Studies on the Resistance to Diminazene Aceturate in *Babesia gibsoni In Vitro*

（体外培養***Babesia gibsoni***におけるジミナゼン耐性に関する研究）

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ABBREVIATIONS

ABC: ATP-Binding Cassette

ATP: adenosine triphosphate

BgHsp70: Babesia gibsoni heat shock protein 70

cDNA: complementary DNA

DA: diminazene aceturate

DMSO: dimethylsulfoxide

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

GIR: growth-inhibitory rate

HCl: hydrochloric acid

HK: high potassium

Hsp70: heat shock protein 70

IC_{50}: 50% inhibitory concentration

IFA: indirect fluorescent antibody

mVYM: modified Vega y Martinez phosphate-buffered saline

NaCl: sodium chloride

PCV: packed cell volume

qRT-PCR: quantitative real-time reverse

transcription-polymerase chain reaction

RBC: red blood cell

Tris: tris(hydroxymethyl)aminomethane

18S rRNA: 18S ribosomal RNA

CONTENTS

GENERAL INTRODUCTION

Canine babesiosis is an important world-wide, tickborne disease caused by hemoprotozoan parasites of the genus *Babesia*. *Babesia canis* and *Babesia gibsoni* have been the two predominant species capable of naturally infecting a dog. *B. canis* is a large (2.4 μm × 5 μm) piriform-shape piroplasm, while *B. gibsoni* is a small(1 μm × 3.2 μm)organism usually observed singly within erythrocytes (Taboada and Lobetti, 2006). Expansive geographic range of *B. canis* includes most of southern Europe, Africa, Asia, North America, Central America and South America. Meanwhile, *B. gibsoni* was initially found primarily in northern Africa and the southern parts of Asia but has now been found in Australia, Europe and the United States (Taboada and Lobetti, 2006). Especially, *B. gibsoni* is the predominant species that causes canine babesiosis in Taiwan (Chuang, 2007), and the incidence of *B. gibsoni* infection has been increasing in Japan (Konishi *et al.*, 2008; Matsuu *et al.*, 2004; Miyama *et al.*, 2005).

Clinical signs of canine babesiosis include anorexia, depression, weakness, pale mucous membranes, icterus, pyrexia, splenomegaly and severe hemolytic anemia (Breitschwerdt, 1990; Groves and Dennis, 1972; Farwell *et al.*, 1982). Fatalities may occur, especially in puppies and occasionally in *B. gibsoni*-infected adults. Laboratory results showed regenerative anemia, thrombocytopenia and leucopenia (Conrad *et al.*, 1991). To diagnose the canine babesiosis, a number of techniques were developed. Definitive diagnosis depends on the demonstration of the organisms within infected erythrocytes, positive serology results and amplification of babesial DNA extracted from infected blood or tissue. Evaluation of Giemsa stained slides requires a significant time commitment on the part of the laboratory technician. Therefore, serologic test of indirect fluorescent antibody (IFA) test is also used to detect babesial antibody (Yamane *et al.*, 1993), while enzyme-linked immunosorbent assay (ELISA) is also available to screen for infected hosts but is used

less commonly in clinical diagnosis (Adachi *et al.*, 1992; Toboada and Lobetti, 2006). Because the organisms vary or are infrequent in blood smears, genetic methods are the most sensitive and specific means of detecting infection. For example, polymerase chain reaction (PCR) is currently recommended (Birkenheuer *et al.*, 2003; Fukumoto *et al.*, 2001).

Many drugs have been utilized for the treatment of canine babesiosis, such as imidocarb dipropionate, diminazene aceturate, phenamidine isethionate, pentamidine isethionate, trypan blue, primaquine phosphate, clindamycin, doxycycline, azithromycin, atovaquone and quinuronium sulfate (Toboada and Lobetti, 2006). Currently the most important antibabesial drug used in *B. gibsoni* infection is diminazene aceturate. Diminazene aceturate (DA), an aromatic diamidine, has been documented as being antibabesial drug and is used worldwide in treating a wide variety of *Babesia* infection. A single intramuscular injection of DA at the dose of 3.5 mg/kg (Breitschwerdt, 1990) or 5 mg/kg (Birkenheuer *et al.*, 1999) is given to treat canine babesiosis. However, drug efficacy

significantly varies between *B. canis* and *B. gibsoni*. For example, no evidence of continued infection was found in the *B. canis*-infected dogs following the treatment of DA or pentamidine, but these drugs failed to eliminate *B. gibsoni* from dogs (Farwell *et al.*, 1982). One of the most effective antibabesial drugs for *B. canis* infection, imidocarb dipropionate, is not effective against *B. gibsoni*. Moreover, because no single drug had successfully eliminated *B. gibsoni* from infected dogs, alternative therapies of additive or synergistic effect have recently been initiated. Combination therapies, including atovaquone/azithromycin and clindamycin/metronidazole/doxycycline, were proposed and showed great effectiveness in treating *B. gibsoni* infection (Birkenheuer *et al.*, 2004; Jefferies *et al.*, 2007; Suzuki *et al.*, 2007). However, those reported therapeutic protocols have several problems, such as taking relatively long time to show clinical effectiveness, possibility of relapse, and emergence of resistant variants (Matsuu *et al.*, 2004; Matsuu *et al.*, 2006; Sakuma *et al.*, 2009). Especially, the alleged

drug resistance of *B. canis* to DA (Collett, 2000) and a single polymorphism mutation in the *cytochrome b* gene of *B. gibsoni* that was suspected as a resistance to atovaquone (Matsuu *et al.*, 2006) were mentioned. In this regard, there is urgent need to clearly elucidate the drug resistance problems in *B. gibsoni*.

Drug resistance is a broad problem in the fight against infectious diseases. After the long exposure to drugs, parasites can frequently develop resistance towards chemotherapeutics. For examples, the *Pfmdr1* gene in *P. falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989), the *LmpgpA* gene in *L. major* (Callahan and Beverley, 1991) and the *TbmrpA* gene in *T. brucei* (Shahi *et al.*, 2002) are involved in drug resistance by the alteration of drug accumulation into parasites. Other mechanisms of drug resistance, such as mutation with mitochondrial genes in *B. gibsoni* (Matsuu *et al.*, 2006; Sakuma *et al.*, 2009), are continuously studied. In *B. gibsoni*, the unsuccessful efficacy of DA has become a serious problem for the treatment of canine babesiosis since

it is one of the most popular antibabesial drugs for the *B. gibsoni* infection. Furthermore, a veterinary practice survey in South Africa indicated that relapse of *B. canis* after DA treatment was observed, although the genetic differences was not detected by molecular biological methods to certify the resistance (Collett, 2000). Hence, this survey did not demonstrate the DA resistance clearly. Though it is essential to identify the genetic differences that are potential drug targets, the mechanism of the action of DA against *B. gibsoni* is currently unknown. In contrast, those against *Trypanosoma* spp. and *Leishmania tarentoloe* were reported to be the inhibition of DNA replication and mitochondrial respiratory activity (Bitonti *et al.*, 1986; Leon *et al.*, 1977; Macadam and Williamson, 1972; Newton and Le Page, 1968). Furthermore, DA has been known to be transported into blood-stream *Trypanosoma equiperdum*, by the P2 aminopurine transporter (Barrett *et al.*, 1995). The *adenosine transporter-1* gene, *TbAT1*, encoding a P2-like nucleoside transporter, has been cloned from *Trypanosoma brucei brucei* (Mäser *et al.*, 1999).

Loss of the *TbAT1* gene activity in *T. brucei* and its analoge, *TevAT1*, in *T. evansi* has been shown to confer resistance to DA (Matovu *et al.*, 2003; Witola *et al.*, 2004). In contrast, the mechanism of DA resistance in *B. gibsoni* is unknown. Even more, the accuracy of DA resistance in *B. gibsoni* is not defined. The purposes of this study, therefore, were to develop and characterize the DA-resistant *B. gibsoni* strain.

CHAPTER 1

Development and Characterization of a Strain of *Babesia gibsoni* Resistant to Diminazene Aceturate *In Vitro*

INTRODUCTION

Canine babesiosis, a tick-borne hematozoan disease, is caused by *Babesia gibsoni* and *Babesia canis*. This disease is characterized by fever, lethargy, anemia and, in severe cases, death (Conrad, 2009; Farwell *et al.*, 1982). Diminazene aceturate (DA), an antibabesial drug, is an aromatic diamidine derivative. Currently, the mechanism of the action of DA on *B. gibsoni* and *B. canis* is not elucidated. DA can temporarily improve the clinical signs of canine babesiosis (Boozer and Macintire, 2003; Fowler *et al.*, 1972). However, this drug is unable to eliminate the parasites from infected dogs, and relapses often occur (Farwell *et al.*, 1982). It is believed that this is due to the development of drug resistance of *B. gibsoni* against DA. Collett (2000) considered that *B. canis* surviving DA treatment could develop drug resistance against DA clinically. However, there is no report proving DA resistance of *B. gibsoni* and *B. canis*. In trypanosomiasis and leishmaniasis, it is reported that DA can inhibit the DNA replication and mitochondrial respiratory activity of those pathogens (Bitonti *et al.*, 1986; Leon *et al.*, 1977). The loss of P2 nucleoside transporter function in *T. brucei brucei* has been implicated in resistance to DA (Carter *et al.*, 1995). Likewise it is possible that *B. gibsoni* could also develop

drug resistance against DA.

In reports about other antibabesial drugs, including atovaquone, clindamycin, metronidazole, doxycycline and pentamidine, almost no single drug treatment or combined treatment could eliminate the parasites from the peripheral blood at the dosages used, and the possibility of relapse and the development of resistant variants remained (Farwell *et al.*, 1982; Fowler *et al.*, 1972; Sakuma *et al.*, 2009; Suzuki *et al.*, 2007; Wilson *et al.*, 1989). From those previous reports, it seems to be difficult to eliminate *B. gibsoni* from infected dogs using the currently available drugs. Therefore, to develop novel and effective antibabesial drugs, it is necessary to clarify the occurrence and the mechanism of drug resistance of *B. gibsoni*.

In the present study, accordingly, an attempt was made to develop a strain of *B. gibsoni* strain resistant to DA, to demonstrate the rise of drug resistance of the parasites. Moreover, the efficacies of clindamycin, doxycycline, metronidazole, and pentamidine against the strain of the parasites resistant to DA were investigated to characterize this strain.

MATERIALS AND METHODS

In vitro culture of *B. gibsoni*

The *B. gibsoni* parasites used in the present study originated from a naturally infected dog in the city of Nagasaki, Japan, in 1973. Since then, this strain has been maintained in cultures as wild-type *B. gibsoni* in the Laboratory of Veterinary Internal Medicine, Graduate School of Veterinary Medicine, Hokkaido University (Yamasaki *et al.*, 2000). The parasites were incubated at 38°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in a culture medium consisting of RPMI-1640 (Invitrogen, California, U.S.A.), 20% dog serum, and canine HK red blood cells (RBCs) that contain the high concentration of potassium (Inaba and Maede, 1984), sufficient to yield a packed cell volume (PCV) of 5%. Every 24 hrs, 60% of the culture supernatant was removed and replaced with an equal volume of fresh culture medium (Yamasaki *et al.*, 2003; Yamasaki *et al.*, 2005). Every 7 days, a half volume of the erythrocyte suspension was removed and replaced with an equal volume of uninfected fresh erythrocyte suspension as a subculture.

Culture of *B. gibsoni* in the medium containing diminazene aceturate

Diminazene aceturate (DA) (Novartis, Tokyo, Japan), which is an antibabesial drug, was used. First, 0, 1, 10, 100, or 1,000 ng/ml of DA was added to the culture medium, and *B. gibsoni*, which is sensitive to DA (wild-type *B. gibsoni*), was cultured in the culture medium. Because the parasites could survive and proliferate only in 1 ng/ml DA, the concentration of DA was gradually increased from 1 to 200 ng/ml using the protocol described below. When the level of parasitemia of *B. gibsoni* increased or was stable, the concentration of DA was raised. If the level of parasitemia decreased, the concentration of DA was reduced. Depending on the proliferation of *B. gibsoni*, the concentration of DA in the culture medium was changed at the time of subculture. Thin smears were prepared at days 0, 3, and 6 after subculture. The level of parasitemia was determined by counting the number of parasitized erythrocytes per 1,000 erythrocytes.

Direct effect of diminazene aceturate on erythrocyte-free parasites *in vitro*

B. gibsoni cultured in 200 ng/ml DA was isolated from infected host cells by the method of Sugimoto *et al.* (1991) with some modifications. A suspension of infected erythrocytes was centrifuged at 800 x g for 5 min at 4°C. After the removal of the supernatant, an equal volume of Tris buffer

(10 mM Tris-HCl, 150 mM NaCl, pH 7.4) was added. After centrifugation, the supernatant was removed and the residue was resuspended with Tris buffer. Then hemolysin was added to a the final concentration of 300 HU/ml. After incubation at 37°C for 10 min, the erythrocyte lysate was cooled on ice and ethylenediaminetetraacetic acid (EDTA) solution (pH 9.0) was added to the final concentration of 5 mM. Then, samples were centrifuged at 5,000 x g for 5 min at 4°C, and the resulting parasite pellet was washed twice with RPMI-1640. The number of parasites was counted using a Burger-Türk counting chamber. Then, 1 x 10^5/ml of erythrocyte-free parasites were cultured with fresh uninfected erythrocytes in the culture medium containing 200 ng/ml DA under the conditions described above. Thin smears were prepared every 24 hrs, and the level of parasitemia was determined by counting the number of parasitized erythrocytes per 1,000 erythrocytes. In addition, *B. gibsoni* cultured without DA was also isolated from infected host cells and cultured with fresh uninfected erythrocytes in the culture medium either with 200 ng/ml DA or without DA as a control.

