ABSTRACT

BIRKENHEUER, ADAM JOSEPH. Canine Babesiosis: Epidemiological, Molecular and Therapeutic Investigations. (Under the direction of Edward B. Breitschwerdt)

Canine babesiosis, caused by *Babesia gibsoni* (Asian genotype), is an emerging infectious disease in the United States (US). A serologic and molecular survey was performed to test the hypothesis that *B. gibsoni* was endemic in NC and to determine the prevalence of infection in affected kennels. Stray dogs (n=359) and kennel dogs (n=149) from NC were tested. This study documented the presence of previously unrecognized *B. gibsoni* infections in NC. In addition, the study documented that the *B. gibsoni* seroprevalence and infection in affected kennels were high.

A second study was performed to identify breed pre-dispositions for canine babesiosis and document the states from which *Babesia* positive blood samples were submitted to the North Carolina State University Vector-Borne Disease Diagnostic Laboratory (NCSU-VBDDL). In addition, a subset of dogs was studied to detect an association between a recent dog bite and *B. gibsoni* infections. Canine *Babesia* infections were identified in dogs from 29 US states and Canada. These cases were the first *B. gibsoni* (Asian genotype) infections documented in 27/29 states. In addition, these states were located in the northeastern, midwestern and western regions of the US where they were previously unrecognized. American pit bull terriers and greyhounds were more likely to test positive for *Babesia* than other breeds. An association was detected between a recent dog bite and *B. gibsoni* (Asian genotype) infection, and the association was even stronger when the bite was inflicted by an APBT. This association implicates direct dog to dog transmission as a major route of infection in the US where a tick vector has not been identified.

Relatively little information exists regarding the molecular characterization of canine *Babesia* spp. When this study began in 1999, only three *Babesia* gene sequences had been deposited in Genbank, isolated from canine blood. Several genes from canine *Babesia* spp. were characterized in this study, including 18S ribosomal RNA (rRNA), internal transcribed spacer regions (ITS), cytochrome B (*cytB*), and rhoptry-associated protein-1 (RAP-1). This genetic data was used to develop a sensitive and specific diagnostic semi-nested PCR test for canine babesiosis. The semi-nested PCR test was designed to amplify a region of the 18S rRNA gene from almost all *Babesia* spp. followed by a second semi-nested PCR reaction to discriminate the species or genotype of the organism. Using this assay, a novel *Babesia* organism was identified in a blood sample obtained from a clinical patient. An amplicon of the expected size was produced with the outer primer pair, but no amplification was detected when primers designed to amplify DNA of all known canine *Babesia* spp. were used. Sequencing of partial and full-length 18S rRNA gene amplicons revealed that the novel organism was genetically distinct from all known *Babesia* spp. for which data exists in Genbank and is most similar to *B. bigemina*.

No treatments tested to date have cleared *B. gibsoni* (Asian genotype) infections from dogs. So in a final study, a double-blind placebo controlled trial was performed to evaluate the efficacy of an atovaquone and azithromycin combination for the treatment of canine *B. gibsoni* (Asian genotype) infections. Eighty percent (8/10) of the treatment group had no detectable *B. gibsoni* (Asian genotype) deoxyribonucleic acid (DNA), by a sensitive and specific polymerase chain (PCR) test, in any of their post-treatment samples. In contrast, *B. gibsoni* (Asian genotype) DNA was detectable by PCR in the post-treatment samples from one hundred percent (11/11) of the placebo treated dogs. The atovaquone and azithromycin

drug combination is the first described treatment that will either eliminate *B. gibsoni* (Asian genotype) infections or suppress the parasitemia below the limit of detection in the majority of treated dogs.

Canine Babesiosis: Epidemiological, Molecular and Therapeutic Investigations

by
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History of Babesiosis

Babesia spp. are tick-transmitted intra-erythrocytic protozoan parasites of the phylum apicomplexa(Ristic, 1988; Ristic et al., 1982). The first intra-erythrocytic piroplasms were described in cattle by Victor Babes in 1888. In 1893 Smith and Kilbourne were subsequently able to document that the causative agent of Texas fever in cattle was transmitted by ticks. They named the organism *Pyrosum bigemina* (believed to be either *Babesia bigemina* or *B. bovis*), making babesiosis the first documented tick-transmitted disease. Since that time over 100 *Babesia* spp. have been phenotypically described(Levine, 1988; Ristic, 1988; Ristic et al., 1982). Babesiosis is the clinical syndrome caused by infection with *Babesia* organisms, and is characterized by fever and hemolytic anemia. Babesiosis is a disease of worldwide distribution with important medical, veterinary and agricultural impact.

Hutcheon is credited with first reporting the presence of intracellular parasites in cases of canine babesiosis in 1896 in South Africa. The syndrome which was previously termed biliary fever, is now presumed to have been caused by *B. canis rossi*. Biliary fever was originally thought to be a hepatic form of canine distemper. *Haemaphysalis leachi* was documented to be the vector for *B. canis rossi* in 1901 by Lousby. In 1910, Patton was the first researcher to describe a small piroplasm infecting canids(Patton, 1910). The first report of canine babesiosis in the US was by Eaton, in 1934, and the strain is presumed to have been *B. canis vogeli*(Eaton, 1934). *Babesia gibsoni*, reported in the US

in 1968, was identified in a dog that was imported from Kuala Lampur, an area of known endemnicity(Groves and Yap, 1968). The first description of a small *Babesia* spp. infecting a dog from North America that did not have a history of travel to an endemic area was in 1979(Anderson et al., 1979). It was more than a decade before another dog was reported to be infected with a small piroplasm in the US. In 1991 Conrad et. al. described an outbreak involving eleven dogs in southern California that were infected with small piroplasms(Conrad et al., 1991). Since no other species of small piroplasms were known to infect dogs at that time, the organisms were presumed to be *B. gibsoni*. In 1999 there was another case series describing nine dogs from North Carolina and one dog from Wisconsin infected with small piroplasms(Birkenheuer et al., 1999). Since that time, there have been increasing numbers of reports of dogs infected with small piroplasms in the US(Irizarry-Rovira et al., 2001; Kocan et al., 2001; Macintire et al., 2002; Stegeman et al., 2003). The number of reports of large piroplasms in dogs in the US does not appear to be increasing.

The first diagnosis of human babesiosis was reported from Yugoslavia in 1957, and the infecting piroplasm presumed to be *B. divergens*(Skrabablo and Deanovic, 1957). Two *Babesia* species, *B. microti* and *B. divergens*, are responsible for the majority of human babesiosis cases reported in the literature(Gelfand and Callahan, 2003; Homer et al., 2000; Krause, 2002). Historically, *Babesia microti* was identified most often infecting rodents or people in North America, but is now being recognized throughout the world, including a recent report in Japan(Tsuji et al., 2001). *Babesia microti*, the primary agent of human babesiosis in the US, causes babesiosis in both immunocompetent and immune

compromised people. *Babesia divergens* is responsible for the majority of cases of human babesiosis in Europe and is most frequently reported in people that are immune compromised. Several novel, and as yet incompletely characterized piroplasms have also been identified as causative agents of human babesiosis(Herwaldt et al., 1996; Herwaldt, 2003; Persing and Conrad, 1995).

Classification

Babesia are classified in the Phylum *Apicomplexa*, Class *Sporozoae*, Order *Piroplasmida*, and family *Babesiidae*. The family *Babesiidae* contains two genera, *Babesia* and *Theileria*. The lack of a pre-erythrocytic stage in the life cycle is considered an important phenotypic feature of the genus *Babesia*. To date over 100 species of *Babesia* have been described(Levine, 1988). Historically *Babesia* spp. have been classified based on their size and their vertebrate host(Euzeby, 1987; Levine, 1988; Ristic, 1988; Ristic et al., 1982). Large *Babesia* are generally considered to be greater than 3 microns in length and small *Babesia* are generally 1-3 microns in length(Ristic, 1988). Table 1 lists the majority of *Babesia* spp. that are known to infect humans or domestic animals.

The classification of the family *Babesiidae* is likely to undergo considerable revision in the future. This is due to the advent of molecular biology and the use of DNA sequences to perform phylogenetic classification. Of the over 100 described *Babesia* species, genetic data is listed in GENBANK for only 15 species(Benson et al., 2003). The most common gene sequence used for phylogenetic studies of piroplasms is the small

ribosomal subunit (18S rRNA)(Allsopp et al., 1994; Caccio et al., 2002; Centeno-Lima et al., 2003; Chansiri and Sarataphan, 2002; Ellis et al., 1992; Kjemtrup et al., 2000a; Kjemtrup et al., 2000b; Zahler et al., 2000a; Zahler et al., 2000b). The small ribosomal subunit is ideally suited for phylogenetic studies because it contains highly conserved, variable, and hypervariable regions(Ludwig and Schleifer, 1994; Woese, 1987). In addition, since 18S rRNA is not translated into protein, it is not subject to the codon wobble like other genes. Lastly since the sequence dictates the structure of the molecule, base changes in many regions can be evaluated for co-variation with their corresponding stem base pair in the proposed rRNA secondary structure to ensure that a plausible pairing exists to retain a functional ribozyme. Phylogenetic analyses based on rRNA gene sequences have resulted in robust separation of three groups of piroplasms(Kjemtrup et al., 2000a; Kjemtrup et al., 2000b; Zahler et al., 2000b; Zahler et al., 2000c). These three groups have been termed the *Babesia* clade, the *Babesia microti*-like group and the *Theileria* group. Some early phylogenetic analyses utilizing 18S rRNA suggested that there was a distinct genetic separation of the large and small *Babesia* species(Homer et al., 2000). However, further phylogenetic studies utilizing more hemoprotozoan organisms and sequences have demonstrated that this assessment is not accurate. Based on the newer phylogenetic studies, both large and small *Babesia* spp. fall within the *Babesia* clade(Kjemtrup et al., 2000a; Zahler et al., 2000c). The nomenclature within the order Piroplasmida and subsequently the literature related to clinical babesiosis can be quite confusing. This is due to the fact that most *Babesia* species were described and named based on phenotypes and the mammalian host before any 18S rRNA sequences

were analyzed. For example, the first "*B. gibsoni*" 18S rRNA sequence deposited in GENBANK by Persing in 1992 was amplified from a canine isolate from California(Persing et al., 1992). Based on that sequence it was believed that *B. gibsoni* was more closely related to *B. microti* than *B. canis*. In 1999 small piroplasms, assumed to be *B. gibsoni*, were found in dogs from North Carolina, but no DNA sequences were reported(Birkenheuer et al., 1999). In April 2000 Zahler concluded that the "*B. gibsoni*" isolated from North America by Persing was genetically distinct from Asian *B. gibsoni* isolates(Zahler et al., 2000c). In addition, in a second publication in April 2000 Zahler reported the detection of a novel small piroplasm identified in dogs in Spain(Zahler et al., 2000b). Subsequent study by Kjemtrup reported in December 2000 concluded that there were at least three genetically distinct small piroplasms that could infect dogs(Kjemtrup et al., 2000a). The study by Kjemtrup included DNA sequences derived from small piroplasms infecting dogs in North America including North Carolina that were identical to the Asian *B. gibsoni* isolates reported by Zahler. This meant that at least two genetically distinct canine small piroplasms existed in North America. Phylogenetic analyses including all three piroplasms consistently place the original "*B. gibsoni*" genotype reported by Persing in the *Theileria* group and the Asian *B. gibsoni* genotype in the *Babesia* clade. Most recently researchers from Spain have identified dogs that are infected with *B. equi*, and another genetically unique *B. gibsoni*-like organism(Criado-Fornelio et al., 2003a; Criado-Fornelio et al., 2003b). Further confusion is added by the name proposed for the canine small piroplasm identified in dogs from Spain, *Theileria annae*(Zahler et al., 2000b). The *T. annae* sequence is consistently placed in the *B.*

microti-like group rather than the *Theileria* group. It is possible that the Asian *B. gibsoni* genotype will remain in the *Babesia* genus, the orignial "*B. gibsoni*" genotype reported by Persing will be re-named and placed in the *Theileria* genus and that *Theileria annea* will be re-named and placed in a new genus for which *B. microti* is the type species. It is interesting that the type species for the *Babesia* genus, *B. bovis*, is difficult to group with the other clades in the current phylogenetic trees based on 18S rRNA gene sequences, and is often placed as a monophyletic species. However, in other cases, such as with *Babesia canis*, differences in 18S rRNA sequences have confirmed that differences in geographic location, vectors and pathogenicity represent distinct organisms as previously suspected.

Unlike prokaryotes, there is no formal candidatus classification for novel eukaryotic organisms for which there is incomplete information for species determination. Recently some researchers have elected to not propose names for genetically unique organisms, until genetic data for all of the phenotypically described *Babesia* species exists(Herwaldt, 2003). Ideally, the proposal of new names for genotypically unique organisms should be withheld to avoid further confusion regarding the nomenclature within the family *Babesiidae*

Biology

Babesia spp. have both vertebrate and invertebrate hosts. The invertebrate host in almost all cases is an ixodid tick. Table 1 lists the tick vector for each *Babesia* spp. of domestic animals and humans. *Babesia* spp. undergo sexual and asexual reproduction. Sexual

reproduction, gamogony, occurs by the combination of two gamonts/ray bodies in the gut of the tick(Homer et al., 2000; Ristic, 1988; Ristic et al., 1982). The resultant zygote transforms into a kinete which enters the hemolymph and eventually penetrates the salivary gland to initiate sporogony. Kinetes are also able to invade other tissues such as the ovaries where they are responsible for transovarial transmission. Asexual reproduction, sporogony, occurs in the tick salivary gland. The kinete develops into a multinucleated sporoblast and will cause the infected salivary gland cell to swell. Sporzoites form by budding off the sporoblast. During tick feeding spozoites are released into the bloodstream of the vertebrate host. These sporozoites invade host erythrocytes by a process called invagination. Initially, the sporozoite resides within a parasitophorous vacuole. Unlike *Plasmodium*, the parasitophorous vacuole eventually disintegrates leaving the trophozoite free within the erythrocyte cytoplasm. These trophozoites then undergo binary fission and form either merozoites or gamonts. Merozoites are released when the erythrocyte is lysed, and can invade other erythrocytes via invagination. Like trophozoites, merozoites may undergo binary fission, called merogony. Gamonts are the developmental stage that once ingested as a component of the tick blood meal during feeding can enter the tick gut and begin the life cycle again. Most *Babesia* spp. only infect erythrocytes in the vertebrate host(Ristic, 1988). This differs from the life cycle of *Plasmodium*, *Theileria* and *Cytauxzoon* which have a pre-erythrocytic stage in which the sporozoites infect hepatocytes, lymphocytes or macrophages(Kier et al., 1987; Mehlhorn and Shein, 1984). Within the invertebrate host, both transovarial and transstadial transmission of *Babesia* can occur(Ristic et al., 1982). Transovarial transmission is

characterized by the ability of an infected tick to produce progeny that are infectious. Transstadial transmission is the characterized by the ability of an individual tick to retain infectivity throughout multiple stages of its lifecycle.

Canine *Babesia* are transmitted by at least three different genera of ixodid ticks. *Babesia canis canis* is transmitted by *Dermacentor reticulatis*. *Babesia c. vogeli* is transmitted by *Rhipicephalus sanguineus*. *Babesia c. rossi* is transmitted by *Haemaphysalis leachi*(Uilenberg et al., 1989). *Babesia gibsoni* (Asian genotype) is transmitted by *Haemaphysalis bispinosa* and *Haemaphysalis longicornis*(Otsuka, 1974; Swaminath, 1937). *Theileria annae* is most likely to be transmitted by *Ixodes hexagonus*(Camacho et al., 2003). An arthropod vector has not been established for the *B. gibsoni* (USA/CA genotype).

Pathology/Immunity

Babesia spp cause disease in the vertebrate hosts through a combination of both direct parasite induced damage and secondary immune mediated effects(Homer et al., 2000; Ristic et al., 1982). As a general rule the small *Babesia* organisms are more virulent than large *Babesia* spp(Homer et al., 2000; Ristic, 1988). Anemia is believed to be responsible for the majority of clinical signs exhibited by animals and people that are infected with *Babesia* spp. Red blood cell destruction occurs when merozoites are released after merogony. This however appears to be a minor component considering that the degree of anemia does not consistently correlate with the degree of parasitemia. An immune-mediated hemolytic anemia is one of the most common disease manifestations

of babesiosis in animals. Many cases are direct Coomb's test positive(Farwell et al., 1982). A direct Coomb's tests for the presence of antibodies and/or complement bound to the red blood cell surface. Either agglutination or flow cytometry can be used detect the presence of these antibodies and/or complement. The exact mechanism by which *Babesia* spp. induce immune mediated hemolytic anemia is unknown. The most commonly accepted theory is that the immune mediated hemolytic anemia is due to soluble parasite antigens binding to the red blood cell surface either through antibody or complement mediated hemolysis(Homer et al., 2000). It has been documented that surface bound complement molecule, C3, is important for RBC invasion by merozoites(Jack and Ward, 1977, 1980a, b; Jack et al., 1981). The importance of C3 in merozoite RBC invasion was elucidated by blocking invasion with trypan blue, a molecule that binds to the C3(Jack and Ward, 1980b). Others have disputed the role of complement in RBC invasion or have found that the role of complement mediated RBC invasion is not necessary for all *Babesia* spp. (Levy et al., 1986; Seinen et al., 1982). Thrombocytopenia is another common manifestation of babesiosis(Birkenheuer et al., 1999; Macintire et al., 2002). Anti-platelet antibodies have been documented via flow cytometry(Wilkerson et al., 2001).

Babesia organisms are well adapted to survive in their vertebrate hosts. In almost all cases examined, animals are able to develop a protective immune response after infection and recovery or immunization(Homer et al., 2000). This protective immune response does not prevent re-infection, but does decrease the degree of parasitemia, morbidity and mortality when animals are re-exposed to the parasite(Homer et al., 2000;

Ristic, 1988; Ristic et al., 1982). Age, sex, and breed/genetic background have all been demonstrated to play a role in determining host susceptibility to *Babesia* infections(Aguilar-Delfin et al., 2001; Langley and Gray, 1987; Levy et al., 1982). In most cases, *Babesia* are able to persist in the vertebrate host for years. In occasional cases the infection appears to be cleared spontaneously, self-perpetuation of the organism stops, or the infection persists below the current limit of detection. Sterilizing immunity or complete resistance to re-infection after primary infection or immunization have rarely been reported. Murine and bovine models have been used to characterize the components of the immune response that contribute to immunity against *Babesia* infections(Abdalla et al., 1978; Ahmed, 2002; Brown et al., 1995; Brown et al., 1993; Callow, 1967; Goff et al., 2003a; Goff et al., 2001; Hemmer et al., 2000a, b; Igarashi et al., 1999; Meeusen et al., 1984; Phillips, 1969; Rodriguez et al., 1996; Rosenblatt-Bin et al., 1998; Van Bemmel et al., 1979; Wood and Clark, 1982; Zivkovic et al., 1984). The relevance of these experimental studies to the many species of *Babesia* that infect animals is unknown. *Babesia microti* which is frequently used in murine studies, unlike most *Babesia* spp., undergoes a lymphocytic stage in its lifecycle prior to the erythrocytic stage. This could have significant effects on the organisms presentation to the host immune system that may not be relevant to other species. Immunity to *Babesia* infections appears to be complex and multi-factorial with no single component identified as the “critical” component necessary for protection or survival. Innate immune responses appear to be the most important factors involved in the resistance to *Babesia* infections. Most studies have determined that a TH-1 type cytokines are important for immunity,(Brown et al.,

1995; Rosenblatt-Bin et al., 1998). but interestingly CD8+ T-cell mediated cytotoxicity does not appear to be the mechanism by which resistance in mediated(Igarashi et al., 1999). Strains of mice that are considered to be TH-1 type responders such a C57Bl/6 are more resistant than TH-2 type responders such as BALB/c mice(Aguilar-Delfin et al., 2001; Moro et al., 1998; Ruebush and Hanson, 1979). This strain associated resistance is maintained in C57Bl/6 mice even when a SCID mutation is introduced, which indicates that B-cell and T-cell responses are not responsible for the resistance(Aguilar-Delfin et al., 2001). Interestingly, in a susceptible strain of mice, survival times were increased by the introduction of the SCID mutation, which suggests that B-cell and T-cell responses may be responsible for some of the pathology associated with *Babesia* infection.

The production of IL-12, interferon γ (IFN-γ), and nitric oxide (NO) have all been shown to be important components of the immune response in mice with babesiosis(Aguilar-Delfin et al., 2003; Ahmed, 2002; Goff et al., 1998; Igarashi et al., 1999; Stich et al., 1998). It is believed that these cytokines are produced by splenic macrophages and exert their effect in the spleen. An age related resistance to babesiosis in young calves was noted and this resistance was determined to be associated with a soluble factor that could be abbrogated by splenectomy(Levy et al., 1982). More recent studies have demonstrated that the soluble factor likely to be associated with parasite death is NO secreted by splenic macrophages(Goff et al., 2001). In addition, young calves have an earlier and greater production of IL-12 and IFN-γ compared to susceptible adult cattle(Goff et al., 2001). The host immune response to *Babesia* infections is not only related to resistance to infection, and may play an important role in pathogenesis. In

one study the increased production of tumor necrosis factor-α (TNF-α) was associated with increased mortality in a murine model(Hemmer et al., 2000a, b).

Diagnosis

Microscopy was the first technique utilized to identify piroplasms and remained the most common diagnostic technique for researchers and clinicians for the diagnosis of babesiosis. Complement fixation tests were utilized for the diagnosis of bovine babesiosis, but have been replaced for more sensitive and specific tests. (Bidwell et al., 1978; Kuttler et al., 1977; Todorovic and Long, 1976). Indirect fluorescent antibody testing to detect the presence of anti-*Babesia* antibodies is commonly used, but is plagued with a lack of sensitivity and specificity(Akinboade and Dipeolu, 1984; Bizzeti et al., 1997; Donnelly et al., 1980; Krause et al., 1994; Kuttler et al., 1977; Plossl, 1984; Weiland and Kratzer, 1979a; Yamane et al., 1993). Seroreactivity against *Babesia* antigens does not definitively prove that an animal is actively infected with *Babesia*, and there are several reports of seronegative animals that were confirmed to be infected by microscopy and or PCR. Other antibody detection based methods, such as enzyme linked immunosorbant assays (ELISA), have been described, but few are commercially available(Bose et al., 1990; Bose and Peymann, 1994; Doherr, 1990; Ebert, 1982; Goff et al., 2003b; Jidaisho, 1986; Kappmeyer et al., 1999; Machado et al., 1997; Makimura et al., 1990; Martins et al., 1996; Nierlich, 1990; Voller et al., 1976; Weiland and Kratzer, 1979a, b). Sensitive and specific tests such as sub-innoculation of blood into rodents or susceptible splenectomized animals have been used in human medicine and research

settings, but are impractical for routine diagnostic purposes. With the advent of molecular biology particularly the polymerase chain reaction (PCR) the diagnosis of babesiosis has been revolutionized. The first PCR test for *Babesia* was described by Fahrimal in 1992. (Fahrimal et al., 1992). Since that time, a number of PCR-based tests have been developed(Bashiruddin et al., 1999; Birkenheuer et al., 2003; Buening et al., 1994; Calder et al., 1996; Carret et al., 1999; Figueroa et al., 1998; Figueroa et al., 1992, 1993; Figueroa et al., 1994; Fukumoto et al., 2001; Gubbels et al., 1999; Lew et al., 1997; Persing et al., 1992; Sahagun-Ruiz et al., 1997; Salem et al., 1999; Santana et al., 2001; Xuan et al., 1998; Zahler et al., 1998). The majority of these tests have been based on 18S rRNA gene sequences, but other genes such as the internal transcribed spacer region, cytochrome b, and P50 have also been used as gene targets. Studies comparing the sensitivity of PCR tests for diagnosis of babesiosis to detection by microscopy have found that PCR is more sensitive in most cases. (Almeria et al., 2001; Krause et al., 1996).

Treatment

Babesia spp. have been difficult to eradicate from infected individuals(Homer et al., 2000; Ristic, 1988; Ristic et al., 1982). Many drugs have been used to treat babesiosis with varying degrees of success(Bauer, 1966; Carbrey et al., 1971; Fowler et al., 1972; Krause et al., 2000; Kuttler, 1980; Kuttler and Johnson, 1986; Roychaudhury and Gautam, 1974; Ryley, 1957; Tadic, 1993; Vulchovski, 1977; Wittner et al., 1996; Wood, 1971). Other drugs have been evaluated for their ability to prevent experimental or

natural infections(Davis, 1982; Decamps, 1982; Morel, 1981; Purnell et al., 1981; Sharma and Bansal, 1984; Uilenberg et al., 1981; Vercammen et al., 1996a, b). Several treatment strategies have been employed including complete elimination of the organism or alternatively incomplete clearance leading to a state of premunition(Kuttler, 1982). Premunition is referred to as the immunity of infection, and is a state in which the animal remains infected, but has very few clinical signs. In many countries where re-infection is nearly impossible to prevent, the goal is to induce a state of premunition through drug treatments that reduce the severity of clinical signs, but do not result in complete sterilization of the *Babesia* infection(Kuttler, 1982; Penzhorn et al., 1995). This strategy has been used in some areas, where animals are purposely infected with a mildly pathogenic strain of the organism (i.e. live vaccine) to achieve protection from severe clinical disease when the animal is exposed to more pathogenic field strains.

Conclusions

Babesiosis remains an important human and animal disease throughout the world. Despite substantial recent advances in knowledge regarding *Babesia* biology, immunopathogenesis, diagnostic testing and chemotherapy, we are still unable to adequately prevent, definitively diagnose and effectively treat *Babesia* infections.

Table 1. List of the *Babesia* species known to infect humans and domestic animals and their merozoite size

Vertebrate Host	***Babesia* spp**	**Large/Small**
Human	***Babesia microti***	**Small**
	Babesia divergens	**Small**
	WA1	**Small**
	MO1	**Small**
	CA1	**Small**
	Non-*B. divergens* sp.	**Small**
	Babesia bovis	**Large**
	Babesia canis	**Large**
Canine	***Babesia canis***	**Large**
	***Babesia gibsoni* (Asian genotype)**	**Small**
	***Babesia gibsoni* (USA/CA genotype)**	**Small**
	Theileria annae	**Small**
	Other spp	**Large**
Feline	***Babesia felis***	**Small**
	Babesia leo	**Small**
Bovine	***Babesia bovis***	**Small**
	Babesia bigemina	**Large**
	Babesia divergens	**Small**
	Babesia major	**Large**
	Babesia jakimovi	**Large**
	Babesia ovata	**Large**
Equine	***Babesia caballi***	**Large**
	Babesia equi	**Small**
Caprine	***Babesia ovis***	**Small**
	Babesia motasi	**Large**

Table 1, continued.

Porcine	***Babesia trautmanii***	**Large**
	Babesia perroncitoi	**Small**

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Serosurvey of Anti-*Babesia* Antibodies in Stray Dogs and American Pit Bull Terriers and American Staffordshire Terriers from North Carolina

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Abstract

Stray dogs (n=359) and kennel dogs (n=149) from North Carolina were tested for evidence of anti-*Babesia* antibodies. Anti-*Babesia* antibodies were detected in 21/359 and 22/149 of the stray and kennel dogs, respectively. A total of 57 dogs from both groups were tested for babesiasis by light microscopy and polymerase chain reaction (PCR). *Babesia* deoxyribonucleic acid (DNA) was detected in 3/28 of the stray dogs and 14/29 of the kennel dogs. When *Babesia* DNA was detected by PCR, the species-specific PCR results differed from the *Babesia* species antibody titer results in 6/17 of the PCR-positive dogs. There was no association between anti-*Babesia* antibodies and the presence of ticks. There are currently *Babesia gibsoni* epizootics affecting American pit bull terrier kennels.

Introduction

Babesiosis is the disease caused by intra-erythrocytic protozoan parasites of the genus *Babesia*. Babesiosis is typically characterized by hemolytic anemia, thrombocytopenia, fever, and splenomegaly. Babesiasis refers to the presence of circulating *Babesia* organisms and does not infer whether or not the organisms are causing clinical disease. Historically, canine babesiosis in the United States (US) has been attributed to either *Babesia canis* (*B. canis*) or *Babesia gibsoni* (*B. gibsoni*), with the vast majority of cases attributed to *B. canis vogeli*. Since Conrad, *et al*., reported the first disease outbreak of canine babesiosis caused by a small *Babesia* in 1991, there have been an increasing number of reports of dogs in the US infected with small *Babesia spp*(Birkenheuer et al., 1999; Conrad et al., 1991; Irizarry-Rovira et al., 2001; Kjemtrup et al., 2000; Kocan et al., 2001; Macintire et al., 2002). *Babesia gibsoni* was presumed to be the only small *Babesia spp.* to infect dogs; however, recent studies demonstrate that at least three genetically distinct, small *Babesia*-like organisms or piroplasms naturally infect dogs(Kjemtrup et al., 2000; Zahler et al., 2000a). Based upon evolving data, the nomenclature of these small *Babesia*-like organisms is likely to undergo revision. In this report, the authors will refer to the Asian genotype as *B. gibsoni* (GenBank Accession Nos. AF271081, AF271082, AF205636, AF175300, and AF175301),(Kjemtrup et al., 2000; Kocan et al., 2001; Zahler et al., 2000b). the North American genotype as the "western piroplasm" (AF158702 and L13729),(Conrad et al., 1992; Zahler et al., 2000a). and the European/Spanish genotype as *Theileria annae* (*T. annae*) (AF188001)(Camacho et al., 2001; Zahler et al., 2000a). Both *B. gibsoni* and the western piroplasm have been identified in dogs from North America, while *T. annae* has only been reported in

Europe(Camacho et al., 2001; Zahler et al., 2000a). To date, the western piroplasm has only been reported in dogs from California(Conrad et al., 1991; Yamane et al., 1994). It is important for the clinician to identify which species and genotype are the cause of babesiosis, since the prognosis for each may be different. *Babesia canis vogeli* is considered to be an intermediately virulent species for which it is presumed that anti-babesial therapy will eliminate the infection. *Babesia gibsoni* is considered to be a virulent species, and to date, no anti-babesial treatment has been able to effectively eliminate the infection in dogs(Yamane et al., 1993a). However, in one report, the majority of *B. gibsoni*-infected dogs displayed no outward signs of clinical illness, despite hematological abnormalities such as thrombocytopenia and anemia. The western piroplasm is also pathogenic(Conrad et al., 1991; Yamane et al., 1994). The responsiveness of the western piroplasm to anti-babesial therapy has not been well characterized. *Theileria annae*-infected dogs were often anemic and thrombocytopenic(Camacho et al., 2001). To the authors' knowledge, the responsiveness to anti-babesial therapy for *T. annae* has not been reported.

