Dedicated to the great state of Minnesota and my family who resides within it.
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TABLE OF CONTENTS

ACKNOWLEDGMENTS ................................................................................................................................. 4

LIST OF TABLES ........................................................................................................................................ 7

LIST OF FIGURES ....................................................................................................................................... 8

ABSTRACT .................................................................................................................................................. 9

CHAPTER

1 BACKGROUND AND SIGNIFICANCE ................................................................................................. 11

Overview and Long Term Objectives ........................................................................................................ 11
Central Hypothesis .................................................................................................................................. 14

2 EXPERIMENTAL INOCULATION OF DOGS WITH ANAPLASMA PHAGOCYTOPHILUM
IN CULTURED MAMMALIAN CELL LINES AND FROM NATURALLY INFECTED BLOOD STABILATE,
EVIDENCE OF INFECTION, AND NECROPSY OF AN INFECTED ANIMAL .................................................. 15

Introduction ............................................................................................................................................... 15
Materials and Methods .............................................................................................................................. 16
Inoculum, Cultures and Mammalian Cell Lines ....................................................................................... 16
Animals, Breed, Identification and Group Designation ............................................................................ 17
Inoculation, Bacterial Concentrations and Infection of Animals .............................................................. 17
Monitoring for Development of Infection .................................................................................................. 19
PCR Detection of A. phagocytophilum DNA in Whole Blood ................................................................. 19
Serological Assays for A. phagocytophilum Infection .............................................................................. 20
Lymphocyte Proliferation Assays ............................................................................................................. 21
Cytokine Analysis of A. phagocytophilum Stimulated T cells ................................................................... 22
Clinical Chemistry and Hematology of A. phagocytophilum Infection .................................................... 23
Necropsy .................................................................................................................................................... 23
Results ....................................................................................................................................................... 24
Physical Exam, Clinical Chemistry and Hematology .............................................................................. 24
Molecular Evidence of Infection ................................................................................................................. 26
Serological Evidence of Infection ............................................................................................................... 27
Lymphocyte Proliferation Assays ............................................................................................................. 27
Cytokine Assays ....................................................................................................................................... 28
Gross Pathology and Histopathology of Necropsy .................................................................................. 28
Discussion .................................................................................................................................................. 29

3 MOLECULAR EVIDENCE OF PERSISTENT ANAPLASMA PHAGOCYTOPHILUM
INFECTION FOLLOWING TREATMENT WITH DOXYCYCLINE, REINOCULATION WITH INFECTED BLOOD STABILATE, AND IMMUNOSUPPRESSION WITH PREDNISONE ............................................................. 43
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Group designation Group1 and Group 2.</td>
<td>39</td>
</tr>
<tr>
<td>2-2</td>
<td>Sample clinical exam form.</td>
<td>40</td>
</tr>
<tr>
<td>2-3</td>
<td>Lymphocyte proliferation assay stimulation index summary Group 1</td>
<td>41</td>
</tr>
<tr>
<td>2-4</td>
<td>Lymphocyte proliferation assay stimulation index summary from Group 2</td>
<td>42</td>
</tr>
<tr>
<td>3-1</td>
<td>Group designation Group A</td>
<td>58</td>
</tr>
<tr>
<td>3-2</td>
<td>PCR detection of <em>A. phagocytophilum</em> for Group A</td>
<td>59</td>
</tr>
<tr>
<td>3-3</td>
<td>PCR detection of <em>A. phagocytophilum msp2</em> gene in 1.0 gram of post–mortem tissues from Group A</td>
<td>60</td>
</tr>
<tr>
<td>3-4</td>
<td>PCR detection of <em>A.phagocytophilum</em>, post reincoulation for Group B</td>
<td>61</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Buffy coat smears of animals from Group 2</td>
<td>33</td>
</tr>
<tr>
<td>2-2</td>
<td>Real time PCR of the <em>msp5</em> gene of Group 2 animals</td>
<td>34</td>
</tr>
<tr>
<td>2-3</td>
<td>Group 2 comparison of neutrophils and lymphocytes</td>
<td>35</td>
</tr>
<tr>
<td>2-4</td>
<td>Lymphocyte proliferation assay for Group 1, animals 8H06 and 8H13</td>
<td>36</td>
</tr>
<tr>
<td>2-5</td>
<td>Lymphocyte proliferation assay for Group 2, animals 8H08 and 8H12</td>
<td>37</td>
</tr>
<tr>
<td>2-6</td>
<td>Cytokine results for Group 2 animals 8H11 and 8H12 for analytes IL-8, IL-10 and TNF-α</td>
<td>38</td>
</tr>
<tr>
<td>3-1</td>
<td>Mean IL-8 Response for Group B throughout the entire study</td>
<td>55</td>
</tr>
<tr>
<td>3-2</td>
<td>Mean IL-10 response for Group B throughout the entire study</td>
<td>56</td>
</tr>
<tr>
<td>3-3</td>
<td>Mean TNF-α response for Group B throughout the entire study</td>
<td>57</td>
</tr>
</tbody>
</table>
A CLINICAL MODEL OF CANINE GRANULOCYTIC ANAPLASMOSIS

By
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December 2009

Organisms within the family Anaplasmataceae are gram-negative, obligate, intracellular bacteria that require eukaryotic cells within the tick or mammalian host for survival and replication. In the limited number of published studies, canine granulocytic anaplasmosis typically presents as an acute, febrile, systemic illness. Clinical signs of infection of A. phagocytophilum in dogs are generally non-specific, including fever, lethargy, depression, anorexia, myalgia, joint pain, and lameness. Abnormal laboratory values are likewise relatively nonspecific, but when combined with clinical presentation may be suggestive of the disease. Abnormal laboratory findings include neutropenia, thrombocytopenia, hyperproteinemia, hyperglobulinemia, mild nonregenerative anemia, and mild hypoalbuminemia. While these signs have been associated with clinical granulocytic anaplasmosis, many of the infected animals had potential co-infection with other pathogens. The presence of additional pathogens may confound or cause synergistically more severe disease.

Presently, doxycycline is the recommended antimicrobial treatment for A. phagocytophilum infection, in both dogs and humans. However, some preliminary studies indicate molecular evidence of persistent infection, following doxycycline therapy. If this is true,
even after treatment with doxycycline, animals may serve as a potential reservoir for the propagation of the bacterium and transmission to other animals or humans.

In the current study, the dogs were infected with an isolate of *A. phagocytophilum* that originated from a clinically ill dog in Minnesota, and infection with *A. phagocytophilum* did not result in the full range of clinical signs reported in the literature. None of the animals showed significant signs of joint pain, lameness, lethargy or anorexia; although a few animals did experience a mild decrease in activity level and mild inappetence that correlated with bacteremia. Additionally, abnormal laboratory values of thrombocytopenia, hyerproteinemia, hyperglobulinemia, and anemia were not seen. The common constellation of signs and abnormal laboratory values began between 7 and 14 DPI and included fever, neutropenia (or a significant decrease in neutrophils), lymphocytosis, mild decrease in activity, and mild inappetence. Severe clinical disease was not seen, but instead a transient mild clinical disease in the absence of potentially confounding co-infection.

Animals from the first study, with documented *A. phagocytophilum* infection were treated with an aggressive regime of doxycycline months after resolution of acute disease and any detection of bacteremia. Immunosuppression with prednisone allowed infections to recrudesce in all animals to significant levels. In some animals, the levels approached those seen in the acute infection. Necropsies done at the time of recrudescence in these animals showed widespread presence of *A. phagocytophilum* in nearly all tissues assayed. These findings have critical implications since treatment with doxycycline may not eliminate *A. phagocytophilum* bacteria from clinically normal dogs with no measurable or detectable bacteremia. Dogs may become life-long *A. phagocytophilum*-carriers, at risk for recrudescence with concurrent illness or immunosuppression.
CHAPTER ONE
BACKGROUND AND SIGNIFICANCE

Overview and Long Term Objectives

Organisms within the family *Anaplasmataceae* are gram-negative obligate intracellular bacteria that require eukaryotic cells within the tick or mammalian host for survival and replication (18). Organisms in the family include infectious agents from the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* (15). Several of these organisms cause potentially fatal tick-transmitted diseases in humans and animals. Important diseases in humans and animals caused by these organisms include human monocytic ehrlichiosis (*Ehrlichia chaffeensis*), human and canine granulocytic ehrlichiosis (*Ehrlichia ewingii*), canine ehrlichiosis (*Ehrlichia canis*), bovine anaplasmosis (*Anaplasma marginale*), and canine anaplasmosis (*A. platys* and *A. phagocytophilum*) (15).

*Anaplasma phagocytophilum* was identified in Minnesota and Wisconsin in 1990 and subsequently the infection in humans, human granulocytic ehrlichiosis (HGE), was first described in 1994 in patients in Wisconsin and Minnesota (1, 4, 40). Canine granulocytic ehrlichiosis was first reported in dogs in 1996 (28), however cases were likely seen as early as 1986 (4). The infectious agent was thought to be a species within the genera of *Ehrlichia* and was initially referred to as granulocytic ehrlichiosis, but a genetic analysis of several organisms in the genera *Ehrlicha* and *Anaplasma* caused reclassification (17). Hence, *Ehrlichia phagocytophila*, *E. equi* and the HGE agent have since been reclassified as *Anaplasma phagocytophilum*; and the disease *A. phagocytophilum* causes has been renamed granulocytic anaplasmosis.

*Anaplasma phagocytophilum* is distributed worldwide throughout North America, Europe, and Asia and is transmitted by hard bodied Ixodid ticks (17, 27, 37). It is the cause of human
granulocytic anaplasmosis and canine granulocytic anaplasmosis; although *A. phagocytophilum* infection has been described in several other animals including cats, rodents, horses, lambs, and sheep (52, 54).

