N	N				
	310105	N	N	N	N	N	N	**P**				
	350302	N	**P**	N	N	N	N	N	N	N	N	N
	350305	N	N	N	N	N	N	N	N	N	N	N

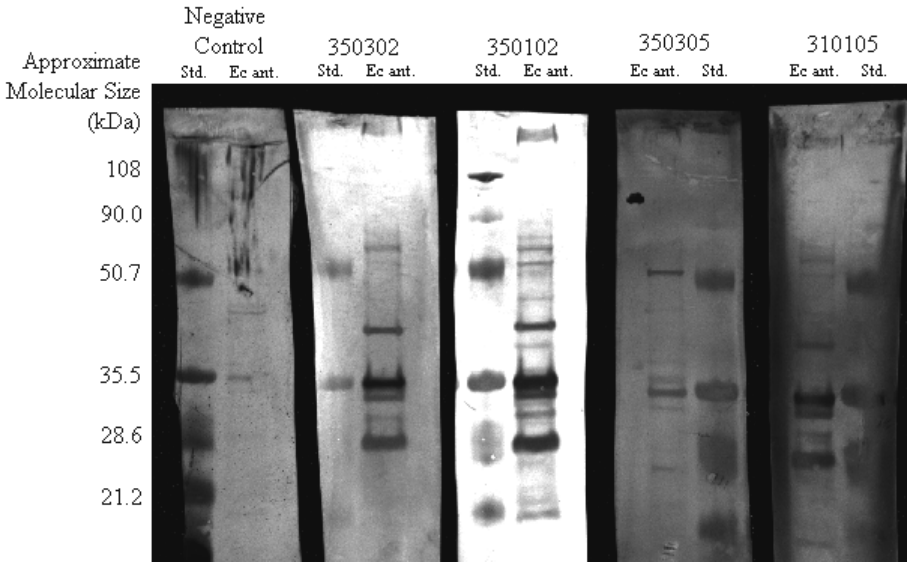

Figure 17: Western blot analysis of second tick challenged dogs. Low range standard is noted as Std. and *E.canis* antigen is noted as Ec ant.

DISCUSSION

This tick transmission study was performed to test the hypothesis that using low passage *E. canis* field isolates will improve the efficiency of transmission in a laboratory setting. It was predicted that by using these isolates *E. canis* transmission would improve and fewer numbers of ticks would be necessary to transmit the pathogen. This study was successful on both accounts. Tick transmission of *Ehrlichia canis* occurred in all 12 dogs challenged. While the level of CME varied from dog to dog, all dogs exhibited multiple characteristics of the disease. These characteristics included thrombocytopenia, clinical signs, high temperatures, and positive results by PCR and/or serology. The use of low passage *E.canis* isolates appeared to improve tick transmission to dogs. The naturally fed tick study demonstrated that transstadial transmission of *E.canis* in a laboratory environment is achieved with as few as 29 infected adults. The number of ticks used in this study represents a more biologically realistic number than the previously

described studies. The bacteria was efficiently transmitted with these numbers of ticks in two independent transmission experiments. All dogs that received these numbers of ticks developed Ehrlichial infections despite the fact that only the New Mexico group of ticks tested PCR positive prior to feeding. The subsequent PCR performed on these groups of ticks after feeding, indicated that both the New Mexico and Arizona E.canis isolates were present in the adult ticks. These results suggest that either too few ticks we tested prior to feeding or that *E. canis* levels increased in the ticks through growth or co-feeding transmission.