The majority of dogs that have been diagnosed with *B. gibsoni* in the US have been American pit bull terriers (APBT) and American Staffordshire terriers (AST), and they have come from kennels housing multiple dogs(Birkenheuer et al., 1999; Irizarry-Rovira et al., 2001; Kjemtrup et al., 2000; Kocan et al., 2001; Macintire et al., 2002). In California, there was no apparent breed predilection for the western piroplasm(Conrad et al., 1991; Yamane et al., 1994). This study was undertaken to determine the prevalence of anti-*Babesia* antibodies in three APBT/AST kennels where *B. gibsoni* had been

previously diagnosed, and to detect anti-*Babesia* antibodies and babesiasis in stray dogs in North Carolina (NC).

Materials and Methods

Sample Collection

Samples were collected between May and December of 1999. The age, breed, sex, and the presence or absence of external parasites at the time of collection were recorded for each dog. Each dog's age was estimated as < or > 6 months based on the presence or absence of deciduous teeth. Collected ticks were stored in 70% ethanol and subsequently identified using a standard key(Hoskins, 1991).

Three regions in NC were sampled for stray dogs. Sera and ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood were collected from stray dogs housed in animal shelters. A total of 359 stray dogs were sampled; 168 were from eastern NC (sampled between July and September 1999), 140 were from central NC (sampled between May and October 1999), and 51 were from western NC (sampled in July 1999). Every available dog was sampled from each location, with the assumption that sampled dogs were representative of the stray dog populations in the respective areas. This number of subjects would allow a 95% chance of detecting at least one infected/exposed dog if the prevalence was 2% to 5.7%(Smith, 1995).

Three APBT/AST kennels located in central NC were selected for study after a single case of *B. gibsoni* had been previously diagnosed in each kennel. Sera and EDTA anticoagulated whole blood were collected from all kennel dogs. Kennel I housed 59 dogs (sampled in May 1999); kennel II housed 43 dogs (sampled in June 1999); and kennel III housed 47 dogs (sampled in December 1999).

Indirect Fluorescent Antibody Testing

Indirect fluorescent antibody (IFA) tests were performed as previously described using anticoagulated whole blood obtained from a splenectomized dog that had been infected with either *B. canis*, *B. gibsoni*, or the western piroplasm as the antigen sources(Levy et al., 1987). For each antigen, reciprocal antibody titers ≥64 were considered seroreactive. Since IFA can be associated with a one dilution error, dogs with reciprocal antibody titers of 32 were included for subsequent microscopic examination and PCR testing to minimize potential false-negative IFA results. To examine the specificity of the IFA, all three antigen slides were tested using sera from dogs that had antibodies against *Toxoplasma gondii* (reactive to a reciprocal antibody titer of 620), *Leishmania infantum* (reactive to a reciprocal antibody titer of 2,048), *Ehrlichia canis* (reactive to a reciprocal antibody titer of 8,192), *Rickettsia rickettsii* (reactive to a reciprocal antibody titer of 2,048), and *Bartonella vinsonii* (reactive to a reciprocal antibody titer of 2,048).

Microscopic Observation

A total of 57 dogs (including 43 seroreactive dogs with reciprocal anti-*Babesia* antibody titers ≥64, 10 dogs with reciprocal antibody titers of 32, and four nonseroreactive anemic dogs) were tested for babesiasis by microscopic observation. The four nonseroreactive dogs tested were kennel dogs without detectable anti-*Babesia* antibodies that were obviously anemic, with red blood cell volumes <25% after erythrocyte sedimentation. Modified Wright's stained thin blood smears were examined by an experienced microscopist at 1,000X for either 30 minutes per animal or until piroplasms were clearly

identified. The microscopist was blinded to the polymerase chain reaction (PCR) results at the time of the microscopic examination.

Polymerase Chain Reaction Testing

A total of 57 dogs (including 43 seroreactors, 10 dogs with reciprocal antibody titers of 32, and four nonseroreactive anemic dogs) were tested for babesiasis by PCR amplification as previously described(Birkenheuer et al., 2003). Total deoxyribonucleic acid (DNA) was extracted from 200 μL of EDTA anticoagulated whole blood using a commercially available kit according to the manufacturer's instructions. In order to prevent PCR amplicon contamination, sample preparation, reaction setup, PCR reaction, and amplicon detection were all performed in separate areas. Positive and negative controls were used in all processing steps, including the DNA extraction.

Statistical Analysis

An odds ratio (OR), when appropriate, or a Fisher's exact test, or both, were used to examine potential associations between the presence of anti-*Babesia* antibodies to the following: age, breed, sex, presence of ticks, and kennel/geographic location(Agresti, 1992). The stray dogs and kennel dogs were evaluated separately, with the presence of anti-*Babesia* antibodies defined as the dependent variable and either age, breed, sex, presence of ticks, or geographic location as the independent variable. A kappa statistic was used to determine the level of agreement between microscopic observation and PCR tests.

Results

A total of 508 dogs were sampled. Of the stray dogs, 270/359 (75.2%) were of mixed breed, and 89/359 (24.8%) appeared to be purebred. The APBT/AST were the most common purebred stray dogs, accounting for 12/359 (3.3%). Of the stray dogs, 161/359 (44.8%) were male, and 198/359 (55.2%) were female. Approximately 20% (71/359) of the stray dogs were estimated to be <6 months of age. Of the kennel dogs, 138/149 (92.6%) were APBT/AST, 7/149 (4.7%) were American bulldogs, and 4/149 (2.7%) were walker hounds. Of the kennel dogs, 68/159 (42.8%) were male, and 91/159 (57.2%) were female. Approximately 12% (18/149) of the kennel dogs were estimated to be <6 months of age. The kennel owners reported that none of the dogs had traveled outside of the US. The percentages of stray dogs parasitized by ticks or fleas at the time of sample collection were 27%, 32%, and 65% from western NC, eastern NC, and central NC, respectively. Ticks were available for identification from 86% of the parasitized western NC dogs, and all were adult *Dermacentor variabilis* (*D. variabilis*). Ticks were available for identification from 41% of the parasitized eastern NC dogs. These ticks were identified as adult *Rhipicephalus sanguineus* (*R. sanguineus*) (52%), nymphal *R. sanguineus* (2.5%), adult *D. variabilis* (27.3%), or adult *Amblyomma americanum* (*A. americanum*) (4.5%), with some dogs parasitized by more than one species (13.7%). Ticks were available for identification from 23% of the parasitized central NC dogs. These ticks were identified as adult *R. sanguineus* (54%), nymphal *R. sanguineus* (4%), adult *D. variabilis* (20%), or adult *A. americanum* (12%), with some dogs parasitized by more than one species (10%). Ticks or fleas were not identified on any kennel dogs, nor did the owners report the historical presence of tick attachment on these dogs. However, it was reported that in all

of the kennels there was a seasonal problem with biting flies. The owners reported no use of external parasite control.

Indirect Fluorescent Antibody Testing

Twenty-one seroreactive stray dogs with reciprocal antibody titers ≥64 were identified (Table 2). Sixteen of the seroreactive stray dogs were mixed-breed dogs; two were foxhounds; and one each was a beagle, pointer, and Labrador retriever. Of the seroreactive stray dogs with reciprocal antibody titers ≥64, four were estimated to be <6 months of age, 17 were >6 months of age, eight were male, 13 were female, and five were parasitized by ticks at the time of sampling. Of the five tick-infested seroreactors, adult male and female *R. sanguineus* were found on two dogs, adult male and female *D. variabilis* were found on two dogs, and one dog was parasitized by adult male and female *D. variabilis* and *A. americanum*. Seven stray dogs had reciprocal antibody titers of 32. Five of these dogs had reciprocal titers of 32 against *B. canis*; one dog had a reciprocal titer of 32 against *B. gibsoni*; and one dog had a reciprocal titer of 32 against the western piroplasm.

Twenty-two seroreactive kennel dogs with reciprocal antibody titers ≥64 were identified (Table 3). The prevalence of dogs with anti-*Babesia* antibodies in kennels I, II, and III were 6.8%, 25.6%, and 14.9%, respectively. Of the seroreactive kennel dogs with reciprocal antibody titers ≥64, two were estimated to be <6 months of age; 20 were estimated to be >6 months of age; eight were male; 14 were female; and none were parasitized by ticks at the time of sampling. All of the seroreactive kennel dogs were

APBT/AST. Three kennel dogs had reciprocal antibody titers of 32. Two dogs had titers of 32 against *B. gibsoni*, and one dog had a titer of 32 against the western piroplasm. Control canine serum samples that were reactive to *Toxoplasma gondii*, *Leishmania infantum*, *Ehrlichia canis*, *Rickettsia ricketsii*, or *Bartonella vinsonii* antigens did not cross-react with any of the three *Babesia* antigens.

Microscopic Observation

Small piroplasms were identified by microscopic observation in 1/28 stray dogs (Table 2) and in 13/29 of the kennel dogs (Table 3). No large piroplasms were observed on blood smears.

Polymerase Chain Reaction Testing

A total of 57 dogs (including 43 seroreactors, 10 dogs with reciprocal antibody titers of 32, and four nonseroreactive anemic dogs) were tested by PCR. Three stray dogs were PCR-positive (Table 2). Discordance between the serological test results and the PCR test results was found in one PCR-positive dog (Table 2; case no. 15) that was only seroreactive against *B. canis* antigens but was PCR-positive for *B. gibsoni*. None of the PCR-positive stray dogs were APBT/AST. Fourteen kennel dogs, all APBT/AST, were PCR-positive for *B. gibsoni* (Table 3). In five PCR-positive kennel dogs, there was discordance between the serological and PCR test results. Two of these dogs (Table 3; case nos. 12, 14) were only seroreactive against the western piroplasm, and one dog (Table 3; case no. 7) was only seroreactive against *B. canis*. The other two PCR-positive

kennel dogs (Table 3; case nos. 3, 4) with discordant IFA test results were anemic and had no detectable anti-*Babesia* antibodies.

Statistical Analysis

There were no associations between the presence of anti-*Babesia* antibodies and age, breed, sex, presence of ticks, or kennel/geographic location within either sample population. There was a strong agreement (Kappa, 0.9) between the microscopic observation and PCR tests. In all cases where there was disagreement between the microscopic results and PCR, the PCR was positive and microscopic testing was negative.

Discussion

This study is unique as it presents the first serological and molecular data for canine *Babesia* in the US that includes simultaneous consideration of *B. canis*, *B. gibsoni*, and the western piroplasm. The results of this study should further alert practitioners in the US to consider *B. gibsoni* as a diagnosis in dogs with signs of babesiosis. Scientific reports have documented *B. gibsoni* infections in dogs from Alabama, Indiana, Oklahoma, and North Carolina(Birkenheuer et al., 1999; Irizarry-Rovira et al., 2001; Kjemtrup et al., 2000; Kocan et al., 2001; Macintire et al., 2002). In addition, the Vector-Borne Disease Testing Laboratory at the North Carolina State University College of Veterinary Medicine has detected *B. gibsoni* DNA by PCR in canine blood samples from Arizona, California, Florida, Georgia, Kentucky, Maryland, Michigan, Minnesota, Mississippi, Missouri, New Jersey, New York, Ohio, Pennsylvania, South Carolina, Tennessee, Texas, Washington, Wisconsin, and Virginia. Based on the results of this study and others, when a *B. gibsoni*-infected dog is identified from a kennel, further investigation is indicated to identify other infected dogs housed on the same property(Macintire et al., 2002).It is important to note that this study demonstrates that there can be discordance between the microscopic, serological, and molecular test results for canine babesiosis. Anti-*Babesia* seroreactivity was not always detectable in infected dogs, and when anti-*Babesia* antibodies were detectable, the highest antibody titer against a given species did not always agree with the PCR detection of DNA in *Babesia* species. This unpredictable seroreactivity and cross reactivity are likely due to antigenic variation between species, strains, and isolates. Since most IFA tests use whole organism preparations, they usually only consist of a single isolate. Therefore, each test is likely to

"favor" isolates that share common or similar immunodominant antigens. It is possible that some *B. gibsoni* strains actually share more common immunodominant antigens with *B. canis* or the western piroplasm than they do with each other. It is also possible that the seronegative, PCR-positive dogs were acutely infected and had not yet developed detectable anti-*Babesia* antibody titers. Lastly, co-infection with more than one type of piroplasm cannot be ruled out in the PCR-positive dogs with discordant serological testing results. The significance of seroreactivity against *Babesia* antigens in conjunction with a negative PCR and microscopic examination result is not known. One possible explanation includes parasitemias below the limit of detection for microscopic examination and PCR, similar to what has been described in bovine babesiosis(Calder et al., 1996). In order to minimize possible false-positive serological test results in an epidemiological study, other serological surveys have used higher reciprocal antibody titers as their cutoff values: 320 for *B. canis* and 1280 for the western piroplasm,(Yamane et al., 1993b). compared to the cutoff value of 64 used in this study. Since the authors' goal was simply to detect any cases in the stray dog and kennel populations, preferably with confirmation by PCR, microscopic observation, or both, they were willing to accept the possibility of some false-positive seroreactors. The use of antibody titers for the diagnosis of infectious diseases only provides indirect evidence of infection, and the results can be difficult to interpret due to cross-reactive antibodies, antigenic variation between isolates/strains, and interlaboratory variation in testing techniques and antigens. The use of cutoff titers for clinical cases can also lead to an incorrect diagnosis, since detectable anti-*Babesia* antibodies are not always present in dogs that are confirmed to be infected with *Babesia spp*. by PCR, microscopic observation, or both.

Interestingly, western piroplasm DNA was not detected in any dog in this study. This organism may not be endemic to North Carolina or dogs may clear the infection spontaneously. The seroreactivity to the western piroplasm that was detected in this study may represent cross-reactivity to other *Babesia* genotypes or other non*Babesia* organisms, or it may represent a level of parasitemia that may be below the limit of PCR detection. While there were two *B. gibsoni* PCR/microscopic observation-positive dogs that were only seroreactive to the western piroplasm, at least the same number of dogs were PCR/microscopic observation-positive and without any detectable anti-*Babesia* antibodies. Since PCR/microscopic observation testing was not performed on all nonseroreactive dogs, statistical analysis cannot be performed to determine whether or not the antiwestern piroplasm antibodies identified in this study were actually associated with detectable *Babesia* DNA by PCR or were simply false-positive results. Since sera from some dogs from Australia, a country where it was presumed that the western piroplasm is not endemic, had reciprocal antibody titers against western piroplasm antigens as high as 160, Yamane, *et al*., suggested using a reciprocal antibody titer of 320 as a cutoff for dogs with clinical signs of babesiosis, and a reciprocal antibody titer of 1,280 for epidemiological studies when using the western piroplasm as an antigen source(Yamane et al., 1993b).

The route of *B. gibsoni* transmission in the US has not been identified. In other endemic areas, tick transmission is believed to be the primary route of infection(Yamane et al., 1993a). Based on the absence of ticks at the time of sampling and the reported historical lack of tick infestation, it does not appear that tick transmission is the primary route of transmission in these kennels. Since detailed histories of the stray dogs infected

with or exposed to *Babesia* were not available, it was impossible to determine how they were infected. Since babesiosis can be chronic, the ticks found on the dogs at the time of sample collection are not necessarily the same ticks that transmitted the infection to the dogs. Other than ticks, the potential routes of transmission for *B. gibsoni* include vertical transmission, direct transmission, and mechanical transmission. Vertical transmission seems plausible(Abu et al., 1973). The authors have also diagnosed *B. gibsoni* in puppies as young as 10 days of age, a time interval that is shorter than the prepatent period following tick transmission. Direct transmission via blood transfer during fighting contact also seems plausible. The authors have diagnosed at least four cases of *B. gibsoni* infection in nonAPBT/AST dogs after they have been in an accidental fight with a carrier animal, and other dogs have been diagnosed with *B. gibsoni* after being attacked by APBT/AST dogs(Irizarry-Rovira et al., 2001). Possible mechanical vectors to consider include biting flies and needles used to vaccinate multiple animals. There is a growing body of evidence that *R. sanguineus* is a potential vector for *B. gibsoni*;(Higuchi et al., 1995; Higuchi et al., 1999a; Higuchi et al., 1999b). however, definitive transmission studies have yet to be performed. Future studies that examine the transmission of *B. gibsoni* among dogs in the US are warranted.

Both *B. canis vogeli* and *B. gibsoni* appear to be endemic to North Carolina. There are *B. gibsoni* epizootics currently affecting APBT and AST kennels. This study also demonstrates that no single diagnostic test will confirm all cases of canine babesiosis. Therefore, if canine babesiosis is suspected, the authors recommend that serology, microscopic observation, and PCR be performed. Serology against both *B. canis* and *B. gibsoni* should be performed, and if serology against the western piroplasm

is performed, the guidelines of Yamane, *et al*., should be considered. Caution is warranted in the use of antibody titers for *Babesia* species identification, since the highest antibody titer did not always correlate with the microscopic and PCR testing results. Lastly, PCR testing should screen for all known genotypes.

Table 2
Results From 21 of the Stray Dogs (n=359) Evaluated for *Babesia* That Had Positive Test Results

Case No.	Location[*]	Microscopy[†]	*Babesia canis* IFA[‡]	*Babesia gibsoni* IFA[‡]	Western piroplasm IFA[‡]	*Babesia canis* PCR[§]	*Babesia gibsoni* PCR[§]	Western piroplasm PCR[§]
1	**ENC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
2	**ENC**	**-**	**256**	**-**	**-**	+	**-**	**-**
3	**ENC**	**-**	**256**	**-**	**-**	**-**	**-**	**-**
4	**ENC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
5	**ENC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
6	**ENC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
7	**ENC**	**-**	**-**	**-**	**64**	**-**	**-**	**-**
8	**ENC**	**-**	**128**	**-**	**-**	**-**	**-**	**-**
9	**CNC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
10	**CNC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
11	**CNC**	**-**	**-**	**-**	**128**	**-**	**-**	**-**
12	**CNC**	**-**	**128**	**-**	**128**	**-**	**-**	**-**
13	**CNC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
14	**CNC**	**-**	**-**	**256**	**-**	**-**	**-**	**-**
15	**CNC**	+	**128**	**-**	**-**	**-**	+	**-**
16	**CNC**	**-**	**-**	**-**	**128**	**-**	+	**-**
17	**CNC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
18	**CNC**	**-**	**64**	**-**	**-**	**-**	**-**	**-**
19	**WNC**	**-**	**-**	**128**	**-**	**-**	**-**	**-**
20	**WNC**	**-**	**-**	**-**	**64**	**-**	**-**	**-**
21	**WNC**	**-**	**64**	**-**	**64**	**-**	**-**	**-**

[*] ENC=eastern North Carolina; CNC=central North Carolina; WNC=western North Carolina
[†] - = No piroplasms observed; + = small piroplasms observed
[‡] IFA=indirect fluorescent antibody; reciprocal antibody titer; - = reciprocal antibody titer <64
[§] PCR=polymerase chain reaction; - = no *Babesia* DNA detected; + = *Babesia* DNA detected

Table 3
Results From 24 of the Kennel Dogs (n=159) Tested for *Babesia* That Had Positive Test Results

Case No.	Location[*]	Microscopy[†]	*Babesia canis* IFA[‡]	*Babesia gibsoni* IFA[‡]	Western piroplasm IFA[‡]	*Babesia canis* PCR[§]	*Babesia gibsoni* PCR[§]	Western piroplasm PCR[§]
1	**I**	+	-	**512**	-	-	+	-
2	**I**	+	-	**512**	-	-	+	-
3	**I**	+	-	-	-	-	+	-
4	**I**	+	-	-	-	-	+	-
5	**I**	-	-	**256**	-	-	-	-
6	**I**	-	-	**256**	-	-	-	-
7	**II**	-	**64**	-	-	-	+	-
8	**II**	+	**64**	**512**	-	-	+	-
9	**II**	+	**64**	**512**	-	-	+	-
10	**II**	+	**64**	**1024**	-	-	+	-
11	**II**	-	**256**	-	-	-	-	-
12	**II**	+	-	-	**128**	-	+	-
13	**II**	-	-	-	**64**	-	-	-
14	**II**	+	-	-	**128**	-	+	-
15	**II**	-	-	-	**64**	-	-	-
16	**II**	-	-	**128**	**128**	-	-	-
17	**III**	-	-	**64**	-	-	-	-
18	**III**	-	-	**1024**	-	-	-	-
19	**III**	-	-	**512**	-	-	-	-
20	**III**	-	-	**512**	-	-	-	-
21	**III**	+	-	**512**	-	-	+	-
22	**III**	+	-	**512**	-	-	+	-
23	**III**	+	-	**1024**	-	-	+	-
24	**III**	+	-	**1024**	-	-	+	-

[*] I=Kennel I; II=Kennel II; III=Kennel III
[†] + = Small piroplasms observed; - = no piroplasms observed
[‡] IFA=indirect fluorescent antibody; reciprocal antibody titer; - = reciprocal antibody titer <64
[§] PCR=polymerase chain reaction; - = no *Babesia* DNA detected; + = *Babesia* DNA detected

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Breed and dog-bite associated *Babesia* infections

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Abstract

This study was performed to identify the breed and geographic location of samples that tested positive for canine babesiosis in the North Carolina State University Vector-Borne Disease Diagnostic Laboratory (NCSU-VBDDL), to characterize the clinical findings in *B. gibsoni* infected dogs that were not American pit bull terriers, and to detect an association between a recent dogbite and *Babesia gibsoni* infection. The (NCSU-VBDDL) database was reviewed for all samples that had undergone *Babesia* specific PCR testing between May 2000 and October 2003. The complete medical records of 12 *B. gibsoni* infected dogs that were not American pit bull terriers (APBT) were reviewed. These 12 B. *gibsoni* infected non-APBT dogs were compared to a group of *Ehrlichia ewingii* infected dogs to detect an association between a recent dogbite and *B. gibsoni* infection.

Twenty-one percent (145/703) of the samples submitted tested positive for the presence of *Babesia* spp. DNA. Ninety percent (131/145) of these samples tested positive for *B. gibsoni*. Ninety-two percent (121/131) of the *B. gibsoni* positive samples were from American pit bull terriers. American pit bull terriers and greyhounds were more likely to test positive for *Babesia*. The clinical findings of the *B. gibsoni* infected dogs that are not American pit bull terriers were similar to the findings previously described with *B. gibsoni* infections. An association was detected between a recent dogbite and *B. gibsoni* infection, and the association was even stronger when the bite was inflicted by an American pit bull terrier.

Introduction

Babesiosis associated with the Asian genotype of *Babesia gibsoni*, is an emerging canine infectious disease in the United States (US)(Birkenheuer et al., 2003b; Birkenheuer et al., 1999; Irizarry-Rovira et al., 2001; Kocan et al., 2001; Macintire et al., 2002). Information about the epidemiology and transmission of *B. gibsoni* in the US is scarce. The tick vectors for *B. gibsoni*, *Haemaphysalis longicornis* and *Haemaphysalis bispinosa*,(Groves and Dennis, 1972; Otsuka, 1974; Swaminath, 1937) have not been reported in the US. *Rhipicephalus sanguineus*, the brown dog tick, is frequently reported to be a competent vector for *B. gibsoni*,(Taboada) and there is some evidence supporting *Rhipicephalus sanguineus* as a competent vector(Higuchi et al., 1995; Higuchi et al., 1999a; Higuchi et al., 1999b; Sen, 1933). However definitive studies under controlled conditions have yet to be performed. The validity of the study performed by Sen (1933) has been questioned since they did not employ the use of tick-proof kennels(Groves and Dennis, 1972; Yamane et al., 1993). Other than tick transmission, the only other route of transmission for *B. gibsoni* that has been documented is the inoculation of the blood from an infected dog into another susceptible canid, and it remains the primary route of infection used in experimental studies. (Groves and Dennis, 1972; Groves and Vanniasingham, 1970; Itoh et al., 1988; Roher et al., 1985; Ruff et al., 1973). There is some evidence to support peri-natal *B. gibsoni* transmission, but controlled studies have not been performed(Abu et al., 1973; Birkenheuer et al., 1999; Itoh and Itoh, 1990).

Two breeds associated with dog fighting, the APBT and Tosa Inu, have been reported to have increased infection rates in other parts of the world(Onishi et al., 1994; Suh et al., 1997). The majority of dogs reported to have *B. gibsoni* infections in the US

have been American pit bull terriers(Birkenheuer et al., 2003b; Birkenheuer et al., 1999; Kocan et al., 2001; Macintire et al., 2002). The reported prevalence of polymerase chain reaction (PCR) positive or seropositive dogs in APBT kennels, selected for study because of prior documentation of *B. gibsoni* in a kennel dog, ranged from 15-55%. (Birkenheuer et al., 2003b; Macintire et al., 2002). In contrast the rate of PCR positive or seropositive dogs housed in non-APBT kennels and animal shelters was 0-1% respectively(Birkenheuer et al., 2003b; Macintire et al., 2002). *Babesia gibsoni* infections have been reported after exposure to APBT dogs or their blood. For example, a mixed-breed dog from Indiana was reported to have been bitten by 3 APBTs prior to developing a *B. gibsoni* infection(Irizarry-Rovira et al., 2001) and a German Shepherd from Michigan was diagnosed shortly after receiving a blood transfusion from a *B. gibsoni* infected APBT(Stegeman et al., 2003).

In this study we reviewed the North Carolina State University College of Veterinary Medicine Vector-Borne Disease Diagnostic Laboratory (NCSU-VBDDL) database and identified the breed and geographic location of samples submitted to the for *Babesia* specific polymerase chain reaction (PCR) testing(Birkenheuer et al., 2003c). We also reviewed the medical records of *B. gibsoni* infected dogs that were not APBTs in order to describe the clinicopathologic findings in these *B. gibsoni* infected non-APBT dogs and determine whether or not there was an association between a recent dogbite by an APBT and *B. gibsoni* infection.

Materials and Methods

Case selection

Cases were selected from all samples submitted to the NCSU-VBDDL between May 2000 and October 2003 by searching the NCSU-VBDDL database. Six additional *B. gibsoni* positive non-APBT dogs were identified through searching the NCSU-VBDDL database prior to 2000, or via consultations through the NCSU-VBDDL

Medical record review

Descriptive data from the database including species, breed, age, sex, and geographic location were recorded for all samples submitted. Complete medical records were available for review for 12 of *B. gibsoni* infected dogs that were not American pit bull terriers. Findings recorded included; age, breed, sex, geographic location, rectal temperature, PCV, nucleated red blood cells (nRBC), reticulocytosis, platelet count, total bilirubin concentration, history of a recent (six months) dogbite or tick attachment prior to the diagnosis of the *B. gibsoni* infection. When the information regarding a recent dogbite or tick attachment was not readily available in the medical record, owners were contacted via the telephone.

Statistical analyses

Comparisons between groups were made using a fisher's exact test or chi square test. All calculations were performed using a statistical program (Statcalc, Epi Info 2002). Only the canine samples were included in the statistical analyses. In the non-APBT dogs infected with *B. gibsoni* a Fisher's exact test or chi square test was used to evaluate

potential associations between the *B. gibsoni* infection and a history of tick attachment or a history of being bitten by an APBT dog or any dog within 6 months of diagnosis. For statistical comparison to the *B. gibsoni* infected non-APBT dogs, a group of dogs that were infected with a different tick-transmitted infection, *Ehrlichia ewingii*, was used. (Goodman et al., 2003). To minimize the bias introduced by living in the same household as an infected APBT, when multiple dogs live in the same household as a *B. gibsoni* infected APBT each household was counted as a single case when setting up the contingency table.

Results

The canine samples submitted for *Babesia* specific PCR testing and other testing are summarized in table 4. Fifteen non-canine samples were submitted for *Babesia* specific PCR testing including: 9 feline samples, 4 equine samples, 1 cetacean sample, and 1 sample for which no species was listed. American pit bull terriers and greyhounds were overrepresented in the samples submitted for *Babesia* specific PCR testing ($p < 0.001$) compared to the canine samples submitted to the NCSU-VBDDL for other testing during the same time period. The samples that tested positive for the presence of *Babesia* spp. DNA by PCR are summarized in table 5. No samples tested positive for multiple *Babesia* spp. The proportion of American pit bull terriers that tested positive for the presence of *Babesia* spp. DNA by PCR (122/282) was significantly greater ($p < 0.001$) than all non-APBT dogs tested including greyhounds (23/406). One greyhound tested positive on three separate samples. The proportion of greyhounds that tested positive for *B. canis vogeli* (6/120) was significantly greater ($p = 0.02$) than the other non-APBT dogs that tested positive for *B. canis vogeli* (3/286). The greyhound that tested positive on multiple samples was treated as a single case. The states from which positive samples were submitted are presented in figure 1. The states from which no positive samples were submitted included AL, CO, HI, MN, MS, RI, TX, and WV. Eleven samples were submitted from TX, 7 from CO, 6 from MS and 1 each from AL, HI, MN, RI, and WV. Positive samples have been submitted to the NCSU-VDBBL from MS and WV prior to May 2000. *B. gibsoni* has been previously reported in AL(Macintire et al., 2002).

Eight percent (10/132) of the *B. gibsoni* positive samples were obtained from non-APBT dogs. One dog tested positive on 2 separate submissions. Six additional *B. gibsoni*

infected non-APBT dogs were identified in the NCSU-VDBBL database prior to May 2000 or via NCSU-VBDDL consultations with practitioners. Breeds represented were 3 boxers, 3 mixed breed, 2 Labrador retrievers 1 each: Rottweiler, English Bulldog, border collie, Boston terrier, Belgian malinois, German Shepherd and cocker spaniel. Five dogs were from Georgia, five from Florida, two from North Carolina, one from Michigan, one from Illinois and one dog was a military working dog for which a geographic location was not submitted. The dog from Michigan was a German shepherd that had received a whole blood transfusion from a *B. gibsoni* APBT prior to diagnosis and has been reported elsewhere(Stegeman et al., 2003). Ages of the non-APBT dogs that tested positive for the presence of *B. gibsoni* DNA, dogs ranged from 2-12 years. Nine were female and six were male. More detailed medical records were available for review from 12 of the *B. gibsoni* infected non-APBT dogs. Rectal temperature greater than 102.5°F was detected in 2/8 dogs for which the body temperature was recorded. Anemia, PCV ranging from 15-27% (Reference range 33-58%), was documented in 11/11 dogs that had a packed cell volume recorded. Reticulocytosis (>60,000 reticulocytes/μl) was detected in 7/7 dogs that had reticulocytes counted. Nucleated RBCs, ranging from 2-16/100 WBC, were observed on blood smear examinations in 11/12 dogs. Thrombocytopenia, platelet counts ranging from 32-147 x 10^3/μl (Reference range 200-400 x 10^3/μl), was detected in 8/9 dogs that had platelet counts measured. Hyperbilirubinemia was detected in 6/8 dogs for which serum biochemical profiles were available for review. A positive Coomb's test was detected in 2/3 dogs tested.

