Studies on the molecular epidemiology and drug therapy for canine *Babesia gibsoni* infection

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2006

Contents

General introduction

Babesia gibsoni (*B. gibsoni*) is a tick-borne hemoprotozoan parasite that causes remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly and hepatomegaly in dogs and that, in some cases, induces the death of infected animals. The parasite was first identified in 1910 in dogs and jackals from India (Patton, 1910), and is now considered endemic in the wild in Asia, Africa, the Middle East, North America (Kjemtrup *et al.*, 2000), and Australia (Muhlnickel *et al.*, 2002). In Japan, *B. gibsoni* had been considered to be endemic in western rather than in eastern Japan (Noda *et al.*, 1977). However, *B. gibsoni* infection has been found in Aomori Prefecture, northeastern Japan, which has a distinctly cold climate and used to have no precious report of *B. gibsoni* infection (Itoh *et al.*, 1987; Onishi *et al.*, 1995). In these reports, the parasite was found in numbers of Tosa dogs. The Tosa dog is a breed of Japanese origin that was produced for dog fighting by mixing several breed, including the Mastiff, Great Dane, Bulldog, and the American Pit Bull Terrier. Dog fighting is a tradition in Aomori Prefecture, where it is especially popular. The reason why *B. gibsoni* has exclusively found in Tosa dogs has not been elucidated.

The clinical severity of babesiosis in dogs is influenced to an extent by the age and the immune status of the affected individual: *B. gibsoni* infection causes progressive anemia in infected animals after surgery of when the animals are receiving immunosuppressive therapy and in general tends to be more severe in young dogs, with a subclinical carrier state arising after recovery from infection. Moreover, host immune responses to the parasite and changes in erythrocyte membrane proteins lead to the development of antierythrocyte antibodies, immune-mediated erythrocyte destruction, and a combination of intravascular and extravascular hemolysis (Adachi *et al.*, 1995; Conrad *et al.*, 1991), with evidence of

immune-mediated hemolysis in blood smears and with many animals having a positive Coomb's test (Jacobsen and Clark, 1994). Thrombocytopenia is also commonly reported, along with variable leukocyte changes, and is thought to be influenced by the effects of stress, systemic inflammation, and bone marrow stimulation (Birkenheuer *et al*, 1999; Yamase *et al.*, 1993), although the mechanisms have not been elucidated.

Some chemotherapeutics, including diminazene aceturate (Fowler *et al.*, 1972), pentamidine isethionate (Farwell *et al.*, 1982) and phenamidine isethionate (Groves and Vanniasingham, 1970), have been reported to reduce the level of parasitemia. However, these agents are unable to eliminate *B. gibsoni* completely (Boozeer and Macintire, 2003). Most dogs that recover from the acute stage are at risk of recurrence and may become a reservoir for tick-born infections (Conrad *et al.*, 1991). Thus, there remains a need for an effective therapeutic agent for the treatment of canine babesiosis caused by *B. gibsoni.* Recently, atovaquone (1,4-hydroxynaphthoquinone) has been reported to be active against a wide range of human protozoan pathogens, including *Plasmodium* spp., *Toxoplasma gondii* (Baggish and Hill, 2002) and some *Babesia* spp.(Weiss, 2002). When used as a single agent in human patients, or animal models infected with these pathogens, atovaquone is effective, but it is associated with unacceptable recrudescence rates and decreased parasite susceptibility following treatment (Chiodini *et al.*, 1995; Looareesuwan *et al.*, 1996; Wittner *et al.*, 1996). Therefore, other drugs have been studied as potential synergistic partners (Baggish and Hill, 2002). However, no detailed evidence has been published on the therapeutic activity of atovaquone alone against *B. gibsoni* in vivo and in vitro.

The purpose in this study was to investigate the actual condition of incidence of *B. gibsoni* infections in Tosa dogs in Aomori Prefecture, and propose new information for the treatment of *B. gibsoni.* In chapter 1, the incidence of acute and subclinical *B. gibsoni*

infection among dogs in Aomori Prefecture was investiagted, and their clinicopathological studies were also carried out. In chapter 2, the therapeutic efficacy of atovaquone against *B. gibsoni* was examined in dogs experimentally infected with *B. gibsoni*, and the appearance of drug resistant parasites after the treatment was also examined. Based on these findings, changes in the *B. gibsoni* mitochondrial DNA sequence for *cytochrome b*, which may bind atovaquone, were studied in chapter 3.

Chapter 1

Incidence of canine *Babesia gibsoni* infection and subclinical infection among Tosa dogs in Aomori Prefecture, Japan.

Introduction

The *Babesia* parasite is generally known to be transmitted by a tick vector such as *Haemaphysalis* and *Rhipicephalus* (Kjemtrup *et al.*, 2000; Taboada and Merchant, 1991). In Japan, *Haemaphysalis* is distributed throughout the country, whereas *Rhipicephalus* is distributed only in tropical areas (Shimada *et al.*, 2003), *B. gibsoni* had been considered endemic in western rather than in eastern Japan (Noda *et al.*, 1977). However, *B. gibsoni* infection has been found in Aomori Prefecture, northeastern Japan, which has a distinctly cold climate and had never had any previous reports of *B. gibsoni* infection (Itoh *et al.*,1987; Onishi *et al.*, 1995). In these reports, the parasite was found in numbers of Tosa dogs. The Tosa dog is a breed of Japanese origin that was produced for dog fighting by mixing several breeds, including the Mastiff, Great Dane, Bulldog, and the American Pit Bull Terrier. Dog fighting is a tradition in Aomori Prefecture, where it is especially popular. The reason for the predilection for Tosa dogs has not been elucidated.

To recognize the incidence of *B. gibsoni* infection in dogs in Aomori Prefecture, dogs diagnosed as having acute *B. gibsoni* infection at the Animal Teaching Hospital, Kitasato University, were investigated between April 2002 and March 2003. Subsequently, to identify the incidence of subclinical *B. gibsoni* infection, 141 Tosa dogs from Aomori Prefecture were tested for the presence of *B. gibsoni* by using a polymerase chain reaction (PCR) assay. In addition, to elucidate the incidence and mechanisms of anemia and thrombocytopenia in subclinical infection, packed cell volumes (PCVs) and platelet counts were measured, followed by an examination for the presence of anti-erythrocyte membrane IgG and anti-platelet IgG.

Materials and Methods

Identification the incidence of B. gibsoni infection in Aomori Prefecture: Between April 2002 and March 2003, dogs diagnosed as having acute *B. gibsoni* infection at the Animal Teaching Hospital, Kitasato University, Aomori Prefecture, were investigated by noting their dog breed, sex, age, parasitemia, PCVs, and platelet counts. Blood samples from each dog were collected with ethylene diamine tetraacetic acid (EDTA) as anticoagrant and thin blood smears were stained with Giemsa stain. Parasitemia was determined by counting the number of parasitized cells per 1,000 erythrocytes and expressing it as a percentage. PCVs and platelet counts were measured with an auto cell counter (Celltaq MEK6400, Nihon Kohden Co., Tokyo).

Investigation of subclinical infection of Tosa dogs: Dogs for this experiment consisted of 141 clinically healthy Tosa dogs that had been bred for dog fighting and were living in Aomori Prefecture between August 2002 and April 2003. There were 87 dogs (85 male, 2 female) with histories of dog fighting and 54 dogs (26 male, 28 female) with no dog fighting history (Table 1). No abnormalities in the general condition of the dogs were observed, and no ticks were found on the dogs. Whole blood samples were collected from the dogs, with either EDTA or heparin being used as the anticoagulant for the PCR assay to screen for *B. gibsoni* infection. Of the 141 samples, 93 were examined for PCVs and anti-erythrocyte membrane IgG. Forty-eight samples from young (<1-year-old) or pregnant dogs were excluded from these tests. Sixty-six samples that were collected with EDTA as the anticoagulant were examined for platelet counts and anti-platelet IgG. Table 1 lists the number of dogs of each test by history of dog fighting and sex. The sera were stored at –20°C until use.

Detection of B. gibsoni by PCR assay: DNA was extracted from 100 μl of whole blood sample using a genomic DNA extraction kit (Gen TLE kit, Takara, Otsu, Japan). PCR was performed using primers designed to amplify the *p18* gene of *B. gibsoni* (DNA Data Bank of Japan database: accession no. AB053292). The forward primer sequence was 5′–TCC GTT CCC ACA ACA CCA GC–3′, and the reverse primer sequence was 5′–CGA ATG AGG ATG ATG AGG AGG A–3′. This PCR is specific for *B. gibsoni*, as Fukumoto *et al.* (2001) found no amplification using DNA from *B. canis* or normal dog leukocytes. PCR was performed on 20 μl of a mixture containing 1 μg of template DNA, 50 pmol of each primer, 200 μM dNTP, and 1.25 units *Taq* DNA polymerase (TaKaRa Taq, Takara) in PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3, Takara). The PCR reaction was repeated for 40 cycles using 30 s of denaturation at 94 °C, 1 min of annealing at 56 °C, and 1 min of extension at 72 °C. Ten-microliter aliquots of the PCR products were analyzed using 2% agarose gel electrophoresis followed by thorium bromide staining and photography. From the results of PCR assays, dogs were divided into groups with subclinical infection and those without infection.

Measurement of PCVs and platelet counts: To compare the difference by dog breed, peripheral blood from six Beagle dogs (all 1-year-old females) without *B. gibsoni* infection were collected and their PCVs and platelet counts were examined.