The effects of other anti-babesial drugs against diminazene aceturate-resistant *B. gibsoni* strain *in vitro*

The growth-inhibitory effects of four drugs,

clindamycin hydrochloride (Pfizer, Tokyo, Japan), doxycycline hydrochloride (MP Biomedicals, LLC CA, U.S.A.), metronidazole (Sigma-Aldrich, Tokyo, Japan) and pentamidine isethionate salt (Sigma-Aldrich, Tokyo, Japan), against the DA-resistant *B. gibsoni* strain were compared with those against the wild-type *B. gibsoni*. These drugs were diluted in a small quantity of dimethylsulfoxide (DMSO) and further diluted in the culture medium. Cultured erythrocytes infected with either the DA-resistant or wild-type *B. gibsoni* strain were collected and washed twice with a modified Vega y Martinez phosphate-buffered saline solution (mVYM solution, pH 7) (Vega *et al.*, 1985) and once with RPMI-1640. After washing, infected erythrocytes were resuspended to the final packed cell volume of 5% in culture medium containing one of the drugs. Final concentrations of clindamycin hydrochloride were 100, 150, 200, and 250 μg/ml. Those of doxycycline hydrochloride were 5, 10, 20, and 40 μg/ml. Those of metronidazole were 200, 400, and 800 μg/ml. Those of pentamidine isethionate salt were 100, 200, 300, and 400 ng/ml. The test was performed in a 48-well culture plate, and each well contained 400 μl of the erythrocyte suspension. The plate was incubated for 7 days. Every 24 hrs, 240 μl of the culture medium in each well was removed and an equal volume of fresh medium containing the appropriate drug concentration was added. Thin smears were

prepared every 24 hrs, and the level of parasitemia was determined by counting the number of parasitized erythrocytes per 1,000 erythrocytes. The growth-inhibitory rates (GIRs) for *B. gibsoni* were calculated from the level of parasitemia by using the formula given below (Eq. 1). The 50% inhibitory concentration (IC_{50}) at day 7 of culture was calculated using probit analysis. One experiment was performed in duplicate, and the same experiment was repeated 3 times. In the analysis, all data from 3 experiments were used in one analysis.

{GIR (%) = (Parasitemia of control group – Parasitemia of drug group) / (Parasitemia of control group) × 100% } Eq. 1

Statistical analysis

Statistical analysis was performed by using a paired *t*-test to compare the levels of parasitemia among different concentrations of each drug. Otherwise, two-sample *t*-tests were used to compare the levels of GIRs between the DA-resistant and wild-type *B. gibsoni* strains.

RESULTS

Development of a strain of *B. gibsoni* resistant to diminazene aceturate

To develop a DA-resistant *B. gibsoni* strain, *B. gibsoni* was cultured in culture media containing various concentrations of DA. When the wild-type *B. gibsoni* was cultured in media containing 10, 100, and 1,000 ng/ml DA, the parasites were almost completely eliminated within 2 weeks (Fig. 1). In contrast, the parasites cultured in 1 ng/ml DA proliferated normally (Fig. 1). Therefore, it was decided that the initial concentration of DA for the culture should be 1 ng/ml. After that, the concentration of DA in the culture medium was increased gradually according to the procedure described in the MATERIALS AND METHODS (Fig. 2). When the concentration of DA was increased from 1 to 5 ng/ml at week 1, the parasites could not proliferate during weeks 2 to 3. Therefore, the DA concentration was decreased to 1 ng/ml at week 4, and then the parasites proliferated in week 5. When the DA concentration was increased from 10 to 15 ng/ml at week 9, the parasites could not proliferate in week 10. Decreasing the DA concentration to 10 ng/ml resulted in the proliferation of parasites in week 11. Except for the two above-mentioned periods, the parasites steadily proliferated as the DA

concentration was increased. In this experiment, the concentration of DA in the culture medium was raised approximately every 2 weeks. Finally, the parasites could proliferate in culture medium containing 200 ng/ml DA after day 420.

Direct effect of diminazene aceturate on erythrocyte-free parasites *in vitro*

To confirm the resistance of *B. gibsoni* against DA, the parasites were removed from erythrocytes and exposed directly to DA. For the positive and negative control cultures, the wild-type *B. gibsoni* removed from erythrocytes was designated wild-type erythrocyte-free parasites. When the wild-type erythrocyte-free parasites were cultured with fresh uninfected erythrocytes without DA as a positive control, the parasites invaded erythrocytes, and proliferated well after day 2 of the culture (Fig. 3). In contrast, when they were cultured with fresh uninfected erythrocytes in 200 ng/ml DA as a negative control, infected erythrocytes were not detected during the culture period (Fig. 3). When the parasites maintained in culture medium containing 200 ng/ml DA were removed from erythrocytes and cultured with fresh uninfected erythrocytes in culture medium containing 200 ng/ml DA, a small number of infected erythrocytes were detected at day

4 of the culture, and then the level of parasitemia increased up to 1.9 ± 0.81% at day 10 of culture (Fig. 3). Because these parasites maintained in culture medium containing 200 ng/ml DA could survive direct exposure to DA, they were considered a DA-resistant *B. gibsoni* strain.

The effects of other antibabesial drugs on the growth of diminazene aceturate-resistant *B. gibsoni* strain *in vitro*

To investigate the characteristics of the DA-resistant *B. gibsoni* strain, the effects of other antibabesial drugs on its growth were observed. In the present study, the GIRs were calculated from the number of parasitized erythrocytes, and those of the DA-resistant were compared with those of wild-type *B. gibsoni* strains. When the DA-resistant and wild-type *B. gibsoni* strains were cultured in medium containing clindamycin, the GIRs were gradually increased through the culture period (Fig. 4). The GIRs for the DA-resistant *B. gibsoni* strain were slightly lower than those for the wild-type *B. gibsoni* at 150, 200, and 250 μg/ml clindamycin (Fig. 4). The GIR for the DA-resistant strain at 250 μg/ml clindamycin at day 7 (43.1 ± 8.96%) was significantly lower than that for the wild-type *B. gibsoni* (68.0 ± 6.12%). The GIRs for the DA-resistant *B. gibsoni* strain at 150 μg/ml clindamycin at days 4 and 6, and those at 200 μg/ml clindamycin

at days 6 and 7 were also significantly lower ($P < 0.05$) than those for the wild-type *B. gibsoni* (Fig. 4). Moreover, the IC_{50} of clindamycin for the DA-resistant *B. gibsoni* strain at day 7 was higher than that for the wild-type *B. gibsoni* (Table 1). The values in the brackets in Table 1 were the calculated IC_{50} of those drugs. Since those values were higher or lower than the used concentration of each drug, it was supposed that those would not be accurate.

When the DA-resistant and wild-type *B. gibsoni* strains were cultured in medium containing doxycycline, the GIRs were also gradually increased during the culture period (Fig. 5). The GIRs for the DA-resistant strain at both 5 and 10 μg/ml doxycycline were almost the same as those for the wild-type *B. gibsoni* through out the culture period. On the other hand, the GIRs for the DA-resistant strain at both 20 and 40 μg/ml doxycycline at days 6 and 7 were significantly lower ($P < 0.05$) than those for the wild-type *B. gibsoni*. The GIR for the DA-resistant strain at 40 μg/ml doxycycline at day 7 (52.4 ± 2.24%) was significantly lower than that for the wild-type *B. gibsoni* (80.3 ± 7.02%). The IC_{50} of doxycycline for the DA-resistant *B. gibsoni* strain at day 7 was higher than that for the wild-type *B. gibsoni* (Table 1).

When the wild-type *B. gibsoni* was cultured in medium containing pentamidine, the GIRs also gradually increased

through out the culture period (Fig. 6). When the DA-resistant *B. gibsoni* strain was cultured in medium containing pentamidine, the GIRs for the DA-resistant strain were obviously lower than those for the wild-type *B. gibsoni* (Fig. 6). Though the GIRs for the DA-resistant strain at 200, 300, or 400 ng/ml pentamidine also gradually increased through out the culture period, that at 100 ng/ml pentamidine hardly increased. The GIRs for the DA-resistant strain at days 4 to 7 at 100 ng/ml pentamidine, those at days 5 to 7 at 200 ng/ml pentamidine, and those at days 3 to 7 at both 300 and 400 ng/ml pentamidine were significantly lower ($P < 0.05$) than those for the wild-type *B. gibsoni* (Fig. 6). The GIR for the DA-resistant strain at 400 ng/ml pentamidine at day 7 (43.5 ± 7.16%) was significantly lower than that for the wild-type *B. gibsoni* (84.7 ± 2.21%). The IC_{50} of pentamidine for the DA-resistant *B. gibsoni* strain at day 7 was over 400 ng/ml, which was higher than that for the wild-type *B. gibsoni* (Table 1).

In this study, even 800 μg/ml metronidazole did not affect the proliferation of the wild-type *B. gibsoni* (data not shown). Furthermore, erythrocytes were lysed in the culture medium containing 800 μg/ml metronidazole. Therefore, the IC_{50} of metronidazole for both the DA-sensitive and wild-type *B. gibsoni* strains were not determined.

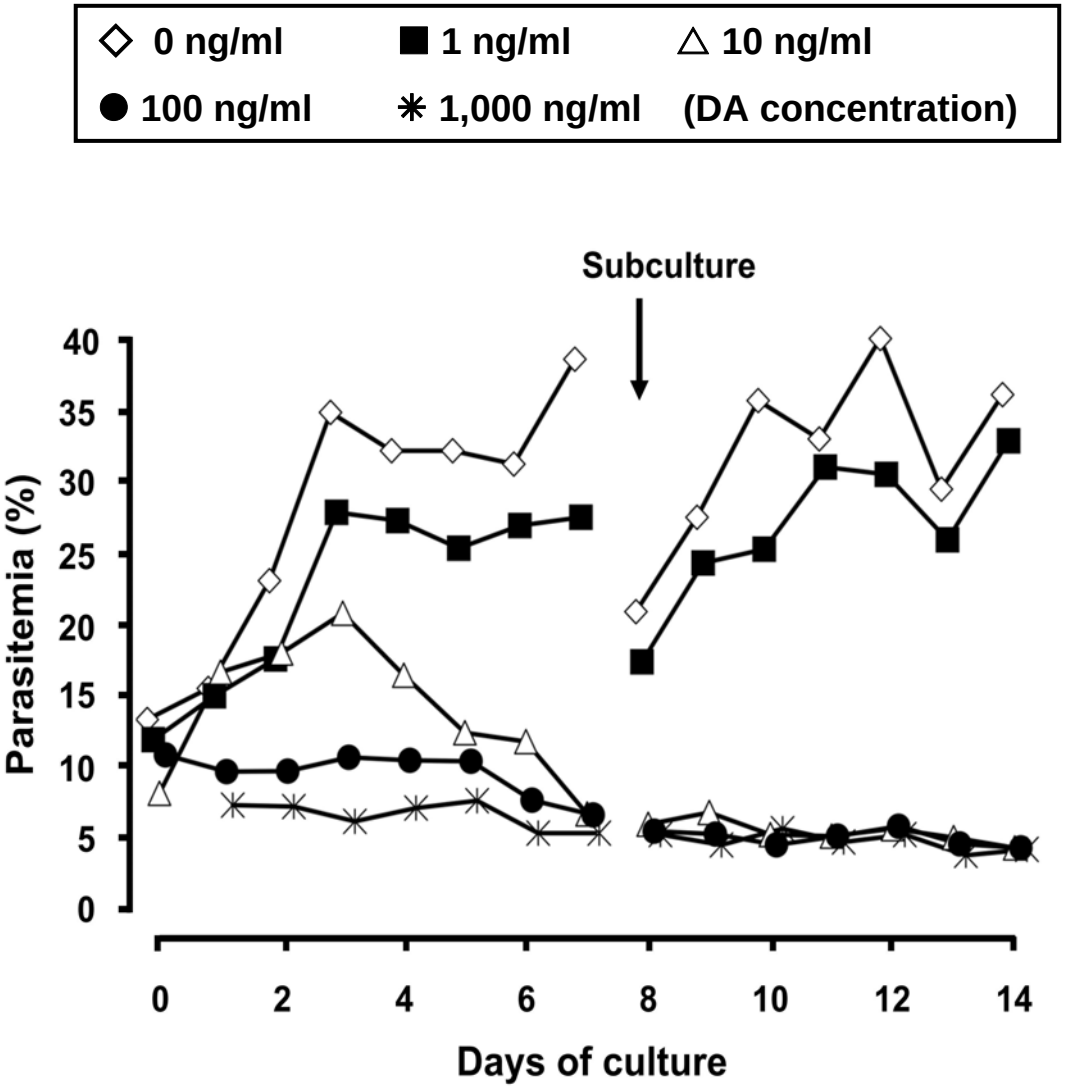

Fig. 1. The levels of parasitemia of *B. gibsoni* cultured with diminazene aceturate (DA) for 2 weeks *in vitro*. The final concentrations of DA were 0 (control, open diamond), 1 (closed square), 10 (open triangle), 100 (closed circle), and 1,000 (asterisk) ng/ml DA in the culture media.

