

**Studies on the establishment of the treatment strategy with atovaquone
for *Babesia gibsoni* infection in dogs**

(犬における *Babesia gibsoni* 感染症に対するアトバコンを中心とした治療法の
確立に関する研究)

Aiko IGUCHI

2014

Contents

General Introduction	1
Chapter 1	4
<i>Development of in vitro atovaquone-resistant Babesia gibsoni with a single-nucleotide polymorphism in cytb</i>	
Introduction	5
Materials and Methods	7
Results	13
Discussion	20
Chapter 2	23
<i>The in vitro interactions and in vivo efficacy of atovaquone and proguanil against Babesia gibsoni infection in dogs</i>	
Introduction	24
Materials and Methods	25
Results	30
Discussion	36
Chapter 3	43
<i>The efficacy of Malarone[®] against 8 naturally Babesia gibsoni infected dogs</i>	
Introduction	44
Materials and Methods	45
Results	47
Discussion	52
General Conclusion	55
Abstract	59
Acknowledgements	63
References	64

General Introduction

Babesia gibsoni (*B. gibsoni*) is a tick-borne parasite infecting the red blood cells of dogs and inducing hemolytic anemia, jaundice, hemoglobinuria, and an enlarged spleen (Groves and Dennis, 1972; Farwell et al., 1982). This parasite has been reported to occur endemically in Asia, Africa, Europe, North America (Kjemtrump et al., 2000), and Australia (Muhlninckel et al., 2002). In Japan, *B. gibsoni* has been distributed in the western part (Inokuma et al., 2003, 2004; Ikadai et al., 2004) and has spread to the eastern part (Miyama et al., 2005).

Early treatment is required for acute *B. gibsoni* infections. Some drugs have been considered, but each treatment has a number of drawbacks. Diminazene aceturate (DA) has been used to treat acute *B. gibsoni* infections in Japan and causes severe adverse effects such as pain at the injection site, and nervous symptoms due to cerebral hemorrhage (Boozer and Macintire, 2003). Some studies have reported the effectiveness of new combination therapies such as clindamycin (CLDM)-metronidazole-doxycycline (DOXY), or DOXY-enrofloxacin-metronidazole, on experimentally infected dogs or clinical patients, but these therapies take a long time to show clinical effectiveness (Suzuki et al., 2007; Lin and Huang, 2010).

Atovaquone (ATV) is a novel antiprotozoal compound that has a

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, 1995). Its mechanism of action that mediated by the inhibition of mitochondrial electron transport has been most completely elucidated for *Plasmodium* spp. (Hudson et al., 1991; Baggish and Hill, 2002). ATV is also effective against acute *B. gibsoni* infections and has generally fewer side effects. However, ATV monotherapy results in relapse as well as the emergence of drug-resistant variants with some single-nucleotide polymorphisms (SNPs) in the cytochrome *b* gene (*cytb*) (Matsuu et al., 2004, 2006). The relationship between SNPs in *cytb* and ATV resistance has been unclear. In order to establish ATV-based treatment strategies against *B. gibsoni* infections, it is necessary to conduct detailed in vitro studies about the behavior of ATV-resistant parasites and the treatment strategies.

To delay or prevent drug resistant development of pathogens, combination drug regimens often achieve a therapeutic efficacy greater than monotherapy. Combination therapy with ATV and azithromycin (AZM) has been reported to be more effective than ATV monotherapy for *B. microti* in hamsters (Wittner et al., 1996), and is also recommended for human *Babesia* spp. infections (Krause et al., 2000), and for *B. gibsoni* infections (Birkenheuer et al., 2004). However, an ATV-resistant parasite

possessing plural SNPs in *cytb* has emerged (Jefferies et al., 2007; Sakuma et al., 2009). Recently, proguanil (PG) has been used in combination with ATV for the treatment of *Plasmodium* spp. infections because of their synergistic interaction (Baggish and Hill, 2002; Fivelman et al., 2004). A tablet formula that combines ATV and PG, Malarone[®], is available for human malaria. Nevertheless, the interaction between ATV and PG and the therapeutic efficacy of Malarone[®] for *B. gibsoni* infection have not been examined.

In chapter 1, an ATV-resistant *B. gibsoni* was developed by in vitro exposure of ATV the uncloned wild type (WT) *B. gibsoni*, which had been maintained at our laboratory since 2004 and mitochondrial gene analysis was performed. Furthermore, the sensitivity of the ATV-resistant *B. gibsoni* to the several anti-babesial drugs was evaluated. In chapter 2, in vitro interactions between ATV and PG against *B. gibsoni* was evaluated. In addition, the clinical efficacy to experimentally *B. gibsoni* infected dogs of this combination therapy using Malarone[®] which is the anti-malarial drug containing ATV and PG was evaluated. Finally, the efficacy of Malarone[®] against *B. gibsoni* in acute stage of naturally infected dogs was studied in chapter 3.

Chapter 1

Development of in vitro atovaquone-resistant *Babesia gibsoni* with a single-nucleotide polymorphism in *cytb*

Introduction

The previous study reported that ATV monotherapy was effective and had few side effects for acute canine *B. gibsoni* infection, but resulted in relapse as well as the emergence of ATV-resistant variant possessing plural SNPs in *cytb* (Matsuu et al., 2004, 2006). Although combination with ATV and AZM has been reported, this therapeutic modality could not completely eliminate parasites in acute *B. gibsoni* infections, and an ATV-resistant parasite possessing plural SNPs in *cytb* has emerged (Jefferies et al., 2007; Sakuma et al., 2009). The additive or synergistic effects of these drugs against *B. gibsoni* have not been fully evaluated. In order to conduct the ATV-based treatment strategies, the in vitro studies including the sensitivity test against ATV-resistant *B. gibsoni* and the evaluation of interaction between ATV and other drugs are required.

In the previous in vivo studies, single-nucleotide substitution at nt363 in *cytb* resulting in the replacement of methionine with isoleucine (M121I) seemed to be the factor responsible for ATV resistance, because M121I was localized in the region thought to be the ATV-binding site in *P. falciparum* (Korsinczky et al., 2000); this substitution was frequently found (Matsuu et al., 2006; Sakuma et al., 2009). However, the relationship between the SNPs including M121I and resistance has not been demonstrated in vitro, and the mechanism underlying ATV treatment-induced resistance

development in the parasite is unclear. It is suggested that mutation in *cytb* following ATV treatment or selective multiplication of parasites with *cytb* SNPs may have taken place. It is necessary to elucidate the relationship between the SNPs in *cytb* and ATV resistance, and to develop a genetic marker for ATV-resistant *B. gibsoni*.

This study was conducted to develop an in vitro ATV-resistant *B. gibsoni* by exposing the parasite culture to ATV for 6 days, and to perform mitochondrial gene analysis. At this time, uncloned WT *B. gibsoni* and WT sibling clones were used for ATV exposure, to confirm how the ATV resistance emerges and *cytb* wild type allele and mutated allele were evaluated using allele-specific SYBR green real-time PCR. Furthermore, this study evaluated the sensitivity of the ATV-resistant *B. gibsoni* to the anti-babesial drugs, including DA, AZM, DOXY, CLDM, and PG, to examine whether the ATV-resistant parasite possessed resistance against those drugs.

Materials and methods

In vitro culture of B. gibsoni: *B. gibsoni* parasites were isolated from a naturally infected Tosa dog in Aomori Prefecture, Japan, in 2004. The parasites were maintained in vitro culture at our laboratory (Matsuu et al., 2008) and were used as the uncloned WT *B. gibsoni* in the present study. Four *B. gibsoni* WT sibling clones were obtained through a modified limiting dilution procedure, as described previously (Korsinczky et al., 2000).

In vitro culture of *B. gibsoni* was performed as reported as previously (Matsuu et al., 2008). Briefly, 200 μ l of packed infected RBCs were dispensed into 1800 μ l of culture medium to obtain a 10% packed cell volume in each well of a 12-well plate. Each parasite was incubated at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 25 mM pyruvic acid, 2 mM L-glutamine, 100 units/mL penicillin G, 100 μ g/mL streptomycin (Invitrogen), and 10% fetal bovine serum (FBS; Funakoshi, Tokyo, Japan). Half the volume of culture medium was replaced with fresh medium every day. Canine erythrocytes were obtained from a healthy beagle, and subculture was carried every 7 days.

In vitro cultivation of B. gibsoni with ATV and isolation of ATV-resistant

parasite: For development of ATV-resistant *B. gibsoni*, different concentrations of ATV were exposed to WT *B. gibsoni* and 4 clones. ATV was obtained from Wako Chemicals (Osaka, Japan), and a stock solution was prepared in dimethyl sulfoxide (DMSO). ATV stock solution was diluted with culture medium to yield final concentrations of 100, 200, 400, 800, and 1600 nM. Twenty-hundred micro litter of each *B. gibsoni* culture suspensions were dispensed per well in 96-well plates in triplicate for each ATV concentration. Identical cultures containing only DMSO and without ATV were prepared to be used as controls. The final concentration of DMSO was adjusted to 0.1%. These plates were incubated at 5% CO₂ and 37°C for 6 days. Every day, half of the medium was replaced with fresh medium, according to the concentration of ATV. The growth inhibition rate of the parasite was calculated by counting the number of parasitized erythrocytes from each of the wells containing drugs and that in the control wells without the drug every 2 days from blood smear (Matsuu et al., 2008). The half-maximal inhibitory concentration (IC₅₀) of ATV was determined after 6 days incubation (Matsuu et al., 2008).

After 6 days of ATV exposure, parasites from each ATV concentration were dispensed into new wells in 12-well culture plates containing ATV-free culture medium and normal dog erythrocytes, and viability was determined by culturing for the next 7

days. Of the parasite-positive wells identified through blood smear observation, the parasites that had been exposed to the highest concentration of ATV were isolated, and examined their ATV sensitivity. The methods were similar to the ATV-exposure step (final ATV concentration: 200-3200 nM), and the growth inhibition rate and IC₅₀ was calculated as described above. If parasites demonstrated low sensitivity following exposure to ATV, they were isolated as ATV-resistant *B. gibsoni*.

Sequence analysis of WT and ATV-resistant *B. gibsoni*: The nucleotide and deduced amino acid sequences of the mitochondrial *cytb* in the each parasite were analyzed. *B. gibsoni* DNA was isolated from 200 µL of each culture by using a genomic DNA extraction kit (QIAamp DNA Mini Kit; Qiagen, USA). To amplify the open reading frame (ORF) region of *cytb*, 2 primer sets were designed on the basis of the reported gene sequence (accession numbers: AB215096 and AB499087) as follows: F1: 5' -AGGGCTTTAACCAAC-3' and R1: 5' -AGTTGGAACACTAACA-3' and F2: 5' -AGTTATTGGGGAGCAAC-3' and R2: 5' -CCTCTGTTTTATAAACGCA-3'. The first primer set (F1 and R1) was used to amplify the anterior region of *cytb*, and the latter (F2 and R2), to amplify the posterior region. A polymerase chain reaction (PCR) was performed using a 25 µL mixture containing 1 µg of template genomic DNA, 10 pmol of each primer, 200 µM

deoxynucleoside triphosphate (dNTP), and 1.25 units of *Taq* polymerase (Takara, Japan). PCR spanned 40 cycles. with denaturation for 30 s at 95°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C. The amplified DNA was purified with commercial kit (QIAamp purification kit, Qiagen, USA) and sequenced directly (ABI PRISM 310 genetic Analyzer, Applied Biosystems, USA). Genetyx Version 8.0 software (Genetyx Co., Tokyo, Japan) was used to characterize the nucleotide sequence data obtained.

