CANINE BABESIASIS IN TEXAS: OCCURRENCE AND MOLECULAR CHARACTERIZATION OF *BABESIA* ISOLATES

A Thesis

by

LAUREN ELYSE LEHTINEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2007

Major Subject: Veterinary Parasitology

CANINE BABESIASIS IN TEXAS: OCCURRENCE AND MOLECULAR CHARACTERIZATION OF *BABESIA* ISOLATES

A Thesis

by

LAUREN ELYSE LEHTINEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee, Patricia Holman
Committee Members, Christine Budke
Thomas Craig
Head of Department, Gerrald Bratton

August 2007

Major Subject: Veterinary Parasitology

ABSTRACT

Canine Babesiasis in Texas: Occurrence and Molecular Characterization of *Babesia* Isolates. (August 2007)

Lauren Elyse Lehtinen, B.S., Baylor University

Chair of Advisory Committee: Dr. Patricia J. Holman

Canine babesiosis is an important worldwide disease caused by protozoan hemoparasites of the genus *Babesia,* which are primarily transmitted to a dog by the bite of an Ixodid tick, although vertical transmission has recently been reported. The disease is typically characterized by hemolytic anemia, fever, splenomegaly, and thrombocytopenia, with clinical signs ranging from clinically normal to acute anemia. Death may even result in some severe cases. Two species of *Babesia*, *Babesia gibsoni* and *Babesia canis,* have long been known to cause babesiosis in dogs. To date, almost all *B. gibsoni* infections in the United States have been reported in American Pit Bull Terriers or in dogs associated with the breed through either transfusion or fighting.

Dog blood samples received from kennels, shelters, and veterinary clinics throughout Texas were tested for the presence of *B. gibsoni* and *B. canis*. A total of 254 samples were tested for *B. gibsoni* and *B. canis* by light microscopy and polymerase chain reaction (PCR). *Babesia gibsoni* was detected in four of the dogs tested and *B. canis* was detected in one of the dogs tested. The average packed cell volumes (PCVs) of infected dogs were compared with those of uninfected dogs, with the infected, on average, having lower PCVs. Molecular characterization of the small subunit ribosomal

RNA gene and the ribosomal RNA internal transcribed spacer regions was performed on all sequences obtained in this study, and results were consistent with those previously reported for *B. gibsoni* and *B. canis*. Also, positive samples and additional samples provided by North Carolinia State University were used to initiate in vitro cultures of the parasites. To date, one isolate of a large unknown *Babesia* sp. from a North Carolina dog was successfully established in vitro. The establishment of *Babesia* spp. parasites in culture may aid in the development of a vaccine for babesiosis and will also be beneficial in improving diagnostic tests for the parasite.

DEDICATION

I would like to dedicate this thesis to my parents Steven and Phyllis Lehtinen. Without their love and support, this would not have been possible. I love you both very much!

ACKNOWLEDGEMENTS

I would like to thank Dr. Patricia Holman for all of her support, guidance, and expertise. I have learned a lot from her and I know that she has invested a great amount of time in my successes. I would also like to thank my committee members, Dr. C. Budke and Dr. T.M. Craig, for their time and effort. I know that they are both very busy and I sincerely appreciate all that they have done.

I would like to thank EunHee Lee for teaching me many of the techniques that I used in my project. She is incredibly patient and a wonderful teacher. My labmates, Issa Baradji and Pamela Garretson, have also been a great support. It has been a privilege to work with them. I wish to say a special thank you to Pam for her friendship, advice, and encouragement. I couldn't have gotten by without her!

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Recently, there has been renewed interest in the ticks of domestic animals due to the emergence and re-emergence of tick-borne diseases caused by bacterial, rickettsial and protozoal pathogens. The term for the disease caused by *Babesia* species is babesiosis (Inokuma et al., 2003).

Many *Babesia* species have been described since Viktor Babés first recognized *Babesia* in the red blood cells of cattle in 1888. The most recent taxonomic classification ranks as follows: Apicomplexa, Anconodasida, Piroplasmorida, Babesiidae (Adl et al., 2005).

Babesia spp. contain subpellicular tubules, which are microtubules lying beneath the unit membrane (pellicle) of many protozoans, often occurring as a fence-like arrangement of longitudinally arranged fibrils connected by thin lateral bridges that support the external cell form. They also have a polar ring, which is a thickened, electron-dense ring at the anterior end, that is part of the apical complex characteristic of these sporozoans. In addition, they contain micronemes, which are small, cordlike, twisted organelles found in the anterior region of many sporozoans (Levine, 1973). In their vertebrate host, *Babesia* spp. primarily infect erythrocytes. Intraerythrocytic forms

---

This thesis follows the style of Veterinary Parasitology.

are oval, round or pyriform, and are approximately 1.5 to 5 m in length. *Babesia* species are differentiated by morphologic appearance and size within the erythrocytes, and the erythrocytic stage parasites are referred to as piroplasms (Kuttler, 1988).

Babesia spp. are transmitted by Ixodid ticks. This was first described by Smith and Kilborne who studied transmission of *Babesia bigemina* by *Boophilus* ticks to cattle (Smith and Kilborne, 1893). Historically, this was an extremely important advance for the field of Parasitology, because it was the first proof of concept that an arthropod could serve as a vector of disease (Levine, 1973). The tick vector is usually specific to each species of *Babesia*. *Babesia canis canis* is transmitted by *Dermacentor reticulatus*, while *Babesia canis vogeli* and *Babesia gibsoni* are transmitted by *Rhipicephalus sanguineus*. *Babesia canis rossi* is transmitted by *Haemaphysalis leachi* (Uilenberg et al., 1989), and in experimental studies, *B. canis* has been transmitted by *Hyalomma marginatum* and *Dermacentor andersoni* (Taboada and Merchant, 1991).

A tick ingests *Babesia* parasites during a blood meal. Upon entering the gut, intraerythrocytic Strahlenkörper (ray bodies), or gametes, may be formed. Gametes then fuse and nuclei merge (Mehlhorn and Schein, 1998). This fusion creates a zygote within 14 to 18 days of tick feeding. During tick molt, the zygote becomes a motile kinete and travels via the hemolymph (the body fluid of ticks that functions as the blood and lymph) to distant sites including the salivary glands, nephrocytes, fat cells, and in some *Babesia* spp., the ovaries. A few days following tick reattachment, the kinetes move to the tick salivary glands where they undergo sporogony, producing up to 100,000 sporozoites. There may be several thousand sporozoites within each salivary alveolus. The infective

sporozoites develop during a blood meal and are introduced to the host following tick molt. Once in the host, sporozoites directly invade erythrocytes and merogony, asexual reproduction, occurs (Melhorn and Schein, 1984). Unlike other *Babesia* species, the sporozoites of *Babesia equi* (*Theileria equi*) and possibly *Babesia microti* must undergo a cycle of multiplication in the vertebrate host lymphocytes before invading erythrocytes; therefore it has been proposed that these species should be included in a separate genus, *Theileria* (Mehlhorn and Schein, 1998; Schein et al., 1981).

Transovarial transmission by the tick vector is an important factor in the successful spread of *Babesia* spp. throughout the world and has been described in most *Babesia* species with the exception of *B. microti* and *B. equi* (*T. equi*) (Mehlhorn and Schein, 1998). Before transmission, *Babesia* parasites enter the developing egg and the emerging larvae are infected (Mehlhorn and Schein, 1984; Telford et al., 1993).

During blood stage, parasites divide within erythrocytes. When two piroplasms are in the erythrocyte, they are called a 'paired form'. When there are four piroplasms, they are referred to as a 'Maltese cross', or tetrad, form (Mehlhorn and Schein, 1984). However, in *B. canis*, the morphological transformation of merozoites into gamonts most likely starts inside the erythrocyte of the vertebrate host (Mehlhorn and Schein, 1998). Occasionally, piroplasms become large and oddly shaped. It is believed that these changed merozoites are gametocytes (specialized haploid cells produced by meiosis) that can only differentiate further into gamonts within the tick gut upon ingestion (Mehlhorn and Schein, 1984). Host erythrocytes may be destroyed when the

parasites reproduce and leave the cell to invade other erythrocytes (Mehlhorn and Schein, 1984).

1.1 Babesia *species and Babesiosis*

In 1971, Levine listed 71 species of *Babesia* (Purnell, 1981). Today, over 100 species have been described (Schuster, 2002). Of those, 18 have been found to cause disease in domestic mammals, including pigs, horses, cattle, sheep, goats, cats, and dogs, with most species occuring in rodents, cattle, and carnivores (Telford et al., 1993). Humans may be infected with certain *Babesia* spp. Generally, *Babesia* spp. are host specific; however, there are exceptions. *Babesia divergens*, primarily a parasite of cattle, may infect rats, gerbils, and humans. In addition, *B. microti*, primarily a rodent parasite, may also infect humans. Finally, *Babesia bovis*, primarily a cattle species, may experimentally induce a parasitemia in sheep and goats (Western et al., 1970; Kuttler, 1988).

In canines, *Babesia* spp. may cause hemolytic anemia in the host (Yamane et al., 1994). Pathogenesis and clinical signs of the disease are variable and the immune status of the host, as well as the species or subspecies (strain) of the infecting parasite, influence how the disease manifests (Jefferies et al., 2001). *Babesia* spp. are capable of producing acute, febrile, and, sometimes, fatal infections. After initial acute infection, the animal may become a chronic carrier (Jefferies et al., 2001).

Two species of *Babesia*, *B. gibsoni* and *B. canis* have classically been described in dogs. However, phylogenetic classification of these canine piroplasms has received recent attention and it is now believed that multiple species and/or subspecies exist throughout the world (Jefferies et al., 2001).

Babesia gibsoni and *B. canis* are differentiated on the basis of their morphology in blood smears (Conrad et al., 1991). *Babesia gibsoni* occurs as small (1 x 3.2 m length) oval or round piroplasms characterized by tetrad or 'Maltese cross' dividing forms (Mehlhorn and Schein, 1984). In contrast, *B. canis* forms larger (2.4 x 5 m length) paired pear-shaped piroplasms within the erythrocyte (Levine, 1973). In Giemsa-stained thin blood smears, *B. gibsoni* is more pleomorphic than *B. canis* and has been described as a delicate ring of bluish cytoplasm, which surrounds a vacuole with one or two chromatin dots located at the periphery (Farwell et al., 1982).

Currently, in the United States, dogs positive for *Babesia* spp. DNA have been identified in 29 states (Birkenheuer et al., 2005). *Babesia gibsoni* is the most commonly detected species and most often is reported in dogs in the southeastern states of Alabama, North Carolina, and Mississippi (Birkenheuer et al., 1999; Kjemtrup et al., 2000; Macintire et al., 2002; Birkenheuer et al., 2003; Birkenheuer et al., 2005). However, it has also been identified in dogs from Indiana, West Virginia, Oklahoma, Missouri, Michigan and Texas suggesting a more widespread distribution of the parasite than previously thought (Irizarry-Rovira et al., 2001; Kocan et al., 2001; Stegeman et al., 2003; Birkenheuer et al., 2005).

1.2 Babesia canis *Nomenclature*

Babesia canis has also been termed *Pyrosoma bigeminum* var. *canis*, *Piroplasma canis*, *Babesia rossi*, *Rossiella rossi*, *Babesia vitalii*, and *Rangelia vitalii* (Levine, 1973). Piana and Galli-Valerio first described *B. canis* in 1895 (Levine, 1973).

1.3 Babesia canis *Distribution and Vectors*

Babesia canis occurs worldwide and is transmitted by *Rhipicephalus*, *Haemaphysalis,* and *Dermacentor* ticks (Farwell et al., 1982; Kuttler, 1988). *Babesia canis* is infective in the larval, nymph, and adult stages of the tick and has been reported not only in domestic canines, but also in a variety of wild Canids including coyotes, wolves, jackals, and foxes (Kakoma and Mehlhorn, 1994; Kuttler and Ristic, 1988). It is possible that wild animals act as subclinical reservoirs of *Babesia* sp. and transmit them to domestic animals through a common tick vector (Ristic and Lewis, 1977). However, the exact epidemiological significance of these potential wild reservoirs is not yet well known (Kuttler, 1988).

1.4 Babesia canis *Genetic Diversity*

Through the analysis of the small subunit ribosomal-RNA gene, there are currently three subspecies of *B. canis* recognized, *B. c. canis*, *B. c. rossi*, and *B. c. vogeli* (Uilenberg et al., 1989). These three subspecies have been discriminated by vector specificity, pathogenicity, and differences in cross-immunity, as well as by a limited

serological cross-reactivity in immunofluorescence, Western blotting, and enzyme-linked immunosorbent assay (ELISA) (Uilenberg et al., 1989; Hauschild et al., 1995; Schetters et al., 1996). However, the antigenic material of the three subspecies is not readily available, so serologic tests have not found a wide application for their discrimination (Zahler et al., 1998). Such discrimination is desirable due to the rising incidence of imported disease resulting from increased travel of dogs, especially in regions where potential tick vector species occur (Uilenberg et al., 1989; Zahler et al., 1998). More recently, genotype differentiation has supported the separation of the three subspecies of *B. canis* (Zahler et al., 1998).

The severity of infections with *B. canis* varies with the subspecies (strain). In some localities, *B. canis* causes a relatively mild disease; in others, it is more pathogenic (Zahler et al., 1998). *Babesia canis rossi* is transmitted by the *Haemaphysalis leachi* tick in southern Africa and usually causes a fatal infection in dogs even after treatment (Uilenberg et al., 1989). In various tropical and subtropical countries, including Australia, the *Rhipicephalus sanguineus* tick transmits *B. c. vogeli*, which leads to mild infections that are usually clinically unapparent (Hill and Bolton, 1966). *Babesia canis canis* is transmitted by the *Dermacentor reticulatus* tick in Europe and shows a level of pathogenicity that generally lies between the virulence of *B. c. vogeli* and *B. c. rossi* (Uilenberg et al., 1989). Differences in pathogenicity among different subspecies (strains) of *B. canis*, as well as the age of the host and immunological response to the parasite, all contribute to a wide variation in severity of clinical manifestation (Martinod et al., 1986).

A new large species of canine *Babesia* was first described by Birkenheuer et al. in 2004 in a seven-year-old female spayed Labrador retriever, and was described as clinically and morphologically indistinguishable from *B. canis*. Analysis of the SSU rRNA gene showed that the new species of *Babesia* shared the highest degree of sequence identity with *Babesia bigemina* (93.9%) and *Babesia caballi* (93.5%). When compared with all three strains of *B. canis*, the isolate only shared a sequence identity of 91.2-91.6% (Birkenheuer et al., 2004b).

1.5 Babesia canis *Pathology*

In naturally infected dogs, the prepatent period of *B. canis* is 10-20 days (Breitschwerdt, 1990). Clinical signs vary with age (puppies most severely affected) and include anorexia, fever, hemoglobinuria, malaise, and icterus (yellowing of the skin and whites of the eyes caused by a build-up of bile pigment, bilirubin, in the blood) (Schetters et al., 1989; Bose et al., 1995; Ano et al., 2001).

The first sign of the disease is fever followed by the presentation of parasitemia roughly three days later. The fever associated with *B. canis* is most likely caused by erythrocyte and parasite debris released during the destruction of the red blood cells. The increasing parasitemia is due to the invasion and multiplication of parasites within the erythrocytes (Kakoma and Mehlhorn, 1994).

In acute cases, usually in puppies, the dog may die of hypotensive shock in a few days. This hypotensive shock occurs early in the infection before the host immune

system has time to mount a response. It is caused by parasitic materials activating the coagulation-complement-kallikrein cascade (Freeman et al., 1994). Potent vasodilators and hypotensors, such as bradykinin and kallikrein, and C3a and C5a from the host complement system contribute to hypotensive shock. A generalized non-specific inflammation may result from early macrophage activation. In addition, when antibody response is at a high level, the deposition of antigen-antibody complexes in the tissues may also contribute to shock (Freeman et al., 1994).