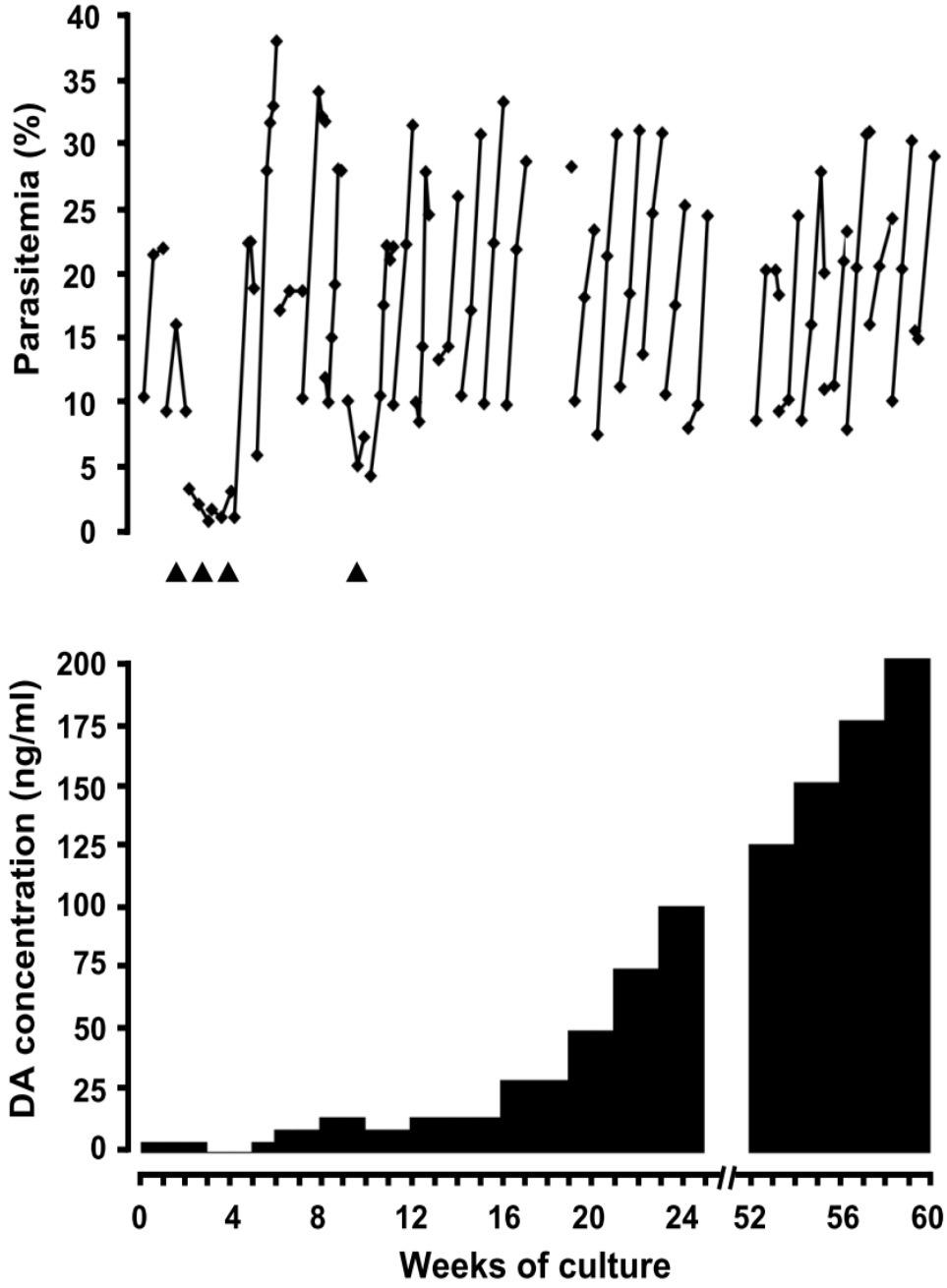

Fig. 2. Development of diminazene aceturate (DA)-resistant *B. gibsoni* strain. Changes in the levels of parasitemia and DA concentration in the culture medium. Arrowheads indicate that the levels of parasitemia decreased.

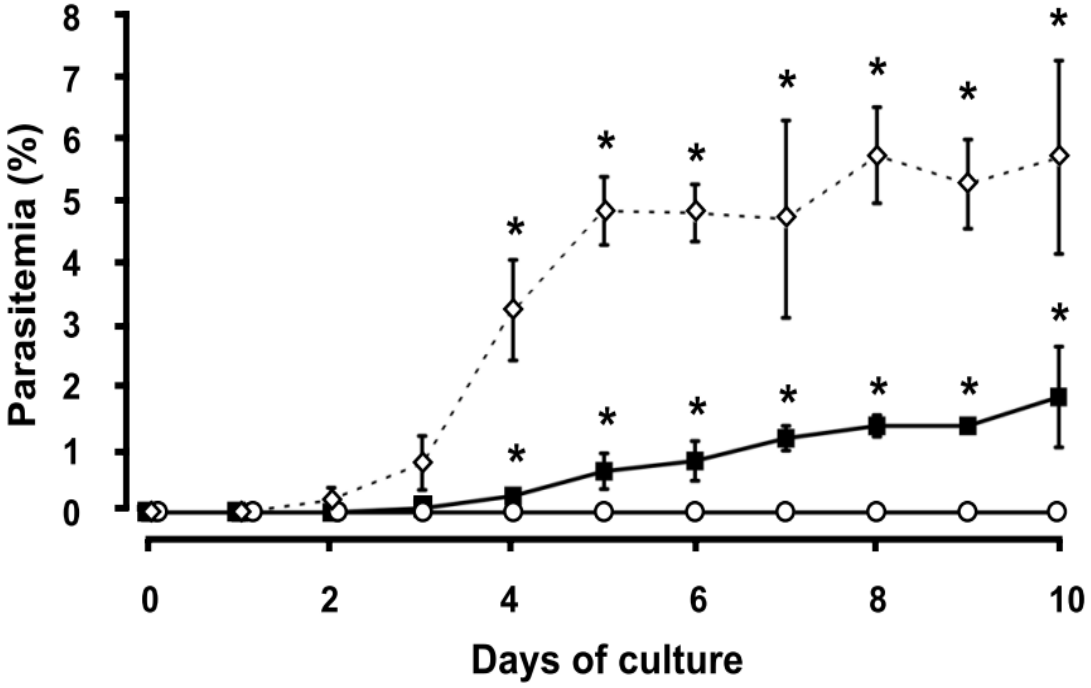

Fig. 3. Direct effect of diminazene aceturate (DA) on erythrocyte-free parasites *in vitro*. The parasites maintained in culture medium including 200 ng/ml DA were removed from erythrocytes and cultured with fresh uninfected erythrocytes in culture medium including 200 ng/ml DA (closed square). As a positive control, wild-type *B. gibsoni* parasites were removed from erythrocytes and cultured with fresh uninfected erythrocytes without DA (open diamond). As a negative control, wild-type *B. gibsoni* erythrocyte-free parasites were cultured with fresh uninfected erythrocytes in culture medium including 200 ng/ml DA (open circle). Data are expressed as mean ± SD (n = 3). *Significantly ($P < 0.05$) different from the value for the negative control.

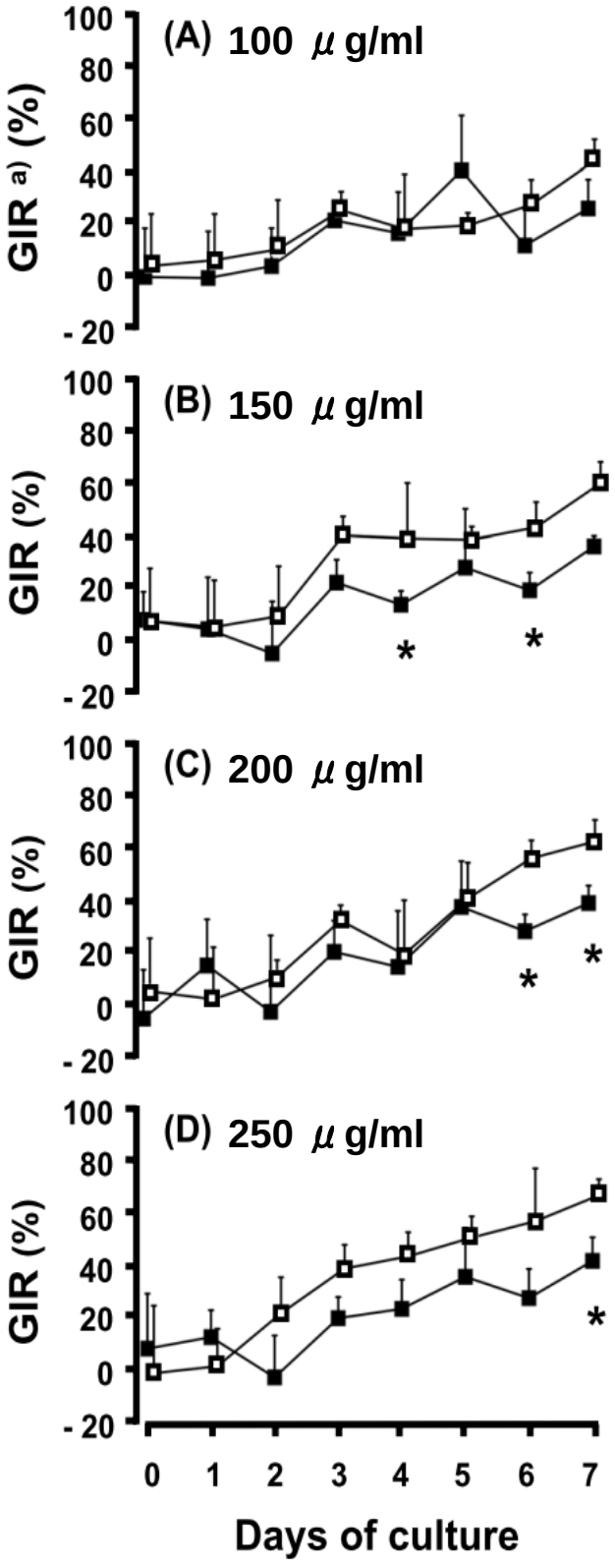

Fig. 4. The growth-inhibitory effects of clindamycin on the DA-resistant and wild-type *B. gibsoni* strains. Clindamycin was added to the culture medium to final concentrations of 100 (A), 150 (B), 200 (C), and 250 μg/ml (D). The growth-inhibitory rates were compared between DA-resistant (closed square) and wild-type (open square) *B. gibsoni* strains by two-sample *t*-test. Data are expressed as mean ± SD (n = 3). *Significantly ($P < 0.05$) different from the value for the DA-resistant *B. gibsoni* strain. a) GIR: growth-inhibitory rate.

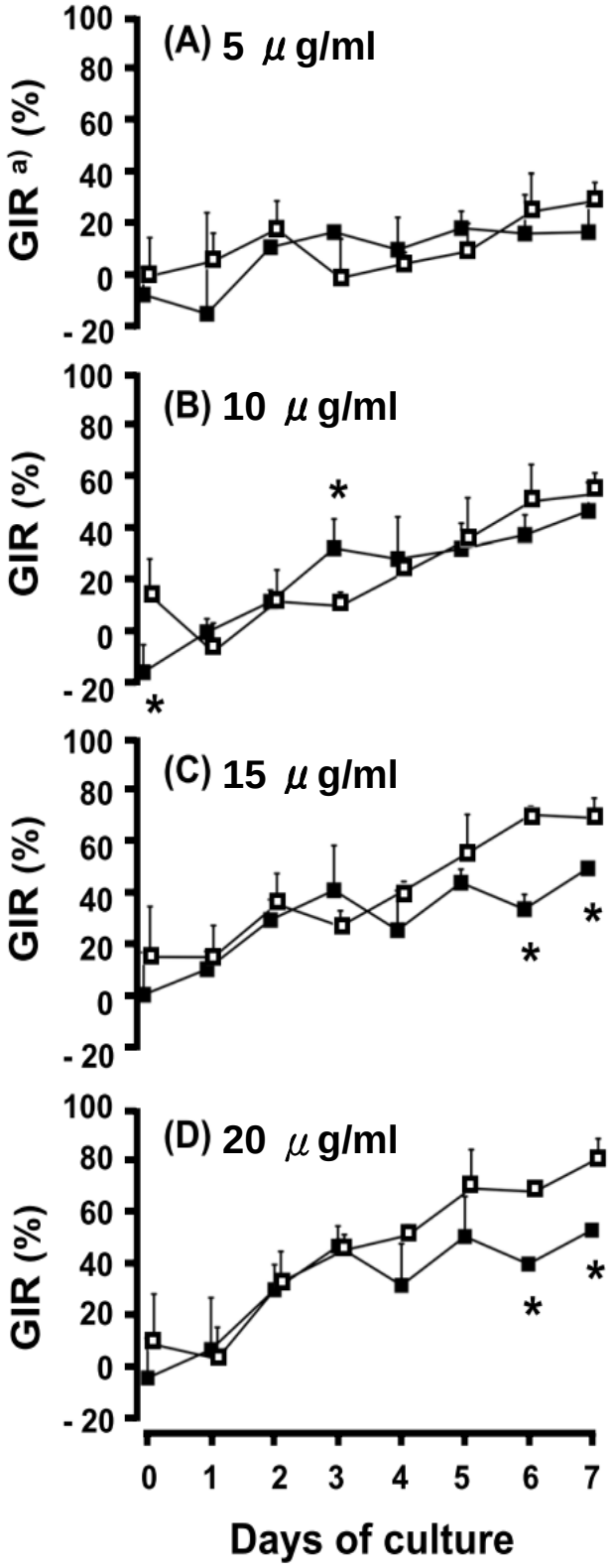

Fig. 5. The growth-inhibitory effects of doxycycline on the DA-resistant and wild-type *B. gibsoni* strains. Doxycycline was added to the culture medium to final concentrations of 5 (A), 10 (B), 20 (C), and 40 μg/ml (D). The growth-inhibitory rates were compared between DA resistant (closed square) and sensitive (open square) *B. gibsoni* strains by two-sample *t*-test. Data are expressed as mean ± SD (n = 3). *Significantly ($P < 0.05$) different from the value for the DA-resistant *B. gibsoni* strain. a) GIR: growth-inhibitory rate.