Quantification of *cytb* wild type allele (363G) and mutated allele (363T) using allele-specific SYBR green real-time PCR: As a single-nucleotide substitution was detected from the ATV-resistant *B. gibsoni* at 1 location on *cytb*, i.e., nt363G to 363T, the population ratio of the 363G and 363T alleles in the parasites was determined using an allele-specific SYBR green real-time PCR. The primer sequence used for this assay is listed in Table 1. Allele-specific forward primers were designed to specifically detect and quantify *cytb* 363G and 363T alleles. The 3' end nucleotides are located on the polymorphic site, and the primers were designed to match 1 of the 2 variants at the nt363 *cytb* position. Moreover, an additional nucleotide mismatch at the third position from the 3' end of each allele-specific primer was introduced to decrease non-specific PCR amplification. The reverse primer was common. Plasmid clones containing the target region, either 363G or 363T, were prepared from DNA samples from the *B.*

gibsoni WT or ATV-resistant, by using a TOPO TA Cloning Kit (Invitrogen). These plasmid clones were used as a standard curve template for quantification of *cytb* 363G or 363T allele. The primer set for the plasmids was designed as follows: 5' -ATTGTTTTAAGTATGGCTATAGCATTCTTAGG-3' and 5' -AATGGATTTGTA CTTGAAGATCTATGCAG-3'. Each plasmid was serially diluted 10-fold to obtain 10^8 – 10^0 molecules/ μ L, and the standard curve was created by plotting the log of initial copy number of input plasmid DNA against the threshold cycle (Ct) value. We extracted 200 μ L of genomic DNA from 200 μ L of each *B. gibsoni* suspension by using a DNA extraction kit (Qiagen), and 1 μ L of genomic DNA was used as template DNA for real-time PCR. To determine the specificity limit of the 363G allele-specific real-time PCR assay, 10^8 copies of plasmids containing the 363T allele were used as template DNA in the negative control. In contrast, we used 10^8 copies of plasmids containing the 363G allele as template DNA for the negative control to determine the detection limit for the 363T allele-specific real-time PCR.

Real-time PCR was performed using a standard protocol recommended by the manufacturer (iQ SYBR Green Supermix, Bio-Rad, Japan). One microliter of template DNA was added to 19 μ L of reaction mixture containing 0.5 μ L of each primer, 8 μ L ultra pure water, and 10 μ L iQ SYBR Green Supermix. The reaction was performed

under the following conditions (MiniOpticon, Bio-Rad): 95°C for 30 s, 95°C for 3 min, and 60°C for 5 s. A thermal melt profile was built following PCR to identify amplicons as specified, and analysis was performed with analysis software (CFX-Manager, Bio-Rad).

Sensitivity test of WT and ATV-resistant B. gibsoni against different drugs:

The sensitivities of WT and ATV-resistant *B. gibsoni* against other different drugs were examined using the same technique as reported previously (Matsuu et al., 2008). Five drugs were used as test compounds, namely CLDM, DA, DOXY, AZM, and PG. CLDM, DA, and DOXY were obtained from Sigma-Aldrich (Tokyo, Japan); AZM and PG were obtained from Wako Chemicals (Osaka, Japan). The final concentrations for the sensitivity tests are as follows: DA at 10–6250 nM, AZM at 2.5–40 µM, DOXY at 4–64 µM, CLDM at 50–800 µM, and PG at 6.25–100 µM. Cultivation continued for 6 days, and monitored the growth inhibition rate and calculated IC₅₀ every 2 days.

Results

***In vitro* generation of ATV-resistant *B. gibsoni*:** The growth of WT *B. gibsoni*, which was the uncloned parasite, was suppressed completely in the presence of 400, 800, and 1600 nM ATV in all the 3 replicates (Figure 1A), and IC₅₀ on day 6 was 164.0 ± 42.0 nM (Table 2). Subsequent cultivation of the parasites without the drug for another 6 days showed no re-growth of the parasite following exposure to 1600 nM but not at lower concentrations of ATV. Therefore, the cultures exposed to 800 nM ATV were isolated and continuously cultured. When the parasite began to grow at a constant rate after about 4 weeks, the sensitivity of this isolated parasite against ATV was examined. The growth inhibition rates of this parasite were 0, 34.2 ± 27.5, and 78.6 ± 17.4 % at the ATV concentration of 400, 800, and 1600 nM respectively (Figure 1B). The IC₅₀ of ATV for this parasite on day 6 was 1021.5 ± 419.7 nM (Table 2). Therefore, the author isolated this parasite as an ATV-resistant *B. gibsoni*.

The *B. gibsoni* WT sibling 4 clones obtained in this study, showed constant growth from about 2 months after the limiting dilution, and the morphology and growth speed were similar to the original *B. gibsoni*. The growth of these 4 clones was completely suppressed in the presence of 400, 800, and 1600 nM ATV. Subsequent cultivation of the parasites without ATV for over 7 days showed no re-growth of the

parasite following exposure to 800 and 1600 nM ATV in all the clones. Therefore, the author isolated the surviving parasites that were exposed to 400 nM ATV. But the sensitivity of these parasites to ATV was similar to that of the original clones (data not shown).

Molecular characteristic of WT and ATV-resistant B. gibsoni: *Cytb* of the ATV-resistant *B. gibsoni* had a single-nucleotide substitution at nt363 (G to T), which resulted in the substitution of methionine with isoleucine (M121I) (Figure 2). This substitution was not detected in the WT *B. gibsoni*, the 4 *B. gibsoni* WT sibling clones and 4 clones exposed ATV 400 nM.

Using the allele-specific real-time PCR assay, the copy number for 363G allele and 363T allele of WT and ATV resistant *B. gibsoni* was calculated (Figure 2). Although no amplification was recorded from the negative controls after PCR for 363G allele, negative and non-specific amplifications were confirmed to have a Ct value of >22 for 363T allele. Therefore, the detection limit for 363T allele was determined at a Ct value of 22. There was a dominant population (>99%) of 363T polymorphism parasites present in the ATV-resistant *B. gibsoni* (Figure 2).

Drug sensitivity test for WT and ATV-resistant B. gibsoni: DA, AZM, DOXY, CLDM, and PG inhibited growth in both WT and ATV resistant *B. gibsoni*. There were

no significant differences in drug sensitivity between the 2 *B. gibsoni* in each cultivation period except against ATV. IC₅₀ for each drug on day 2, 4, and 6 for WT and ATV-resistant *B. gibsoni* are listed in Table 2.

Table 1. Primer sequence for *cytb* allele-specific PCR

Target allele	Forward primer sequence(5'-3') (location in <i>cytb</i>)	Reverse primer sequence(5'-3') (location in <i>cytb</i>)
363G allele	ggttatgtttaccaa <u>at</u> gg <u>t</u> caag <u>t</u> <u>G</u> * (<u>nt337-363</u>)	ttggaacactaacactataaccacct (nt457-432)
363T allele	ggttatgtttaccaa <u>at</u> gg <u>t</u> caag <u>t</u> <u>T</u> (<u>nt337-363</u>)	

*Underline shows mismatch at the third nucleotide from 3' terminal, and capital shows polymorphic site on 3' end.

Figure 1

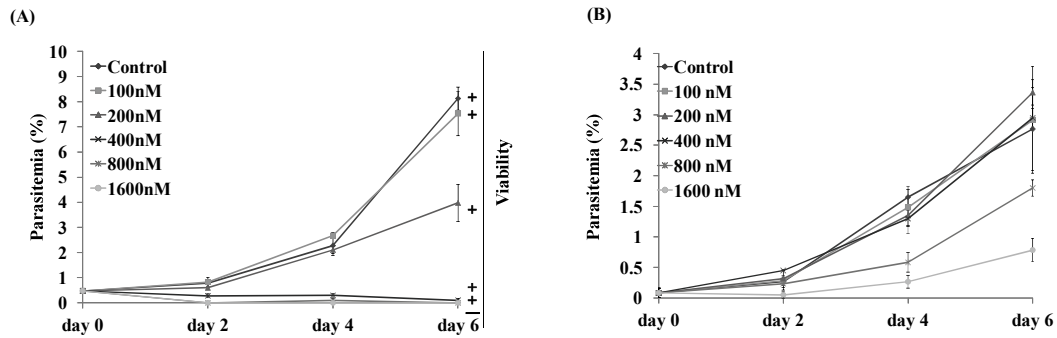


Figure 1. (A) Growth curves of WT *B. gibsoni* in vitro culture treated with different concentrations of ATV and the determination of parasite viability or recrudescence after withdrawal of the drug. Data are representative of one experiment of three separate experiments. (B) Growth curves of *B. gibsoni* isolated after 800 nM ATV treatment. Parasite growth was not inhibited completely even in the presence of 400, 800 or 1600 nM ATV. Data are representative of one experiment of three separate experiments.

Table 2. IC₅₀s for 6 drugs on days 2, 4 and 6 of *B. gibsoni* WT and ATV-resistant strain.