Clinical signs and abnormal laboratory findings associated with human and canine anaplasmosis typically present during the acute, bacteremic phase of infection. Signs and symptoms of disease are quite variable and include lethargy, inappetence, fever, splenomegaly, malaise, headache, and myalgia (15, 36, 52, 50). Rare signs and symptoms include arthralgia, gastrointestinal signs such as nausea, vomiting, and diarrhea, mild to severe respiratory signs and central nervous system signs linked to meningitis, which can result in seizures, ataxia, or expressions such as dullness or stupor. Common abnormal laboratory findings include thrombocytopenia, leukopenia, anemia, neutropenia and increased serum concentrations of liver enzymes (15, 27, 36, 51, 53). Many of these signs and symptoms were often associated with secondary infections (55). Common serum biochemical abnormalities include elevated serum alkaline phosphatase activity and mild to moderate hypoalbuminemia and hyperfibrinogenemia (36). Evaluation of buffy coat smears at times of high bacteremia reveal membrane-bound vacuoles of intracytoplasmic organisms (morulae) circulating within the neutrophils of an acutely infected dog (29). Acute infections and potential subclinical persistent infections have been documented in dogs naturally and experimentally inoculated with isolates of *A. phagocytophilum* (21, 23, 24, 25, 36, 37, 48).

Definitive signs of disease and pathogenesis previously described for naturally occurring *A. phagocytophilum* are likely complicated by the ability of a single tick to harbor and transmit multiple pathogens, and by the fact that dogs can be infected with multiple tick species. It is not uncommon to have positive serological test results to multiple *Ehrlichia spp.*, as well as
Anaplasma spp. and Neorickettsia spp. (13). Whether a single tick infected with multiple pathogens or multiple ticks on a single animal, either could serve as a vector for the simultaneous infection of multiple pathogens in a single host. Concurrent infections in naturally infected dogs with multiple arthropod-borne pathogens, including Ehrlichia spp. and Borrelia spp., have been documented in case reports and in epidemiologic studies (40). Co-infection with multiple pathogens complicates disease diagnosis and treatment, and confounds reports from previous studies that attempt to describe the clinical course of naturally occurring clinical infections (15). Often, dogs with A. phagocytophilum will present with clinical signs of fever, polyarthritis and a history of recent or previous tick exposure. Diagnosis can be further complicated by variation in abnormalities in laboratory test results during the acute phase of the disease. Furthermore, CBC and serum chemistry test results in persistently infected subclinical carriers are often normal (Alleman, personal communication).

Presently, doxycycline is the recommended antimicrobial treatment for A. phagocytophilum infection, in both dogs and humans (1, 28, 30, 43) and rapid clinical improvement within one to two days of treatment is typically observed in most uncomplicated cases (1, 10, 22, 28). However, some preliminary reports indicate molecular evidence of persistent infection, following doxycycline therapy (2, 3). If this is true, even after treatment with doxycycline, animals may serve as a potential reservoir for the propagation of the bacterium and transmission to other animals or humans.

Controlled studies investigating the optimal duration of treatment or the dosage required to completely clear viable A. phagocytophilum bacteria from infected individuals have not been reported. It is not certain whether the current recommended treatment for A. phagocytophilum
infection merely alleviate clinical signs and potentially result in a carrier state of infection. This knowledge is currently lacking in our understanding of this zoonotic pathogen.

The first objective of this study is to develop an infection model for canine anaplasmosis using an isolate of *A. phagocytophilum* from a naturally infected dog in order to evaluate the infection and clinical disease parameters of an infection free of confounding co-infections. The second objective is to determine if doxycycline is efficacious towards the eradication of *A. phagocytophilum* in experimentally infected dogs.

**Central Hypothesis**

The central hypothesis of this study is that *Anaplasma phagocytophilum* causes measurable disease in dogs that can be eradicated with treatment by doxycycline. The hypothesis was tested using the following specific aims.

- characterize the canine disease and immunological response caused by *A. phagocytophilum* grown in cultured mammalian cell lines and from infected blood stabilate.

- determine whether treatment with doxycycline will clear *A. phagocytophilum* bacteria in experimentally infected dogs.

Developing a more thorough definition of the clinical disease, the host immune response and the efficacy of treatment, will provide veterinarians with better parameters in order to diagnosis and effectively treat canine granulocytic anaplasmosis and potentially limit transmission of this zoonotic pathogen.
CHAPTER TWO
EXPERIMENTAL INOCULATION OF DOGS WITH *ANAPLASMA PHAGOCYTOPHILUM* IN CULTURED MAMMALIAN CELL LINES AND FROM NATURALLY INFECTED BLOOD STABILATE, EVIDENCE OF INFECTION, AND NECROPSY OF AN INFECTED ANIMAL

Introduction

Naturally acquired canine granulocytic anaplasmosis typically presents as an acute, febrile, systemic illness (50). Clinical symptoms of infection with *Anaplasma phagocytophilum* in dogs are generally non-specific, including fever, lethargy, depression, anorexia and myalgia (15, 36, 51). Abnormal laboratory evaluations are likewise nonspecific, but when combined with clinical presentation may be suggestive of the disease. Abnormal laboratory findings have included thrombocytopenia, hyperproteinemia, hyperglobulinemia, mild nonregenerative anemia, and mild hypoalbuminemia (15, 27, 51).

Concurrent infections in naturally infected dogs with multiple arthropod-borne pathogens, including *Ehrlichia spp.* and *Borrelia spp.*, have been documented, which have the potential to confound clinical signs (40). It is possible and even probable that many signs often associated with *A. phagocytophilum* infection are due to an additional pathogen or alternatively a synergistic effect of the co-infections. Due to the potential of confounding results, it is imperative that a controlled experimental infection ruling out co-infection be done in order to confidently delineate the true clinical signs attributable to *A. phagocytophilum*. Only a few experimental infections have been reported. One of the studies was done with blood taken from a dog with clinical disease, but co-infection was not ruled out (38). Other experimental infections were done using human isolates (NY18) of *A. phagocytophilum* (2, 3). It is known that different strains of *A. phagocytophilum* may have different host tropisms. For example, the AP1 strain of *A. phagocytophilum* cannot infect humans (44). Recently Morresette et. al. have shown some significant differences in the *msp2* genes that have been described as adhesions likely important
in cellular invasion and thus infection (44). These animals had inconsistent and minimal clinical signs, but were not extensively evaluated beyond clinical exam, serology, and PCR.

In our study, we set out to develop a complete canine model of the disease caused by a dog strain of A. phagocytophilum in the absence of co-infection to be used in evaluating strain virulence and potentially in vaccine efficacy trials. A dog strain of A. phagocytophilum referred to as the “Baxter” strain was derived from the blood of a clinically diseased animal from Minnesota. The sample did not contain co-infections of B. burgdorferi and E. canis, two of the most common co-infections with clinical disease similar to that associated with natural infection with A. phagocytophilum.

In order to standardize the infectious material in variables such as dose, infectivity, and source, the Baxter strain of A. phagocytophilum was cultured in vitro utilizing two cell lines. The first is derived from a human leukemia and is considered a monocyte-neutrophil precursor (HL-60 – American Type Culture Collection, [ATCC] CCL-240™, Manassas, VA). Following establishment of the bacteria in the HL-60 cell line, the bacteria was transferred to a commercial endothelial cell line derived from Rhesus macaque (RF/6A– ATCC CCL-240™, Manassas, VA). Complete physical exams, laboratory tests, PCR, buffy coat smears, and immunological studies were done to give the most definitive characterization of the disease.

**Materials and Methods**

**Inoculum, Cultures and Mammalian Cell Lines**

Uninfected human (Homo sapiens) HL-60 myeloblastic leukemia cells (ATCC CCL-240™, Manassas, VA) were maintained in DMEM medium (HyClone, Logan, UT). Uninfected fetal rhesus monkey (Macaca mulatta) RF/6A endothelial cells (ATCC CRL-1780™, Manassas, VA) were maintained in RPMI-1640 medium (HyClone, Logan, UT). All medium was supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 4 mM Gibco L-Glutamine
(Invitrogen, Carlsbad, CA), 0.25% NaHCO₃ (Sigma, St. Louis, MO), 25 mM HEPES (Sigma, St. Louis, MO), at a pH of 7.5, a temperature of 37°C and 5% CO₂.

Blood stabilate from the index case of the Baxter isolate of A. phagocytophilum was spun down and the buffy coat inoculated into uninfected HL-60 cells at a concentration of 1 x 10⁶ per ml in a 25mm² flask. The cells were grown to greater than 50% infectivity before splitting the cells into three 25mm² flasks. Using a hemocytometer, the infected cells were counted and diluted to a concentration of 1 x 10⁶ per ml using uninfected HL-60 cells. The bacteria were transferred to RF/6A cells by lysing the infected HL60 cells and passing them through a 3 um filter to eliminate any HL-60 contamination. When RF/6A cells were greater than 50% infected, as determined by cyto spin, the cells were split by a ratio of 1:3. To form a cell suspension, the RF/6A endothelial cell monolayers were detached from the culture flask using 0.25% trypsin (HyClone, Logan, UT).

**Animals, Breed, Identification and Group Designation**

Ten dogs, intact males and females, of four to six months in age (on study day 0) were used in this study. Dog breeds included three golden retrievers, three beagles, and four mixed breed. Animals were identified by number tattooed in the ear, except for the golden retrievers, which were identified by different colored collars. Dogs were confirmed to be sero-negative for infection with A. phagocytophilum, B. burgdorferi, E. canis and D. immitis using the Snap Test 4Dx (SNAP®4Dx®, IDEXX, Westbrook, ME) on study day -14.

To test different inoculum preparations, animals were divided into two groups, such that each group had all three breeds represented. Group designations are listed in table 2-1.