Hemoglobinuria may ensue as a result of the massive destruction of infected erythrocytes. This destruction is not only due to the infection itself, but is also due to the host immune response. When erythrocytes are destroyed, hemoglobin from the erythrocytes is released. In severe pathology, due to the destruction of erythrocytes, babesiosis may present as a hemorrhagic disease with bleeding of the edges of the ears and from the muzzle. Additionally, internal hemorrhages may be found on the heart, bronchi, pleura, and intestines (Levine, 1973).

During acute episodes, *B. canis* may cause in icterus, a loss of appetite, weakness, increased thirst, prostration (complete exhaustion or weakness), and, possibly, death. In chronic episodes, fever resulting from infection is not markedly high and does not usually last more than a few days. There is little icterus, but anemia is severe and the dog becomes very weak and emaciated (Levine, 1973; Loretti and Barros, 2005).

Inflammation of the iris and of the cornea may be seen when the eyes are affected and rheumatic signs with inflammation of muscle may be present when muscles are involved. The involvement of the respiratory system may cause shortness of breath,

difficult or laboured breathing, and catarrh, an inflammation of mucous membranes. Also, any involvement of the circulatory system causes ascites (an effusion and accumulation of serous fluid in the abdominal cavity), edema, or purpura (a condition characterized by hemorrhages in the skin and mucous membranes resulting in the appearance of purplish spots or patches). In addition, inflammation of the oral mucosa and the stomach may be present (Levine, 1973).

The central nervous system may also be involved in cases of *B. canis*, resulting in paresis, locomotor disturbances and epileptiform fits (Levine, 1973). These manifestations result from the blockage of the local circulation and corresponding anoxia (lack of oxygen) that in turn, are a result of the mechanical and immune-mediated destruction of erythrocytes. The cerebral form of babesiosis yields clinical signs that include lack of coordination, muscle tremors, seizures, coma, aggression, and hindquarter paresis, which may be confused with rabies. This may lead to a mis-diagnosis of the disease (Levine, 1973; Lobetti, 1998).

Some dogs recovering from *Babesia* spp. infections do not completely clear themselves of the parasite. Instead, they may remain permanent carriers with low levels of parasitemia their entire life. This is a significant problem because chronically infected dogs can serve as reservoirs for future infection (Fowler et al., 1970; Farwell et al., 1982; Yamane et al., 1993).

1.6 Babesia canis *Diagnosis and Treatment*

Babesia spp. may be found in thin, Giemsa-stained blood films during the early stages of infection (Yamane et al., 1994). Due to their low numbers in chronic carriers, parasites are difficult to visualize in a blood smear. To detect these infections, other methods may be used, including indirect immunofluorescent antibody (IFA) test, agglutination, complement fixation, indirect hemagglutination, and Enzyme-Linked ImmunoSorbent Assay (ELISA). In the past, the IFA test was the preferred method of diagnosis in chronic and active infections of *B. canis* and *B. gibsoni* (Anderson et al., 1980; Levy et al., 1987; Bose et al., 1995). However, the test has poor specificity as a result of antigen cross-reactivity and fails to identify recent infection (Yamane et al., 1994; Jefferies et al., 2003).

Recent advances in automated DNA sequencing have led to an ability to detect and identify piroplasms with better specificity and sensitivity than past methods (Birkenheuer et al., 2002; Jefferies et al., 2003). Current methods include polymerase chain reaction (PCR), PCR- restriction fragment length polymorphism (RFLP) and reverse line blot hybridization (Carret et al., 1999; Matjila et al., 2004). In some cases, sequencing single PCR products is a reliable diagnostic technique (Criado-Fornelio et al., 2003).

Treatment of *B. canis* includes the administration of the drugs diminazene aceturate, imidocarb diproprionate, isometamidium, and amicarbalide. These are used in treatment of acute cases and result in the disappearance of the detectable parasitemia.

However, some treated dogs may have a secondary relapsing parasitemia. The dogs showing the secondary infection are generally immune to a later virulent challenge, but the dogs not showing the chronic infection are susceptible (Kuttler, 1988). Imidocarb diproprionate is the choice treatment for *B. canis* infection in dogs (Adeyanju and Aliu, 1982).

1.7 Babesia gibsoni *Nomenclature*

Babesia gibsoni has also been known as *Piroplasma gibsoni*, *Achromaticus gibsoni*, *Babesiella gibsoni*, *Pattonella gibsoni*, and *Nuttallia bauryi* (Levine, 1973). Patton first identified *B. gibsoni* in jackals in India in 1910.

1.8 Babesia gibsoni *Distribution and Vectors*

At one time, *B. gibsoni* was thought to occur only in India, Africa, Sri Lanka, and Malaysia. However, it is now known to occur in Asia, North America, northern and eastern Africa, Australia, and Europe, with the major distribution of occurrence in Asia and Africa. It has become an important parasite in the United States as well (Kuttler, 1988; Birkenheuer et al., 1999). *Babesia gibsoni* may occur in many different canine hosts including domestic dogs, jackals, wolves, Indian wild dogs, foxes, as well as ferrets, badgers and coyotes (Kuttler, 1988). These wild animals may serve as carriers of *B. gibsoni* and the possibility of coyotes serving as reservoir hosts has been supported by

the experimental infection of coyote populations with resulting mild clinical signs (Roher et al., 1985).

1.9 Babesia gibsoni *Genetic Diversity*

Recent genetic characterizations have shown that small piroplasms known to infect dogs represent a greater diversity than previously thought. Thus far, three genetically distinct small *Babesia*-like piroplasms have been reported to naturally infect dogs (Kjemtrup et al., 2000). They include *B. gibsoni* (Asian, Okinawa, or Oklahoma genotype), *Theileria annae* (Spanish/European genotype) and *Babesia conradae* (Kjemptrup et al., 2006), the Western piroplasm (formerly called *B. gibsoni*); (Kjemptrup et al., 2000; Zahler et al., 2000). *Theileria annae* was first identified by Zahler and others in 2000 in a dog from Spain. Analysis of the SSU rRNA gene showed that its identity with *B. gibsoni* was approximately 90% and its identity with *B. canis* was 88.6%. Additionally, it was most similar to *B. microti*, with an identity of 96.7% (Zahler et al., 2000). *Babesia conradae* was first described by Kjemtrup et al., in 2006 as a renaming of the *B. gibsoni* California (or Western piroplasm) genotype (Conrad et al., 1991). Morphologically, *B. conradae* is similar to *B. microti*, and analysis of the SSU rRNA gene demonstrates that it is most closely related to the WA-1 type *Babesia*, originally isolated from a human in Washington State (Quick et al., 1993; Birkenheuer et al., 2006). *Babesia gibsoni* and *B. conradae* have been identified in dogs in the United States. To date, *B. conradae* has been reported only in dogs from California and

T. annae has only been reported in Spain. The nomenclature of these small *Babesia*-like organisms will be modified when their taxonomy is better understood (Birkenheuer et al., 2003).

Babesia gibsoni is transmitted by both *Haemaphysalis* and *Rhipicephalus* ticks in regions outside of the United States. To date, the tick vector has not yet been confirmed in the United States. *Rhipicephalus sanguineus* is the suspected vector, but no study has definitively confirmed its competency as a vector in the United States. Ixodid ticks are usually responsible for transmission; however, in experimental studies, the parasite was also transmitted transplacentally (Farwell et al., 1982; Kuttler, 1988; Taboada, 1996; Birkenheuer et al., 2005; Fukumoto et al., 2005). In addition, transmission may occur via blood transfusion or through contamination from previously used equipment such as tail docking devices or needles. Finally, there is good evidence that *B. gibsoni* may be transmitted from direct blood contamination during dogfights (Groves and Dennis, 1972; Botros et al., 1975; Wozniak et al., 1997; Birkenheuer et al., 1999; Irizarry-Rovira et al., 2001; MacIntire et al., 2002; Stegeman et al., 2003; Birkenheuer et al., 2005).

1.10 Babesia gibsoni *Pathology*

Dogs with *B. gibsoni* often present with anorexia, vomiting, fever, splenomegaly (an enlargement of the spleen), lethargy, and acute hemolytic anemia (Taboada, 1991; Yamane et al., 1993; Zahler et al., 2000). Also, various degrees of hemolysis are seen with *B. gibsoni* (Taboada, 1998). Hyperglobulinaemia (an abnormally large amount of

globulins in the circulating blood plasma) may be observed. This indicates antigenic stimulation by the parasite resulting in erythrocyte destruction (Taboada, 1998). Thrombocytopenia (a decrease in the number of platelets in the blood, resulting in the potential for increased bleeding and decreased ability for clotting) is also a consistent finding in *B. gibsoni* infections. It may develop before parasitemia is detectable and persist after the resolution of anemia (Meinkoth et al., 2002). The thrombocytopenia found in animals infected with *B. gibsoni* may be due to immune-mediated destruction of platelets, scattered intravascular coagulation, or aggregation and sequestration of platelets in the spleen (Wozinak et al., 1997). Neutropenia may also be seen in a *B. gibsoni* infection (Taboada, 1998; Meinkoth et al., 2002).

The anemia caused by *B. gibsoni* is due to destruction of erythrocytes, resulting from a combination of the direct mechanical disruption caused by the parasite as it leaves the red blood cell, along with intravascular hemolysis, which may be immune-mediated or nonimmune-mediated (Farwell et al., 1982; Onishi et al., 1990). In addition, oxidative damage may contribute to the destruction of erythrocytes by making them more susceptible to phagocytosis by macrophages (Murase and Maede, 1990).

Infections of *B. gibsoni* may be misdiagnosed as idiopathic (self-causing) immune-mediated hemolytic anemia. In *B. gibsoni* infections, there may be immune-mediated hemolytic anemia along with red blood cell agglutination and a positive direct Coomb's test. The latter is a haemagglutination test in which Coombs' reagent (antiglobulin, or anti-human globulin rabbit immune serum) is added to detect incomplete (non-agglutinating, univalent, blocking) antibodies coating erythrocytes.

However, these are also indicative of idiopathic immune-mediated anemia (Farwell et al., 1982; Taboada, 1998; Birkenheuer et al., 1999; Irizarry-Rovira et al., 2001).

In the United States, the majority of dogs diagnosed with *B. gibsoni* are American Staffordshire terriers and American Pit Bull Terriers (Birkenheuer et al., 2003). These breeds display aggressive tendencies and are more likely to bite than other dogs, resulting in the direct transmission of *B. gibsoni*. They are often used as fighting dogs and fighting may be a way of parasite transmission (Birkenheuer et al., 2005).

1.11 Babesia gibsoni *Diagnosis and Treatment*

Babesia gibsoni is small, usually oval or annular in shape, and less than 3 m in length, thus appearing less than one-eighth of the diameter of the host erythrocyte (Kuttler et al., 1988). It is pleomorphic and exhibits linear, reticulate (network forming), pyriform, amoeboid, and signet ring forms, with the latter form most common (Fowler et al., 1970; Fukumoto et al., 2000).

Babesia gibsoni infects erythrocytes via an invagination of the host cytoplasmic reticulum (Levine, 1988). It is efficient in its digestion of hemoglobin, so no hemozoin pigment (an iron-containing pigment) is formed (Rudzinska et al., 1981). Lack of pigment formation is a distinguishing characteristic for *B. gibsoni* identification in blood smears (Rudzinska et al., 1981). *Babesia gibsoni* divides synchronously into four merozoites. It is uncertain whether this division occurs by schizogony or by budding (Rudzinska et al., 1981).

Since *B. gibsoni* is so small, and infections display low levels of parasitemia, it is difficult to detect in erythrocytes on blood smear examination and may be overlooked (Conrad et al., 1991). In addition, serologic cross-reactivity between *Babesia* spp. may cause problems in diagnosis when an IFA test for IgG antibodies is used (Taboada, 1998; Irizarry-Rovira et al., 2001). Therefore, PCR analysis may be necessary to make a differentiating diagnosis (Birkenheuer et al., 2002).

With drug therapies presently available, complete elimination of *B. gibsoni* parasites from infected dogs is not expected (Birkenheuer et al., 2005). Imidocarb is the choice treatment for *B. canis* infection in dogs, but cannot eliminate the parasite when used to treat *B. gibsoni* (Adeyanju and Aliu, 1982). Other treatments, including phenamidine isethionate and diminazine aceturate, also have the ability to resolve clinical signs, but are not currently available in the United States (Groves and Dennis, 1972; Farwell et al., 1982; Taboada, 1998; Birkenheuer et al., 1999). Metronidazole, clindamycin, and doxycycline also have some efficacy in the treatment of babesiosis (Farwell et al., 1982; Taboada, 1998). In the treatment of *B. microti*, a *Babesia* species infecting rodents as well as humans, a combination of azithromycin and atovaquone was effective in reducing parasitemia (Herwaldt et al., 1995; Hughes and Oz, 1995; Stegeman et al., 2003). Based on the results of Birkenheuer (2004a), an atovaquone-azithromycin combination treatment is also effective in suppressing *B. gibsoni* levels below PCR detection in dogs. However, it has been suggested that the atovaquone allows recrudescence of parasites with decreased susceptibility to this drug (Matsuu et al., 2004).

The opinions on administration of corticosteroids in dogs with babesiosis vary (Stegeman et al., 2003). Steroids may be beneficial in reducing immune-mediated erythrocyte destruction in acute stages of the disease, but long-term effects include a reduced ability to clear the parasite by the spleen (Taboada, 1998). Splenic function is very important in limiting the parasitemia as shown by the high parasitemia of splenectomized dogs experimentally infected with *B. gibsoni* (Groves and Dennis, 1972; Botros et al., 1975; Meinkoth et al., 2002).

Mutation of the *cytochrome b* (*cytb*) gene, located in the mitochondrial genome, has been described in atovaquone-resistant isolates of human protozoan pathogens, including *Toxoplasma gondii*, *Pneumocystis carinii*, and *Plasmodium* spp. (Walker et al., 1998; Syafruddin et al., 1999; McFadden et al., 2000; Suswam et al., 2001). Recently, Matsuu et al., 2006 described similar mutations in *B. gibsoni*. This indicates that single nucleotide polymorphisms in the sequence for the *cytb* gene may be associated with the resistance of *B. gibsoni* to atovaquone treatment. Knowledge of these genetic mutations may be valuable as markers for clinical application and for the selection of possible drugs to be used treatment along with atovaquone (Matsuu et al., 2006).

1.12 Differentiation of Babesia canis *and* Babesia gibsoni

Differentiation of the infecting *Babesia* sp. is important for prognosis and treatment of babesiosis (Stegeman et al., 2003). However, the pathogenesis and disease caused by *B. canis* and *B. gibsoni* are very similar (Martinod et al., 1986). This leads to

a difficulty in diagnosis of the causative organism of babesiosis. The serologic cross-reactivity between *B. gibsoni* and *B. canis* may cause problems in diagnosis when an IFA test is used (Taboada, 1998; Irizarry-Rovira et al., 2001).

Both *B. canis* and *B. gibsoni* cause some degree of hemolysis. However, unlike *B. canis*, there is a lack of severe bilirubinemia despite extreme anemia in *B. gibsoni* infections (Taboada, 1998).

1.13 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) is a molecular biology technique developed in the early 1980's by Mullis and published in 1985 by Cetus Corporation (Saiki et al., 1985). Through the use of PCR, genetic regions of interest may be amplified in large quantities and then used for analysis (Guyer and Koshland, 1989).

PCR uses a target segment of DNA or a gene, from which primers are designed. Primers are synthetic oligonucleotides (usually ~20 nucleotides long) that complement a segment on either end of the target DNA. Specific primers are added to the DNA, which contains the sequence to be amplified, along with a thermostable DNA polymerase and the four deoxyribonucleotide triphosphates in a suitable buffer. Heating pulls the double stranded DNA apart in a process called denaturing, and the primers then bind to their specific complementary sequence on either side of the target DNA sequence. DNA polymerase extends the primers, synthesizing complementary strands of DNA using deoxynucleotide triphosphates (Prescott et al., 2005).

The DNA polymerase used in PCR must be heat stable so that the enzyme is not denatured by the repeated denaturing heat cycles. Two popular enzymes used are Taq polymerase from the thermophilic bacterium *Thermus aquaticus* and Vent polymerase from *Thermococcus litoralis (*Prescott et al., 2005).