The history of being bitten by any dog, being bitten by an APBT, and history of tick attachment for the *B. gibsoni* infected non-APBT dogs was obtained either from the medical record or via the telephone for 12 of the *B. gibsoni* infected dogs and 12 *E. ewingii* infected dogs and are summarized in table 6. Two of the *B. gibsoni* infected non-APBT dogs had been obtained as strays within 6 months of the diagnosis. Another dog had no medical record available for review and the owner could not be contacted via the telephone. So it was unknown whether or not these 3 dogs had been bitten recently by another dog or had a history of tick attachment. These 3 dogs were excluded from the statistical analyses detecting potential associations between a recent dogbite or tick attachment and *B. gibsoni* infection. There was an association ($p = 0.014$) between a recent dogbite and *B. gibsoni* infections when breed of dog inflicting the bite was not taken into consideration. There was a stronger association ($p < 0.001$) between a recent bite by an APBT and *B. gibsoni* infection. Four of the *B. gibsoni* infected non-APBT dogs were housed on the same property as a *B. gibsoni* infected APBT and represented 2 separate households. The association remained ($p = 0.045$) when each household was counted as a single case and breed of dog inflicting the bite was not taken into consideration. Three of the APBT dogs that had inflicted bite-wounds were available for *Babesia* specific PCR testing, and 2 of them tested positive for *B. gibsoni*.

Discussion

The samples submitted to the NSCU-VBDDL for *Babesia* specific PCR testing were primarily canine in origin. This is not surprising since feline babesiosis has not been reported in the US, which is the primary region from which our samples are submitted. The overrepresentation of APBT and greyhound samples is probably due to the clinicians submitting samples having an increased index of suspicion for babesiosis in these breeds, as they are the primary breeds reported in the literature to have babesiosis in the US(Birkenheuer et al., 2003b; Birkenheuer et al., 1999; Breitschwerdt et al., 1983; Macintire et al., 2002; Taboada et al., 1992). Additionally the owners of these breeds frequently request that their dogs be tested for babesiosis.

Babesia gibsoni is the most commonly detected *Babesia* spp by PCR in the NCSU-VBBDL representing 90% of the *Babesia* positive samples. The results of the current study indicate that *B. gibsoni* infections are widespread and not limited to the Southeastern US. We did not evaluate travel history and this cannot be excluded as a possible confounder. The results of this study indicate that American pit bull terriers were significantly more likely to test positive for the presence of *Babesia* spp. DNA than the non-APBT dogs. This overrepresentation may be due to an increased susceptibility to infection or may be due to increased exposure to potential risk factors. It is the authors' opinion that increased exposure to potential risk factors is more likely than a genetic condition leading to increased susceptibility of infection. Tick transmission among the APBTs in the US seems unlikely. No ticks were detected in 3 kennels housing a total of 149 dogs with a high prevalence of seropositive dogs (14.9%) and a history of tick attachment was not reported by the owners(Birkenheuer et al., 2003b). This report also

indicates that greyhounds are significantly more likely to test positive for *B. canis vogeli* than non-greyhound dogs. This may be due to increased susceptibility or increased exposure to risk factors. Further studies are indicated to identify the risk factors associated with *Babesia* infections in both APBT dogs and greyhounds.

The clinical and hematological manifestations of *B. gibsoni* infections in non-APBT dogs does not appear to be different from those reported in APBT dogs or in *B. gibsoni* infections in other dogs that have been naturally or experimentally infected(Birkenheuer et al., 1999; Groves and Dennis, 1972; Irizarry-Rovira et al., 2001; Kocan et al., 2001; Meinkoth et al., 2002). These findings are identical to those associated with immune-mediated hemolytic anemia (IMHA) and immune-mediated thrombocytopenia (ITP). Without specific testing, dogs with babesiosis can easily be misdiagnosed as having IMHA or ITP. It has been our experience that *Babesia* infected dogs that have been treated with prolonged immunosuppressive therapy prior to anti-babesial therapy, do not respond as well as dogs that are treated with anti-babesial therapy early in their disease course(Birkenheuer et al., 2003a).

We have previously suggested that bite wounds or blood transmission during fighting contact as possible routes of transmission for *B. gibsoni*. This potential route of transmission is unlikely to ever be tested under controlled conditions. American pit bull terriers are frequently aggressive towards other dogs and are the most common breed associated with organized dog fighting in the US. American pit bull terriers owners are often reluctant to report whether or not their dogs were bitten by another dog prior to developing babesiosis. We did not believe that reliable results could be obtained regarding whether or not the *B. gibsoni* infected APBT dogs had been bitten by another

dog prior to developing babesiosis could be obtained and chose not to pursue further investigation within that population of dogs. In the non-APBT dogs there was an association between having been recently bitten by another dog, particularly an APBT, and *B. gibsoni* infection. This association may be questioned from the point of view that dogs living in the same household as a *B. gibsoni* infected dog are or could be more likely to be infected. However, the association between the dogbite and *B. gibsoni* infection remained significant when attempts were made to minimize this bias. The authors acknowledge that the historical control group used for statistical comparison is not ideal. However, due to the retrospective nature of the study, age, breed, sex, temporal and geographically matched controls were not readily available.

Interestingly three samples tested positive in a screening test that was designed to amplify the DNA from most piroplasms but tested negative for the presence of DNA from all known canine piroplasms when subjected to specific testing. One of these samples has been subjected to further testing including DNA sequencing and was determined to represent a genotypically unique organism(Birkenheuer, 2003). The other two samples have not been subjected to further testing.

This is the first study to document that APBT dogs are significantly more likely to be infected with *B. gibsoni* than other breeds of dogs. It is also the first study to document an association between *B. gibsoni* infection and having been bitten by an APBT. This study should prompt clinicians to question clients whether or not there is a history of a recent dogbite, especially by an APBT, in dogs that are presenting with anemia, thrombocytopenia, hyperbilirubinemia and fever.

Table 4. Overview of the samples submitted to the North Carolina State University Vector Borne Disease Diagnostic Laboratory (2000-2003).

		APBT*	Greyhound
Total number of samples submitted	7744		
Total number of canine samples submitted	5986	352	447
Total number of samples submitted for *Babesia* specific PCR	703		
Total number of canine samples submitted for *Babesia* specific PCR	688	282	120

* APBT, American pit bull terrier

Table 5. Overview of samples that tested positive for the presence of *Babesia spp.* DNA by PCR

	Positive for *B. gibsoni* DNA by PCR	Positive for *B. canis vogeli* DNA by PCR	Positive for "other" piroplasm DNA by PCR
Total	131	11	3
APBT*	121	0	1
Greyhound	0	8	0
Other breed	10	3	2

* APBT, American pit bull terrier

Figure 1. States from which *Babesia* PCR positive samples were submitted to the NCSU-VBDDL

† Positive samples were submitted from the states that are shaded gray
* *Babesia gibsoni* positive samples had been submitted from MS and WV to the NCSU-VBDDL prior to May 2000
** *Babesia gibsoni* has been reported in AL (Macintire et al., 2002).

Table 6. Overview of exposure to potential risk factors for infection.

	Dog bite	APBT* bite	Ticks	Housed with infected dog
Babesia gibsoni	9/12	9/12	2/12	4/12
Ehrlichia ewingii	3/12	0/12	5/12	2/12

* APBT, American pit bull terrier

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Molecular Characterization of Canine *Babesia* Isolates

Adam J. Birkenheuer

Abstract

Relatively little information exists in regards to the molecular characterization of canine *Babesia* spp. When this study began in 1999, there were only three gene sequences deposited in Genbank that were isolated from canine *Babesia* spp. We characterized several genes from canine *Babesia* spp. that have potential uses in phylogenetics, diagnostics, and as vaccine candidate antigens including 18S ribosomal RNA (rRNA), internal transcribed spacer regions (ITS), cytochrome B (*cytB*), and rhoptry-associated protein-1 (RAP-1). Analyses of these genes confirm the presence of three morphologically identical but genetically distinct small canine piroplasms. Regions of conservation and variability within these genes have been identified that can be used for screening tests as well as specific molecular diagnostic tests to differentiate species/strains.

Introduction

Prior to the beginning of this study there were relatively few gene sequences available in Genbank from canine *Babesia* isolates. In 1999 we described a series of dogs from North Carolina (NC) that were infected with small *Babesia* organisms that were presumed to be *B. gibsoni*(Birkenheuer et al., 1999). The North Carolina State University Vector-Borne Disease Diagnostic Laboratory (NCSU-VBDDL) had designed oligonucleotide primers to amplify either *B. canis* (Genbank acc. L13727) or *B. gibsoni* (Genbank acc. L13729) by PCR. Our preliminary investigations determined that when subjected to PCR, the small *Babesia* spp. samples isolated from dogs in NC produced amplicons with the *B. canis* primers but not the *B. gibsoni* primers. This prompted us to hypothesize that there were at least two genetically distinct small *Babesia* spp. capable of infecting dogs. In order to address this hypothesis we sought to characterize genes such as rRNA, ITS, *cytB*, RAP-1 that can be used for phylogenetic studies, diagnostic testing, and as potential vaccine candidate antigens.

Ribosomal Ribonucleic Acid (rRNA)

The 18S rRNA genes of canine *Babesia* spp. isolates were targeted for analysis for their usefulness in phylogenetics and diagnostic test development. Analysis of rRNA genes has become one of the most powerful tools available to characterize and infer phylogenetic relationships of both prokaryotic and eukaryotic organisms(Woese, 1987). The small ribosomal subunit (18S rRNA) gene is the most commonly utilized genes for these phylogenetic analyses. Ribosomal RNA genes are transcribed and their RNA products form ribozymes. The rRNA sequence dictates the structure and function of the

molecule. This means that they are not subjected to codon usage variability and wobble. In addition, rRNA gene sequences can be evaluated for co-variation to determine whether or not a change is sequence is likely to be a real mutation or sequencing error. Co-variation is assessed by evaluating an rRNA molecules proposed secondary structure. The secondary structures are predicted to consist of a series of stems and loops. If a base is involved in a stem structure a change in that position should result a corresponding change in the base that is predicted to be it's pair in the stem. We developed oligonucleotide primers to amplify full-length 18S rRNA genes from all known piroplasms. These 18S rRNA gene sequences can be used to infer phylogenetic relationships and develop oligonucleotide primers for diagnostic testing.

Internal transcribed spacer regions

The internal transcribed spacer (ITS) region of the ribosomal gene unit is located between the 18S and 28S (16S and 23S) rRNA subunit genes in most organisms. The ITS1 and ITS2 regions flank the 5.8S rRNA gene. In many species the ITS regions encode the gene sequences for transfer RNA (tRNA). The ITS regions usually have a higher degree of sequence variability compared to the rRNA genes themselves. We isolated *B. gibsoni* ITS regions to compare them to the known canine *Babesia* ITS sequences. As demonstrated above, the 18S rRNA gene sequences between species vary by only a few percent, and the difference between subspecies is often less than 2%. In contrast, the ITS regions of the same subspecies of *B. canis* only shared 70-82% sequence similarity. This variability makes the ITS region an attractive region for the development of DNA probes that can be used to differentiate closely related organisms.

Cytochrome *B*

The phylum Apicomplexa are a unique and diverse group of organisms. They have a unique small multi-copy mitochondrial DNA (mtDNA) sequence. The mtDNA typically include the genes for cytochrome B (*cytB*) and cytochrome c oxidase. These genes occur in relatively high copy numbers in most apicomplexans. Ranging in copy number from 15 to 150 copies per cell(Wilson and Williamson, 1997). This makes them excellent candidates as targets for molecular testing. For comparison, the most common target gene for PCR testing, the 18S rRNA gene subunit, has only three copies per cell(Dalrymple et al., 1992; Reddy et al., 1991). The sensitivity of a *cytB* based PCR test was better than that of an 18S rRNA based PCR test when compared using identical samples from a group of experimentally infected cattle(Salem et al., 1999). We sought to isolate and characterize *B. gibsoni cytB* genes as a potential target gene for molecular diagnostic testing.

The *cytB* molecule is also believed to be a target of the anti-protozoal drug atovaquone(Korsinczky et al., 2000; Srivastava et al., 1999; Syafruddin et al., 1999). Mutations in the putative drug binding site of this molecule have been associated with drug resistance in apicomplexans(Kazanjian et al., 2001; Korsinczky et al., 2000; McFadden et al., 2000; Srivastava et al., 1999; Syafruddin et al., 1999; Vaidya and Mather, 2000). Atovaquone in combination with azithromycin is the treatment of choice of *B. microti*, and the use of this drug combination for the treatment of *B. gibsoni* infections in dogs is described in chapter 6. We also sought to characterize *B. gibsoni cytB* genes to screen this gene sequence for mutations in isolates that were resistant to atovaquone and azithromycin.

Rhopty associated protein- 1 (RAP-1)

Babesia species infect the red blood cells (RBC) of mammals in a host-specific manner. Merozoites, the stage of the organism that infect the host RBC's, have structures called rhoptries that are involved in red blood cell invasion(Ristic, 1988). The Rap-1 proteins are associated with these rhoptries(Brown et al., 1998; Dalrymple, 1993; Dalrymple et al., 1993; Dalrymple et al., 1996; Skuce et al., 1996; Suarez et al., 1991). Immunization with Rap-1 proteins and rhoptries are immunoprotective against bovine *Babesia* infections, and antibodies against Rap-1 proteins prevent *Babesia* RBC invasion in vitro(Dalrymple, 1993; Machado et al., 1993). Unlike other merozoite surface proteins the Rap-1 protein sequences are conserved within a given *Babesia* species(Suarez et al., 1991). Therefore, the Rap-1 proteins are considered leading candidate antigens for anti-*Babesia*l vaccines. The Rap-1 genes are ≅1.5 kilobase (kb) multi-copy, tandem repeat genes, that do not contain introns and have been identified in all species of *Babesia* that have been examined to date.

Materials and methods Materials and Methods

Samples

18S rRNA: Whole blood from dogs naturally or experimentally infected with *B. gibsoni* (Asian genotype), *B. gibsoni* (California genotype), *B. canis vogeli*, *B. canis canis*, *B. canis rossi* and *Theileria annae*. Whole blood from one dog experimentally infected with a cryostabilate of whole blood from one dog that had been experimentally infected with a two different isolates, a "Gulf Coast" *B. canis* isolate and a *B. canis* isolate from North Carolina.

ITS: Whole blood from dogs naturally and experimentally infected with *B. gibsoni* (Asian genotype) (n=2), *B. gibsoni* (California genotype) and *B. canis canis*.

Cytochrome B: Whole blood from a dog experimentally infected with *B. gibsoni* (Asian genotype).

RAP-1: Whole blood from dogs naturally and experimentally infected with *B. gibsoni* (Asian genotype). Whole blood from a dog experimentally infected with *B. canis canis*.

Preparation of DNA

DNA was isolated from canine whole blood samples using the QIAamp DNA blood mini kit according to the manufacturer's instructions. Some PCR amplicons were column purified to remove unincorporated PCR primers and primer dimers. Plasmid DNA was isolated with the QIAprep spin miniprep according to the manufacturer's instructions.

Oligonucleotide Primers

The DNA sequences for the oligonucleotide primers used in these studies are listed in table 7.

18S rRNA:

Oligonucleotide primers were designed to amplify partial and nearly full-length 18S rRNA genes based on alignments of the known piroplasm 18S rRNA gene sequences (Clustal, DNAStar) *B. canis rossi* (L19079), *B. gibsoni* (Asian genotype) (AF271081), *B. gibsoni* (California genotype) (AF158702), *T. annae* (AF188001), *B. bigemina* (X59607), *B. caballi* (Z15104), *B. odocoilei* (U16369), *B. divergens* (U07885), *B. bovis* (L19077), *B. microti* (U09833), *B. rodhaini* (M87565) *T. equi* (Z15105) and human 18S rRNA gene sequence. Care was take to ensure that the 3' end of the primers did not match mammalian 18S rRNA gene sequences.

ITS:

Oligonucleotide primers RIB13 and RIB3 designed by Zahler were used to amplify the ITS1, 5.8S rRNA, and ITS2 regions(Zahler et al., 1998).

Cytochrome B:

Oligonucleotide primers JD279and JD280 designed by Salem were used to amplify partial *cytB* genes(Salem et al., 1999).

RAP-1:

Degenerate oligonucleotide primers designed by Dalrymple were used to amplify putative partial RAP-1 gene sequences(Dalrymple et al., 1996).

Polymerase chain reaction conditions

In order to prevent PCR amplicon contamination, sample preparation, reaction setup, PCR amplification, and amplicon detection were all performed in separate areas. Positive and negative controls were used in all processing steps including the DNA extraction. The presence of PCR inhibitors in DNA samples that tested negative in our PCR test was excluded by the amplification of a fragment of the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene. Specific cycling conditions and reagent concentrations for each PCR reaction are listed in the appendices.

Amplicon detection

All PCR products were visualized after electrophoresis in a 1 or 2% agarose gel containing 0.2μg/ml concentration of ethidium bromide by transillumination with an ultraviolet light.

Cloning and DNA Sequencing

All amplicons were cloned into the TOPO PCR 2.1 or P-GEM T-easy vectors and competent *E. coli* were transformed according to the manufacturer's instructions. Recombinants were selected by blue/white color of colonies and plasmid DNA from at least three clones, for each isolate, were sequenced. Recombinant plasmid DNA was

sequenced bi-directionally with infrared fluorescent-labeled primers M13R-700 (5'CAGGAAACAGCTATGACCATG) and T7-800 (5'TAATACGACTCACTATAGGGCGA) synthesized by LI-COR In*c.*, Lincoln, NE, USA. Previously described internal sequencing primers, 515F and 1391R, were also used for full-length 18S rDNA sequencing(Pitulle et al., 1999). The sequencing reaction conditions were: 2 min. at 92 °C, followed by 30 amplification cycles (30 se*c.* at 92° C, 15 se*c.* at 55° C, 30 se*c.* at 72° C) (Hybaid PCR Express). The sequencing reactions were analyzed by polyacrylamide gel electrophoresis (3.75%) on an automated DNA sequencer (LI-COR 4200 DNA Sequencer, LI-COR In*c.*, Lincoln, NE, USA).

Results

18S rRNA:

The samples tested yielded amplicons ranging in size from 1600-1800 bp, when the oligonucleotide primer pair 5-22F/1661R was used. Figure 2 shows amplicons from *B. gibsoni* (Asian genotype), *B. gibsoni* (California genotype), and *B. c. canis*. Data not shown for *B. c. vogeli*, *B. c. rossi*, and *T. annae*. A DNA alignment of the sequences generated in this study and those from Genbank is shown in Figure 3. A sequence identity matrix comparing the sequences generated in this study to the sequences in Genbank utilized for the original alignment is presented in table 8.

ITS:

The *B. gibsoni* (Asian genotype) samples produced ≈ 900 base pair (bp) products, the *B. c. canis* sample yielded an 870bp product and the *B. gibsoni* (CA genotype) yielded an ≈ 1000 bp product after amplification. A DNA sequence alignment of the ITS sequences characterized in this study to the *Babesia* ITS sequences in Genbank is presented in figure 4. A sequence identity matrix comparing the sequences identified in this study to each other and the previously published canine *Babesia* ITS sequences is presented in table 9.

CytB

The *B. gibsoni* (Asian genotype) sample yielded an ≈ 740 bp product after PCR amplification. A DNA sequence alignment comparing the *B. gibsoni* (Asian genotype)

ctyB partial gene sequence to the *Piroplasmida cytB* gene sequences in Genbank is shown in figure 5. A sequence identity matrix comparing the sequence identified in this study to the previously published *Babesia* spp. *cytB* gene sequences is presented in table 10

RAP-1

The *B. gibsoni* (Asian genotype) samples yielded an ≈ 250 bp amplicon and the *B. c. canis* sample yielded an ≈ 240 bp after PCR amplification. (Figure 6) A 250bp product was amplified from non-infected canine DNA at lower annealing temperatures, and was no longer visible at the final annealing temperature of 58*C.* The proposed reading frame was utilized to translate the DNA sequences and an alignment of the putative amino acid sequences is presented in figure 7. A sequence identity matrix comparing the sequences identified in this study to the previously published RAP-1 sequences is presented in table 11.

Conclusions

18S rRNA

The oligonucleotide primer pair designed to amplify the nearly full-length 18S rRNA genes from all known *Babesia* spp. produced robust PCR products of the expected size from all of the samples tested without amplifying any other detectable PCR products. The *B. gibsoni* (Asian genotype) 18S rRNA gene sequences characterized in this study were 99.9-100% identical to the previously published *B. gibsoni* (Asian genotype) 18S rRNA gene sequences. The fact that the *B. gibsoni* (Asian genotype) 18S rRNA gene sequences are highly conserved, including the hypervariable regions, among isolates from all over the world indicates that this gene is an excellent target for diagnostic test development. Sequence comparisons between canine small *Babesia* isolates confirmed the presence of not two, but three genetically distinct organisms.

Interestingly the 18S rRNA gene sequence from the whole blood of the dog experimentally infected with a cryostabilate of whole blood from another dog that had been experimentally infected with a two different isolates, a "Gulf Coast" *B. canis* isolate and a *B. canis* isolate from North Carolina, was 100% identical to that of *B. canis canis*. *Babesia c. canis* has not been reported in the US. It cannot be determined whether or not both isolates were *B. c. canis* or if one isolate thrived during passages and infection. Unfortunately, the original isolates are no longer available for testing. The presence of *B. c. canis* in a sample that was presumed to originate in the US is interesting and the presence of this subspecies in the US could easily go undetected because the disease manifestations in natural disease are indistinguishable, and the organisms are morphologically indistinguishable via light microscopy.

ITS

The internal transcribed spacer region sequences characterized in this study, as expected, contained less sequence similarity between organisms compared to the 18S rRNA gene sequences. As is the case with other species, the ITS regions can be used to differentiate closely related organisms. There does however appear to greater intra-species variation in the ITS regions compared to the 18S rRNA genes. More isolates of each species should be sequenced to identify highly conserved and variable regions that might be used to design oligonucleotide probes to differentiate species and discriminate isolates of the same species. Some of the intra-species sequence variability may be due to sequence differences between gene copies within an individual isolate. More work needs to be performed to characterize the copy number and microheterogeneity of ribosomal gene units in canine *Babesia* spp. Since the 18S rRNA and ITS regions occur in the genome as a continuous gene unit there is no obvious advantage to using ITS as a target for molecular diagnostic testing over 18S rRNA in terms of gene copy number and test sensitivity. The higher degree of sequence dissimilarity could prove useful however for increasing specificity of molecular tests using the ITS region as a target.

The Asian and Californian genotypes shared a higher degree of sequence similarity (57.1%) than the Asian *B. gibsoni* genotype shared with the *B. canis* subspecies (51.3-54.5%). In contrast, the Asian *B. gibsoni* genotype shares a higher degree of sequence similarity with the *B. canis* subspecies (94-95.5%) than it does with the California/USA genotype of *B. gibsoni* (85.8%) when comparing nearly full-length 18S rRNA gene sequences. Although this may have significance in regards to the

development of oligonucleotide probes for diagnostic testing, the phylogenetic implications are unclear. It is well accepted that phylogenetic relationships of most organisms can be inferred from 18S rRNA analysis. However the usefulness of ITS gene sequences for phylogenetic analyses has not been evaluated as extensively.

CytB

The sequence of the amplicons derived from the *B. gibsoni* (Asian genotype) using the primers designed to amplify apicomplexan *cytB* genes had a high degree of similarity to the previously described apicomplexan *cytB* sequences. The gene sequence did not contain any stop codons when translated using the open reading frame deduced from the other sequences. This sequence is likely to represent the partial gene sequence for the *cytB* gene of *B. gibsoni* (Asian genotype). The *cytB* gene has both diagnostic and therapeutic implications in the study of canine babesiosis. In all apicomplexan organisms studied to date, the *cytB* gene has been located in the extrachromasomal mitochondrial DNA. This is a high-copy gene which makes it an attractive candidate for molecular diagnostic testing. The use of *cytB* as a target gene for a PCR test has potential advantages regarding test sensitivity. The specificity of developing a PCR test based on this gene sequence is not easily predicted because the *cytB* gene sequences for the closely related organisms are not yet known. The *cytB* genes from more isolates and more species should be characterized to determine whether or not they are as well conserved as the rRNA and ITS genes before *cytB* based molecular tests are developed.

The partial *cytB* gene sequence from the *B. gibsoni* (Asian genotype) contains the sequence for one of the putative drug binding sites for atovaquone that is associated with

drug resistance in *Plasmodium* spp. As described in chapter 6 an atovaquone and azithromycin combination therapy is the only treatment to successfully eliminate or suppress *B. gibsoni* infections below the limit of detection. The partial *cytB* sequence can be amplified from atovaquone resistant isolates to screen directly for mutations or used as probe to identify, clone and sequence the full-length *cytB* gene for analysis.

RAP-1

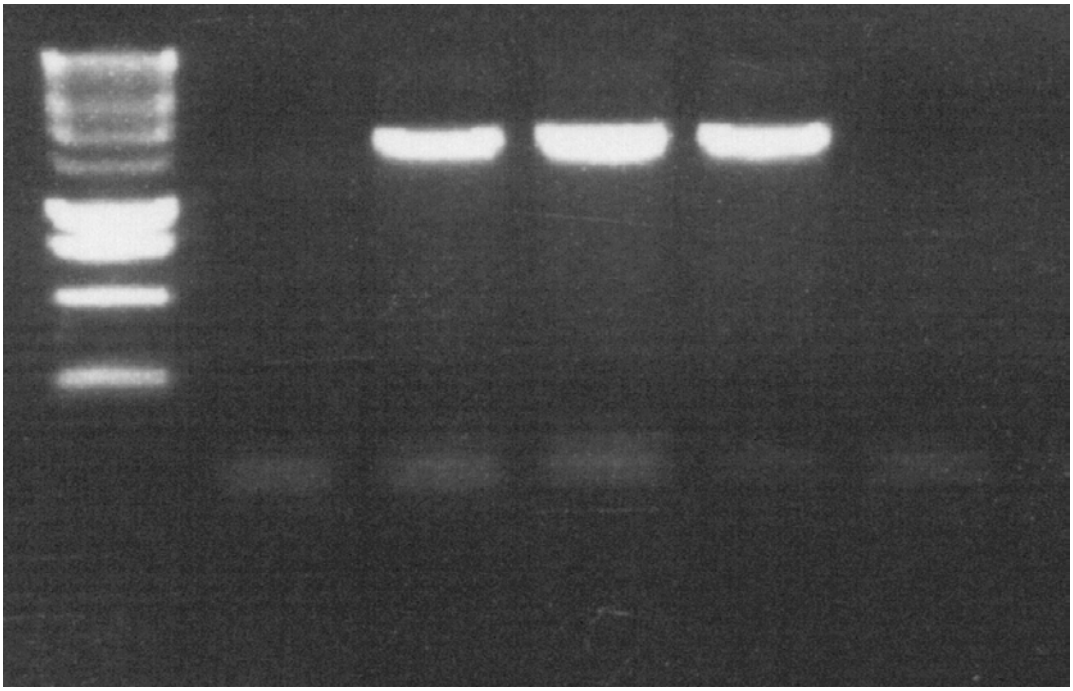
The degenerate oligonucleotide primers designed by Dalrymple generated a ≈250 bp product from *B. gibsoni* (Asian genotype) and ≈240 bp product from *B. c. canis*. When translated using the reading frame deduced from the known RAP-1 sequences, the *B. c. canis* sequence shared a high degree of sequence similarity to the *B. canis* sequences published by Dalrymple (73.7%). The proposed amino acid sequence derived from the *B. gibsoni* (Asian genotype) sequences shared the highest degree of sequence similarity to the *B. c. canis* sequence (31.2%). The degree of similarity of the putative *B. gibsoni* (Asian genotype) RAP-1 amino acid sequence to the other *Babesia* spp. RAP-1 sequences was lower (16.6-28.1%), but was more similar than the putative *B. divergens* RAP-1 sequence was to the other sequences (9.5-22.2%). These putative partial RAP-1 gene sequences are of potential diagnostic and therapeutic importance for canine babesiosis. A number of serodiagnostic tests for other *Babesia* spp. have been developed using RAP-1 genes as an antigen. They have been shown to be sensitive and specific for the detection of anti-*Babesia* antibodies. At this time, serologic testing for canine babesiosis in most laboratories is based on whole organism preparations which r*equi*re

either dogs for infection or dogs to donate blood for in vitro culture. The development of an enzyme linked immunosorbant assay based on recombinant RAP-1 proteins offers an attractive alternative to the current testing modalities.

In addition to its potential uses in serodiagnostics, RAP-1 proteins are leading canidates for inclusion in recombinant *Babesia* vaccines. The RAP-1 protein was determined to be at least partially protective by Wright (1992) during an experiment in which cattle were immunized with fractions of whole *B. bovis* parasites. It has been studied extensively since then and although it does not appear to confer complete protection when used as a single antigen, it is still considered a leading candidate for inclusion in a mulit-valent vaccine. The only vaccine available for canine babesiosis consists of a supernatant obtained from in vitro *B. c. canis* cultures and is only available in Europe. This vaccine does not confer complete protection from infection, but does reduce clinical signs and parasitemia. It is only protective against *B. c. canis*. The high degree of similarity between the *B. c. canis* RAP-1 sequence identified in this study with the *B. canis* sequence in Genbank, (presumably *B. canis vogeli*) might indicate that it could be used as an vaccine antigen for all subspecies of *B. canis*. More isolates should be sequenced for comparison.