Failure to induce a severe form of Ehrlichiosis in all but three (85306, 350102, 310105) of the dogs that where challenged with ticks, suggests that only a mild acute phase of the disease was induced by these *E. canis* isolates. The *E.canis* was then maintained as a sub-clinical disease that was occasionally detectable. This type of result would not be unexpected. *Ehrlichia canis* can persists in the canine host as a sub-clinical disease for month to years and may only become evident during immunosuppression of the host such as with the occurrence of a secondary infection (18). This would explain the persistence of the disease in the field while the clinically diagnosed cases are seen sporadically. The PCR results from all dogs that were infected by tick transmission follow the sporadic pattern of a sub-clinical infection. Many weeks passed between positive PCR tests in these dogs and only one dog maintained positive results for more than four consecutive weeks (350102). This suggests that the amount of *E. canis* present in the blood may vary from week to week. This point should be investigated further to determine the impact it would have on the tick's ability to acquire the bacterium when the disease is in a sub-clinical state. Additionally, to clinically characterize CME by tick transmission, future studies should extend the length of animal monitoring to capture the chronic phase of the disease (6 months – 1 year) or consider co-infection with another disease and/or using

immunosuppressive therapy. These techniques may lead to more severe forms of Canine Monocytic Ehrlichiosis.

The use of temperature, platelet count, and PCR data to predict parasitemic peaks with the tick-infected dogs was not successful. No correlations could be drawn between spikes in temperatures or drops in platelet counts and days when dogs tested PCR positive. This is unfortunate because the ability to predict when high levels of *Ehrichia* are in the blood might identify the most appropriate times to allow ticks to acquisition feed on these hosts.

The serological responses of the tick challenged dogs in these studies were also very inconsistent. The serology was tested by western immunoblot and dogs were considered positive if a 30kDa protein was recognized by the sera from the infected dogs. The 30kDa protein has been routinely described in *E.canis* serological studies and was used as the key indicator of positive serum in this study (6). There was dog-to-dog variation in the levels of antibody responses generated against the *Ehrlichia* antigen in the tick challenged dogs, while the IV infected dogs developed consistently high antibody titers against the antigen. The variation of antibody responses by the tick challenged dogs might be a consequence of using *Ehrlichia* antigen that was produced from a high passed cell cultured isolate. Antigenic variation between strains has been described for other rickettsial organisms such as *Anaplasma marginale* (20). It could be speculated that the strain to strain variation illustrated with *Anaplasma marginale* may also be present with *E. canis*. It has also been described that the major outer membrane proteins of *Anaplasam marginale* can change throughout the course of an infection (21). The organism expresses different variants of the outer membrane protein to avoid immunological control by the infected host. It has been demonstrated that *E.canis* can alter its outer membrane proteins as well. A study has described that *E.canis* can express different proteins while in the tick that are

not expressed when the organism is in the canine host (22). Due to potential antigenic variation and expression, it would not be surprising that the dogs, which were tick infected with a low passed *Ehrlichia*, did not generate high antibody responses against a much different high passed cell culture isolate. Further studies would need to be conducted to identify any antigenic differences between low passage *E. canis* field isolates and high passage cell culture isolates.

The results from the second transmission study of naturally fed nymphs, confirms the results that were obtained in the first transmission study. Interestingly, an improvement of *E.canis* transmission was observed in the second naturally fed transmission study. The results of this study revealed a quicker onset of clinical signs as compared to the first transmission study. Decreased platelet counts and PCR positive tests occurred one week earlier than in the previous transmission study. Additionally, the onset of clinical signs appeared to be more pronounced than in the first transmission study. The dogs tested in the second transmission study had multiple weeks in succession when clinical signs were observed, while the first study had most clinical signs sporadically distributed throughout the study. This second transmission study further validates the need to use low pass isolates when conducting tick transmission studies with *E.canis*. The most ideal method for studying *E.canis* or another Rickettsial disease may be to use an isolate that is routinely passed from tick-to-dog-to-tick. The aforementioned passage method may avoid the issue of lost recognition to the tick that is associated with passing the isolate only in cell culture or the mammalian host.