Preparation of antigen for enzyme-linked immunosorbent assay (ELISA): For erythrocyte membrane antigen and platelet antigen, blood samples from clinically healthy 3-year-old male Beagles were collected with EDTA as the anticoagulant. For erythrocyte membrane antigen, after being washed with phosphate-buffered saline solution (PBS), erythrocytes were lysed with 30 volumes hypooncotic buffer (5 mM Tris-HCl, 1 mM EDTA) and 1/1000 volume 0.8 M phenylmethylsulfonyl fluoride in dimethyl sulfoxide. After

incubation for 5 min on ice, the lysed solution was centrifuged for 15 min at 4°C. Then, the prepared erythrocyte membrane was washed 3 times with hypooncotic buffer and suspended in sucrose buffer (250 mM sucrose, 1 mM EDTA, pH 7.4 with Tris-HCl).

For platelet antigens, the techniques of Campbell *et al.* (1984) were employed. After being isolated from the blood and washed 3 times with PBS-EDTA, the platelets were suspended with PBS-EDTA.

ELISA procedure: The ELISA was performed by a slight modification of the method of Adachi *et al.* (1992). The 96-well ELISA plates (ELISA-Plate, Greiner Bio-one, Germany) were coated with 50 μl of erythrocyte membrane antigens (80 μg protein/ml) or platelet antigens (5×10^7/ml) in 50 mM carbonate buffer (pH 9.6). After blocking the reaction with 100 μl of PBS containing of 2% skim milk, we added 100 μl of the test serum, which was diluted 1:50 for the erythrocyte membrane antigen and diluted 1:100 for the platelet antigen with PBS containing 2% skim milk. Fifty microliters of anti-dog IgG peroxidase conjugate (anti-dog IgG, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:1000 with PBS containing 2% skim milk was added as the second antibody. After adding 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), the optical absorbance at 405 nm was recorded using spectrophotometers (Opsys MR Microplate reader, Dynex, London, UK).

Statistical analysis: The Chi-squared test was used to evaluate the difference in *B. gibsoni* infection between dogs with a history of fighting and dogs without a history of fighting. The other data were evaluated using Student's t-test. Values of $P < 0.05$ were considered statistically significant.

Results

Among the dogs seen at the Animal Teaching Hospital, Kitasato University, between April 2002 and March 2003, 18 dogs were diagnosed as having *B. gibsoni* infection. These dogs were all male Tosa dogs and showed mild to severe Babesiosis syndrome and had a wide range of parasitemia. Their PCV values varied. In all but one dog, the platelet count was lower than $10 \times 10^4/\mu l$ (Table 2).

Forty-two of the 141 dogs (29.8%) were positive for *B. gibsoni* in PCR assay. Forty-one of 87 dogs (47.1%) with histories of dog fighting were positive and one of 54 dogs (1.9%) without histories of dog fighting were positive for *B. gibsoni* (Table 3). There was a significant difference between dogs with a history of fighting and those without a history of fighting in the rate of *B. gibsoni* infection ($P < 0.05$).

The mean ± SD PCVs of dogs with subclinical infection and dogs without infection were 46.74 ± 8.03 and 43.96 ± 8.31, respectively (Table 2). There was no significant difference in PCVs between dogs with subclinical infection and dogs without infection. The mean ± SD platelet counts of dogs with subclinical infection and dogs without infection were 13.75 ± 8.45 and 22.35 ± 11.41, respectively. Dogs with subclinical infection had significantly lower mean platelet counts than did the dogs without infection ($P < 0.05$). The mean ± SD PCVs and platelet counts of the six Beagle dogs were 47.57 ± 2.98% and 33.98 ± 18.29 $\times 10^4/\mu l$; there was no significant difference in PCVs or platelet counts between Beagle dogs and Tosa dogs without *B. gibsoni* infection.

Individual sera exhibited a wide range of ELISA levels (optical densities) for anti-erythrocyte membrane antigen (Figure 1). The mean ± SD ELISA level of dogs with subclinical infection was 0.20 ± 0.08, which was significantly higher than that of dogs

without infection (0.12 ± 0.06, $P < 0.05$). There was a wide range of ELISA levels for anti-platelet antigen exhibited by the individual sera (Figure 2). The mean ± SD ELISA level of dogs with subclinical infection was 0.33 ± 0.15, which was significantly higher than that of dogs without infection (0.16 ± 0.07, $P < 0.05$).

Table 1. Number of Tosa dogs in each examination

		History of dog fighting			
		+		−	
	Total	male	female	male	female
PCR	141	85	2	26	28
PCVs and anti-erythrocyte membrane IgG	93	54	1	20	18
Platelet count and anti-platelet IgG	67	49	1	10	7

Table 2. Dogs with acute babesiosis infected with *B. gibsoni* at Kitasato University Animal Teaching Hospital between April 2002 and March 2003

Case No.	Year / Sex / Breed	Parasitemia (%)	PCVs (%)	Platelet count ($\times 10^4/\mu l$)
1	4 / male / Tosa	Low[a)]	27	4.7
2	2 / male / Tosa	11.4	15.2	0
3	1 / male / Tosa	Low	41.3	1.9
4	unknown / male / Tosa	Low	30.2	1
5	2 / male / Tosa	Low	49.7	8.7
6	3 / male / Tosa	Low	23.8	31.4
7	1 / male / Tosa	3.1	29.1	1.3
8	2 / male / Tosa	4.0	28.6	0.2
9	4 / male / Tosa	2.5	18.9	9.8
10	2 / male / Tosa	2.3	20.8	3.1
11	1 / male / Tosa	1.3	29.4	2.1
12	2 / male / Tosa	1.0	30.3	0
13	1 / male / Tosa	Low	28.9	0.3
14	2 / male / Tosa	1.0	31.3	7.2
15	1 / male / Tosa	1.1	23	5.9
16	3 / male / Tosa	0.4	36.9	0
17	8 / male / Tosa	Low	34.8	1.8
18	2 / male / Tosa	1.1	46.6	0.3

[a)]Parasites detected, but present in $< 0.1\%$ of parasitemia.

Table 3. Subclinical *B. gibsoni* infection of 141 Tosa dogs in Aomori Prefecture, Japan

		B. gibsoni infection detected with PCR assay	
	No. of examined	Positive	Negative
Dog fighting (+)	87	41(47.1%)*	46(52.9%)
Dog fighting (-)	54	1(1.9%)	53(98.2%)
Total	141	42(29.8%)	99(70.2%)

* Significant difference between dogs with history of fighting and dogs without history of fighting in positive results for *B. gibsoni* ($P < 0.05$)

Table 4. PCVs and Platelet counts in Tosa dogs with or without *B. gibsoni* subclinical infection

	Total No.	Mean Value ± SD	
		Non-infection	Subclinical infection
PCVs	(93)	43.96 ± 8.31 % (67)	46.74 ± 8.03 % (26)
Platelet counts	(67)	22.35 ± 11.41 $\times 10^4/\mu l$ (45)	13.75 ± 8.45 $\times 10^4/\mu l$ (22)*

* $P < 0.05$ versus non-infection

( ) : number of dogs examined

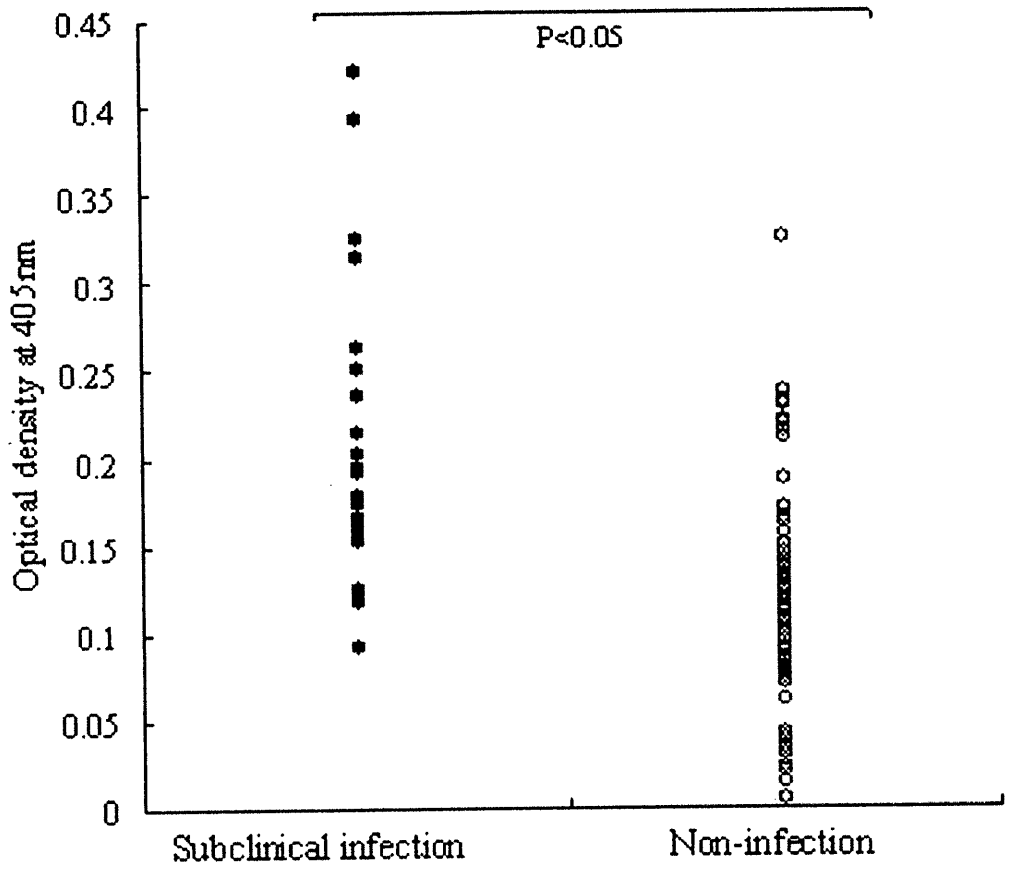

Figure 1. Reactivity of sera from dogs with *B. gibsoni* subclinical infection (●) and dogs without infection (○) to erythrocyte membrane antigen.