Table 7. Sequences for the oligonucleotide primers used in this chapter

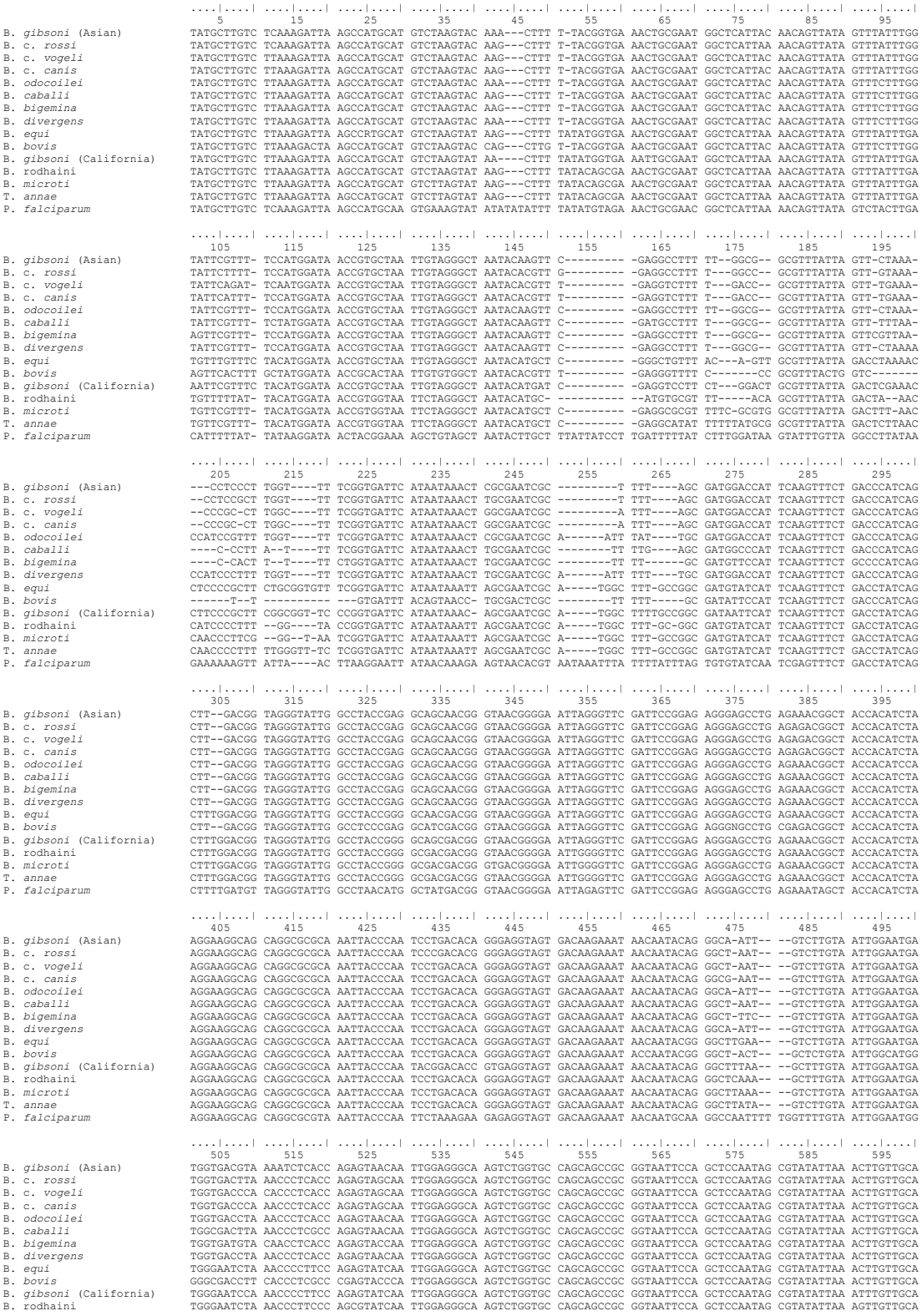
Primer Name	Sequence (5'-3')	Reaction/Use
5-22F	GTTGATCCTGCCAGTAGT	Full-length 18S rRNA Forward Primer
1661R	AACCTTGTTACGACTTCTC	Full-length 18S rRNA Reverse Primer
RIB-13	CCGAATTCTTTGTGAACCTTATCA	ITS Forward Primer
RIB-3	CGGGATCCTTCRCTCGCCGYTACT	ITS Reverse Primer
JD279	TGGAAYTTWGGGTTT	CYTB Forward Primer
JD280	CCATTCTGGWAWWATRTG	CYTB Reverse Primer
RAP-1FD	NCCNRANCCTAYCARTTGGANGCNGC	RAP-1 Forward Primer
RAP-1RD	CCGNTGSTRSATRATGTTYTGNATRRAN	RAP-1 Reverse Primer
G3PDH-F	CCTTCATTGACCTCAACTACAT	Detection of PCR inhibitors
G3PDH-R	CCAAAGTTGTCATGGATGACC	Detection of PCR inhibitors



1 2 3 4 5 6

Figure 2. Analysis of 18S rRNA PCR products by 1% agarose gel electrophoresis and ethidium bromide staining. From left to right the lanes contain the following: 1KB Molecular weight marker (lane 1), canine DNA (lane 2), *B. gibsoni* (Asian genotype) (lane 3), *B. c. canis* (lane 4), *B. gibsoni* (California/USA genotype) (lane 5), negative (no DNA) control (lane 6).

Figure 3. Alignment of 18S rRNA gene sequences



```
B. microti                TGGGAATCTA AACCCTTCCC AGAGTATCAA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC GGTAATTCCA GCTCCAATAG CGTATATTAA AGTTGTTGCA
T. annae                  TGGGAATCTA AACCCTTCCC AGAGTATCAA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC GGTAATTCCA GCTCCAATAG CGTATATTAA AGTTGTTGCA
P. falciparum             TGGGAATTTA AAACCTTCCC AGAGTAACAA TTGGAGGGCA AGTCTGGTGC CAGCAGCCGC GGTAATTCCA GCTCCAATAG CGTATATTAA AATTGTTGCA

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                             605        615        625        635        645        655        665        675        685        695
B. gibsoni (Asian)        GTTAAAAAGC TCGTAGTTGA ATTTCTGCG- ---------- ---------- ---------- ---------- --TTGCCCGA CT----CGGC T-----ACTT
B. c. rossi               GTTAAAAAGC TCGTAGTTGT ATTTTTGC-- ---------- ---------- ---------- ---------- --TTGGCGGT TTG--TTGCC TT----TGTG
B. c. vogeli              GTTAAAAAGC TCGTAGTTGA ATTTTAGCG- ---------- ---------- ---------- ---------- --TGTTCGAG TTT--GCCAT TC----GTTT
B. c. canis               GTTAAAAAGC TCGTAGTTGT ATTTTTGCG- ---------- ---------- ---------- ---------- --TTGACGGT TTG--ACCAT TT----GGTT
B. odocoilei              GTTAAAAAGC TCGTAGTTGA ATTTCTGCG- ---------- ---------- ---------- ---------- --TCACCGTA TT---TTGAC T-----TTTG
B. caballi                GTTAAAAAGC TCGTAGTTGA ATTTCTGCG- ---------- ---------- ---------- ---------- --TTGCGTTG TTC--TTGCT TT-----TTG
B. bigemina               GTTAAAAAGC TCGTAGTTGT ATTTCAGCC- ---------- ---------- ---------- ---------- --TCGCGTTT TTT--CCCTT TT----GTTG
B. divergens              GTTAAAAAGC TCGTAGTTGA ATTTTTGCG- ---------- ---------- ---------- ---------- --TGGTGTTA ATA--TTGAC T-----AATG
B. equi                   GTTAAAAAGC TCGTAGTTGA ATTTCTGCT- ---------- ---------- ---------- ---------- --GTTTCGTT GAC-TGCGCT T-----GGCG
B. bovis                  GTTAAAAAGC TCGTAGTTGA ATCTC-ACG- ---------- ---------- ---------- ---------- --TCCCC--- ------CGCT T------G-G
B. gibsoni (California)   GTTAAAAAGC TCGTAGTTGA ACTTCTGCC- ---------- ---------- ---------- ---------- --GCCGGGAC TTCGTTCCCT TCGG--GGCT
B. rodhaini               GTTAAGAAGC TCGTAGTTGA ATTTCTGCC- ---------- ---------- ---------- ---------- --TCGTCGTT TATGCTCGCT TCCA--AGCG
B. microti                GTTAAGAAGC TCGTAGTTGA ATTTCTGCC- ---------- ---------- ---------- ---------- --TTGTCATT AAT-CTCGCT TCCG--AGCG
T. annae                  GTTAAGAAGC TCGTAGTTGA ATTTCTGCC- ---------- ---------- ---------- ---------- --TTATCATT AAT-TTCGCT TCCG--AACG
P. falciparum             GTTAAAACGC TCGTAGTTGA ATTTCAAAGA ATCGATATTT TATTGTAACT ATTCTAGGGG AACTATTTTA GCTTTTGGCT TTAATACGCT TCCTCTATTA

                          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                             705        715        725        735        745        755        765        775        785        795
B. gibsoni (Asian)        GC-CTTGTCT GGTTTCGCT- ---TTTG--- ----G----- ---------- ---GGTTTTC CC----CTTT TTACTTTGAG AAAATTAGAG TGTTTCAAGC
B. c. rossi               GC-TTGATTC CGCTTGGCT- ---TTTG--- ----G----- ---------- ---CTT-TTT GC----CTTA TTACTTTGAG AAAATTAGAG TGTTTCAAGC
B. c. vogeli              GG-CTTTTTC GAGTTCGCT- ---TTTG--- ----G----- ---------- ---GT--TTT CC----CTTT TTACTTTGAG AAAATTAGAG TGTTTCAAGC
B. c. canis               GG-TTATTTC GTTTTCGCT- ---TTTG--- ----G----- ---------- ---GAA-TTT CC----CTTT TTACTTTGAG AAAATTAGAG TGTTTCAAGC
B. odocoilei              TC-GACTGTC GGTTTCGCT- ---TTTG--- ----G----- ---------- ---GATTTAT CC----CTTT TTACTTTGAG AAAATTAGAG TGTTTCAAGC
B. caballi                CT-TGATTTT CGCTTCGCT- ---TTTT--- ----G----- ---------- ---------- -------TTT TTACTTTGAG AAAATTAGAG TGTTTATCGC
B. bigemina               GG-TCTTTTC G--CTGGCT- ---TTT---- ---------- ---------- ---------- -------TTT TTACTTTGAG AAAATTAGAG TGTTTCAAGC
B. divergens              TC-GAGATTG CACTTCGCT- ---TTTG--- ----G----- ---------- ---GATTTAT CC----CTTT TTACTTTGAG AAAATTAGAG TGTTTCAAGC
B. equi                   TT-TGTCATC G---TTGCG- ---GCTT--- ----G-GTTG GGTTTCGATT ATTCGTTTCC CG----GCGT TTACTTTGAG AAAATTAGAG TGCTTGAAGC
B. bovis                  TCCTTTCCTC GCCGGGACG- ---CCT---- ---------- ---------- ---------- --------CG TTACTTTGAG AAAATTAGAG TGTTTCAAGC
B. gibsoni (California)   TCGTTTTCTC GG--TGGCA- ---TCCCTCT GGTTAATTTG GGCCTCGGCC CTCTTTTTCC AG----TTTT T-ACTTTGAG AAAATTAGAG TGTTTCAAGC
B. rodhaini               TT-TGTAATT GACTTGGCA- ---TCTT--- ---TGTGGAT TAGTGCGCAA GCATTTTTCC AC----TATT T-ACTTTGAG AAAACTAGAG TGTTTCAAAC
B. microti                TTTTTTTATT GACTTGGCA- ---TCTTCTG GA--TTTGGT GCCTTCGGGT ACTATTTTCC AG----GATT T-ACTTTGAG AAAACTAGAG TGTTTCAAAC
T. annae                  TAATTTTATT GATTTGGCA- ---TCTTCTG GATTATATAT TATTTTTAAT ATATTTTTCC AG----TATT TTACTTTGAG AAAACTAGAG TGTTTCAAAC
P. falciparum             TTATGTTCTT TAAATAACAA AGATTCTTTT TAAAATCCCC ACTTTTGCTT TTGCTTTTTT GGGGATTTTG TTACTTTGAG TAAATTAGAG TGTTCAAAGC

                          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                             805        815        825        835        845        855        865        875        885        895
B. gibsoni (Asian)        AGA------- --CTTGTGTC ----TTGAAT ACTTCAGCAT GGAATAATAA AGTAGGACT- TTGGTTCTAT TTTGTTGGTT TGTGAACCTT A-----G-TA
B. c. rossi               AGA------- --CTTTTGTC ----TTGAAT ACTGTAGCAT GGAATAATAG AGTAGGACT- TTGGTTCTAT TTTGTTGGTT TGGGAACCTT G-----GGTA
B. c. vogeli              AGA------- --CTTTTGTC ----TTGAAT ACTTCAGCAT GGAATAATAG AGTAGGACT- TTGGTTCTAT TTTGTTGGTT ATTGAACCTT A-----G-TA
B. c. canis               AGA------- --CTTTTGTC ----TTGAAT ACTTCAGCAT GGAATAATAG AGTAGGACT- TTGGTTCTAT TTTGTTGGTT ATTGAACCTT A-----G-TA
B. odocoilei              AGA------- --CTTTTGTC ----TTGAAT ACTTCAGCAT GGAATAATAG AGTAGGACT- TTGGTTCTAT TTTGTTGGTT TGTGAACCTT A-----G-TA
B. caballi                AGA------- --CTTTTGTC ----TTGAAT ACTTCAGCAT GGAATAACAT AGTAGGACC- TTGGTTCTAT TTTGTTGGTT TGGGACCTTG -------GTA
B. bigemina               AGA------- --CTTTTGTC ----TTGAAT ACTTCAGCAT GGAATAATAG AGTAGGACC- TTGGTTCTAT TTTGTTGGTT TTGAGCCTTG -------GTA
B. divergens              AGA------- --CTTTTGTC ----TTGAAT ACTTCAGCAT GGAATAATAG AGTAGGACT- TTGGTTCTAT TTTGTTGGTT TGTGAACCTT A-----G-TA
B. equi                   AGG------- --CTTTTGCC ----TTGAAT ACTTTAGCAT GGAATAACAG AGTAGGACT- TTGGTTCTAT TTTGTTGGTT TTAGGAGCCA G-----AGTA
B. bovis                  AG-------- ---GTTTCGC ----CTGTAT AATTGAGCAT GGAATAACCT TGTATGACCC TGTCGTACCG TTGGTTGGCT TTGG------ ------GTA
B. gibsoni (California)   AGG------- --CTTTTGCC ----TTGAAT ACTTCAGCAT GGAATAATAA AGTAGGACT- TTGGTTCTAT TTTGTTGGTT TCAG-GACCA A-----AGTA
B. rodhaini               AG-------- --CAATTGCC ----TTGAAT ACTACAGCAT GGAATAATAA AGTAGGACT- TTGGTTCTAT TTTGTTGGTT TTTG-AGCCA T-----AGTA
B. microti                AGG------- --CATTCGCC ----TTGAAT ACTACAGCAT GGAATAATGA AGTAGGACT- TTGGTTCTAT TTTGTTGGTT ATTG-AGCCA G-----AGTA
T. annae                  AGG------- --CTTTTGCC ----TTGAAT ACTACAGCAT GGAATAATGA AGTAGGACTA TTGGTTCTAT TTTGTTGGTT ATCG-AACCA T-----AGTA
P. falciparum             AAACAGTTAA AGCATTTACT GTGTTTGAAT ACTATAGCAT GGAATAACAA AATTGAACAA GCTAAAATTT TTTGTTCTTT TTTCTTATTT TGGCTTAGTT

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                             905        915        925        935        945        955        965        975        985        995
B. gibsoni (Asian)        ATGGTTAATA GGAACGG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. c. rossi               ATGGTTANTA GGAACGG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. c. vogeli              ATGGTTAATA GGAACGG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. c. canis               ATGGTTAATA GGAACGG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. odocoilei              ATGGTTAATA GGAACGG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. caballi                ATGGTTAATA GGAACGG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAACGA TTTGCCAAGG
B. bigemina               ATGGTTAATA GGAACGG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACCAC TGCGAAAGCA TTTGCCAAGG
B. divergens              ATGGTTAATA GGAACGG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. equi                   ATGGTTAATA GGAACAG-TT GGGGGCATTC GTATTTGACT GTCAGAGGTG AAATTCTTAG ATTTGTCAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. bovis                  ATGGTTAATA GGAACGG-TT GGGGGCATTC GTACTCGACT GTCAGAGGTG AAATTCTTAG ATTTGTCGAT GACGCACGAC TGCGAAAGCA TTTGCCAAGG
B. gibsoni (California)   ATGGTTAATA GGAGCAG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. rodhaini               ATGGTTAATA GGAGCAG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
B. microti                ATGGTTAATA GGAGCAG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
T. annae                  ATGGTTAATA GGAGCAG-TT GGGGGCATTC GTATTTAACT GTCAGAGGTG AAATTCTTAG ATTTGTTAAA GACGAACTAC TGCGAAAGCA TTTGCCAAGG
P. falciparum             ACGATTAATA GGAGTAGCTT GGGGACATTC GTATTCAGAT GTCAGAGGTG AAATTCTTAG ATTTTCTGGA GACGAACAAC TGCGAAAGCA TTTGTCTAAA

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                            1005       1015       1025       1035       1045       1055       1065       1075       1085       1095
B. gibsoni (Asian)        ACGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACCATGCC GACTAGGGAT TGGA------
B. c. rossi               ACGTTTCCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGTGAT TGGA------
B. c. vogeli              ACGTTTCCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACCATGCC GACTAGTGAT TGGA------
B. c. canis               ACGTTTCCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGTGAT TGGA------
B. odocoilei              ACGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGGGAT TGGA------
B. caballi                ACGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGGGAT TGGA------
B. bigemina               ACGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGGGAT TGGA------
B. divergens              ACGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGGGAT TGGA------
B. equi                   ATGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACGATGCC GACTAGAGAT TGGA------
B. bovis                  ACGCTTCCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCTT AAACTATGCC GACTAGGGAT TGGT------
B. gibsoni (California)   ATGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGCTCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACTAT AAACTATGCC GACTAGAGAT TGGA------
B. rodhaini               ATGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGAGAT TGGA------
B. microti                ATGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGAGAT TGGA------
T. annae                  ATGTTTTCAT TAATCAAGAA CGAAAGTTAG GGGATCGAAG ACGATCAGAT ACCGTCGTAG TCCTAACCAT AAACTATGCC GACTAGAGAT TGGA------
P. falciparum             ATACTTCCAT TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTAGGTGT TGGATGAAAG

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                            1105       1115       1125       1135       1145       1155       1165       1175       1185       1195
B. gibsoni (Asian)        ---------- ---GGTCGTC ATTTTT-C-G ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. c. rossi               ---------- ---GGTCGTC GTTTGT-TTG ACC------- -----CCTTC AGGAGCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. c. vogeli              ---------- ---GGTCGTC GTTTTG-CTG ACC------- -----CCTTC AGGAACTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. c. canis               ---------- ---GGTCGTC GTTTT--TTG ACC------- -----CCTTC AGGAACTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. odocoilei              ---------- ---GGTCGTC ATTTTT-CCG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. caballi                ---------- ---GGTCGTC ATTTTT-CTG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. bigemina               ---------- ---GGTCGTC ATTTT--CCG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. divergens              ---------- ---GGTCGTC ATTTTT-CCG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. equi                   ---------- ---GGTCGTC AGTTTGAACG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. bovis                  ---------- ---GGTCGTC GCCTC----G ACT------- -----CCGTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. gibsoni (California)   ---------- ---GGTCGTC ATTTTAAACG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. rodhaini               ---------- ---GGTCGTC AGTTTAAACG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
B. microti                ---------- ---GGTCGTC AGTTTAAACG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
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T. annae                    ---------- ---GGTCGTC AGTTTAAACG ACT------- -----CCTTC AGCACCTTGA GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG
P. falciparum               TGTTAAAAAT AAAAGTCATC TTTCGAGGTG ACTTTTAGAT TGCTTCCTTC AGTACCTTAT GAGAAATCAA AGTCTTTGGG TTCTGGGGCG AGTATTCGCG

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                               1205       1215       1225       1235       1245       1255       1265       1275       1285       1295
B. gibsoni (Asian)          CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA AACTCACCAG GTCCAGACAA
B. c. rossi                 CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA AACTCACCAG GTCCAGACAA
B. c. vogeli                CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA AACTCACCAG GTCCAGACAA
B. c. canis                 CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA AACTCACCAG GTCCAGACAA
B. odocoilei                CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA AACTCACCAG GTCCAGACAA
B. caballi                  CAAGTCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA AACTCACCAG GTCCAGACAG
B. bigemina                 CAAGTCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA ACCTCACCAG GTCCAGACAG
B. divergens                CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGCA TG-GCGGCTT AATTTGACTC AACACGGGGA AACTCACCAG GTCCAGACAA
B. equi                     CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA AACTCACCAG GTCCAGACAG
B. bovis                    CAAGTCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA ACCTCACCAG GTCCAGACAG
B. gibsoni (California)     CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA ACCTCACCAG GTCCAGACAT
B. rodhaini                 CAAGTCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGG-- CAGGCGGCTT AATTTGACTC AACACGGGAA ACCTCACCAG GTCCAGACAT
B. microti                  CAAGTCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGAA ACCTCACCAG GTCCAGACAT
T. annae                    CAAGTCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGAA ACCTCACCAG GTCCAGACAT
P. falciparum               CAAGCGAGAA AGTTAAAAGA ATTGACGGAA GGGCACCACC AGGCGTGGAG CTTGCGGCTT AATTTGACTC AACACGGGGA AACTCACTAG TTTAAGACAA

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                               1305       1315       1325       1335       1345       1355       1365       1375       1385       1395
B. gibsoni (Asian)          A-GTTAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. c. rossi                 ACGGTAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. c. vogeli                ACGGTAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. c. canis                 ACGGTAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. odocoilei                T-GTTAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. caballi                  A-GGTAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. bigemina                 A-GTAAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. divergens                T-GTTAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. equi                     A-GGAAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTT TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. bovis                    C-GTAAGGAT TGACAGGTTG ATTGTCCTTT CTTGATTCTC TGGGTAGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. gibsoni (California)     A-GTTAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTA TGGGTAGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. rodhaini                 A-GAAATGAT TGACAGATTG ATAGCTCTTT CTTGATTCTA TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
B. microti                  A-GAGAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTA TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
T. annae                    A-GAGAGGAT TGACAGATTG ATAGCTCTTT CTTGATTCTA TGGGTGGTGG TGCATGGCCG TTCTTAGTTG GTGGA-GTGA TTTGTCTGGT TAATTCCGTT
P. falciparum               G-AGTAGGAT TGACAGATTA ATAGCTCTTT CTTGATTTCT TGGATGGTGA TGCATGGCCG TTTTTAGTTC GTGAATATGA TTTGTCTGGT TAATTCCGAT

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                               1405       1415       1425       1435       1445       1455       1465       1475       1485       1495
B. gibsoni (Asian)          AACGAACGAG ACCTTAACCT GCTAACT--- ---------- ---------- ---------- ---------- ---------- ------AGTT GCCGTTA---
B. c. rossi                 AACGAACGAG ACCTTAACCT GCTAACT--- ---------- ---------- ---------- ---------- ---------- ------AGCG CTGGTTA---
B. c. vogeli                AACGAACGAG ACCTTAACCT GCTAACT--- ---------- ---------- ---------- ---------- ---------- ------AGCG GCGGTTA---
B. c. canis                 AACGAACGAG ACCTTAACCT GCTAACT--- ---------- ---------- ---------- ---------- ---------- ------AGTG CCGGTTA---
B. odocoilei                AACGAACGAG ACCTTAACCT GCTAACT--- ---------- ---------- ---------- ---------- ---------- ------AGTG CCCGTGAGAA
B. caballi                  AACGAACGAG ACCTTAACCT GCTAACT--- ---------- ---------- ---------- ---------- ---------- ------AGCT TCCCTTT---
B. bigemina                 AACGAACGAG ACCTTAACCT GCTAACT--- ---------- ---------- ---------- ---------- ---------- ------AGCT GCTTGGA---
B. divergens                AACGAACGAG ACCTTAACCT GCTAACT--- ---------- ---------- ---------- ---------- ---------- ------AGTG TCCGTAAAAA
B. equi                     AACGAACGAG ACCTTAACCT GCTAAAT--- ---------- ---------- ---------- ---------- ---------- ------AGGG TGTGAGACTT
B. bovis                    AACGGACGAG ACCTTAACCT GCTATTT--- ---------- ---------- ---------- ---------- ---------- ------AGTC GCTCGGT---
B. gibsoni (California)     AACGAACGAG ACCTTAACCT GCTAAAT--- ---------- ---------- ---------- ---------- ---------- ------AGCA GCTGAGAATA
B. rodhaini                 AACGAACGAG ACCTTAACCT GCTAAATT-- ---------- ---------- ---------- ---------- ---------- ------AGGA TATGGGACTA
B. microti                  AACGAACGAG ACCTTAACCT GCTAAATT-- ---------- ---------- ---------- ---------- ---------- ------AGGA TCTGGGACAA
T. annae                    AACGAACGAG ACCTTAACCT GCTAAATT-- ---------- ---------- ---------- ---------- ---------- ------AGGA TCTGAGACAA
P. falciparum               AACGAACGAG ATCTTAACCT GCTAATTAGC GGCGAGTACA CTATATTCTT ATTTGAAATT GAACATAGGT AACTATACAT TTATTCAGTA ATCAAATTAG

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                               1505       1515       1525       1535       1545       1555       1565       1575       1585       1595
B. gibsoni (Asian)          ----T----- ---------- ---------- ---------- ---TTCAGTT TCGGCCAGCT TCTT------ ---------- ---------- ----------
B. c. rossi                 ----C----- ---------- ---------- ---------- ---TT-GGTT TCCCGCTGCT TCTT------ ---------- ---------- ----------
B. c. vogeli                ----C----- ---------- ---------- ---------- ---TGTGGTT TCCGGTTGCT TCTT------ ---------- ---------- ----------
B. c. canis                 ----T----- ---------- ---------- ---------- ---TTGAGTT TCCGGTTGCT TCTT------ ---------- ---------- ----------
B. odocoilei                GGTTC----- ---------- ---------- ---------- ---GTCCGTT ACGGGTTGCT TCTT------ ---------- ---------- ----------
B. caballi                  ---------- ---------- ---------- ---------- ---TTTGTT TGGGTTTGCT TCTT------ ---------- ---------- ----------
B. bigemina                 ---------- ---------- ---------- ---------- ---TTGTGTC CTCGTTTGCT TCTT------ ---------- ---------- ----------
B. divergens                GGTTC----- ---------- ---------- ---------- ---GTCCGTT ACGGTTTGCT TCTT------ ---------- ---------- ----------
B. equi                     GGTTT----- ---------- ---------- ---------- ---------- CATTTCCGCT TCTT------ ---------- ---------- ----------
B. bovis                    ----C----- ---------- ---------- ---------- ----TCTGTC CGTGCGCACT TCAT------ ---------- ---------- ----------
B. gibsoni (California)     AACTTT---- ---------- ---------- ---------- --GTTGTTTT CAGCATTGCT TCTT------ ---------- ---------- ----------
B. rodhaini                 CATTTC---- ---------- ---------- ---------- --A--GTCT- CATTATCGCT TCTTG----- ---------- ---------- ----------
B. microti                  GCTTT----- ---------- ---------- ---------- --GCTGTTC- CAGTATCGCT TCTT------ ---------- ---------- ----------
T. annae                    ACTTCT---- ---------- ---------- ---------- --GCTGTTT- CAGTATCGCT TCTT------ ---------- ---------- ----------
P. falciparum               GATATTTTTA TTAAAATATC CTTTTCCCTG TTCTACTAAT AAATTGTTTT TTACTCTATT TCTCTCTTCT TTTAAGAATG TACTTGCTTG ATTGAAAAGC

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                               1605       1615       1625       1635       1645       1655       1665       1675       1685       1695
B. gibsoni (Asian)          -----AGAGG GACTTTGGGG CTCTAAGCCA CAAGGAAGAT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. c. rossi                 -----AGAGG GACTTTGGGG CTTGAAGCTC CAAGGAAGAT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. c. vogeli                -----AGAGG GACTTTGGGG CTCTAAGCCC TGAGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. c. canis                 -----AGAGG GACTTTGGGG CGCTAAGCCC TGAGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. odocoilei                -----AGAGG GACTTTGCGG CTCTAAGCCG CAAGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. caballi                  -----AGAGG GACTTTACAG CGACAAGCTG TAGGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. bigemina                 -----AGAGG GACTCCTGTG CTTCAAGCGT GGGGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. divergens                -----AGAGG GACTTTGCGG CTCTAAGCCG CAAGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. equi                     -----AGAGG GACTTTGCGG TCATAAATCG CAAGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. bovis                    -----AGAGG GACTCTGCGG CGTCAAGCTG CGGTGAGGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. gibsoni (California)     -----AGAGG GACTTTGCGG TCATAAATCG CAAGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. rodhaini                 -----AGAGG GACTTTGCGT TCATAAAACG CAAGGAAGTG TAAGGCAATA ACAGGTCTGT GAT-CCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
B. microti                  -----AGAGG GACTTTGCGT TCATAAAACG CAAGGAAGTG TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
T. annae                    -----AGAGG GACTTTGCGT TCATAAAACG CAAGGAAGTT TAAGGCAATA ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCTGCACGCG CGCTACACTG
P. falciparum               TTCTTAGAGG AACATTGTGT GTCTAA--CA CAAGGAAGTT TAAGGCAACA ACAGGTCTGT GATGTCCTTA GATGAACTAG GCTGCACGCG TGCTACACTG

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                               1705       1715       1725       1735       1745       1755       1765       1775       1785       1795
B. gibsoni (Asian)          ATGCATTCAT CGAGTGT--- ---------- ---------- ---------- ---------- ------TATC CCTGGCCGAG AGGTCCGGGT AATCTT---T
B. c. rossi                 ATGCATTCAT CGAGTGT--- ---------- ---------- ---------- ---------- ------TGCC CCTGGCCGAG AGGTCTGGGT AATCTT---T
B. c. vogeli                ATGCATTCAT CGAGTT---- ---------- ---------- ---------- ---------- ------GTTC CTTGGCCGAG AGGTCTTGGT AATCTT---T
B. c. canis                 ATGCATTCAT CGAGTTT--- ---------- ---------- ---------- ---------- ------ATTC CTTGGCCGAG AGGTCTAGGT AATCTT---T
B. odocoilei                ATGCATTCAT CGAGTTT--- ---------- ---------- ---------- ---------- ------TATC CCTGCCCGAA AGGGCTGGGT AATCTT---T
B. caballi                  ATGCATTCAC TAAGTTT--- ---------- ---------- ---------- ---------- ------TTCC TGC-TCCGAA AGGTGTGGGT AATCTG---T
B. bigemina                 ATGCATCCAT CGAGTTT--- ---------- ---------- ---------- ---------- ------GTCC TGT-CCCGAA AGGGTTGGGT AATCTT---T
B. divergens                ATGCATTCAT CGAGTTT--- ---------- ---------- ---------- ---------- ------TATC CCTTCCCGAA AG--CTGGGT AATCTT---T
B. equi                     ATGCATTCAC TGAGTG---- ---------- ---------- ---------- ---------- ------TATC CTTGGCTGAG AGGCTTGGGT AATCTT---T
B. bovis                    ATGCTTGCCT CGTGTT---- ---------- ---------- ---------- ---------- ------TACC CTCGGCCGAT AGGCCCTGGT AACCCC---T
B. gibsoni (California)     ATGCATTCAT CGAGTTT--- ---------- ---------- ---------- ---------- ------TATC CTTGCCCGAA AGGGTTAGGT AATCTT---T
B. rodhaini                 ATGCATTCAA CGAGTT---- ---------- ---------- ---------- ---------- ------T-TC CTTGGCCGG- AAGTCCGGGT AATCTTA--C
B. microti                  ATGCATTCAA CGAGTTT--- ---------- ---------- ---------- ---------- ------T-TC CTTGGCCGTC GGGTCCGGGT AATCTTA--C
T. annae                    ATGCATTCAA CGAGTTC--- ---------- ---------- ---------- ---------- ------T-TC CTTGGCCGAT GGGTCCGGGT AATCTTA--C
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P. falciparum              ATATATATAA CGAGTTTTTA AAAATATGCT TATATTTGTA TCTTTGATGC TTATATTTTG CATACTTTTC CTCCGCCGAA AGGCGTAGGT AATCTTTATC

                           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                              1805       1815       1825       1835       1845       1855       1865       1875       1885       1895
B. gibsoni (Asian)         AGTATGCATC GTGAC-GGGG ATTGATTTTT GTAATTCTAA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. c. rossi                AGTATGCATC GTGAC-GGGG ATTGATTTTT GTAATTCTAA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. c. vogeli               AGTATGCATC GTGAC-GGGG ATTGATTTTT GCAATTCTAA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. c. canis                AGTATGCATC GTGAC-GGGG ATTGATTTTT GTAATTCTAA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. odocoilei               AGTATGCATC GTGAC-GGGG ATTGATTTTT GCAATTCTAA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. caballi                 AGTATGCATC GTGTC-GGGG ATTGATTTTT GCAATTCTAA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. bigemina                AGTGTGCATC GTGTC-GGGG ATTGATTTTT GCAATTCTAA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. divergens               AGTATGCATC GTGACTGGGG ATTGATTTTT GCAATTCTAA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. equi                    AGTATGCATC GTGAT-GGGG ATTGATTATT GTAATTCTTA ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAC TACGTCCCTG
B. bovis                   AGTCTGCATC GTGTT-TGGG ATTGATCTTT GCAATTCTAG ATCATGAACG AGGAATGCCT AGTATGCGCA AGTCATCAGC TTGTGCAGAT TACGTCCCTG
B. gibsoni (California)    AGTATGCATC GTGAT-GGGG ATTGATTATT GCAATTATTA ATCATGAACG AGGAATGCCT AGTAGGCGCG AGTCATCAGC TCGTGCCGAC TACGTCCCTG
B. rodhaini                AGTATGCATC GTGAT-GGGG ATAGATTATT GCAATTATTA ATCTTGAACG AGGAATGCCT AGTAGGCGCG AGTCATCAGC TCGTGCC-AC TACGTCCCTG
B. microti                 AGTATGCATC GTGAT-GGGG ATAGATTATT GCAATTATTA ATCTTGAACG AGGAATGCCT AGTAGGCGCG AGTCATCAGC TCGTGCCGAC TACGTCCCTG
T. annae                   AGTATGCATC GTGAT-GGGG ATAGATTATT GCAATTATTA ATCTTGAACG AGGAATGCCT AGTAGGCGCG AGTCATCAGC TCGTGCCGAC TACGTCCCTG
P. falciparum              AATATATATC GTGAT-GGGG ATAGATTATT GCAATTATTA ATCTTGAACG AGGAATGCCT AGTAAGCATG ATTCATCAGA TTGTGCTGAC TACGTCCCTG

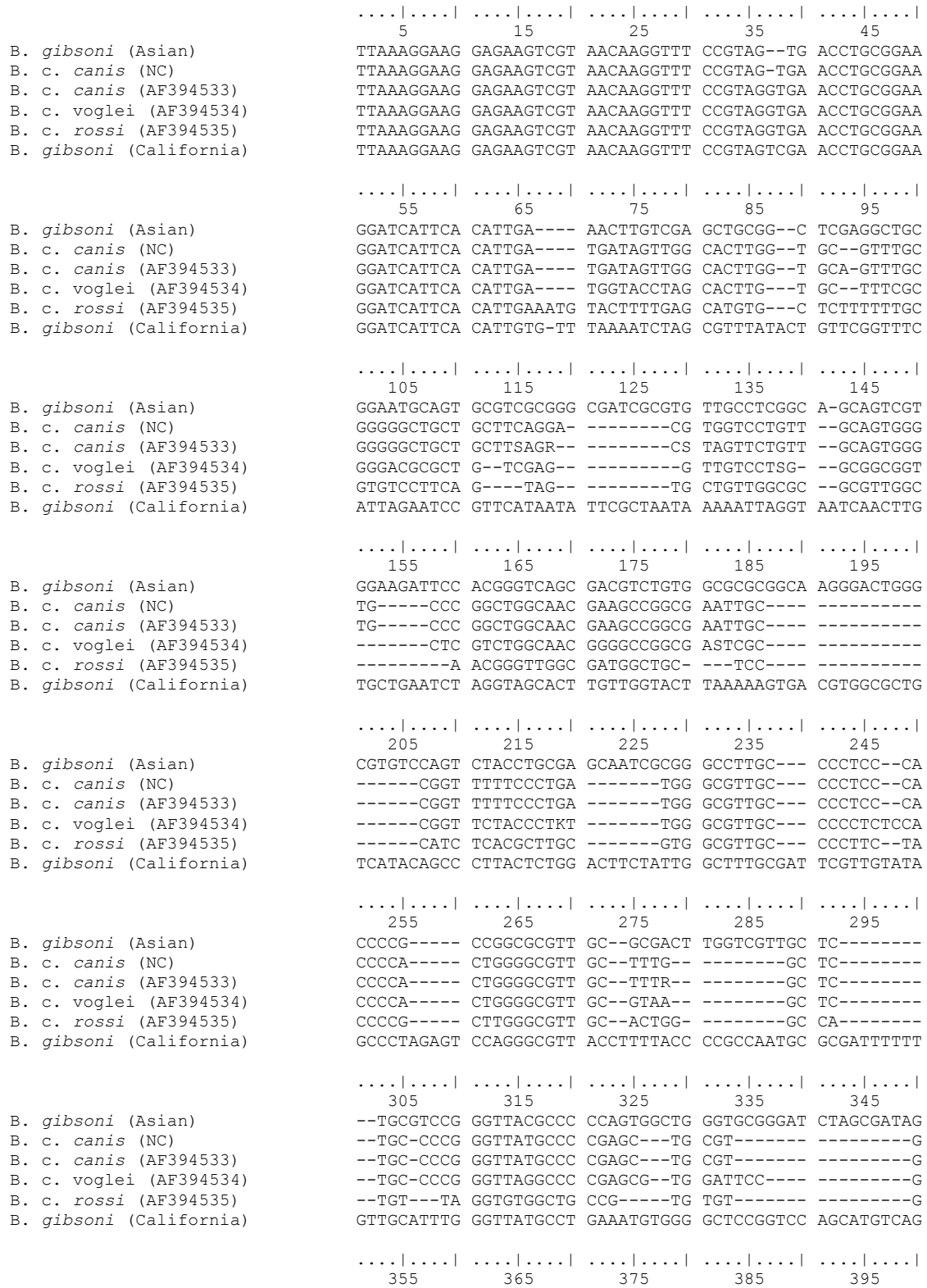
                           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                              1905       1915       1925       1935       1945       1955       1965       1975       1985       1995
B. gibsoni (Asian)         CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC CGTGGCTTTT CCGATTCGTC GGTTTTGCCT AGGGAAGTTT
B. c. rossi                CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC CGTGGCTATT CCGATTCGTC GGTTTTGCCG TGGAAAGTTT
B. c. vogeli               CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC TGTGGCTTTT CCGATTCGTC GGTTTAGCTT CGGAAAGTTT
B. c. canis                CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC TGTCGCTTTT CCGATTCGTC GGTTTAGCTT CGGAAAGTTT
B. odocoilei               CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC CGTGGCTTTT CCGATTCGTC GGTTTTGCCT AGGGAAGTCT
B. caballi                 CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC CGTGGCTTTT CCGATTCGTC GGTTTTGCCT TGGGAAGTTT
B. bigemina                CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC CGTGGCTTTT CCGATTCGTC GGTTTTGCCT AGGGAAGTTT
B. divergens               CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC CGTGGCCTTT CCGATTCGTC GGCTTGGCCT AGGGAAGTCT
B. equi                    CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC TGTGATGTTT CCTTTTCTTT GGAGACGTCT AGGGAAGTTT
B. bovis                   CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAA CCTG-CTTGC GCGATCCGTC GCCGCTCGCG GGCCAAGTTT
B. gibsoni (California)    CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC CGTGACGTTT CTAATTCGTT AGCAACGTCT AGGGAAGTTT
B. rodhaini                CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC TGAAGAACTT A-GATTCGTC TAAAGTTTTT TGGAA-GTTT
B. microti                 CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC CAAGAAACGT G-GATTCGTC CTTCGTTTTT TGGAAAGTTT
T. annae                   CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATCGAGTG ATCCGGTGAA TTATTCGGAC TGAAAAACGT G-GATTCGTC CTTCGTTTTT TAGAAAGTTT
P. falciparum              CCCTTTGTAC ACACCGCCCG TCGCTCCTAC CGATTGAAAG ATATGATGAA TTGTTTGGAC AAGAAAAATT G-AATTATAT TCTTTTTTTT CTGGAAAAAC

                           ....|....| ....|....| ....|
                              2005       2015       2025
B. gibsoni (Asian)         TGTGAACCTT ATCACTTAAA GGAAG
B. c. rossi                TGTGAACCTT ATCACTTAAA GGAAG
B. c. vogeli               TGTGAACCTT ATCACTTAAA GGAAG
B. c. canis                TGTGAACCTT ATCACTTAAA GGAAG
B. odocoilei               CGTGAACCTT ATCACTTAAA GGAAG
B. caballi                 TGTGAACCTT ATCACTTAAA GGAAG
B. bigemina                TGTGAACCTT ATCACTTAAA GGAAG
B. divergens               TGTGAACCTT ATCACTTAAA GGAAG
B. equi                    TGTGAACCTT ATCACTTAAA GGAAG
B. bovis                   TGTGAACCTT ATCACTTAAA GGAAG
B. gibsoni (California)    TGTGAACCTT ATCACTTAAA GGAAG
B. rodhaini                TGTGAACCTT ATCACTTAAA GGAAG
B. microti                 TGTGAACCTT ATCACTTAAA GGAAG
T. annae                   TGTGAACCTT ATCACTTAAA GGAAG
P. falciparum              CGTAAATCCT ATCTTTTAAA GGAAG
```