**Inoculation, Bacterial Concentrations and Infection of Animals**

For animals in Group 1, RF/6A cells were grown in 75mm² flasks and split until 10 flasks were approximately 65% infected as determined by light microscopic examination of cyto spin.
To form a cell suspension, the RF/6A endothelial cell monolayers were detached from the culture flask using 0.25% trypsin (HyClone, Logan, UT). The cell suspension from each flask was combined and the total suspension was centrifuged at 250g for 10 minutes at 21°C. The pellet was washed 2 times with fresh, sterile Hanks Balanced Salt Solution (HBSS) (20-021-CV, Mediatech, Inc., Manassas, VA). After the second wash, the cellular pellet was resuspended in 5 mL of sterile saline. Two hundred microliters were used to estimate the concentration of viable bacteria using the BAC live-dead kit utilizing a standard curve of plasmid DNA, previously calibrated to bacterial numbers verified with PCR. The cell suspension was diluted with sterile saline to give a final concentration of $1 \times 10^9$ cells/mL. One milliliter of infected cell suspension was used to intravenously inoculate each of the 5 dogs, using the cephalic vein, in Group 1 (8H04, 8H06, 8H09, 8H13 and 8H14). One milliliter of infected cell suspension was stored at -80°C for DNA extraction and to confirm bacterial concentration by Real-Time PCR. One milliliter was used to infect a culture of uninfected bacteria RF/6A cells.

For Group 2, the animals received one of the following infection preparations. One milliliter of blood stabilate, collected in ethylenediaminetetraacetic acid (EDTA) and prepared by adding 10% dimethyl sulfoxide (DMSO), from index dog from which the Baxter strain was derived was used to intravenously inoculate one dog (8H12). Infected HL-60 cells were propagated in 75 mm2 flasks and split when the cell concentration exceeded 5 x 10^6 cells/mL until 5 flasks of infected cells were obtained; however, the infection was low between 10 and 20 percent as estimated by cytospin analysis. Two hundred microliters were used to estimate the concentration of viable bacteria using the BAC live-dead kit. The number of bacteria was approximately $1 \times 10^8$ enough to infect only 1 dog (8H10). One milliliter of infected cell
suspension was stored at -80°C for DNA extraction and to confirm bacterial concentration by Real-Time PCR. One milliliter was used to infect a culture of uninfected HL-60 cells.

The remaining three dogs (8H05, 8H08, and 8H11) were infected with one milliliter of infected blood collected from 8H12, at a concentration of $4.6 \times 10^4$ bacteria/mL as determined by PCR post-inoculation.

**Monitoring for Development of Infection**

Post inoculation clinical observations based on previously reported clinical signs and rectal temperatures were recorded once daily. Clinical parameters used to detect disease included the presence/absence of fever (>103.5°F) lameness, and enlarged lymph nodes. Malaise, anorexia and joint pain/swelling were also monitored on a scale of 1-3 with 3 being most severe (Table 2-2). Lymph node swelling and joint pain/swelling were assessed by palpation of the lymph nodes and diarthroidal joints.

Group 1 dogs were examined daily and phlebotomized 2 times per week for the duration of the trial. Group 2 animals were examined daily and phlebotomized 2 x per week until 9 DPI when blood was collected daily for 2 weeks and 3 x per week after. Blood was collected in purple top EDTA tubes for CBC, PCR analysis, and lymphocyte proliferation assays, and red top sterile tubes, from which serum was collected for serum chemistry and serology.

**PCR Detection of* A. phagocytophilum* DNA in Whole Blood**

DNA was extracted from anticoagulated, EDTA-whole blood using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) in accord with the manufacturer instructions. DNA was extracted from 200 µL of anticoagulated whole blood and used as the template for real-time quantitative PCR amplification of the single copy *msp5* gene and the multicopy *msp2* gene.

For *msp5* PCR, 25µL DNA amplification reactions were performed in a MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode covered with MicroAmp® 96-Well Optical
Adhesive Film on a 7500 Fast Real-Time PCR System using 12.5 µL TaqMan® Fast Universal PCR Master Mix (Applied Biosystems Inc, Foster City, CA) plus 5 µL of extracted DNA template, 0.8 µM of each primer (forward and reverse), and 0.2 µM of fluorescently labeled oligonucleotide probe (MWG Biotech, High Point, NC). The primer set was 5’-AGA TGC TGA CTG GGG ATG AG-3’ (forward) and 5’-TCG GCA TCA ACC AAG TAC AA-3’ (reverse). The oligonucleotide probe was labeled with 5’ 6-carboxyhexafluorescein (HEX) as a reporter dye and 3’ 6-carboxy-N, N’, N’-tetramethylrhodamine (TAMRA) as a quencher. The sequence of the probe was 5’-HEX-CGT AGG TGA GTC TGA TAG TGA AGG TAMRA-3’.

For msp2 PCR, 25µL DNA amplification reactions were performed, as for msp5, using primers 5’-AGC TAA GGA GTT AGC TTA TGA-3’ (forward) and 5’-GGC AAG AGC AGC AGC AAG-3’ (reverse). The oligonucleotide probe was labeled with 5’ 6-carboxyfluorescein (FAM) as a reporter dye and 3’ 6-carboxy-N, N’, N’-tetramethylrhodamine (TAMRA) as a quencher. The sequence of the probe was 5’-FAM-TGT TGT TAC TGG ACA GAC TGA TAA TAMRA-3’.

Thermocycler conditions used to quantify both msp5 and msp2 genes were 95°C for 20 sec activation followed by 40 cycles of 95°C for 30 sec denaturation and 60°C for 30 sec annealing, extension, and data collection. Copy numbers for samples were calculated based on a plasmid standard curve by using the 7500 Fast Real-Time PCR System software (Applied Biosystems Inc, Foster City, CA).

Serological Assays for A. phagocytophilum Infection

The IDEXX SNAP®4Dx® (IDEXX, Westbrook, ME) test was used to determine the time of seroconversion. The A. phagocytophilum test spot on the lateral-flow immunoassay, uses a synthetic peptide based on the immunodominant p44 protein of A. phagocytophilum as the antigen, with 98% specificity and 96% sensitivity. To document the development of antibodies
directed towards *A. phagocytophilum*, serum was monitored twice weekly using a commercially available sandwich ELISA based upon the *msp2* (p44) protein (SNAP®4Dx®, IDEXX, Westbrook, ME), according to manufactures specifications.

**Lymphocyte Proliferation Assays**

Lymphocyte proliferation tests (LPT) were conducted once weekly during the infection trials, to monitor the cell mediated immune response. Proliferation assays were conducted in replicate wells of round-bottom 96-well plates for 6 days. Peripheral blood mononuclear cells (PBMC) at a concentration of 2 x 10⁵ cells per well isolated from whole blood collected in 0.5 M EDTA were cultured for six days in triplicate wells with dilutions of 10, 1, and 0.1 µg/ml *A. phagocytophilum* whole antigen. Bacterial antigen was prepared from either HL-60 or RF/6A cell cultures. The bacteria were released from the cells by repeated shearing 20 times through a 25 gauge needle, then passed through a 3 micron filter to remove any whole cells and large cellular debris. The resultant material was centrifuged at 450xg for 5 min to pellet any remaining cellular debris. The supernatant was centrifuged at 12,000xg for 30 minutes to pellet the bacteria. The bacteria were used whole or sonicated for 2 minutes at 50 Watts with a 4 mm titanium probe to disrupt the bacterial walls. The organisms were resuspended in PBS containing the protease inhibitors antipain and E-64 (Boehringer Mannheim, Indianapolis, IN) at 25 µg/ml and PMSF (Sigma, St. Louis, MO) at 300 µg/ml. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA) and verified with Western blot using known infected serum.

Proliferation assays were conducted in replicate wells of round-bottom 96-well plates (Costar). PBMC (2 x 10⁵ cells/mL) were cultured for 6 days in triplicate wells with dilutions of 10, 1, and 0.1 µg/ml. In addition, ConA (5 µg/ml) was included as a positive control for proliferation. Uninfected RF/6A or uninfected HL-60 antigen at a concentration of 1 µg/ml, depending on which cell type with which the animals had been infected, and medium only were
used as negative controls. The PBMC were radiolabeled for the last 18 h of culture with 0.25 µCi of [³H]thymidine (DuPont, New England Nuclear) and harvested onto glass filters, and radionucleotide incorporation was determined using a Betaplate 1205 liquid scintillation counter. Results are presented as the mean cpm of triplicate cultures ± 1 SD. In addition, a stimulation index (SI) was determined by dividing the cpm of the antigen stimulated cells by the highest negative control. A positive result is considered a SI greater than 2 and a total CPM greater than 200.

Statistical analysis was performed using NCSS for Windows 1997 (NCSS, Kaysville, Utah, USA). A one-way ANOVA was used to analyze data from the multiple dates, with Bonferroni tests to correct for multiple comparisons.

**Cytokine Analysis of *A. phagocytophilum* Stimulated T cells**

Cytokine assays were conducted twice from the infection trial, to monitor the cell mediated immune response in animals from Group 2. Peripheral blood mononuclear cells (PBMC) were isolated from frozen PBMC’s collected at -69 DPI and 14 DPI. PBMCs were incubated using Con A (5µg/ml), complete RPMI 1640 medium, and *A. phagocytophilum* at concentrations of 10 µg/ml, 1 µg/ml, and 0.1 µg/ml overnight. The following day, PBMC’s were centrifuged at 220g for 5 minutes. Supernatant was collected and 150µl of TRIzol® (Invitrogen, CA) was added to the remaining cells. Cells and supernatant were frozen at -80°C until further use.