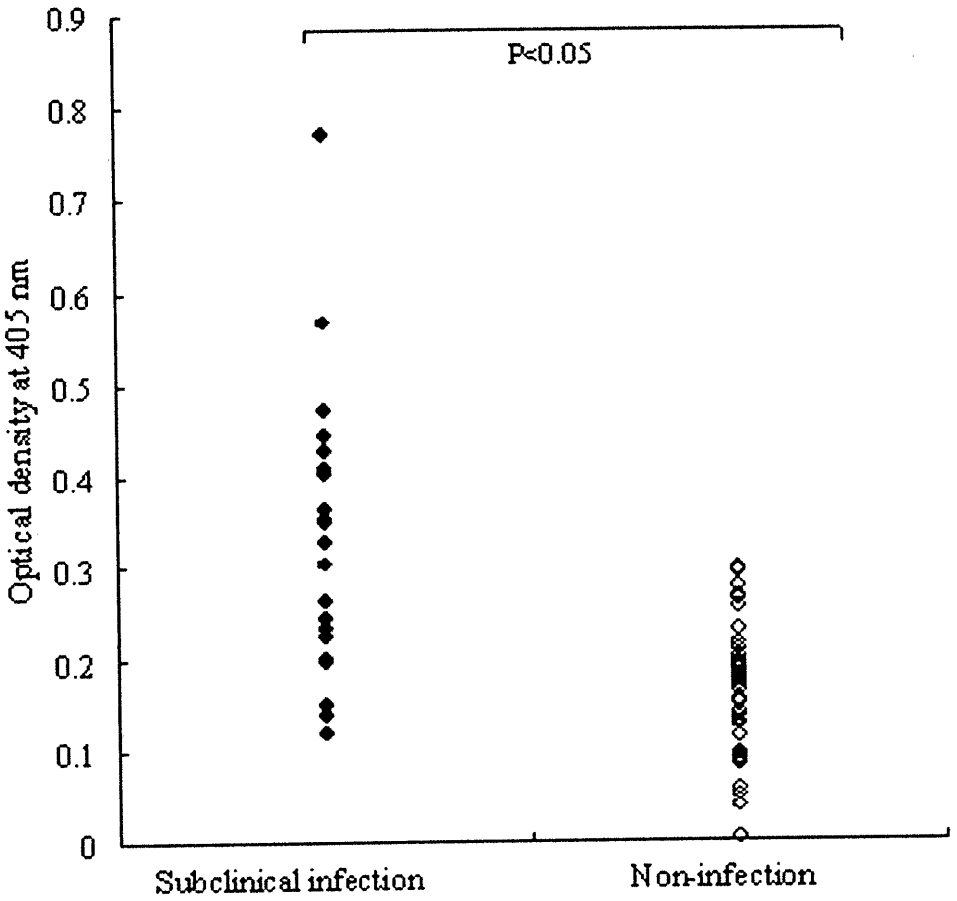

Figure 2. Reactivity of sera from dogs with *B. gibsoni* subclinical infection (●) and dogs without infection (○) to platelet antigen.

Discussion

The present study demonstrates that only dogs of the Tosa breed showed acute *B. gibsoni* infection for the year of observation at the Animal Teaching Hospital, Kitasato University. This finding is similar to that found in a study reported 19 years ago (Itoh *et al.*, 1987). In addition, a significant proportion of Tosa dogs (29.8%) was carriers of *B. gibsoni* in Aomori Prefecture, Japan. All of the infected dogs, except for one female, had a history of dog fighting.

The *Babesia* parasite is generally known to be transmitted by ticks such as *Haemaphysalis* and *Rhipicephalus* (Kjemtrup *et al.*, 2000; Taboada and Merchant, 1991). *Haemaphysalis* species has an especially wide geographical distribution, including Aomori Prefecture (Shimada *et al.*, 2003); therefore, the vector ticks might have contributed to the spread of *B. gibsoni* infection among Tosa dogs. However, dogs with acute *B. gibsoni* infection were not recognized in dogs of any other breed. Furthermore, natural infection of this pathogen, as identified by PCR assays, has been found only in dogs of the Tosa breed, and has not been identified in tests of 872 dogs of other breeds in dogs of any other breeds in Aomori Prefecture (Ikadai *et al.*, 2004). It has been reported in 2003, that *B. gibsoni* parasite was not detected in ticks from eastern Japan, including Aomori Prefecture, in an epidemiological survey (Inokuma *et al.*, 2003). In the light of these data, the result of this study suggests the possibility that *B. gibsoni* in Tosa dogs might be transmitted through blood contamination during dog fighting, rather than through ticks. Many recent studies in the United States and Australia have reported *B. gibsoni* infections and subclinical infections in bull terriers, including American Pit Bull Terriers and American Staffordshire Bull Terriers (Birkenheuer *et al.*, 1999; Macintire *et al.*, 2002; Muhlnickel *et al.*, 2002). Although the

route of infection is not clear in these reports, the fact that these countries had not been considered endemic regions previously (Birkenheuer *et al.*, 1999; Macintire *et al.*, 2002; Muhlnickel *et al.*, 2002) and that the incidence is limited to dogs of a specific breed point to similarities to our findings in Aomori Prefecture. This study is the first to demonstrate that *B. gibsoni* may spread among dogs in Japan through a peculiar incident, namely, dog fighting.

Only one dog that had no history of fighting was positive for *B. gibsoni*, and the route of transmission was uncertain. Generally, only adult male dogs participate in official dog fighting, and younger dogs or female dogs have no history of fighting. One of the possibilities of the infection route could be blood contamination during a situation such as breeding or unofficial fighting. In fact, this animal had been mated with an infected dog two weeks before blood sampling. Some reports of dogs being infected with the *B. gibsoni* parasite imply transplacental transmission (Abu *et al.*, 1973; Harvey *et al.*, 1988). Dog fighting is performed throughout Japan, and Tosa dogs may be moved to many places for breeding or fighting tours. This dog might be infected through ticks at some places where is endemic area. To investigate the possibility that the pathogen might be transmitted by ticks that are naturally distributed around places where Tosa dogs live, a more detailed survey of ticks is needed.

The present study also showed that both thrombocytopenia incidence and anti-platelet IgG levels were significantly higher in dogs with subclinical infections than in dogs without infection. Thrombocytopenia in dogs infected with *B. gibsoni* has been reported previously (Taboada and Marchant, 1991; Wilkerson *et al.*, 2001; Wonzniak *et al.*, 1997); however, the mechanisms were not elucidated. The presence of a platelet-surface associated IgG in experimental *B. gibsoni* infected dogs at the acute and chronic stages was demonstrated by Wilkerson *et al.* (2001). In the present study, natural subclinically infected

dogs developed higher levels of anti-platelet IgG than did dogs without infection. This suggests the possibility that the thrombocytopenia in subclinical *B. gibsoni* infection is due to autoimmune responses, and the presence of anti-platelet IgG accelerates the platelet destruction in *B. gibsoni* infected dogs. Anemia is the most common symptom of infection with *B. gibsoni* (Conrad *et al.*, 1991; Groves *et al.*, 1972; Taboada and Marchant, 1991), and is thought to be caused by mechanical destruction by the parasite, immune-mediated intravascular or extravascular hemolysis, or immune-mediated extravascular hemolysis (Adachi *et al.*, 1992; Adachi *et al.*, 1994; Morita *et al.*, 1994; Morita *et al.*, 1996). Oxidative damage may contribute to the destruction of erythrocytes by increasing their susceptibility to phagocytosis by macrophages (Otsuka *et al.*, 2002). The anemia associated with *B. gibsoni* may be due to the interaction of several factors. In the present study, the serum from dogs with subclinical infections had higher levels of antibodies against the erythrocyte membrane, although the incidence of anemia in dogs with subclinical infections was not high. The development of subclinical *B. gibsoni* infection may depend on a delicate balance between the proliferation of the parasite and the host immune response against the parasites.

In conclusion, Tosa dogs from Aomori Prefecture, Japan, were highly infected by *B. gibsoni* subclinically and this pathogen might be successfully transmitted during dog fighting. Dogs with subclinical infections were at risk of chronic thrombocytopenia, which may due to autoimmune mechanisms. Tosa dogs may be a useful breed for the study of the mechanisms of clinicopathological abnormalities associated with infection with *B. gibsoni*.

Chapter 2

Efficacy of atovaquone against *Babesia gibsoni* in vivo and in vitro.

Introduction

The findings in chapter 1 have demonstrated that *Babesia gibsoni* may spread among dogs in Japan through dog fighting and these infected dogs may become a reservoir for tick-borne infections. Moreover, these dogs are at risk of chronic thrombocytopenia, which may be due to autoimmune mechanisms. Thus, this remains a need for an effective therapeutic agent for the treatment of canine babesiosis caused by *B. gibsoni*. Some chemotherapeutics, including diminazene aceturate (Fowler *et al.*, 1972), pentamidine isethionate (Farwell *et al.*, 1982) and phenamidine isethionate (Groves and Vanniasingham, 1970), have been reported to reduce parasitemia, but these are unable to eliminate *B. gibsoni* completely (Boozer *et al.*, 2003).

Atovaquone (1,4-hydroxynaphthoquinone) is active against a wide range of human protozoan pathogens, including *Plasmodium* spp., *Toxoplasma gondii* (Baggish and Hill, 2002), and *Babesia* spp. (Weiss *et al.*, 2002). However, recurrence of the disease and decreased sensitivity of the protozoa to therapy have been reported when atovaquone was used alone (Chiodini *et al.*, 1995; Looareesuwan *et al.*, 1996; Wittner *et al.*, 1996). Atovaquone combined with azithromycine has therapeutic efficacy for dogs naturally infected with *B. gibsoni* (Birkenheuer *et al.*, 2004). However, no detailed evidence has been published on the therapeutic activity of atovaquone alone against *B. gibsoni* in vivo or in vitro. In this study, the sensitivity of *B. gibsoni* against atovaquone in experimentally infected dogs was evaluated. Subsequently, the in vitro sensitivity of parasites obtained from untreated and atovaquone-treated dogs was examined.