Table 8 Sequence identity table comparing 18S rRNA genes

	B. gibsoni (Asian)	*B. c. rossi*	*B. c. vogeli*	*B. c. canis*	*B. odocoilei*	*B. caballi*	*B. bigemina*	*B. divergens*	*B. equi*	*B. bovis*	*B. gibsoni* (California)	*B. rodhaini*	*B. microti*	*T. annae*	*P. falciparum*
B. gibsoni (Asian)	1	0.942	0.94	0.944	0.955	0.926	0.918	0.946	0.865	0.831	0.858	0.841	0.85	0.851	0.627
B. c. rossi	---	1	0.944	0.952	0.931	0.922	0.91	0.926	0.858	0.829	0.848	0.837	0.842	0.84	0.625
B. c. vogeli	---	---	1	0.977	0.931	0.915	0.914	0.925	0.861	0.831	0.852	0.84	0.841	0.844	0.626
B. c. canis	---	---	---	1	0.934	0.917	0.914	0.926	0.862	0.829	0.854	0.84	0.844	0.845	0.626
B. odocoilei	---	---	---	---	1	0.925	0.916	0.976	0.87	0.829	0.868	0.847	0.855	0.858	0.631
B. caballi	---	---	---	---	---	1	0.939	0.922	0.854	0.846	0.844	0.833	0.84	0.838	0.625
B. bigemina	---	---	---	---	---	---	1	0.91	0.851	0.851	0.852	0.832	0.838	0.838	0.616
B. divergens	---	---	---	---	---	---	---	1	0.864	0.822	0.859	0.846	0.851	0.853	0.628
B. equi	---	---	---	---	---	---	---	---	1	0.798	0.903	0.887	0.893	0.892	0.636
B. bovis	---	---	---	---	---	---	---	---	---	1	0.79	0.783	0.784	0.782	0.585
B. gibsoni (CA)	---	---	---	---	---	---	---	---	---	---	1	0.886	0.893	0.898	0.641
B. rodhaini	---	---	---	---	---	---	---	---	---	---	---	1	0.939	0.934	0.643
B. microti	---	---	---	---	---	---	---	---	---	---	---	---	1	0.962	0.647
T. annae	---	---	---	---	---	---	---	---	---	---	---	---	---	1	0.654
P. falciparum	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1

Figure 4. Sequence alignment of *Babesia* internal transcribed spacer regions



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B. gibsoni (Asian)              TTCTCGGCCT GCGCCGCTGG GGGATGCTGC TGGTGGTCGC TCTGGCGGCC
B. c. canis (NC)                TT----GAGC GC--GGCCTG GGGATGTT-- -GGTG--TGC GTGTGCGCTC
B. c. canis (AF394533)          TT----GAGC GC--GGCCTG GGGATGTT-- -GGTG--TGC GTGTGCRCTC
B. c. voglei (AF394534)         TT----GGTT TCCGGGCTTG GGGATGT--- -GGCG--CGC CTGGGCGCGT
B. c. rossi (AF394535)          CT------CT GC---CTTGG GTTATGCC-- ---TGGGAGT TTGGGCTCCT
B. gibsoni (California)         GATGCGATTT AGTCGTACTG TGCGTACTTC TGGATTGCGC CGGACGTTAT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   405        415        425        435        445
B. gibsoni (Asian)              TGAAGGCGGC TCGGCTGTGT -AAAGCAGTC GCTGCGGGTC ACGTTGGTGA
B. c. canis (NC)                CGAC--CGGT ----CTGTG- -------ACC ACTGCGGGCC -------TGT
B. c. canis (AF394533)          CGAC--CGGT ----CTGTG- -------AC- ACTGCGGGCC -------TGT
B. c. voglei (AF394534)         CAG----GGT ----CTYTG- -------ACC TCTGCGG--C -------TTG
B. c. rossi (AF394535)          GGGATGCCGT -C--TTGTGG -CGGTTCGCG ATTGTCGGCT ---------G
B. gibsoni (California)         TAGCTATGTT TTTAGCGAAT GCGTTTGGTA TTTGCTTGTT GCATTTTTAT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   455        465        475        485        495
B. gibsoni (Asian)              CGCGTTGAAG CAACTCCG-- CTTGAGTT-T TCAAGTTGAT GACAGAATTT
B. c. canis (NC)                CCCGATTA-G CAACTCCA-- CTTGACTC-T GTCGAGTAAC GA--AATTCT
B. c. canis (AF394533)          CCCGACTA-G CAACTCCA-- CTTGACTY-T GTCGAGTAWC GA--AATTCT
B. c. voglei (AF394534)         GCCGATTT-G CAACTCCG-- CTTGACG--T GTCGAGTATT GA--AATTCT
B. c. rossi (AF394535)          AGTGCGTAAG CAACTCC--- TTTGATT--- ------TGAT GA--AATTTG
B. gibsoni (California)         TAAAACCCTG CGACAATGGC TTCGGCTGTT GTCGCGTTTT GTATGAATCC

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   505        515        525        535        545
B. gibsoni (Asian)              AAACTTTCAG CGATGGATGT CTTGGCTCAC ACAACGATGA AGGACGCAGC
B. c. canis (NC)                AAACTTTCAG CGATGGATGT CTTGGCTCAC ACAACGATGA AGGACGCAGC
B. c. canis (AF394533)          AAAATTTCAG CGATGGATGT CTTGGCTCAC ACAACGATGA AGGACGCAGC
B. c. voglei (AF394534)         AAAATTTCAG CGATGGATGT CTTGGCTCAC ACAACGATGA AGGACGCAGC
B. c. rossi (AF394535)          CAAATTTCAG CGATGGATGT CTTGGCTCAC ACAACGATGA AGGACGCAGC
B. gibsoni (California)         AAACTTTCAG CGATGGATGT CTTGGCTCAC ACAACGATGA AGGACGCAGC

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   555        565        575        585        595
B. gibsoni (Asian)              AAATTGCGAT ATGCATTATG ACTTGCAGAC TTCTGCGATT -AACAGACCT
B. c. canis (NC)                GAAATGCGAT ATGCATTATG ACTTGCAGAC TTCTGCGATT TAACAGACCT
B. c. canis (AF394533)          GAAATGCGAT ATGCATTATG ACTTGCAGAC TTCTGCGATT TAAC-GACCT
B. c. voglei (AF394534)         GAAATGCGAT AT-CATTATG ACTTGCAGAC TTCTGCGATT TAAC-GACCT
B. c. rossi (AF394535)          GAAATGCGAT ATGCATTATG ACTTGCAGAC TTCTGCGATT TAAC-GACCT
B. gibsoni (California)         GAATTGCGAT AACCATTGTG AATTGCACGC TCCTGCGAAT CAACAGACCT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   605        615        625        635        645
B. gibsoni (Asian)              CCGAACGTAA CCAACACACC GCCTCTGCTC GCATGCGGTA CTCCCGTTTC
B. c. canis (NC)                CCGAACGTAA CCAACACACC GCCTCTGCTC GCACGCGGTA CTCCCGTTTC
B. c. canis (AF394533)          CCGAACGTAA CCAACACACC GCCTCTGCTC GCACGCGGTA CTCCCGTTTC
B. c. voglei (AF394534)         CCGAACGTAA CCAACACACC GCCTCTGCTC GCACGCGGTA CTCCCGTTTC
B. c. rossi (AF394535)          CCGAACGTAA CCAACACACC GCCTCTGCTC GCACGCGGTA CTCCCGTTTC
B. gibsoni (California)         CTGAACGTAT CATACACACC GCCTCTGCTT GCATGCGGTA CTCCCATTTC

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   655        665        675        685        695
B. gibsoni (Asian)              AGTGAGC-CC TCTGTCCTAA GGGCGCAACC CTTTTGGTG- -GCCCTTGCG
B. c. canis (NC)                AGTGAGC-GC CCTTTACTGA GG-CTTAGCC TTTTTGGCA- -GTC-TCGCG
B. c. canis (AF394533)          AGTGAGC-GC CCTTTACTGA GG-CTTAGC- TTTTTGGCA- -GTC-TCGCG
B. c. voglei (AF394534)         AGTGAGC-GC CCTTTACTGA GG-CTTKGC- TTTTTGGCA- -GTC-TCGCG
B. c. rossi (AF394535)          AGTGAGCTCC TTTTTCCTGC GGTTAACAAG GTTTTGGAA- -GCC-GCGCG
B. gibsoni (California)         AGTGAAC-TT TAATTCCTAA AAACAACCAC TTTGTGGTAA GGTTTTTGAG

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   705        715        725        735        745
B. gibsoni (Asian)              AGTGGGCGA- --TTGGCGTC GCCCGATTGC GCAGACG--- ---CTTGCCT
B. c. canis (NC)                AGTGGGTG-- --CTGTAGGC GCCTGATTAC TTAGAGGGAT -GGCTGGCCA
B. c. canis (AF394533)          AGTGGGTG-- --CTGTGGGC GCCTGATTAC TTAGAGGGAT -GGCTGGCCA
B. c. voglei (AF394534)         AGTGGGTG-- --CTGTGGGC GCCCGATTGC GTGGAGGAGT TGGCTGGCCA
B. c. rossi (AF394535)          ATTGGGCT-- ---TTGGATG GCCTGAA-GT TCTGAGC--- -----TGCCC
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B. gibsoni (California)          AGTGGATGTA TCTTTTGAGC ATCTCAATTT CCGCAAGCGA -GAGTCGCCT

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                    755        765        775        785        795
B. gibsoni (Asian)               GGGACTTG-- ---------- --------TG CGGCGACCGC C-GCCTCGGA
B. c. canis (NC)                 AGGTCGTGGC TGCTGGGCTA CTGGGTAGTG CGGCGGCTAT TTGCCTCGAA
B. c. canis (AF394533)           AGGTCGTGGC TGCTGGGCTA CTGGGTAGTG CGGCGGCTAT TTGCCTCGAA
B. c. voglei (AF394534)          AGGTCGTGGY KGTRGTTCYA CTCTGTGGAG STGCGGCCAT TTGCCTCGAA
B. c. rossi (AF394535)           AGCTTGTGGC TG--G----- ---------G CGGTTTGCCG TTGCCTCGAA
B. gibsoni (California)          GTGGTTTGGC GGGAAGTTTG GTCTCTAGGG ACTTTACTTT CGATCTCGGA

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                    805        815        825        835        845
B. gibsoni (Asian)               GCTGCCTGTT GTAA--CTCG TTACTCTGGC TCCTGATTGG C-GTCAGCAG
B. c. canis (NC)                 GTTTCCTGT- GTA---CTTG TTACCGAATC TTCTGTGAGG CTGTCGGGCG
B. c. canis (AF394533)           GTTTCCTGT- GTA---CTTG TTACCGAATC TTCTGTGAGG CTGTCGGGCG
B. c. voglei (AF394534)          GCATTTTGT- GTA---TGTG ATACCGAATC TTCTGCGAGG CTGTTGGGCT
B. c. rossi (AF394535)           GAGCGTTGT- GTAT--ATTA TGCATGAAAC TTC-GTGAGG CTGTTGACTT
B. gibsoni (California)          GCTTCTTGTT GTATATATTC TACTTGATGC TTCCTTGTTG GTCCCATCTT

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                    855        865        875        885        895
B. gibsoni (Asian)               GT---CC-GG GCGCTGCTTT GCAGTGGCCG --------TC TGAGAGGTTT
B. c. canis (NC)                 TTG--CC-CG AGACTGTGGC GGGCTGTGTG -C------TC TGTGTAGTTT
B. c. canis (AF394533)           TTG--CC-CG AGACTGTGGC GGGCTGTGTG -C------TC TGTGTAGTTT
B. c. voglei (AF394534)          CTG--CC-CA AGACTGCGGT GAGCCGTCTG AC------TC CGTGTC-WTC
B. c. rossi (AF394535)           TGT--CAATG ACGCTGTATG GTTGTGGATG AA------AG CTCGAT-CGT
B. gibsoni (California)          CTATGCATTA GTATAGTAGA TCAGTGCGCC ACCATAATCG TTCGAGCTTC

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                    905        915        925        935        945
B. gibsoni (Asian)               AA-------- ---------- --ACTCGCTC CTGAAATCGG GTGAGGCTAT
B. c. canis (NC)                 GA-------- ---------- --TTTTGCTC CTGAAATCGG GTGAGGCTAT
B. c. canis (AF394533)           GA-------- ---------- --TTTTGCTC CTGAAATCGG GGTAGGCTAT
B. c. voglei (AF394534)          TA-------- ---------- --TTCCGCTC CTGAAATCGG GGTAGGCTAT
B. c. rossi (AF394535)           GA-------- ---------- --CCTTGCTC CTGAAATCGG GTGAGAGGAT
B. gibsoni (California)          AAATTTAGGT TTTAATACCA TTTTTCATTC CTGAAATTGG GTGAGGCCAT

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                    955        965        975        985        995
B. gibsoni (Asian)               CCGCTGAATT TAAGCATATA ATTAAGCGGA AGAAAAGA-- -TAACTATGA
B. c. canis (NC)                 CCGCTGAATT TAAGCATATA ATTAAGCGGC AGAAAAGAAA ATAACCATGA
B. c. canis (AF394533)           CCGCTGAATT TAAGCATATA ATTAAGCGGC AGAAAAGAAA ATAACCATGA
B. c. voglei (AF394534)          CCGCTGAATT TAAGCATATA ATTAAGCGGC AGAAAAGAAA ATAACCATGA
B. c. rossi (AF394535)           CCGCTGAATT TAAGCATATA ATTAAGCGGC AGAAAAGAAA ATAACCATGA
B. gibsoni (California)          CCGCTGAATT TAAGCATATA ACTAAGCGGA AGAAAAGAAA ATAACAATGA

                                 ....|..
                                    1005
B. gibsoni (Asian)               TTCCCCC
B. c. canis (NC)                 TTCCCTT
B. c. canis (AF394533)           TTCCCTT
B. c. voglei (AF394534)          TTCCCTC
B. c. rossi (AF394535)           TTCCCTT
B. gibsoni (California)          TTCCCTT
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Table 9. Sequence identity table comparing the internal transcribed spacer region gene sequences of canine *Babesia* spp.

Sample	B. gibsoni (Asian genotype) Isolate-1	B. gibsoni (Asian genotype) Isolate-2	B. c. canis NA isolate	B. c. canis Genbank Acc. No.	B. c. vogeli Genbank Acc. No.	B. c. rossi Genbank Acc. No.	B. gibsoni (California genotype)
B. gibsoni (Asian genotype) Isolate-1	1.000	0.993	0.539	0.534	0.545	0.513	0.571
B. gibsoni (Asian genotype) Isolate-2	---	1.000	0.543	0.540	0.551	0.519	0.575
B. c. canis NA isolate	---	---	1.000	0.975	0.838	0.677	0.493
B. c. canis Genbank Acc. No.	---	---	---	1.000	0.839	0.676	0.488
B. c. vogeli Genbank Acc. No.	---	---	---	---	1.000	0.669	0.483
B. c. rossi Genbank Acc. No.	---	---	---	---	---	1.000	0.474
B. gibsoni (California genotype)	---	---	---	---	---	---	1.000

Figure 5. Alignment of *Pirolplasmida* cytochrome *b* gene sequences

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                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                    5         15         25         35         45
B. gibsoni (Asian)              AAAGTGGGTT TTGTATTTGT ATTTCAGATA ATGTCTGGAC TGATGCTAAC
B. bovis (AF053002)             ATAGTCGGTT TTGTATTTGT ATTTCAAATA CTATCAGGAC TTTTGTTGAC
B. bigemina (AF109354)          ATCGTGGGTT TCGTTTTTGT ATTCCAGATT TTCTCTGGTT TACTTATGAC
T. annulata (M63015)            ATACTTGGAA TATTGTTAGT ACTCCAAATT ATATCCGGGT TGATGCTTTC
P. falciparum (M99416)          CTTTTAGGAA TAATATTTTT TATTCAAATT ATAACAGGTG TATTTTTAGC
P. berghei (AF146076)           CTTTTAGGTA TAATATTCTT TATTCAAATA TTAACAGGTG TATTTTTAGC

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                    55        65         75         85         95
B. gibsoni (Asian)              TTTTTATTAT ATTCCAGGAG GAATGGAATC TTTTAATAGT GTTATTAGAG
B. bovis (AF053002)             ATTTTATTAT GTTCCTGGAA GCGTTGATTC TTTTGAATGT GTAATTAGAG
B. bigemina (AF109354)          CTTTTATTAT GTTCCAGGAG ATGTTGATTC TTTCGAATGT GTTATCAGAG
T. annulata (M63015)            TTTCTTTTAT GTGCCAGCAA AAGGTATGGC TTTTGAAAGT ACTTTGGCCG
P. falciparum (M99416)          AAGTCGATAT ACACCAGATG TTTCATATGC ATATTATAGT ATACAACACA
P. berghei (AF146076)           AAGTCGTTAT TCTCCTGAAA TATCATATGC TTACTATAGT ATCCAACATA

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   105       115        125        135        145
B. gibsoni (Asian)              TATTAACAGA AGTTAATATG GGTTGGGCAG TTCGTTATTT TCATGCTCAA
B. bovis (AF053002)             TGCTTGCTGA AGTCAATATG GGTTGGGCAA TGCGTTATTT TCACGCTCAA
B. bigemina (AF109354)          TATTAACTGA GGTTAATATG GGTTGGGCAC TTCGTTATTT CCATGCTCAA
T. annulata (M63015)            TAATGTTAAA CATTTGTTTC GGTTGGTTTG TTCGTCTTTA TCACTCGTTT
P. falciparum (M99416)          TTTTAAGAGA ATTATGGAGT GGATGGTGTT TTAGATACAT GCACGCAACA
P. berghei (AF146076)           TTTTAAGAGA ATTATGGAGT GGATGGTGTT TTAGATATAT GCATGCTACT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   155       165        175        185        195
B. gibsoni (Asian)              TGTGTTTCTT TCTGTTTTTT CTTTATGTTT TTACATATGT TAAAAGGATT
B. bovis (AF053002)             TGTGTTTCTT TTTGTTTCTT TTTCATGATG CTACATATGC TAAAGGGATT
B. bigemina (AF109354)          TGTGTTTCTT TTTGCTTTTT CTTCATGATG TTACATATGT TAAAAGGTTT
T. annulata (M63015)            GGAGTTTCGT TTTATTTCTT CTTTATGTTT CTACATATCA TGAAAGGTAT
P. falciparum (M99416)          GGTGCTTCTC TTGTATTTTT ATTAACATAT CTTCATATTT TAAGAGGATT
P. berghei (AF146076)           GGTGCATCAC TTGTATTTTT CTTAACTTAT CTTCATATTT TAAGAGGATT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   205       215        225        235        245
B. gibsoni (Asian)              ATGGTATTCA AGTAAATATT TACCTTGGTC ATGGTATTCT GGAATGACTA
B. bovis (AF053002)             GTGGTACTCA AGCAGATATC TACCATGGTC ATGGTATTCT GGAATGGTTA
B. bigemina (AF109354)          ATGGTACTCA AGTAAACATT TACCTTGGTC CTGGTATTCA GGAATGGTTA
T. annulata (M63015)            GTGGTATTCT AGTAATCATT TACCTTGGTC TTGGTATTCT GGTGTTGTTA
P. falciparum (M99416)          AAATTACTCA TATATGTATT TACCATTATC ATGGATATCT GGATTGATTT
P. berghei (AF146076)           AAATTATTCA TACTTATATT TACCATTATC ATGGATATCA GGACTAATAA

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   255       265        275        285        295
B. gibsoni (Asian)              TATTTGTTTT AAGTATGGCT ATAGCATTCT TAGGTTATGT TTTACCAAAT
B. bovis (AF053002)             TATTTATTTT AAGTATGGCT ATTGCTTTCT TAGGTTATGT TCTTCCTTAC
B. bigemina (AF109354)          TATTTATTTT GAGTATGGCA ATTGCTTTCT TAGGTTATGT TCTTCCAAAT
T. annulata (M63015)            TTTTCGTTTT AAGTATAGCA ACTGCTTTTG TTGGTTATGT ATTACCAGAT
P. falciparum (M99416)          TATTTATGAT ATTTATTGTA ACTGCTTTCG TTGGTTATGT CTTACCATGG
P. berghei (AF146076)           TTTTCGCATT ATTTATAGTT ACAGCTTTTA TAGGTTATGT TTTACCTTGG

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   305       315        325        335        345
B. gibsoni (Asian)              GGTCAAATGA GTTATTGGGG AGCAACAGTT ATAACAAACC TGTTTTATTG
B. bovis (AF053002)             GGACAAATGA GTTATTGGGG AGCAACAGTT ATTATAAACT TGTTCTACTG
B. bigemina (AF109354)          GGACAAATGA GTTACTGGGG AGCAACAGTT ATAATTAACT TATTCTATTG
T. annulata (M63015)            GGTCAAATGA GCTTCTGGGG AGCTACAGTC ATAGGTGGTT TATTGAAATT
P. falciparum (M99416)          GGTCAAATGA GTTATTGGGG TGCAACTGTA ATTACTAACT TGTTATCCTC
P. berghei (AF146076)           GGACAAATGA GTTACTGGGG TGCAACTGTT ATTACTAATT TATTATCTGG