The new fragments of target DNA serve as templates, synthesizing additional new DNA strands. In this manner, additional cycles of PCR will predominantly yield many copies of the desired DNA fragment (Prescott et al., 2005). The products of PCR may then be loaded into wells of an agarose gel and elecrophoresed to yield a visual product upon staining with a dye such as ethidium bromide (Prescott et al., 2005).

PCR is advantageous because it offers high sensitivity and specificity when compared to other methods of diagnosis (Ikadai et al., 2004). It is particularly helpful when parasitemias are low or infections are subclinical (Persing et al., 1992). In addition, species identification is possible using the sequence of the DNA product (Persing et al., 1995). Species identification may be based on species-specific primer design so that only the target species is amplified, when used as a diagnostic test.

1.14 Culture

Several different species of Babesia have been cultured in vitro with success (Erp et al, 1980; Vega et al., 1985). Early attempts to culture *B. gibsoni* were made by Rao in 1926; three different temperatures and 14 different medium formulations were tried unsuccessfully. In 1980, Levy and Ristic introduced the cultivation method for *B. bovis*,

which was modeled after *Plasmodium* culture systems; the microaerophilous stationary phase (MASP) culture. Murase et al. (1991) modified the aforementioned system and cultured parasites in a microtiter plate that allowed for an efficient oxygen supply. Cultures were initiated from dogs infected with *B. gibsoni*. Parasites were cultured in α-minimal essential medium (MEM) supplemented with 40% normal dog serum in a humidified atmosphere containing 5% CO_2 at 37 °C. However, the culture period was only 15 days (Murase et al., 1991).

Onishi et al. (1993) were able to extend the survival of *B. gibsoni* parasites in vitro to 28 days by exchanging a portion of the red blood cells (RBCs) every seventh day after initiation. Parasites were cultured in RPMI-1640 medium with 20% normal dog serum in a humidified atmosphere containing 5% CO_2 at 37° C. However, the number of parasites gradually decreased one week after incubation and by four weeks of incubation, parasites did not grow, even though fresh medium and RBCs were supplied. Many factors were examined, including the effect of serum concentration of different culture media including M-199, F-12, L-15, α-MEM, and RPMI, the gaseous environment, as well as the interval of medium exchange on the in vitro cultivation of *B. gibsoni*. They concluded that a higher parasitemia could be obtained by using RPMI-1640 supplemented with 20% normal dog serum in a humidified atmosphere containing 5% CO_2 at 37 °C with medium exchange every 24 hours.

Zweygarth and Lopez-Rebollar (2000) cultured *B. gibsoni* of Sri Lanka and Bangladesh stock in HL-1 medium that had been supplemented with 40% normal dog serum with a maximum parasitemia from 1.3 to 5.6%. They concluded that a

concentration of 40% serum is important during the early stages of culture, and could be lowered to 20% after the initiation phase. In addition, it was reported that RPMI-1640 medium did not support the growth of the parasites, while HL-1 medium did. Finally, they reported that *B. gibsoni* cultures required an O_2-reduced atmosphere for initiation of culture from blood with a low parasitemia (<0.01%), while blood with a higher parasitemia (2.6%) could be used to initiate cultures in a normal atmosphere with 5% CO_2. It was also suggested that actively dividing parasites required normal oxygen levels.

Sunaga et al. (2002) reported the longest maintenance of in vitro cultures of *B. gibsoni*. They cultivated *B. gibsoni* at a high level of parasitemia continuously in vitro for 814 days with an average parasitemia of 18.2 ± 2.4% on day 3 of culture, in RPMI-1640 medium supplemented with 7.5% normal dog serum in a humidified atmosphere containing 5% CO_2 at 37 ºC.

2. MATERIALS AND METHODS

2.1 Canine Blood Samples

Canine blood samples from dogs of any breed, age, or gender were submitted by various kennels, shelters, rescue groups and clinics throughout Texas. Blood was drawn by cephalic venipunture into 3-5 ml tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). The animal's age, breed, gender, and the presence of any clinical signs of babesiosis were noted for each sample. The blood samples were immediately refrigerated and kept cold until transported to the Department of Veterinary Pathobiology, Texas A&M University, College Station, TX. Upon arrival, the samples were refrigerated until use. For each sample, the packed cell volume (PCV) was determined by microhematicrit centrifugation. Thin blood films were prepared and stained with Giemsa stain and then microscopically examined for the presence of *Babesia* spp.

2.2 DNA Extraction

Blood collected in EDTA was centrifuged at 500 *X g* for 15 min and the serum was removed. A 200 l aliquot of packed red blood cells was transferred to a 2 ml microcentrifuge tube and washed 2X in at least 5 volumes of 0.15 M phosphate buffered saline (PBS) containing 15 mM EDTA (PBS/EDTA) by centrifugation at 935 *X g* for 3 min. The pellet was then similarly washed twice with PBS. Next, 100 l PBS was

added to the final red blood cell (RBC) pellet and the mixture was used in DNA extraction.

Genomic DNA (gDNA) was purified from each blood sample following the protocol of a Flexi-Gene DNA Kit (Qiagen, Valenia, CA.). First, 750 l FG1 (lysis buffer) was added to each sample, mixed well by inversion, and centrifuged 30 s at 12,000 *X g* in a fixed-angle rotor. Most of the supernatant was removed using a P1000 pipettor and any residual supernatant was removed using a P200 pipettor so that the pellet was as dry as possible. A 1:100 Protease (Qiagen) and FG2 (denaturation buffer) mixture was prepared, and 150 l of the mix was added to each tube with vortexing immediately upon addition (3-4 rounds of high speed vortexing for 5 s each). The tubes were briefly centrifuged and placed in a 65 C waterbath for 5 min. Next, 150 l 100% isopropyl alcohol was added to each tube and mixed by inversion 20 times. Tubes were labeled and centrifuged 3 min at 12, 000 *X g*, noting the position of the tube in the centrifuge so that the location of the pellet was known. Supernatant was discarded in two steps, as described above, with care not to disturb the pellet. Then, 150 l 70% ethanol was added and tubes were vortexed 5 s. Tubes were centrifuged 3 min at 12,000 *X g* and the position of the tubes in the centrifuge was noted as before. Supernatant was once again removed in 2 steps, as described above, and the pellet was air dried (at least 5 min) until all liquid evaporated. Finally, approximately 30 l Buffer FG3 (hydration buffer) was added to each tube (more FG3 was added if the pellet was very large, but no

less than 30 l was used) and tubes were vortexed 5 s at a low speed with an incubation of at least 10 min at 65 C to resuspend the DNA.

2.3 SSU rRNA Gene Sequencing

The SSU rRNA genes were amplified from approximately 50-100 ng template genomic DNA using 1pmol each primers A (5'-AACCTGGTTGATCCTGCCAGT-3') and Bcnvo 1686R (5'-ATCCTTCTGCAGGTTCACCTACG-3') in a 12 l reaction volume according to manufacturer's instructions (AdTAQ 2 PCR Kit, BD Biosciences Clontech, Palo Alto, CA). The amplication profile for the primary PCR reaction was: initial denaturation at 96 C for 3 min, followed by 60 cycles of denaturation at 96 C 10 s, annealing at 60 C for 10 s and extension at 72 C for 2 min, with a final extension at 72 C for 10 min and then hold at 4 C (PCR Express or Sprint thermocycler; Hybaid, Ashford, UK). A nested reaction followed the primary PCR reaction with the reaction volume and reagents the same as above with the following exceptions. the template for the nested PCR reaction was 0.5 l of a 1:20 dilution of the primary PCR product, primers for nested amplification of the *B. gibsoni* SSU rDNA sequence were BabV1650R (5'-AACCGACGAATCGGAAAA-3') and BgibFN

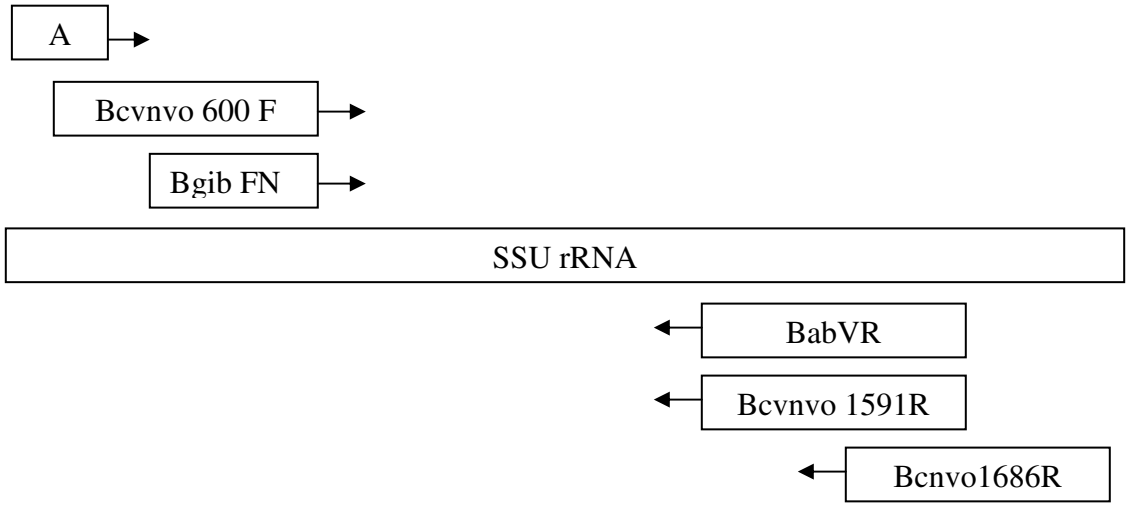

Fig. 2.1. Schematic drawing illustrating positions and directions of primers used to amplify the SSU rRNA gene region. Primers for primary PCR include A and Bcnvo 1686R. Primers for *B. gibsoni* nested PCR include BgibFN and BabVR. Primers for *B. canis* nested PCR include Bcvno 600F and Bcnvo 1591R.

(5'-CGGCTACTTGCCTTGTCTG-3'); and primers for amplification of the *B. canis* gene sequence were Bcnvo 1591R (5'- GTGTTCGAGTTTGCCATTC-3') and Bcnvo 600F (5'-GTGTTCGAGTTTGCCATTC-3') (Fig. 2.1).

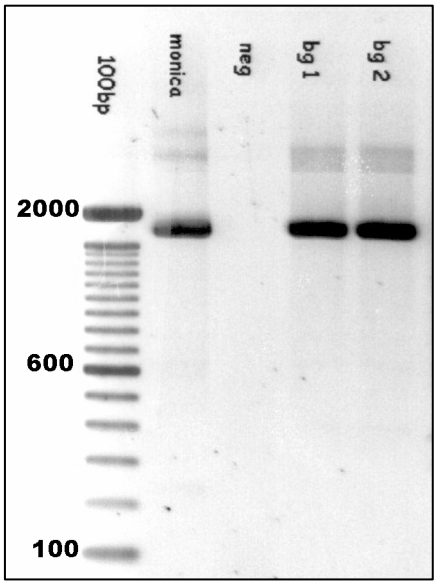

Fig. 2.2. Primary PCR products separated by electrophoresis through an agarose gel. Neg, negative water control; bg1 and bg2, positive *B. gibsoni* controls; Monica, test sample.

The amplification profile for the *B. gibsoni* nested reaction was initial denaturation at 96 C, followed by 45 cycles of denaturation at 96 C, annealing at 63 C for 10 s and extension at 72 C for 2 min, with a final extension at 72 C for 10 min and then held at 4 C (PCR Express or Sprint thermocycler; Hybaid, Ashford, UK). The amplification profile for *B. canis* was the same as above, but with an annealing temperature of 60 C. Primary and nested PCR products were separated by electrophoresis through a 1% agarose gel and their positions were marked by a 100 base pair (BP) marker (Invitrogen, Carlsbad, CA). The agarose gel was then stained with ethidium bromide and bands were visualized by UV transillumination (Fig. 2.2 and Fig. 2.3).

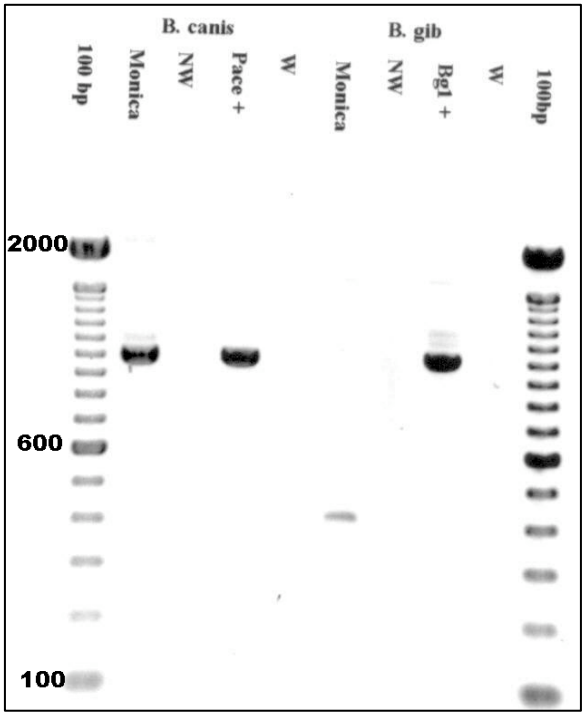

Fig. 2.3. *Babesia gibsoni* and *B. canis* nested PCR products separated by electrophoresis through an agarose gel. W and NW, negative water controls, Bg1+, positive *B. gibsoni* control; Pace+, positive *B. canis* control; Monica, test sample.

Each amplicon was ligated into a plasmid vector, pCR®4-TOPO, and chemically competent *Escherichia coli* cells (TOP10F, One Shot; Invitrogen) were transformed following the protocol of the manufacturer (TA Cloning®Kit; Invitrogen.). If the PCR product was more than 24 h old at the time of ligation, then 2X Qiagen *Taq* PCR Master Mix was added to the sample and incubated at 72 C for 15 min. This step added single deoxyadenosine (A) overhangs to the 3' ends of the SSU-DNA amplicon to make sure that it would be ligated properly with a plasmid vector. If the amplicon was less than 24 hrs old, it was ligated directly into the vector. *Escherichia coli* cells were thawed on ice

and a TOPO cloning reaction was prepared containing 4 l of the PCR product, 1 l salt solution, 1 l TOPO and 1 l sterile water. The TOPO cloning reaction was incubated 30 min at room temperature and 2 l of the reaction were added to the *E. coli* cells. Cells were then placed on ice for 30 min. Cells were heat shocked at 42 C for 30 s and placed back on ice. Then, 250 l room temperature SOC Medium was added to the cells and the mixture was incubated 1 hr in an incubator-shaker 37 C at 200 rpm (Queue Orbital Shaker, Queue Systems Inc., Columbia, SC). Cells were spread on two LB (Luria Broth Agar, Sigma-Aldrich Co.-Aldrich., St. Louis, MO) plates containing 50 µg/ml Kanamycin (Sigma-Aldrich Co.-Aldrich) and X-Gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside, Fisher Scientific, Fair Lawn, NJ) according to manufacturer's instructions (TA Cloning kit, Invitrogen). Finally, plates were incubated at 37 C overnight. The next day, transformed colonies were color selected and colony amplification was performed to make sure the correct sized inserts were present. A minimum of 9 white (transformed with insert) and 1 blue (transformed without insert) colonies were checked for each sample. For every 10 colonies, 9 l ultrapure water was dispensed into each of 10 PCR tubes and a master mix of 100 l 2X Qiagen PCR buffer [*Taq* DNA Polymerase, Qiagen PCR Buffer (with 3mM $MgCl_2$), and 400 l of each dNTP], 4 l M13F (-20) (5'-GTAAAACGACGGCCAG-3'), 4 l M13R (5'-CAGGAAACAGCTATGAC-3') and 2 l ultrapure water were prepared. A new pipet tip was touched to each colony and mixed into separate tubes containing the ultrapure water. Tubes were then placed into a thermocycler at 94 C for 10 min. Next, the

thermocycler was paused, and samples were placed on ice. Finally, 11 l of the master mix was added to each tube and the program was continued for 30 cycles with a denaturation of 94 C for 30 s, annealing at 50 C, and extension at 72 C for 2 min, final extension at 72 C for 10 min, and then held at 4 C (PCR Express or Sprint thermocycler, Hybaid). Products were checked on an ethidium bromide stained 1% agarose gel, as described above, alongside 100 BP DNA ladder.