Materials and Methods

Test compound: Atovaquone (Mepron®, Glaxo Welcome, NC, USA) was used as the test compound.

In vivo sensitivity of atovaquone against B. gibsoni: *B. gibsoni* parasites were isolated from a naturally infected Tosa dog in Aomori Prefecture, Japan in 2003. The 18S rDNA sequences of this parasite show 100% homology to *B. gibsoni* isolated from dogs in Asia, Oklahoma, North Carolina, and Okinawa (Ikadai *et al.*, 2004). The parasite was maintained in author's laboratory by passage through a beagle (dog A, a three-year-old female). The Kitasato University's Animal Care and Ethics Committee approved the use of animals. Three one-year-old, parasite-free, female beagles (dogs B, C, and D) were obtained from Japan Clea (Tokyo, Japan), and maintained in a closed environment in the author's laboratory for one month before the study. These dogs were vaccinated against distemper and parvovirus and received a physical examination, and no *Babesia* spp. was detected in initial peripheral blood smears. All dogs were held in separate cages and fed a commercial dog food (Japan Hills Colgate, Tokyo, Japan) according to the manufacturer's instructions; tap water was available *ad libitum*. Three dogs (B, C, and D) were experimentally inoculated intravenously (day 1) with 5×10^7 fresh *B. gibsoni*-parasitized erythrocytes. When parasitemia reached 10%, atovaquone was administered orally at 30 mg/kg twice a day for 7 days, to each dog. Blood samples were collected with EDTA as the anticoagulant at 1–7 days intervals until 80 days after the induction of the infection. After atovaquone therapy, a PCR assay was followed to detect the presence of parasite DNA (see below).

Blood smears from each dog were examined microscopically after Giemsa staining. The level of parasitemia was determined and measurements of PCVs and platelet counts were

performed as mentioned at chapter 1. PCR assay was performed on 100 µl samples of peripheral blood from dogs B, C, and D. In the PCR assay, DNA from *B. gibsoni* from each blood sample was extracted using a genomic DNA extraction kit (Gen TLE kit, Takara). The PCR was performed by using the method as described in chapter 1.

In vitro sensitivity testing: An in vitro atovaquone sensitivity test was performed using parasites collected from the untreated, subclinically infected control animal (dog A) and two of the experimentally infected animals after the therapy (dogs B and C).

An additional blood sample was collected from each of dogs A, B, and C. The parasites were cultured in canine erythrocytes in a culture medium with various concentrations of atovaquone. The culture medium was based on that used by Sunaga *et al.* (2002). It contained RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 25 mM *N*-2-hydroxyethyl piperazine *N'*-2-ethanesulfonic acid, 2 mM L-glutamine, 24 mM $NaHCO_4$, penicillin G (100 units/ml), streptomycin (100 µg/ml), and canine serum at a final concentration of 20%. Atovaquone was dissolved in dimethyl sulfoxide (DMSO) and was then diluted in the culture medium to give final concentrations of 0.1, 1, 10, 100, and 1,000 nM. This range of drug concentrations was based on the results of preliminary assays conducted in our laboratory. The final concentration of DMSO used in dilution of the drugs was 1% in the culture medium.

The sensitivity test was performed in 24-well plates, and 50 µl of packed parasitized erythrocytes was dispensed into each well. Aliquots of 450 µl of the serially diluted atovaquone in culture medium, or in complete medium with 1% DMSO without drug as a control for normal parasite growth were added to each well. The plates were incubated in a humidified 5% CO_2 incubator at 37 °C. Every 24 hour, the medium in each well was aspirated and a fresh 450 µl of solution, containing the appropriate test drug concentration,

was added. A 1 μl sample was taken from each well and a thin smear was made, fixed with absolute methanol, and stained with Giemsa solution to count the number of parasitized erythrocytes.

Analysis of results: The sensitivity against atovaquone was evaluated by measuring the rate of the parasite growth inhibition. This was calculated by counting the number of parasitized erythrocytes from each of the wells containing atovaquone and that in the control wells without the drug. The 50% inhibitory concentration (IC_{50}) of the drug was defined as the concentration required for 50% reduction in the mean number of parasitized erythrocytes from that of the control culture after 48 hour of incubation. An IC_{50} was calculated using probit analysis. In each of the three dogs, the test was performed on three occasions, and the inhibition rate at each concentration of atovaquone was calculated and expressed as the mean percentage ± standard deviation.

Results

After induction of the *B. gibsoni* infection, atovaquone treatment was commenced 26, 24 and 24 days after infection in dogs B, C, and D, respectively, when parasitemia reached >10% (Figure 3). No clinical side effects were observed following treatment in any dogs. The parasitemia decreased quickly and organisms were no longer detected in blood smears within 2 days. The PCVs and platelet counts, which started to decrease after induction of the infection, began to increase soon after treatment commenced (Figure 3). PCR assays showed that *B. gibsoni* DNA was intermittently present in the peripheral blood and that it had not been eliminated (Figure 4). Moreover, parasites reappeared in the blood smears at 32, 35, and 31 days after the last treatment in dogs B, C, and D, respectively (mean 33 ± 2 days). At the time of recurrence of the parasite, slight anemia and thrombocytopenia were recognized. Although the anemia disappeared spontaneously, chronic thrombocytopenia and low parasitemia of *B. gibsoni* in peripheral blood persisted in the dogs, but without any clinical symptoms (data not shown).

In the untreated animal (dog A), the in vitro culture commenced when the mean parasitemia was 2.6 ± 0.2% and complete growth inhibition of *B. gibsoni* occurred in the in vitro culture that contained atovaquone at a concentration of 1,000 nM after 48 hours of incubation (Figure 5). The IC_{50} value of atovaquone against *B. gibsoni* was 89.02 ± 17.29 nM. At the time of recurrence of the parasite after atovaquone treatment, blood samples taken from dogs B and C had mean parasitemia values of 1.6 ± 0.2% and 1.3 ± 0.3%, respectively. At 1,000 nM atovaquone, the original *B. gibsoni* growth was completely inhibited, but the inhibition rates of recurrent parasite were 39.52 ± 8.34 % (dog B) and 31.31 ± 8.14 % (dog C) (Figure 6). Complete growth inhibition was not observed at the range of

the concentrations used in this study and the IC_{50} values could not be determined.

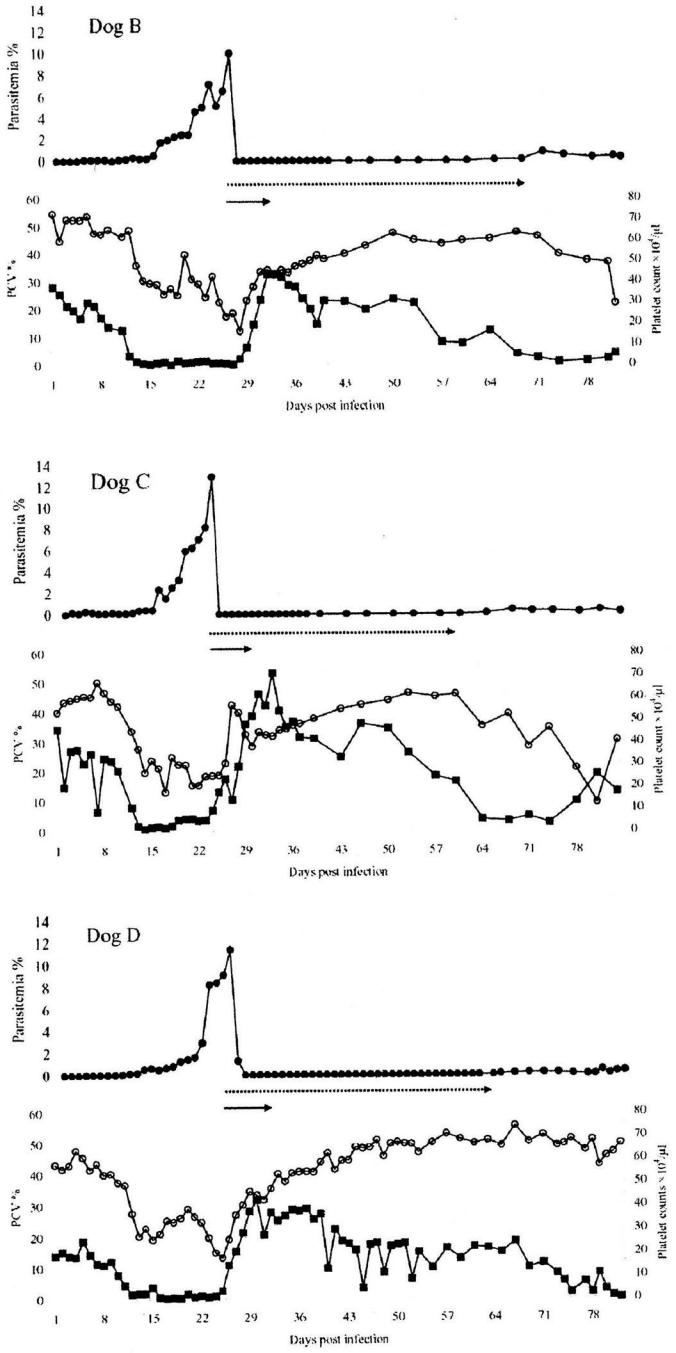

Figure 3. Changes in parasitemia (●), PCV (○), and platelet counts (■) in atovaquone - treated dogs (B, C, and D) during 80 days after infection with *B. gibsoni*. The dogs were inoculated intravenously with 5×10^7 parasitized erythrocytes. The time of atovaquone treatment (solid arrows) and the time that *B. gibsoni* organisms were absent on microscopic examination (dotted arrows) are shown.