CHAPTER 3

TRANSMISSION OF *EHRLICHIA CANIS* TO DOGS BY EXPERIMENTALLY-INJECTED *RHIPICEPHALUS SANGUINEUS* TICKS

Ehrlichia canis tick to dog transmission studies have always been conducted using a parasitemic donor dog to infect the ticks. While this method of infecting ticks is effective, it would be useful to have an alternative method that avoids the complications associated with natural feeding. For example, convalescent donors must be consistently monitored to determine peaks in parisitemia. The timing of tick application to capture these spikes may prove critical in efficient transmission of *E. canis* from dog to tick. Also, there is no method for determining individual tick uptake of infectious material without sacrificing the replete ticks, which were intended for use as vectors for transmission studies. An alternative to this method of feeding ticks on a donor dog has been described for other pathogens. It has been shown that pathogens such as *Anaplasma marginale*, *Hepatozoon canis*, and *Theileria* can be introduced into ticks through experimental injection and the ticks will then become competent transmitters of these pathogens. It was hypothesized that this same technique of experimental injection would also apply to *E. canis.* The hypothesis was tested by developing a method of experimentally inoculating engorged ticks with *E. canis* infected white blood cells and then allowing the infected ticks to feed on dogs. It was expected that the injected engorged nymphs would successfully molt and subsequently become competent vectors of *E.canis*. This injection method would avoid the previously described complications for natural infection of ticks with *E. canis*. The ticks would be inoculated with a known amount of infectious material and each tick would receive the same amount of material with out question. The need for maintaining donor dogs

and predicting peaks in parasitemia could also be eliminated by the use of frozen or cell cultured material.

MATERIALS AND METHODS

Preparation of Experimentally-Injected Nymphs

Fully blood-fed nymphs were prepared at Oklahoma State University and shipped immediately after harvest. Within 6-8 days after repletion with the blood meal, the nymphs were percutaneously- injected with *E. canis* infected WBCs. The WBCs were obtained from the two dogs infected with the low passage *E. canis* isolates by IV. The infected WBCs were isolated from whole blood from these dogs by centrifugation. The isolated WBCs were resuspended in sterile PBS at a concentration of $1.0x10^6$ WBCs/ml. Immediately after isolation, the WBCs were injected into the engorged nymphs. Each nymph was placed dorsal side down on double-sided tape and a 30-guage needle was used to inject the WBC suspension into the nymphs (7). The injection site was located under the integument in the central part of the alloscutum (Figures 18, 19). The material was injected at approximately 0.5-1.0ul per engorged nymph. Once the material was administered, the needle was not withdrawn until several seconds had passed. This reduced the possibility of material escaping from the hole caused by the injection. The injection site formed a plug as the needle was withdrawn and no material or gut contents were lost. The nymphs remained on the tape for approximately one hour before being removed. The ticks were then returned to the vented tubes and incubated at 27°C and 95% humidity. Once the nymphs molted into adults, a random sample (5 to 10%) of the molted ticks were tested for *E. canis* infection, using a standard PCR assay. The remaining ticks were used to transmit *E. canis* to naive dogs.

Figure 18: Engorged nymph injected with E. canis infected white blood cells.

Figure19: Engorged nymph after injection with white blood cells. Note the formation of a seal at the injection site.

Analysis of a 1µl sample of White Blood Cell suspension

White blood cells were isolated from *E. canis* positive dog 16604 (strain New Mexico), that had been IV challenged with infected blood. The method of WBC isolation described below was used to prepare a sample of $1.0x10^6$ WBCs/ml. DNA was extracted from multiple volumes of the WBC suspension. The following volumes were used: 1µl, 10µl, 25µl, 50µl, 100µl. The DNA extractions were set up as described below and were followed by 16S nested PCR reactions to detect *E. canis*.

Dog and Tick Follow-Up Testing

Dog

Weekly blood samples and temperatures were taken from the tick challenged dogs to monitor the *E. canis* infection. The infection was monitored by temperature, 16S nested PCR, CBC counts, and western blots. A dog was diagnosed as successfully infected after exhibiting multiple symptoms including, high temperatures, low platelet counts, and PCR positive.