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                                355        365        375        385        395
B. gibsoni (Asian)              GATTCCGGAT TTTGTTATTG TACTACTAGG TGGTTATAGT GTTAGTGTTC
B. bovis (AF053002)             GTTCCCAGAT TTAGTCGCTC TTGTTTTGGG TGGTTATGGA GTTGCATTTC
B. bigemina (AF109354)          GTTCCCTGAT ATGGTATCTC TTGTACTAGG AGGATTTGGT GTTGGATTCC
T. annulata (M63015)            TTTCGGAAAA GCTAATGTTC TAATTTTTGG AGGCCAAACA GTTGGTCCAG
P. falciparum (M99416)          TATTCCAGTA GCAGTAATTT GGATATGTGG AGGATATACT GTGAGTGATC
P. berghei (AF146076)           TATACCATCA TTAGTAATTT GGTTATGTGG AGGATACACT GTTAGTGATC

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   405        415        425        435        445
B. gibsoni (Asian)              CAACTTTACA AAGATTTTAT ATATTACACT TTATTTTACC TTTTGTATTA
B. bovis (AF053002)             CAACTCTACA AAGATTTTAC ATATTACATT TCATATTACC TTTTGTATTG
B. bigemina (AF109354)          CAACATTACA AAGATTCTAT ATTCTACATT TTATATTGCC TTTTGTTTTA
T. annulata (M63015)            AGACATTAGA GAGATTCTTT TCTATACATG TTATATTGCC TGTGATTATA
P. falciparum (M99416)          CTACAATAAA ACGATTTTTT GTACTACATT TTATCTTACC ATTTATTGGA
P. berghei (AF146076)           CAACAATTAA AAGATTTTTT GTATTACATT TTATATTACC ATTTGTAGCT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   455        465        475        485        495
B. gibsoni (Asian)              TTGGGTGTAG TAGTAGTTCA TATTTATTAT CTGCATAGAT CTTCAAGTAC
B. bovis (AF053002)             TTAATTGTTG TTTTAATTCA TATTTATTAC CTTCACAGGT CTTCAAGTAC
B. bigemina (AF109354)          TTGTTTATAG TCATTATTCA CATTTATTAT CTACACAGGT CTTCAAGTAC
T. annulata (M63015)            TTGTTAGTTG TTATATTTCA TCTTTATGTT CTTCATAGAG ATGGAAGTTC
P. falciparum (M99416)          TTATGTATTG TATTTATACA TATATTTTTC TTACATTTAC ATGGTAGCAC
P. berghei (AF146076)           TTATGTATTG TATTTATACA TATTTTCTTT TTACATTTAC ATGGTAGCAC

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   505        515        525        535        545
B. gibsoni (Asian)              AAATCCATTA TCAGGTGTAG AT-TCTTGGT ATGTATCAAG ATTTTATCCT
B. bovis (AF053002)             TAATCCTTTG TCAGGAGTAG AT-TCTTGGT TAGTTTCAAG ATTTTATCCA
B. bigemina (AF109354)          TAATCCATTA TCTGGAATAG AT-TCTTGGT TAGTTTCTAG ATTCTATCCT
T. annulata (M63015)            AAATCCATTG GCAGTTATAG ACATGCTAGC TA-TATTTAG ATTTCATCCA
P. falciparum (M99416)          AAATCCTTTA G--GGTAT-G ATACAGCA-T TAAAAATACC CTTTTATCCA
P. berghei (AF146076)           TAATCCTTTA G--GGTAT-G ATACAGCA-T TAAAAATACC CTTCTATCCA

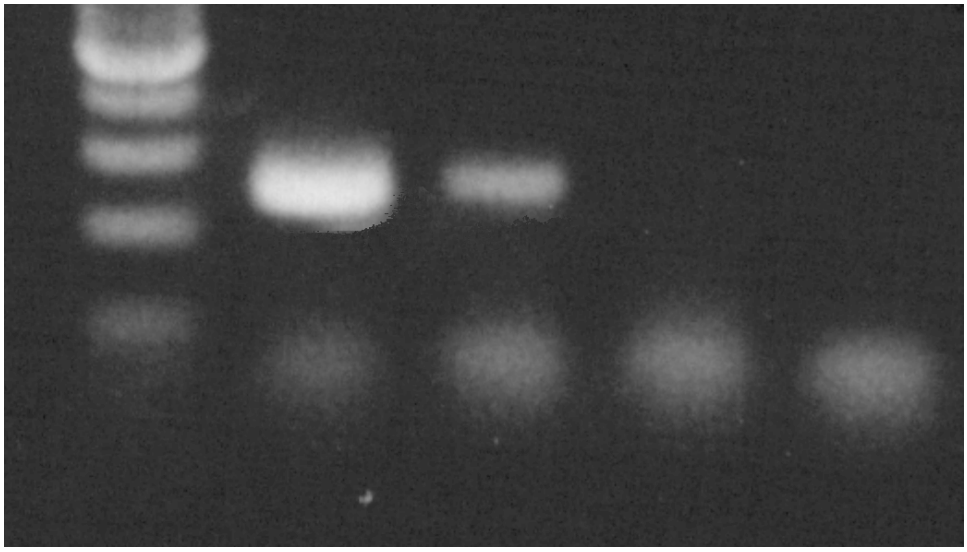
                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   555        565        575        585        595
B. gibsoni (Asian)              GTTATAATAT TCAGTGATTT AAAGATGTTG ACTATGCTAT TTGCTGCTTT
B. bovis (AF053002)             GTAATTATAT TCAGTGATAT TAGAATGCTA ACAATGTTGT TTATATTGTT
B. bigemina (AF109354)          GTTATCATAT TTAGTGATAT AAAAATGTTG ACTATGTTGT TTATATTGCT
T. annulata (M63015)            GTGGTACTTT TCAGTGATAT TAGATTTATA GTTATAGTAA TTTTATTAAT
P. falciparum (M99416)          AATCTATTAA GTCTTGATGT TAAAGGATTT AATAATGTTA TAATTTTATT
P. berghei (AF146076)           AATCTATTAA GTCTTGATGT AAAAGGATTT AATAATATAT TAATATTATT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                   605        615        625        635        645
B. gibsoni (Asian)              GGGTGTTCAA TTAACTTATG GTATAATACC ATTGTTTCAG GGAGATGTTG
B. bovis (AF053002)             AGGTGTTCAA TTAACTTATG GAGTAATACC TTTATTCCAA GGAGATTGTG
B. bigemina (AF109354)          TGGAGTTCAA TTAACTTATG GAGTAATTCC ATWGTTCCAA GGTGACTGTG
T. annulata (M63015)            TGGAGTGCAA TCTGGTTATG GATTCATTTC TATTTTTCAA GCAGATCCTG
P. falciparum (M99416)          TCTAATACAA AGTTTATTTG GAATTATACC TTTATCACA- -----TCCTG
P. berghei (AF146076)           TTTAATACAA AGTATTTTTG GAGTTATACC ACTATCTCA- -----TCCAG