Plasmid DNA was purified from overnight cultures of selected clones (QIAprep Spin Miniprep Kit; Qiagen). Next, 6 ml LB broth and 6 l ampicillin (50 mg/ml stock) were pipetted into a 50 ml centrifuge tube and inoculated with a single white colony known to contain the insert DNA. Tubes were then incubated overnight at 37 C, 200 rpm (~ 16 hr growth). Then, tubes were centrifuged at 1000 *X g* for 30 min to pellet bacteria. All supernatant was removed and discarded so that pellet was as dry as possible. The pellet was resuspended in 250 l Buffer P1 (resuspension buffer) and transferred into a 2.0 ml centrifuge tube. Then, 250 l Buffer P2 was added and the tubes were inverted 7 times to mix. Next, 340 l Buffer N3 was added and the tube was immediately inverted 7 times to mix. Tubes were centrifuged 10 min at 10,000 *X g*. The supernatant was pipetted into a Qiagen spin prep column with care taken to avoid pipetting the precipitate with the liquid. The spin column was centrifuged 1 min and the liquid was discarded into a microtube. Next, 500 l Buffer PB was added to the column and the column was centrifuged 1 min at 10,000 *X g*. The liquid in the microtube was discarded and 750 l Buffer PE was added with a following centrifugation of 1 min at

10,000 *X g*. The liquid in the microtube was discarded and the column was centrifuged again for 1 min at 10,000 *X g*. Next, the spin column was placed into a clean 1.5 ml microfuge tube and 50 l ultrapure water was added directly onto the middle of the Qiagen column membrane. The tube was allowed to stand 1 min before it was centrifuged 1 min at 10,000 *X g* and the spin column was removed. Then, 1 l plasmid DNA was electrophoresed through a 1% agarose gel alongside 460 ng of a pTZ plasmid marker (Sigma-Aldrich). The gel was stained with ethidium bromide, viewed by ultraviolet transillumination as described above and the pDNA concentration estimated.

Inserts from appropriate clones were sequenced using forward primer M13F (-20) and reverse primer M13R (primer sites positioned within the plasmid) (Davis Sequencing, Davis, CA). BLAST searches (NCBI) were performed for all sequences and sequences were aligned using ClustalW 1.8 Program (EMBL-EBI, 2007).

DNA purification, preparation of PCR master mix reactions, DNA template addition to each PCR master mix aliquot and cloning were each performed in different rooms with dedicated equipment to prevent any cross-contamination of samples. In addition, each PCR experiment included distilled water template controls to ensure that no carry-over contamination had occurred.

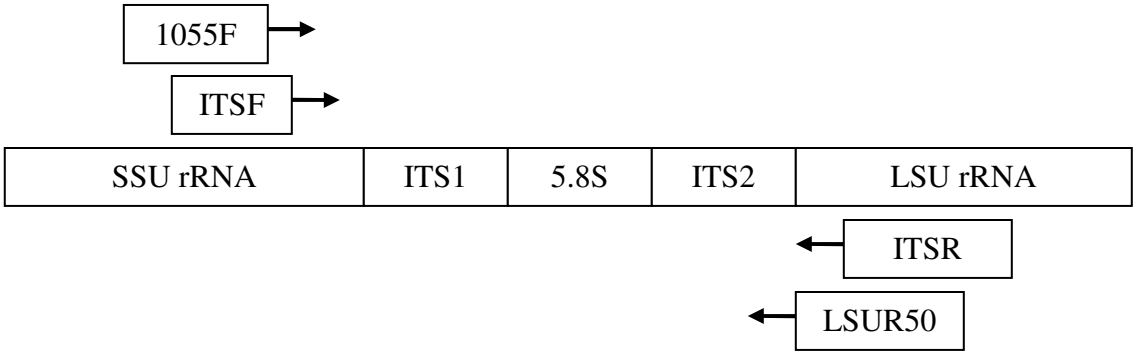

Fig. 2.4. Schematic drawing illustrating positions and directions of primers used to amplify the ITS1-5.8S-ITS2 gene region. Primers for primary PCR include 1055F and ITSR. Primers for nested PCR include ITSF and LSUR50.

2.4 ITS Gene Sequencing

Similar methods to those given above were used to amplify the Internal Transcribed Spacer (ITS) region (ITS1-5.8s-ITS2) (Fig. 2.4). The forward strand primer, 1055F (5'-GGTGGTGCATGGCCG-3'), derived from the SSU rRNA gene, and a reverse strand primer, ITSR (5'-GGTCCGTGTTTCAAGACGG-3'), derived from the large subunit (LSU) rRNA gene were used in the primary PCR (Fig. 2.4). The amplification profile for the ITS primary PCR was: initial denaturation at 96 C for 3 min, followed by 35 cycles of denaturation at 96 C for 10 s, annealing at 55 C for 10 s, extension at 72 C for 2 min, with a final extension of 72 C for 10 min and then hold at 4 C. An ITS nested PCR of the primary product followed using forward strand primer ITS F (5'-GAGAAGTCGTAACAAGGTTTCCG-3'), derived from the SSU rRNA

gene, and reverse strand primer LSUR50 (5'-GCTTCACTCGCCGTTACTAGG-3'), derived from the LSU rRNA gene (Fig. 2.4). The amplification profile for the nested ITS PCR was the same as that for the primary reaction. All primary and nested ITS PCR products were eletrophoresed through an agarose gel as previously described, alongside a 100 BP marker (Invitrogen). Nested ITS PCR products were cloned and sequenced as described above.

2.5 Parasite Cultures

Erythrocyte stage *Babesia* sp. cultures were initiated from blood samples that tested positive for *B. gibsoni* or *B. canis* by PCR, and from infected blood or blood cryostabilates kindly provided by Dr. A. Birkenheuer, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC. Donor canine red blood cells (RBCs) were from a 4-yr-old American Pointer mixed breed neutered male dog. Blood samples were drawn into EDTA as described above. Prior to use in culture, all blood samples were centrifuged 15 min at 1000 *X g* to pellet the cells and the plasma and buffy coat were removed. The erythrocyte pellet was washed twice by centrifugation (3 min at 885 *X g*) in 0.15 M Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich) with 15 mM EDTA and once in DPBS without EDTA. At initiation, 0.2 ml packed and washed erythrocytes were added to 0.8 ml of complete culture medium per well of a 24-well culture plate. For 48-well plates, 80 µl packed washed RBC was added to each well containing 720 µl of medium. Cryopreserved

B. gibsoni and *B. canis* infected blood samples were thawed quickly at 37 C. Aliquots of 0.2-0.25 ml were added to 24-well plate wells containing 0.1 ml packed donor RBC in 0.9 ml complete medium for a final volume of 1.20-1.25 ml/well.

Complete culture media tested included: HL-20, which contained HL-1 (BioWhittaker, Walkersville, MD, USA) medium supplemented with 20% canine serum (PelFreeze, Rogers, Arkansas), 2 mM L-glutamine (GibcoBRL), 20 mM hypoxanthine and 3.0 mM thymidine (HT; Invitrogen Corp. Corp.), 2 µg/ml ALBUMAX I (Lipid Rich Bovine Serum Albumin; Invitrogen); HL-20+, prepared the same as HL-20 with the addition of 1% HB101®; R40, containing RPMI-1640 (with L-glutamine and $NaHCO_3$, Sigma-Aldrich) medium supplemented with 40% canine serum, 50 mM Hepes (Cambrex Bio Science, Walkersville Inc., Walkersville, MD), HT, and ALBUMAX; R40+, prepared the same as R40 with the addition of 1% HB101®; R40T, prepared the same as R40 medium, but with addition of 10 mM TES ((N-tris[Hydroxymethyl]-methyl-2-aminoethane-sulforic acid); Sigma-Aldrich); R40TH, prepared the same as R40T medium but with addition of 1% HB101® (Lyophilized supplement rehydrated according to manufacturer's instructions; Irvine Scientific®, Santa Ana, CA) (Table 2.1). All cultures were incubated at 37 C in a humidified atmosphere of 2% oxygen, 5% carbon dioxide and 93% nitrogen.

Table 2.1

Media and duration of culture for *B. gibsoni*, *B. canis* and Lg. *Babesia* sp. isolates.

	Isolate ID	R40	R40+	R40T	R40TH	HL20	HL20+	Duration	Subcultures
B. gibsoni	2.19.07	x		x		x		40 days	0
	5.11.07-B	x			x			32 days	0
	5.11.07-C	x		x	x	x		32 days	0
	5.11.07-LM	x		x	x	x		32 days	0
	11.29.06	x	x	x		x	x	24 days	0
B. canis	3.15.07-B	x		x		x		34 days	0
	3.15.07-A	x		x		x		34 days	0
	3.30.07	x		x		x		33 days	0
	4.21.07	x		x		x		42 days	0
	4.27.07	x		x		x		36 days	0
Large *Babesia* sp.	5.11.07-L	x		x	x	x		32 days	0
	5.11.07-D	x			x			5.11.07-	5

Each day, 0.65 (48-well plate well) or 1 ml (25-well plate well) of medium overlying the cell layer was removed without disturbing the RBC layer and < 1 µl removed and used to make a blood film. An equal volume of fresh medium to that removed was then added to each well. Each week, 50 µl packed uninfected donor canine RBC (or 20 µl for 48-well plates), washed as above, was added to each culture well. Giemsa-stained thin RBC films were prepared to view the proliferation of *Babesia* spp. If no parasite establishment or growth was seen after 4-6 wk, cultures were terminated. When proliferation of the parasites was sufficient, 48-well plate subcultures were prepared by resuspending the well contents and transferring 200 µl into a new well with 540 µl medium and 60 µl packed donor erythrocytes. Stocks were cryopreserved in 10% PVP and stored in liquid nitrogen (Holman et al., 1994). DNA from successful

culture isolates was molecularly characterized by amplification and sequencing of the SSU rRNA gene along with the ITS region.

3. RESULTS

3.1 Polymerase Chain Reaction (PCR)

3.1.1 Babesia gibsoni

Canine blood samples were received from 254 dogs throughout Texas (Figure 3.1). Submissions were obtained from kennels, shelters, veterinary clinics and rescue groups, and were grouped by the Texas region in which they originated (Table 3.1).

Blood samples were received from areas from the Prairies and Lakes region of Texas. A total of 128 samples were obtained from 24 pit bull terriers, 42 greyhounds and 62 dogs of other breeds. Of these dogs, 2 tested positive for *B. gibsoni* by PCR. Both positive dogs were American Pit Bull Terriers. Case no. 1 was a 7 year old American Pit Bull Terrier from a rescue group near Dallas, TX. It had a PCV of 30 and was positive on Giemsa-stained thin blood smears. At the time of blood sampling, the dog demonstrated no clinical signs of babesiosis. Case no. 2 was a 2 year old female American Pit Bull Terrier mixed breed stray found in College Station, TX. This dog had a PCV of 33 and had mild clinical signs consistent with those of babesiosis. Parasites were visualized on Giemsa-stained thin blood smears.

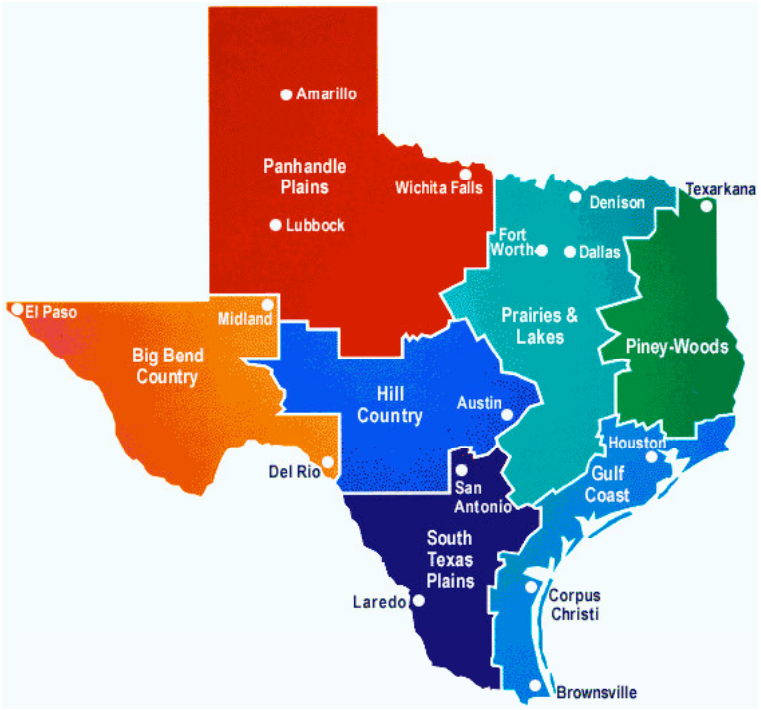

Fig. 3.1. Map of regions of Texas. (Obtained from texasoutside.com.)

A total of 43 blood samples were obtained from the Gulf Coast region of Texas, including, 2 greyhounds, 33 American Pit Bull Terriers, and 8 dogs of other breeds. Two American Pit Bull Terriers were positive for *B. gibsoni* by PCR. Case no. 3 was an 8 week old female American Pit Bull Terrier that originated in a breeding kennel near Houston, TX. The PCV was not determined due to the clotting of the blood sample and the dog did not exhibit clinical signs of babesiosis at the time of blood draw. Parasites were visualized on Giemsa-stained thin blood smear. Case no. 4 was a 9 year old female American Pit Bull Terrier mixed breed whose blood was submitted by a veterinary clinic near Houston, TX. The dog had a PCV of 41 and, at the time of blood

draw, demonstrated clinical signs consistent with those of babesiosis and showed parasites on Giemsa-stained thin blood smears.

Blood samples were obtained from dogs in the South Texas Plains region of Texas. A total of 83 samples included 4 from American Pit Bull Terriers and 79 from dogs of other breeds. None of these dogs tested positive for *B. gibsoni* by PCR.

3.1.2 Babesia canis

Of all submitted samples, only 1 dog tested positive for *B. canis*. Case no. 5 was a Mastiff mixed breed male whose blood was submitted from a veterinary clinic in Mission, TX (South Texas Plains). The PCV could not be determined due to clotting of the blood (autoagglutination). Upon time of blood draw, the dog exhibited clinical signs of babesiosis. Parasites were visualized on Giemsa-stained thin blood smears and the PCR results were positive for *B. canis*.