Tick

Adult ticks that were collected after 10-14 days of blood feeding were housed in the tick incubation chamber. The adult ticks were tested 3 weeks post blood meal for presence of *E. canis*. Pools of 5-10 ticks were prepared to assess the presence of *E. canis* post blood meal. The ticks were tested by 16S-nested PCR.

RESULTS

Experimental-Injection of WBCs into Nymphs

White blood cells were isolated from both IV-challenged dogs on days 18 and 21 post-challenge. On these days, the isolated white blood cells were injected into engorged *Rhipicephalus sanguineous* nymphs at a concentration of $2.5X10^6$ WBCs/ml. The engorged nymphs were 6 or 8 days removed from completion of blood meals when injected with the WBC suspensions. Each nymph received 0.5-1.0µl of the WBC suspension. An attempt was made to extract *E. canis* DNA from a 1.0µl sample of WBC suspension prior to tick injection. The results from the 16S nested PCR indicate that there is not enough material in a 1.0µl sample of $1.0X10^6$ WBC suspension to detect *E. canis*. The lowest volume of this WBC suspension that yielded a positive PCR result for *E. canis* was 25.0µl.

The injected ticks molted to adults at an average of 86% survival rate (Table 21). A pool of 5 molted ticks from each experimentally injected group tested negative for *E. canis* infection by 16S nested PCR. The decision was made not to sacrifice further molted ticks for PCR analysis and to move forward with the transmission study using the remaining ticks.

Table 21: Molting results of experimentally injected nymphs

E. canis Strains	AZ Dog	NM Dog	NM Dog
WBC Harvest Day	18	18	21
Days Post Engorgement	6	6	8
Number Inoculated	150	150	200
Volume per tick	0.5-1ul	0.5-1ul	0.5-1ul
WBCs per ml	$2.5X10^6$	$2.5X10^6$	$2.5X10^6$
Number Molted	136	119	173
% Molted	91%	79%	87%

Transmission Feeding of Adult Injected Ticks on Dogs

Four SPF beagles were used to feed the adult ticks that had been experimentally injected with white blood cells from dogs in the acute stages of Ehrlichiosis. The ticks were applied to the dogs in the following order: Dogs-85304 and 85305 received Arizona strain injected ticks at 68 and 68 ticks per dog; Dogs-53802 and 53705 received New Mexico strain injected ticks at 142 and 150 ticks per dog (Table 22). The ticks were collected from the dogs after feeding for 10-14 days. The fed ticks were counted and then tested for *E. canis* infection by 16S nested PCR after 3 weeks in the incubation chamber. To test the ticks for the presence of E.canis, groups of 5 and 10 ticks were assembled from each dog and processed as described in the materials and methods in chapter 2 for DNA extraction. All dogs yielded ticks that tested positive for *E.canis* by PCR after transmission feeding (Table 23). The positive results from the PCR test of the ticks was only observed in the 10 tick pools and not in the pools of 5 ticks (Figure 10, 11). Further analysis of the groups of ticks was not made after obtaining positive PCR results with the pools of 10 ticks.

All four of the tick challenged dogs showed clinical signs and 3 of 4 dogs became PCR positive during the study. An increase in white blood cells, mean platelet volume, monocytes and

lymphocytes were observed between days 42 and 49, while a decrease in platelet counts was observed between days 42 and 49 (Tables 27,28,29,31,32). Results from temperature monitoring revealed decreases between days 35 and 38 and increases in average temperatures between days 42 and 63 (Tables 25,26). Hemoglobin analysis of the groups during the 91 day study did not reveal any changes (Table 30). The first dog became 16S nested PCR positive on day 49 and all dogs, except 53705, became PCR positive at least once during the study (Table 24). None of the PCR positive dogs maintained positive results for more than one week. The serological analysis by western blot of the four dogs indicated an antibody response against *E.canis* antigen by all dogs at day 91 except 53705 (Figure 20). Only dogs 85304 and 53802 developed strong antibody response as compared to the IV challenged dogs. Dog 85305 had a mild antibody response against the *E.canis* antigen.