                                ....|....| ....|....| ....|....|
                                   655        665        675
B. gibsoni (Asian)              ATAATTCAAT TGAATCAAAC CCATTACAAA
B. bovis (AF053002)             ATAATTCAAT TATGGCAAAT CCTTTGCAAA
B. bigemina (AF109354)          ATAATTCTAT CATGGCAAAC GCTTTGCAGA
T. annulata (M63015)            ATAATTCGAT ATTATCTGAT CCTTTAAACA
P. falciparum (M99416)          ATAATGCTAT CGTAGTAAAT ACATATGTTA
P. berghei (AF146076)           ATAATGCTAT AGTTGTTAAT ACATATGTTA
```

Table 10. Sequence identity table comparing the proposed partial cytochrome *b* amino acid sequences

Sample	*Babesia bovis*	*Babesia bigemina*	*Babesia gibsoni (Asian genotype)*	*Theileria annulata*	*Plasmodium berghei*	*Plasmodium falciparum*
Babesia bovis	1.000	0.915	0.836	0.522	0.473	0.442
Babesia bigemina	---	1.000	0.823	0.522	0.455	0.433
Babesia gibsoni (Asian genotype)	---	---	1.000	0.508	0.473	0.455
Theileria annulata	---	---	---	1.000	0.402	0.420
Plasmodium berghei	---	---	---	---	1.000	0.910
Plasmodium falciparum	---	---	---	---	---	1.000



1 2 3 4 5

Figure 6. Analysis of RAP-1 PCR products by 2% agarose gel electrophoresis and ethidium bromide staining. From left to right the lanes contain the following: 100bp Molecular weight marker (lane 1), *B. c. canis* (lane 2), *B. gibsoni* (Asian genotype) (lane 3), canine DNA (lane 4), negative (no DNA) control (lane 5).

Figure 7. Alignment of putative partial RAP-1 amino acid sequences

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                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                    5         15         25         35         45
B. gibsoni (Asian 1)            YVLKHCEGD- PFRRQVKEKM ALFRGDGQYA EFHTFLLNLA INNNQFYRLT
B. gibsoni (Asian 2)            YVLKHCEGD- PFRRQVKEKM ALFRGDGQYA EFHTFLLNLA INNNQFYRLT
B. canis canis (NC)             YAFRNCESN- PMKXPSEAFW MRFRHGGRYG AYHNFLVNMF NRN---LSQT
B. canis vogeli                 YVFRNSESN- PIKNPTEAFW MRFRHGGRYG AYHNFLVNIL YKN---LSDS
B. divergens 1                  YIFGLVLKDY RIDNGIARFK LSSKSKSNF- -YRIFMAALV RSN---LSFS
B. divergens 2                  YIFGLVLKDY RIDNGIARFK LSSKSKSNF- -YRIFMAALV RSN---LSFS
B. divergens 3                  QLFKECDSN- ATKEPIDEFL MRFKHGGRYG AYQSFIMNVV TSS---YTQL
B. caballi                      ILFKESDAN- PANSTEKRFW MRFRRGKNHS YFHDLVFNLL EKN---VTRD
B. ovis 1                       TLFKNSASN- PAKNGLKGQW MRFRNGKKHG DYHRFLIGLL AKG---VTRK
B. ovis 2                       TLFKNSASN- PAKNGVKGQW MRFRNGKKHG DYHRFLIGLL AKG---VTRK
B. ovis 3                       TLFKNSASN- PAKNGVKGQW MRFRNGKKHG DYHRFLIGLL AKG---VTRK
B. bovis 1                      RLFKESASN- PAKNSVKREW LRFRNGANHG DYHYFVTGLL NNN---VVHE
B. bovis 2                      RLFKESASN- PAKNSVKREW LRFRNGANHG DYHYFVTGLL NNN---VVHE
B. bovis 3                      RLFKESASN- PAKNSVKREW LRFRNGANHG DYHYFVTGLL NNN---VVHE
B. bigemina 1                   LLFKNCRRN- ESRHWMDRFW MRFKRGGRYA AYYSFSLNLL RRN---LFLG
B. bigemina 2                   LLFKNCRRN- ESRHWMDRFW MRFKRGGRYA AYYSFSLNLL RRN---LFLG
B. bigemina 3                   LLFKNCRRN- ESRHWMDRFW MRFKRGGRYA AYYSFSLNLL RRN---LFLG

                                ....|....| ....|.
                                    55         65
B. gibsoni (Asian 1)            AE-EQDLDAF VRRYVF
B. gibsoni (Asian 2)            AE-EQDLDAF VRRYVF
B. canis canis (NC)             VE-EENLEGF VSKYAY
B. canis vogeli                 MV-DDNLEGF VRKYAY
B. divergens 1                  AQFRQHINAF LTRYLY
B. divergens 2                  AQFRQHINAF LARYLY
B. divergens 3                  SD-NTEVEDL VNRYLY
B. caballi                      AD-ATDIENF ASRYLY
B. ovis 1                       DG-ATDLEFL VNK---
B. ovis 2                       DG-ATDLEFL VNK---
B. ovis 3                       DG-ATDLEFL VNK---
B. bovis 1                      EG-TTDVEYL VNKVLY
B. bovis 2                      EG-TTDVEYL VNKVLY
B. bovis 3                      EG-TTDVEYL VNKVLY
B. bigemina 1                   DD-ENALHGF VQKYFY
B. bigemina 2                   DD-KNALHGF VQKYFY
B. bigemina 3                   DD-KNALHGF VQKYFY
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Table 11. Sequence identity table comparing the proposed partial RAP-1 amino acid sequences for *Babesia* spp.

Sample	*Babesia divergens*	*Babesia divergens*	*Babesia divergens*	*Babesia caballi*	*Babesia ovis*	*Babesia ovis*	*Babesia canis*	*Babesia canis canis* NA iso.	*Babesia gibsoni* (Asia) NA iso. 1	*Babesia gibsoni* (Asian) NA iso. 2	*Babesia bovis*	*Babesia bigemina*	*Babesia bigemina*
Babesia divergens	1.000	0.983	0.190	0.206	0.095	0.095	0.222	0.174	0.166	0.166	0.174	0.206	0.206
Babesia divergens	---	1.000	0.190	0.206	0.095	0.095	0.222	0.174	0.166	0.166	0.174	0.206	0.206
Babesia divergens	---	---	1.000	0.327	0.311	0.311	0.377	0.409	0.187	0.187	0.344	0.393	0.393
Babesia caballi	---	---	---	1.000	0.442	0.442	0.327	0.295	0.234	0.234	0.459	0.327	0.327
Babesia ovis	---	---	---	---	1.000	0.982	0.409	0.344	0.187	0.187	0.590	0.295	0.295
Babesia ovis	---	---	---	---	---	1.000	0.409	0.344	0.203	0.203	0.606	0.295	0.295
Babesia canis	---	---	---	---	---	---	1.000	0.737	0.281	0.281	0.360	0.426	0.426
Babesia canis canis NA iso.	---	---	---	---	---	---	---	1.000	0.312	0.312	0.327	0.459	0.442
Babesia gibsoni (Asia) NA iso. 1	---	---	---	---	---	---	---	---	1.000	1.000	0.203	0.281	0.265
Babesia gibsoni (Asian) NA iso. 2	---	---	---	---	---	---	---	---	---	1.000	0.203	0.281	0.265
Babesia bovis	---	---	---	---	---	---	---	---	---	---	1.000	0.278	0.278
Babesia bigemina	---	---	---	---	---	---	---	---	---	---	---	1.000	0.983
Babesia bigemina	---	---	---	---	---	---	---	---	---	---	---	---	1.000

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Development and Evaluation of a Semi-nested PCR for the Detection and Differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in Canine Blood Samples

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Abstract

Canine babesiosis has been recently recognized as an emerging infectious disease of dogs in North America. We sought to develop a semi-nested PCR to detect and differentiate *Babesia gibsoni* (Asian genotype), *B. canis vogeli, B. c. canis*, and *B. c. rossi* DNA in canine blood samples. An outer primer pair was designed to amplify an ≈ 340 base pair fragment of the 18S rRNA genes from *B. gibsoni* (Asian genotype), *B. c. vogeli*, *B. c. rossi* and *B. c. canis*, but not mammalian DNA. Forward primers were designed that would specifically amplify a smaller fragment from each organism in a semi-nested PCR. The practical limit of detection was 50 organisms/ml of mock-infected EDTA anti-coagulated whole blood. The primer pair also amplified a ≈ 370 b.p. fragment of the *B. gibsoni* (USA/California genotype) 18S rRNA gene from the blood of an experimentally infected dog with a high percent parasitemia. Amplicons were not detected when DNA extracted from the blood of a dog that was naturally infected with *Theileria annea* at a low percent parasitemia was amplified. Due to limited sensitivity, this test is not recommended for the routine diagnosis of *B. gibsoni* (USA/California genotype) or *T. annae*. The PCR test did not amplify *Toxoplasma gondii*, *Neospora caninum*, *Leishmania infantum, Cryptosporidium parvum* or canine DNA under any of the conditions tested. The semi-nested PCR test was able to detect and discriminate *B. gibsoni* (Asian genotype), *B. c. vogeli*, *B. c. canis*, and *B. c. rossi* DNA in blood samples from infected dogs.

Introduction

Babesiosis is an important disease of domestic dogs in the United States caused by intra-erythrocytic protozoan parasites of the genus *Babesia*. These hemoprotozoan parasites, along with *Theileria* spp., are often referred to as piroplasms. Babesiosis is typically characterized by hemolytic anemia, thrombocytopenia, fever and splenomegaly. Historically, canine babesiosis has been attributed to infection with either *B. canis* or *B. gibsoni*, based on parasite size and the geographic location in which the infection was acquired. Recently an increased number of genetically unique piroplasms have been identified, and the recognized geographic ranges of various canine piroplasms appear to be expanding. Since Conrad et. al. reported the first outbreak of canine babesiosis caused by a small *Babesia* in the United States in 1991, there have been an increasing number of reports of dogs infected with small *Babesia sp.* (Birkenheuer et al., 1999; Camacho et al., 2001; Irizarry-Rovira et al., 2001; Kjemtrup et al., 2000; Kocan et al., 2001; Macintire et al., 2002; Zahler et al., 2000a). During the California outbreak in 1991, *Babesia gibsoni* was presumed to be the only small *Babesia sp*. to infect dogs. However, recent studies demonstrate that there are at least three genetically distinct small *Babesia*-like organisms or piroplasms that can infect dogs(Kjemtrup et al., 2000; Zahler et al., 2000a; Zahler et al., 2000b). Based on evolving data, the nomenclature of these small piroplasms is likely to undergo revision.

It is diagnostically important to determine the species, subspecies and genotype that causes canine babesiosis, as the virulence, prognosis and response to anti-babesial drugs may be different for each organism. In this report we will refer to the Asian genotype of *B. gibsoni* (GenBank Accesion Nos. AF271081, AF271082, AF205636,

AF175300 and AF175301) as *Babesia gibsoni* (Asian genotype),(Kjemtrup et al., 2000; Kocan et al., 2001; Zahler et al., 1998) the North American genotype of *B. gibsoni* (AF158702, AF231350 and L13729) as *B. gibsoni* (USA/California genotype)(Conrad et al., 1991; Kjemtrup et al., 2000; Zahler et al., 2000b) and the European small canine piroplasm (AF188001) as *Theileria annae*, as has been proposed by Zahler et. al(Zahler et al., 2000a). Both *B. gibsoni* (Asian genotype) and the *B. gibsoni* (USA/California genotype) have both been identified in dogs from North America, while *T. annae* has only been reported in Europe(Camacho et al., 2001; Zahler et al., 2000a). *Babesia gibsoni* (Asian genotype) is considered to be virulent in dogs and to date no anti-babesial treatment has been able to eliminate the infection(Yamane et al., 1993). The *B. gibsoni* (USA/California genotype) is also virulent, but it's susceptibility to anti-babesial therapy has not been well characterized(Wozniak et al., 1997; Yamane et al., 1994). To our knowledge, the comparative pathogenicity or the responsiveness to anti-babesial therapy of *T. annae* has not been studied.

There is support for the existence of three subspecies of large canine piroplasms, *B. c. vogeli*, *B. c. canis*, and *B. c. rossi* based on genetic data, vector specificity, and variations in pathogenicity(Carret et al., 1999; Uilenberg et al., 1989; Zahler et al., 1998). *Babesia c. vogeli*, is found in North America, Europe, and Asia, is considered to be a moderately virulent species, and it is presumed that anti-babesial therapy will eliminate the infection. *Babesia c. canis*, which is mostly found in Europe, has somewhat variable virulence, and the organism is generally considered to respond to anti-babesial drugs(Carret et al., 1999; Uilenberg et al., 1989; Zahler et al., 1998). *Babesia c. rossi*, which has only been identified in Africa, is considered to be a highly virulent piroplasm

that may not be susceptible to the currently available drugs(Carret et al., 1999; Uilenberg et al., 1989; Zahler et al., 1998).

The definitive diagnosis of canine babesiosis, as well as the visual differentiation of the species of piroplasms, can be difficult for the clinician. The generally accepted gold standard(s) to rule out babesiosis are splenectomy with or without immune suppression, or blood transfusion from the suspect dog into a splenectomized dog(Bose et al., 1995). For obvious reasons, these procedures are rarely, if ever, performed in the clinical setting achieve a diagnosis. Historically, light microscopic examination of stained blood smears and serology have provided the primary means of diagnosing babesiosis in dogs. Light microscopic examination can not be used to establish the genotype of any piroplasms. Antibodies to *Babesia* spp. are often cross-reactive; therefore serology may not definitively discriminate species or subspecies. In addition, there are reports of canine *Babesia* infections in which piroplasms were not identified by light microscopic examination and/or serologic testing yielded false negative results in dogs that were infected with *Babesia*(Birkenheuer et al., 1999; Breitschwerdt et al., 1983; Macintire et al., 2002). Since the geographic range of specific piroplasms appears to be expanding, location should not be used as the sole criterion for species or subspecies identification. Although not without limitations, the polymerase chain reaction (PCR) offers a practical and non-invasive means to detect and differentiate infections with various *Babesia* spp., and also provides a sensitive tool to assess treatment outcomes. PCR is likely to be more sensitive than light microscopic examination of stained blood smears based on the reported limits of detection for each test(Bose et al., 1995). Since infected dogs may have antibodies that are unpredictably cross-reactive against other

Babesia species or sub-species, PCR is more specific than serology. To our knowledge there are no studies directly comparing all of the available diagnostic tests for babesiosis in a canine population in which the true disease prevalence is known. In this study we sought to develop a PCR test for canine babesiosis that can detect and differentiate *B. gibsoni* (Asian genotype), *B. c. vogeli, B. c. canis* and *B. c. rossi* and to define the test's limits of detection in canine blood samples.

Materials and Methods

Samples

Babesia gibsoni (Asian genotype) infected whole blood samples were either obtained from a specific pathogen free splenectomized dog that was infected intravenously with blood from a dog that was confirmed to be infected with *B. gibsoni* (Asian genotype), or dogs (n=5) from North America that were confirmed to be infected with a small piroplasm. *Babesia c. vogeli* infected canine whole blood samples were obtained from dogs (N=3) from North America that were confirmed via light microscopy to be infected with large piroplasms. *Babesia c. canis* infected canine whole blood sample was obtained from a specific pathogen free splenectomized dog that was infected intravenously with blood from a dog that was confirmed to be infected with *B. c. canis*. *Babesia c. rossi* infected canine whole blood samples, kindly provided by George Moore (United States Army Medical Department Center and School, Fort Sam Houston, Texas), were obtained from dogs (n=2) from South Africa that were confirmed via light microscopy to be infected with large piroplasms. *Babesia gibsoni* (California/USA genotype) infected canine whole blood samples were obtained from a specific pathogen free splenectomized dog that was infected intravenously with blood from a dog that was confirmed to be infected with *B. gibsoni* (California/USA genotype). The original *B. gibsoni* (California/USA genotype) isolate was kindly provided by Patricia Conrad (University of California, Davis, CA). *Theileria annae* infected canine whole blood sample from Spain, kindly provided by Sam Telford III (Harvard University, Boston, MA), was obtained from a dog confirmed via light microscopy to be infected with small piroplasms. *Toxoplasma gondii* and *Neospora caninum* DNA was kindly provided by Nick Sharp

(Animal Critical Care Group of Vancouver, Burnaby, British Columbia). DNA extracted from the anticoagulated whole blood of a dog that was naturally infected with *Leishmania infantum*(Gaskin et al., 2002). *Cryptospridium parvum* DNA was kindly provided by Lance Perryman (Colorado State University, Fort Collins, CO).

Primer design

Oligonucleotide primers were designed based on the canine *Babesia* 18S rRNA genes as reported in Genbank(Benson et al., 2000). For the amplification of the nearly full-length *Babesia* 18S rRNA genes primers (5-22F and 1661R) were designed to amplify *Babesia* 18S rRNA genes but not mammalian 18S rRNA genes.

For the semi-nested PCR, an outer primer pair (455-479F and 793-772R) was designed that would amplify an approximately ≈340 base pair (b.p.) fragment from *B. gibsoni* (Asian genotype) (AF271081, AF271082, AF205636, AF175300 and AF175301), *B. c. vogeli* (AJ009796 and AY072925), *B. c. canis* (AJ009795 and AY072926), and *B. c. rossi* (L19079) that spanned a hypervariable region of the 18S rRNA gene. Then specific internal primers were designed for *B. gibsoni* (Asian genotype) (BgibAsia-F), *B. c. vogeli* (BCV-F), *B. c. canis* (BCC-F), and *B. c. rossi* (BCR-F) that were paired with the outer reverse primer in the semi-nested secondary reaction to amplify 185 b.p., 192 b.p., 198 b.p., and 197b.p. amplicons respectively. The sequences of the oligonucleotide primers used in this study are listed in table 12. All primers were synthesized by Integrated DNA Technologies, Coralville, IA.

Preparation of DNA

DNA was isolated from canine whole blood samples using the QIAamp DNA blood mini kit according to the manufacturer's instructions. Plasmid DNA was isolated with the QIAprep spin miniprep according to the manufacturer's instructions.

PCR

The nearly full-length *B. gibsoni* (Asian genotype) and *B. c. canis* 18S rRNA genes were amplified by PCR. The amplification of the nearly full-length 18S rRNA genes was performed using 25-µl reactions. Each 25-µl reaction contained 1X concentration of PCR Buffer II (Perkin Elmer, USA), 0.625 U of *Taq* polymerase, 0.5 ul of DNA template, 1.5 m*M* $MgCl_2$, 12.5 pmol of each primer, and 200 µ*M* of each dNTP. Cycling conditions were 95 C for 5 minutes, followed by 35 amplification cycles (95 C for 1 minute, 56 C for 1 minute, and 72 C for 1 minute), and a final extension step at 72 C for 5 minutes. (PCR Express; Thermo Hybaid, Middlesex, United Kingdom)

The PCR reaction conditions were optimized for annealing temperature (55 to 62ºC by increments of ≈1.4ºC) and MgCl concentration (1.0mM to 5.0mM increments of 0.5mM) using the experimentally infected *B. gibsoni* (Asian genotype) and non-infected canine DNA as positive and negative controls respectively. The optimal conditions were used with a 50-µl reaction volume containing 1.25U Amplitaq gold (Perkin Elmer, USA)/reaction, 25pmol of each primer, and 200µM of each dNTP, 1.5mM MgCl concentration, and a 1X concentration of PCR Buffer II (Perkin Elmer, USA). DNA amplification using the outer primer pair was performed in a thermal cycler (PCR

Express; Thermo Hybaid, Middlesex, United Kingdom) at the following temperature profiles: initial denaturation at 95º C for 5 minutes, followed by 50 amplification cycles (95º C for 45 seconds, 58º C for 45 seconds, and 72º C for 45 seconds), and a final extension step at 72º C for 5 minutes. (PCR Express; Thermo Hybaid, Middlesex, United Kingdom)

Semi-nested PCR reactions (i.e. specific forward primers paired with the outer reverse primer) were each carried out in separate tubes under the same conditions as the outer primer pair except for the following; 0.5µl from the initial reaction was used as a DNA template and the reactions were amplified for 30cycles. In order to prevent PCR amplicon contamination, sample preparation, reaction setup, PCR amplification, and amplicon detection were all performed in separate areas. Positive and negative controls were used in all processing steps including the DNA extraction. The presence of PCR inhibitors in DNA samples that tested negative in our PCR test was excluded by the amplification of a fragment of the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene. The presence of *T. annae* DNA in the sample from Spain was confirmed by amplification of the nearly full-length 18S rRNA gene as described above.

Amplicon detection

All PCR products were visualized after electrophoresis in a 2% agarose gel containing 0.2µg/ml concentration of ethidium bromide by transillumination with an ultraviolet light.

Cloning and sequencing of PCR Products

The PCR products were cloned into a plasmid vector (PCR 2.1®, Invitrogen, Carlsbad, CA, USA) and *Escherichia coli* (TOP10'®, Invitrogen, Carlsbad, CA, USA) strain was transformed following the protocol of the supplier. Recombinants were selected by blue/white color of colonies and plasmid DNA from at least three clones, for each isolate, were sequenced. Recombinant plasmid DNA was sequenced bi-directionally with infrared fluorescent-labeled primers M13R-700 (5'CAGGAAACAGCTATGACCATG) and T7-800 (5'TAATACGACTCACTATAGGGCGA) synthesized by LI-COR Inc., Lincoln, NE, USA. Previously described internal sequencing primers, 515F and 1391R, were also used for full-length 18S rDNA sequencing(Pitulle et al., 1999). The sequencing reaction conditions were: 2 min. at 92 °C, followed by 30 amplification cycles (30 sec. at 92° C, 15 sec. at 55° C, 30 sec. at 72° C) (Hybaid PCR Express). The sequencing reactions were analyzed by polyacrylamide gel electrophoresis (3.75%) on an automated DNA sequencer (LI-COR 4200 DNA Sequencer, LI-COR Inc., Lincoln, NE, USA).

Limit of detection

The DNA concentrations of the purified plasmids containing nearly full length *B. gibsoni* (Asian genotype) (sequence identical to AF271081) and *B. c. canis* (sequence identical to AY072926) 18S rRNA genes were determined by spectrophotometry (Biospec-mini, Shimadzu corporation, Columbia, MD, USA). These plasmid clones were individually serially diluted ten-fold in tris-EDTA buffer to concentrations ranging from 1,000,000 to

10 plasmids/ml. One microliter of each dilution was then used as a template for the primary PCR reaction.

To determine the practical limit of detection of the semi-nested PCR, leukocyte reduced packed red blood cells from *B. gibsoni* (Asian genotype) or whole blood from a *B. c. canis* infected dog with parasitemias of known percentages were added to whole blood obtained from a non-infected dog. For the *B. gibsoni* (Asian genotype) mock infected samples, five hundred milliliters of whole blood was sterilely collected, using CPDA-1 as an anticoagulant, from the jugular vein of a splenectomized dog that had been experimentally infected with *B. gibsoni* (Asian genotype) via intravenous injection. The blood was leukoreduced using a commercially available leukocyte reduction filter. (Purecell, Pall corporation, East hills, NY) The leukoreduced blood was centrifuged at 4000 rpm and the plasma was removed. The red blood cell count was determined in an automated cell counter, and a thin smear was stained with a modified Wright's stain to determine the percent parasitemia. After leukoreduction and concentration the red blood cell count was 12.5 x 10^6 cell/µl with a parasitemia of 10% based on a one thousand cell count. Forty microliters (5x10^7 organisms) of these leukoreduced *B. gibsoni* (Asian genotype) infected concentrated red blood cells were added to 960µl of non-infected EDTA anti-coagulated canine whole blood. These samples were then ten-fold serially diluted using the non-infected canine whole blood. The red blood cell count of the non-infected dog was 6.8 x10^6 cells/µl. For the mock infected *B. c. canis* samples 3 ml of EDTA anticoagulated whole blood was collected via jugular venopuncture from a splenectomized dog that had been infected intravenously with *B. c. canis*. The *B. c. canis* infected dog's red blood cell count was 5.7 x 10^6 cell/µl with a parasitemia of 9% based

on a one thousand-cell count. One hundred microliters ($5x10^7$ organisms) of these *B. c. canis* infected red blood cells were added to 900µl of non-infected EDTA anti-coagulated canine whole blood. These samples were then ten-fold serially diluted using the non-infected canine whole blood. The red blood cell count of the non-infected dog was 6.8 $x10^6$ cells/µl. The final test concentrations of both mock infected samples ranged from 500,000 to 5 organisms/ml. Total DNA was extracted from 200µl aliquots of these dilutions using a commercially available DNA extraction kit. Five µl of each dilution was used as a template for the primary PCR reaction.

Specificity

Five microliters of DNA extracted from *Toxoplasma gondii*, *Neospora caninum*, , *Cryptosporidium parvum*, and *Leishmania infantum* infected canine whole blood and a non-infected dog were used as template the PCR reactions.

DNA sequence analysis

The DNA sequences generated in this study were aligned and compared to the DNA sequences in Genbank(Benson et al., 2000). using a computer program. (MegAlign™, DNAStar, Inc., Madison, WI, USA)

Results

PCR

Nearly full-length (≈ 1.7 kb) 18S rRNA genes were amplified from the experimentally infected *B. gibsoni* (Asian genotype), *B. c. canis* samples and the naturally infected *T. annae* sample from Spain (data not shown).

During the primary reaction of the semi-nested PCR, a ≈ 340 b.p. product was amplified from *B. gibsoni* (Asian genotype), *B. c. vogeli*, *B. c. canis* or *B. c. rossi* infected canine whole blood samples. An ≈ 370 b.p. product was amplified from the *B. gibsoni* (USA/California genotype) infected canine whole blood samples. No amplicons were detected when DNA from the *T. annae* infected canine whole blood sample was used as template for the PCR. (Figure 7) The PCR tests did not produce amplicons when canine DNA or DNA extracted from *T. gondii*, *N. caninum*, or *C. parvum* were used as template (data not shown).

During the secondary semi-nested reaction, the test was able to differentiate *B. gibsoni* (Asian genotype), *B. c. vogeli*, *B. c. canis* and *B. c. rossi* when the specific internal primers were paired with the reverse primer in the secondary reactions (Figure 8). Occasionally after the second round of amplification both the ≈ 340 b.p., the and the 185-200 b.p. amplicons were visualized on the gel, but test interpretation was not affected (Figure 9). An additional ≈ 300 b.p. amplicon of unknown significance was also detectable after the secondary semi-nested PCR reactions. In both the primary and secondary reactions, low molecular weight bands (≈ 25-50 b.p.) were often detected in both positive and negative samples, including the no DNA controls. These low molecular weight bands were presumed to be "primer-dimers."

Amplicon contamination was not detected in any of the negative control samples at any time. As determined by amplification of G3PDH, no PCR inhibitors were detected in any of the DNA samples that were negative when using our PCR test.

Limit of detection

The absolute limit of detection, using plasmid clones of nearly full length *B. gibsoni* (Asian genotype) and *B. c. canis* 18S rRNA genes diluted in tris-EDTA buffer, was 1 molecule/reaction (Data not shown). The practical limit of detection of *B. gibsoni* (Asian genotype) and *B. c. canis* organisms diluted in non-infected canine whole blood was 50 organisms/ml. (Figure 10) The second round of amplification using a specific internal primer paired with the reverse primer did not improve the limit of detection, but did improve ease of interpretation due to the enhanced visualization of bands on the gel.

Sequencing

The sequences of the nearly full-length 18S rRNA gene clones for *B. gibsoni* (Asian genotype) and *B. c. canis* were 100% identical to Genbank accession numbers AF271081 and AY072926 respectively. The sequences of the ≈340 b.p. amplicons from the North American dogs naturally infected with small piroplasms were identical to Genbank accession numbers AF271081, AF271082, AF205636, AF175300 and AF175301 (*B. gibsoni* Asian genotype). The sequences of the ≈340 b.p. amplicons from the North American dogs naturally infected with large piroplasms were ≥ 99 % identical to Genbank accession numbers AJ009795 and AY072926 (*B. c. vogeli*). The sequences of the ≈340 b.p. amplicons from the dog experimentally infected with *B. c. canis* was

identical to Genbank accession number AY072926 (*B. c. canis*). The sequences of the ≈ 340 b.p. amplicons from the South African dogs naturally infected with large piroplasms were ≥ 99 % identical to Genbank accesion number L19079 (*B. c. rossi*). The sequence of the 369 b.p. amplicon from the dog experimentally infected with *B. gibsoni* (USA/California) was 98% identical to Genbank accession numbers AF158702 and AF231350 (*B. gibsoni* USA/California genotype). The sequence of the nearly full-length *T. annae* 18S rRNA amplicon was ≥ 99 % identical to Genbank accesion number AF188001 (*T. annae*).

Discussion

For several reasons, a definitive diagnosis of canine babeisiosis can be difficult to achieve in the clinical setting. Light microscopic examination can not consistently differentiate species or subspecies. The lack of standardized serologic assays, the presence of cross-reactive antibodies, and recent changes in the geographic ranges of several canine piroplasms have also further complicated the diagnosis of babesiosis in dogs. In this study we describe a semi-nested PCR that has a practical limit of detection of 50 organisms/ml of canine whole blood. The PCR is specific for the diagnosis and differentiation of *B. gibsoni* (Asian genotype), *B. c. vogeli, B. c. canis* and *B. c. rossi*. Based on lowest number of detectable organisms/ml of whole blood, the limit of detection test is 180 fold lower than the reported limit of detection for a previously described PCR based test for *B. gibsoni* (Asian genotype)(Ano et al., 2001). Unfortunately, the majority of reports describing PCR based tests for the diagnosis of canine babesiosis have not reported a limit of detection or in the case of one study(Fukumoto et al., 2001) only reported the limit of detection in terms of lowest detectable percent parasitemia(Caccio et al., 2002; Carret et al., 1999; Irizarry-Rovira et al., 2001; Kjemtrup et al., 2000; Kocan et al., 2001; Macintire et al., 2002; Zahler et al., 1998). Although the lowest detectable percent parasitemia is often reported for the detection of piroplasmosis, comparisons of tests based on percent parasitemia can be difficult to interpret. For example, *Babesia* infected animals can have red blood cell counts ranging from severely anemic to normal (1.5×10^6 to 7.6×10^6 red blood cells per microliter) therefore the degree of anemia could result in as much as a five fold difference in the total number of parasites/volume when examining samples from

different animals with identical percent parasitemias. The lowest estimated percent parasitemia that was detectable by our test was 0.00000073%, which is about 1,300 fold lower than the accepted limit of detection (0.001% parasitemia) for light microscopic examination of stained blood smears(Bose et al., 1995). Despite the aforementioned difficulty in interpreting differences in test sensitivities based on percent parasitemia, it is seems unlikely that differences in red blood cell counts would account for a 1300 fold difference.

The clinical sensitivity of the PCR could not be determined in this study, and to our knowledge has not been described for any other PCR tests for canine babesiosis. In order to determine the clinical sensitivity, a population of animals in which the true prevalence of infection is known (i.e. experimental infection) is required. The detection limit of our test is superior to or comparable with the tests that have been described for the detection of piroplasmosis in other species(Bashiruddin et al., 1999; Calder et al., 1996; Salem et al., 1999). A blinded study comparing PCR and serologic testing, has been performed evaluating the clinical sensitivity of a PCR test in cattle experimentally infected with *B. bovis*(Calder et al., 1996). The PCR test used in that study had an absolute sensitivity that was similar to our PCR for canine babesiosis. That study demonstrated false negative PCR tests in 30% of the samples, especially when the samples were obtained from chronically infected cattle with low percent parasitemia. However, the clinical sensitivity was improved to >90% by re-testing cattle with negative PCR tests 10-14 days later. A similar clinical sensitivity would be expected for the PCR test described here.

The approach used to develop the outer primer pair of our semi-nested PCR should permit the detection of previously unrecognized/unsequenced piroplasms with genetic variation in this hypervariable region of the 18S rRNA gene. If the ≈ 340b.p. amplicon is detected, but species-specific amplification not possible using the internal primers, then the ≈ 340b.p. can be sequenced directly for genotype/species identification. Surprisingly, despite a four base pair difference on the 3' end of the forward primer when compared to the *B. gibsoni* (USA/California genotype) (AF216496 and AF175300) rRNA gene sequences, an 18S rRNA gene amplicon was detected when a sample containing high numbers of parasites (30% parasitemia) was used as a template for the outer primer pair reaction. Despite an identical sequence in the primer regions, amplicons were not detected when a *T. annae* (AF188001) infected sample with a lower percent parasitemia (0.01%) was used as template. Since there were no detectable amplicons in the non-infected canine whole blood samples under the testing conditions, we chose not to increase the annealing temperature to eliminate the mispriming of the *B. gibsoni* (USA/California genotype). Although the test might be useful for identifying some *B. gibsoni* (USA/California genotype) or *T. annae* infected samples in which there are large numbers of organisms. Due to limited sensitivity, we do not recommend the use of our test for the routine diagnosis of *B. gibsoni* (USA/California genotype) or *T. annae*. In these instances when a 370 b.p. amplicon is detected, we recommend that either specific tests for *B. gibsoni* (USA/California genotype) or *T. annae* are performed or that the amplicons are sequenced to confirm the genotype of the organism.

In conclusion, we describe a PCR test for the diagnosis of canine babesiosis that can detect and differentiate *B. gibsoni* (Asian genotype), *B. c. vogeli, B. c. canis* and *B. c.*

rossi with a defined limit of detection. This test should improve diagnostic capabilities for the detection and differentiation canine *Babesia* in clinical samples and facilitate future research studies that assess canine infection with these organisms.

Table 12. Sequences for the primers used in the semi-nested PCR test.

Primer Name	Sequence (5'-3')	Reaction/Use
5-22F	GTTGATCCTGCCAGTAGT	Full-length 18S rRNA Forward Primer
1661R	AACCTTGTTACGACTTCTC	Full-length 18S rRNA Reverse Primer
455-479F	GTCTTGTAATTGGAATGATGGTGAC	Semi-nested PCR Outer Forward Primer
793-772R	ATGCCCCCAACCGTTCCTATTA	Semi-nested PCR Outer Reverse Primer
BgibAsia-F	ACTCGGCTACTTGCCTTGTC	Semi-nested PCR *B. gibsoni* (Asian genotype) Specific Forward Primer
BCV-F	GTTCGAGTTTGCCATTCGTT	Semi-nested PCR *B. c. vogeli* Specific Forward Primer
BCC-F	TGCGTTGACGGTTTGACC	Semi-nested PCR *B. c. canis* Specific Forward Primer
BCR-F	GCTTGGCGGTTTGTTGC	Semi-nested PCR *B. c. rossi* Specific Forward Primer
G3PDH-F	CCTTCATTGACCTCAACTACAT	Detection of PCR inhibitors
G3PDH-R	CCAAAGTTGTCATGGATGACC	Detection of PCR inhibitors

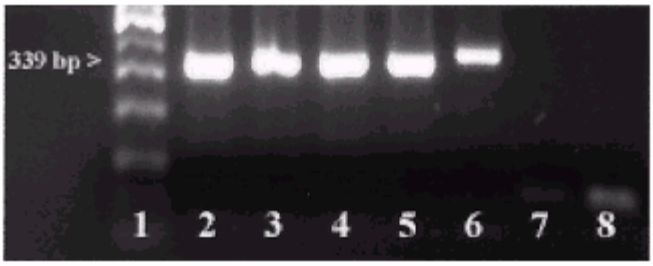

Figure 8.

Analysis of semi-nested PCR products (outer primer pair only) by 2% agarose gel electrophoresis and ethidium bromide staining. From left to right the lanes contain the following: Molecular weight marker (lane 1), *B. gibsoni* (Asian genotype) (lane 2), *B. c. vogeli* (lane 3), *B. c. canis* (lane 4), *B. c. rossi* (lane 5), *B. gibsoni* (California/USA genotype) (lane 6), *T. annae* (lane 7), Negative (no DNA) control (lane 8).

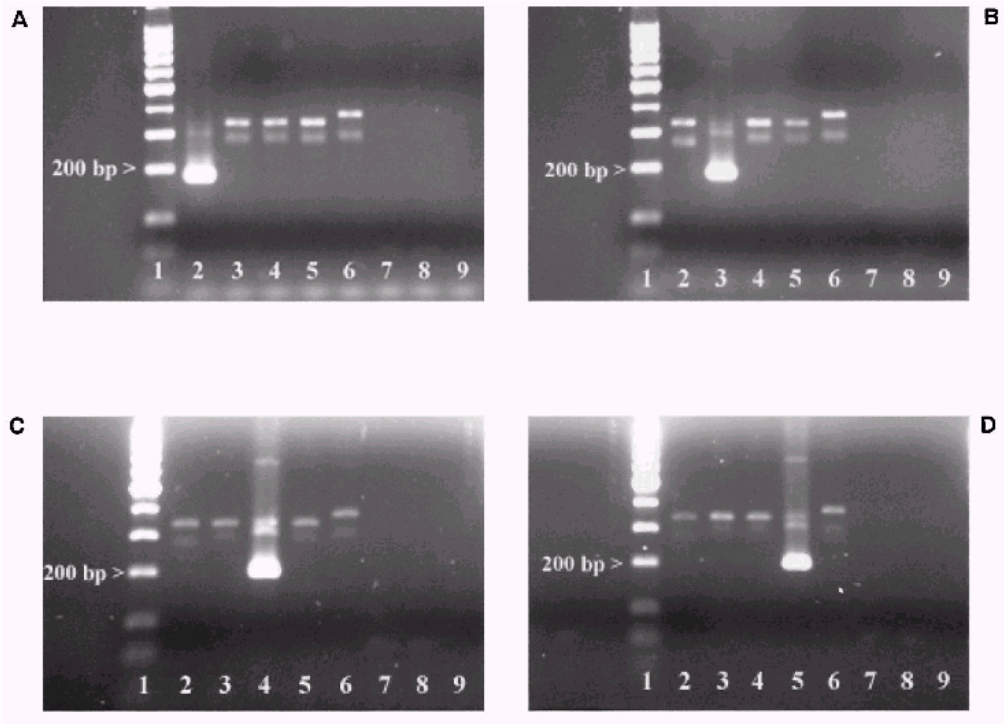

Figure 9.

Analysis of the specificity of the semi-nested PCR by 2% agarose gel electrophoresis and ethidium bromide staining. 2A: Analysis of semi-nested *B. gibsoni* (Asian genotype) specific secondary reactions. 2B: Analysis of semi-nested *B. c. vogeli* specific secondary reactions. 9). 2C: Analysis of semi-nested *B. c. canis* specific secondary reactions. 2D: Analysis of semi-nested *B. c. rossi* specific secondary reactions. In figures 2A through 2D from left to right the lanes contain the following: Molecular weight marker (lane 1), *B. gibsoni* (Asian genotype) (lane 2), *B. c. vogeli* (lane 3), *B. c. canis* (lane 4), *B. c. rossi* (lane 5), *B. gibsoni* (California/USA genotype) (lane 6), *T. annae* (lane 7), Canine genomic DNA (lane 8), Negative (no DNA) control (lane 9).

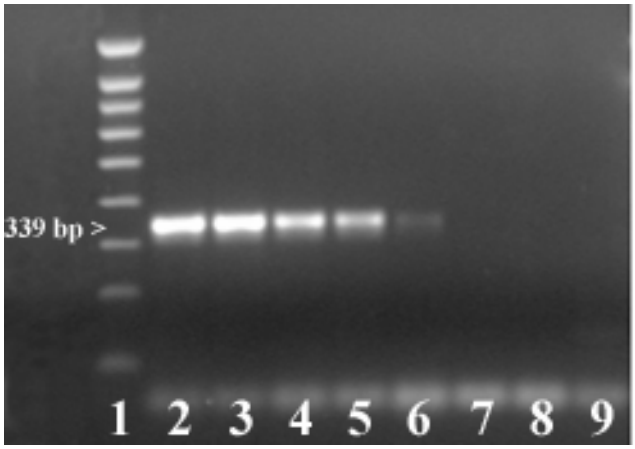

Figure 10.

Analysis of the limit of detection of the semi-nested PCR (outer primer pair amplicons only) by 2% agarose gel electrophoresis and ethidium bromide staining. From left to right the lanes contain the following: Molecular weight marker (lane 1), 500,000 *B. gibsoni* (Asian genotype) organisms/ml (lane 2), 50,000 *B. gibsoni* (Asian genotype) organsims/ml (lane 3), 5,000 *B. gibsoni* (Asian genotype) organisms/ml (lane 4), 500 *B. gibsoni* (Asian genotype) organisms/ml (lane 5), 50 *B. gibsoni* (Asian genotype) organisms/ml (lane 6), 5 *B. gibsoni* (Asian genotype) organisms/ml (lane 7), canine genomic DNA (lane 8), Negative (no DNA) control (lane 9).

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Detection and molecular characterization of a novel large *Babesia* species in a dog

Adam J. Birkenheuer

Abstract