Drugs (measure)	WT strain			ATV resistant strain		
	day 2	day 4	day 6	day 2	day 4	day 6
ATV (nM)	173.1±23.7	245.0±76.2	164.8±42.3	1205.2±338.5*	1342.9±443.0*	1021.5±419.7*
DA (nM)	994.4±338.8	228.1±20.7	40.6±12.5	759.6±82.3	208.9±51.0	47.5±8.5
AZM (µM)	14.1±437	11.5±2.8	9.4±3.9	11.8±4.0	8.6±0.8	7.2±0.2
DOXY (µM)	17.3±1.1	14.9±3.3	9.1±5.5	21.0±3.1	13.5±8.9	12.4±9.8
CLDM (µM)	290.3±139.5	462.9±280.6	203.0±45.1	295.0±108.4	326.3±66.3	204.0±52.3
PG (µM)	41.3±8.9	34.1±16.4	42.3±18.6	65.5±45.6	37.0±6.3	44.9±8.5

* A significantly different result in comparison with the determination for WT (P<0.05)

Figure 2

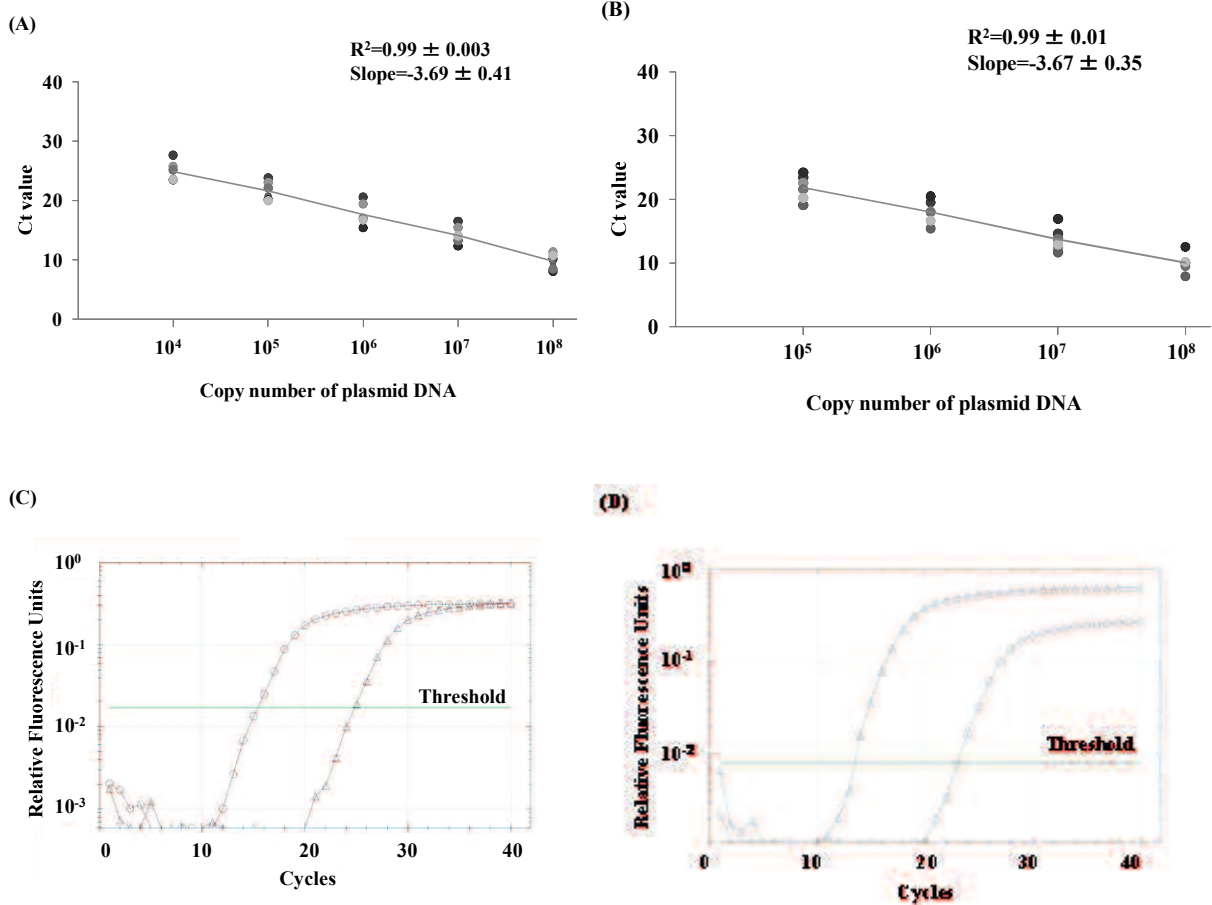


Figure 2. (A) Standard curves from 6 different experiments for 363G allele specific real-time PCR. Standard curves showed 10^4 -fold dynamic range (10^8 – 10^4 copy number of plasmid DNA). (B) Standard curves from 6 different experiments for 363T allele specific real-time PCR. Standard curves demonstrated a 10^3 -fold dynamic range (10^8 – 10^5 copy number of plasmid DNA). (C) The amplification curve in the 363G allele-specific real-time PCR. For the WT *B. gibsoni* (○), 363G allele was amplified at the Ct value of 14.2 ± 0.8 , and the copy number was calculated as $7.4 \times 10^7 \pm 5.2 \times 10^7$ per microliter of suspension. For the ATV-resistant *B. gibsoni* (Δ), 363G allele was amplified at the Ct value of 25.1 ± 3.0 , and the copy number was $8.6 \times 10^4 \pm 1.6 \times 10^4$ per microliter of suspension. (D) The amplification in the 363T allele-specific real-time PCR. For the WT *B. gibsoni* (○), because the 363T allele was amplified at the Ct value >22 , the copy number was not determined. For the ATV-resistant *B. gibsoni* (Δ), 363T allele was amplified at the Ct value of 13.7 ± 0.9 , and the copy number was $7.3 \times 10^6 \pm 0.5 \times 10^6$ per microliter of suspension.

Discussion

In this study, an ATV-resistant *B. gibsoni* was developed successfully by exposing a WT uncloned *B. gibsoni* to 800 nM ATV for 6 days. The *B. gibsoni* originated from naturally infected dogs in Japan without therapeutic experience with ATV. A SNP was detected in *cytb* of the ATV-resistant *B. gibsoni*, resulting in the amino acid substitution M121I. Allele-specific real-time PCR revealed that the M121I variant population comprised over 99% of the population. On the other hand, development of ATV resistance or gene polymorphisms on *cytb* was not confirmed by exposing 4 WT sibling clones to ATV. These results suggested that the M121I variant had selectively multiplied after 6 days of ATV exposure.

In a previous study, plural polymorphisms resulting in the substitution of the amino acid residue at *cytb* in parasites were isolated from dogs experiencing recurrent infections following ATV treatment. Of these polymorphisms, only 1 substitution on nt363, resulting in M121I, is localized in the region believed to be the ATV-binding site in *P. falciparum* (Korsinczky et al., 2000) and emerged most frequently in vivo studies (Matsuu et al., 2006; Sakuma et al., 2009). Therefore, M121I appeared to be responsible for the development of ATV resistance. In this study, the M121I variant grew and dominated over the ATV-resistant cultures in vitro. This finding strongly suggests that

the M121I substitution is associated with ATV resistance in *B. gibsoni*. However, the relation between other polymorphisms detected from previous in vivo studies (Matsuu et al., 2006; Sakuma et al., 2009) and ATV resistance are yet to be revealed. In addition, ATV-resistant *P. falciparum* mutants containing single or double amino acid mutations have been derived from ATV-sensitive cloned lines by culturing parasites in medium containing ATV at gradually increasing concentrations (Korsinczky et al., 2000). More detailed studies would require for the clarification of which factor is involved.

Allele-specific real-time PCR was employed for the quantification of M121I variant population among ATV-resistant *B. gibsoni* in this study. This method is based on the positioning of the 3' base of a PCR primer that match only one SNP allele and accurately extend only the correctly matched primer under stringent conditions (Bottema and Sommer, 1993; Wilhelm et al., 2002). The study could specifically detect and quantify 10^5 – 10^8 copy numbers of polymorphic alleles. Due to non-specific amplification, the quantitative sensitivity was not very high. It was actually unable to detect the 363T allele from the WT *B. gibsoni*, which suspected forms a small minority of M121I variants. However, this method is easy, quick, and cost-effective. This might be useful to assess the monitoring of M121I variant for in vitro or in vivo studies.

In this study, the sensitivity of WT *B. gibsoni* to that of 5 other candidate

anti-babesial drugs (DA, AZM, DOXY, CLDM, and PG) was compared with that of ATV-resistant *B. gibsoni*. This study showed that the declining ATV sensitivity to M121I did not affect the mechanism of action of these drugs. The individual activities of these drugs against *B. gibsoni*, except PG, have been reported in a previous in vitro study (Matsuu et al., 2008). These drugs are more commonly used among clinical veterinarians and have been used solely or in combination with other drugs for clinical cases (Wulansari et al, 2003; Suzuki et al., 2007; Lin et al., 2010). The findings of this study may be important for clinical veterinarians with respect to the selection of alternative therapy against recurrent infections in dogs after ATV treatment. In this study, the activity of PG against *B. gibsoni* was demonstrated for the first time. PG is a highly protein-bound molecule that has been used in combination with ATV for treatment of *Plasmodium* spp. infections because of their synergistic interaction (Baggish and Hill, 2002; Fivelman et al., 2004). A medication that combines ATV and PG, i.e., Malarone, is commercially available for treatment of human malaria. The interaction between ATV and the other drugs, including PG, against *B. gibsoni* is unknown. Although further studies are required to analyze the correlation between ATV and these drugs, the agents used in this study could be candidates for ATV basic combination therapy.

Chapter 2

The in vitro interactions and in vivo efficacy of atvaquone and proguanil against

***Babesia gibsoni* infection in dogs**

Introduction

In chapter 1, ATV-resistant *B. gibsoni* in *cytb* with M121I displayed the same sensitivity as the WT *B. gibsoni* against 5 drugs, including DA, AZM, DOXY, CLDM, and PG. Although further studies are required to analyze the correlation between ATV and these drugs, these 5 drugs could be candidate for ATV basic combination therapy.

In the present study, the author analyzed the interaction of ATV and PG against two in vitro culture strains of *B. gibsoni* (WT and ATV-resistant strain with M121I in *cytb*). Subsequently, the author evaluated the efficacy of ATV and PG against *B. gibsoni* in acute and chronic stage of experimentally infected dogs. The author assessed whether Malarone[®] can inhibit the recurrence and emergence of resistance against ATV or not.

Materials and Methods

Interaction of ATV and PG against B. gibsoni in vitro: The interaction of ATV and PG against the uncloned WT *B. gibsoni* and ATV-resistant *B. gibsoni* was examined.

In vitro drug interactions were assessed using a modified fixed ratio isobologram method (Fivelman et al., 2004). ATV and PG were six-point twofold diluted, and the IC₅₀ that evaluated in chapter 1 fell near the midpoint of a dilution series. That is, ATV was diluted as 1.6, 0.8, 0.4, 0.2, 0.1, and 0 μM against WT *B. gibsoni*, 3.2, 1.6, 0.8, 0.4, 0.2, and 0 μM, against ATV-resistant *B. gibsoni*, and 100, 50, 25, 12.5, 6.25, and 0 μM of PG against both types of *B. gibsoni*. Combination solution for WT *B. gibsoni* were prepared in fixed ratio solutions at ratio of 0.6:0, 0.8:6.25, 0.4:12.5, 0.2:25, 0.1:50 and 0: 100 of ATV and PG (μM). Combination solution for ATV-resistant *B. gibsoni* of that were prepared at ratio of 3.2:0, 1.6:6.25, 0.8:12.5, 0.4:25, 0.2:50 and 0: 100 of ATV and PG (μM). Each combination solution was serially diluted five times in twofold dilutions (Fivelman et al., 2004). Combination solution for 16:6.4μM of ATV and PG was also prepared and this was serially diluted in fivefold dilution to assess interactions of ATV and PG at the ratio of 2.5:1 as same as Malarone[®]. The final concentration of DMSO was adjusted to 0.05%. Packed infected RBCs (20

μL) were dispensed into 180 μL of culture medium with each concentration of drug to obtain a 10% PCV in each well of a 96-well plate. The parasites were incubated at 5% CO₂ and 37°C for 6 days. Each day, half of the medium was replaced with fresh medium containing ATV and PG. The rate of growth inhibition of the parasitized erythrocytes from each of the wells was calculated 6 days from the blood smear (Matsuu et al., 2008).