Table 22: Numbers of adult ticks applied to each naïve dog.

Dog ID	Type of Tick Applied	Number of ticks	Engorged	Partially Fed
85304	AZ Injected	68 Adults	40	28
85305	AZ Injected	68 Adults	13	55
53802	NM Injected	142 Adults	81	61
53705	NM Injected	150 Adults	70	80

Table 23: Post challenge PCR screen of adult injected ticks.

	Post Challenge PCR	16S Nested PCR	
Dog ID	Type of Tick Applied	5 Tick Pool	10 Tick Pool
85304	AZ Injected	Negative	Positive
85305	AZ Injected	Negative	Positive
53802	NM Injected	Negative	Positive
53705	NM Injected	Negative	Positive
Tick Control	Uninfected Control Ticks	Negative	Negative
	Positive Control	Positive	Positive
	No DNA Control	Negative	Negative

Table 24: PCR data for injected tick challenged dogs. PCR positive days are noted in bold.

		Study Day												
ID	Type of Tick	-3	7	14	28	35	42	49	56	63	70	77	84	91
85304	AZ Lab Injected	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg
85305	AZ Lab Injected	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg
53802	NM Lab Injected	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg
53705	NM Lab Injected	Neg	Neg	Neg	Neg	Neg	Neg							
	Positive Control	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**
	No DNA Control	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Table 25: Temperatures for injected tick challenged dogs °F. High temperatures are noted in bold.

		Days Post Tick Challenge															
Type of Tick	ID	-3	1	2	3	4	7	10	14	17	22	24	28	31	35	38	42
NM Injected	53705	102.4	102.8	102.8	102.6	102.5	102.8	102.7	103.3	102.5	102.0	102.7	102.7	102.1	102.5	101.7	**104.6**
	53802	102.7	102.1	102.4	102.5	102.6	103.4	103.1	102.5	102.4	101.8	102.7	101.3	102.3	102.6	100.6	102.4
Average		102.6	102.5	102.6	102.6	102.6	103.1	102.9	102.9	102.5	101.9	102.7	102.0	102.2	102.6	101.2	103.5
AZ Injected	85304	102.6	103.6	103.4	102.8	102.4	102.5	102.7	102.7	102.2	101.8	102.9	102.7	102.5	102.3	101.6	102.6
	85305	102.6	103.0	102.7	102.0	101.6	102.5	102.5	103.5	102.8	101.9	101.6	101.9	102.5	102.5	99.7	101.8
Average		102.6	103.3	103.1	102.4	102.0	102.5	102.6	103.1	102.5	101.9	102.3	102.3	102.5	102.4	100.7	102.2

Table 26: Temperatures continued for injected tick challenged dogs.

		Days Post Tick Challenge												
Type of Tick	ID	45	49	52	56	59	63	66	70	73	77	80	84	91
NM Injected	53705													
	53802	102.2	103.4	102.2	101.4	102.4	102.1	102.7	102.2	102.1	102.3	102.4	102.6	101.6
Average		102.2	103.4	102.2	101.4	102.4	102.1	102.7	102.2	102.1	102.3	102.4	102.6	101.6
AZ Injected	85304	103.0	101.7	102.1	102.8	101.9	101.8	102.3	101.7	103.3	102.5	101.8	101.9	102.3
	85305	102.0	102.4	102.0	102.6	102.4	101.6	101.6	102.3	102.8	102.1	101.8	101.9	102.2
Average		102.5	102.1	102.1	102.7	102.2	101.7	102.0	102.0	103.1	102.3	101.8	101.9	102.3