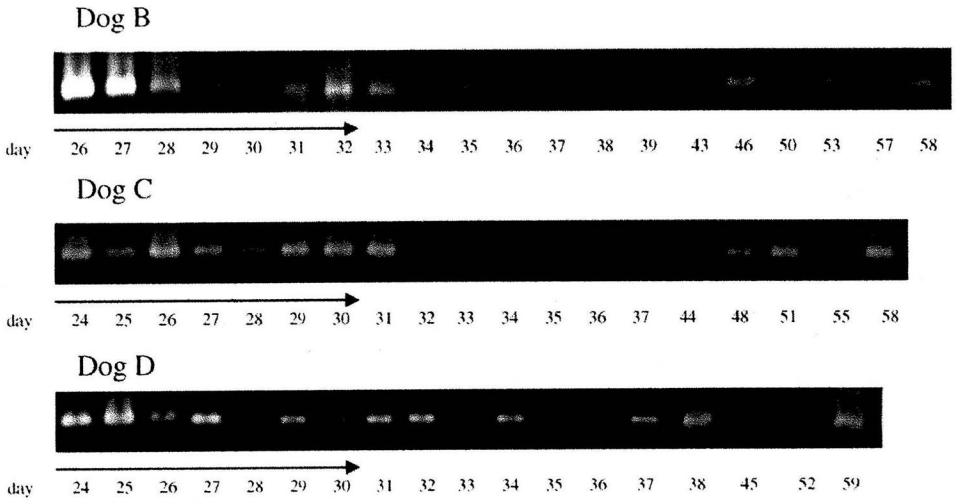

Figure 4. Result of PCR assay in dogs (B, C, and D). The PCR assays were performed following treatment with atovaquone (see Figure 3). The size of bands detected was 182 bp. The time of atovaquone treatment (slid arrows) is shown.

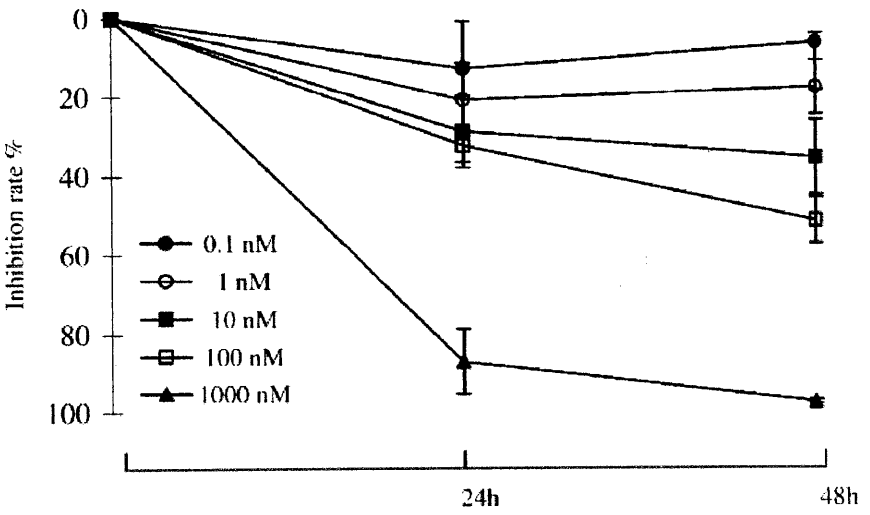

Figure 5. Change in inhibition rate in the atovaquone sensitivity test made in in vitro cultures of *B. gibsoni*. The data were obtained from an untreated dog (A) and the in vitro culture commenced when the mean parasitemia was 2.6 ± 0.2%. Each value represents the mean and standard deviation from three separate experiments.

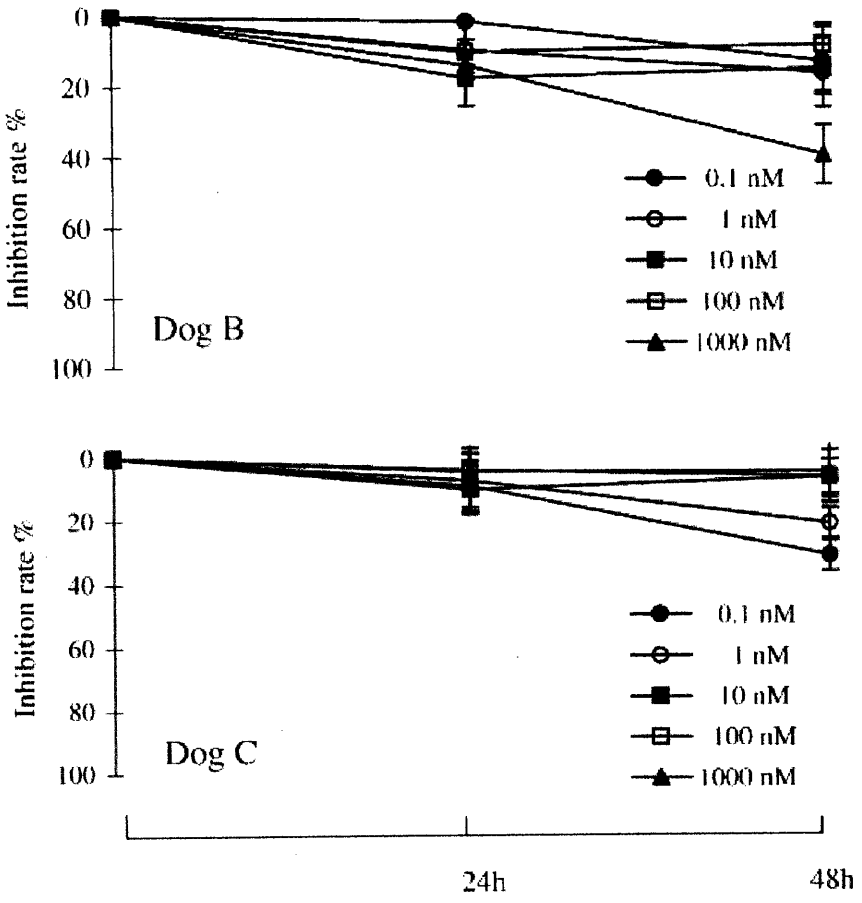

Figure 6. Change in inhibition rate in the atovaquone sensitivity test made in in vitro cultures of *B. gibsoni*. The data were obtained from atovaquone-treated dogs and the in vitro culture commenced when the mean parasitemia was 1.6 ± 0.2% (dog B) and 1.3 ± 0.3% (dog C). Each value represents the mean and standard deviation from three separate experiments.

Discussion

The results in this study indicate that atovaquone has potential therapeutic effectiveness against acute *B. gibsoni* infections in dogs. When given orally as a single dose (30 mg/kg twice daily for 7 days), it quickly reduced the parasitemia without any clinical side effects. At times, the response to atovaquone therapy was so rapid that *B. gibsoni* DNA could not be detected by the PCR assay, despite the well-recognized sensitivity of this assay for detecting organisms in the peripheral blood (Fukumoto *et al.*, 2001). Anemia and thrombocytopenia, which are the major clinical symptoms accompanying *B. gibsoni* infection (Conrad *et al.* 1991; Meinkoth *et al.* 2002) recovered sharply soon after treatment.

Atovaquone is structurally similar to the inner mitochondrial protein ubiquinone which is an integral component of electron flow in aerobic respiration of protozoa. Therefore, atovaquone competitively inhibit the mitochondrial electron transport of protozoa, it leads the collapse of the mitochondrial membrane potential. (Baggish and Hill, 2002; Hudson *et al.*, 1991). Atovaquone is active against a wide range of human protozoan pathogens, including *Plasmodium* spp., *Toxoplasma gondii* (Baggish and Hill, 2002), and some *Babesia* spp. (Gray and Pudney, 1999; Hughes and Oz, 1995; Pudney and Gray, 1997; Weiss *et al.*, 2002). When used as a single agent in human patients or animal models infected with these pathogens, atovaquone is effective, but it is associated with unacceptable recrudescence rates and decreased parasite susceptibility following treatment (Chiodini *et al.*, 1995; Looareesuwan *et al.*, 1996; Wittner *et al.*, 1996). Therefore, other drugs have been studied as potential synergistic partners (Baggish and Hill, 2002). In human trials, atovaquone has been used for the treatment of patients with *P. falciparum* infections in combination with proguanil (Baggish and Hill, 2002).

In this study, although atovaquone significantly combated *B. gibsoni* infections, infections recurred in all of the experimentally infected dogs. Moreover, the parasites that reappeared about two months after therapy were less sensitive to atovaquone than the original isolate. This was shown using in vitro proliferation assays. Because the parasite used in this study was obtained from field isolates and it had not been cloned, whether the parasite developed resistance against atovaquone in the laboratory or if the parasite had been selected from a mixed population of resistant and sensitive organisms could be not defined. Other drugs combined with atovaquone might be required to prevent both recurrence and the reduction in sensitivity. The author found an IC_{50} of 89.02 ± 17.29 nM for atovaquone against *B. gibsoni* parasites not previously exposed to atovaquone, which is higher than the range of 0.9–23 nM reported for *P. falciparum* strains using different in vitro methodologies (Basco *et al.*, 1995; Gupta *et al.*, 2002). The IC_{50} value obtained in the present study might be useful for isolating *B. gibsoni* organisms that are atovaquone-resistant and thus could be applied to the development of combination drug therapies.

In conclusion, atovaquone significantly combated *B. gibsoni* infections in dogs. However, the parasite could not be eliminated from host blood and its sensitivity to atovaquone decreased. The use of atovaquone alone might allow the development of atovaquone-resistant parasites or select for parasites with reduced sensitivity to atovaquone from a mixed population. Further detailed investigations are needed for the development of effective clinical applications of atovaquone against *B. gibsoni*.

Chapter 3

Cloning of the *Babesia gibsoni cytochrome b* gene and isolation of three single nucleotide polymorphisms from parasites present after atovaquone treatment.