Assessment of drug interaction is based on calculation of the sum of the fractional inhibitory concentrations (Σ FICs) at the given IC₅₀ by the formula

(IC₅₀ of ATV in the mixture/IC₅₀ of ATV alone) + (IC₅₀ of PG in the mixture/IC₅₀ of PG alone).

Σ FICs <1 denote synergism, Σ FICs \geq 1 and <2 denote additive interaction, Σ FICs \geq 2 and <4 denote slight antagonism, and Σ FICs \geq 4 denote marked antagonism (Gupta et al., 2002).

Efficacy of Malarone[®] against experimentally infected dogs: Malarone[®]

(GlaxoSmithKline, Buckinghamshire, UK), which is a medication containing 250 mg

ATV and 100 mg PG, was used as the test compound. Before starting the infectious experiments, Malarone[®] were administered to 4 healthy dogs (2 Beagles and 2 American Cocker Spaniels) for 10 days (17-25 mg/kg ATV and 7-10 mg/kg PG) to assess the incidence of side effects. General conditions were checked every day and blood samples were collected pre-treatment, during treatment (on day 8), and post treatment (on day 14) for complete blood count (CBC) (Cell Alpha, Nihon Kohden, Tokyo, Japan) and biochemical analyses; alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (Cre), total protein (TP), albumin/globulin ratio (A/G), and total bilirubin (T-bil) (DRI-CHEM 3500i, Fuji Film, Tokyo, Japan).

The experimental infections were undertaken at Kitasato University and the Animal Care and Ethics Committee of the university approved the use of animals. Five beagles (dogs 1-5; two females and three males, 2-7 years old) were obtained from Oriental Yeast Company (Tokyo, Japan) and maintained in an air controlled and isolated room to prevent another infection for two weeks before the study starting. All dogs were maintained in separate cages and fed a commercial dog food according to the manufacturer instructions; tap water was available *ad libitum*. These dogs had been vaccinated against distemper. The dogs were confirmed to be healthy by physical

examination and blood examination. By blood Gimsa stained smears observations, any intraerythrocytic pathogens were not detected. By a polymerase chain reaction (PCR) assay, *B. gibsoni p18* gene was not detected (Matsuu et al., 2004).

Dog 1 was inoculated intravenously (day 1) with 1×10^7 *B. gibsoni*-parasitized erythrocytes isolated from a naturally infected Tosa dog in Aomori Prefecture. Dog 2 was inoculated in the same manner with 2×10^8 *B. gibsoni*-parasitized erythrocytes isolated from dog 1. Dogs 3-5 were inoculated with 5×10^7 *B. gibsoni*-parasitized erythrocytes isolated from dog 2.

Half a tablet of Malarone[®] was orally administered twice a day to each dog for 10 days (17–25 mg/kg ATV and 7–10 mg/kg PG). Blood samples were collected with ethylenediaminetetraacetic acid (EDTA) as the anticoagulant at 1 to 7-day intervals until 80 days after the administration was completed. Blood smears were made to examine parasitemia microscopically after Giemsa staining. PCV was measured using an automated blood cell counter. Serum samples were isolated for biochemical analyses: ALT, ALP, BUN, Cre, TP, A/G, and T-bil. Genomic DNA was extracted from samples of whole blood after Malarone[®] therapy at 7 days intervals for a PCR assay to detect the parasite *p18* gene (Matsuu et al., 2004) and an allele-specific SYBR green real-time PCR assay to quantify the M121I variant population as mentioned in chapter 1.

Analysis of results: Student's *t*-test was used to evaluate the difference in blood chemicals and PCVs between pre and post treatment. Values of $P < 0.05$ were considered statistically significant.

Results

Interaction of ATV and PG against B. gibsoni: The Σ FICs against WT *B. gibsoni* were 0.93, 0.82, 0.83, and 0.96 at a ratio of 4:1, 3:2, 2:3, and 1:4 of ATV and PG, respectively. Σ FICs against ATV-resistant *B. gibsoni* were 0.88, 0.60, 0.77, and 0.88 at each ratio. Based on the denotation above, the interaction between ATV and PG against WT and ATV-resistant *B. gibsoni* was defined as synergism. The isobolograms for the interactions between ATV and PG are shown in Figure 3. The FICs against WT and ATV-resistant *B. gibsoni* at ratio of 2.5:1 of ATV and PG were 0.76 and 0.48, respectively, and the interaction between ATV and PG at the ratio was also defined as synergism.

Efficacy of Malarone[®] against experimentally infected dogs: Malarone[®] treated 4 healthy dogs did not show any clinical signs during and after the treatment period. And there were not any significant differences between pre, during, and post administration of Malarone[®] in CBC and blood chemical analysis.

Dogs 1 and 2 showed low parasitemia and no symptoms even after day 40: they were used as models of chronic infection. Dogs 3-5 showed high parasitemia and typical clinical signs such as anemia, fever, and choloplasia by day 21; they were used

as models of acute infection. Malarone[®] treatments were started on day 84 and day 46 for dogs 1 and 2, respectively. For dogs 3-5, treatments were started on day 21 when PCV decreased to <20%. After Malarone[®] treatment, PCV began to increase rapidly and the parasitemia began to decrease in all the dogs (Figure 4). The parasites disappeared in the blood smears between day 85-123, 47-86, 28-35, 28-44, and 29-44 in dog 1, 2, 3, 4, and 5 respectively. In dog 3, the parasite re-increased to 2.1% on day 55, and PCV decreased to <20%, then Malarone[®] treatment was restarted. After second treatment, PCV increased and parasitemia decreased, and the parasite was disappeared again in the blood smears between day 86-97 (Figure 4).

Changes in biochemical parameters after treatment initiation are shown in Figure 5. T-bil levels in dogs 3 and 5 were 5.1 and 5.2 mg/dL (reference range: 0.1–0.5 mg/dL), respectively, before treatment and decreased after treatment. In dogs 1 and 5, ALT activity increased transiently up to 178 IU/L and 218 IU/L, respectively (reference range: 21–102 IU/L) after the start of treatment. In dog 5, ALP activity increased markedly up to 2807 IU/L (reference range: 20–156 IU/L) and subsequently decreased slowly by day 56 after treatment initiation. In dog 4, Cre levels increased up to 4.5 mg/dL (reference range: 0.5–1.5 mg/dL) at day 7 after treatment initiation. TP levels increased and A/G decreased slowly after treatment initiation in all the dogs. When the

biochemical levels were compared to the pre date, BUN was slightly increased on 37 and 51 day after inoculation (21.7 ± 3.9 and 23.3 ± 6.0 mg/dl) compared to pre treatment (14.6 ± 2.6 mg/dl) ($P < 0.05$). There was not any statistical significance in other serum biochemical pre and post treatment.

During the treatment period, dogs 1 and 5 exhibited self-limiting vomiting on day 2 but did not require treatment, and Malarone[®] treatment continued. PCR assays revealed the *B. gibsoni p18* to be detected intermittently even after Malarone[®] treatments (data not shown). Allele-specific SYBR green real-time PCR assays showed that the M121I variant population comprised a maximum 80% of the population soon after Malarone[®] treatments, but the population fluctuated during this experiment. In dogs 2 and 4, the percentage of the M121I allele decreased to below half, whereas in dog 5, only a temporary decrease was observed (Figure 4).

Figure 3

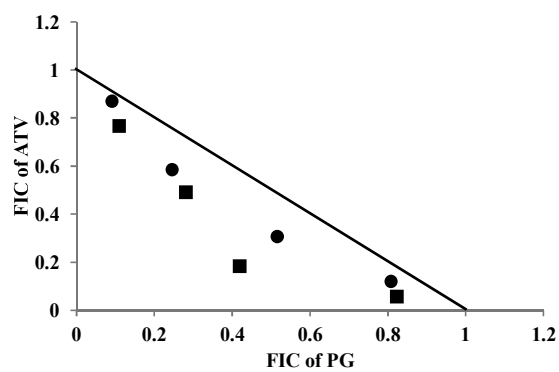


Figure 3. Isobolograms showing interactions between ATV and PG against WT *B. gibsoni* (●) and ATV-resistant *B. gibsoni* (■). The numbers on the axes represent normalized FICs.

Figure 4

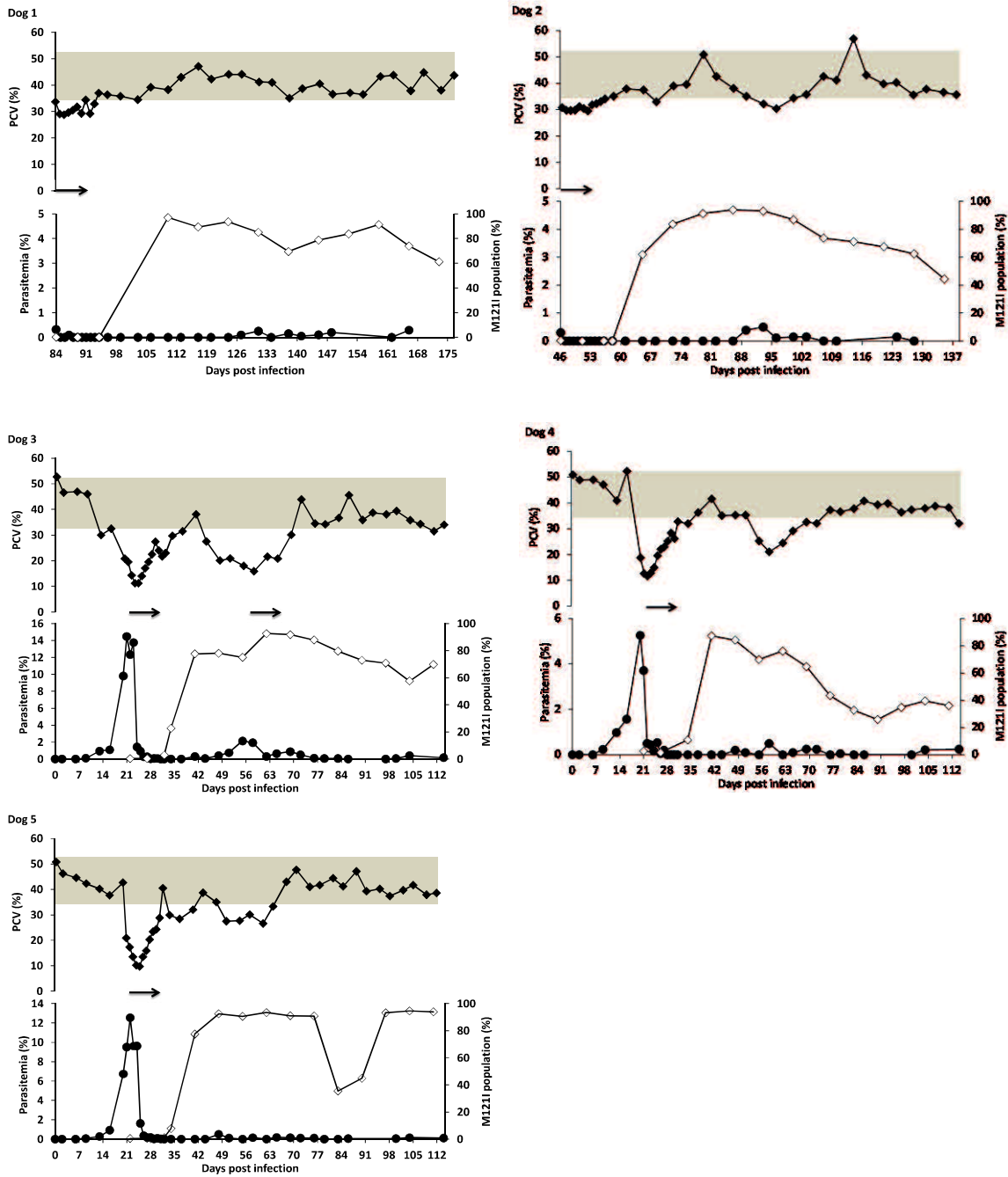


Figure 4. Changes in PCV (◆), parasitemia (●), and M121I population (◇) in Malarone[®]-treated dogs (1-5) after infection with *B. gibsoni*. Dogs were inoculated intravenously with 5×10^7 (dog 1), 2×10^8 (dog 2), or 5×10^8 (dogs 3-5) parasitized erythrocytes on day 0. The period of Malarone[®] treatment is shown as arrows. The gray zone means reference range of PCV.