Table 27: White blood cell counts (K/ul) for injected tick challenged dogs. High counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Injected	85304	12.28	12.96	10.60	9.86	11.18	6.52	7.80	8.52	9.50	9.04	8.88	10.82	7.96
	85305	**21.40**	14.78	15.64	14.48	14.44	**20.38**	10.36	**17.30**	13.98	13.98	13.86	16.58	13.52
AZ Injected Average		16.84	13.87	13.12	12.17	12.81	13.45	9.08	12.91	11.74	11.51	11.37	13.70	10.74
NM Injected	53705	11.80	10.60	14.04	13.76	11.56	15.94							
	53802	**19.68**	15.16	10.62	11.80	13.36	16.00	16.08	10.68	12.98	11.86	15.48	13.32	12.60
NM Injected Average		15.74	12.88	12.33	12.78	12.46	15.97	16.08	10.68	12.98	11.86	15.48	13.32	12.60

Table 28: Lymphocyte counts (K/ul) for injected tick challenged dogs. High counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Injected	85304	4.35	4.14	3.19	2.76	3.71	2.53	2.91	2.08	2.39	2.61	2.56	2.86	1.86
	85305	**6.18**	4.63	4.65	4.78	**5.27**	**6.11**	3.83	4.57	3.81	3.96	3.07	3.98	2.91
AZ Injected Avg		5.27	4.39	3.92	3.77	4.49	4.32	3.37	3.33	3.10	3.29	2.82	3.42	2.39
NM Injected	53705	4.99	3.69	3.36	4.41	4.53	3.09							
	53802	**6.93**	**5.39**	4.27	4.87	**6.82**	**6.50**	**5.35**	3.15	3.13	4.12	4.01	3.82	3.29
NM Injected Avg		5.96	4.54	3.82	4.64	5.68	4.80	5.35	3.15	3.13	4.12	4.01	3.82	3.29

Table 29: Monocyte counts (K/ul) for injected tick challenged dogs. High counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Injected	85304	1.17	0.83	0.51	0.59	1.01	1.11	0.48	0.72	0.72	0.63	0.63	0.73	0.51
	85305	1.44	1.14	0.89	0.79	0.94	**3.23**	0.62	1.15	0.91	0.65	0.70	1.21	0.75
AZ Injected Average		1.31	0.99	0.70	0.69	0.98	2.17	0.55	0.94	0.82	0.64	0.67	0.97	0.63
NM Injected	53705	1.67	0.57	0.78	0.82	0.80	1.91							
	53802	1.83	0.90	0.77	0.82	1.98	**2.82**	0.97	0.48	0.65	0.72	0.44	0.75	0.74
NM Injected Average		1.75	0.74	0.78	0.82	1.39	2.37	0.97	0.48	0.65	0.72	0.44	0.75	0.74

Table 30: Hemaglobin counts (K/ul) for injected tick challenged dogs.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Injected	85304	15.10	15.60	15.80	15.40	14.50	15.30	14.90	17.10	16.10	17.00	16.30	17.00	17.80
	85305	16.00	16.60	16.80	15.00	16.30	15.60	16.10	17.00	16.60	17.10	15.80	17.30	16.80
AZ Injected Average		15.55	16.10	16.30	15.20	15.40	15.45	15.50	17.05	16.35	17.05	16.05	17.15	17.30
NM Injected	53705	12.90	14.20	14.20	15.10	14.20	12.80							
	53802	13.50	13.80	13.10	12.90	13.50	12.80	12.90	13.10	13.10	13.20	12.90	14.30	12.60
NM Injected Average		13.20	14.00	13.65	14.00	13.85	12.80	12.90	13.10	13.10	13.20	12.90	14.30	12.60