Table 3.1.
Summary of all dogs tested for *B. gibsoni* and *B. canis.*

Prairies & Lakes							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
1	Pit Bull	M	1.5 y	55	neg	neg	ND 1
2	Pit Bull	M	18 m	72	neg	neg	ND 2
5	Pit Bull	F	3 y	45	neg	neg	ND 3
Case #1	**Pit Bull**	**F**	**7 y**	**30**	**pos**	**pos**	**ND 4**
2 WM	Pit Bull	M	6 y	54	neg	neg	ND 5
3 WM	Pit Bull	M	3 y	39	neg	neg	ND 6
4 WM	Pit Bull	F	10 y	37	neg	neg	ND 7
5 WM	Pit Bull X	M	7 y	41	neg	neg	ND 8
1 M	Greyhound	F	3 y	56	neg	neg	ND 9
Case #2	**Pit Bull X**	**F**	**2 y**	**33**	**pos**	**pos**	**Pos 10**
1 K	Greyhound	M	2 y	55	neg	neg	ND 11
2 K	Greyhound	M	2 y	47	neg	neg	ND 12
3 K	Greyhound	M	3 y	47	neg	neg	ND 13
4 K	Greyhound	M	4 y	57	neg	neg	ND 14
5 K	Greyhound	M	6 y	62	neg	neg	ND 15
6 K	Greyhound	M	3 y	65	neg	neg	ND 16
7 K	Greyhound	M	4 y	46	neg	neg	ND 17
8 K	Greyhound	M	3 y	51	neg	neg	ND 18
9 K	Greyhound	M	3 y	74	neg	neg	ND 19
10 K	Greyhound	M	2 y	54	neg	neg	ND 20
11 K	Greyhound	M	3 y	55	neg	neg	ND 21
12 K	Greyhound	M	5 y	45	neg	neg	ND 22
13 K	Greyhound	M	3 y	65	neg	neg	ND 23
14 K	Greyhound	M	2 y	48	neg	neg	ND 24
15 K	Greyhound	M	2 y	49	neg	neg	ND 25
16 K	Greyhound	M	2 y	56	neg	neg	ND 26
17 K	Greyhound	M	2 y	45	neg	neg	ND 27
18 K	Greyhound	M	5 y	51	neg	neg	ND 28
19 K	Greyhound	M	6 y	49	neg	neg	ND 29
20 K	Greyhound	F	2 y	48	neg	neg	ND 30
21 K	Greyhound	F	2 y	48	neg	neg	ND 31
22 K	Greyhound	F	9 y	47	neg	neg	ND 32
23 K	Greyhound	F	6 y	41	neg	neg	ND 33
24 K	Greyhound	F	6 y	53	neg	neg	ND 34
25 K	Greyhound	F	4 y	50	neg	neg	ND 35
26 K	Greyhound	F	2 y	48	neg	neg	ND 36
27 K	Greyhound	F	6 y	37	neg	neg	ND 37
28 K	Greyhound	F	3 y	35	neg	neg	ND 38

Table 3.1. Continued.

Prairies & Lakes							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
29 K	Greyhound	F	8 y	49	neg	neg	ND 39
30 K	Greyhound	F	9 y	43	neg	neg	ND 40
31 K	Greyhound	F	9 y	41	neg	neg	ND 41
32 K	Greyhound	F	8 y	46	neg	neg	ND 42
33 K	Greyhound	F	4 y	50	neg	neg	ND 43
34 K	Greyhound	F	2 y	50	neg	neg	ND 44
35 K	Greyhound	F	4 y	51	neg	neg	ND 45
36 K	Greyhound	F	2 y	52	neg	neg	ND 46
37 K	Greyhound	F	3 y	60	neg	neg	ND 47
38 K	Greyhound	F	2 y	48	neg	neg	ND 48
39 K	Greyhound	F	7 y	51	neg	neg	ND 49
40 K	Greyhound	F	7 y	50	neg	neg	ND 50
A	Rottweiler X	M	2 y	42	neg	neg	ND 51
B	Golden Lab	F	1 y	41	neg	neg	ND 52
C	Border Collie	U	1 y	44	neg	neg	ND 53
D	Rottweiler X	M	2 y	54	neg	neg	ND 54
E	Dachshund	U	4 y	34	neg	neg	ND 55
F	U X	U	6 m	47	neg	neg	ND 56
G	Border Collie	F	1 y	41	neg	neg	ND 57
H	Rottweiler X	M	2 y	45	neg	neg	ND 58
I	Terrier X	U	5 y	45	neg	neg	ND 59
J	Yellow Lab	U	U	40	neg	neg	ND 60
K	Brown Lab X	U	U	40	neg	neg	ND 61
L	Shepherd X	U	2 y	51	neg	neg	ND 62
M	Lab X	U	2 y	43	neg	neg	ND 63
N	Lab x	U	2 y	45	neg	neg	ND 64
O	Chihuahua	U	4 y	46	neg	neg	ND 65
P	Poodle X	U	1 y	49	neg	neg	ND 66
Q	Corgi X	U	4 y	50	neg	neg	ND 67
R	Terrier X	U	U	47	neg	neg	ND 68
S	Pit Bull	M	2 y	37	neg	neg	ND 69
T	Terrier	U	2 y	38	neg	neg	ND 70
U	Border Collie	U	1 y	21	neg	neg	ND 71
V	Pit Bull	F	3 m	33	neg	neg	ND 72
W	Lab X	F	2 y	27	neg	neg	ND 73
X	Pit Bull	F	2 y	39	neg	neg	ND 74
Y	Pit Bull X	M	6 m	31	neg	neg	ND 75
Z	Greyhound	F	5 y	36	neg	neg	ND 76
27 BS	Collie X	F	1 y	40	neg	neg	ND 77
28 BS	Lab X	F	2 y	45	neg	neg	ND 78

Table 3.1. Continued.

Prairies & Lakes							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
29 BS	Golden Retriever	F	1.5 y	74	neg	neg	ND 79
30 BS	Golden Retriever	F	2 y	60	neg	neg	ND 80
31 BS	Pit Bull	M	3 m	35	neg	neg	ND 81
32 BS	Pit Bull X	M	5 y	55	neg	neg	ND 82
33 BS	Boxer	U	U	53	neg	neg	ND 83
34 BS	U X	U	U	37	neg	neg	ND 84
35 BS	Foxhound	F	4 y	36	neg	neg	ND 85
36 BS	Shepherd X	U	4 m	47	neg	neg	ND 86
37 BS	Lab X	U	10 m	55	neg	neg	ND 87
38 BS	Lab	U	1 y	54	neg	neg	ND 88
39 BS	Lab X	U	1 y	62	neg	neg	ND 89
40 BS	Dachshund	U	5 y	43	neg	neg	ND 90
41 BS	Rottweiler X	U	10 m	41	neg	neg	ND 91
42 BS	German Shepherd X	U	10 y	47	neg	neg	ND 92
43 BS	Shepherd X	U	4 y	59	neg	neg	ND 93
44 BS	Lab X	U	1.5 y	61	neg	neg	ND 94
45 BS	Rottweiler	U	2 y	50	neg	neg	ND 95
46 BS	Chihuahua	U	5 y	68	neg	neg	ND 96
47 BS	Springer Spaniel	U	2 y	61	neg	neg	ND 97
48 BS	Rottweiler	M	5 y	65	neg	neg	ND 98
49 BS	English Pointer	M	7 y	66	neg	neg	ND 99
50 BS	English Pointer	F	6 y	62	neg	neg	ND 100
51 BS	Dachshund X	U	3 y	70	neg	neg	ND 101
52 BS	Wolfhound	U	9 m	49	neg	neg	ND 102
53 BS	Terrier X	M	1.5 y	60	neg	neg	ND 103
54 BS	Lab X	U	3 y	59	neg	neg	ND 104
55 BS	U X	F	9 y	47	neg	neg	ND 105
56 BS	Husky X	M	4 y	35	neg	neg	ND 106
57 BS	Dachshund	U	2 y	50	neg	neg	ND 107
58 BS	Chihuahua	U	2 y	44	neg	neg	ND 108
59 BS	Lab	U	2.5 y	45	neg	neg	ND 109
60 BS	Pit Bull	U	1 y	33	neg	neg	ND 110
61 BS	U X	U	1 y	31	neg	neg	ND 111
62 BS	Rottweiler X	U	2 y	37	neg	neg	ND 112
63 BS	Catahoula	U	3 y	41	neg	neg	ND 113
64 BS	U X	U	1 y	29	neg	neg	ND 114

Table 3.1. Continued.

Prairies & Lakes							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
65 BS	Lab	U	3 y	25	neg	neg	ND 115
66 BS	Lab X	U	2 y	32	neg	neg	ND 116
67 BS	Blue Heeler	U	1 y	32	neg	neg	ND 117
68 BS	Dachshund X	M	1 y	35	neg	neg	ND 118
69 BS	Dachshund X	F	1 y	29	neg	neg	ND 119
70 BS	Great Dane	F	3.5 y	37	neg	neg	ND 120
71 BS	Pit Bull	F	2 y	23	neg	neg	ND 121
72 BS	Pit Bull	F	1.5 y	41	neg	neg	ND 122
74 BS	Pit Bull	F	6 m	41	neg	neg	ND 123
75 BS	Pit Bull	U	1 y	44	neg	neg	ND 124
76 BS	Pit Bull X	U	U	47	neg	neg	ND 125
1 J	Pit Bull	F	3 y	41	neg	neg	ND 126
2 J	Pit Bull	F	8 y	45	neg	neg	ND 127
Pinky	Pit Bull	F	6 y	30	neg	neg	ND 128
South Texas Plains							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
Case #5	**Mastiff X**	**F**	**U**	**U**	**pos**	**pos**	**ND 129**
1 M	French Bulldog	M	10 m	39	neg	neg	ND 130
2 M	Bull Terrier	M	3 y	40	neg	neg	ND 131
1 TC	Maltese X	M	1 y	50	neg	neg	ND 132
2 TC	Pit Bull	M	3 m	37	neg	neg	ND 133
3 TC	Lab	M	6 m	39	neg	neg	ND 134
4 TC	Pit Bull	M	6 y	25	neg	neg	ND 135
1 BP	German Shepherd	M	U	52	neg	neg	ND 136
2 BP	German Shepherd	M	U	45	neg	neg	ND 137
3 BP	German Shepherd	M	5 y	50	neg	neg	ND 138
4 BP	German Shepherd	M	4 y	59	neg	neg	ND 139
5 BP	German Shepherd	M	8 y	51	neg	neg	ND 140
6 BP	German Shepherd	F	3.5 y	47	neg	neg	ND 141
7 BP	German Shepherd	M	7 y	51	neg	neg	ND 142
8 BP	Belgian Mal	F	2.5 y	56	neg	neg	ND 143
9 BP	German Shepherd	M	3 y	55	neg	neg	ND 144

Table 3.1. Continued.

South Texas Plains							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
10 BP	German Shepherd	F	5 y	58	neg	neg	ND 145
11 BP	Belgian Malinois	M	5 y	40	neg	neg	ND 146
12 BP	Belgian Malinois	F	2.5 y	56	neg	neg	ND 147
13 BP	Groenedael	M	5 y	58	neg	neg	ND 148
14 BP	Belgian Malinois	F	5 y	71	neg	neg	ND 149
15 BP	Belgian Malinois	F	3 y	50	neg	neg	ND 150
16 BP	German Shepherd	F	5 y	52	neg	neg	ND 151
17 BP	German Shepherd	F	2 y	47	neg	neg	ND 152
18 BP	Belgian Malinois	M	5 y	55	neg	neg	ND 153
19 BP	German Shepherd	M	3 y	51	neg	neg	ND 154
20 BP	Belgian Malinois	F	4 y	51	neg	neg	ND 155
21 BP	Dutch Shepherd	M	4 y	33	neg	neg	ND 156
22 BP	German Shepherd	F	2 y	49	neg	neg	ND 157
1 ADL	Lab X	F	1.5 y	32	neg	neg	ND 158
2 ADL	Shepherd X	F/s	3 y	33	neg	neg	ND 159
3 ADL	Shepherd X	F	1.5 y	33	neg	neg	ND 160
4 ADL	Pit Bull X	M/n	1 y	45	neg	neg	ND 161
5 ADL	Lab X	F/s	5 m	48	neg	neg	ND 162
6 ADL	Mtn. Curr X	M	5 m	41	neg	neg	ND 163
7 ADL	Hound X	M/n	4 m	38	neg	neg	ND 164
8 ADL	Lab X	M/n	6 m	42	neg	neg	ND 165
9 ADL	Lab X	M/n	6 y	22	neg	neg	ND 166
10 ADL	U X	F/s	5 m	36	neg	neg	ND 167
11 ADL	Lab X	M/n	1 y	47	neg	neg	ND 168
12 ADL	Springer Spaniel	F/s	7 m	42	neg	neg	ND 169
13 ADL	German Shepherd	M/n	8 y	41	neg	neg	ND 170
14 ADL	Lab X	F/s	1 y	33	neg	neg	ND 171

Table 3.1. Continued.

South Texas Plains							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
15 ADL	Collie X	M/n	2 y	36	neg	neg	ND 172
16 ADL	Springer Spaniel X	M/n	8 y	43	neg	neg	ND 173
17 ADL	Terrier X	M/n	8 y	49	neg	neg	ND 174
18 ADL	Shepherd X	M/n	2 y	48	neg	neg	ND 175
19 ADL	Heeler X	M/n	1 y	40	neg	neg	ND 176
20 ADL	Catahoula X	M/n	5 y	43	neg	neg	ND 177
21 ADL	Terrier	M/n	1 y	51	neg	neg	ND 178
22 ADL	Heeler X	M/n	3 y	43	neg	neg	ND 179
23 ADL	Spitz X	F/s	1.5 y	44	neg	neg	ND 180
24 ADL	Lab X	M/n	2 y	46	neg	neg	ND 181
25 ADL	Shepherd X	M/n	4 y	52	neg	neg	ND 182
26 ADL	Lab X	F/s	3 y	31	neg	neg	ND 183
27 ADL	Collie X	M/n	1.5 y	41	neg	neg	ND 184
28 ADL	Shepherd X	F/s	1.5 y	47	neg	neg	ND 185
29 ADL	Shepherd X	F/s	7 y	38	neg	neg	ND 186
30 ADL	Shepherd X	F/s	3 y	46	neg	neg	ND 187
31 ADL	Basset Hnd X	F/s	2 y	49	neg	neg	ND 188
32 ADL	Lab X	F/s	7 y	51	neg	neg	ND 189
33 ADL	Hound X	F/s	3 y	45	neg	neg	ND 190
34 ADL	Collie X	M/n	2 y	49	neg	neg	ND 191
35 ADL	Terrier X	F/s	6 m	33	neg	neg	ND 192
36 ADL	Lab X	M/n	4 m	37	neg	neg	ND 193
37 ADL	Shepherd X	F/s	3 y	51	neg	neg	ND 194
38 ADL	Mtn. Cur X	M/n	5 m	33	neg	neg	ND 195
39 ADL	Shepherd X	M/n	6 m	36	neg	neg	ND 196
40 ADL	Rottweiler X	M/n	3.5 y	47	neg	neg	ND 197
41 ADL	Pit Bull	F/s	4 y	45	neg	neg	ND 198
42 ADL	Staffordshire Terrier X	M/n	1 y	47	neg	neg	ND 199
43 ADL	Heeler X	M/n	4.5m	39	neg	neg	ND 200
44 ADL	Lab X	F/s	4 y	46	neg	neg	ND 201
45 ADL	Lab X	M/n	10 y	38	neg	neg	ND 202
46 ADL	Lab X	M/n	7 y	45	neg	neg	ND 203
47 ADL	Boxer X	F/s	1.5 y	34	neg	neg	ND 204
48 ADL	Boxer X	M/n	1.5 y	41	neg	neg	ND 205
49 ADL	Border Collie	M/n	1.5 y	33	neg	neg	ND 206
50 ADL	Terrier X	F/s	1.5 y	38	neg	neg	ND 207
51 ADL	Lab X	M/n	2 y	46	neg	neg	ND 208
52 ADL	Lab X	F/s	2 y	39	neg	neg	ND 209
53 ADL	Bassenji X	F/s	1 y	34	neg	neg	ND 210

Table 3.1. Continued.