Figure 5

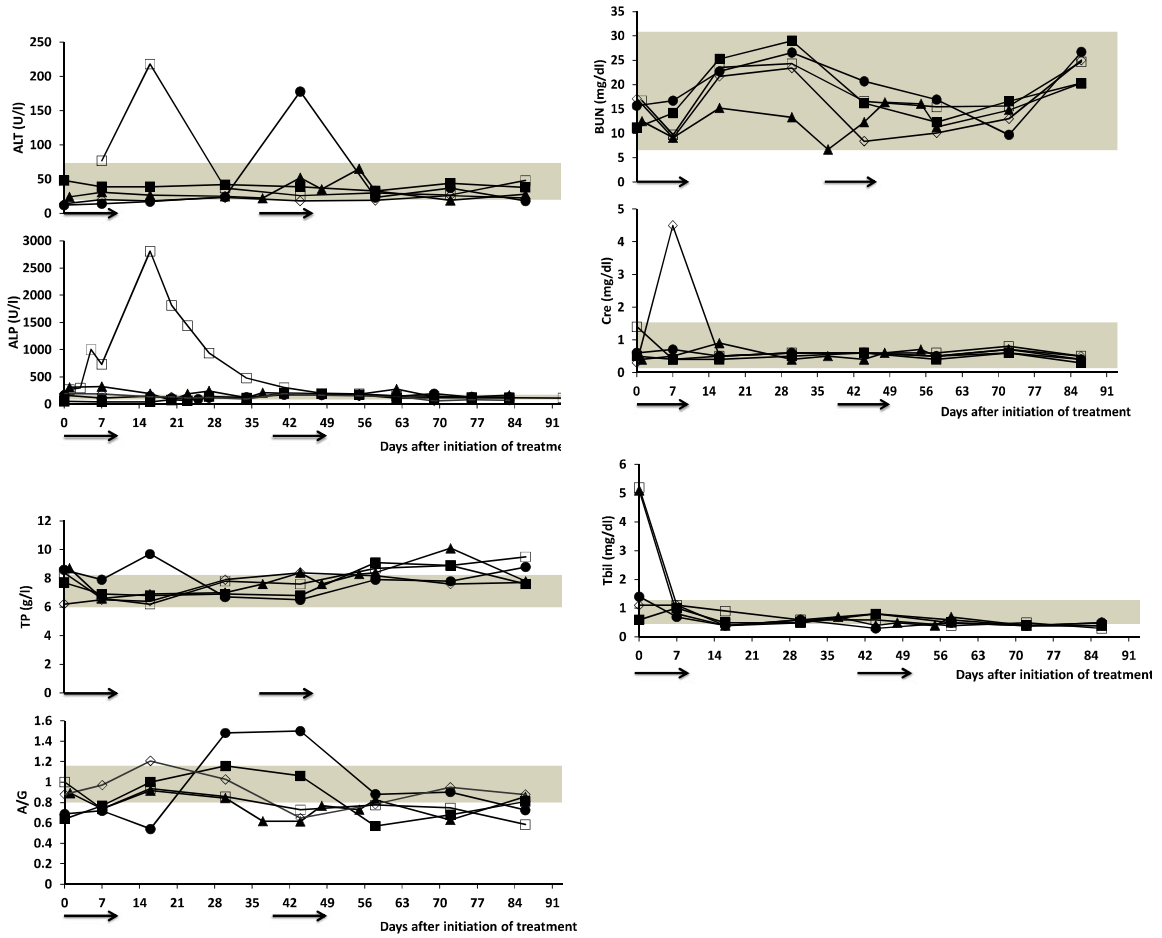


Figure 5. Changes in biochemical parameters after initiation of Malarone[®] treatment in dogs 1 (●), 2 (■), 3 (▲), 4 (◇), and 5 (□). The day when treatment was started is shown as day 0. The period of the treatment is shown as arrows. The second treatment was only for dog 3. The gray zone means reference range of each parameter.

Discussion

In this study, the author evaluated the efficacy of a combination of ATV and PG against *B. gibsoni* in vitro and in vivo. ATV has broad spectrum activity against *Plasmodium* spp., *Toxoplasma gondii* (Baggish and Hill, 2002), and *Babesia* spp. (Weiss et al., 2002). The mechanism of action involves selective inhibition of electron transport in parasite mitochondria (Hudson et al., 1991; Baggish and Hill, 2002). PG is a highly protein-bound molecule. PG by itself has weak anti-Plasmodium activity (Pudney et al., 1999), but when it was used with ATV against *P. falciparum*, PG enhanced the mitochondrial membrane collapse by ATV (Srivestave et al., 1999).

In the present study, the combination of ATV and PG showed comparable synergism against WT and ATV-resistant *B. gibsoni* in vitro. In chapter 1, the ATV sensitivity of ATV-resistant *B. gibsoni* was about fivefold less than that of WT *B. gibsoni*. Canfield et al. (1995) reported that the combination of ATV and PG had a similar synergistic effect against *P. falciparum* ATV-sensitive W2 and D6 strains and ATV-resistant C2B strains. The C2B strain had 95 times ATV resistance compared with that of the ATV-sensitive strain (Canfield et al., 1995). On the other hand, a study reported that the synergy against the ATV-resistant NGATV01 strain the sensitivity was more than 2000-fold less than that of the ATV-sensitive strain, was significantly lower

than that against the ATV-sensitive T996 and K1 strains (Fivelman et al., 2004). With respect to *P. falciparum*, the sensitivity of ATV may affect the synergism of ATV and PG. With regard to *B. gibsoni*, the synergism may be similar to the result obtained in the study by Canfield et al. However, it is not known if the results against ATV-resistant *B. gibsoni* support the same mechanism as that observed in *Plasmodium* spp. The detailed mechanisms of action of ATV and PG against *B. gibsoni* multiplication need to be elucidated in further studies.

The synergistic interaction of ATV and PG against *B. gibsoni* at the ratio of 2.5:1 as well as other ratio was shown in vitro, and thus, the author examined the clinical efficacy of this combination against *B. gibsoni* in experimentally infected dogs. After administration of Malarone[®] against *B. gibsoni*-infected dogs, anemia recovered immediately and parasitemia began to decrease in acute and chronic models. However, Malarone[®] could show neither eliminate of the parasite nor inhibit its reappearance in the blood. One dog developed severe anemia as PCV decreased <20% again and the second treatment with Malarone[®] was started. This second treatment showed immediate effects, similar to that seen the first time, although the M121I parasite presented at >80%. This result may reflect the synergistic interaction between ATV and PG, but this short administration has risk of the M121I parasites.

The M121I variant population in host blood was measured during experiments. After treatment with Malarone[®], the M121I population increased in all dogs immediately and showed various changes in each dog. Interestingly, the M121I population decreased to less than half in some dogs. Chapter 1 showed that the emergence of ATV resistance was suspected to be because of the selective multiplication of *B. gibsoni* M121I variants. There may be an optimum consistency at which *B. gibsoni* M121I variants can increase significantly. However, the reasons why the M121I population changed are unknown. Although the multiplication speed of WT and ATV-resistant *B. gibsoni* seems to be similar in in vitro culture, this might alter if the parasites invade host dogs. It cannot be denied the possibility that the parasites without M121I after treatment with Malarone[®] may acquire tolerance by other mechanisms. To elucidate the possibility that the decrease of M121I is depend on dosage of ATV or the presence of PG, further study is needed. And although a longer observation period will be needed to elucidate whether the M121I allele disappears after long period or not, the ATV resistance due to M121I may decrease after a certain period.

In the present study, some side effects were confirmed during and after Malarone[®] treatment in *B. gibsoni* infected dogs. The treatment of human malaria with higher doses of ATV and PG showed gastrointestinal side-effects and elevations of liver

transaminase and bilirubin levels in approximately 15% patients (Baggish et al., 2002). It is also reported that the addition of PG to ATV did not substantially alter the side effect profile by ATV alone (Paul et al., 2003). For dogs, there are only few reports describing the toxicity of ATV and PG. It has been reported that a 2.5:1 combination with ATV and PG results in vomiting and diarrhea and that the treatment could not be continued in dogs although the dose for each drug has not been described (Brikenheuer et al., 2004). On the other hand, acute toxicological studies in rats and dogs that received high doses of the combination of ATV and PG did not show significant adverse effects on the liver (Pudney et al., 1999). In the present study, two dogs experienced self-limiting vomiting during Malarone[®] treatment. And slightly high levels of ALT in two dogs (Dog 1 and 5) and significantly high levels of ALP in one dog (dog 5) were observed soon after the start of administration (Figure 5). The other biochemical parameters including cholesterol, triglyceride, lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT) in dog 5 were checked. On day 5 and 16 after initiation of treatment, when the ALP reached its peak, the LDH activities were also elevated to 815 and 705 IU/L respectively. On the other hand, GGT activities was elevated to 207 IU/L on the day 0, just before starting treatment, and decreased to < 1 IU/L on day 5 after initiation of treatment. Cholesterol and triglyceride levels were

normal. These abnormalities in liver enzymes were not shown in any healthy dogs in our preliminary experiment. In *B. gibsoni* infected dogs, it was reported that acute hepatocyte injury may occur (Wozniak EJ et al., 1997). Considering to the elevation of GGT before treatment, there was a possibility that dog 5 had already been damaged in liver by *Babesia* infection and it might induce the elevation of ALT, ALP, and LDH after Malarone[®] treatment.