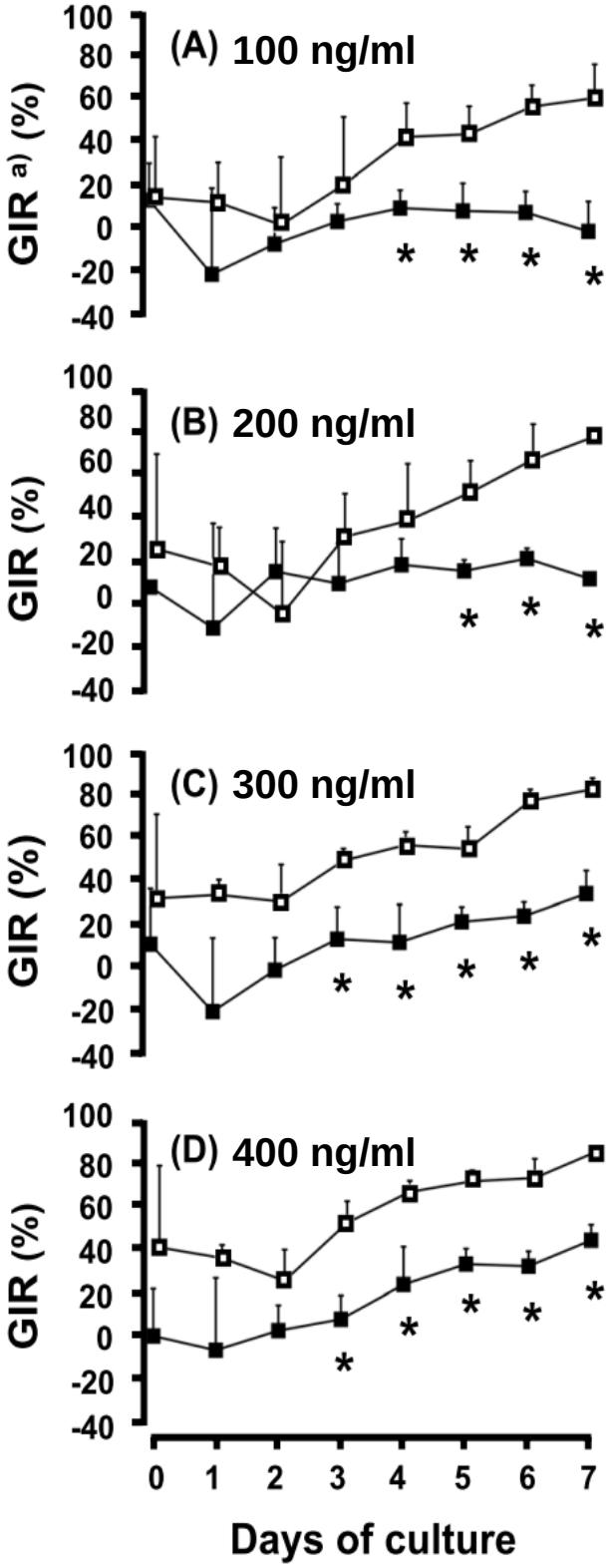

Fig. 6. The growth-inhibitory effects of pentamidine on the DA-resistant and wild-type *B. gibsoni* strains. Pentamidine was added to the culture medium to final concentrations of 100 (A), 200 (B), 300 (C), and 400 ng/ml (D). The growth-inhibitory rates were compared between DA-resistant (closed square) and wild-type (open square) *B. gibsoni* strains by two-sample *t*-test. Data are expressed as mean ± SD (n = 3). *Significantly ($P < 0.05$) different from the value for the DA-resistant *B. gibsoni* strain. a) GIR: growth-inhibitory rate.

Table 1. The 50% inhibitory concentrations (IC_{50}) of clindamycin, doxycycline and pentamidine for the DA-resistant and wild-type *B. gibsoni* strains at day 7 of culture.

Antibabesial drugs	DA-resistant *B. gibsoni* strain	Wild-type *B. gibsoni*
Clindamycin (μg/ml)	> 250 (336.8) [a)]	120.42
Doxycycline (μg/ml)	24.49	10.01
Pentamidine (ng/ml)	> 400 (487.08) [a)]	< 100 (40.09) [a)]

a) Values are calculated using probit analysis, but were larger or smaller than the tested concentration of each drug.

DISCUSSION

In the present study, a DA-resistant *B. gibsoni* strain was developed. First the wild-type *B. gibsoni* was cultured in culture medium with 1 ng/ml DA, and the DA concentration was gradually raised. Finally, the parasites proliferated in culture medium containing 200 ng/ml DA after day 420 of the culture. Those parasites were maintained in 200 ng/ml DA thereafter. Since IC_{50} values of DA for *B. gibsoni* previously reported were 88.43 ± 10.94 nM, which corresponds to 45.59 ng/ml (Matsuu *et al.*, 2008), 89.02 ± 17.29 nM, which corresponds to 45.89 ng/ml (Matsuu *et al.*, 2004), and 103 ± 12 ng/ml (Subeki *et al.*, 2007), respectively, the parasites, which IC_{50} values of DA are higher than the wild-type *B. gibsoni*, will be considered to have resistance against DA. The 200 ng/ml DA in the culture medium in this study was much higher than the IC_{50} values reported. Thus, this *B. gibsoni* strain maintained in 200 ng/ml DA could survive and proliferate in the concentration of DA adequate to inhibit the proliferation of the wild-type *B. gibsoni in vitro*. Because *B. gibsoni* is an intraerythrocytic protozoan, it invades erythrocytes and multiplies in them. Therefore, if DA does not penetrate through the erythrocyte membrane, it is possible that the parasites will not contact DA directly, and survive in the

infected erythrocytes. Accordingly, the parasites were removed from erythrocytes and exposed directly to DA. In the present study, hemolysin was used to remove *B. gibsoni* from erythrocytes. When wild-type erythrocyte-free parasites were cultured with fresh uninfected erythrocytes without DA, the parasites were able to invade the erythrocytes. It was thus demonstrated that the parasites removed from erythrocytes by using hemolysin could remain alive and infective. On the other hand, when the wild-type erythrocyte-free parasites were cultured with DA, all of the parasites were destroyed, indicating that the wild-type parasites could not resist direct exposure to DA. Furthermore, when the parasites maintained in culture medium containing 200 ng/ml DA were removed from erythrocytes and cultured with uninfected erythrocytes with 200 ng/ml DA, these parasites remained alive and infective. These results clearly showed that this *B. gibsoni* strain maintained in culture medium containing 200 ng/ml DA was a DA-resistant *B. gibsoni* strain.

Though atovaquone, which is an antiprotozoal drug, combats *B. gibsoni* infection in dogs, the sensitivity of *B. gibsoni* to atovaquone decreases (Matsuu *et al.*, 2004). Moreover, Matsuu *et al.* (2006) and Sakuma *et al.* (2009) demonstrated that variant strains of *B. gibsoni* could be obtained from clinical cases treated with atovaquone. However,

these variant strains were not proved to have resistance to atovaquone *in vitro*. Additionally, there is no report proving the resistance of *B. gibsoni* to any antibabesial drugs. Accordingly, this is the first report clearly demonstrating drug resistance of *B. gibsoni in vitro*. However, It could not be determined whether all the parasites developed resistance to DA or the DA-resistant parasites were selected from a mixed population of the parasites. Nevertheless, according to the results from the present study, it can be suspected that the parasites, which will be strong against DA, might be selected within one week when they are exposed to even 1 ng/ml DA. This indicates that *B. gibsoni* would acquire some resistance against DA in a short-period exposure to the low concentration of DA. Consequently, the effect of DA on *B. gibsoni in vivo* will be decreasing, if the DA concentration in the peripheral blood might be maintained over 1 ng/ml for a few days in clinical cases. The further studies will be necessary to clarify this hypothesis.

To investigate the characteristics of the DA-resistant *B. gibsoni* strain, the GIRs of clindamycin, doxycycline, and pentamidine for the DA-resistant *B. gibsoni* strain were compared with those for the wild-type *B. gibsoni*. It was found that the DA-resistant *B. gibsoni* strain showed strong resistance against pentamidine, and weak resistance against

clindamycin and doxycycline. Those results suggested that pentamidine would have inhibitory effects against *B. gibsoni* similar to DA.

The mechanism of the action of DA against *B. gibsoni* is currently unknown. In contrast, those against *Trypanosoma* spp. and *Leishmania* spp. were reported to be the inhibition of DNA replication and mitochondrial respiratory activity (Bitonti *et al.*, 1986; Leon *et al.*, 1977; Macadam and Williamson, 1972; Newton and Le Page, 1978). On the other hand, pentamidine inhibits the DNA replication and mitochondrial respiratory activity of *Pneumocystis carinii* (Tidwell *et al.*, 1993), breaks the double-stranded DNA of Lewis lung carcinoma in a mouse tumor model *in vivo* (Chow *et al.*, 2004), inhibits protein biosynthesis in a cell-free rat liver system *in vitro* (Bielawski *et al.*, 2000), and alters the lipidic metabolism in *Leishmania donovani* and *L. amazonesis* (Basselin and Robert-Gero, 1998). The present results and those previous reports suggest that DA and pentamidine might affect *B. gibsoni* via the mechanisms of the action, such as inhibition of DNA replication, and mitochondrial respiration activity. Therefore, observation and comparison of metabolic pathways such as the DNA replication, mitochondrial respiration, and protein biosynthesis, of the DA-resistant and wild-type *B. gibsoni* strains should result in the elucidation of the

mechanism of DA resistance of the parasites. In addition, in the present study, it could not be determined whether the resistance of *B. gibsoni* against DA will be reversible or not. However, it is reported that mutations of ATP-Binding Cassette (ABC) transporter superfamily gene would relate to the pentamidine resistance of *Leishmania major* (Coelho *et al.*, 2003).The mutations of certain genes seem to be irreversible changes for the pathogens. Therefore, the analysis of some genes of the DA-resistant *B. gibsoni* strain will lead us to the elucidation of both the mechanism and reversibility of DA resistance of the parasites. Moreover, clindamycin and doxycycline inhibit protein synthesis in bacteria (Plumb, 2005a; Plumb, 2005b), and target the apicoplast of some Apicomplexan parasites (Dahl *et al.*, 2006; Wiesner *et al.*, 2008). In the present study, the DA-resistant *B. gibsoni* strain showed only weak resistance against those drugs. In this regards, the upregulation or downregulation of a certain transporter molecule, which transports those drugs, might result in the development of the drug resistance as a possible mechanism of drug resistance. For example, resistance of *Plasmodium falciparum* to chloroquine is associated with increased drug efflux, and the mediation of drug efflux is by an ATP-dependent efflux pump (Krogstad *et al.*, 1987). Moreover, overexpression of genes of the pump in some

chloroquine-resistant lines greatly adds to the circumstantial evidence that these genes mediate chloroquine resistance in these lines (Foote *at al.*, 1989; Wilson *et al.*, 1989). Therefore, it is possible that a certain transporter, which will transport DA, would be upregulated or downregulated in the DA-resistant *B. gibsoni* strain. In this instance, since pentamidine shares a similar structure with DA, that will be actively removed from the parasites, resulting in the strong resistance of the DA-resistant strain against pentamidine. In contrast, because clindamycin and doxycycline have a different structure from DA, those will be transported slightly. In the future, the discoveries of the characteristics, alternations, and gene mutations of DA-resistant strains will result in the development of novel and easy techniques for detecting the DA-resistant *B. gibsoni* strain. This will greatly contribute to decide the treatment strategies in clinical practice.

In the present study, a DA-resistant *B. gibsoni* strain were successfully developed *in vitro*. This resistant strain was continuously maintained in culture medium containing 200 ng/ml DA. Since the DA-resistant *B. gibsoni* strain exhibited strong resistance against not only DA but also pentamidine, these drugs appear to have shared mechanisms of action against *B. gibsoni*. Investigation of the metabolic pathways inhibited

by DA and pentamidine could lead to elucidation of the mechanism of DA resistance of *B. gibsoni*, resulting in the development of novel and effective antibabesial drugs.

Summary

An attempt was made to develop a strain of *Babesia gibsoni* resistant to diminazene aceturate (DA), an antibabesial drug, *in vitro*. Since the wild-type *B. gibsoni* survived and proliferated in culture medium containing 1 ng/ml DA, the concentration of DA was gradually increased from 1 to 200 ng/ml. As a result, the parasites survived and proliferated in the medium containing 200 ng/ml DA, which was much higher than the 50% inhibitory concentration (IC_{50}) of DA for *B. gibsoni*. Subsequently, these parasites were removed from erythrocytes and exposed directly to 200 ng/ml DA. They survived and invaded fresh erythrocytes, though the wild-type *B. gibsoni* did not survive. Based on these results, the parasites cultured in 200 ng/ml of DA were determined to be a DA-resistant *B. gibsoni* strain. In addition, IC_{50} levels of clindamycin, doxycycline, and pentamidine for the DA-resistant *B. gibsoni* strain were determined. The IC_{50} levels of clindamycin, doxycycline and pentamidine for the DA-resistant strain were higher than those for the wild-type *B. gibsoni*. The IC_{50} of pentamidine for the resistant strain was much greater than that for the wild-type *B. gibsoni*. These results indicated that the DA-resistant *B. gibsoni* strain could have resistance not only to DA, but also to other antibabesial drugs. In conclusion, a DA-resistant *B. gibsoni*

strain was successfully developed *in vitro*.