Table 31: Mean Platelet Volume (fL) for injected tick challenged dogs. High counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Injected	85304	**15.40**	13.90	13.20	**15.10**	14.80	**19.20**	**16.40**	**16.40**	15.00	14.80	**17.70**	**15.20**	**15.70**
	85305	13.30	10.80	13.20	14.80	11.80	**15.10**	13.00	14.00	13.30	12.70	12.10	**15.60**	13.00
AZ Injected Average		14.35	12.35	13.20	14.95	13.30	17.15	14.70	15.20	14.15	13.75	14.90	15.40	14.35
NM Injected	53705	14.20	10.70	10.90	10.70	11.20	14.80							
	53802	17.10	13.20	12.80	13.60	12.30	**18.40**	**18.60**	14.70	14.80	13.90	14.70	**15.10**	14.80
NM Injected Average		15.65	11.95	11.85	12.15	11.75	16.60	18.60	14.70	14.80	13.90	14.70	15.10	14.80

Table 32: Platelet counts (K/ul) for injected tick challenged dogs. Low counts are noted in bold.

		Days Post Tick Challenge												
Type of Tick	ID	7	14	22	28	35	42	49	56	63	70	77	84	91
AZ Injected	85304	328.0	367.0	384.0	361.0	284.0	**71.0**	310.0	363.0	312.0	354.0	331.0	303.0	365.0
	85305	243.0	451.0	444.0	147.0	427.0	**134.0**	418.0	201.0	450.0	418.0	390.0	**166.0**	384.0
AZ Injected Avg		285.5	409.0	414.0	254.0	355.5	102.5	364.0	282.0	381.0	386.0	360.5	234.5	374.5
NM Injected	53705	408.0	446.0	429.0	518.0	447.0	**198.0**							
	53802	319.0	415.0	350.0	296.0	311.0	**111.0**	**121.0**	286.0	302.0	276.0	241.0	336.0	332.0
NM Injected Avg		363.5	430.5	389.5	407.0	379.0	154.5	121.0	286.0	302.0	276.0	241.0	336.0	332.0

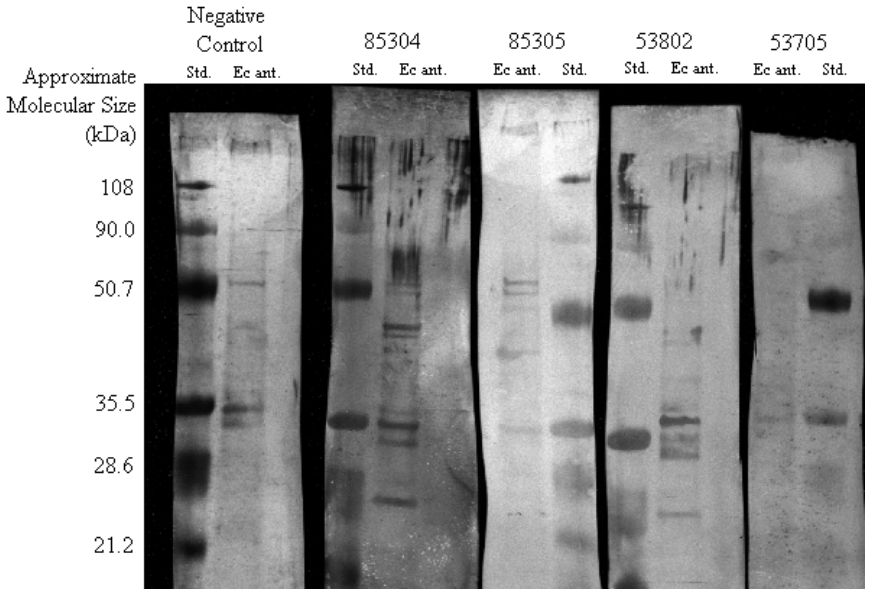

Figure 20: Western blot analysis of dogs challenged with injected ticks. Low range standard is noted as Std. and *E.canis* antigen is noted as Ec ant.