In two dogs, hyperbilirubinemia was confirmed before treatment (day 0). Because the hyperbilirubinemia disappeared after treatment, it may have been due to hemolysis by the *Babesia* parasites rather than a side effect of Malarone[®]. Also, all five dogs showed elevations of TP and decrease in A/G ratio after treatment initiation. It is reported that the total protein concentrations with *B. gibsoni* and *B. canis* were unremarkable and the gamma globulins were broad polyclonal increases (Farwell et al., 1982). Changes in TP and A/G in the present study may have been due to chronic *B. gibsoni* infection. However, the cause of a temporally hypercreatinemia in dog 4 without azotemia is not known. In this study, the administration of Malarone[®] for dogs at the described dose seemed to have potential for hepatotoxicity or gastrointestinal side effect especially in *B. gibsoni* infected dogs. At this time, because the number of dogs used in the present study was small, the accurate incidence rate of side effect were

unknown and the safety of ATV and PG for dogs was not established. For the safety clinical application, further detailed study using more number of dogs is needed.

ATV can inhibit *B. gibsoni* immediately, but the problem of this drug is that it cannot eliminate the parasite from host completely and it occur the emergence of ATV resistant parasite. In the previous study, the therapeutic efficacy of higher dose of ATV alone (30 mg/kg BID, 7days) against *B. gibsoni* in three experimentally infected dogs was evaluated. Anemia and thrombocytopenia were significantly improved. The parasite was disappeared from blood smears within 2 days of ATV treatment started, and reappeared in blood smears 33 ± 2 days after the last treatment. The reappeared parasites developed ATV resistant (Matsuu et al., 2004). Recently, ATV has been used combined to AZM (Birkenheuer et al., 2004, Jefflies et al., 2007, Sakuma et al., 2009). However, this combination therapy could not eliminate the parasite completely, and ATV resistant parasite with M12II was emerged after treatment. Sakuma et al. tried ATV (13.3mg/kg TID, 10 days) and AZM (10mg/kg SID, more than 10 days) treatment to eight dogs that were naturally infected with *B. gibsoni*. In this study, clinical signs and hematological signs improved soon. But five dogs relapsed with M12II after cessation of the first 10-day ATV/AZM treatment. In this study, Malarone[®] treatment also could not eliminate the parasite from host dog and inhibit M12II parasite, both in

acute and even in chronic stage of infection. The treatment efficacy of Malarone[®] in acute stage was quickly, but comparing the present results to the previous studies, the duration which the parasites were inhibited completely were even shorter than the higher dose of ATV monotherapy. The low dose of ATV in this study might cause early relapse of M121I population.

Malarone[®] is a tablet containing ATV and PG, and one of the benefits is its ease to administration. And in vitro evidence supported the synergistic of ATV and PG to both WT and ATV-resistant *B. gibsoni*, however, this combination therapy can not eliminate parasite and drug resistance might develop. The careful monitoring about side effects is needed, and administration of Malarone[®] should be avoided when the infected dogs are accompanied by abnormal liver enzymes.

Chapter 3

The efficacy of Malarone[®] against 8 naturally *Babesia gibsoni* infected dogs

Introduction

In chapter 2, the interaction of the combination of ATV and PG showed synergism against both of ATV-sensitive and ATV-resistant *B. gibsoni* in vitro. In addition, Malarone[®], containing ATV and PG, showed clinical efficacy for the experimentally *B. gibsoni* infected dogs. However, it was also suggested that this combination therapy can not eliminate parasite, and drug resistance might develop. The further combination of the other anti-babesial drugs may need to inhibit the reappearance of parasite, and further detailed study using more number of dogs was needed.

In the present study, the author evaluated the efficacy of Malarone[®] against *B. gibsoni* in acute stage of naturally infected dogs. Additionally, the author assessed whether the addition of DOXY to Malarone[®] treatment can inhibit the recurrence and emergence of resistance against ATV or not.

Materials and methods

Eight dogs with acute onset of Babesiosis, exhibiting anemia and with microscopic evidence of parasitemia were enrolled in this study. The 8 dogs (dog 6-13) were diagnosed as canine Babesiosis at Shiranaga Animal Hospital during 2012-2013 in Yamaguchi Prefecture, Japan. The breed of dogs, age, sex, symptoms and blood parameters were shown in Table 3.

Small piroplasma in red blood cells were confirmed by thin blood smear observation in all the dogs, and they were diagnosed as canine Babesiosis. They had not received any antiprotozoal drugs before their admission to the Shiranaga Animal Hospital. After diagnosis, the 4 dogs (dog 6-9) received Malarone[®] therapy (ATV 17-25 mg/kg, PG 7-10 mg/kg, twice per day for 10 days) and another 4 dogs (dog 10-13) received combination therapy with Malarone[®] for 10 days and DOXY for 30 days (5 mg/kg, twice per day).

After the initiation of therapy, blood samples were collected with EDTA as the anticoagulant at 1 to 30-day intervals until 90 days after the administration was completed. Blood smears were made to examine the parasitemia microscopically. PCV was measured using an automated blood cell counter. Serum samples were isolated for biochemical analysis: ALT, ALP, BUN, Cre, TP, A/G, and T-bil. Genomic DNA was

extracted from samples for a PCR assay to detect the parasite *p18* gene and an allele-specific SYBR green real-time PCR assay to quantify the M121I variant population.

Relapse of Babesiosis was defined in this study as the reappearance of *B. gibsoni* in blood smear and the decrease of PCV less than 25%. At the time of relapse, either Malarone[®] therapy or DOXY combination therapy was reinitiated, at the same dosages and period mentioned above. When vomiting for more than three times or anorexia was found, the treatment was stopped.

Results

In 3 of 4 cases (dog 6, 7, and 8) with Malarone[®] therapy and in all cases with the DOXY combination (dog 10-13), clinical signs and anemia improved soon after initiation of the treatment (Figure 6). Because dog 9 showed anemic development although parasitemia decreased during Malarone[®] therapy, the observation was stopped and this dog was treated with DA (2mg/kg, every other day). After this therapy, PCV increased to 34%. Two dogs (dog 10 and 11) with DOXY combination did not relapse, on the other hand, other 3 dogs with Malarone[®] therapy (dog 6, 7, and 8) and 2 dogs with DOXY combination (dog 12 and 13) relapsed during observation period. These 5 relapsed dogs received second Malarone[®] therapy and they responded well. However, dog 8 relapsed again after the second treatment. This dog received third Malarone[®] administration but had a progression of anemia. Then, the observation was stopped. In all dogs, supportive therapies such as blood transfusion were not required, and obvious adverse effects resulted to these treatment protocols were not detected. At biochemical analysis, dog 9 showed high ALP activity (996 U/l) before the Malarone[®] therapy. There were no significant changes in other serum biochemical. PCR assays showed that *B. gibsoni p18* gene was not detected a few times but was detected on last day in all dogs. The M121I population in host blood was measured at pre and post of Malarone[®]

administration. It showed under 0.1% in all dogs at the start of Malarone[®] administration, but relapsed dogs showed 15-96% of M121I population at the initiation of the second treatment (Table 4).

Table 3. The breed of dogs, age, sex, clinical signs, and blood parameters

No	Breed	age	sex	PCV (%)	Parasitemia (%)	Clinical signs	biochemical analysis							treatment protocol
							ALT	ALP	BUN	Cre	TP	AG	T-Bil	
6	Welsh Corgi Pembroke	6y	male	23	2	anorexia, depression	40	134	12.4	0.3	7.7	0.5	0.4	
7	Pug	2y	female	19	4.5	anorexia	16	317	15.4	0.6	5.2	0.6	0.4	Malarone® therapy (ATV 17-25 mg/kg, PG 7-10 mg/kg), twice per day, for 10 days
8	Miniature Dachshund	unknown	male	35	1.5	anorexia	33	88	22.5	0.7	6	0.8	0.3	
9	Shiba	14y	male	35	3	anorexia, depression	36	996	24.3	0.9	5.8	0.8	0.3	
10	Mixed breed	8y	male	20	5.5	anorexia, hemoglobinuria	19	176	14.7	0.6	5.9	0.5	0.2	
11	American Cocker Spaniel	3y	female	17	3	anorexia, depression	19	152	17.1	0.6	4.9	0.6	0.3	Combination therapy (Malarone® for 10 days and DOXY for 30 days (5 mg/kg, twice pre day))
12	West Highland White Terrier	6y	male	25	2	anorexia, depression	20	209	14	0.4	6.8	0.7	0.2	
13	Chihuahua	7y	male	21	3	pale mucus membrane	30	210	10.5	0.4	8.2	0.5	0.4	

Figure 6

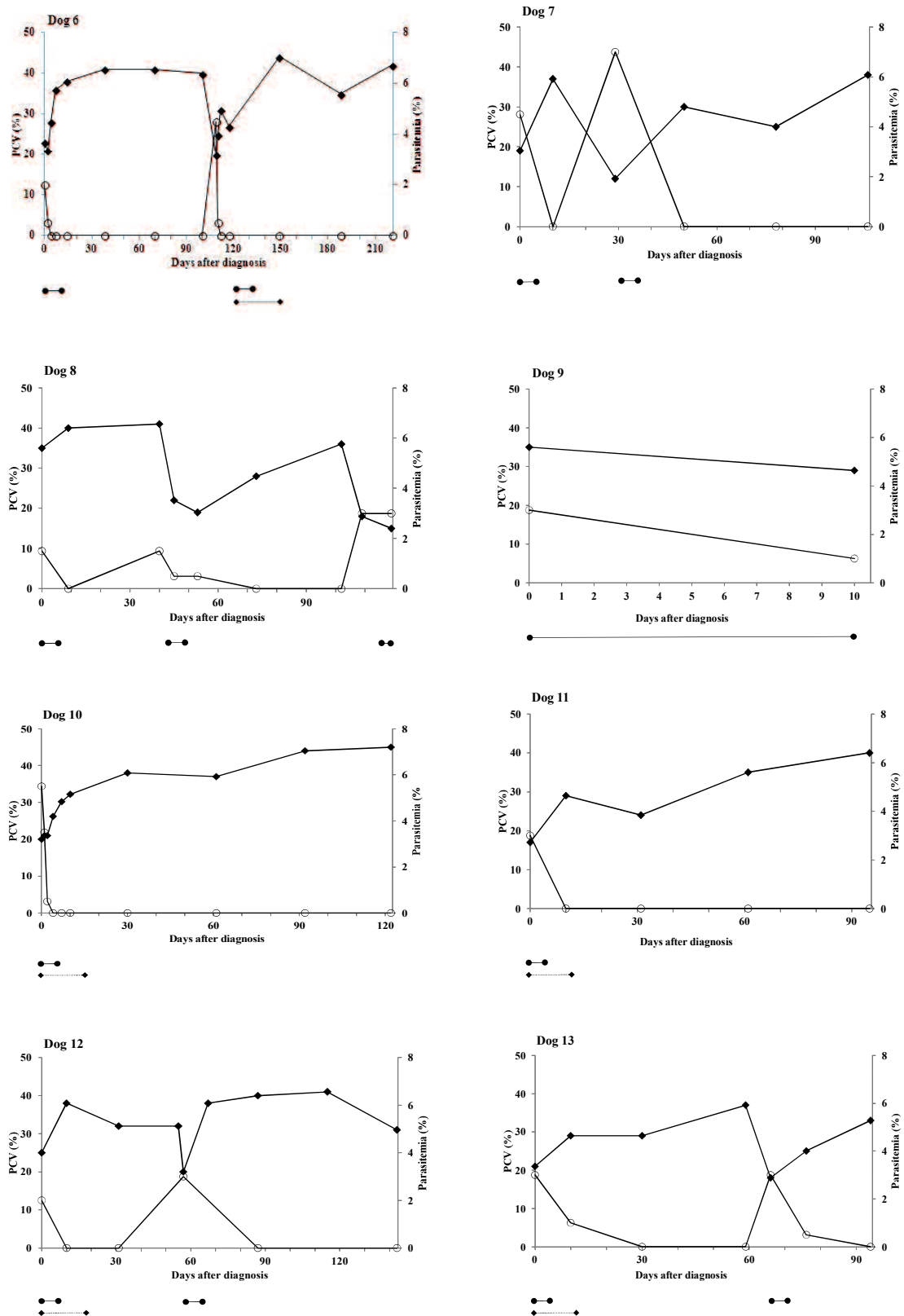


Figure 6. Changes in PCV (◆) and parasitemia (○) in the 8 dogs. The period Malarone[®] (●) and/or DOXY (◆) treatment was shown as arrows.