A commercially available canine cytokine/chemokine kit from Millipore (Canine Cytokine/Chemokine Panel, Millipore, Billerica, MA), was used to test the following analytes, IL-2, IL-4, IL-8, IL-10, IFN-γ and TNF-α. The samples were processed according to the manufactures recommendations, as previously described. 25µL of each sample was added to
25µL of the provided assay buffer and 25µL of the provided matrix solution in a 96 well plate. Cytokine detection beads were premixed in an opaque bottle, to avoid contact with light, and 25µL of the mixture was added to each sample well. The plate was allowed to incubate at 4°C overnight. The following day, the mixture was removed by vacuum and washed twice using the provided wash buffer. 25µL of the detection antibodies were then added to each well and allowed to incubate for one hour. 25µL of Streptavidin-Phycoerythrin was then added and allowed to incubate for 30 minutes. The plate was then washed three times using the provided wash buffer. 150µL of sheath fluid as added to the wells and the plate was read on a Luminex 100\textsuperscript{TM} IS.

Data was analyzed using Milliplex Analyst software (Millipore, Billerica, MA), a weighted 5-parameter logistic method for calculating cytokine concentrations in samples. Statistical data was analyzed using NCSS for Windows 1997 (NCSS, Kaysville, Utah, USA), using paired t-tests.

**Clinical Chemistry and Hematology of *A. phagocytophilum* Infection**

In Group 1 animals, CBC, biochemical profiles, and buffy coat smears were performed on blood collected twice weekly to document hematological and biochemical changes. In Group 2, CBC, biochemical profiles, and buffy coat smears were done twice weekly until 9 DPI when samples tests were done daily.

**Necropsy**

Postmortem examination was performed on the dog 8H10 just following the peak of infection in order to maximize the potential of seeing significant pathology. A complete set of tissues was collected and fixed for 24 hours in 10 percent buffered formalin including trachea, lung, liver, kidney, spleen, lymph nodes, pancreas, joint capsule, lymph nodes, and brain.
Results
Physical Exam, Clinical Chemistry and Hematology

Group 1 dogs were inoculated with *A. phagocytophilum* infected in vitro cultured RF/6A cells. Patent infection of the animals in Group 1 was not demonstrated by detection of morulae using the buffy coat smears. Other than periodic, transient febrile episodes, the physical exam parameters remained normal. The only other potentially significant clinical sign seen in the Group 1 dogs was a transient drop in neutrophil count or true neutropenia (< 3000/µL). Dog 8H04, experienced several febrile events (103.6°F at 13 DPI, 103.9°F at 20 DPI, 103.7°F at 29 DPI, 103.8°F at 33 DPI and 104.2°F at 36 DPI) however, none of these events correlated with the neutropenic event. Three animals did experience one true neutropenic events, 8H04 (2600/µl at 42 DPI), 8H09 (2100/µl at 39 DPI) and 8H13 (2400/µl at 28 DPI), none of which corresponded with a fever.

Two animals in Group 1 experienced a transient increase in lymphocytes (lymphocytosis). Animal 8H04 had an increase from 2850/µl to 5680/µl between 35 and 39 DPI. 8H14 had an increase from 2760/µl to 5310/µl between 46 and 49 DPI. However, animals in Group 1 experienced a *Giardia* infection around the time of inoculation with *A. phagocytophilum*, resulting in diarrhea, fever, and increased size of lymph nodes. Concurrent giardiasis may have masked true febrile events and may have resulted in the increased variability of the clinical parameters in Group 1.

Group 2 dogs were inoculated with either blood stabilate (8H12, 8H05, 8H08 and 8H11) or in vitro cultured HL-60 cells (8H10). All animals in Group 2 were confirmed positive by the detection of morulae via buffy coat smears (Fig 2-1). All of the animals of Group 2 had transient febrile events that lasted from 1 to 3 days. Animal 8H05 reached a peak temperature of 103.9°F at 12 DPI; 8H12 a peak value of 103.4°F at 11 DPI; and 8H08 a peak value of 102.8°F at 9 DPI.
Animal 8H11 never experienced a true febrile event; however it reached a peak temperature of 102°F at 10 DPI. This value was significantly elevated from temperature recorded on other days and the data is consistent with the animal experiencing a true febrile event that happened concurrently with the time of peak bacteremia. All of the Group 2 animals had slight to mild decreases in activity level at the time of peak infection, but not enough to be considered depressed or lethargic. One animal, 8H10, ate more slowly for 2 days correlating with fever and peak bacteremia, but was not considered anorexic. None of the animals in Group 2 showed clinical signs of anorexic, lameness, or joint pain or swelling.

All of the animals in Group 2 experienced a significant decrease in neutrophil counts. Not all of the neutrophil values were considered to be truly neutropenic (3,000/µl) however, the nadir values did constitute a significant decrease from the previous reading and were associated with a concurrent or subsequent increase in band neutrophils (regenerative response). Animals 8H12 and 8H08 experienced true neutropenia with values of 920/µl at 14 DPI and 2200/µl at 9DPI, respectively. 8H05 had a drop in neutrophil counts from 6100/µl to 4200/µl at 17 and 18DPI. 8H10 experienced a drop in neutrophil count from 5510/µl to 3380/µl on 13 and 14 DPI. 8H11 experienced a drop in values from 9300/µl to 5900/µl on 16 and 17 DPI.

Peak temperatures often loosely correlated with the time of neutropenia or nadir, 8H05 experienced neutropenia from 17 and 18 DPI had a peak febrile event at 12 DPI. 8H08 had true neutropenia at 9 DPI and its peak temperature at 10 DPI. 8H10 experienced a drop in neutrophil count on days 13 to 14 DPI and was febrile at 14 DPI. 8H11 had a drop in neutrophil counts on 16 to 17 DPI and had its peak temperature at 10 DPI. Lastly, 8H12 experienced true neutropenia on 14 DPI and its peak temperature on 11 DPI. Animals in Group 2 appeared to have occasional
variations in clinical chemistry values; however, these were not consistent and could be correlated to the neutropenia or febrile events.

The three non-beagle dogs (8H05, 8H11 and 8H12) experienced an increase in lymphocytes that appeared to correlate with neutropenia (Figure 2-3). Animal 8H05 lymphocytes increased from 2100/µl to 5900/µl between 17 and 18 DPI. Animal 8H11 experienced an increase of 2300/µl to 5700/µl at 17 and 18 DPI. Lastly, 8H12 experienced an increase of 1450/µl to 6500/µl between 12 and 14 DPI.

**Molecular Evidence of Infection**

Using PCR analysis, targeting both the *msp2* and *msp5* gene, none of the animals in Group 1 had detectable levels of *A. phagocytophilum*. All animals in Group 2 had detectable levels of the *msp5* gene (Figure 2-2). Peak infection levels, as determined by *msp5* PCR data, loosely correlated with neutropenic events. Animals 8H12 and 8H10 had detectable levels of the *msp5* gene at 4 DPI with values 676 and 92 copies, per 5 µL of whole blood, respectively. 8H12 reached its peak value at 11 DPI, with a value of $9.8 \times 10^5$ copies, per 5 µL of whole blood, and experienced neutropenia at 14 DPI. 8H10 reached its peak at 14 DPI, with a value of $2.5 \times 10^4$ copies, per 5 µL of whole blood, and experienced nadir neutrophil count at 14 DPI. 8H05 had detectable levels of bacterial DNA by 7 DPI of 86 copies, per 5 µL of whole blood, and reached its highest value at 14 DPI with a value of $1.2 \times 10^4$ copies, per 5 µL of whole blood; 8H05 experienced nadir neutrophil count at 18 DPI. 8H08 was positive at 10 DPI, with 15 copies, per 5 µL of whole blood, and reached its peak value later than the other animals in Group 2 at 27 DPI, with a value of 7,567 copies, per 5 µL of whole blood; 8H08 experienced true neutropenia at 9 DPI. 8H10 had detectable levels at 4 DPI with a value of 31 copies, per 5 µL of whole blood. It reached its peak value at 14 DPI with $2.5 \times 10^4$ copies, per 5 µL of whole blood, and experienced nadir neutrophil count at 14 DPI. Lastly, 8H11 first had detectable levels at 10 DPI with at value
of 92 copies, per 5 µL of whole blood. It reached its peak at 15 DPI with 3857 copies, per 5 µL of whole blood, and experienced nadir neutrophil count at 17 DPI. Interestingly, 8H08 (beagle) reached its peak infection 10 to 12 days later than 8H05 (golden retriever) and 8H11 (mixed breed), even though animals were inoculated with identical material.

**Serological Evidence of Infection**

One animal (8H09) from Group 1 seroconverted via Snap Test 4Dx (SNAP®4Dx®, IDEXX, Westbrook, ME) at 35 DPI, much later than the two week seroconversion typically seen in *A. phagocytophilum* infections. All animals in Group 2 seroconverted via Snap Test (SNAP®4Dx®, IDEXX, Westbrook, ME). Animal 8H10 seroconverted first (10 DPI); the remainder of the animals seroconverted within two weeks of inoculation (8H05, 8H08 and 8H11 at 16DPI, 8H12 at 14 DPI). Once seroconversion occurred, animals in Group 2 remained seropositive for the entirety of the study.

**Lymphocyte Proliferation Assays**

All but one (8H04) of the animals from Group 1 had at least one positive lymphocyte proliferation test result (Table 2-3). Dogs 8H06, 8H09, 8H13, and 8H14 all had significant positive results between 28 DPI and 35 DPI, and these events roughly correspond to febrile events, nadir neutrophil count, and seroconversion (8H09). Dogs 8H06 and 8H13 each had additional positive response at 73 DPI, which did not correlate with a febrile or neutropenic event.

All but one of the animals from Group 2 had at least one positive lymphocyte proliferation test result (Table 2-4). Dog 8H11 responded as early as 7 DPI and had a positive test for the remaining time points tested. These time points, in 8H11, correspond to the clinical timing of detection of bacteria, fever, and neutropenia between days 9 and 25. Dogs 8H05, 8H08, and 8H11 all had positive LPT results by day 27 DPI. Dog 8H10 was euthanized at 19 DPI and did
not have positive LPT. Interestingly, those animals with earlier and more consistent LPT responses (8H11) had considerably lower peak bacteremia, as detected by *msp5* gene copy numbers, and less severe decreases in neutrophil numbers (a drop from 9300/µl to 5900/µl on 16 and 17 DPI for 8H11). Dog 8H12 did not mount a consistent LPT response until 37 DPI and experienced the highest peak bacteremia (9.8 x 10⁵ at 11 DPI, per 5 µL of whole blood) and had the greatest drop in neutrophil numbers (920/µl at 14 DPI).