Chapter 2

Reduced transcript levels of the heat shock protein 70 gene in diminazene aceturate-resistant *Babesia gibsoni* variants under the low concentrations of diminazene aceturate

INTRODUCTION

Babesia gibsoni is an intraerythrocytic protozoan parasite that infects dogs and causes canine babesiosis. It is difficult to eliminate this parasite from infected dogs, though a number of drugs, including clindamycin, diminazene aceturate, metronidazole and pentamidine, are used for the treatment of the disease (Farwell *et al.*, 1982; Fowler *et al.*, 1972; Wulansari *et al.*, 2003). Recently, a new treatment strategy using atovaquone and azithromycin has been proposed (Matsuu *et al.*, 2004; Matsuu *et al.*, 2006; Sakuma *et al.*, 2009; Suzuki *et al.*, 2007); however, possible relapses and the development of atovaquone-resistant variants are also matters of concern.

Diminazene aceturate (DA), one of the most common antibabesial drugs, is an aromatic diamidine derivative. In chapter I, a DA-resistant *B. gibsoni* strain was developed *in vitro* (Hwang *et al.*, 2010). While developing the DA-resistant *B. gibsoni* strain, DA-resistant *B. gibsoni* variants, which were maintained in culture with DA from 1 to 175 ng/ml for more than 8 weeks were also obtained. Finally, the parasites cultured with 200 ng/ml of DA were determined to be a DA-resistant *B. gibsoni* strain. This DA-resistant strain could survived direct exposure to 200 ng/ml of DA. The DA-resistant strain had higher tolerance to antibabesial drugs such as clindamycin, doxycycline and pentamidine, than wild-type *B. gibsoni*;

however, the mechanism of resistance against DA in *B. gibsoni* remains to be elucidated.

Heat shock protein 70 (Hsp70), a 70-kDa Hsp, acts as a protein chaperone (Heike *et al.*, 1996) and plays important roles in cell proliferation and the control of cellular functions (Folgueira *et al.*, 2008; Lindquist, 1986; Song *et al.*, 2008). Previously, the sequence of the *Hsp70* gene of *B. gibsoni* (*BgHsp70*) was determined (Terkawi *et al.*, 2009; Yamasaki *et al.*, 2007; Yamasaki *et al.*, 2002). BgHsp70 is constitutively expressed at the erythrocyte stage. Moreover, when the temperature was elevated from 37°C to 42°C for 1 hr, both the gene transcription and protein synthesis of BgHsp70 were increased (Yamasaki *et al.*, 2008). Although the role and function of BgHsp70 remain largely unknown, the Hsp70 of *Plasmodium*, *Trypanosoma* and *Leishmania* species, which are important pathogens for humans and animals, might play important roles in survival and proliferation of these parasites within the host (Linquist, 1986). In *Plasmodium falciparum*, Hsp70 has also been proposed to play a role during the adaptation of those parasites to different environments such as elevation of temperature (Kumar *et al.*, 1991), suggesting that Hsp70 also contributes to the development of drug resistance. Indeed, Witkowski *et al.* (2010) showed the overexpression of Hsp70 in an artemisinin-tolerant *Plasmodium falciparum* strain. Previous studies also suggested that Hsp70 was involved in drug resistance in breast cancer cells (Fuqua *et al.*,

1994; Vargas-Roig *et al.*, 1998). A high nuclear proportion of Hsp70 in tumor cells correlated significantly with drug resistance. In bacteria, the Hsp70 family also has significant antibiotic action. A mutation in *dnaK* (*Hsp70*) in *Staphylococcus aureus* leads to significantly reduced survival after oxacillin treatment (Singh *et al.*, 2007). In addition, in *Escherichia coli*, the bactericidal action of fluoroquinolone is moderately affected by DnaK and GroEL (Hsp60) chaperones (Yamaguchi *et al.*, 2003). Considering these reports, it was expected that BgHsp70 is related to the development of resistance against DA in *B. gibsoni*. Thus, in the present study, changes in the transcription of the *BgHsp70* gene were examined during the development of the DA-resistant *B. gibsoni* strain, and it was found that the expression of the gene might be reduced when the parasites are exposed to the low concentration of DA.

MATERIALS AND METHODS

In vitro cultivation of wild-type *B. gibsoni*

The *B. gibsoni* parasites used in the present study was cultured as described in Chapter 1. Wild-type *B. gibsoni* were cultured in culture medium containing 1 ng/ml of DA for 14 days. Thin smear samples were made at days 0, 3, 6, 7, 10, 13 and 14, and parasitemia was calculated by counting the number of parasitized cells per 1,000 red blood cells (RBCs). Total RNA of cultured *B. gibsoni* was extracted at days 7 and 14 for the preparation of samples for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). This experiment was conducted 3 times.

In vitro cultivation of DA-resistant *B. gibsoni* variant

The DA-resistant *B. gibsoni* strain was developed in Chapter I. Briefly, the wild-type *B. gibsoni* strain was cultured the culture medium containing 1 ng/ml DA, and then the concentration of DA was gradually increased from 1 to 200 ng/ml (Hwang *et al.*, 2010). Thereafter, the DA-resistant *B. gibsoni* strain was maintained in culture medium containing 200 ng/ml DA and survived direct exposure to 200 ng/ml DA.

During the development of the DA-resistant *B. gibsoni* strain, DA resistant *B. gibsoni* variants were separated. When the concentration of DA was increased, the parasites needed roughly

2 weeks to adapt to each of the increased concentration of DA. These parasites, which adapted to 1, 30, 50, 75, 100, 125, 150, 175 and 200 ng/ml DA, were separated as DA1, DA30, DA50, DA75, DA100, DA125, DA150, DA175 and DA200 variants, respectively. The DA-resistant variants were maintained in culture with each concentration of DA for over 8 weeks, and were used for RNA extraction. The DA200 variant was determined to be the DA-resistant *B. gibsoni* strain as described above. Total RNA of each variant was extracted at day 7 after subculture for the preparation of samples for qRT-PCR. Experiments using DA1, DA30, DA50, DA75 and DA100 variants were conducted 3 times. Experiments using DA125, DA150, DA175 and DA200 variants were conducted 4 times.

Moreover, to examine the rapid response of the *BgHsp70* transcription against the high concentration of DA, the DA-resistant *B. gibsoni* strain (DA200 variant) and wild-type *B. gibsoni* were exposed to 500 ng/ml DA for 24 hr. Briefly, each culture medium was replaced with a new culture medium containing 500 ng/ml DA for 24 hr. Measurement of parasitemia and the extraction of total RNA of both wild-type *B. gibsoni* and DA-resistant strains were performed at hours 0, 1, 2, 4, 6, 12 and 24 of incubation.

Analysis of the transcription of the *BgHsp70* gene by qRT-PCR

The infected RBCs described above were harvested and lysed, and then the liberated parasites were pelleted and RNA was extracted

using an RNeasy mini kit (QIAGEN, Valencia, CA, U.S.A.). Consequently, cDNA was synthesized from the total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Tokyo, Japan). Reaction mixtures made according to the instruction manual were reacted (25°C for 4 min, 42°C for 30 min and 85°C for 5 min) using a Veriti™ 96 Well Thermal Cycler (Applied Biosystems, Tokyo, Japan). The quantity of the *BgHsp70* transcripts in a cDNA sample was measured by qRT-PCR according to the method of Yamasaki *et al.* (2008) with some modifications. PCR was performed with the resulting cDNA as a template and specific oligonucleotide primers (BgHsp70-1, 5'-AGGGTCGTCTTAGCACGAG-3'; BgHsp70-2, 5'-GTGCTTGGCTTCGACACAGC-3'). The reaction mixture containing SYBR® *Premix Ex Taq*™ (Perfect Real Time; TAKARA BIO Inc., Otsu, Japan) (2 x conc.), the PCR primers described above, ROX Reference Dye (50 x conc.) and 400 ng cDNA as a template was amplified with the ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Tokyo, Japan). After initial incubation for 10 sec at 95°C, the cDNA was subjected to 37 cycles of amplification. The program was as follows: denaturation at 95°C for 5 sec and reannealing and extension at 60°C for 31 sec. Continuous observation of amplifying DNA was performed with SYBR® *Premix Ex Taq*™. To confirm the specificity of the amplification product, melting curve analysis was performed. In addition, to check for DNA contamination in the water, primers and enzymes for PCR, PCR with water as a template was performed

every time. The quantity of *B. gibsoni* 18S ribosomal RNA (18S rRNA) was also measured by qRT-PCR using specific oligonucleotide primers (Bg18S-1, 5′-TCGTATTTAACTGTCAGAGG-3′; Bg18S-2, 5′-ACGGTATCTGATCGTCTTCG-3′). The copy numbers of the *BgHsp70* transcripts and the amounts of 18S rRNA in each cDNA sample were calculated by the method of Yamasaki *et al.* (2008). The relative amount of the copy number of the *BgHsp70* transcripts against the amount of *B. gibsoni* 18S rRNA was also measured.

Statistical analysis

Two-sample *t*-tests were used to compare the copy numbers of the *BgHsp70* transcripts, the amounts of 18S rRNA, the relative amounts of the *BgHsp70* transcripts and the levels of parasitemia in each *B. gibsoni* variant.

RESULTS

Change in the transcription of the *BgHsp70* gene, and the amount of 18S rRNA in wild-type *B. gibsoni* cultured with 1 ng/ml DA for 14 days

When wild-type *B. gibsoni* was cultured with 1 ng/ml DA, the parasites proliferated just like those cultured without DA (Fig. 7). The amount of 18S rRNA of *B. gibsoni* cultured with 1 ng/ml DA was significantly ($P < 0.05$) lower than that without DA at day 14 (Fig. 8A). In addition, the copy number of the *BgHsp70* transcripts was decreased, though not significantly, at day 14 when the parasites were cultured with 1 ng/ml DA (Fig. 8B). The relative number of the *BgHsp70* transcripts was also decreased at day 14 (Fig. 8C).

Transcription of the *BgHsp70* gene and the amount of 18S rRNA in DA-resistant *B. gibsoni* variants

While developing the DA-resistant *B. gibsoni* strain, the DA-resistant *B. gibsoni* variants were separated. Each DA-resistant variant was maintained in culture with the respective concentration of DA for more than 8 weeks before the examination of the transcription of the *BgHsp70* gene. To observe transcription in these variants, the copy numbers of the *BgHsp70* transcripts and the amounts of 18S rRNA were measured (Figs. 9 and 10, respectively).

The amounts of 18S rRNA in those variants cultured with various concentrations of DA were almost the same as in wild-type *B. gibsoni* (Figs. 9A and 10A).

The copy number of the *BgHsp70* transcripts in the DA1 variant was significantly ($P < 0.05$) lower than in wild-type *B. gibsoni* (Fig. 9B). The copy numbers of the *BgHsp70* transcripts in DA30, DA50 and DA75 variants appeared to increase with escalating doses of DA while that in the DA100 variant was slightly decreased; however, the copy numbers of the *BgHsp70* transcripts in these variants were lower than in wild-type *B. gibsoni*. The relative amounts of the *BgHsp70* transcripts in DA1 and DA30 variants were significantly ($P < 0.05$) lower than in wild-type *B. gibsoni* (Fig. 9C). The copy numbers of the *BgHsp70* transcripts in DA125, DA150, DA175 and DA200 variants were almost the same as in wild-type *B. gibsoni* (Fig. 10B); therefore, the relative numbers of the *BgHsp70* transcripts in those variants were almost the same as in wild-type *B. gibsoni* (Fig. 10C).

Transcription of the *BgHsp70* gene and the amount of 18S rRNA in wild-type *B. gibsoni* and DA-resistant *B. gibsoni* stains in the presence of 500 ng/ml DA for 24 hrs

To examine the rapid responses of the *BgHsp70* transcripts to a high concentration of DA, wild-type *B. gibsoni* and DA-resistant strains (DA200) were incubated in culture medium containing 500 ng/ml DA. When wild-type *B. gibsoni* and DA-resistant strains were

exposed to 500 ng/ml DA, their levels of parasitemia were not decreased within 24 hrs (Fig. 11A and 11B, respectively). In wild-type *B. gibsoni*, the amount of 18S rRNA was almost constant throughout the incubation period (Fig. 12A). The copy number of the *BgHsp70* transcripts appeared to have slightly decreased at hour 24, although it was nearly constant until hour 12 (Fig. 12B); therefore, the relative numbers of the *BgHsp70* transcripts were slightly, but not significantly, decreased at hour 24 (Fig. 12C).

In the DA-resistant *B. gibsoni* strain, the amount of 18S rRNA was also almost constant throughout the incubation period (Fig. 13A). There was a significant ($P < 0.05$) difference in the copy number of the *BgHsp70* transcripts at hour 6 between parasites cultured with 500 ng/ml DA and without DA (Fig. 13B). Otherwise, the copy number of the *BgHsp70* transcripts in the DA-resistant strain incubated with 500 ng/ml DA was nearly constant throughout the incubation period (Fig. 13B); therefore, the relative numbers of the *BgHsp70* transcripts were also almost constant (Fig. 13C).