Introduction

Atovaquone is a novel antiprotozoal compound that has broad-spectrum activity against human protozoan pathogens, including *Plasmodium* spp., *Toxoplasma gondii* (Baggish and Hill, 2002), and *Babesia* spp (Hughes and Oz, 1995; Pudney and Gray, 1997; Gray and Pudney, 1999; Weiss, 2002). This compound is an analogue of ubiquinone, and its mechanism of action is via inhibition of mitochondrial electron transport (Baggish and Hill, 2002; Hudson *et al.*, 1991). However, recurrence of the disease and decreased sensitivity of the protozoa to therapy have been reported when atovaquone alone was used. Mutation of the *cytochrome b* (*cytb*) gene, which is located in the mitochondrial genome, has been described in atovaquone-resistant isolates of *Plasmodium* spp. (Syafruddin *et al.*, 1999; Suswan *et al.*, 2001), *T. gondii* (McFadden *et al.*, 2000), and *Pneumocystis carinii* (Walker *et al.*, 1998).

In chapter 2, the author described the therapeutic efficacy of atovaquone against *Babesia gibsoni* using experimentally infected dogs, and suggested that the atovaquone allows recrudescence of parasites with decreased susceptibility to this drug. However, the mechanisms responsible have not been investigated. Genetic changes associated with decreased sensitivity of *B. gibsoni* to atovaquone may be valuable as markers for clinical application and for selection of drugs for use in combination with atovaquone. In this chapter, a nucleotide coding sequence for *B. gibsoni cytb,* which might be the atovaquone target, was identified. Subsequently, the sequence of the *cytb* gene from atovaquone-treated animals with recurring parasitemia was determined and compared with that of the pretreatment parasite.

Materials and Methods

Parasites: The original parasite used in this study was isolated from a naturally infected Tosa dog in Aomori Prefecture, Japan, and was identified as an Asian genotype. This parasite was maintained by passage through a beagle (Dog A) that was not exposed to drug treatment. Whole blood samples were collected from this dog, and EDTA was used as anticoagulant. Blood samples that had been stored in our laboratory were used to compare the nucleotide sequence of *B. gibsoni cytb* before and after atovaquone treatment. The blood samples were collected during previous study (chapter 2), from three experimentally infected dogs (B, C, and D) before atovaquone treatment and during the recurrence of *B. gibsoni* infection after atovaquone treatment (30 mg/kg twice daily for seven days). The recurrent parasites showed less sensitivity to atovaquone than those obtained before treatment.

Cloning of the B. gibsoni cytb gene: *B. gibsoni* DNA was isolated from blood samples using a genomic DNA extraction kit (GFX Genomic Blood Purification Kit, Amersham, Buckinghamshire, UK). Total RNA from *B. gibsoni* was isolated using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Reverse transcription to obtain cDNA was performed as follows: 10 μg of total RNA, denatured at 65 °C, was reverse transcribed in a total volume of 40 μl using 2 μg oligo dT primer and 200 U Superscript II reverse transcriptase (Takara) in a solution containing 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, and 75 mM KCl (RT Buffer) with 100 μM of each dNTP at 42 °C for 1 hour.

The primer pair was designed according to the sequence of nucleotides from *Babesia bovis* (accession number AF053002), *Babesia bigemina* (accession number F109354) and *Theileria annulata* (accession number M63015) as follows: JD279, 5′-TGG AA(C/T)

TT(A/T) GGG TTT -3′; ROR8BG, 5′-A(A/T)G G(A/T)A TTA CTC CAT AAG TTA-3′. A PCR was performed on 20 μl of a mixture containing 1 μg of template genomic DNA, 10 pmol of each primer, 200 μM dNTP, and 1.25 units Taq Gold DNA polymerase (Invitrogen, Carlsbad, CA, USA). The PCR reaction was repeated for 40 cycles using 30 sec denaturation at 94 °C, 1 min annealing at 40 °C, and 1 min extension at 72 °C to obtain a 533 bp fragment. The PCR product was ligated into the pCR2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen). The entire ligation reaction was used to transform *Escherichia coli* DH5α competent cells. Plasmid DNA from two positive transformants was used to sequence the DNA of the insert. Both strands of the plasmid insert DNA were sequenced using the Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing analysis was performed using GENETYX-MAC (version 10; Software Development, Co. Ltd., Tokyo, Japan). The similarities of this nucleotide sequence to those of *B. bovis*, *B. bigemina*, and *T. annulata* were 82.2%, 80.3%, and 64.5%, respectively.

Subsequently, 3′ rapid amplification of cDNA ends (RACE) was performed to determine the sequence of the gene at the 3′ end of *cytb*. A primer was designed as follows: Ky1, 5′-GAG TAT TAA CAG AAG TTA ATA TGG -3′. We performed PCR on 1 μl cDNA using 50 pmol primer and 2.5 U Taq polymerase (Takara) in a solution containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, and 200 mM of each dNTP. The thermal cycling conditions were: one cycle for 5 min at 94 °C; five cycles of 1 min at 94 °C, 48 °C, and 72 °C; final incubation at 72 °C for 10 min. The resulting 670-bp fragment was cloned using the method described earlier into the pCR2.1-TOPO vector and the sequence was then determined.

A commercial primer kit (DNA Walking *Speed up* kit, Seegene, CA, USA) was used to sequence the 5′ end of *cytb*, and three primers were designed corresponding to the

sequences of the *cytb* fragments obtained from JD279 and ROR8BG: Ky4, 5′- CAG GTT TGT TAT AAC TGT TGC TCC -3′; Ky6, 5′-ACG AAC TGC CCA ACC CAT AT -3′; Ky2, 5′-CCC ATA TTA ACT TCT GTT AAT ACT C-3′. A primer set of CYTb 1, 5′-TGT ATT ACT ATA CTG TGA GT-3′ and CYTb 2, 5′-AAC TCC CCT CTG TTT TA-3′ was designed corresponding to the nucleotide sequences obtained for the 3′ and 5′ ends (Fig. 7). Finally, PCR was performed using primers CYTb 1 and CYTb 2 with *B. gibsoni* genomic DNA and the resulting fragment of 1,228 bp was cloned and sequenced as described earlier.

Comparison of cytb between pre treatment and recurrent parasite: Pretreatment parasite DNA and recurrent parasite DNA extracted from three dogs was subjected to PCR using the primer pair CYTb 1 and CYTb 2. The fragment of 1,228 bp was then sequenced directly. In instances where no synonymous mutation was observed, a second PCR was run on the original template DNA, which was then sequenced to ensure that the change was not a PCR artifact.

Results

B. gibsoni cytb sequences for the open reading frame (ORF) were obtained from at least three independent PCR amplifications. Then, the complete sequence of the *B. gibsoni cytb* gene (1,228 bp long, including 1,071 bp of the ORF encoding a protein of 357 amino acid residues) was obtained (accession number AB215096; Figure 7). Comparison of the deduced amino acid sequence against those of *B. bovis*, *B. bigemina*, and *T. annulata*, which are defined partially (Salem *et al.*, 1998: Megson *et al.*, 1991) showed 79.7%, 82.5%, and 52.1% identity, respectively, within their defined partial sequences. Comparison of the deduced amino acid sequence against those of *Theileria parva* (accession number Z23263), *P. falciparum* (accession number AU086218), and *T. gondii* (accession number AF023246) showed 53.8%, 41.4%, and 41.5% identity, respectively (Figure 8). The nucleotide sequence of the *cytb* gene amplified from blood samples taken from two dogs in Okinawa Prefecture (Ikadai *et al.*, 2004) showed > 99% identity to that obtained from dogs in Aomori Prefecture. At amino acid 309, tyrosine was substituted by cysteine in *B. gibsoni* isolated in Okinawa Prefecture.

B. gibsoni cytb genes obtained before and after atovaquone treatment were successfully amplified and sequenced from samples obtained from all three dogs (Figure 9). G-to-T or A substitution at nucleotide 363, resulting in methionine-to-isoleucine substitutions (M121I) were observed in the *cytb* gene of *B. gibsoni* from all three dogs after atovaquone treatment. Two other single polymorphisms, a G-to-A substitution at nucleotide 658, which resulted in valine-to-isoleucine substitution (V220I), and an A-to-G substitution at nucleotide 907, which resulted in isoleucine-to-valine substitution (I303V), were observed in the *cytb* gene of *B. gibsoni* from two of three dogs after atovaquone treatment. V220I was a mixed

population with wild-type nucleotide in *B. gibsoni* from Dog B and Dog C, but was not detected in *B. gibsoni* from Dog D. I303V was a complete substitution in *B. gibsoni* from Dog B, a mixed population with wild-type nucleotide in *B. gibsoni* from Dog C, and was not detected in Dog D (Figure 9).