DISCUSSION

The hypothesis that low passage *E. canis* isolates can be successfully introduced to engorged nymphs by experimental injection was tested in this study. It was discussed that injection of engorged nymphs with *E. canis* infected white blood cells would produce competent vectors of the pathogen. As predicted, after injection with the infected WBCs, the low passage isolates were successfully introduced into and maintained in engorged nymphs through the molt. These ticks were then demonstrated to be competent vectors of *E.canis* as adults as they successfully transmitted the bacteria to four *E.canis* negative dogs. The transmission study was performed using high numbers of ticks (68, 142, and 150) to ensure successful transmission of *E.canis* from tick to dog. Future studies need to be performed using biologically realistic numbers of ticks to validate this technique as an alternative to using naturally fed ticks for *E.canis* transmission studies. It would also be useful to examine experimental injection of engorged nymphs with infected cell culture and infected frozen WBCs. Experimental injection of these infectious materials would avoid the need for maintaining an infected donor to provide the infectious material. Ultimately, this technique would provide a humane and cost effective alternative to exposing donor dogs to *E. canis*. This method will also allow transmission studies to be conducted without the issues encountered with natural tick infection previously mentioned.

As with chapter 2, the severe form of CME failed to develop in these four dogs. Dog 53705 was removed from the study on day 42 after developing fever (104.6) and a low platelet count (198.0). However, this dog was removed prior to becoming PCR positive and further analysis of this dog was not made beyond day 42. The PCR results and serological responses of these four dogs where also similar to dogs in chapter 2. In comparison, the two studies had very

similar results. One could make the observation that the experimentally injected ticks transmitted *E.canis* as well as the naturally infected ticks. However, more ticks were applied to each dog in the injection tick study and fewer dogs were tested. It is possible that further testing of the experimental injection method may produce *E. canis* transmission results equal to those for naturally fed ticks.

CONCLUSIONS

The use of low passage *E.canis* isolates effectively improved the natural feeding method of infecting ticks. The transmission studies using the naturally fed ticks demonstrated a specific and precise representation of the field model of tick transmission. This was observed by successful transmission of the pathogen in all tick challenged dogs and by using biologically realistic numbers of tick. While the results of these studies add to the current understanding of the *E. canis* dog-tick-dog transmission cycle, further analysis of this model needs to be tested. Future studies could include using a low passage isolate to identify the lowest limit of ticks needed for successful transmission to occur. Additionally, the first hypothesis could be tested by maintaining a low passage isolate in continuous cell culture and then testing to see if infectivity in the tick decreases over time. This could lead to the genetic comparison of the high versus low passage of *E.canis* to identify genes that would be needed by the organism to infect ticks.

The experimental injection study demonstrated that low passage *E.canis* can infect engorged nymphs when introduced by artificial injection. This study also demonstrates that the injected ticks are competent vectors of the pathogen as adults. The injected ticks were able to infect four of four dogs through blood feeding as adults. The injection method of artificially infecting ticks may prove to be a useful tool for future transmission studies. Specifically by avoiding the limitations associated with needing parasitemic hosts to complete transmission cycles. Future studies will need to identify sources other than acutely infected dogs for obtaining infectious material. For example, cell cultured field isolates and frozen banks of WBCs from infected dogs could be alternative sources. Ultimately, this may enable more vector transmission studies to be carried out that would otherwise not have taken place. One area that could be

extensively studied using this technique would be to study the biology of the pathogen in the tick. Studies can be conducted identifying tissues infected by *E. canis* and the process by which the organism travels from the gut of the tick to transmission through blood feeding. These studies may lead to the identification of when transmission occurs from the feeding tick to the host. This time of transmission has been identified for pathogens such as *Borrelia burgdorferi*, the causative agent of lyme disease, and is about 36 hours from the time feeding is initiated. This may or may not be the same for *E .canis* transmission. The use of the experimental injection technique may lead to the determination of when *E. canis* transmission occurs during the blood meal.

In conclusion, these studies document new information with regard to vector transmission of *E.canis* as well as pathogenesis of CME. The significance of using a low-passage isolate for *E. canis* transmission studies was highlighted with both the naturally fed and experimentally injected tick studies. Overall, the data obtained from these studies will add to the knowledge of *E.canis* transmission, as well as improve the methods in which future transmission studies are conducted.

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