Gulf Coast							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
Case # 3	**Pit Bull X**	**F**	**9 y**	**41**	**pos**	**pos**	**Pos 211**
L 1	Shih Tzu	F	2 y	U	neg	neg	ND 212
L 2	Schnauzer	M	6 y	U	neg	neg	ND 214
1 HS	Pit Bull	M	8 wk	U	neg	neg	ND 215
2 HS	Pit Bull	M	8 wk	26	neg	neg	ND 216
3 HS	Pit Bull	M	8 wk	U	neg	neg	ND 217
4 HS	Pit Bull	M	8 wk	20	neg	neg	ND 218
5 HS	Pit Bull	M	8 wk	25	neg	neg	ND 219
6 HS	Pit Bull	M	8 wk	29	neg	neg	ND 220
7 HS	Pit Bull	M	12wk	23	neg	neg	ND 221
8 HS	Pit Bull	M	9 wk	21	neg	neg	ND 222
9 HS	Pit Bull	F	9 wk	24	neg	neg	ND 223
10 HS	Pit Bull	M	6 wk	18	neg	neg	ND 224
Case #4	**Pit Bull**	**F**	**8 wk**	**U**	**pos**	**pos**	**ND 225**
12 HS	Pit Bull	M	6 wk	U	neg	neg	ND 226
13 HS	Pit Bull	M	6 wk	U	neg	neg	ND 227
14 HS	Pit Bull	M	8 wk	U	neg	neg	ND 228
15 HS	Pit Bull	F	6 wk	U	neg	neg	ND 229
16 HS	Pit Bull	F	6wk	U	neg	neg	ND 230
17 HS	Pit Bull	F	4 m	32	neg	neg	ND 231
18 HS	Pit Bull	M	4 m	25	neg	neg	ND 232
19 HS	Pit Bull	F	4 m	26	neg	neg	ND 233
20 HS	Pit Bull	F	4 m	29	neg	neg	ND 234
21 HS	Pit Bull	M	4 M	27	neg	neg	ND 235
22 HS	Pit Bull	F	6 wk	26	neg	neg	ND 236
23 HS	Pit Bull	M	8 wk	U	neg	neg	ND 237
3	Lab	M	3 y	37	neg	neg	ND 238
4	Golden Retriever	F	17 wk	38	neg	neg	ND 239
6	Boxer	M	10 m	39	neg	neg	ND 240
7	Greyhound	U	3 y	41	neg	neg	ND 241
8	Lab	F/s	8 y	49	neg	neg	ND 242
9	Dachshund	M	1 y	54	neg	neg	ND 243
1 A	Greyhound	F	7 y	25	neg	neg	ND 244
8 J	Pit Bull	M	4 y	45	neg	neg	ND 245
9 J	Pit Bull	M	7 m	38	neg	neg	ND 246
10 J	Pit Bull	F	6 y	35	neg	neg	ND 247
11 J	Pit Bull	F	5 y	32	neg	neg	ND 248
3 J	Pit Bull	M	6 y	36	neg	neg	ND 249
4 J	Pit Bull	M	3 m	34	neg	neg	ND 250

Table 3.1. Continued.

Gulf Coast							
Dog I.D. #	**Breed**	**Sex**	**Age**	**PCV**	**Giemsa**	**PCR**	**Culture**
5 J	Pit Bull	M	6 m	39	neg	neg	ND 251
6 J	Pit Bull	M	3 y	46	neg	neg	ND 252
7 J	Pit Bull	F	4 y	36	neg	neg	ND 253
2 A	Lab	F	3 y	U	neg	neg	ND 254

U = Unavailable
ND = Not Done
All positive dogs were positive for *Babesia gibsoni*, except for Case #5, who tested positive for *Babesia canis vogeli.*

3.2 Packed Cell Volume (PCV)

A correlation between the PCV of the canine and the status of *B. gibsoni* infection was shown. Normal dogs had a higher average PCV of 43%, while dogs infected with *B. gibson*i had a lower average PCV of 35% (Fig. 3.2).

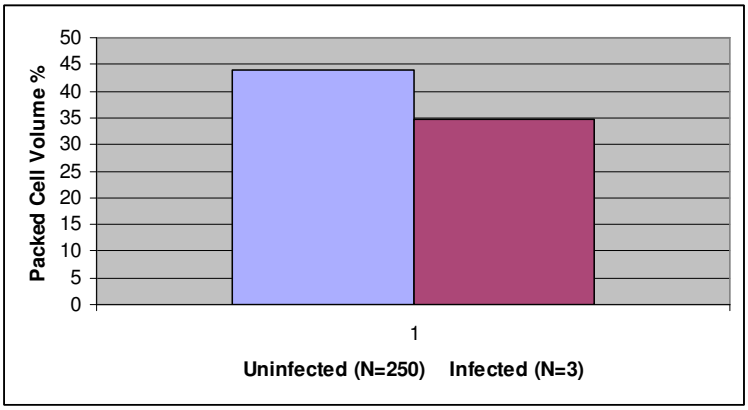

Fig. 3.2. Comparison of the mean packed cell volume of uninfected and infected dogs.

3.3 SSU rRNA

Babesia gibsoni isolates from all positive dogs shared the same SSU rRNA gene sequence, in the appropriate 1000 bp fragment (base positions 615-1670, based on GenBank accession no. DQ184507.1 for *B. gibsoni*). The obtained sequence matched the sequence for the *B. gibsoni* Oklahoma genotype (GenBank accession no. AF205636.1), which is synonomous with the Asian and Okinawa genotypes of *B. gibsoni* (100% identity) (Figure 3.3).

3.4 ITS

All *B. gibsoni* ITS sequences were consistent with those reported for the *B. gibsoni* Asian genotype (GenBank Accession number EF185065.1) (Fig. 3.4) and the *B. canis ITS* sequence was consistent with that reported for *B. canis vogeli* (GenBank Accession number EF180055.1) (Fig. 3.5). Some minor variation was evident among the aligned ITS clones that appeared to be due to real sequence differences, and some random variation, most likely due to PCR and/or sequencing errors, was also seen.

```
BgibATM     CGGCTACTTGCCTTGTCTGGTTTCGCTTTTGGGGTTTTCCCCTTTTTACTTTGAGAAAAT 60
BgibOK      CGGCTACTTGCCTTGTCTGGTTTCGCTTTTGGGGTTTTCCCCTTTTTACTTTGAGAAAAT 60

BgibATM     TAGAGTGTTTCAAGCAGACTTGTGTCTTGAATACTTCAGCATGGAATAATAAAGTAGGAC 120
BgibOK      TAGAGTGTTTCAAGCAGACTTGTGTCTTGAATACTTCAGCATGGAATAATAAAGTAGGAC 120

BgibATM     TTTGGTTCTATTTTGTTGGTTTGTGAACCTTAGTAATGGTTAATAGGAACGGTTGGGGGC 180
BgibOK      TTTGGTTCTATTTTGTTGGTTTGTGAACCTTAGTAATGGTTAATAGGAACGGTTGGGGGC 180

BgibATM     ATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAA 240
BgibOK      ATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAA 240

BgibATM     AGCATTTGCCAAGGACGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATC 300
BgibOK      AGCATTTGCCAAGGACGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATC 300

BgibATM     AGATACCGTCGTAGTCCTAACCATAAACCATGCCGACTAGGGATTGGAGGTCGTCATTTT 360
BgibOK      AGATACCGTCGTAGTCCTAACCATAAACCATGCCGACTAGGGATTGGAGGTCGTCATTTT 360

BgibATM     TCGACTCCTTCAGCACCTTGAGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTC 420
BgibOK      TCGACTCCTTCAGCACCTTGAGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTC 420

BgibATM     GCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCT 480
BgibOK      GCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCT 480

BgibATM     TAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAGTTAGGATTGACAGATTG 540
BgibOK      TAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAGTTAGGATTGACAGATTG 540

BgibATM     ATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGAT 600
BgibOK      ATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGAT 600

BgibATM     TTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAACTAGTTGCCGTTATTT 660
BgibOK      TTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAACTAGTTGCCGTTATTT 660

BgibATM     CAGTTTCGGCCAGCTTCTTAGAGGGACTTTGGGGCTCTAAGCCACAAGGAAGATTAAGGC 720
BgibOK      CAGTTTCGGCCAGCTTCTTAGAGGGACTTTGGGGCTCTAAGCCACAAGGAAGATTAAGGC 720

BgibATM     AATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCTGCACGCGCGCTACACTGATGCAT 780
BgibOK      AATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCTGCACGCGCGCTACACTGATGCAT 780

BgibATM     TCATCGAGTGTTATCCCTGGCCGAGAGGTCCGGGTAATCTTTAGTATGCATCGTGACGGG 840
BgibOK      TCATCGAGTGTTATCCCTGGCCGAGAGGTCCGGGTAATCTTTAGTATGCATCGTGACGGG 840

BgibATM     GATTGATTTTTGTAATTCTAAATCATGAACGAGGAATGCCTAGTATGCGCAAGTCATCAG 900
BgibOK      GATTGATTTTTGTAATTCTAAATCATGAACGAGGAATGCCTAGTATGCGCAAGTCATCAG 900

BgibATM     CTTGTGCAGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATCGAGT 960
BgibOK      CTTGTGCAGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATCGAGT 960

BgibATM     GATCCGGTGAATTATTCGGACCGTGGCTTTTCCGATTCGTCGGTTTTGCCTAGGGAAGTT 1020
BgibOK      GATCCGGTGAATTATTCGGACCGTGGCTTTTCCGATTCGTCGGTTTTGCCTAGGGAAGTT 1020

BgibATM     TTGTGAACCTTATCACTTAAAGGAAGGAGAAGTCG 1055
BgibOK      TTGTGAACCTTATCACTTAAAGGAAGGAGAAGTCG 1055
```

Fig. 3.3. Clustal alignment of *B. gibsoni* SSU rRNA sequence (615-1670) with *B. gibsoni* Oklahoma genotype SSU rRNA sequence (GenBank accession no. AF205636.1) showing 100% identity.

```
B.gibATM    ACATTGAAACTTGTCGAGCTGCGGCTCGAGGCTGCGGAATGCAGTGCGTCGCGGGCGATC 60
B.gib       ACATTGAAACTTGTCGAGCTGCGGCTCGAGGCTGCGGAATGCAGTGCGTCGCGGGCGATC 60

B.gibATM    GCGTGTTGCCTCGGCAGCAGTCGTGGAAGATTCCACGGGTCAGCGACGTCTGTGGCGCGC 120
B.gib       GCGTGTTGCCTCGGCAGCAGTCGTGGAAGATTCCACGGGTCAGCGACGTCTGTGGCGCGC 120

B.gibATM    GGCAAGGGACTGGGCGTGTCCAGTCTACCTGCGAGCAATCGCGGGCCTTGCCCCTCCCAC 180
B.gib       GGCAAGGGACTGGGCGTGTCCAGTCTACCTGCGAGCAATCGCGGGCCTTGCCCCTCCCAC 180

B.gibATM    CCCGCCGGCGCGTTGCGCGACTTGGTCGTTGCTCTGCGTCCGGGTTACGCCCCCAGTGGC 240
B.gib       CCCGCCGGCGCGTTGCGCGACTTGGTCGTTGCTCTGCGTCCGGGTTACGCCCCCAGTGGC 240

B.gibATM    TGGGTGCGGGATCT 254
B.gib       TGGGTGCGGGATCT 254
```

Fig. 3.4. Clustal alignment of *B. gibsoni* ITS partial sequence obtained in this study (B.gibATM) with *B. gibsoni* ITS sequence of *B. gibsoni* Oklahoma genotype (B.gib; GenBank accession no. EF 18065.1) showing 100% identity.

```
BcanATM   ACATTGATGGTACCTAGCACTTGTGCTTTCGCGGGACGCGCTGTCGAGGTTGTCCTGGGC 60
Bcvo      ACATTGATGGTACCTAGCACTTGTGCTTTCGCGGGACGCGCTGTCGAGGTTGTCCTSGGC 60
          ********************************************************* ***

BcanATM   GGCGGTCTCGTCTGGCAACGGAGCCGGCGACTCGCCGGTTCTACCCTGTTGGGCGTTGCC 120
BcvZ      GGCGGTCTCGTCTGGCAACGGGGCCGGCGASTCGCCGGTTCTACCCTKTTGGGCGTTGCC 120
          ********************* ******** **************** ************

BcanATM   CCCTCTCCACCCCACTGGGGCGTTGCGTAAGCTCTGCCCCGGGTTAGGCCCCGAGCGTGG 180
BcvZ      CCCTCTCCACCCCACTGGGGCGTTGCGTAAGCTCTGCCCCGGGTTAGGCCCCGAGCGTGG 180
          ************************************************************

BcanATM   ATTCCGTCGGTTTCCGGGCTTGGGGATGTGGCGCGCCTGGGCGCGTCAGGGTCTTTGACC 240
BcvZ      ATTCCGTTGGTTTCCGGGCTTGGGGATGTGGCGCGCCTGGGCGCGTCAGGGTCTYTGACC 240
          ******* ****************************************** *****

BcanATM   TCTGCGGCTTGGCCGATTTGCAACTCCGCTTGACTGTGTCGAGTATTGAAATTCTAAACT 300
BcvZ      TCTGCGGCTTGGCCGATTTGCAACTCCGCTTGACTGTGTCGAGTATTGAAATTCTAAACT 300
          ************************************************************

BcanATM   TTCAGCGATGGATGTCTTGGCTCACACAACGATGAAGGACGCAGCGAATTGCGATACGCA 360
BcvZ      TTCAGCGATGGATGTCTTGGCTCACACAACGATGAAGGACGCAGCGAATTGCGATACGCA 360
          ************************************************************

BcanATM   TTATGACTTGCAGACTTCTGCGATTTAACAGACCTCCGAACGTAACCAACACACCGCCTC 420
BcvZ      TTATGACTTGCAGACTTCTGCGATTTAACAGACCTCCGAACGTAACCAACACACCGCCTC 420
          ************************************************************

BcanATM   TGCTCGCACGCGGTACTCCCGTTTCAGTGAGCCCCCTTTTCTGAGGCTTTGCCTTTTTGG 480
BcvZ      TGSTCGCACGCGGTACTCCCGTTTCAGTGAGCCCCCTTTTCTGAGGCTTKGC-TTTTTGG 479
          ** ******************************************** ** *******

BcanATM   CAGCCTTGCGAGTGGGTGGTTGTGCCGCCCGATTGCGTGGAGGAGTTGGCTGGCCAAGGT 540
BcvZ      CAGCCTTGCGAGTGGGTGGTTGTGCCGCCCGATTGCGTGGAGGAGTTGGCTGGCCAAGGT 539
          ************************************************************


BcanATM   CGTGGCTGTGGTTCTACTCTGTGGAGCTGCGGCCATTTGCCTCGAAGCATTTTGTGTATG 600
BcvZ      CGTGGYKGTRGTTCYACTCTGTGGAGSTGCGGCCATTTGCCTCGAAGCATTTTGTGTATG 599
          *****  ** **** *********** ********************************

BcanATM   TGATAC-GAATCTTCTGCGAGGCTGTTGGGCTCTGCCCAAGACTGCGGTGAGCCGTCTGA 659
BcvZ      TGATACCGAATCTTCTGCGAGGCTGTTGGGCTCTGCCCAAGACTGCGGTGAGCCGTCTGA 659
          ****** ****************************************************

BcanATM   CTCCGTGTCATCTAT 674
BcvZ      CTCCGTGTCWTCTAT 674
          ********* *****
```

Fig. 3.5. Clustal alignment of *B. canis* ITS sequence obtained in this study (BcanATM) with *B. canis vogeli* ITS sequence (BcvZ; GenBank accession no. EF180055.1).

3.5 Culture

In total, cultures were initiated for of 4 *B. gibsoni* infected blood samples, 1 *B. gibsoni* cryostock, 5 *B. canis* cryostocks, and 2 dog blood samples infected with an unnamed large *Babesia* sp. (Birkenjeuer et al., 2004). A number of the *B. gibsoni* and *B. canis* cultures showed parasites for up to 6 weeks; however, none became established. In all cases, rapidly decreasing numbers of parasites were seen after 2-3 wk in culture. A *Babesia* sp.-infected blood sample from North Carolina resulted in a successful established culture (Fig. 3.6). Culture was attempted in R40 and R40TH media, however, the cells cultured in the R40TH media did not thrive, while those in the R40 media did. The initial culture was set up with 80 l infected dog blood and 720 l media. Parasites were observed on Day 23, first passage was made on Day 28 (1:2 split), second passage on Day 31 (1:4 split). Passage 3 cultured parasites were cryopreserved on Day 30 of culture.

Fig. 3.6. *Babesia* sp. piroplasms at sequential stages of division cultured in R40 medium (1000X).