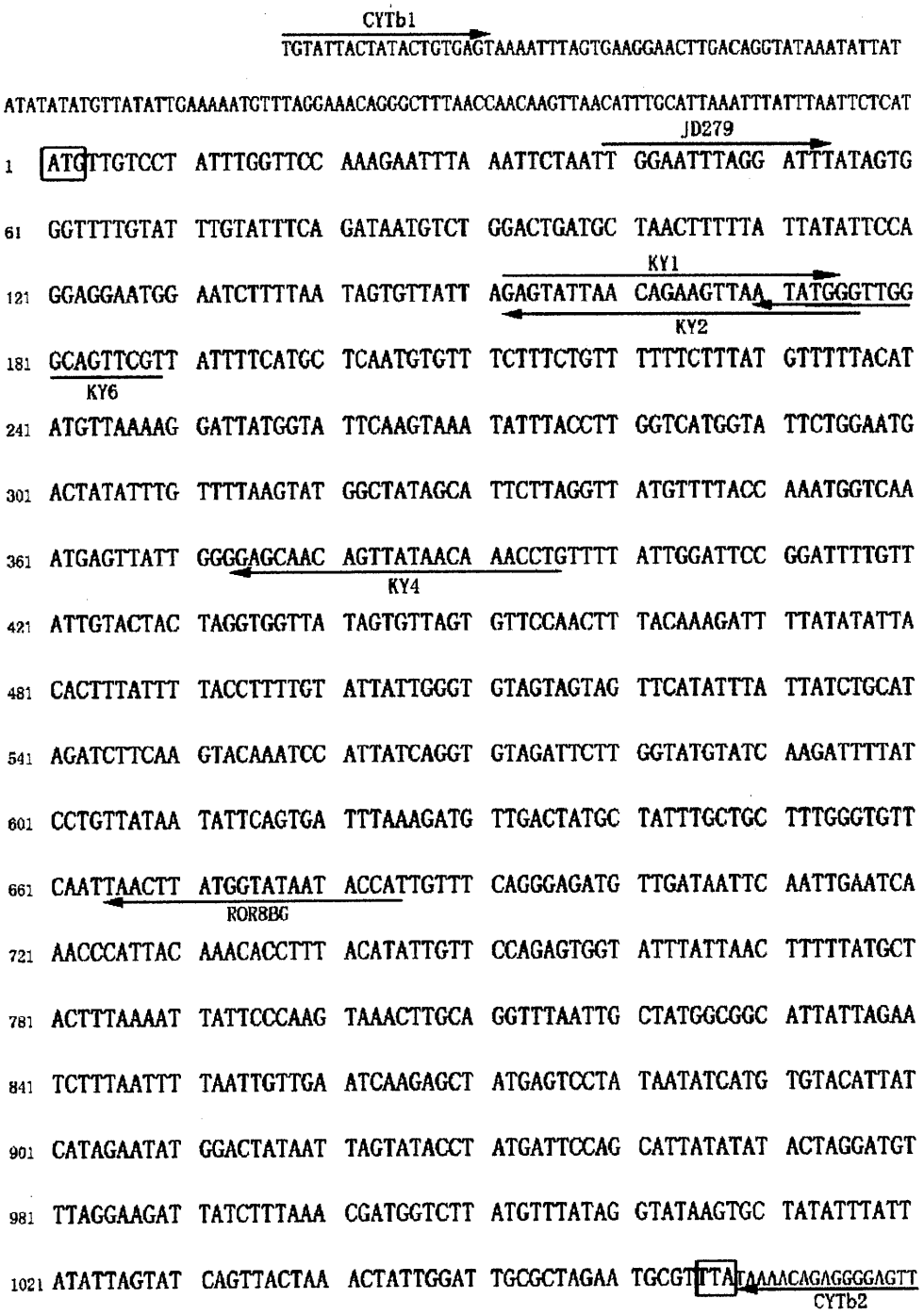

Figure 7. The complete nucleotide sequence of *B. gibsoni cytb*. Nucleotides are numbered beginning with the first methionine of the open reading frame, which is indicated by a box. The lines represent the primer sites used in this study.

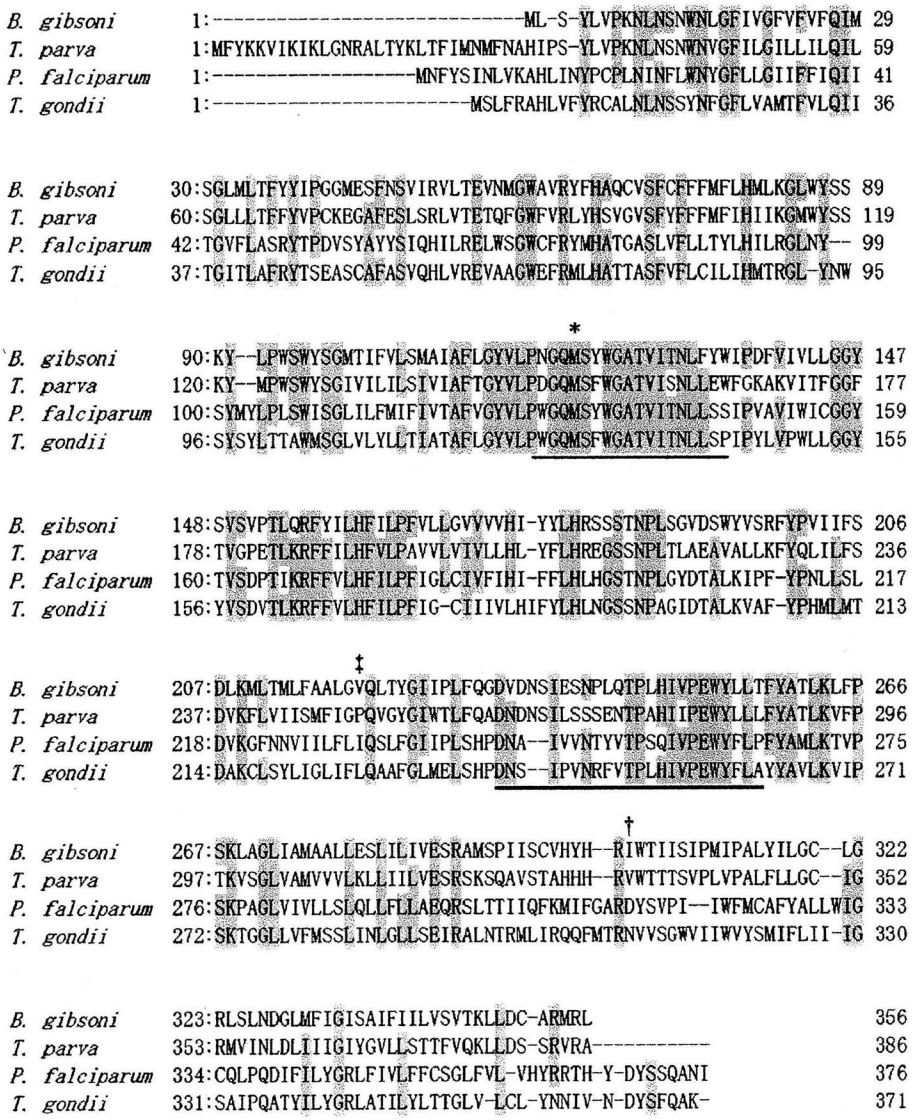

Figure 8. Alignment of deduced amino acid sequences of the *cytb* gene of *B. gibsoni* with other apicomplexan parasites. The deduced amino acid sequence of *B. gibsoni cytb* was aligned with those of *T. parva* (accession number Z23263), *P. falciparum* (accession number AU086218), and *T. gondii* (accession number AF023246). Converse amino acid residues are shaded. Lines represent putative atovaquone binding site for *P. falciparum*. The mutation points observed in recurrent *B. gibsoni* are indicated by * (M121I), ‡ (V220I), and † (I303V).

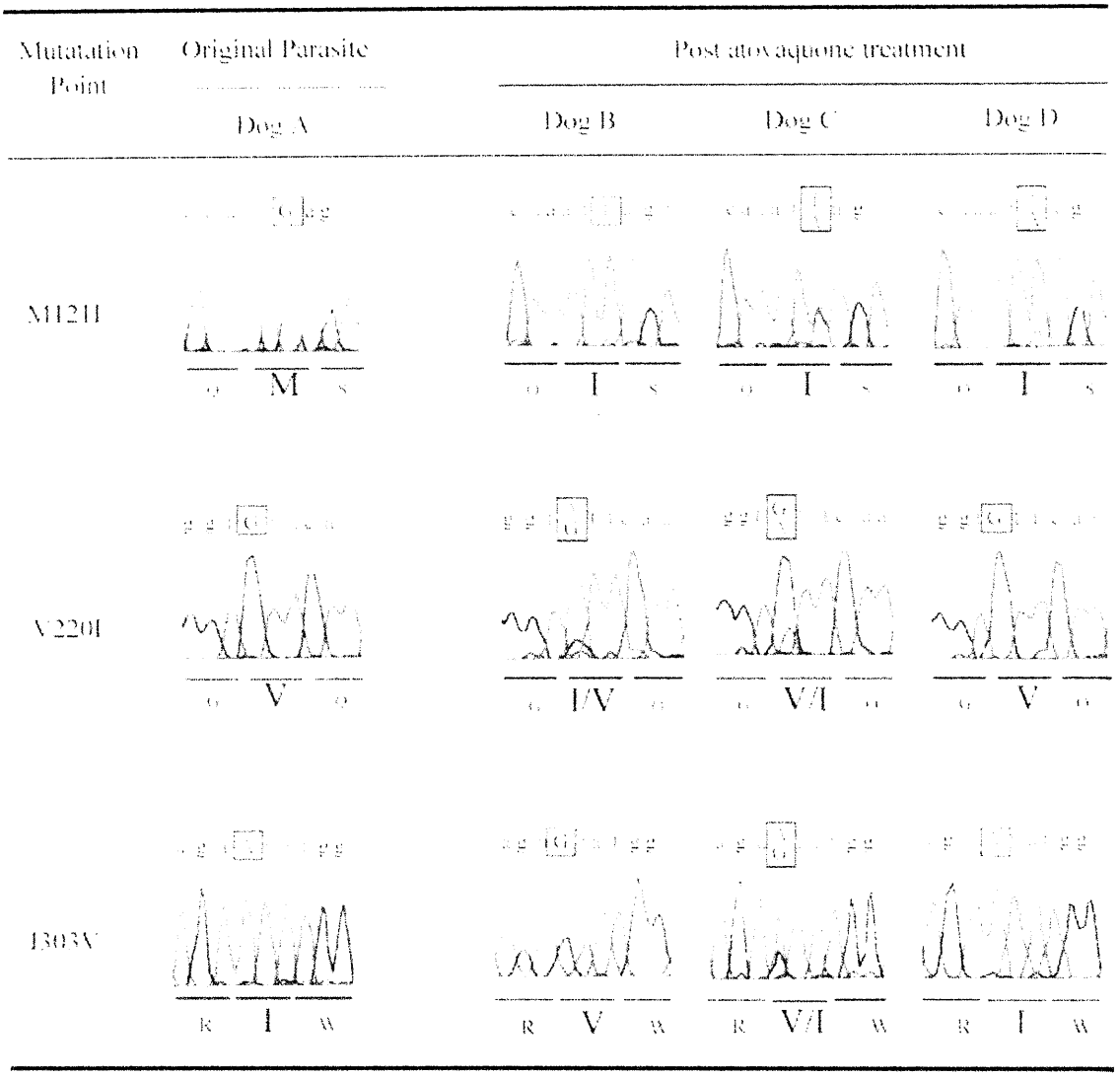

Figure 9. Direct sequencing of the original and recurrent *B. gibsoni cytb* gene. The mutation sites of M121I, V202I, and I303V in the original parasite (Dog A) and atovaquone treated parasites from three experimental dogs (Dogs B, C, and D) are shown. Pretreatment sequences of each of the polymorphism sites in the gene of *B. gibsoni* from all three dogs (Dog B, C, and D) corresponded to those of the original parasite (Dog A).