Table 4 M12II populations pre and after the treatment

Dogs	First treatment		Second treatment		Third treatment	
	Pre	Post	Pre	Post	Pre	Post
1	0.02	ND	ND	ND	-	-
2	0.06	ND	14.5	13.9	-	-
3	0.06	ND	58.7	ND	91.3	84.4
4	0.33	0.34	-	-	-	-
5	0.61	ND	-	-	-	-
6	0.09	ND	-	-	-	-
7	0.52	ND	80.1	80.1	-	-
8	0.70	ND	96.1	95.9	-	-

ND: Not detected

Discussion

Clinical signs in 7 of 8 dogs were well controlled by the Malarone[®] therapy or DOXY combination therapy without supportive therapies. In these 7 dogs, parasitemia decreased, and PCV increased on day 10 when the administration of Malarone[®] was completed. Therefore, the administration of Malarone[®] seems to be clinically useful strategy for treating canine Babesiosis. Three of 4 dogs that were treated with Malarone[®] relapsed. On the other hand, 2 of 4 dogs with DOXY combination therapy did not relapse. DOXY has been used for canine Babesiosis as combination therapies with CLDM-metronidazole or enrofloxacin-metronidazole. In chapter 1, DOXY inhibited growth in both WT and ATV-resistant *B. gibsoni* and the IC₅₀ was lower than that of CLDM. It is suggested that the new combination therapy with Malarone[®] and DOXY may prevent some of the recurrence of the canine *B. gibsoni* infections.

Even though an allele-specific SYBR green real-time PCR assay showed various rate of M121I population (15-96%) at the initiation of the second treatment in each relapsed dog, these dogs responded well to the second administration of Malarone[®]. In chapter 1, the M121I variant population comprised over 99% in ATV-resistant *B. gibsoni* that showed low sensitivity to ATV. This indicated that the ATV dosage might be needed to increase when the second treatment is needed to relapsed dog, but this is

not evaluated until today. On the other hand, the combination of ATV and PG showed synergism against ATV-resistant *B. gibsoni* in vitro, and the second Malarone[®] treatment of same dosage was effective to relapsed dog with M12II in chapter 2. It is suggested that the combination of ATV and PG might be effective regardless of M12II population. However, dog 8 developed anemia during the third Malarone[®] therapy in this study. There is no report about repeated dosing of Malarone[®] against human or dogs. The study using large number of dogs is required to resolve this concern.

In this study, dog 9 developed anemia during the first Malarone[®] therapy. It is reported that a disadvantage of ATV is its bioavailability. In persons with decreased intestinal absorption the effectiveness of ATV may be decreased, particularly during management of AIDS patients who have PCP or Toxoplasmosis (Baggish et al., 2002). Although dog 9 in this study did not show digestive symptom, it is suggested that this dog decreased absorption and did not response well. And also, because the mechanism of ATV against *B. gibsoni* is not elucidated completely, other factors expect M12II might be instrumental in the effectiveness. Additionally, dog 9 had high ALP activity before the therapy. In the previous study, it was reported that acute hepatocyte injury may occur in *B. gibsoni* infected dogs (Wonzniak et al., 1997). In chapter 2, it was also suggested that the elevation of liver enzymes after the Malarone[®] treatment might be

induced in the dogs that have hepatocyte injury by *B. gibsoni* infection. The careful monitoring is required when Malarone[®] is administrated to patients especially with the elevation of liver enzyme levels.

This study suggested that Malarone[®] therapy, the combination of ATV and PG, had clinical efficacy in naturally infected dogs with *B. gibsoni*. Furthermore, at least twice administration for the relapsed patient Malarone[®] could improve their anemia as first treatment. Combination of Malarone[®] and DOXY may prevent the recurrence of canine Babesiosis.

General conclusion

B. gibsoni is a tick-borne hemoprotozoan parasite causing hemolytic anemia in dogs. For canine *B. gibsoni* infection, various therapeutic modalities, including DA, antibiotics, and their combination therapies have been described, but are not definitive treatment strategy. In this study, the author focused on ATV, which is effective against acute *B. gibsoni* infections. However, this drug has a critical disadvantage of the emergence of ATV resistant parasite. Until now, there are few reports about the mechanism for ATV resistance or the appropriate treatment strategy to inhibit the recurrence of ATV resistant parasite. The purpose in this study was to establish the treatment strategy with ATV based on in vitro and in vivo studies.

Firstly, the author successfully developed an in vitro ATV-resistant *B. gibsoni* by exposing the parasite culture to 800 nM ATV for 6 days, and performed mitochondrial gene analysis. The sensitivity of this isolate was about fivefold less than that of WT *B. gibsoni*. An SNP was detected in *cyt b* of the ATV-resistant *B. gibsoni*, resulting in the amino acid substitution M121I. Allele-specific real-time PCR revealed that the M121I variant population comprised over 99% of the population. On the other hand, neither ATV resistance nor gene polymorphisms appeared in the same manner when cloned isolate. Thus, the emergence of ATV resistance was likely due to the

selective multiplication of *B. gibsoni* M121I variants. Furthermore, the sensitivity of WT *B. gibsoni* to that of 5 other candidate anti-babesial drugs (i.e, DA, AZM, DOXY, CLDM, and PG) was compared with that of ATV-resistant *B. gibsoni*. The present study showed that the declining ATV sensitivity to M121I did not affect the mechanism of action of these drugs.

A medication that combines ATV and PG, i.e., Malarone[®] is commercially available for treatment of human malaria. Malarone[®] might also be available for treatment of *B. gibsoni* infection, so, in vitro interactions between ATV and PG against *B. gibsoni* and the clinical efficacy of this combination therapy using Malarone[®] were evaluated in chapter 2. In chapter 2, in vitro study demonstrated that the combination of ATV and PG showed synergism against uncloned WT and ATV-resistant *B. gibsoni* using a modified fixed ratio method. In vivo study, administration of Malarone[®] resulted in decrease parasitemia and clinical improvements in two and three dogs in chronic and acute stage of *B. gibsoni* experimentally infection, respectively. The second Malarone[®] treatment to one dog showed anemia was effective as the first treatment. Thus, the combination of ATV and PG might be available for canine *B. gibsoni* infections, but there are some concerns. First, this combination could not fully eliminate *B. gibsoni*, and would increase parasites with M121I. The further combination of the other

anti-babesial drugs may be need to inhibit the reappearance of parasite. Second, the number of dogs in this study was low. For the safety clinical application, further detailed study using more number of dogs is needed.

In chapter 3, the therapeutic efficacy of Malarone[®] alone and Malarone[®] with DOXY against *B. gibsoni* was examined in eight dogs naturally infected by *B. gibsoni*. In 7 of 8 dogs, clinical signs and anemia improved soon after initiation of the first treatment. Two dogs treated with DOXY combination inhibited the relapse, on the other hand, three dogs treated with Malarone[®] alone relapsed, and one of them relapsed three times. The M121I population showed under 0.1% in 8 treated dogs at the start of Malarone[®] administration, but 15-96% in 5 relapsed dogs at the start of the second treatment. Five relapsed dogs responded well to the second treatment, but one dog received the third treatment did not responded. It is suggested that the combination of ATV and PG would be effective regardless of M121I population, and at least twice of the repetitive treatment Malarone[®] might be effective. Further combination of Malarone[®] and DOXY may prevent the recurrence of canine Babesiosis.

This is the first report on the in vitro establishment of an ATV-resistant *B. gibsoni* culture strain with gene polymorphisms. The information obtained from the in vitro study may be important with respect to the selection of treatment strategies for

canine *B. gibsoni* infection. Nevertheless, it has been unknown about the mechanism of the antibabesial drugs including ATV against *B. gibsoni*. WT and ATV-resistant *B. gibsoni* have been maintained via in vitro culture in ATV-free normal culture medium. The ATV-resistant *B. gibsoni* isolated in this study could be useful for further research, not only in developing a treatment strategy against the emergence of ATV resistance, but also for studying the mechanism of action of ATV against *B. gibsoni*.

The author developed the in vivo study based on the in vitro evaluation using those culture strains. The combination of ATV and PG, Malarone[®], would be available for clinical treatment and could decrease *B. gibsoni* with M121I. The additional combination with DOXY may course the more effective result. The combination of ATV and other antibabesial drugs would be more useful than ATV monotherapy.

Abstract

Babesia gibsoni infects the red blood cells of dogs and induces hemolytic anemia, jaundice, hemoglobinuria, and enlarged spleen. Although diminazene aceturate (DA) has been used to treat acute *B. gibsoni* infections, it often fails to eliminate the parasite from the affected dogs and causes severe adverse effects such as pain at the injection site, and nervous symptoms due to cerebral hemorrhage. Some studies have reported the effectiveness of new combination therapies such as clindamycin (CLDM)-metronidazole-doxycycline (DOXY), or DOXY-enrofloxacin-metronidazole, on experimentally infected dogs or clinical patients, but these therapies take a long time to show clinical effectiveness. As mentioned above, early treatment is required for acute *B. gibsoni* infections, but this has a number of drawbacks. Atovaquone (ATV) is effective against acute *B. gibsoni* infections, but ATV monotherapy results in relapse as well as the emergence of drug-resistant variants with some single-nucleotide polymorphisms (SNPs) in the cytochrome *b* (*cytb*). It is necessary to elucidate the relationship between the SNPs and ATV resistance.

It is required to elucidate relationship between ATV resistance and SNPs in *cytb* and to develop strategies for ATV resistance when ATV would be used for *B. gibsoni* infection. Therefore, ATV-resistant *B. gibsoni* was developed in vitro, and the

mitochondrial genes were analyzed. Subsequently the efficacy of combination therapy with ATV was evaluated based on pharmacological evaluations using ATV-sensitive and ATV-resistant *B. gibsoni*.