**Cytokine Assays**

Data was collected from 8H05, 8H08, 8H11 and 8H12 at -69 DPI and 14 DPI (Figure 2-6). Only IL-10 and TNF-α had statistically significant increases (p < 0.05) between the two dates, at both concentrations of *A. phagocytophilum* (10 and 1.0 µg/mL). Statistical differences were not observed in any other analytes.

**Gross Pathology and Histopathology of Necropsy**

Animal 8H10 was necropsied on 19 DPI shortly following the peak of infection to maximize the chances of seeing any transient pathology during the acute phase of infection. Submandibular, left prescapular and mesenteric lymph nodes were considered minimally to mildly enlarged. Other organs examined and considered grossly normal include oral cavity, nose, pharynx, trachea, esophagus, heart, lungs, diaphragm, liver, gall bladder, spleen, stomach, small intestine, colon, kidneys, adrenal glands, ureters, urinary bladder, prostate, urethra, testicles, calvarium, and brain.

Histopathology was done on all major organs evaluated grossly. A description of significant pathological findings follows: There are small numbers of multifocal randomly scattered small areas of inflammation and necrosis. The areas of inflammation and necrosis are characterized by 1 to 4 pale fragmented hepatocytes with absent to karyorrhectic nuclei surrounded by small to moderate numbers of neutrophils, macrophages, and fewer lymphocytes.
and plasma cells. Many of the blood vessels have large numbers of neutrophils in the lumens that occasionally extravasate and infiltrate the perivascular tissue. Similar to large blood vessels in the liver, many of the large vessels in the lung contain large numbers of neutrophils. However, unlike findings in the liver, extravasation of neutrophils into the surrounding tissues is not seen.

Numerous, well developed primary and secondary follicles and thickened cortex are present in the submandibular, left prescapular, and mesenteric lymph nodes indicative of reactive nodes and consistent with the enlarged appearance. The right prescapular lymph node has moderate to large numbers of large foamy round cells (macrophage) in the subcapsular and trabecular sinuses. The right prescapular lymph node has moderate numbers of macrophages in the medullary sinuses that contain moderate numbers of neutrophils. The left prescapular and popliteal lymph nodes also have primary and secondary follicles although somewhat less prominent than the other nodes consistent with their smaller size. Synovial samples from both carpal joints and both stifle joints were evaluated. No significant pathology was noted.

Other organs evaluated and considered histologically normal include heart, esophagus, trachea, adrenal gland, small intestine, colon, kidney, prostate, and brain.

**Discussion**

In the current study, the dogs were infected with an isolate of *A. phagocytophilum* that originated from a clinically ill dog in Minnesota. Animals included in this study were determined to have no concurrent infections with *B. burgdorferi* and *E. canis*, the most typical co-infections seen with *A. phagocytophilum* infection (40). In Group 1, inoculations with infected RF/6A cultured endothelial cells did not result in obvious patent infections, as reflected by PCR and buffy coat analyses. Although no infection or clinical disease was noted, some of the inoculated dogs in Group 1 showed immunologic responses specific for *A. phagocytophilum*. One animal
(8H09) seroconverted at 35 DPI and several of the animals had positive LPT starting on 28 DPI (8H06, 8H09, 8H13), which may point to a low level, non-detectable, non-clinical infection.

There have been previous successful infections utilizing infected RF/6A cells inoculated into naïve animals (2, 3). *A. phagocytophilum* lacks appropriate genes for LPS and peptidoglycan biosynthesis (39). Membrane cholesterol is required for the survival of these bacteria, and depletion or alteration of cholesterol disrupts their structural integrity, rendering bacteria unable to infect their host cells (39). It is possible that during the purification process, the structural integrity of these cells were compromised, rendering the bacteria unable to infect animals in Group 1. Future studies may need to investigate the purification procedure further, or rely on the direct injection of the cells into animals.

Blood stabilate used to infect RF/6A endothelial cells, for Group 1 inoculations, originated from a naturally infected dog in Minnesota. Previous successful inoculations using endothelial cells relied on the use of human strains (NY18) of *A. phagocytophilum* (2, 3). While both strains of *A. phagocytophilum* were virulent, there may be implications of culturing a human strain versus a dog strain in primate derived endothelial cells and inoculating a non-human host, which may have resulted in the lack of detectable infection in the current study.

Group 2 animals were inoculated using blood stabilate from the index case (8H12) or blood passaged through dog 8H12 (8H05, 8H08, 8H11) or with infected HL-60 cultured promyelocytic cells (8H10). Inoculations in this group resulted in clinical infection or patent infections in all animals corroborated by positive PCR results beginning at 4 DPI and peaking around 11-16 DPI, with 8H08 peaking later at 27 DPI, and visualization of morulae in neutrophils in buffy coat smears, typically corresponding to the peak bacteremia.
Experimental infection with *A. phagocytophilum* did not result in the full range of clinical signs reported in the literature including lethargy, anorexia, and lameness and joint pain (15, 36, 52, 50). Although a few animals had a mild decrease in activity level and mild inappetence, these signs were mild and only experienced by a few of the animals. Inspection and palpation of joints did not reveal any physical abnormalities and reluctance to move associated with joint pain, occasionally reported in acutely infected animals, and was not observed in any animals in acute infection or after 5 months.

In Group 2, clinical disease due to *A. phagocytophilum*, was characterized by signs of infection beginning at 7 DPI (8H08 at 4 DPI). Peak infectivity was typically seen between 11-15 DPI, lasting from 1 to 3 days and correlating with the peak of bacteremia, as determined by PCR. The clinical signs of infection included fever in 5 of 5 animals (5/5), neutropenia (3/5) or a drop in neutrophil numbers (5/5), lymphocytosis (3/5), mild decrease in activity (3/5), and mild inappetence (1/5).

All dogs in Group 2 seroconverted between 14-16 DPI and all but one animal had a strong LPT between 14 and 27 DPI. Cytokine profiles during the acute infection of Group 2 included significant increases in IL-10 and TNF-α from -69 to 14 DPI. These cytokine changes are suggestive of an inflammatory profile and a potential Th17 suppressive response in the absence of increases also in IL-4.

Earlier literature describing natural *A. phagocytophilum* infection in dogs typically does not describe co-infection with other organisms including *E. canis* and *B. burgdorferi* (22, 23). Therefore, it is difficult to distinguish whether clinical signs are due solely to *A. phagocytophilum* infection or another organism or possibly a synergistic effect between the two (or more) bacteria. This study has documented that clinical signs often reported in dogs with
suspected *A. phagocytophilum* infection, including anorexia, myalgia, lameness, lethargy and joint pain, are not the result of *A. phagocytophilum* infection alone. This will have important implications for clinicians when diagnosing dogs with previous tick exposure, presenting with these clinical signs; other organisms must be ruled out as a potential co-infection.

Furthermore, storage of the blood stablilate used to prepare inoculum for animals in this study may have attenuated the virulence of its infectivity. Even though the index case showed signs of severe clinical disease, animals in this study did not present with the same signs of clinical disease. Also, previous studies that did result in animals with severe signs used different strains of bacteria as a source of inoculum. Different strains and differences in inoculum preparation may also account for difference documented. This may have important implications on future studies, as researchers may want to investigate virulence regarding inoculum preparation and differences among bacterial strains.
Figure 2-1. Buffy coat smears of animals from Group 2. The red arrows point to morulae within the cytoplasm of the neutrophils, characteristic of *A. phagocytophilum* infection. Animals in Group 2 were inoculated on 04 Dec 08 and these photos are taken approximately 2 weeks DPI.
Figure 2-2. Real time PCR of the *msp5* gene of Group 2 animals, concentrations are per 5 µL of whole blood. Animals in this group were inoculated on 04 Dec 09 (study day 0), except for 8H12, which was inoculated on 06 Nov 08. Detection of *A. phagocytophilum* bacteria peaks around 2 weeks DPI and tapers off, reaching zero copy numbers by 4 weeks DPI for most animals. The peak of bacteremia in one animal (8H08) reached its peak infection late, at 27 DPI, significantly delayed as compared to the other 4 animals.
Figure 2-3. Group 2 comparison of neutrophils and lymphocytes. The straight red lines indicate the normal canine ranges for neutrophils (3000/µL is the low end 11,500/µL is the high end). The straight blue lines indicate the normal range for canine lymphocytes (1000/µL to 4500/µL). The black boxes show concurrent increases in lymphocyte numbers and significant decreases in neutrophil numbers.
Figure 2-4. Lymphocyte proliferation assay for Group 1, animals 8H06 and 8H13. The figures are representative of data collected during the trial period. Stimulation as measured by CPM for the antigens on 74 DPI was considered statistically different from the negative control, which is indicated by the stars, (p < 0.05). The figures on the right are representative of the stimulation index of *A. phagocytophilum* at 10 µg/mL and 1.0 µg/mL weekly throughout the study (0 to 74 DPI). Notice the titrated responses to the different concentrations of *A. phagocytophilum* antigen and the significant response at 74 DPI.
Figure 2-5. Lymphocyte proliferation assay for Group 2, animals 8H08 and 8H12. The figures on the left are representative of positive responses during the trial as measured by CPM. The stars above *A. phagocytophilum* 10µg/mL and 1.0µg/mL indicate that the counts per minute, calculated from stimulation with these antigens, are statistically different from the counts per minute when stimulated with medium alone (p < 0.05). The figures on the right are representative of the stimulation indices determined weekly throughout infection. All but one animal in Group 2 had significant specific *A. phagocytophilum* stimulation.
Figure 2-6. Cytokine results for Group 2 animals 8H11 and 8H12 for analytes IL-10 and TNF-α. The silver bars on the left are *A. phagocytophilum* at 1.0µg/mL at -69 DPI and 14 DPI. The blue bars on the right are *A. phagocytophilum* at 10µg/mL at -69 DPI and 14 DPI. There was a statistically significant (p < 0.05) increase for both IL-10 and TNF-α at both 1.0 and 10µg/mL between the two dates.
<table>
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<tr>
<th>Parameter</th>
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<th>8H06 (Golden Retriever)</th>
<th>8H09 (Beagle)</th>
<th>8H13 (Mixed Breed)</th>
<th>8H14 (Mixed Breed)</th>
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<th>8H08 (Beagle)</th>
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Table 2-1. Group designation. Group 1 consisted of five dogs inoculated with bacteria grown the RF/6A endothelial cell lines on 02 Oct 2008. Group 2 consisted of five dogs. One animal was inoculated with naturally infected blood stabilate (8H12). Blood stabilate from the acute infection of animal 8H12 was subsequently used to inoculate 8H05, 8H08 and 8H11. Animal 8H10 was inoculated with bacteria grown in the HL-60 mammalian cell line.
Table 2-2. Sample clinical exam form. Lameness, anorexia, joint pain/swelling, enlarged lymph nodes, malaise, and rectal temperature were checked daily in all animals post-inoculation. Anorexia, joint pain/swelling and malaise were ranked on a scale of 1-3, with 1 being the least severe and 3 being the most severe.
<table>
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<th>14 DPI</th>
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Table 2-3. Lymphocyte proliferation assay stimulation index summary from Group 1. All of the animals in Group 1 had at least one positive response, beginning at 25 DPI through 74 DPI.
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<tr>
<td></td>
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<td>---</td>
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Table 2-4. Lymphocyte proliferation assay stimulation index summary from Group 2. All but one of the animals in Group 2 had a positive response, starting at 7 DPI and monitored through 27 DPI (for 8H05, 8H08 and 8H11) and through 56 DPI (for 8H12).
CHAPTER THREE
MOLECULAR EVIDENCE OF PERSISTENT *ANAPLASMA PHAGOCYTOPHILUM* INFECTION FOLLOWING TREATMENT WITH DOXYCYCLINE, REINOCULATION WITH INFECTED BLOOD STABILATE, AND IMMUNOSUPRESSION WITH PREDNISONE