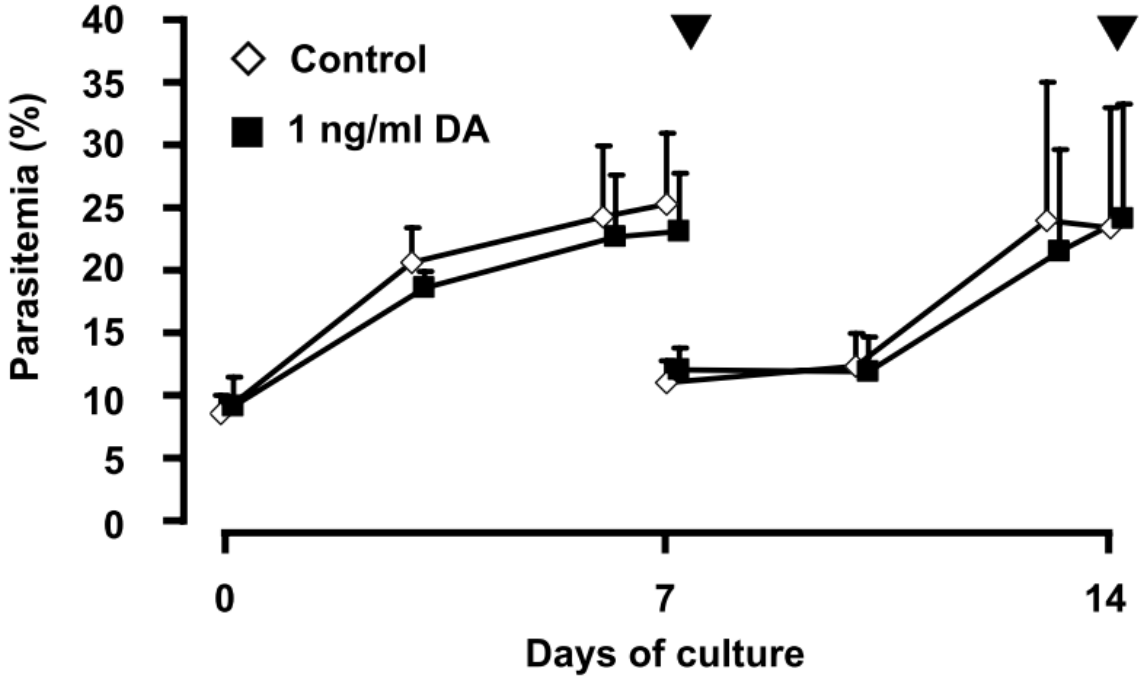

Fig. 7. Levels of parasitemia of wild-type *B. gibsoni* cultured with 1 ng/ml DA (closed square) and without DA (control, open diamond) for 14 days. Arrowheads indicate the points of subculture and sampling. Data are expressed as the means ± SD (n = 3).

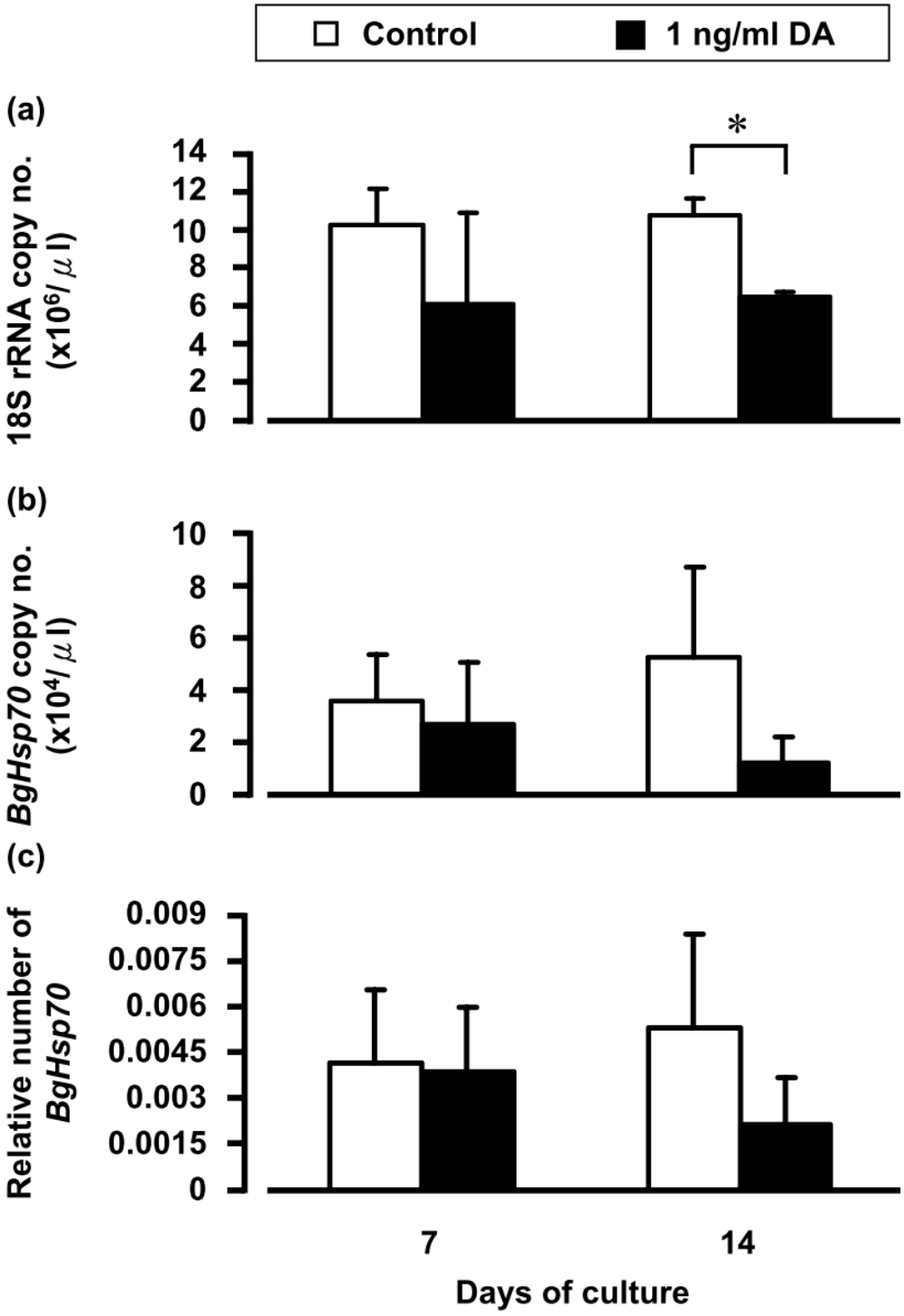

Fig. 8. Changes of the amount of *B. gibsoni* 18S rRNA (A), copy number of the *BgHsp70* transcripts (B) and relative numbers of the *BgHsp70* transcripts (C) in wild-type *B. gibsoni* cultured in culture medium containing 1 ng/ml DA (closed bar) and without DA (control, open bar) for 14 days. Data are expressed as the means ± SD (n = 3). *Significantly ($P < 0.05$) different from the control group.

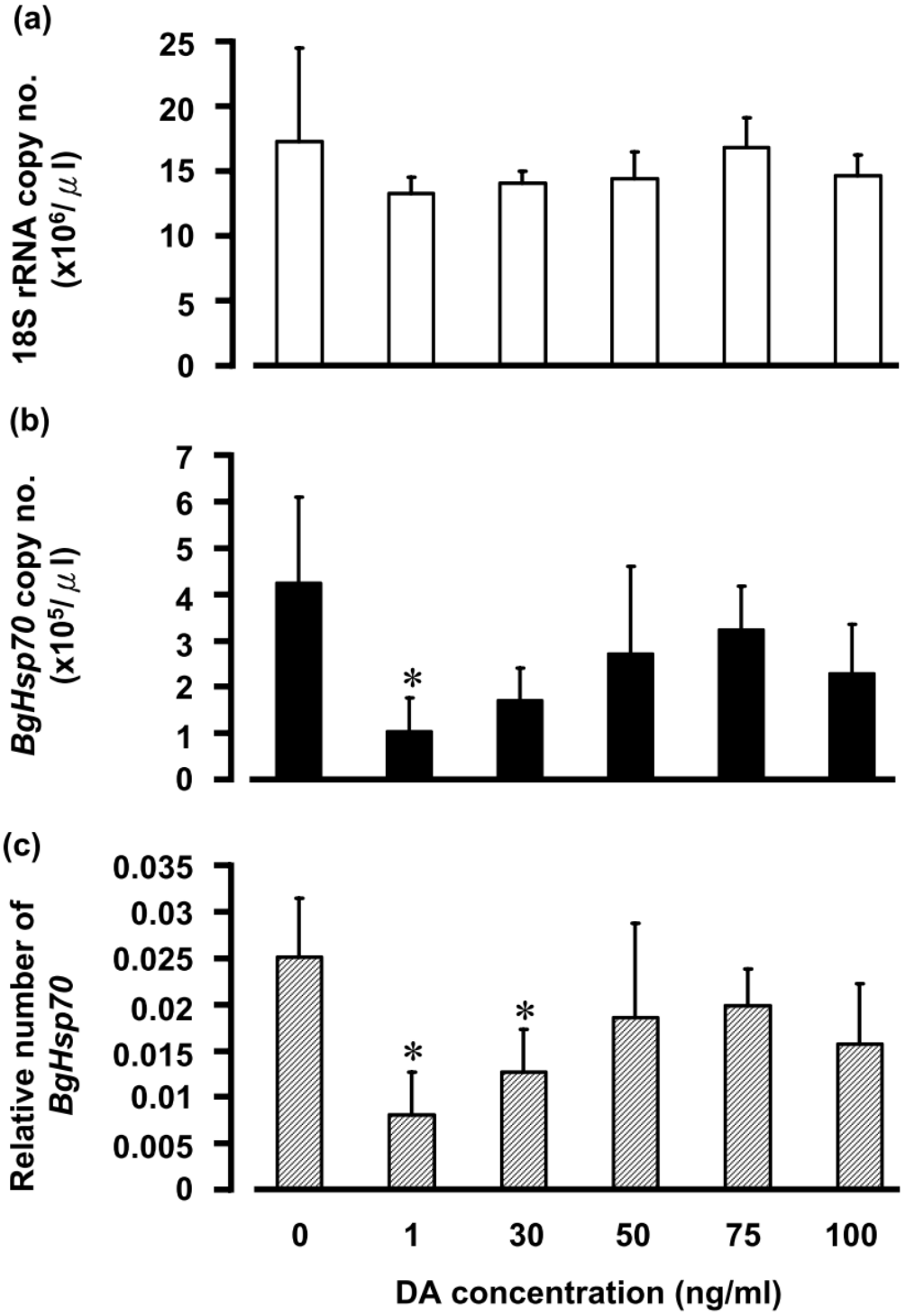

Fig. 9. The amounts of *B. gibsoni* 18S rRNA (A), copy numbers of the *BgHsp70* transcripts (B) and relative numbers of the *BgHsp70* transcripts (C) in DA0 (wild-type *B. gibsoni*), DA1, DA30, DA50, DA75 and DA100 variants of *B. gibsoni*, which were maintained in culture medium containing 0, 1, 30, 50, 75 and 100 ng/ml DA, respectively. Data are expressed as the means ± SD (n = 3). *Significantly ($P < 0.05$) different from the control group.

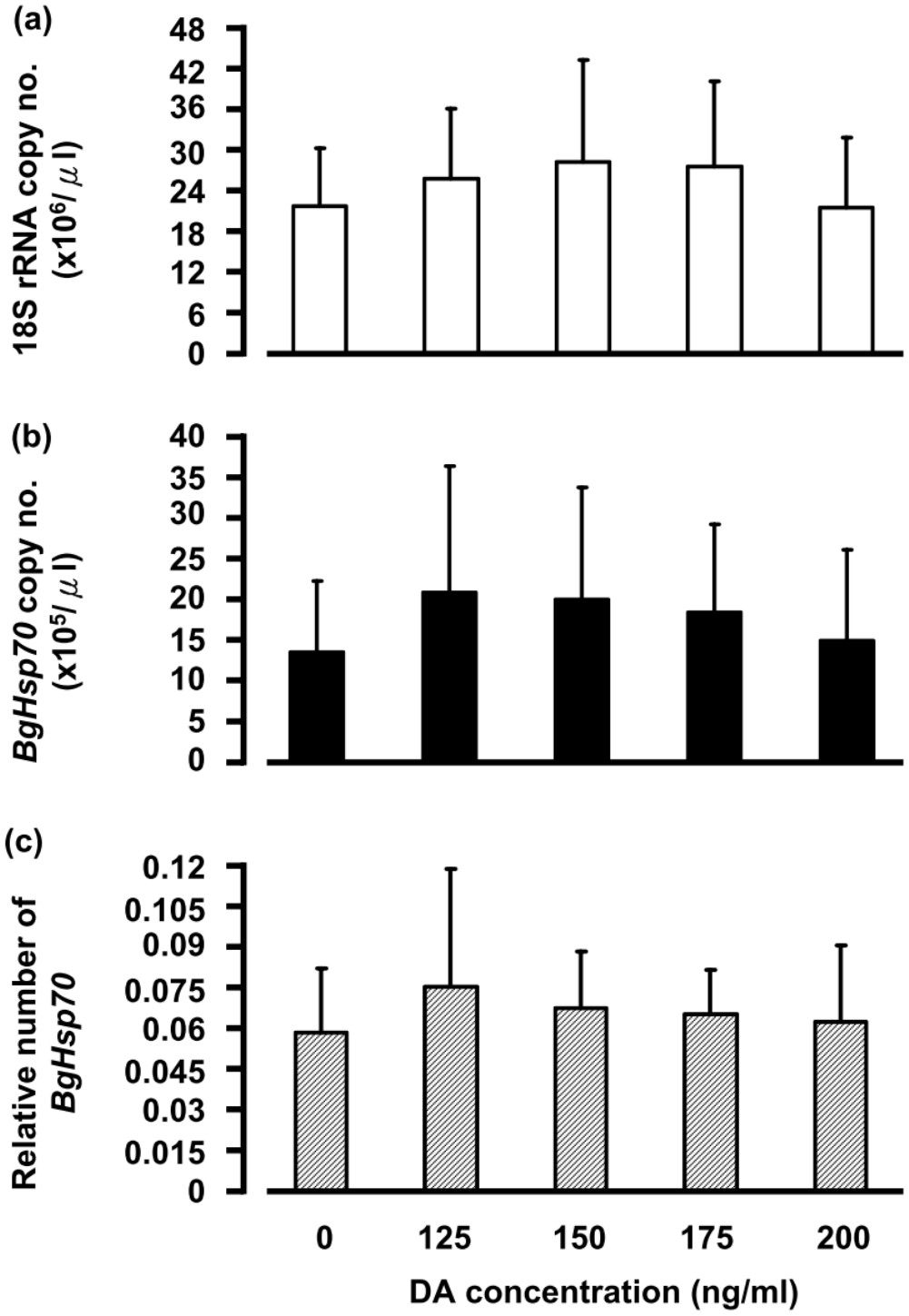

Fig. 10. The amounts of *B. gibsoni* 18S rRNA (A), copy numbers of the *BgHsp70* transcripts (B) and relative numbers of the *BgHsp70* transcripts (C) in DA0 (wild-type *B. gibsoni*), DA125, DA150, DA175 and DA200 (DA-resistant strain) variants of *B. gibsoni*, which were maintained in culture medium containing 0, 125, 150, 175 and 200 ng/ml DA, respectively. Data are expressed as the means ± SD (n = 4).