Babesia canis has generally been considered the only large *Babesia* to infect dogs. Here we describe the molecular characterization of a large *Babesia* species that was detected in the blood and bone marrow of a dog with clinical and hematological abnormalities consistent with babesiosis. Analysis of the 18S rRNA genes revealed a unique sequence that shared 93.9% sequence identity with *B. bigemina* and 93.5% sequence identity with *B. caballi*, compared to 91.2-91.6% identity with *B. canis canis*, *B. c. vogeli*, and *B. c. rossi*. Cross-reactive antibodies against *B. canis*, *B. gibsoni* (Asian genotype), or *B. gibsoni* (USA genotype) antigens were not detected in acute or convalescent serum samples. The dog was treated with imidocarb diproprionate, which resulted in the resolution of clinical signs, and subsequently *Babesia* DNA was not detectable by PCR in post-treatment samples. The organism described in this report represents a genetically unique large *Babesia sp.* that is capable of infecting the domestic dog and the eighth genetically distinct piroplasm that can be a canine pathogen.

Introduction

Babesia canis is presumed to be the only large *Babesia* species to infect dogs throughout the world. Historically, the microscopic identification of large (≥ 3-5μm) piroplasms in canine erythrocytes was sufficient for the diagnosis of a *B. canis* infection. Geographic location, differences in pathogenicity, antigenic variation, and differences in vector specificity have all been used to support the existence of three subspecies of *B. canis*; *B. c. canis*, *B. c. rossi*, and *B.c. vogeli*(Hauschild and Schein, 1996; Hauschild et al., 1995; Lewis et al., 1996; Uilenberg et al., 1989). There are only a few reports that describe the molecular characterization of the large *Babesia* organisms infecting dogs(Caccio et al., 2002; Carret et al., 1999; Zahler et al., 1998 Criado-Fornelio, 2003 #479). Molecular evidence from these reports also supports the existence of three genetically distinct subspecies, namely *B. c. canis*, *B. c. rossi*, and *B. c. vogeli*.

The advent of molecular biology, particularly the polymerase chain reaction (PCR), has resulted in the identification and differentiation of many genetically unique organisms that are morphologically indistinguishable. For example, it was previously accepted that *B. gibsoni* was the only small piroplasm that infected dogs. However, recent studies on the molecular characterization of the small piroplasms of dogs revealed there that are at least three genetically distinct organisms(Kjemtrup et al., 2000a; Zahler et al., 2000b). It is likely that this data will result in the reclassification of these small canine piroplasms into three distinct *Babesia* species or possibly two *Babesia* species and one *Theileria* species (*T. annae*). Additionally a recent report described the presence of DNA from a fourth small piroplasm, namely *B. equi* (*T. equi*), amplified by PCR from the blood from dogs in Spain(Criado-Fornelio et al., 2003).

In this report, we describe the 18S rRNA gene sequence of a unique large piroplasm identified in the blood and bone marrow of a dog from North America with clinical babesiosis.

Materials and methods

Patient

A seven-year-old female spayed Labrador retriever undergoing chemotherapy for lymphoma was evaluated for fever (T= 105.3º F), anemia, thrombocytopenia, and leukopenia. The dog received vincristine (oncovin) 0.7 mg/m2 IV 1 week prior to presentation. According to the owner, there was no recent history of tick exposure. The dog had not of traveled outside of the state of North Carolina. The dog had acquired a wound on the face prior to presentation that was believed to be an animal bite 6 weeks prior to the presentation for babesiosis. Microscopic examination of Giemsa stained blood smears and bone marrow aspirates resulted in the identification of single or paired intra-erythrocytic pyriform, oval or ameboid shaped piroplasms (Figure 11).

Indirect fluorescent antibody (IFA) testing

Acute and convalescent serum samples were evaluated by IFA for the presence of antibodies against *Babesia canis, B. gibsoni* (Asian genotype), *B*. *gibsoni* (USA genotype), *Ehrlichia canis*, *Bartonella vinsonii* (*berkhoffii) Rickettsia rickettsii*, or *Borrelia burgdorferi* antigens as previously described(Kordick et al., 1999).

DNA isolation, PCR amplification, cloning and sequencing

DNA was isolated from EDTA anticoagulated whole blood samples using the QIAamp DNA blood mini kit according to the manufacturer's instructions. Partial and

nearly full-length 18S rRNA genes were amplified, cloned, and sequenced as previously described(Birkenheuer et al., 2003).

Sequence analysis

Seven clones from two independent PCR reactions were used to determine the nearly full-length 18S rRNA gene consensus sequence. For comparison, the corresponding 18S sequences of *B. canis rossi* (L19079), *B. canis vogeli* (AY072925), *B. canis canis* (AY072926), *B. gibsoni* (Asian genotype) (AF271081), *B. gibsoni* (USA genotype) (AF158702), *T. annae* (AF188001), *B. bigemina* (X59607), *B. caballi* (Z15104), *B. odocoilei* (U16369), *B. divergens* (U07885), *B. bovis* (L19077), *B. microti* (U09833), *B. rodhaini* (M87565) *T. equi* (Z15105), and *Plasmodium falciparum* (M19172) were also included in a alignment. Sequences alignments were constructed using Clustal V in the Bioedit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). One alignment mask, referred to as the edited alignment, consisted of only positions at which there was at least a 50% consensus, and consisted of 1596 nucleotide positions. A second alignment, referred to as the un-edited alignment, contained all nucleotide positions and consisted of 2026 nucleotide positions. Cladogram construction and bootstrap analyses were performed using the PHYLIP 3.6 package. A rooted tree was constructed using distance methods using the DNADIST and KITCH programs. Bootstrap analyses (1000 replicates/tree) were performed using the SEQBOOT and CONSENSE programs. Trees were edited using Microsoft word.

Results

Amplification of the nearly full-length 18S rRNA gene from the canine piroplasm resulted in a 1666bp product. Based on the un-edited alignment, this sequence shared the highest degree of sequence identity with *B. bigemina* (93.9%) and *B. caballi* (93.5%). When compared to *B. c. vogeli*, *B. c. canis* and *B. c. rossi* the isolate only shared 91.2-91.6% sequence identity (Table 13). Phylogenetic analyses resulted in the organism being placed in the *Babesia spp. sensu stricto* clade with good statistical support (99.8-100%). Our analyses further supported a distinct separation of the *Theileria* clade and the *B. microti*-like group from the *Babesia spp. sensu stricto* clade (Figures 12 and 13). The new *Babesia sp.* was placed as a sister species to *B. bigemina* in the trees inferred from both the edited and un-edited alignments (69.6% and 74.1% of the time respectively). The phylogeny of *B. caballi* could not be reliably resolved. The tree inferred using the edited alignment placed *B. caballi* in a group distinct from both *B. bigemina* and the new *Babesia sp.*, while the tree inferred from the un-edited alignment placed *B. caballi* in a monophyletic group with *B. bigemina* and the new *Babesia sp.* However, there was relatively low statistical support with either alignment, with *B. caballi* being placed in a separate group from the new *Babesia sp.* 61.3% of the time in the edited alignment and *B. caballi* being placed in the same group as the new *Babesia sp.* 50.5% of the time in the un-edited alignment. In our analyses, *B. bovis* was placed as a monophyletic group separate from the *Theileria clade*, the *B. microti*-like group and the *Babesia spp. sensu stricto* clade.

The dog in this report had a rapid resolution of the fever, anemia and thrombocytopenia following treatment with imidocarb diproprionate (6.6mg/kg IM). After treatment, *Babesia* DNA was no longer detectable by PCR.

Discussion

In this report, the authors have described and characterized a novel large *Babesia sp.* detected in a dog with clinical and hematological abnormalities consistent with babesiosis. The clinical signs identified in this dog were consistent with those described with canine babesiosis caused by other species of *Babesia*. The leukopenia in this dog was difficult to interpret. Although transient leukopenia has been described with babesiosis (Meinkoth et al., 2002), it is unclear in this case whether or not it was related to the babesiosis, or the chemotherapy since the dog had a history of transient leukopenia after chemotherapy. The detection of genetically unique piroplasms that are clinically and morphologically indistinguishable from known organisms highlights the need for molecular diagnostics in clinical medicine(Caccio et al., 2002; Kjemtrup et al., 2000a; Zahler et al., 2000b).

Based on the phylogenetic analyses, the organism described in this report is placed in the *Babesia spp. sensu stricto* clade and is a probably sister species to *B. bigemina*. It is unknown whether or not the organism described in this report represents a new species of *Babesia* or is one of the existing *Babesia spp*. for which no genetic data are available. There were some differences in the phylogenetic relationships inferred in this study compared to previous studies. To the authors' knowledge a "definitive" molecular phylogenetic analysis of the order *Piroplasmida* does not exist. The representative organisms, genes analyzed, alignment methods, and phylogenetic analyses are not the same in all of the reports described to date. These differences often result in the placement of certain organisms within different groups. Some of the phylogenetic branches within the *Babesia spp. sensu stricto* clade are often associated with low

bootstrap values. This indicates that the relationships of the organisms within this group cannot be consistently defined with the current data sets. As determined in our analyses, low bootstrap values associated with the placement of *B. caballi* have been observed in other studies(Holman et al., 2000; Zahler et al., 2000b). Although *B. bovis* has been placed in the *Babesia spp. sensu stricto* clade in other studies, it is often not included (Caccio et al., 2002; Zahler et al., 2000a; Zahler et al., 2000b), or is a monophyletic species within that group(Allsopp et al., 1994; Kjemtrup et al., 2000a; Kjemtrup et al., 2000b). We were able to place *B. bovis* as a monophyletic species in the *Babesia spp. sensu stricto* clade when *Neospora caninum* (U17345) was included in the alignment as an additional outgroup (data not shown). Despite these differences there does however appear to be a consistent separation of the *Babesia spp. sensu stricto* clade, the *B. microti*-like group and the *Theileria spp.* clade(Allsopp et al., 1994; Kjemtrup et al., 2000a; Kjemtrup et al., 2000b; Zahler et al., 2000a; Zahler et al., 2000c; Zahler et al., 1998). The final taxonomic position of all of the piroplasms is likely to be better resolved once more species and more genes have been characterized. The organism described in this report either represents a new *Babesia sp*. or is one of the nearly 100 *Babesia sp*. described for which no genetic data has been reported.

As this *Babesia sp.* was detected in an immunosuppressed dog, it is unclear whether or not the organism is a host-specific canine pathogen or was only pathogenic in an immunocompromised dog, for which the piroplasm is not host-specific. This clinical scenario would be similar to human infections with *B. divergens* in Europe.

In conclusion, this report describes a novel large *Babesia sp.* that is capable of infecting the domestic dog and the eighth genetically distinct piroplasm that can be a

canine pathogen. This report further emphasizes the superiority of molecular testing compared to morphologic and serologic testing for the accurate identification of piroplasms in both clinical and research settings.

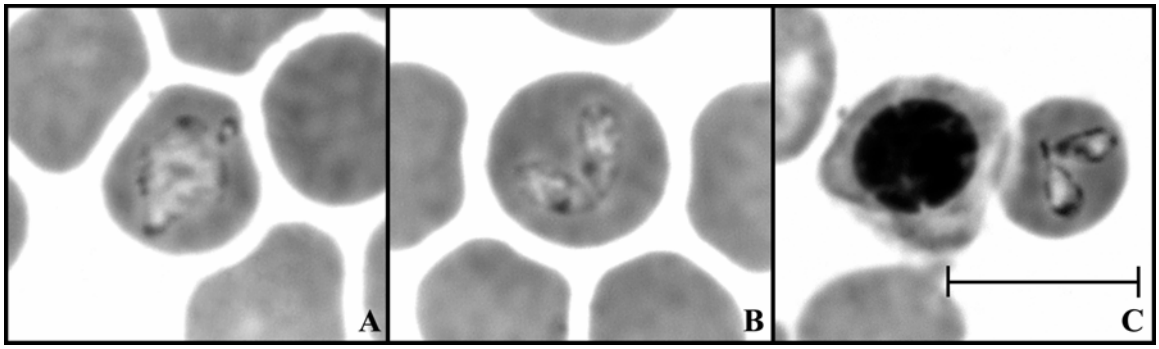

Figure 11. Photomicrograph of the novel large *Babesia*. A: Ameboid form. B and C: Paired pyriform forms.

Table 13. Sequence identity table comparing 18S rRNA gene sequences.

	New	*B. c. rossi*	*B. c. vogeli*	*B. c. canis*	*B. odocoilei*	*B. gibsoni (Asia)*	*B. caballi*	*B. bigemina*	*B. divergens*	*B. equi*	*B. bovis*	*B. gibsoni (CA)*	*B. rodhaini*	*B. microti*	*T. annae*	*P. falciparum*
New	100	91.6	91.2	91.2	91.6	92	93.5	93.9	91	85.6	84.6	85.1	83.8	84.6	84.7	61.9
B. c. rossi	---	100	94.4	95.2	93.1	94.2	92.2	91	92.6	85.8	82.9	84.8	83.7	84.2	84	62.5
B. c. vogeli	---	---	100	97.7	93.1	94	91.5	91.4	92.5	86.1	83.1	85.2	84	84.1	84.4	62.6
B. c. canis	---	---	---	100	93.4	94.4	91.7	91.4	92.6	86.2	82.9	85.4	84	84.4	84.5	62.6
B. odocoilei	---	---	---	---	100	95.5	92.5	91.6	97.6	87	82.9	86.8	84.7	85.5	85.8	63.1
B. gibsoni (Asia)	---	---	---	---	---	100	92.6	91.8	94.6	86.5	83.1	85.8	84.1	85	85.1	62.7
B. caballi	---	---	---	---	---	---	100	93.9	92.2	85.4	84.6	84.4	83.3	84	83.8	62.5
B. bigemina	---	---	---	---	---	---	---	100	91	85.1	85.1	85.2	83.2	83.8	83.8	61.6
B. divergens	---	---	---	---	---	---	---	---	100	86.4	82.2	85.9	84.6	85.1	85.3	62.8
B. equi	---	---	---	---	---	---	---	---	---	100	79.8	90.3	88.7	89.3	89.2	63.6
B. bovis	---	---	---	---	---	---	---	---	---	---	100	79	78.3	78.4	78.2	58.5
B. gibsoni (CA)	---	---	---	---	---	---	---	---	---	---	---	100	88.6	89.3	89.8	64.1
B. rodhaini	---	---	---	---	---	---	---	---	---	---	---	---	100	93.9	93.4	64.3
B. microti	---	---	---	---	---	---	---	---	---	---	---	---	---	100	96.2	64.7
T. annae	---	---	---	---	---	---	---	---	---	---	---	---	---	---	100	65.4
P. falciparum	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	100

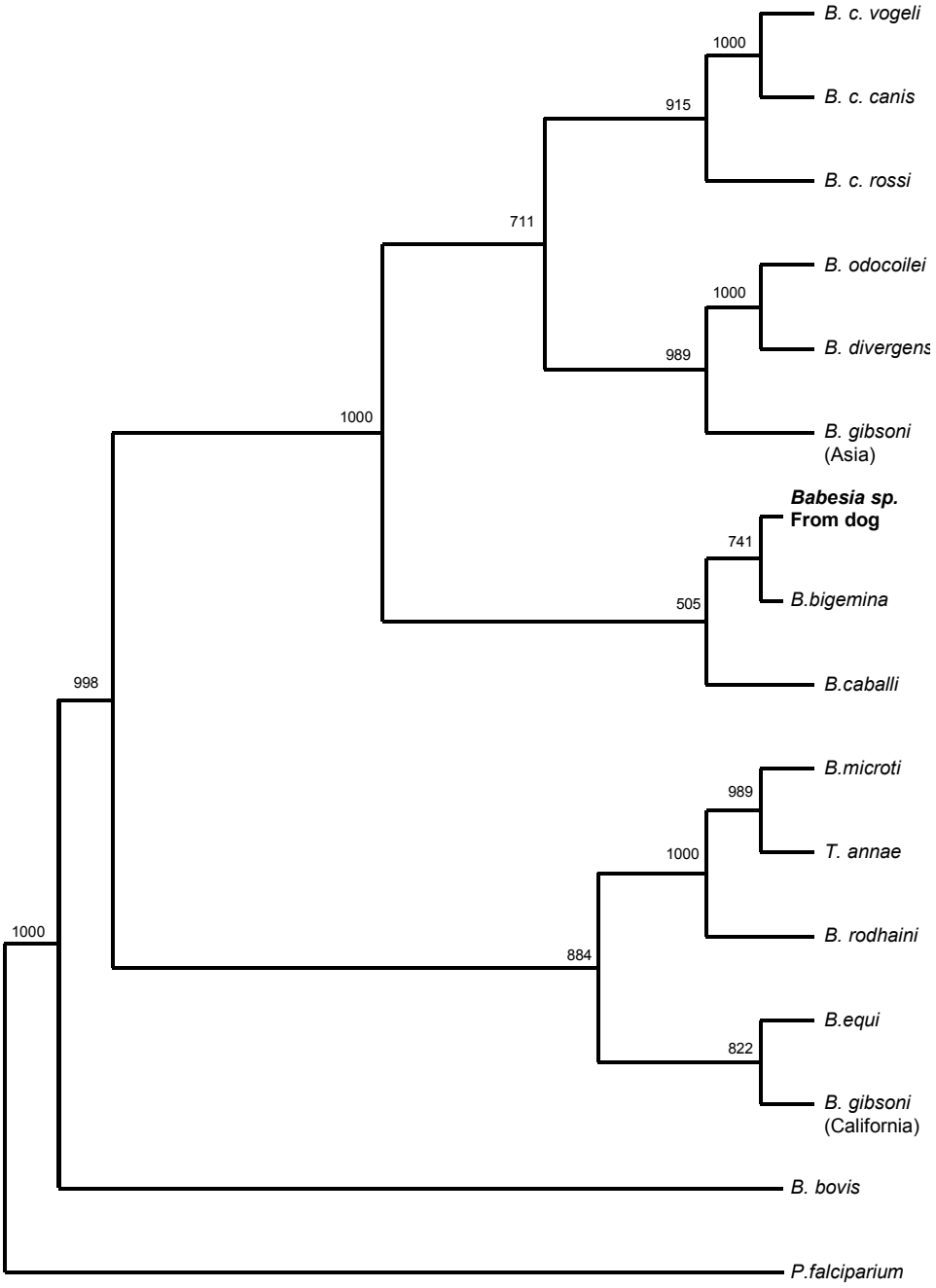

Figure 12: Phylogenetic tree inferred using the edited alignment.

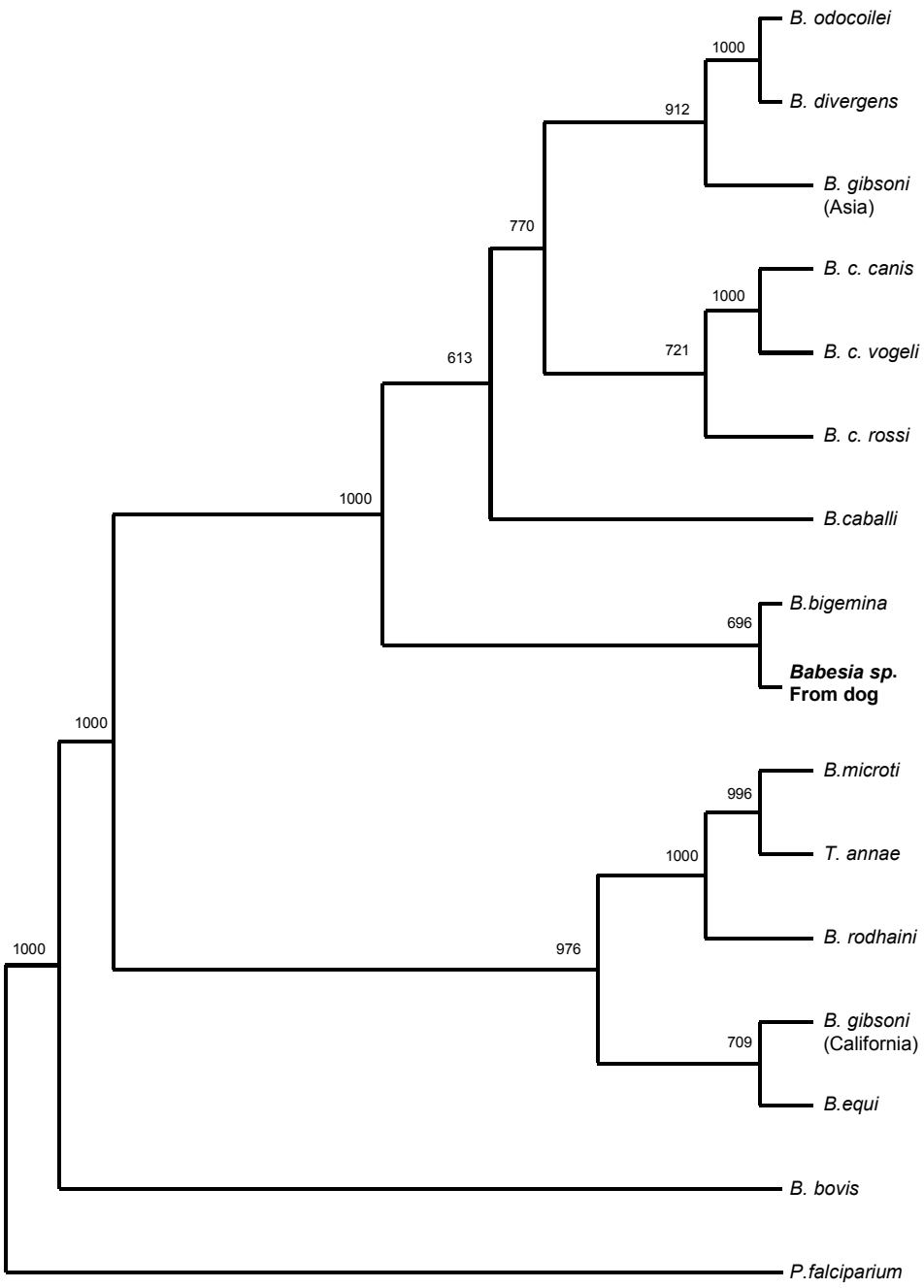

Figure 13: Phylogenetic tree inferred using the un-edited alignment.

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Double-blind Placebo-Controlled Trial Evaluating the Efficacy of An Atovaquone and Azithromycin Combination Therapy for Chronic *Babesia gibsoni* (Asian genotype) Infections

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Abstract

Babesiosis caused by *Babesia gibsoni* (Asian genotype) is an emerging canine disease in the United States. To date, no drugs have been shown to eliminate *B. gibsoni* (Asian genotype) infections from dogs. Twenty-two dogs that remained persistently infected with *B. gibsoni* (Asian genotype) after either imidocarb diproprionate and or diminazine aceturate therapy were identified and randomly and evenly distributed into two groups. One group was treated with atovaquone and azithromycin combination therapy and the other group received placebo. Eighty percent (8/10) of the treatment group had no detectable *B. gibsoni* (Asian genotype) deoxyribonucleic acid (DNA), by a sensitive and specific polymerase chain (PCR) test, in any of their post-treatment samples. In contrast, *B. gibsoni* (Asian genotype) DNA was detectable by PCR in the post-treatment samples from one hundred percent (11/11) of the placebo treated dogs. One dog in the treatment group was excluded from the treatment outcome analysis. The excluded dog had two consecutive negative PCR tests and was euthanatized due to ongoing degenerative joint disease prior to completion of the study. No adverse effects were reported in any dog during the study period. An atovaquone and azithromycin drug combination is the first described treatment that will either eliminate *B. gibsoni* (Asian genotype) infections or suppress the parasitemia below the limit of detection in the majority of treated dogs.

Introduction

Babesia gibsoni (Asian genotype) is an emerging infectious disease with an expanding region of endemicity(Birkenheuer et al., 2003a; Birkenheuer et al., 1999; Irizarry-Rovira et al., 2001; Kocan et al., 2001; Macintire et al., 2002). Infection with *B. gibsoni* (Asian genotype) can result in severe clinical manifestations in some dogs, while others only exhibit mild clinical signs and minimal hematologic or biochemical abnormalities(Birkenheuer et al., 1999; Botros et al., 1975; Farwell et al., 1982; Groves and Dennis, 1972; Macintire et al., 2002). There is no vaccine available to prevent *B. gibsoni* (Asian genotype) infections, and since the parasites are not eliminated by any of the anti-babesial therapies tested to date, pets that survive acute infections are at risk for recurring clinical disease and serve as reservoir hosts. (Farwell et al., 1982; Fowler and Ruff, 1970; Groves and Vanniasingham, 1970; Yamane et al., 1993). Canine babesiosis caused by *B. gibsoni* (Asian genotype) is very common in areas where tick-transmission is occurring. For example, in Japan 39% of the veterinarians surveyed had treated dogs for *B. gibsoni* (Asian genotype) infections between 1991 and 1992(Onishi et al., 1994). Seroprevalence studies in Malaysia, Taiwan, Korea, Japan, and Okinawa reported seroprevalences of 17.7%, 17%, 16.4%, 10.6%, and 9.4% respectively(Chang and Tu, 1992; Inokuma et al., 1998a; Inokuma et al., 1998b; Rajamanickam et al., 1985; Suh et al., 1997).

Recent molecular investigations have determined that there are at least three genetically distinct small piroplasms that are capable of infecting dogs,(Kjemtrup et al., 2000a) two have which have had 18S ribosomal ribonucleic acid sequences submitted to Genbank(Benson et al., 2003) as *B. gibsoni*. One of these genotypes has been identified

in dogs from Asia and North America and is presumed to be the same as the *B. gibsoni* originally described by Patton in 1910(Patton, 1910). The other genotype has only been reported in dogs in California. The genotype of the majority of small *Babesia* organisms reported in the United States have been of the Asian genotype(Birkenheuer et al., 2003a; Irizarry-Rovira et al., 2001; Kjemtrup et al., 2000a; Kocan et al., 2001; Macintire et al., 2002) not the California genotype(Conrad et al., 1991; Conrad et al., 1992; Kjemtrup et al., 2000b). The number of *B. gibsoni* (Asian genotype) infections reported in the United States is increasing. Prior to 1999 there were only two cases presumed to be *B. gibsoni* (Asian genotype) infections in the US, and one of those was a dog that had been imported from an endemic region(Anderson et al., 1979; Groves and Yap, 1968). Since 1999 the number of reports of *B. gibsoni* (Asian genotype) infections in the U.S. has increased considerably. (Birkenheuer et al., 2003a; Birkenheuer et al., 1999; Irizarry-Rovira et al., 2001; Kocan et al., 2001; Macintire et al., 2002; Stegeman et al., 2003).

No drugs have been demonstrated to clear *B. gibsoni* (Asian genotype) infections from dogs including diminazine aceturate and imidocarb diproprionate(Birkenheuer et al., 1999; Farwell et al., 1982; Fowler et al., 1972; Groves and Dennis, 1972; Groves and Vanniasingham, 1970; Itoh et al., 1988; Ruff et al., 1973; Stegeman et al., 2003; Takahashi, 1984; Yamane et al., 1993). The anti-protozoal drug atovaquone has shown excellent anti-babesial activity against two *Babesia* species, *B. microti* and *B. divergens*(Gray and Pudney, 1999; Hughes and Oz, 1995; Pudney and Gray, 1997). Atovaquone combined with the azalide antibiotic, azithromycin, has eliminated *B. microti* parasitemia in both humans and hamsters(Krause et al., 2000; Wittner et al., 1996). These two drugs appear to have an additive effect since neither drug alone will not

eliminate *Babesia* spp. parasitemia(Pudney and Gray, 1997; Weiss et al., 1993; Wittner et al., 1996). The mechanism of action of either atovaquone or azithromycin against *Babesia* spp. is unknown. The mechanism of action of atovaquone against other protozoa is believed to involve the inhibition of cytochrome *b* and electron transport(Baggish and Hill, 2002; Srivastava et al., 1999; Syafruddin et al., 1999; Vaidya and Mather, 2000). We have performed a controlled pilot study evaluating an atovaquone and azithromycin drug combination in *B. gibsoni* infected dogs. (Birkenheuer, et. al., Research Abstract #6, ACVIM Forum, 2003. Charlotte, NC) Both atovaquone and azithromycin have been shown to be safe in a large number of vertebrate species, and throughout our pilot study or in follow-up phone calls, up to five months after the end of the study period, no adverse effects were reported in any of the treated dogs. To our knowledge this is the first report on the use of atovaquone in dogs, and there is no information about its pharmacokinetics or safety in dogs. In humans atovaquone is excreted primarily in the feces after extensive enterohepatic re-circulation resulting in a long half-life. The pharmacokinetics of azithromycin in dogs has been studied and it has a very large safety margin and a long tissue half-life(Shepard and Falkner, 1990). The most commonly reported adverse effects in people treated with an atovaquone and azithromycin combination was diarrhea and rash in 8% of the recipients(Krause et al., 2000). We report the results of a double-blind placebo-controlled trial to evaluate the safety and efficacy of an atovaquone and azithromycin combination for the treatment of *B. gibsoni* (Asian genotype) infections in dogs.

Materials and Methods

Case Enrollment Criterion and Study Population

Dogs were selected for inclusion in the study if they had persistent *B. gibsoni* (Asian genotype) infection documented by PCR and light microscopy after failure of conventional anti-babesial therapy (imidocarb diproprionate and/or diminazine aceturate). *B. gibsoni* (Asian genotype) infection had to be documented 30 days or more after the last anti-babesial treatment. Dogs were excluded if they were considered ill enough to require hospitalization by the attending clinician. Seventeen dogs had been treated with imidocarb diproprionate alone (dose range 6 to 6.6mg/kg IM) for two doses one to two weeks apart. Four dogs had been treated with diminazene aceturate (3.5mg/kg IM) for two doses two weeks apart. One dog was treated with both imidocarb diproprionate (6.6mg/kg IM) for two doses two weeks apart followed by dimiazene aceturate (3.5mg/kg IM) for two doses two weeks apart. After randomization (data collected after study completion) there were eight dogs in the treatment group that had been treated with imidocarb diproprionate alone and three dogs that had been treated with diminazene aceturate alone. The placebo group consisted of nine dogs that had been treated with imidocarb diproprionate alone, one dog that had been treated with diminazene aceturate alone, and one dog that had been treated with both drugs. No concurrent anti-infective therapy other than heartworm prophylaxis could be administered during the study period. Twenty-two dogs satisfied the case enrollment criteria and were randomly and evenly divided into two groups (treatment and placebo). Eleven dogs were male and eleven were female. Ages ranged from one to twelve years. Breeds included seventeen American pit bull terriers, and one each; American staffordshire terrier, German shepherd, rottweiler,

boxer and mixed-breed. Owners of the dogs were required to sign an informed consent including information regarding the use of placebo and that the side effects of the atovaquone and azithromycin drug combination were unknown.

Study Protocol

The study was conducted as a randomized, double-blind, placebo-controlled therapeutic trial, which was designed to detect at least a 55% difference between the two groups with a power (1-β) of 0.8 and an α level of 0.05(Machin and Campbell, 1987). Owners, clinicians, and laboratory technicians were blinded throughout the study until all of the post-treatment PCR results had been analyzed. The treatment group received atovaquone (Mepron®, GlaxoSmithKline, Research Triangle Park, NC) and azithromycin (Zithromax ®, Pfizer, New York, NY). The atovaquone dosage was 13.3mg/kg orally three times daily and was administered with a fatty meal. The azithromycin dosage was 10mg/kg (range 10-11.6mg/kg) orally once daily. The dosages and course of therapy are extrapolated from published veterinary and human dosages and based on our pilot study(Krause et al., 2000; Shepard and Falkner, 1990; Taboada)(Birkenheuer, et. al., Research Abstract #6, ACVIM Forum, 2003. Charlotte, NC). For ease of dosing, the azithromycin was reformulated by the North Carolina State University, College of Veterinary Medicine Pharmacy, as 50mg gelatin capsules. Both drugs were administered simultaneously for ten days. The placebo group received color and consistency matched encapsulated and liquid placebos consisting of dextrose in gelatin capsules and white liquid food color (Professional Compounding Centers of America, Houston, TX) mixed with yellow food color (McCormick and Company Inc., Sparks, MD). Both the treatment

and placebo groups were treated with fipronil (Frontline®, Merial Ltd, Duluth, GA) throughout the course of the study. To avoid transmission of *B. gibsoni* (Asian genotype) via bite wounds and/or blood transfer, physical contact with other dogs was prohibited. (Birkenheuer, et. al., Research Abstract #4, ACVIM Forum, 2003. Charlotte, NC) Each dog was scheduled to be evaluated a total of five times pre-treatment (Day 0), end of treatment (Day 10) and at three post-treatment evaluations, one each, at approximately 60, 90, and 120 days. A physical examination, complete blood count and *Babesia* specific PCR was performed at each sample collection time. A serum biochemical profile was performed at days 0, 10, and 120. Based on pharmacokinetic studies in dogs and other mammals, there should be no detectable amounts of either drug by the 60-day post-treatment visit(Riviere et al., 1998; Shepard and Falkner, 1990). This study was performed in compliance with the North Carolina State University Animal Care and Use protocol 01-092.

DNA Extraction and PCR

Deoxyribonucleic acid (DNA) was isolated from EDTA anticoagulated whole blood samples using a comercial kit according to the manufacturer's instructions. *Babesia gibsoni* (Asian genotype) DNA was amplified as previously described(Birkenheuer et al., 2003b). The presence of PCR inhibitors in DNA samples that tested negative in our PCR test was excluded by the amplification of a fragment of the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene as previously described(Birkenheuer et al., 2003b). In order to prevent PCR amplicon contamination sample preparation, reaction setup, PCR amplification, and amplicon detection were all performed in separate areas. Positive and negative controls were used in all processing steps including the DNA extraction.

Statistical Analyses

A dog was considered to have a positive treatment outcome if no *B. gibsoni* (Asian genotype) DNA was detected by PCR in any post-treatment sample. A dog was considered to have a negative treatment outcome if *B. gibsoni* (Asian genotype) DNA was detected by PCR in any post-treatment sample. Difference in treatment outcome between the two groups was assessed using a Fisher's exact test. Statistical analyses were carried out using a statistical program

Results

Babesia gibsoni (Asian genotype) DNA was detectable by PCR in post-treatment samples from 2/10 dogs in the treatment group compared to 11/11 dogs in the placebo group. There was a statistically significant difference in outcome ($p < 0.001$) between the treatment and placebo groups. The results of the PCR tests are displayed in table 14. The G3PDH gene fragment was amplified from all DNA samples that tested negative by PCR for the presence of *Babesia* DNA, excluding the presence of PCR inhibitors in these samples. A total of five dogs did not complete all post-treatment visits; three dogs in the treatment group and two dogs in the placebo group. Of the 3 dogs in the treatment group, one dog was euthanatized due to progressive degenerative joint disease, one dog died from a heat stroke after being left in a car, and one dog was lost to follow-up due to lack of owner compliance. In the placebo group, two dogs were lost to follow-up due to lack of owner compliance. Only dog #3 (table13) was excluded from the outcome analyses. This dog was in the treatment group and had two consecutive negative PCR tests prior to being euthanatized for progressive degenerative joint disease. The remaining four dogs were included in the outcome analyses since they were considered to have a negative treatment outcome based on at least one positive PCR tests in a post-treatment sample.

The hematological and biochemical values for both the treatment and placebo groups for which the mean values were outside of the reference range are presented in table 15. No adverse effects were reported in either the treatment or placebo groups.

Discussion

Based on the results of the present study, atovaquone and azithromycin combination therapy is an effective treatment for *B. gibsoni* (Asian genotype) infections in dogs. To the author's knowledge this is the first report of a treatment that has been successful in either eliminating *B. gibsoni* (Asian genotype) infections or suppressing parasitemias below the limit of detection. Since the dogs in this study did not undergo splenectomy and drug-induced immune suppression, it cannot be definitively concluded that the *B. gibsoni* (Asian genotype) infections were sterilized by the combination therapy. However, even if complete parasite elimination did not occur, and *B. gibsoni* (Asian genotype) infections were only suppressed below the limits of PCR detection, reduction of the levels of parasitemia has been shown to significantly reduce the transmission of tick-transmitted protozoan pathogens such as *Babesia* and *Theileria*(Randolph, 1995; Smith, 1984; Young et al., 1996). Atovaquone is commercially available in the United States in two formulations. A single drug formulation, and a 2.5:1 combination with proguanil hydrochloride. The single drug formulation was used in the current study. The use of the 2.5:1 combination with proguanil hydrochloride in dogs has resulted in vomiting and diarrhea. (Birkenheuer, Unpublished observations, 2003) The efficacy of an atovaquone and azithromycin combination therapy in acutely or critically ill dogs has not been evaluated. The lack of adverse clinical, hemtological and biochemical abnormalities reported in the treatment group is promising, but larger studies will need to be performed to fully assess the safety of the atovaquone and azithromycin drug combination in dogs.

It is unknown whether or not the two dogs in the treatment group that tested positive in the post-treatment samples represented treatment failures, re-infection, or lack of dosing compliance on the part of the owner. Plasma concentrations of atovaquone and azithromycin were not evaluated to assess treatment compliance or drug absorption. Resistance to atovaquone in other protozoa has been reported and is associated with mutations in the cytochrome *b* gene(Korsinczky et al., 2000; McFadden et al., 2000; Srivastava et al., 1999; Syafruddin et al., 1999; Vaidya and Mather, 2000; Wittner et al., 1996). It is possible that some *B. gibsoni* (Asian genotype) isolates are resistant to or will acquire resistance against atovaquone and azithromycin combination therapy. It is unclear what effect, if any was introduced by the overrepresentation of American pit bull terriers in the study population. This is the most commonly reported breed affected in the US(Birkenheuer et al., 1999; Kocan et al., 2001; Macintire et al., 2002). and represents over 85% of the *B. gibsoni* (Asian genotype) cases identified in the North Carolina State University, College of Veterinary Medicine, Vector-Borne Disease Diagnostic Laboratory Database. (Breitschwerdt and Levy, Unpublished Data, 2003)

In conclusion the present study demonstrates that an atovaquone and azithromycin drug combination is an effective treatment for dogs that are chronically infected with *B. gibsoni*. Additionally it is the only treatment for *B. gibsoni* (Asian genotype) infections to have resulted in either elimination of infection or the suppression of parasitemia below the limit of detection. An effective treatment for *B. gibsoni* (Asian genotype) will have important implications for dogs around the world.

Table 14.
Results of the PCR tests for the treatment and placebo groups

Dog	Group	Day 0	Day 60	Day 90	Day 120
1	Treatment	+	-	-	-
2	Treatment	+	-	-	-
3	Treatment	+	-	-	ND
4	Treatment	+	-	-	-
5	Treatment	+	-	-	-
6	Treatment	+	+	+	ND
7	Treatment	+	-	-	-
8	Treatment	+	-	-	-
9	Treatment	+	-	-	-
10	Treatment	+	-	-	-
11	Treatment	+	-	+	ND
12	Placebo	+	+	+	+
13	Placebo	+	+	ND	ND
14	Placebo	+	+	+	-
15	Placebo	+	+	+	+
16	Placebo	+	+	+	ND
17	Placebo	+	-	+	+
18	Placebo	+	+	+	+
19	Placebo	+	-	+	+
20	Placebo	+	+	+	+
21	Placebo	+	+	-	+
22	Placebo	+	+	+	+

+, positive PCR test
-, negative PCR test
ND, not determined

Table 15A.
The hematological and biochemical variables (number out of range, mean and standard deviation) for treatment group dogs for which the mean values were outside of the reference range

Parameter	Reference range	Day 0 mean	Day 10 mean	Day 60 mean	Day 90 mean	Day 120 mean
Plasma protein	6.1-7.5 (g/dl)	6/11 8.0 (1.0)	6/11 7.9 (0.7)	6/11 7.6 (0.6)	4/10 7.6 (0.2)	5/8 7.9 (0.6)
Mean cell volume	63.9-72.8 (fl)	7/11 74.4 (4.7)	7/11 73.4 (3.6)	5/11 73.3 (3.3)	WRR	5/8 74.2 (2.6)
Mean corpuscular hemoglobin	21.9-25.7 (g/dl)	4/11 26.5 (3.5)	WRR	WRR	WRR	WRR
Red cell distribution width	14.0-17.0 (%)	8/11 19.0 (2.4)	9/11 19.1 (3.0)	10/11 21.6 (2.9)	7/10 19.8 (3.6)	6/8 20.6 (3.9)
Total Protein	5.1-7.9 (g/dl)	5/11 8.0 (1.2)	WRR	ND	ND	WRR
Globulin	1.8-4.4 (g/dl)	5/11 4.7 (1.2)	5/11 4.4 (1.0)	ND	ND	WRR

WRR, Within reference range
ND, not determined

Table 15B.
The hematological and biochemical variables (number out of range, mean and standard deviation) for placebo group dogs for which the mean values were outside of the reference range

Parameter	Reference range	Day 0 mean	Day 10 mean	Day 60 mean	Day 90 mean	Day 120 mean
Plasma protein	6.1-7.5 (g/dl)	9/11 8.7 (0.8)	11/11 8.5 (0.8)	9/11 8.1 (0.7)	9/9 8.8 (0.8)	9/9 8.9 (0.9)
Mean cell volume	63.9-72.8 (fl)	4/11 71.9 (3.5)	6/11 73.4 (5.0)	6/11 72.9 (2.9)	WRR	5/9 73.9 (3.8)
Red cell distribution width	14.0-17.0 (%)	6/11 19.6 (3.8)	10/11 19.7 (3.1)	7/11 18.7 (3.4)	7/9 19.5 (3.4)	7/9 19.2 (3.8)
Total Protein	5.1-7.9 (g/dl)	8/11 8.7 (1.1)	7/11 8.3 (0.9)	ND	ND	6/9 8.7 (1.0)
Globulin	1.8-4.4 (g/dl)	8/11 5.5 (1.3)	9/11 5.1 (1.0)	ND	ND	6/9 5.6 (1.2)

WRR, Within reference range
ND, not determined

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Conclusions

The work performed in this study identified a national epidemic of canine babesiosis caused by *B. gibsoni*, an organism that was previously unrecognized in the US. A sensitive and specific diagnostic test for canine babesiosis was developed, and this test was used to characterize the risk factors for *B. gibsoni* infections and elucidate a possible route of transmission in the US. Lastly, I developed and evaluated a treatment strategy that clears *B. gibsoni* parasitemias from infected dogs. This is perhaps the most important finding, since all prior treatments tested had failed to clear *B. gibsoni* parasitemias from infected dogs. These findings should allow aid in the diagnosis, prevention and treatment of canine babesiosis throughout the world. Future studies of canine babesiosis will include disease prevention, vaccine development, and mechanisms of resistance to anti-protozoal drugs.

Appendices

18S rRNA PCR

The amplification of the nearly full-length 18S rRNA genes was performed using 25-µl reactions. Each 25-µl reaction contained 1X concentration of PCR Buffer II (Perkin Elmer, USA), 0.625 U of *Taq* polymerase, 0.5 ul of DNA template, 1.5 m*M* $MgCl_2$, 12.5 pmol of each primer, and 200 µ*M* of each dNTP. Cycling conditions were 95 C for 5 minutes, followed by 35 amplification cycles (95 C for 1 minute, 56 C for 1 minute, and 72 C for 1 minute), and a final extension step at 72 C for 5 minutes. (PCR Express; Thermo Hybaid, Middlesex, United Kingdom)

Internal transcribed spacer region PCR

The amplification the internal transcribed spacer region was performed using 50-µl reactions. Each 50-µl reaction contained 1X concentration of PCR Buffer II (Perkin Elmer, USA), 1.25 U of *Taq* polymerase, 1 ul of DNA template, 1.5 m*M* $MgCl_2$, 50 pmol of each primer, and 200 µ*M* of each dNTP. Cycling conditions were 95 C for 5 minutes, followed by 35 amplification cycles (95 C for 45 seconds, 45 C for 45 seconds, and 72 C for 45 seconds), and a final extension step at 72 C for 5 minutes. (PCR Express; Thermo Hybaid, Middlesex, United Kingdom)

Cytochrome *b* PCR

The amplification the internal transcribed spacer region was performed using 50-µl reactions. Each 50-µl reaction contained 1X concentration of PCR Buffer II (Perkin

Elmer, USA), 1.25 U of *Taq* polymerase, 1 ul of DNA template, 1.5 m*M* $MgCl_2$, 50 pmol of each primer, and 200 μ*M* of each dNTP. Cycling conditions were 95 C for 5 minutes, followed by 35 amplification cycles (95 C for 45 seconds, 41 C for 45 seconds, and 72 C for 1 minute), and a final extension step at 72 C for 5 minutes. (PCR Express; Thermo Hybaid, Middlesex, United Kingdom)

RAP-1 PCR

The amplification the internal transcribed spacer region was performed using 50-μl reactions. Each 50-μl reaction contained 1X concentration of PCR Buffer II (Perkin Elmer, USA), 1.25 U of *Taq* polymerase, 1 ul of DNA template, 1.5 m*M* $MgCl_2$, 50 pmol of each primer, and 200 μ*M* of each dNTP. Cycling conditions were 95 C for 5 minutes, followed by 35 amplification cycles (95 C for 45 seconds, 55.5 C for 45 seconds, and 72 C for 45 seconds), and a final extension step at 72 C for 5 minutes. (PCR Express; Thermo Hybaid, Middlesex, United Kingdom)