**Introduction**

The vector-borne, zoonotic organism *Anaplasma phagocytophilum*, was first reported in humans in Minnesota and Wisconsin in 1994 (1, 4, 40) and later in dogs from Minnesota and Wisconsin in 1996 (28). Since then, the organism has been recognized as having a worldwide geographic distribution and is now the third most common tick-borne infection of humans in the United States (19). When diagnosed early, patients tend to show rapid clinical improvement within one to two days of treatment. However, rarely prolonged disease or death is possible in those who are heavily infected or otherwise immunocompromised (19).

Doxycycline, which is a bacteriostatic antibiotic, is the most common recommended treatment for *A. phagocytophilum* infections (2). An oral dosage of 5 to 10 mg/kg given twice a day for 14 to 30 days has been recommended and clinical signs tend to resolve rapidly, often within 24 to 48 hours. However recent studies have reported dogs experimentally inoculated with a human strain of *A. phagocytophilum* have remained persistently infected and in subclinical carrier states following doxycycline treatment (2, 3). Controlled studies investigating the optimal duration of treatment or the dosage have not yet been conducted and it is not fully understood whether treatment merely alleviates clinical signs, potentially inducing a low-level, subclinical carrier state of infection.

Chronically infected carriers could act as potential reservoirs for further transmission of the bacteria and disease. These animals could be stimulated to recrudesce by therapeutic agents or by concurrent illnesses that suppresses the immune system. Steroids used to treat chronic dermatitis
or chemotherapy have been anecdotally associated with occurrence/recurrence of *A. phagocytophilum* infection (Alleman, personal communication).

The first purpose of this investigation was to investigate whether treatment with doxycycline will clear *A. phagocytophilum* bacteria in experimentally infected dogs by administering corticosteroids following treatment of an aggressive regimen of doxycycline and a grace period allowing for the clearance of the antibiotic from the animals’ systems. The second purpose of the investigation was to determine if animals inoculated with infected RF/6A cells were subclinically infected and remained persistently infected at undetectable levels as seen in the chronic phase of the Group 2 animals and whether inoculation with the RF/6A cells imparted protective immunity against challenge with blood stabilate. In order to test these hypotheses, steroids were administered to two animals initially inoculated with bacteria grown in RF/6A endothelial cells to determine whether bacterial levels were detectable by PCR or buffy coat smears. Lastly, three animals from Group 1 were re-inoculated, using infected blood, to determine whether the initial inoculation using cultivated endothelial cells provided animals with immunity from *A. phagocytophilum*. 
Materials and Methods

Animals and Group Designation

All of the animals from the first part of this study, with the exception of 8H10 which was necropsied, were utilized for the studies in this section. Animals were re-designated into three groups. Group A consisted of animals originally inoculated with infected blood, corresponding to Group 2 of the previous study. Animals from this group were all confirmed positively infected by seroconversion, PCR of the *msp5* gene and visualization of morulae in buffy coat smears. Group A (8H05, 8H08, 8H11, and 8H12) was utilized for antibiotic therapy trials. Group B consisted of two seronegative animals (8H04 and 8H13) originally inoculated with RF/6A endothelial cells (Group 1 of the previous study), which were not previously confirmed positively infected by seroconversion, PCR or buffy coat smears. Group C consisted of two seronegative animals (8H06 and 8H14) and one animal that transiently seroconverted (8H09) also originally inoculated with RF/6A endothelial cells (Group 1 of the previous study), but not confirmed infected by PCR or visualization of morulae. Group designation and treatment plans are listed in table 3-1.

Treatment with Doxycycline and Immunosuppression with Prednisone

To determine whether doxycycline is efficacious in clearing *A. phagocytophilum* from previously infected dogs, Group A was treated with doxycycline at the current recommended dose of 10 mg/kg, twice daily for 28 days, beginning at 113 DPI for 8H05, 8H08 and 8H11 and 142 DPI for 8H12 (8H12 was originally inoculated before other animals included in Group A). Group A animals had remained seropositive from the first study, however they were repeatedly PCR negative before doxycycline treatment. No drugs were administered two weeks following the antibiotic treatment to allow for clearance of the antibiotic. Following the two week period, all four dogs were administered an immunosuppressive dose of prednisone (1 mg/kg, twice
daily) for two weeks, beginning at 161 DPI, for animals 8H05, 8H08 and 8H11, and 190 DPI for 8H12. Serial blood samples for PCR targeting the *msp2* and *msp5* gene were taken daily during prednisone treatment. If an animal had > 100 copy numbers from real-time PCR for *msp2* or *msp5*, PBMCs from blood collected in EDTA or heparinized sterile tubes from that day were isolated and put into HL-60 cells (American Type Culture Collection, ATCC CCL-240™, Manassas, VA) for verification of the presence of viable organisms. Dogs were euthanized 7 (8H05, 8H08 and 8H11) and 8 (8H12) months post-infection.

**Inoculation via Blood Stabilate**

Animals from Group B (8H04 and 8H13) were used to determine if previous inoculation with *A. phagocytophilum* grown in RF/6A endothelial cells provided immunity towards re-inoculated with infected blood. Group B was re-inoculated using infected blood from 8H12, which was initially inoculated from the Baxter strain of *A. phagocytophilum*. Animals were phlebotomized daily for PCR detection, clinical chemistry and hematology, using EDTA and heparinized sterile tubes. To document hematological and biochemical changes, blood and serum were collected twice weekly, using heparinized and sterile tubes, to perform complete blood counts and biochemical profiles.

Cytokine assays were conducted once weekly from animals in Group B, to monitor the cell mediated immune response. A commercially available canine cytokine/chemokine kit from Millipore (Canine Cytokine/Chemokine Panel, Millipore, Billerica, MA), was used to test the following analytes, IL-2, IL-4, IL-8, IL-10, IFN-γ and TNF-α. The samples were processed according the manufactures recommendations, previously described in chapter 2.

Data was analyzed using Milliplex Analyst software (Millipore, Billerica, MA), a weighted 5-parameter logistic method for calculating cytokine concentrations in samples. Statistical data
was analyzed with SAS (SAS Institute Inc., Cary, NC), using a generalized linear mixed model, to account for repeated measures with T-test comparing individual animals or days.

**Immunosuppression with Prednisone and Monitoring**

To determine if animals inoculated with *A. phagocytophilum* grown in RF/6A endothelial cells had bacteria reside at copy numbers too low to detect, animals in Group C were immunosuppressed using prednisone (1 mg/kg, 2 x daily) for 14 days. The animals were phlebotomized daily for PCR detection, clinical chemistry and hematology. Complete blood counts and biochemical profiles were performed on Group 2 animals, twice weekly, on heparinized blood and sterile serum to document hematological and biochemical changes.

**PCR Detection of *A. phagocytophilum* DNA in Whole Blood**

DNA was extracted from anticoagulated, EDTA-whole blood and using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and was used as the template for real-time quantitative PCR amplification of the single copy *msp5* gene, using the techniques previously described in chapter 2.

**Necropsy**

Postmortem examinations were carried out on the dogs from all groups, and specimens from the trachea, lung, liver, kidney, spleen, lymph nodes, pancreas, joint capsule, lymph nodes, and brain were fixed in 10 per cent buffered formalin within one hour after death.