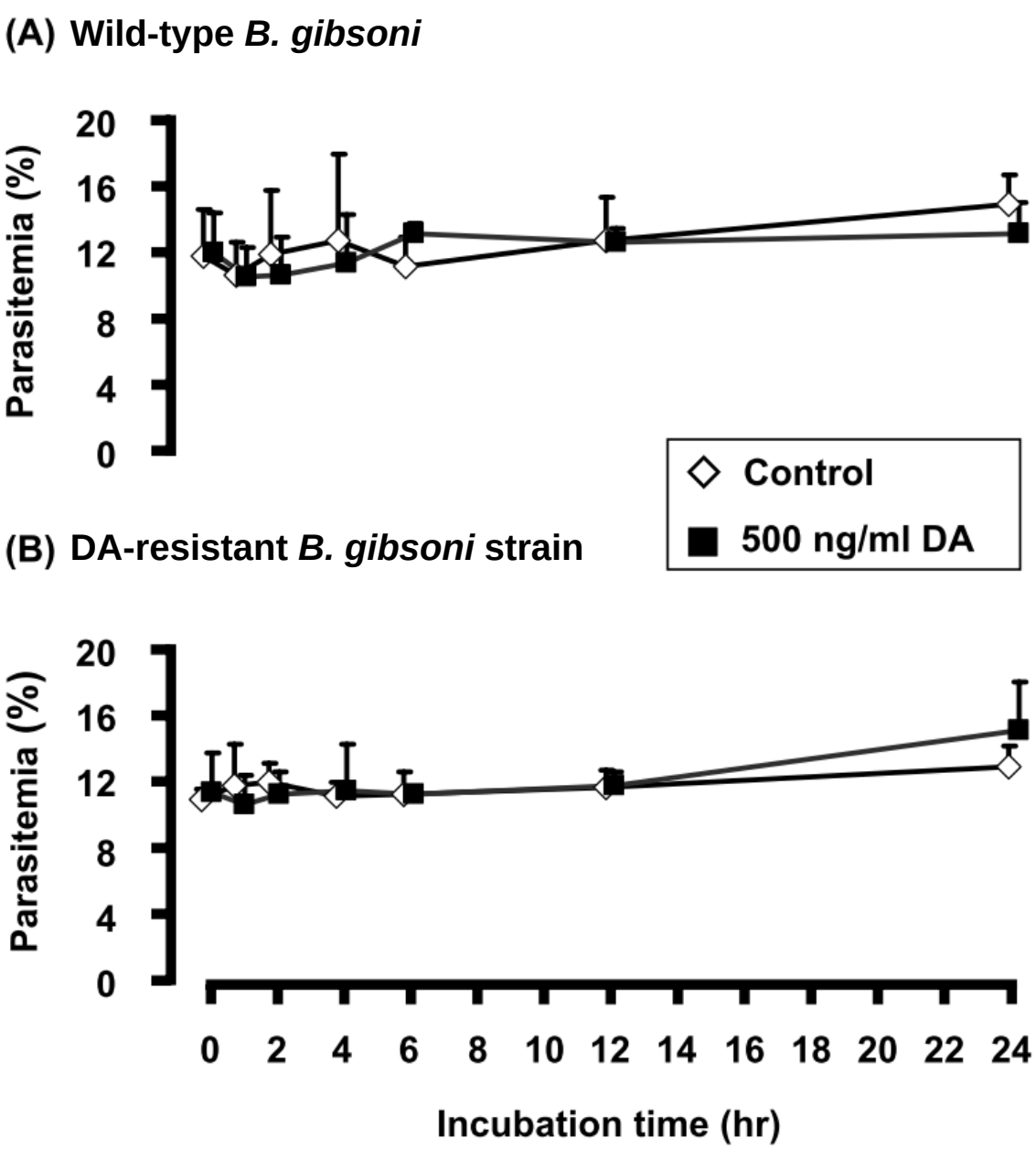

Fig. 11. Levels of parasitemia of wild-type *B. gibsoni* (A) and DA-resistant (B) *B. gibsoni* strains cultured with 500 ng/ml DA (closed square) and without DA (control, open diamond) for 24 hrs. Data are expressed as the means ± SD (n = 3).

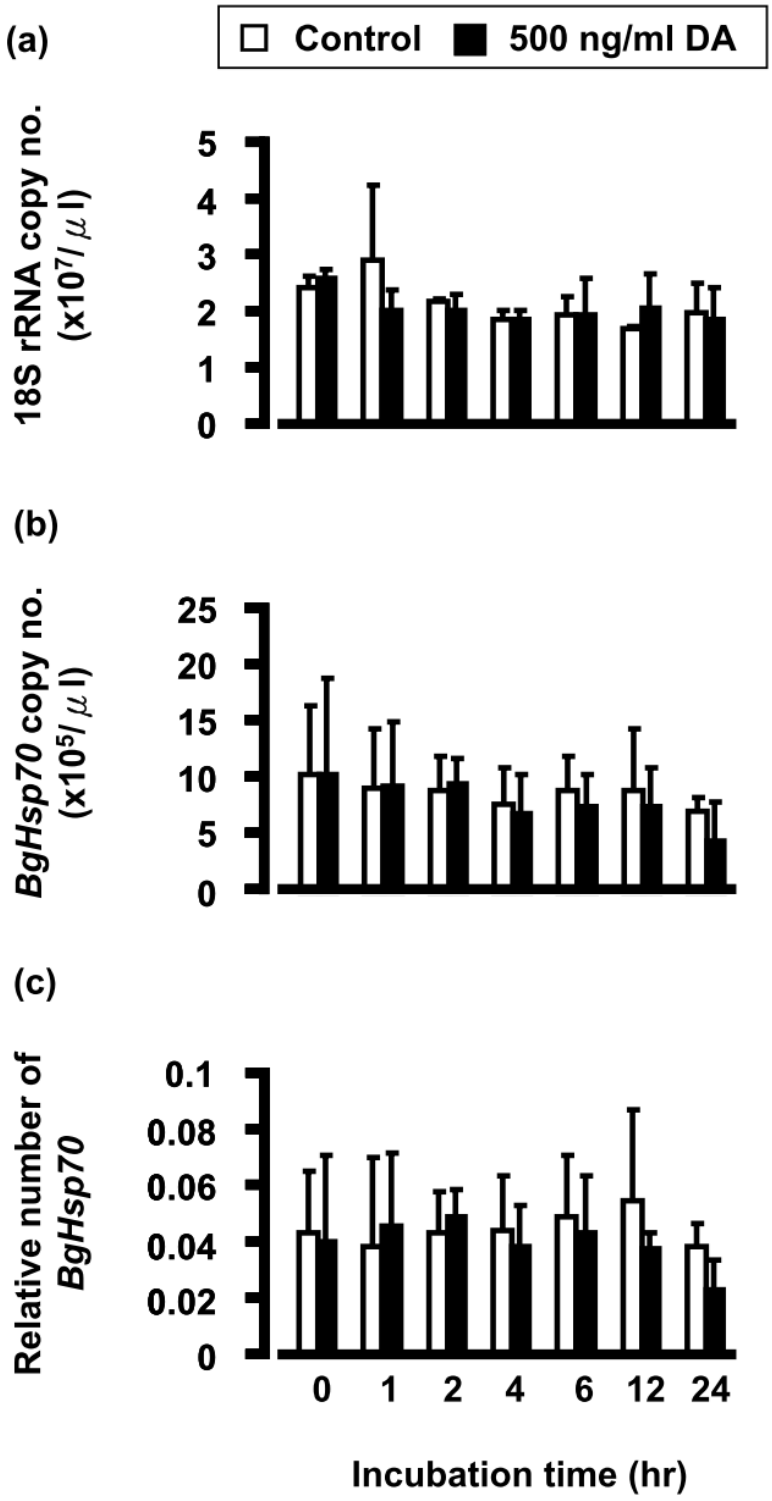

Fig. 12. The amounts of *B. gibsoni* 18S rRNA (A), copy numbers of the *BgHsp70* transcripts (B) and relative numbers of the *BgHsp70* transcripts (C) in wild-type *B. gibsoni* cultured with 500 ng/ml DA (closed bar) and without DA (control, open bar) were observed for 24 hrs. Data are expressed as the means ± SD (n = 3).

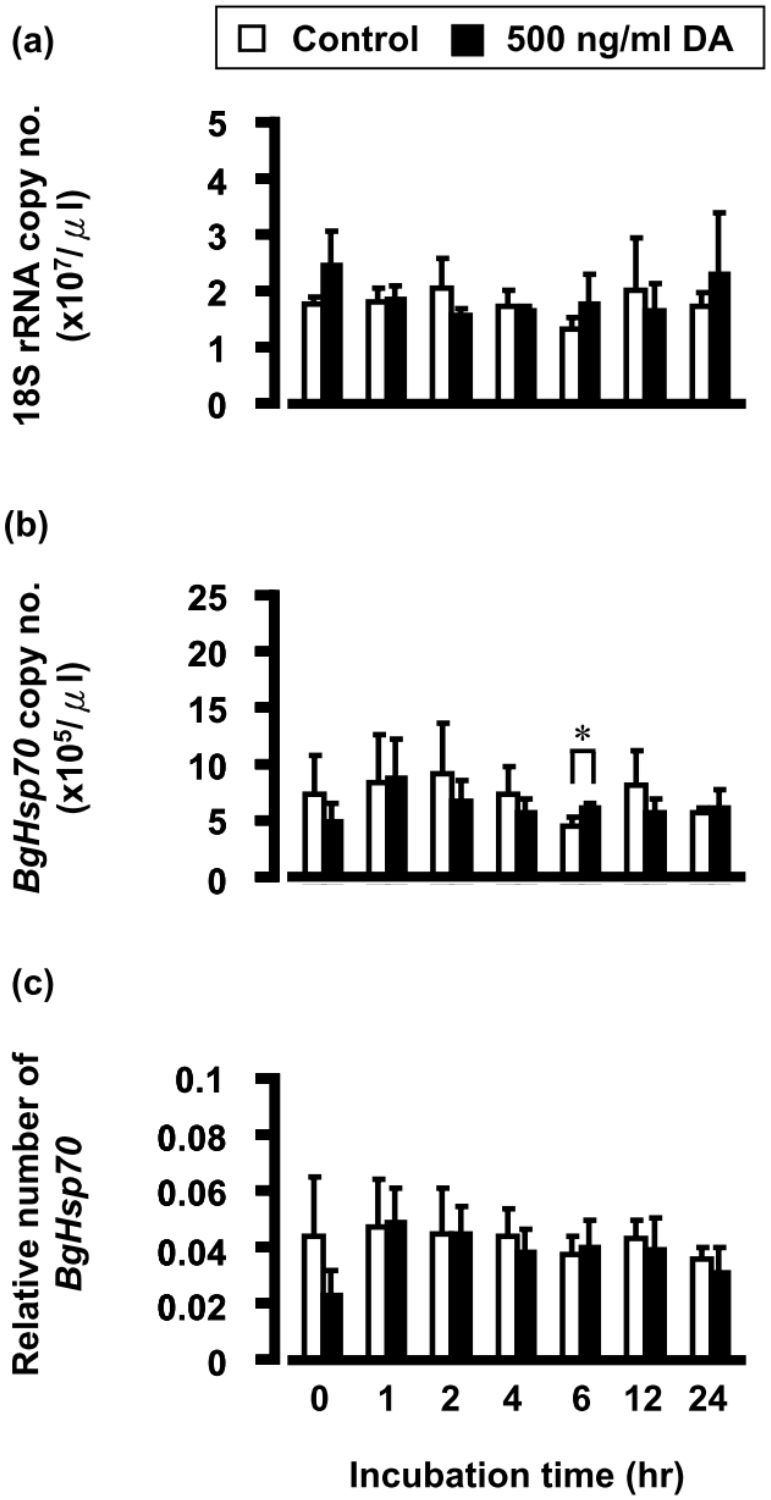

Fig. 13. The amounts of *B. gibsoni* 18S rRNA (A), copy numbers of the *BgHsp70* transcripts (B) and relative numbers of the *BgHsp70* transcripts (C) in the DA-resistant *B. gibsoni* strain cultured with 500 ng/ml DA (closed bar) and without DA (control, open bar) were observed for 24 hrs. Data are expressed as the means ± SD (n = 3). *Significantly ($P < 0.05$) different from the control group.