In chapter 1, an ATV-resistant *B. gibsoni* was developed by in vitro exposure of uncloned wild-type (WT) *B. gibsoni* to 800 nM ATV for 6 days. The ATV sensitivity of this parasite was six-fold less than that of WT *B. gibsoni*. Sequence analysis of mitochondrial genes showed a SNP at *cytb* nt363 (G to T) that resulted in the substitution of methionine with isoleucine (M121I). This ATV-resistant *B. gibsoni* displayed the same sensitivity as the WT *B. gibsoni* against 5 drugs, including DA, azithromycin, DOXY, CLDM, and proguanil (PG). When the parasites re-increased, the PCV decreased to <20% in one dog. This dog received the second Malarone[®] treatment, and it was effective as the first treatment.

Malarone[®], likewise ATV monotherapy, could inhibit *B. gibsoni*, but this drug could not inhibit the reappearance of parasites with the M121I allele. In chapter 3, the efficacy of Malarone[®] against *B. gibsoni* in acute stage of naturally infected dogs was evaluated. Additionally whether the addition of DOXY to Malarone[®] treatment can inhibit the recurrence and emergence of resistance against ATV or not was evaluated. After diagnosis of canine Babesiosis for 8 dogs, the 4 dogs received Malarone[®] therapy

for 10 days and another 4 dogs received combination therapy with Malarone[®] for 10 days and DOXY for 30 days. In 3 of 4 cases with Malarone[®] therapy and in all cases with the DOXY combination, clinical signs and anemia improved soon after initiation of the treatment. One dog showed anemic development, although parasitemia decreased during Malarone[®] therapy. Two of 4 dogs with DOXY combination did not relapse, on the other hand, other 3 dogs with Malarone[®] therapy and 2 dogs with DOXY combination relapsed during observation period. These 5 relapsed dogs received second Malarone[®] therapy, and they responded well. However, one dog relapsed again after the second treatment. This dog received third Malarone[®] administration but had a progression of anemia. Real-time PCR assays showed under 0.1% in all dogs at the start of Malarone[®] administration, but relapsed dogs showed 15-96% of M121I population at the initiation of the second treatment.

In this study, the new therapeutic strategy was tried for experimentally or naturally infected dogs, based on the data from in vitro study. The ATV-resistant *B. gibsoni* isolated in this study could be useful for further research, not only in developing a treatment strategy against the emergence of ATV resistance, but also for studying the mechanism of action of ATV against *B. gibsoni*. The in vivo study suggested that combination with ATV, PG and DOXY might be effective for canine Babesiosis,

although even this combination could not eliminate the parasites from host completely.

The further detailed studies about mechanism of ATV against *B. gibsoni* are required.

Acknowledgement

The author wishes to acknowledge Dr. Yoshiaki Hikasa (Tottori University) and Dr. Aya Matsuu (Kagoshima University) for their appropriate suggestion and critical reading of the manuscript. The author gratefully appreciates Dr. Hiromi Ikadai (Kitasato University) and Dr. Kazuki Harada for their technical advises and suggestions on the present study, and critical reading of the manuscript. The author thanks Dr. Yasuyuki Endo (Kagoshima University), Dr. Nobuyuki Shiranaga (Shiranaga Animal Hospital) for their contribution to this study.

References

- Baggish, A.L., Hill, D.R., 2002. Antiparasitic agent atovaquone. *Antimicrob. Agents Chemother.* 46, 1163–1173.
- Boozer, A.L., Macintire, D.K., 2003. Canine babesiosis. *Vet. Clin. North Am. Small Anim. Pract.* 33, 885–904.
- Bottema, D.D., Sommer, S.S., 1993. PCR amplification of specific alleles: rapid detection of known mutations and polymorphisms. *Mutat. Res.* 288, 93–102.
- Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2004. Efficacy of combined atovaquone and azithromycin for therapy of chronic *Babesia gibsoni* (Asian genotype) infections in dogs. *J. Vet. Intern. Med.* 18, 494–498.
- Canfield, C. J., Pundey, M., Gutteridge, W. E., 1995. Interactions of atovequone with other antimalarial drugs against *Plasmodium falciparum* in vitro. *Exp. Parasitol.* 80, 373–381.
- Farwell, G.E., Legrand, E.K., Cobb, C.C., 1982. Clinical observations on *Babesia gibsoni* and *Babesia canis* infections in dogs. *J. Am. Vet. Med. Assoc.* 180, 507–511.
- Fivelman, Q.L., Adagu, I.S., Warhurst, D.C., 2004. Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or

- dihydroartemisinin against drug resistant strains of *Plasmodium falciparum*.
Antimicrob. Agents Chemother. 48, 4098–4102.
- Gray, J., Pudney, M., 1995. Activity of atovaquone against *Babesia microti* in the Mongolian gerbil, *Meriones unguiculatus*. *J. Parasitol.* 85, 723–728.
- Groves, M.G., Dennis, G.L., 1972. *Babesia gibsoni*: field and laboratory studies of canine infections. *Exp. Parasitol.* 31, 153–159.
- Gupta S., Thapar M. M., Wernsdorfer W. H., Bjorkman A., 2002. In vitro interactions of artemisinin with atovaquone, quinine, and mefloquine against *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* 46, 1510–1515.
- Hudson, A.T., Dickins, M.M., Ginger, C.D., Gutteridge, W.E., Holdich, T., Hutchinson, D.B., 1991. 566C80: A broad spectrum anti-infective agent with activity against malaria and opportunistic infections in AIDS patients. *Drug Exp. Clin. Res.* 17, 427–435.
- Hughes, W.H., Oz, H., 1995. Successful prevention and treatment of babesiosis with atovaquone. *J. Infect. Dis.* 172, 1042–1046.
- Ikadai, H., Tanaka, H., Shibahara, N., Matsuu, A., Uechi, M., Itoh, N., Oshiro, S., Kudo, N., Igarashi, I., Oyamada, T., 2004. Molecular evidence of infections with *Babesia gibsoni* parasites in Japan and evaluation of the diagnostic potential of a

loop-mediated isothermal amplification method. *J. Clin. Microbiol.* 42, 2465–2469.

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

Inokuma, H., Yoshizaki, Y., Matsumoto, K., Okuda, M., Onishi, T., Nakagome, K., Kosuqi, R., Hirakawa, M., 2004. Molecular survey of *Babesia* infection in dogs in Okinawa, Japan. *Vet. Parasitol.* 121, 341-346.

Jefferies, R., Ryan, U.M., Jardine, J., Robertson, I.D., Irwin, P.J., 2007. *Babesia gibsoni*: detection during experimental infections and after combined atovaquone and azithromycin therapy. *Exp. Parasitol.* 117, 115–123.

Kjemtrump, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Moudreaux, M.K., Stockham, S.L., Iruzarry-Rovira, A., Conrad, P. A., 2000. There are at least three genetically distinct small piroplasms from dogs. *Int. J. Parasitol.* 30, 1501–1505.

Korsinczky, M., Chen, N., Kotecka, B., Saul, A., Rieckmann, K., Cheng, Q., 2000.

Mutations in *Plasmodium falciparum* cytochrome *b* that are associated with atovaquone resistance are located at a putative drug-binding site. *Antimicrob. Agents Chemother.* 44, 2100–2108.

Krause, P.J., Lepore, T., Sikand, V.K., Gadaw, J.J., Burke, G., Telford, S.R., Brassard, P., Pearl, D., Azlanzadeh, J., Chistianson, D., McGrath, D., Spielman, A., 2000. Atovaquone and azithromycin for the treatment of babesiosis. *N. Engl. J. Med.* 343, 1454–1458.

Lin, M.T., Huang, H.P., 2010. Use of doxycycline-enrofloxacin-metronidazole combination with/without diminazene diaceturate to treat naturally occurring canine babesiosis caused by *Babesia gibsoni*. *Acta Vet. Scand.* 52, 27.

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

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

Matsuu, A., Yamasaki, M., Xuan, X., Ikadai, H., Hikasa, Y., 2008. In vitro evaluation

- of the growth inhibitory activities of 15 drugs against *Babesia gibsoni* (Aomori strain). *Vet. Parasitol.* 157, 1–8.
- Miyama, T., Sakata, Y., Shimada, Y., Ogino, S., Watanabe, M., Itamoto, K., Okuda, M., Verdida, R. A., Xuan, X. N., Nagasawa, H., Inokuma, H., 2005. Epidemiological survey of *Babesia gibsoni* infection in dogs in eastern Japan. *J. Vet. Med. Sci*, 67, 467-471.
- Muhlninckel, C.J., Jefferies, R., Morgan-Ryan, U.M., Irwin, P.J., 2002. *Babesia gibsoni* infection in three dogs in Victoria. *Aust. Vet. J.* 80, 606–610.
- Paul, M. A., McCarthy, A. E., Gibson, N., Kenny, G., Cook, T., Gray, G., 2003. The impact of Malarone and primaquine on psychomotor performance. *Avit. Space Environ. Med.* 74, 238-245
- Pudney, M., Gray, J.S., 1997. Therapeutic efficacy of atovaquone against the bovine intraerythrocytic parasite, *Babesia divergens*. *J. Parasitol.* 83, 307–310.
- Pudney, M., Gutteridge, W., Zeman, A., Dickins, M., Wolley, J. L., 1999. Atovaquone and proguanil hydrochloride: a review of nonclinical studies. *J. Travel. Med.* 6 (Suppl. 1), S8-S112.
- Sakuma, M., Setoguchi, A., Endo, Y., 2009. Possible emergence of drug-resistant variants of *Babesia gibsoni* in clinical cases treated with atovaquone and

azithromycin. *J. Vet. Intern. Med.* 23, 493–498.

Srivastava, I. K., Vaidya, A. B., 1999. A mechanism for the synergistic antimalarial action of atovaquone and proguanil. *Antimicrob. Agents Chemother.* 43,1334–1339.

Suzuki, K., Wakabayashi, H., Takahashi, M., Fukushima, K., Yabuki, A., Endo, Y., 2007. A possible treatment strategy and clinical factors to estimate the treatment response in *Babesia gibsoni* infection. *J. Vet. Med. Sci.* 69, 563–568.

Weiss, L., 2002. Babesiosis in humans: a treatment review. *Expert. Opin. Pharmacother.* 3, 1109–1115.

Wihelm, J., Reuter, H., Tews, B., Pingound, A., Hahn, M., 2002. Detection and quantification of insertion/deletion variations by allele-specific real-time PCR: application for genotyping and chimerism analysis. *Biol. Chem.* 383, 1423–1433.

Wittner, M., Lederman, J., Tanowitz, H.B., Rosenbaum, G.S., Weiss, L.M., 1996. Atovaquone in the treatment of *Babesia microti* infections in hamsters. *Am. J. Trop. Med. Hyg.* 55, 219–222.

Wozniak, E. J., Barr, B. C., Thomford, J. W., Yamane, I., McDonough, S. P., Moore, P. F., Naydan, D., Robinson, T. W., Conrad, P. A., 1997. Clinical, anatomic, and

immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (*Canis familiaris*). J. Parasitol. 83, 692-699.

Wulansari, R., Wijaya, A., Ano, H., Horii, Y., Makimura, S., 2003. Lymphocyte subsets and specific IgG antibody levels in clindamycin-treated and untreated dogs experimentally infected with *Babesia gibsoni*. J. Vet. Med. Sci. 65, 579–584.