For PCR detection of *A. phagocytophilum* DNA in post-mortem tissues, DNA was extracted from unfixed post-mortem tissue samples using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) in accord with the manufactures instructions, which indicated that the following amounts of biological material be used for DNA extractions: 25mg of most tissues or 10mg of spleen. Control DNA samples, including positive DNA extracted from *A. phagocytophilum* grown in RF/6A endothelial cells and negative control DNA extracted from an uninfected dog.
were similarly extracted. Fifty microliter DNA amplification reactions were performed for msp2 previously described in chapter 2.

Results

Doxycycline and Prednisone Trial

Group A animals were used to determine the efficacy of doxycycline treatment of animals infected with *A. phagocytophilum*. This group consisted of seropositive animals originally inoculated with naturally infected blood and confirmed positively infected by *msp2* and *msp5* PCR and morulae visualization in buffy coat smears (8H05, 8H08, 8H11, and 8H12). Although the animals remained seropositive, multiple negative *msp2* and *msp5* PCR tests were documented prior to the antibiotic therapy. Following doxycycline treatment, from 113 DPI and 142 DPI (8H12, which was inoculated before the other animals), and a 14 day period to clear the antibiotic, the animals were given immunosuppressive doses of prednisone. During antibiotic treatment and the two weeks prior to prednisone treatment all of the animals remained PCR negative. There was one day during doxycycline therapy at 144 DPI, that 8H11 had detectable levels of the *msp2* gene of *A. phagocytophilum* (Table 3-2). However, this response may have been artifactual, as this number was significantly lower than peak bacteremia levels detected during prednisone therapy.

*A. phagocytophilum* was detected in all of the animals during prednisone treatment. Animals 8H08 and 8H11 had the greatest numbers detected beginning around day five of prednisone treatment. 8H08 had the highest detectable level of *msp2* gene copy numbers (4 x 10^4 copies, per 5 µL of whole blood) on day five of prednisone therapy. By day six of prednisone therapy, the *msp2* copy numbers had decreased substantially to no longer significant levels (8 copies, per 5 µL of whole blood). The *msp2* values fluctuated throughout the study, but the never reached the peak level achieved on day five. On day 21, the *msp2* values became undetectable
and stayed negative for the remainder of the study. The animal never had significant detectable levels of the *msp5* gene.

8H11 also had *A. phagocytophilum* bacteremia detectable by the *msp2* (1.9 x 10^4 copies, per 5 µL of whole blood) and *msp5* (30 copies, per 5 µL of whole blood) assays on day five of prednisone therapy. By day six, the *msp2* copy number had dropped substantially (33 copies, per 5 µL of whole blood) and the *msp5* gene was undetectable. The *msp2* values fluctuated for the remainder of the study, but never reached came close to the peak value achieved at day five, *msp5* remained negative.

Dogs 8H05 and 8H12 had detectable levels of the *msp2* gene, but not as great of a response as 8H08 and 8H11. 8H12 had detectable levels of *msp2* by day six (968 copies, per 5 µL of whole blood) and 8H05 had detectable levels by day 10 (2339 copies, 5 µL of whole blood). The *msp2* values fluctuated for the remainder of the study, but did not achieve the peak levels again. 8H12 had only one positive *msp5* response on day 12 (26 copies, 5 µL of whole blood), 8H05 had none.

Fresh PBMC’s from 8H08 and 8H11 were collected at the peak of infection (5 DPI) and were placed into fresh HL-60 cultured promyelocytic cells, to verify viable infective material during the infection. No successful cultures were obtained from these animals. Cultures from dogs 8H05 and 8H12 were not attempted due to low levels of bacterial DNA in the blood, as estimated by PCR.

Animals 8H05, 8H08 and 8H12 were euthanized and necropsied on 08 June 2009; 8H11 was euthanized and necropsied on 01 June 2009. PCR was performed on liver, lung, heart, brain, left and right kidney, pancreas and spleen, targeting the *msp2* gene. Detectable levels were
detected in all tissues from all animals (Table 3-3). The heart and brain were not collected from 8H12.

**Inoculation via Blood Stabilate**

Group B consisted of two seronegative animals (8H04 and 8H13) originally inoculated with *A. phagocytophilum* cultured in RF/6A endothelial cells, but not confirmed positively infected by PCR or examination of buffy coat smears. These animals were used to determine if the previous inoculation with RF/6A cells may have resulted in protective immunity to reinoculation. Group B (8H04 and 8H13) was inoculated a second time on 16 April 2009 (0 DPI), using infected blood stabilate from animal 8H12. 8H13 had detectable levels of bacteria by 11 DPI (Table 3-4), and seroconverted, as determined by the Snap Test 4Dx, by 18 DPI. 8H13 reached its peak bacteremia, at 19 DPI, with *msp2* assay peaking at 2 x 10^5 copies, per 5 µL of whole blood. 8H13 was determined to have a patent infection, as determined by PCR detection of *A. phagocytophilum* and seroconversion.

Animal 8H04 remained seronegative for the entirety of the study. At 5 and 27 DPI, the *msp2* copy number exceeded 150 copies, per 5 µL of whole blood; therefore, 8H04 was considered to have a possible latent infection, as determined by PCR detection of *A. phagocytophilum* by the *msp2* gene.

Cytokine concentrations for IL-2, IL-4, IL-8, IL-10, IFN-γ, and TNF-α were examined weekly during the challenge of Group B. Significant statistical differences were seen in the two dogs in IL-8, IL-10 and TNF-α concentrations. Animals 8H13 and 8H04 had significantly different (p < 0.05) concentrations of IL-8 at 0, 12, 20, 27 and 41 DPI (Figure 3-1). Animal 8H04 also experienced a significant drop in IL-8 levels (p < 0.05) between 0 and 20 DPI. Animal 8H13 did not experience a significant decrease. There were no significant differences between the two dogs in IL-10 concentrations (Figure 3-2). However, 8H04 experienced a significant drop (p <
0.05) in concentration of IL-10 between 0 and 20 DPI, which may signify a significant decrease in Th2 humoral or Th17 suppressive response, as IL-10 is known to play a significant role in each. Statistically significant differences (p < 0.05) in TNF-α levels between the two dogs occurred at 12 DPI (Figure 3-3) when the mean concentration in 8H04 increased to almost triple the concentration of 8H13. TNF-α concentration then significantly decreased (p < 0.05) in 8H04 reaching its nadir value at 20 DPI.

**Immunosuppression with Prednisone**

Group C consisted of two seronegative animals (8H06 and 8H14) and one animal that transiently seroconverted (8H09), after being inoculated with infected RF/6A endothelial cells, but were not confirmed to have become infected. These animals had significant drops in neutrophil counts and intermittent positive lymphocyte proliferative responses months after inoculation and were considered the most likely of all animals in the original Group 1 to have had potential subclinical and undetectable infections. The purpose of this study was to determine if these animals, previously inoculated with *A. phagocytophilum* grown in RF/6A cultures, which showed no previous signs of infection, actually had low lying levels of infection too low to detect by conventional methods. Animals in group C (8H06, 8H09 and 8H14) were administered prednisone and monitored using PCR and Snap Test 4DX (SNAP®4Dx®, IDEXX, Westbrook, ME) for signs of infection. Infection was not detected in any of the animals in this study, as none of the animals in this group had positive PCR values during the time of immunosuppression.

**Discussion**

Antimicrobial treatment of chronically infected animals did not clear the bacterial infection. Before and during doxycycline therapy, animals in Group A did not have detectable levels of *A. phagocytophilum* in the peripheral blood as measured by PCR of both the *msp2* and *msp5* genes, once the animals had recovered from acute infection; with the exception of animal...
8H11 which had one positive msp2 result at 144 DPI. Immunosuppression with prednisone allowed bacteremia to recrudesce to significant levels in all animals. In some animals, the levels approached those seen in the acute infection. Necropsies of these animals showed that chronic, persistent infection exist in animals infected with *A. phagocytophilum*. Even after receiving doxycycline therapy, all of the animals in this group had positive msp2 copy numbers in many post-mortem tissues. This has critical implications since treatment with doxycycline may not eliminate *A. phagocytophilum* bacteria from clinically normal dogs with no measurable or detectable bacteremia. Dogs may become life-long *A. phagocytophilum*-carriers, at risk for recrudescence with concurrent illness or immunosuppression (2, 3). Also, chronic carriers may act as an environmental source of organisms for ticks during acquisition-feeding. The numbers in this trial are small and more animals will need to be tested to make definitive conclusions. In addition, future trials investigating different doxycycline dosages with precise quantitation of plasma drug concentration may be warranted.

We also attempted to determine whether inoculation with organisms cultured in RF/6A endothelial cells provide immunity towards re-inoculation via infected blood stabilate. Animal 8H13 showed the similar disease signs in a similar time frame as the previous Group 2 infected dogs, including seroconversion after two weeks and detection of the msp2 gene using PCR which reached levels similar to those seen in the Group 2 animals. The other animal (8H04) in Group B was considered to not have evidence of patent infection. Only two msp2 PCR samples were greater than 100 copy numbers per 5 µL of blood, and this animal did not seroconvert.

IL-8 and IL-10 cytokine data show an initial drop followed by a gradual increase, reaching values equal to or greater than pre-inoculation levels in both animals. The decrease, which occurred at 20 DPI for both analytes, correlated with the peak of infectivity for 8H13 as
determined by PCR, which may signify either a significant decrease in Th2 humoral response, or a Th17 suppressive response as IL-10 is known to play a significant role in each (35). Throughout the entire study, animal 8H04 had a significantly higher concentration of IL-8 than 8H13. IL-8 is a chemotactic that attracts neutrophils, basophils, and T-cells. The mobilization and recruitment of neutrophils and Th2 humoral response may have contributed to the protection seen in this animal.