DISCUSSION

Heat shock proteins (Hsps) are considered to play a major role in the development of stress resistance and adaption to the environment. Elevated levels of Hsps have been proven to enhance tolerance to all kinds of stress (Lindquist, 1986). Furthermore, Hsp27 and Hsp90 were reported to be related to drug resistance. The increased expression of these Hsps participates in oncogenesis and in resistance to chemotherapy (Didelot *et al.*, 2007; Fuqua *et al.*, 1994; Vargas-Roig *et al.*, 1998); however, in the present study, the transcription of the *BgHsp70* gene of wild-type *B. gibsoni* was reduced by the relatively lower concentrations of DA such as that of 1 ng/ml. Similarly, the reduction of Hsps was reported for various organisms. Sørensen *et al.* (2003) reported in a review that the direction of evolutionary adaption of the Hsp levels seemed to be divergent. The lower Hsp70 expression in *Daphnia magna*, which is a standard test organism in aquatic toxicology, was associated with less stress sensitivity under long-term selection pressure exerted by environmental pollution (Haap and Köhler, 2009). In selection experiments with different species of *Drosophila*, the expression of Hsp70 was lower in lines that had previously been exposed to stressful conditions for many generations (Lansing *et al.*, 2000; Sørensen *et al.*, 1999). An experiment using North Sea mussels also showed that phenotypes displaying low Hsp levels appeared to be

evolutionarily favored (Brown *et al.*, 1995). From those reports and the present results it is hypothesized that the transcript levels of the *BgHsp70* gene would be reduced during the selection of the DA-resistant *B. gibsoni* strain under the long-term selection pressure of DA. It is also presumed that the transcription levels of the *BgHsp70* gene would return to the normal level after achieving resistance to DA; however, since the mechanism for the reduction of the *BgHsp70* transcription is still not clear, further study will be necessary to confirm this hypothesis.

In addition, the rapid response of the *BgHsp70* transcripts against the strong selection pressure of DA was examined. Yamasaki *et al.* (2008) reported that the gene transcription and protein synthesis of BgHsp70 were increased by a temperature shift for 1 hr. Therefore, a rapid response of the *BgHsp70* transcription was expected against the strong pressure of DA; however, the present results suggested that there was no rapid response of the *BgHsp70* transcription to this DA exposure in either wild-type *B. gibsoni* or the DA-resistant *B. gibsoni* strain. Since DA does not show antibabesial activity within 24 hrs, it would need more than 24 hrs to affect the proliferation of the parasites.

In the present study, the amounts of 18S rRNA in the DA-resistant variants were almost the same as in wild-type *B. gibsoni*. The amounts of 18S rRNA in parasites incubated with 500 ng/ml DA were also almost constant for 24 hrs; therefore, the

relative numbers of the *BgHsp70* transcripts were well consistent with the copy numbers of the *BgHsp70* transcripts in those studies. The amount of 18S rRNA has been used to adjust the transcript levels of the *BgHsp70* gene in *B. gibsoni* (Yamasaki *et al.*, 2008) and several genes in *Plasmodium falciparum* (Blair *et al.*, 2002; Nirmalan *et al.*, 2002; Yano *et al.*, 2005) by qRT-PCR. However, the amount of 18S rRNA was decreased when wild-type *B. gibsoni* were cultured with 1ng/ml DA. Yamasaki *et al.* (2008) reported that the amount of 18S rRNA was also decreased at a lower temperature. This previous report and the present results indicate that the amount of 18S rRNA changes with specific conditions. It is thus necessary to confirm the constancy of the amount of 18S rRNA in every experiment.

The mechanism of DA resistance in *B. gibsoni* remains to be clarified. Further analysis of the function of BgHsp70 will lead to elucidation of the mechanism of DA resistance in the parasites.

SUMMARY

In Chapter I, a diminazene aceturate (DA)-resistant *Babesia gibsoni* strain that was maintained in culture with 200 ng/ml DA was developed. While developing this strain, DA-resistant *B. gibsoni* variants, which were maintained in culture with DA from 1 to 175 ng/ml for more than 8 weeks were also obtained. Because heat shock protein 70 (Hsp70) seems to play important roles in adaptation to a stress environment in protozoan parasites, in the present study, the copy number of the *B. gibsoni Hsp70* (*BgHsp70*) transcripts of those DA-resistant variants were examined using quantitative real-time reverse transcription-polymerase chain reaction. It was found that when wild-type *B. gibsoni* was exposed to 1 ng/ml DA, the level of the *BgHsp70* transcripts was decreased at day 14. The copy number of the *BgHsp70* transcripts in the DA-resistant variant cultured with 1 ng/ml DA was significantly lower than in wild-type *B. gibsoni*, while those in DA-resistant variants increased with escalating doses of DA from 1 to 75 ng/ml, although they were lower than in wild-type *B. gibsoni*. However, those in DA-resistant variants cultured with > 125 ng/ml DA were almost the same as wild-type *B. gibsoni*. These results indicated that the transcripttion levels of the *BgHsp70* gene might be reduced when the parasites are exposed to a lower concentration of DA, and then might recover to the normal level after achieving resistance

against DA. It was expected that further study of the function of BgHsp70 will elucidate the mechanism of drug resistance against DA in *B. gibsoni*.

GENERAL CONCLUSION

Canine babesiosis caused by *Babesia gibsoni* is treated with diminazene aceturate (DA). DA can temporarily improve the clinical signs of canine babesiosis but is unable to eliminate the parasites from infected dogs, and relapses often occur. Therefore, it is believed that *B. gibsoni* might achieve the resistance against DA. However, there is no report clearly demonstrating the DA resistance in *B. gibsoni*. Therefore, in this study, a DA-resistant *B. gibsoni* strain was developed and the differences between the DA-resistant *B. gibsoni* strain and wild-type *B. gibsoni* were compared. First, a DA-resistant *B. gibsoni* strain was developed *in vitro* by the gradual increase of the DA concentration from 1 to 200 ng/ml. The parasites survived and proliferated in the medium containing 200 ng/ml DA, which is much higher than the 50% inhibitory concentration (IC_{50}) of DA for *B. gibsoni*. Subsequently, these parasites were removed from erythrocytes and exposed directly to 200 ng/ml DA. They survived and invaded fresh erythrocytes,

though wild-type *B. gibsoni* did not survive. Based on these results, the parasites cultured with 200 ng/ml DA were determined as a DA-resistant *B. gibsoni* strain. Thereafter, to investigate the characteristics of the DA-resistant *B. gibsoni* strain, the effects of other antibabesial drugs, including clindamycin, doxycycline, metronidazole and pentamidine, on the DA-resistant *B. gibsoni* strain were examined. The DA-resistant *B. gibsoni* strain showed strong resistance against pentamidine, and weak resistance against clindamycin and doxycycline. Moreover, the IC_{50} values of clindamycin, doxycycline and pentamidine for the DA-resistant strain at day 7 were higher than those for the wild-type *B. gibsoni*, respectively. These results indicated that the DA-resistant *B. gibsoni* strain could have resistance not only to DA, but also to other antibabesial drugs. Especially the DA-resistant *B. gibsoni* strain exhibited resistance against pentamidine, which shares similar structure with DA. In other protozoan, the mechanisms of drug resistance through mutations and/or amplification in drug transporters or drug

targets were demonstrated. Therefore, the analysis for those metabolic pathways in the DA-resistant *B. gibsoni* strain will lead to elucidate the mechanism of the action of DA against *B. gibsoni*.

Consequently, to characterize the DA-resistant *B. gibsoni* strain, the transcription levels of *B. gibsoni* heat shock protein 70 (*BgHsp70*) gene, which plays important roles in cell proliferation and the control of cellular function, was measured by quantitative real-time reverse transcription-polymerase chain reaction. In *Plasmodium falciparum*, Hsp70 has been proposed to contribute to the development of drug resistance. Therefore, the change in the transcription levels of the *BgHsp70* gene was analyzed in DA resistance. During the development of the DA-resistant *B. gibsoni* strain, DA-resistant *B. gibsoni* variants, which were maintained in culture with DA from 1 to 175 ng/ml for more than 8 weeks, were also obtained. The copy number of the *BgHsp70* transcripts in the DA-resistant variant cultured with 1 ng/ml DA was significantly lower than in wild-type *B. gibsoni* while

those in DA-resistant variants increased with escalating doses of DA from 1 to 75 ng/ml, though they were lower than in wild-type *B. gibsoni*. Moreover, those in DA-resistant variants cultured with > 125 ng/ml DA were almost the same as wild-type *B. gibsoni*. It is hypothesized that the transcription levels of the *BgHsp70* gene would be reduced during the selection of the DA-resistant *B. gibsoni* strain under the long-term weak pressure of DA, and then would be returned to the normal level after achieving resistance against DA. However, since the reason why the transcription levels of the *BgHsp70* gene was reduced is still unclear, further study will be necessary to confirm this hypothesis.

In conclusion, it was clearly demonstrated the development of DA resistance of *B. gibsoni in vitro*. The DA-resistant *B. gibsoni* strain obtained resistance against other antibabesial drugs. Moreover, the transcription levels of the *BgHsp70* gene was reduced by the weak DA pressure and then recovered when *B. gibsoni* had achieved resistance against DA. However, the role of BgHsp70 for the DA resistance in *B.*

gibsoni remains unclear. Further studies of BgHsp70 might prove to determine the mechanism of the DA resistance of *B. gibsoni*. Finally, the results obtained from this study could contribute to a better understanding of the DA resistance in *B. gibsoni in vitro*.

JAPANESE SUMMARY

Babesia gibsoni により引き起こされる犬バベシア症は抗バベシア原虫薬の酢酸ジミナゼン(diminazene aceturate, DA)の投与により治療するが、原虫を犬体内から完全に排除することは困難であり、再発を繰り返すとされている。この原因として、原虫が DA 耐性を獲得することが疑われているが、今まで実験的に DA 耐性株の作製を行った例や、その解析を行った報告はない。そこで本研究では、DA に対して薬剤耐性を示す *B. gibsoni* の作製を試み、その耐性株の特性を解析した。

初めに、培養にて維持している *B. gibsoni* を用いて、DA 耐性 *B. gibsoni* 株の作製を行った。このために、培養液に含まれる DA 濃度を 1 ng/ml から開始して除々に増加させ、最終的に 200 ng/ml まで増加させた。さらに、200 ng/ml の DA を含んだ培養液中で維持している原虫を感染赤血球から分離して 200 ng/ml の DA を含む培養液に直接曝露し、原虫の赤血球への再侵入と増殖を観察した。その結果、DA に耐性を持たない *B. gibsoni* (野生株)は、同様の実験で生存できなかったが、200 ng/ml の DA で維持している原虫は、新しい赤血球に侵入し、増殖した。*B. gibsoni* に対する DA の 50%の阻害濃度(IC_{50})は 45.89 ng/ml あるいは 103 ng/ml と報告されているため、これは DA 耐性株であると考えられた。以上より、この *B. gibsoni* は DA に対する耐性を獲得したと考えられた。

次に、この DA 耐性 *B. gibsoni* 株の特徴を明らかにするため、様々な種類の作用機序を持つ抗バベシア原虫薬に対する DA 耐性 *B. gibsoni* 株の抵抗性を比較した。すなわち、作製した DA 耐性 *B. gibsoni* 株及び野生株をクリンダマイシン、ドキシサイクリン、メトロニダゾール及びペンタミジンをそれぞれ含む

培養液中にて 7 日間培養し、各薬剤に対する抵抗性を比較した。その結果、DA 耐性 *B. gibsoni* 株はクリンダマイシン、ドキシサイクリンに対して弱い抵抗力を示し、ペンタミジンに対して強い抵抗性を示した。特に DA と類似の作用機序を持つペンタミジンに対しては高い抵抗性を示し、同系統の薬剤に対して強い耐性を示すと考えられた。さらに、それぞれの薬剤に対する DA 耐性 *B. gibsoni* 株の IC_{50} は上昇した。よって、以上の薬剤の作用する代謝経路を比較することで、DA の *B. gibsoni* に対する作用機序や、DA 耐性の機序が明らかになることが期待できる。

さらに、この DA 耐性 *B. gibsoni* 株の特徴を明らかにするため、細胞機能に重要な役割を果たすとされている熱ショックタンパク質 70 (heat shock protein 70, Hsp70)遺伝子の転写量を定量的リアルタイム PCR 法(quantitative real-time reverse transcription-polymerase chain reaction; qRT-PCR)で測定した。*Plasmodium falciparum* において Hsp70 は薬剤耐性の獲得に貢献しているとの報告があり、*B. gibsoni* の Hsp70 (BgHsp70)も DA 耐性の獲得に貢献していることが期待された。DA 耐性 *B. gibsoni* 株を作製する過程で、1 ng/ml から 175 ng/ml までの DA 存在下で 8 週間以上維持している DA 耐性 *B. gibsoni* 変異株を分離した。これらの変異株の *BgHsp70* 遺伝子の転写量を測定したところ、1 ng/ml の DA 存在下で維持している変異株の *BgHsp70* 遺伝子転写量は野生株に比べて有意に低く、DA 濃度が 1 ng/ml から 75 ng/ml まで増加するにつれて *BgHsp70* 遺伝子の転写量が上昇した。さらに、培養液中の DA 濃度が 125 ng/ml 以上で維持している変異株では、*BgHsp70* 遺伝子の転写量が野生株と同程度であった。以上の結果より、DA 耐性 *B. gibsoni* 変異株の *BgHsp70* 遺伝子の転写量は低濃度での DA に長時間曝されることで減少し、DA 耐性を獲得した後に野生株と同程度に回復することが推測された。しかしながら、*BgHsp70* 遺伝子の転写量が減少

した原因はまだ解明されていない。今後、BgHsp70 の機能を明らかにすることで、DA 耐性の機序が明らかになるかもしれない。

本研究では、DA 耐性 *B. gibsoni* 株の作製に成功した。この DA 耐性 *B. gibsoni* 株は、DA のみならず、他の抗バベシア原虫薬に対する抵抗性も増加していた。さらに、低濃度の DA により、*BgHsp70* 遺伝子の転写量が減少することも明らかになった。しかしながら、DA 耐性の機序は明らかになっておらず、さらなる研究が必要である。本研究で得られた成績は、今後 *B. gibsoni* における DA 耐性の機序を解明する上で重要な知見と考えられる。

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