4. DISCUSSION

This study utilized PCR to analyze blood samples from various dog breeds, with American Pit Bull Terriers and greyhounds of particular interest, throughout different regions of Texas for the presence of *B. canis* and *B. gibsoni*. American Pit Bull Terriers were of particular interest because they are known to have a higher prevalence of infection with *B. gibsoni* than other breeds (Birkenheuer et al., 2003). A possible explanation for this high prevalence is that these dogs are often used as fighting dogs and fighting is a possible means of transmission (Birkenheuer et al., 2005). Also, vertical transmission has been reported, so in kennel situations, it is possible that the parasite is able to persist throughout the generations of dogs originating from a kennel where the parasite is endemic (Birkenheuer et al., 2005; Fukumoto et al., 2005). Dogs in kennels are also more likely to be exposed to the tick vector of *B. gibsoni* than dogs living in other conditions (Birkenheuer et al., 1999). In addition, greyhounds have long been known to have a higher prevalence of infection with *B. canis*. In 1992, Taboada et al., found that, in Florida, 46% of greyhounds tested for *B. canis* were positive for the parasite.

PCR was ideal for this study because unlike serosurveys, which indicate an exposure to the parasite, PCR detects DNA from the parasite, indicating the actual infection status of the host. A previous study of several American Pit Bull Terrier kennels along with sample submissions from veterinary clinics, showed that out of 152 East Texas dogs of various breeds, 24 American Pit Bull Terriers were positive for

B. gibsoni, while all other breeds were negative for the parasite (Table 4.1) (P.J. Holman, unpublished results).

The results of this study support those of previous studies indicating that *B. gibsoni* is most commonly found in American Pit Bull Terriers, since all dogs testing positive in this study were of this breed (Birkenheuer et al., 1999; Macintire et al., 2002; Birkenheuer et al., 2005). No dogs of any other breed were positive for *B. gibsoni* in this study. Additionally, the mode of transmission of *B. gibsoni* is suspect. There is no history of any tick infestation at any of the long-term shelters or kennels studied. This suggests that means of transmission other than via the tick vector, including vertical transmission, dog fighting, or the use of contaminated needles or other equipment, may occur more commonly than is currently thought (Stegeman et al., 2003; Birkenheuer et al., 2005; Fukumoto et al., 2005).

It has been reported that subclinical infections with *B. gibsoni* are not uncommon. Macintire et al. (2002) reported that out of 18 American Pit Bull Terriers testing positive for *B. gibsoni,* only one dog demonstrated clinical signs consistent with babesiosis. Additionally, the combined data of the current studies indicate that *B. gibsoni* subclinical infections are not uncommon in Texas dogs. The majority of dogs studied were clinically normal. From the unpublished results (P.J. Holman), only five dogs showed clinical signs out of 22 positive dogs. Out of the four dogs positive for *B. gibsoni* in the latter study, two dogs demonstrated clinical signs of babesiosis at the time of blood draw. The number of subclinical infections between these two studies is Table 4.1. *Babesia* spp. test results from East Texas dogs (unpublished results).

Table 4.1.
Babesia spp. test results from East Texas dogs (unpublished results).

Breed	N	Giemsa Stain			*B. gibsoni* PCR		Culture	
		# Pos		# Neg	# Pos	# Neg	# Pos	# Neg
		B.gibsoni	*B.canis*					
American Bulldog	6	0	0	6	0	6	ND*	
American bulldog mix	1	0	0	1	0	1	ND	
American Staffordshire	1	0	0	1	0	1	ND	
Australian cattle dog	1	0	0	1	0	1	ND	
Aust cattle dog mix	2	0	0	2	0	2	ND	
Basset hound	1	0	0	1	0	1	ND	
Basset hound mix	1	0	0	1	0	1	ND	
Beagle mix	1	0	0	1	0	1	ND	
Bischon Fr	1	0	0	1	0	1	ND	
Blue heeler	1	0	0	1	0	1	ND	
Border collie	1	0	0	1	0	1	ND	
Border collie mix	3	0	0	3	0	3	ND	
Boxer	2	0	0	2	0	2	ND	
Chihuahua	2	0	0	2	0	2	ND	
Chihuahua mix	1	0	0	1	0	1	ND	
Chow mix	1	0	0	1	0	1	ND	
Cocker mix	1	0	0	1	0	1	ND	
Collie mix	1	0	0	1	0	1	ND	
Cur, Mountain	2	0	0	2	0	2	ND	
Cur, Mixed breed	4	0	0	4	0	4	ND	
Dachshund	4	0	0	4	0	4	ND	
Dachshund mix	1	0	0	1	0	1	ND	
Dalmatian	1	0	0	1	0	1	ND	
Golden Retriever	1	0	0	1	0	1	ND	
German Shepherd	4	0	0	4	0	4	ND	
Great Dane	2	0	0	2	0	2	ND	
Great Pyrenees	1	0	0	1	0	1	ND	
Great Pyrenees mix	1	0	0	1	0	1	ND	
Hound, English	2	0	0	2	0	2	ND	
Hound, Plott	2	0	0	2	0	2	ND	
Hound, Mixed breed	4	0	0	4	0	4	ND	
Husky	1	0	0	1	0	1	ND	
Husky mix	2	0	0	2	0	2	ND	
Jack Russell Terrier mix	1	0	0	1	0	1	ND	
Labrador Retriever mix	2	0	0	2	0	2	ND	
Mastiff	2	0	0	2	0	2	ND	
Mastiff, mix	1	0	0	1	0	1	ND	
Miniature Pinscher	1	0	0	1	0	1	ND	
Miniature Pinscher mix	1	0	0	1	0	1	ND	

Table 4.1. Continued.

Breed	**N**	**Giemsa Stain**			***B. gibsoni* PCR**		**Culture**	
		# Pos		# Neg				
		B.gibsoni	*B.canis*		# Pos	# Neg	# Pos	# Neg
Miniature schnauzer	2	0	0	2	0	2	ND	
Pekingese	1	0	0	1	0	1	ND	
Pekingese mix	1	0	0	1	0	1	ND	
Pit Bull Terrier	**67**	**16**	**0**	**56**	**22**	**45**	**19**	**3**
Pit Bull Terrier mix	2	0	0	2	0	2	ND	
Pointer	1	0	0	1	0	1	ND	
Poodle	3	0	0	3	0	3	ND	
Rat Terrier	3	0	0	3	0	3	ND	
Rottweiler	2	0	0	2	0	2	ND	
St Bernard	2	0	0	2	0	2	ND	
Schnauzer	5	0	0	5	0	5	ND	
Shepherd mix	5	0	0	5	0	5	ND	
Sheltand Sheepdog	1	0	0	1	0	1	ND	
Terrier mix	6	0	0	6	0	6	ND	
West Highland Whitel	1	0	0	1	0	1	ND	
Whippet	1	0	0	1	0	1	ND	
Unknown mix	7	0	0	7	0	7	ND	
Vizsla	1	0	0	1	0	1	ND	
Yorkshire terrier	1	0	0	1	0	1	ND	
Total	195	16	0	179	22	173	22 cultured	

of particular concern because the carrier animal, while not suffering from signs of the disease, may serve as reservoir of infection for other dogs.

All dogs postive for *B. gibsoni* in this study and the previous unpublished study were found in East Texas. However, due to a much larger number of dogs sampled from this area and the fact that kennel populations were included, no conclusion may be drawn in regards to in which part of Texas the parasite is most common.

In this study, there was a correlation between PCV and infection with *B. gibsoni*. In 2002, Macintire and others also confirmed this observation. In their study, the average PCV of *B. gibsoni* infected dogs was 31%, while the average PCV of uninfected dogs was 52%. The average PCV for *B. gibsoni* infected dogs from this study (infected-35%, uninfected-45%) and the previous study (infected-33%, uninfected-45%) was also lower than the average PCV for uninfected dogs. However, these correlations may be spurious because PCVs are variable among dogs. Carrier animals infected with *B. gibsoni* may have normal PCVs and uninfected animals may have low PCVs.

The SSU rRNA gene was ideal for molecular characterization of *Babesia* spp. due to its tight structural and functional constraints. It also has low subsitiution rates and distinct conserved and variable regions. In addition, there is a huge database of sequences for the SSU rRNA gene. This gene is most useful for distinguishing genus and species differences; however, it may not always be possible to discriminate between closely related species or subspecies (Prichard and Tait, 2001).

Currently, three genetically distinct small piroplasms are known to infect dogs in the United States. They include *B. gibsoni* (Asian genotype), *T. annae*, and *B. conradae* (Kjemtrup et al., 2000, 2006). Results of the SSU rRNA gene molecular characterization for all positive samples in the study showed that all *B. gibsoni* positive samples had a consensus SSU rRNA sequence with 100% identity to the commonly reported *B. gibsoni* Oklahoma genotype (synonymous with the Asian and Okinawa genotypes). The *B. canis* positive sample had an SSU rRNA sequence matching the commonly reported *B. canis vogeli,* which is the most commonly identified genotype of

the canine large piroplasms in the United States. An alignment of the individual cloned SSU rRNA sequences obtained in this study showed that some variations existed among them. However, these variations were random and inconsistent, so it is probable that they are due to PCR and/or sequencing errors.

The rRNA ITS region is more subject to mutation than the rRNA genes. The primary transcript is located between precursor ribosomal subunit genes and it is removed by splicing when the structural RNA precursor molecule is processed into a ribosome; therefore, it has a higher degree of variation than the SSU rRNA gene and is more useful for distinguishing organisms at either species or subspecies levels (Prichard and Tait, 2001).

All positive samples had ITS sequences matching those commonly reported for either *B. gibsoni* (Oklahoma genotype) or *B. canis vogeli*, depending on if they were positive for *B. gibsoni* or *B. canis.* Some minor variation was evident among the aligned ITS clones that appeared to be due to real sequence differences, and some random variation, most likely due to PCR and/or sequencing errors, was also seen.

Currently, the production of parasites used for research and diagnostic tests involves experimental infection of dogs. Establishing continuous cultures of these parasites will help eliminate this procedure and provide an unlimited source of parasites to be used as a format for testing efficacy of new anti-babesial pharmaceuticals, for the generation of improved diagnostic tests, as well as for vaccine development.

Samples testing positive for *B. gibsoni* or *B. canis* were used to initiate in vitro cultures of the parasites. In addition, infected blood was provided by Dr. Birkenheuer

from Northa Carolina State to use in culture. The culture methodology followed previously published protocols based on the microaerophilous stationary phase (MASP) system (Levy and Ristic, 1980; Vega et al., 1985; Holman et al., 1994). Despite numerous attempts to culture the canine *Babesia* spp., all were eventually terminated due to lack of parasite growth after 4-6 weeks, except for one. An unnamed large species of *Babesia* (Birkenheuer et al., 2004b) from a dog in North Carolina was established in culture and successfully subcultured several times. The culture of this large unnamed *Babesia* sp. is still being maintained and subcultures have been cryopreserved for later use.

5. CONCLUSION

Babesia gibsoni subclinical infections are not uncommon in Texas American Pit Bull Terriers and, due to the lack of known tick infestation in any of the kennels or shelters submitting samples, the mode of transmission is suspect. In addition, the molecular characterization of all positive samples yielded results consistent with commonly reported species and/or subspecies of both *B. gibsoni* and *B. canis*. No new or uncommon species or subspecies of *Babesia* were identified in any dogs studied. Attempts to establish continuous cultures of the parasites were largely unsuccessful. However, one new large unnamed *Babesia* sp. found in a North Carolina dog was successfully established in culture, which is promising for the establishment of other *Babesia* spp. parasites in the future. Future aims include screening more American Pit Bull Terrier kennels in other regions of Texas, raising awareness of the parasites through owner education, and further attempts to establish in vitro cultures of both *B. gibsoni* and *B. canis*.

REFERENCES

Adl, S.M., Simpson, A.G.B., Farmer, M.A., Andersen, R.A., Anderson, O.R., Barta, J.R., Bowser, S.S., Brugerolle, G., Fensome, R.A., Fredericq, S., James, T.Y., Karpov, S., Kugrens, P., Krug, J., Lane, C.E., Lewis, L.A., Lodge, J., Lynn, D.H., Mann, D.G., Mccourt, R.M., Mendoza, L., Moestrup, O., Mozley-Standridge, S.E., Nerad, T.A., Shearer, C.A., Smirnov, A.V., Spiegel, F.W., Taylor, M.F., 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J. Eukaryotic Microbiol. 52, 399-451.

Adeyanju, B.J., Aliu, Y.O., 1982. Chemotherapy of canine ehrlichiosis and babesiosis with imidocarb dipropionate. J. Am. Anim. Hosp. Assoc. 18, 827-830.

Anderson, J.F., Magnarelli, L.A., Sulzer, A.J., 1980. Canine babesiosis: Indirect fluorescent antibody test for a North American isolate of *Babesia gibsoni*. Am. J. Vet. Res. 41, 2102-2105.

Ano, H., Makimura, S.O., Harasawa, R., 2001. Detection of *Babesia* species from infected dog blood by polymerase chain reaction. J. Vet. Med. Sci. 63, 111-113.

Birkenheuer, A.J., Levy, M.G., Savary, K.C.M., Gager, R.B., Breitschwerdt, E.B., 1999. *Babesia gibsoni* infection in dogs from North Carolina. J. Am. Anim. Hosp. Assoc. 35, 125-128.

Birkenheuer, A.J., Levy, M.G., Stebbins, M., 2002. Development of a hemi-nested PCR test for canine babesiosis and its use in a serologic and molecular survey of pit bull terrier kennels and animal shelters. In: Proceedings. 20th Ann. Meet. Am. Coll. Vet. Intern. Med. Forum. 779.

Birkenheuer, A.J., Levy, M.G., Stebbins, M., Poore, M., Breitschwerdt, E., 2003. Serosurvey of anti*Babesia* antibodies in stray dogs and American Pit Bull Terriers and American Staffordshire Terriers from North Carolina. J. Am. Anim. Hosp. Assoc. 39, 551-557.

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2004a. Efficacy of combined atovaquone and azithromycin for therapy of chronic *Babesia gibsoni* (Asian genotype) infections in dogs. J. Vet. Intern. Med. 18, 494-498.

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004b. Detection and Molecular characterization of a novel large *Babesia* species in a dog. Vet. Parasitol. 124, 151-160.

Birkenheuer, A.J., Correa, M.T., Levy, M.G., Breitschwerdt, E.B., 2005. Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2000-2003). J. Am. Vet. Med. Assoc. 227, 942-947.

Bose, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T., de Vos, A.J., 1995. Current state and future trends in the diagnosis of babesiosis. Vet. Parasitol. 57, 61-74.

Botros, B.A., Moch, R.W., Barsoum, I.S., 1975. Some observations on experimentally induced infection of dogs with *Babesia gibsoni*. Am. J. Vet. Res. 36, 293-296.

Breitschwerdt, E.B., 1990. Babesiosis. In: Green (Ed.) Clinical Microbiology and Infectious Diseases of the Dog and Cat. WB Saunders Co, Philadelphia, pp.796-805.

Carret, C., Wakas, F., Carcy, B., Grande, N., Precigout, E., Moubri, K., Schetters, T.P., Gorenflot, A., 1999. *Babesia canis canis*, *Babesia canis vogeli*, *Babesia canis rossi*: Differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. J. Euk. Microbiol. 46, 298-303.

Conrad, P.A., Thomford, J., Yamane, I., Whitig, J., Bosma, L., Uno, T., Holshuh, H.J., Shelly, S., 1991. Hemolytic anemia caused by *Babesia gibsoni* infection in dogs. J. Am. Vet. Med. Assoc. 199, 601-605.

Criado-Fonelio, M.A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carreto, J.C., 2003. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. Vet. Parasitol. 113, 189-201.

EMBL-EBI, 2007. ClustalW sequence analysis tool. Accessed on June 15, 2007. Available at: http://www.ch.embnet.org/software/LALIGN_form.html.

Erp, E.E., Smith, R.D., Ristic, M., Osorno, B.M., 1980. Continuous in vitro cultivation of *Babesia bovis*. Am. J. Vet. Res. 41, 1141-1142.

Farwell, G.E., LeGrand, E.K., Cobb, C.C., 1982. Clinical observations on *Babesia gibsoni* and *Babesia canis* infections in dogs. J. Am. Vet. Med. Assoc. 180, 507-511.

Fowler, J.L., Ruff, M.D., Hornof, W.J., 1970. Modification of Field's stain for examination of growth forms of *Babesia gibsoni*. Am. J. Vet. Res. 31, 1079-1083.