TNF-α data showed a significant difference between the two animals at 12 DPI, when the concentration in 8H04 tripled the amount in 8H13. This early spike in systemic inflammation or innate immune response may have resulted in the protection observed for 8H04. TNF-α levels are considered indicators of general inflammation including both innate and adaptive immune responses. TNF-α is secreted by macrophages, monocytes, neutrophils, helper T-cells and NK-cells following their stimulation by bacterial lipopolysaccharides (35). This early increase in systemic inflammation or innate immune response may be due to the reaction neutrophils or memory T helper cells. Alternatively, the response may be the result of exposure to LPS from the inoculum; however, this seems more likely to have occurred earlier than 12 DPI. Regardless of the source, the increased in TNF-α may have resulted in the protection seen in animal 8H04.

The two animals used in this study were different breeds (8H04 was a golden retriever, while 8H13 was a mixed breed); however, data from the first part of this study suggest that breed plays no obvious role in *A. phagocytophilum* infection. The subject numbers are minimal and obviously need to be repeated with a larger group as the differences may be due to individual animal differences rather than protection imparted by the inoculation. However, data are intriguing and warrant future studies as endothelial cells may be suitable for a potential vaccine.
Animals in Group C were tested to determine if the inoculation of infected cultured RF/6A cells caused a subclinical and undetected *A. phagocytophilum* infection. Evidence of a subclinical infection included the seroconversion of 8H09, significant decreases in neutrophil numbers, and multiple specific lymphocyte proliferation weeks after a single inoculation. None of the immunosuppressed animals had detectable levels of *A. phagocytophilum* as measured by PCR or seroconversion. Either the animals were never infected or a low-level infection was cleared by the host. We do not have sufficient data to definitively answer this question with these data, but the evidence supporting a possible low-level infection is intriguing.
Figure 3-1. Mean IL-8 Response for Group B throughout the entire study. The blue line represents dog 8H04, the red line represents 8H13. Significantly different (p < 0.05) values of IL-8 between dogs occur at 0, 12, 20, 27 and 41 DPI. 8H04 also experienced a significant drop in IL-8 levels (p < 0.05) between 0 and 20 DPI.
Figure 3-2. Mean IL-10 response for Group B throughout the entire study. There was no significant difference between the two dogs in IL-10 concentrations. However, 8H04 experienced a significant drop (p < 0.05) in levels of IL-10 between 0 and 20 DPI.
Figure 3-3. Mean TNF-α response for Group B throughout the entire study. At 12 DPI, there was a statistically significant difference (p < 0.05) in concentrations between the two dogs. Animal 8H04 also experienced a significant drop in concentration between 12 and 20 DPI.
Table 3-1. Group designation. Group A consisted of 8H12, 8H05, 8H08 and 8H11, which correlated with Group 1 from the first study. Animals in Group A were treated with doxycycline and then immunosuppressed with prednisone. Group B consisted of 8H04 and 8H14. Animals in this group were originally inoculated with RF/6A endothelial cells. In this study, animals were re-inoculated with infected blood stabilate to determine whether RF/6A cells provided animals with immunity. Group C was comprised of animals 8H06, 8H09 and 8H13. Animals in this group were originally inoculated with RF/6A endothelial cells and were determined not to be infected using our detection methods. Animals were immunosuppressed with prednisone to determine whether animals were actually infected at levels too low to detect by conventional methods.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>8H08 (Beagle)</th>
<th>8H11 (Mixed breed)</th>
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<td>04Dec08</td>
<td>04Dec08</td>
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<td>Blood from 8H12</td>
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<td>Doxy (10mg/kg-2x daily, 28 days); Pred (1mg/lb- 2x daily 14 days)</td>
<td>Doxy (10mg/kg-2x daily, 28 days); Pred (1mg/lb- 2x daily 14 days)</td>
<td>Doxy (10mg/kg 2x daily, 28 days); Pred (1mg/lb- 2x daily 14 days)</td>
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<tr>
<th>Parameter</th>
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<th>8H13 (Mixed breed)</th>
<th>8H06 (Golden retriever)</th>
<th>8H09 (Beagle)</th>
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<td>Plan</td>
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<td>Infection via blood stabilate</td>
<td>Pred 1mg/lb 2x daily for 14 days</td>
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Table 3-2. PCR detection of *A. phagocytophilum* for Group A (which correlates to a portion of Group 1 from the first study), beginning on study day 0 through study day 175 (study day 204 for 8H12, which was inoculated before the 8H05, 8H08 and 8H11). Numbers are bacterial copy numbers per 5µL of whole blood. Peak bacteremia from the first study and from the immunosuppression with prednisone are in bold.
Table 3-3. PCR detection of *A. phagocytophilum* *msp2* gene in 1.0 gram of post-mortem tissues from Group A. Significant quantities of *A. phagocytophilum* DNA was detectable several months after inoculation, in the peripheral blood and in post-mortem tissues of immunosuppressed animals, even after an extended course of antibiotic therapy. The heart and brain were not collected from 8H12.

<table>
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<th>8H11 (Mixed breed)</th>
<th>8H12 (Mixed breed)</th>
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<td>2360</td>
<td>2810</td>
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<td>900</td>
<td>24620</td>
<td>1281</td>
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<td>10560</td>
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</tr>
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<td>860</td>
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<td>1581</td>
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</tr>
</tbody>
</table>


Table 3-4. PCR detection of *A. phagocytophilum*, post reinoculation for Group B. Animals in this group were initially inoculated with bacteria cultured in RF/6A endothelial cells, and were determined not infected by our detection methods. Animals were re-inoculated using infected blood from 8H12 (Group 1, from the first study) on 16 April 2009. Copy numbers are per 5 µL of whole blood. 8H13 showed signs of infection beginning at 11 DPI, and peaking at 19 DPI. 8H04 had positive *msp2* copy numbers starting at 5 DPI, which was its peak bacteremia level.

<table>
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</tr>
<tr>
<td>5 DPI</td>
<td>183</td>
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</tr>
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<td>7 DPI</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>15 DPI</td>
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</tr>
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<td>18 DPI</td>
<td>23</td>
<td>1.2 x 10⁵</td>
</tr>
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CHAPTER IV
CONCLUSIONS AND FURTHER RESEARCH

Four *Anaplasma* infection trials using immunocompetent dogs were performed to investigate usage of different mammalian cell lines for experimental infection and to further describe clinical aspects of *Anaplasma* pathogenesis. Dogs were inoculated with *A. phagocytophilum* from two different mammalian cell lines (RF/6A and HL-60) and from blood stablilate, prepared from parasitemic blood of a naturally infected dog or experimentally passaged through a naïve dog, allowed to develop chronic infection, and treated with doxycycline. Following resolution of clinical disease and loss of the ability to detect the bacteria, significant quantities of *A. phagocytophilum* DNA was detectable several months later in the peripheral blood and in post-mortem tissues (heart, spleen, lungs, kidney) of immunosuppressed animals even after an extended course of antibiotic therapy.

Together these findings represent the following:

- infection of dogs using cultured HL-60, promyelocytic cells or blood stablilate as the source of inoculum,
- molecular evidence of chronic, persistent *A. phagocytophilum* infection in blood and tissues of subclinical dogs despite doxycycline treatment.

Studies documented here have helped to further research of *A. phagocytophilum* infection in canines, by exploring the clinical disease and immunological response dogs experimentally inoculated with various mammalian cells and blood stablilate. Experiments described here also elucidate a carrier-state of infection post-antibiotic therapy. The culmination of these studies helps to lay the groundwork for future investigations defining the clinical disease caused by *A. phagocytophilum*. Potential future studies may include:

- determining differences in the severity or quality of disease caused by different strains of *A. phagocytophilum*
- determining if and how culturing in mammalian cells influences experimental *A. phagocytophilum* infections
• varying the dosage of infection to determine the outcome

In addition, these studies invite future investigations in the areas of treatment and persistence of *A. phagocytophilum* in the canine host and the potential that a subclinical reservoir could be a risk of transmission to other pets or owners in a household. Potential future studies may include:

• determine if longer treatment or higher dosages of doxycycline may be more effective for treatment of *A. phagocytophilum* infection or
• determine if alternative antibiotic therapies may be warranted
• determine if viable *A. phagocytophilum* organisms can be cultured from postmortem collected tissues or from the PBMC’s of recrudescing animals
• determine if inoculation of dogs with homogenized postmortem tissues from the infected animals can cause a patent infection in a naïve dog.

Applications of these studies will likely influence future research regarding *A. phagocytophilum* using the defined model to more fully elucidate the pathophysiology of the disease. *A. phagocytophilum* infection trials described in Chapter 2 demonstrated that acute-febrile disease was demonstrated, however, rather than severe clinical disease we documented a transient, mild disease in the absence of co-infection. Infection trials described here also work to more fully dissect the immunological response to *A. phagocytophilum* infection from stabilate and bacteria cultivated in various mammalian cell lines, which will have important implications on future vaccine development. Future strategies for *Anaplasma* vaccine development may rely on the use of genetically modified bacteria that have been cultivated in endothelial cells since these cells are easier to manipulate and perform plaque isolation of mutants compared to other *in vitro* host cell types. However, *A. phagocytophilum* infection trials described in Chapter 2 demonstrated that infection was not seen in dogs inoculated with RF/6A endothelial cells, and other strategies may need to be investigated. Finally, trials described in Chapter 3 demonstrated
persistent *A. phagocytophilum* infection despite doxycycline therapy. This will have implications on future recommendations for the treatment of *A. phagocytophilum* infections.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Melissa M. Clark graduated from Blaine High School in Blaine, Minnesota and began her undergraduate career studying natural sciences at the University of Wisconsin-Madison. In the spring of 2006, she received her Bachelor of Science in zoology and natural science, wildlife ecology major. While earning her undergraduate degree, Melissa worked as a student intern at the National Wildlife Health Center and as an emergency medical technician for the city of Madison, Wisconsin.

After graduation, Melissa accepted a position with the University of Florida, working as a field technician on a National Science Foundation project. Melissa began her graduate studies in the spring of 2008. She received her Masters’ degree from the University of Florida, College of Veterinary Medicine, Department of Infectious Diseases and Pathology in the fall of 2009.