Discussion

In this study, the complete sequence of the *B. gibsoni cytb* gene was determined. This is the first such determination in the *Babesia* genus. The ORF shows a highly conserved protein with strong identity throughout to that of other apicomplexan parasite *cytb* amino acids (Figure 8).

In the malaria parasite, atovaquone is thought to inhibit the mitochondorial *cytochrome* bc_1 complex by competitive binding with coenzyme Q. M121I, which was identified in *B. gibsoni* from all three atovaquone treated dogs, was localized in the region thought to be the target region responsible for the effect of atovaquone in *P. falciparum* (Figure 7). Moreover, the same substitution has been reported for atovaquone-resistant *Plasmodium* spp (Syafruddin *et al.*, 1999: Korsinczkey *et al.*, 2000). As the recurrent parasites displayed less atovaquone sensitivity after atovaquone treatment in chapter 2, the M121I identified in this study may be associated with the loss of sensitivity.

Two other single polymorphisms V220I and I303V were observed in the *cytb* gene of *B. gibsoni* from two of three dogs after atovaquone treatment. Whether these polymorphisms induce less sensitivity against atovaquone and how sensitivity is reduced in *B. gibsoni* are not known. Further study is needed to establish a direct correlation between the three single polymorphisms and atovaquone resistance.

Direct sequencing in *B. gibsoni* DNA extracted from pretreatment dogs and of parasite DNA extracted from the Okinawa Prefecture isolate, which had not been exposed to atovaquone, failed to detect these three nucleotide polymorphisms. Additional screening of *B. gibsoni* isolated from two naturally infected Tosa dogs in Aomori Prefecture that had not atovaquone treatment also revealed that these polymorphisms were absent (data not shown).

In these findings, a single seven days course of atovaquone treatment might induce changes in the population of *B. gibsoni* with variant polymorphisms.

Atovaquone becomes a major component of a new antibabesial treatment in which azithromycine is used as a combination drug for canine babesiosis (Birkenheuer *et al.*, 2004). This information on polymorphisms of the atovaquone binding site in *B. gibsoni* may be useful not only for treatment of canine babesiosis, but also for treatment of human babesiosis. These single polymorphisms in the *cytb* gene may be useful as molecular markers for monitoring the development and spread of drug-resistant parasites in the field.

General conclusion

B. gibsoni is a tick-borne hemoprotozoan parasite causing hemolytic anemia in dogs. This parasite is now considered endemic in the world wild. In Japan, this parasite is found in numbers of Tosa dogs. In this study, the predilection of incidence of canine *B. gibsoni* infection for Tosa dogs in Japan was studied firstly.

Blood samples from 141 Tosa dogs from Aomori Prefecture during August 2002 to April 2003 were collected and the *p18* gene of *B. gibsoni* were detected from these samples using PCR assay to investigate the incidence of subclinical *B. gibsoni* infection. Forty-one of 87 dogs (47.1%) with histories of dog fighting and one dogs of 54 without a history of dog fighting were positive for *B. gibsoni*; that is, 42 of 141 dogs (29.8%) showed a positive result. Tosa dogs in Aomori Prefecture, Japan, were highly infected with *B. gibsoni* but had no symptoms, and this pathogen might be transmitted through dog fighting. These dogs may become a reservoir for tick-born infections to other companion animals. In addition, dogs with subclinical infections were also at risk of chronic thrombocytopenia which may be due to autoimmune mechanisms. Thus, it remains a need for an effective agent for eliminate this parasite. Subsequently, the therapeutic efficacy of atovaquone, which is active against a wide range of human protozoan pathogens were examined.

In chapter 2, the therapeutic efficacy of atovaquone against *B. gibsoni* was examined in three dogs experimentally infected with this parasites isolated from naturally infected dogs in Aomori Prefecture, Japan. Once parasitemia reached 10%, atovaquone was administered orally (30 mg/kg twice daily for 7 days). Within 2 days of atovaquone treatment, the parasite disappeared from blood smears without any clinical side effects. Anemia and thrombocytopenia were markedly improved in all the dogs. However, a PCR assay revealed

that one of the *B. gibsoni* marker gene was intermittently present in peripheral blood after atovaquone therapy indicating that the organism had not been eliminated, and parasites reappeared in blood smears 33 days after the last treatment. To investigate the change in the susceptibility to atovaquone, an in vitro sensitivity test was performed using peripheral blood obtained from an untreated dog that was infected with the original parasite isolate, and from two of the experimentally infected and atovaquone-treated animals. The recurring parasites were less susceptible to atovaquone than the parasites isolated from the dog. Although atovaquone treatment against *B. gibsoni* was highly effective, our results indicated that the use of atovaquone therapy alone allows the recrudescence of parasites and decreases parasite susceptibility to this drug. However, the mechanisms responsible for the decreased susceptibility have not been investigated. Genetic changes associated with decreased sensitivity of *B. gibsoni* to atovaquone may be valuable as markers for clinical application and for selection of drugs for use in combination with atovaquone.

In chapter 3, nucleotide coding sequence for *B. gibsoni cytb,* which might be the atovaquone target, was identified firstly. Subsequently, the sequence of the *cytb* gene of *B. gibsoni* isolated from atovaquone-treated animals with recurring parasitemia was determined and compared with that of the parasite obtained from the dogs before the treatment.

DNA was extracted from *B. gibsoni* isolated from Aomori Prefecture, Japan, and 1,288 bp of *cytb*, including 1,071 bp of open reading frame, were sequenced. The sequence showed high homology to that of other apicomplexan parasites. *B. gibsoni cytb* DNA from three dogs that had been experimentally infected with *B. gibsoni* and treated with atovaquone were also sequenced. The *cytb* genes from all three atovaquone-treated dogs contained a single mutation resulting in an amino acid change (M121I) in one of the putative ubiquinone binding sites of *P. falciparum*, which was homologous to mutations in other apicomplexan

protozoa that exhibit resistance to atovaquone. Two other mutations (V220I and I303V) were observed in two of the dogs. These results indicate that point mutations in the sequence for mitochondrial *cytb* may be associated with decreased susceptibility of *Babesia* species to atovaquone.

In conclusion, the present study demonstrated that *B. gibsoni* may spread among dogs in Japan through a peculiar incident, namely, dog fighting. In addition, atovaquone significantly combated this parasite. However, the use of atovaquone therapy alone allows the recrudescence of parasites and decreases parasite susceptibility to this drug. Point mutations in the sequence for parasite's mitochondrial *cyt b* might be associated with these results.

Abstract

To identify the incidence of *Babesia gibsoni* infection among dogs in Aomori Prefecture, Japan, blood samples from 141 Tosa dogs from Aomori Prefecture were collected and polymerase chain reaction (PCR) assay were performed to investigate the incidence of subclinical *B. gibsoni* infection. 47.1% with histories of dog fighting, and 1.9% without any histories of dog fighting were positive for *B. gibsoni*; that is, 29.8% showed a positive result. Tosa dogs from Aomori Prefecture were highly infected with *B. gibsoni* but showed no symptoms, and this pathogen might be successfully transmitted during dog fighting. These dogs may become a reservoir for tick-born infections to other companion animals. Furthermore, dogs with subclinical infections were also at risk of chronic thrombocytopenia which may be due to autoimmune mechanisms. Thus, it remains a need for an effective agent for eliminate this parasite.

The therapeutic efficacy of atovaquone, which is active against *Plasmodium* spp. and *T. gondii*, against this parasite was examined in three dogs experimentally infected with *B. gibsoni*. Atovaquone (30mg/kg 7 days orally) showed potential therapeutic effectiveness against acute *B. gibsoni* infection in dogs. However, infections recurred in all treated dogs. The parasites that reappeared about two months after therapy were less sensitive to atovaquone than the original isolate. We identified a single-nucleotide mutation in the *cytb* gene of the recurring parasite that resulted in an M121I substitution at the amino acid level. This mutation was identified in *B. gibsoni* obtained from all three atovaquone-treated dogs. This amino acid substitution is localized in the region corresponding to that proposed as the target region for atovaquone action in *Plasmodium* spp.

Acknowledgements

The author wishes to acknowledge Dr. Yoshiaki Hikasa (Tottori University) for his appropriate suggestion and critical reading of the manuscript. The author gratefully appreciates Dr. Hiromi Ikadai (Kitasato University) for his technical advisees and suggestions on the present study, and critical reading of the manuscript. The author thanks Dr. Seiichi Higuchi and Dr. Shozo Okano (Kitasato University) for their contribution to this study, and Drs. Yushi Koshida, Akemi Kawabe, Kenichi Inoue, Megumi Kawahara, Kayoko Miyamoto, Satomi Ono (Kitasao University) for their technical assistances.

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