Freeman, M.J., Kirby, B.M., Panciera, D.L., Henik, R.A., 1994. Hypotensive shock syndrome associated with acute *Babesia canis* infection in a dog. J. Am. Vet. Med. Assoc. 204, 94-96.

Fukumoto, S., Xuan, X., Igarashi, I., Zhang, S., Mugisha, J., Ogata, T., Nagasaw, H., Fuijsaki, K., Suzuki, N., Mikami, T., 2000. Morphological changes of *Babesia gibsoni* grown in canine red blood cells-substituted severe combined immune deficiency mice. J. Parasitol. 86, 956-959.

Fukumoto, S., Hiroshi, S., Igarashi, I., Xuan, X., 2005. Fatal experimental transplacental *Babesia gibsoni* infections in dogs. Int. J. Parasitol. 35, 1031-1035.

Groves M.G., Dennis G.L., 1972. *Babesia gibsoni*: Field and laboratory studies of canine infections. Exp. Parasitol. 31, 153-159.

Guyer, R.L., Koshland, D.E. Jr., 1989. The molecule of the year. Science. 246, 1543-1546.

Hauschild, S., Shayan, P., Schein, E., 1995. Characterization and comparison of merozoite antigens of different *Babesia canis* isolates by serological and immunological investigations. Parasitol. Res. 81, 638-642.

Herwaldt, B.L., Springes, F.E., Roberts, P.P., Eberhard, M.L., Case, K., Persing, D.H., Agger, W.A., 1995. Babesiosis in Wisconsin: A potentially fatal disease. Am. J. Trop. Med. Hyg. 53, 146-151.

Hill, M.W.M., Bolton, B.L., 1966. Canine babesiosis in Queensland. Aust. Vet. J. 42, 391-392.

Holman, P.J., Chieves, L., Olson, D., Wagner, G.G., 1994. *Babesia equi* erythrocytic stage continuously cultured in an enriched medium. J. Parasitol. 80, 232-236.

Hughes, W.T., Oz, H.S., 1995. Successful prevention and treatment of babesiosis with atovaquone. J. Infect.Dis. 172, 1042-1046.

Ikadai, H., Tanaka, H., Shibahara, N., Matsuu, A., Uechi, M., Itoh, N., Oshiro, S., Kudo, N., Igarashi, I., Oyamada, T., 2004. Molecular evidence of infections with *Babesia gibsoni* parasites in Japan and evaluation of the diagnostic potential of a loop-mediated isothermal amplification method. J. Clin. Microbiol. 42, 2465-2469.

Inokuma, H., Yoshizaki, Y., Shimada, Y., Sakata, Y., Okuda, M., Onishi, T., 2003. Epidemiological survey of *Babesia* species in Japan performed with specimens from ticks collected from dogs and detection of new *Babesia* DNA closely related to *Babesia odocoilei* and *Babesia divergens* DNA. J. Clin. Microbiol. 41, 3494-3498.

Irizarry-Rovira, A.R., Stephens, J., Christian, J., Kjemtrup, A., DeNicola, D.B., Widmer, W.R., Conrad, P.A., 2001. *Babesia gibsoni* infection in a dog from Indiana. Vet. Clin. Path. 30, 180-188.

Jefferies, R., Ryan, U.M., Muhlnickel, C.J., Irwin, P.J., 2003. Two species of canine *Babesia* in Australia: Detection and characterization by PCR. J. Parasitol. 89, 409-412.

Kakoma, I., Mehlhorn, H., 1994. *Babesia* of domestic animals. In: Kreier, J.P., Baker J.F. (Eds.), Parasitic Protozoa 2nd Ed. Academic Press, San Diego, pp.141-216.

Kjemtrup, A.M., Conrad, P.A., 2000. Human babesiosis: An emerging tick-borne disease. Int. J. Parasitol. 30, 1323-1337.

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cumings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000. There are at least three genetically distinct small piroplasms from dogs. Int. J. Parasitol. 30, 1501-1505.

Kjemptrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006. *Babesia conradae*, sp. nov., a small canine *Babesia* identified in California. Vet. Parasitol. 138, 103-111.

Kocan, A.A., Kjemtrup, A., Meinkoth, J., Whitworth, L.C., Murphy, G.L., Decker, L., Lorenz, M., 2001. A genotypically unique *Babesia gibsoni*-like parasite recovered from a dog in Oklahoma. J. Parasitol. 87, 437-438.

Kuttler, K.L., 1988. World-wide impact of babesiosis. In: Ristic M. (Ed.), Babesiosis of Domestic Animals and Man. CRC Press, Boca Raton, FL, pp.1-22.

Levine, N.D., 1971. Taxonomy of piroplasms. Trans. Am. Microsc. Soc. 90, 2-33.

Levine, N.D., 1973. Protozoan Parasites of Domestic Animals and Man. Burgess Publishing Company, Minneapolis, MN, 406 pp.

Levine, N.D., 1988. Blood parasites: the piroplasms. In: The Protozoan Phylum Apicomplexa. Vol. II. CRC Press, Boca Raton, FL, p. 35.

Levy, M.G., Ristic, M., 1980. *Babesia bovis*: continuous cultivation in a microaerophilous stationary phase culture. Sci. 207, 1218-1220.

Levy, M.G., Breitschwerdt, E.B., Monocol, D.J., 1987. Antibody activity to *Babesia canis* in dogs in North Carolina. Am. J. Vet. Res. 48, 339-341.

Lobetti, R.G., 1998. Canine Babesiosis. Compendium on Continuing Education for the Practicing Veterinarian. 20, 418-431.

Loretti, A.P., Barros, S.S., 2005. Hemorrhagic disease in dogs infected with an unclassified intraendothelial prioplasm in southern Brazil. 134, 193-213.

Macintire, D.K., Boudreaux, M.K., West, G.D., Bourne, E.C., Wright, J.C., Conrad, P.A., 2002. *Babesia gibsoni* infection among dogs in the southeastern United States. J. Am. Vet. Med. Assoc. 220, 325-329.

Martinod, S., Laurent, N., Moreau, Y., 1986. Resistance and immunity of dogs against *Babesia canis* in an endemic area. Vet. Parasitol. 19, 245-254.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P.J., Nijof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa, Vet. Parasitol. 122, 119-125.

Matsuu, A., Koshida, Y., Kawahara, M., Inoue, K., Ikadai, H., Hikasa, Y., Okano, S., Higuchi, S., 2004. Efficacy of atovaquone against *Babesia gibsoni* in vivo and in vitro. Vet. Parasitol. 124, 9-18.

Matsuu, A., Miyamoto, K., Ikadai, H., Okano, S., Higuchi, S., 2006. Short report: Cloning of the *Babesia gibsoni cytochrome b* gene and isolation of three single nucleotide polymporphisms from parasites present after atovaquone treatment. Am. J. Trop. Med. Hyg., 74 (4), 593-597.

McFadden, D.C., Tomavo, S., Berry, A., Boothroyd, J.C., 2000. Characterization of *cytochrome b* from *Toloplama gondii* and Q (o) domain mutation as a mechanism of atovaquone resistance. Mol. Biochem. Parasitol. 108, 1-12.

Mehlhorn, H., Schein, E., 1984. The piroplasms: life cycle and sexual stages. Adv. Parasitol. 23, 37-103.

Mehlhorn, H., Schein, E., 1998. Redescription of *Babesia equi* (Laveran, 1901) as *Theileria equi* (Mehlhorn, Schein 1998). Parasitol. Res. 84, 467-475.

Meinkoth, J.H., Docan, A.A., Loud, S.D., Lorenz, M.D., 2002. Clinical and hematologic effects of experimental infection of dogs with recently identified *Babesia gibsoni*-like isolates from Oklahoma. J. Am. Vet. Med. Assoc. 220, 185-186.

Murase, T., Maede, Y., 1990. Increased erythrophagocytic activity of macrophages in dogs with *Babeisa gibsoni* infection. Jpn. J. Vet. Sci. 52, 321-327.

Murase T., Hashimoto T., Ueda T., Maede Y., 1991. Multiplication of *Babesia gibsoni* in in vitro culture and its relation to hemolysis of infected erythrocytes. J. Vet. Med. Sci. 53, 759-760.

Onishi, T., Ueda, K., Horie, M., Kajikawa, T., Ohishi, I., 1990. Serum hemolytic activity in dogs infected with *Babesia gibsoni*. J. Parasitol. 76, 564-567.

Onishi, T., Morita, M., Ando, T., 1993. In vitro cultivation and infectivity of *Babesia gibsoni*. Jpn. J. Parasitol. 42, 340-344.

Persing, D.H., Mathiesen, D., Marshall, W. F., 1992. Detection of *Babesia microti* by polymerase chain reaction. J. Clin. Microbiol. 30, 2097-3103.

Persing, D.H., Conrad, P.A., 1995. Babesiosis: new insights from phylogenetic analysis. Infect. Agents Dis. 4, 182-195.

Prescott,L.M., Harley, J.P., Klein, D.A. (Eds.), 2005. Microbiology 6th ed., McGraw-Hill, New York, pp. 316-319.

Prichard, R., Tait, A., 2001. The role of molecular biology in veterinary parasitology. Vet. Parasitol. 98, 169-194.

Purnell, R.E., 1981. Babesiosis in various hosts. In: Ristic M., Kreier J.P. (Eds.), Babesiosis, Academic Press, New York, pp.25-63.

Quick, R.E., Herwaldt, B.L., Thomford, J.W., Garnett, M.E., Eberhard, M.L., Wilson, M., Steingart, K.R., 1993. Babesiosis in Washington State: a new species of *Babesia*? Ann. Intern. Med. 119, 284-290.

Ristic, M., Lewis, G.E., 1977. *Babesia* in man and wild and laboratory-adapted mammals. In: J.P. Kreier (Ed.), Parasitic Protozoa Vol. IV, Academic Press, New York, pp. 53-76.

Roher, D.P., Anderson, J.F., Nielsen, S.W., 1985. Experimental babesiosis in coyotes and coydogs. Am. J. Vet. Res. 46, 256-262.

Rudinska, M.A., 1981. Morphologic aspects of host-cell-parasite relationships in babesiosis. In: Ristic M., Kreier J.P. (Eds.), Babesiosis. Academic Press, New York, pp. 87-143.

Saiki, R. K., Scharf, S., Faloona F., Mullis K. B., Horn, G. T., Erlich H., A., Arnheim N., 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 230, 1350-1354.

Schein, E., Rehbein, G., Voigt, W.P., Zwegarth, E., 1981. *Babesia equi* (Laveran 1901). Development in horses and in lymphocyte culture. Tropenmed. Parasitol. 32, 223-227.

Schetters, T.P.M., Kleuskens, J.A.G.M., Scholtes, N.C., Pasman, J.W., Goovaerts, D., 1989. Vaccination of dogs against *Babesia canis* infection. Vet. Parasitol. 127, 81-85.

Schetters, T.P.M., Scholtes, N.C., Kleuskens, J.A.G.M., Bose, H.J., 1996. Not peripheral parasitaemia but the level of soluble antigen in plasma correlates with vaccine efficiacy against *Babesia canis*. Parasitol. 115, 485-493.

Schetters, T.P.M., Moubri, K., Précigout, E., Kleuskens, J., Scholtes, N.C., Gorenflot, A., 1997. Different *Babesia canis* isolates, different diseases. Parasitology. 115, 485-493.

Schuster, F.L., 2002. Cultivation of *Babesia* and *Babesia*-like blood parasites: Agents of an emerging zoonotic disease. Clin. Microbiol. Rev. 15, 365-373.

Smith, T., Kilborne, F.L., 1893. Investigation into the Nature, Causation and Prevention of Texas or Southern Cattle Fever. Washington, DC: US Dept Ag.

Stegeman, J.R., Birkenheuer, A.J., Kruger, J.M., Breitschwerdt, E.B., 2003. Transfusion-associated *Babesia gibsoni* infection in a dog. Am. Vet. Med. Assoc. 222, 959-963.

Sunaga, F., Namikawa, K., Kanno, Y., 2002. Continuous in vitro culture of erythrocytic stages of *Babesia gibsoni* and virulence of the cultivated parasite. J. Vet. Med. Sci. 64, 571-575.

Suswam, E., Kyle, D., Lang-Unnasch, N., 2001. *Plasmodium falciparum*: the effect of atovaquone resistance on respiration. Exp. Parasitol. 98, 180-187.

Syafruddin, E., Siregar, J.E., Marzuki, S., 1999. Mutations in the *cytochrome b* gene of *Plasmodium berghei* conferring resistance to atovaquone. Mol. Biochem. Parasitol. 104, 185-194.

Taboada, J., 2006. Babesiosis. In: Greene C.E., (Ed.), Infectious Diseases of the Dog and the Cat. 3rd Ed. WB Saunders, Philadelphia, pp. 722-736.

Taboada, J, Merchant, SR. 1991. Babesiosis of companion animals and man. Vet. Clin. North Am. Small Anim. Pract. 21, 103-123.

Taboada, J., Harvey, J.W., Levy, M.G., Breitschwerdt, E.B., 1992. Seroprevalence of babesiosis in greyhounds in Florida. J. Am. Vet. Med. Assoc. 200, 47-50.

Telford III, S.R., Gorenflot, A., Brasseur, P., Spielman, A., 1993. Babesial infections in humans and wildlife. In: Krier J. (Ed.), Parasitic Protozoa, vol 5. Academic Press, New York, NY, pp.1-47.

Texasoutside.com. Accessed on June 11, 2007. Available at www.texasoutside.com/.../texas-regions01.gif.

Uilenberg, G., Franssen, F.F.J., Perié, N.M., Spanjer, A.A.M., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Vet. Q. 11, 33-40.

Vega, C.A., Buening, G.M., Green, T.J., Carson, C.A., 1985. In vitro cultivation of *Babesia bigemina*. Am. J. Vet. Res. 46, 416-420.

Walker, D.J., Wakefield, A.E., Dohnn, M..N., Miller, R.F., Baughman, R.P., Hossler, P.A., Bartlett, M.S., Smith, J.W., Kazanjian, P., Meshnick, R., 1998. Sequence polymorphisms in the *Pneumocystis carinii cytochrome b* gene and their association with atovaquone prophylaxix failure. J. Infect. Dis. 178, 1767-1775.

Western, K.A., Benson, G.D., Gleason, N.N., Healy, G.R., Schultz, M.G., 1970. Babesiosis in a Massachusetts resident. N. Eng. J. Med. 283, 854-856.

Wozinak E.F., Barr B.C., Thomford J.W., Yamane I., McDonough S.P., Moore P.F., Naydan D., Robinson T.W., Conrad P.A., 1997. Clinical, anatomic, and immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (*Canis familiaris*). J. Parasitol. 83, 692-699.

Yamane, I., Conrad, P.A., Gardner, I.A., 1993. *Babesia gibsoni* infection in dogs. J. Protozool. Res. 3, 111-125.

Yamane, I., Gardner, I.A., Ryan, C.P., Levy, M., Urrico, J., Conrad, P.A., 1994. Serosurvey of *Babesia canis*, *Babesia gibsoni*, and *Ehrlichia canis* in pound dogs in California, USA. Prev. Vet. Med. 18, 293-304.

Zahler, M., Schein, E., Rinder, H., Gothe, R., 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitol. Res. 84, 544-548.

Zahler, M., Rinder, H., Zweygarth, E., Fukata, T., Maede, Y., Schein, E., Gothe, R., 2000. *'Babesia gibsoni'* of dogs from North America and Asia belong to different species. Parasitology. 120, 365-369.

Zwegarth, E., Lopez-Rebollar, L.M., 2000. Continuous in vitro cultivation of *Babesia gibsoni*. Parasitol. Res. 86, 905-907.

VITA

Lauren Elyse Lehtinen
1310 Arrowspring
San Antonio, TX 78258
USA

EDUCATION

2007	M.S. Veterinary Parasitology, Texas A&M University, College Station, Texas, USA
2005	B.S. Biology, Baylor University, Waco, Texas, USA