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

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**ABSTRACT.** We attempted to develop a strain of *Babesia gibsoni* resistant to diminazene aceturate (DA), an anti-babesial drug, *in vitro*. Since the DA-sensitive *B. gibsoni* strain could survive and proliferate in culture medium containing 1 ng/ml DA, the concentration of DA was gradually increased from 1 to 200 ng/ml. The results showed that the parasites could survive and proliferate in the medium containing 200 ng/ml DA, which was much higher than the 50% inhibitory concentration (IC₅₀) of DA for *B. gibsoni*. Subsequently, these parasites were removed from erythrocytes and exposed directly to 200 ng/ml DA. They were able to survive and invade fresh erythrocytes, though the DA-sensitive *B. gibsoni* strain did not survive. Based on these results, the parasites cultured within 200 ng/ml DA were determined to be a DA-resistant *B. gibsoni* strain. In addition, the IC₅₀ levels of clindamycin, doxycycline and pentamidine for the DA-resistant *B. gibsoni* strain were determined. The IC₅₀ levels of clindamycin, doxycycline and pentamidine for the DA-resistant strain were higher than those for the DA-sensitive strain. The IC₅₀ of pentamidine for the resistant strain was much greater than that for the DA-sensitive strain. These results indicated that the DA-resistant *B. gibsoni* strain could have resistance not only to DA, but also to other anti-babesial drugs. In conclusion, we successfully developed a DA-resistant *B. gibsoni* strain *in vitro*.

**KEY WORDS:** *Babesia gibsoni*, diminazene aceturate, drug resistance.

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Canine babesiosis, a tick-borne hematozoan disease, is caused by *Babesia gibsoni* and *B. canis*. This disease is characterized by fever, lethargy, anemia and, in severe cases, death [9, 11]. Diminazene aceturate (DA), an anti-babesial drug, is an aromatic diamidine derivative. Currently, the mechanism of action of DA on *B. gibsoni* and *B. canis* is unknown. DA can temporarily improve the clinical signs of canine babesiosis [4, 13]. However, this drug is unable to eliminate the parasites from infected dogs, and relapses often occur [11]. We believe that this is due to the development of drug resistance of *B. gibsoni* against DA. Collett [8] 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 has been reported that DA can inhibit the DNA replication and mitochondrial respiratory activity of these pathogens [3, 16]. The loss of P2 nucleoside transporter function in *Trypanosoma brucei brucei* has been implicated in resistance to DA [6]. Likewise, it is possible that *B. gibsoni* could develop drug resistance against DA.

In reports about other anti-babesial 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 development of resistant variants remained [11, 13, 24, 27, 32]. 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 anti-babesial drugs, it is necessary to clarify the occurrence and mechanism of drug resistance of *B. gibsoni*.

In the present study, we attempted to develop a strain of *B. gibsoni* 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 parasites resistant to DA were investigated to characterize the 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 in our laboratory [33]. The parasites were incubated at 38°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ in a culture medium consisting of RPMI-1640 (Invitrogen, Carlsbad, CA, U.S.A.), 20% dog serum and canine HK red blood cells (RBCs) that contain a high concentration of potassium [14], sufficient to yield a packed cell volume (PCV) of 5%. Every 24 hr, 60% of the culture supernatant was removed and replaced with an equal volume of fresh culture medium [34]. 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. gibboni in the medium containing diminazene aceturate: Diminazene aceturate (DA; Novartis, Tokyo, Japan), which is an anti-babesial drug, was used. First, 0, 1, 10, 100 or 1,000 ng/ml DA was added to the culture medium, and B. gibboni, which is sensitive to DA (DA-sensitive B. gibboni strain), was cultured in the culture medium. Because the parasites were only able to survive and proliferate 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. gibboni 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. gibboni, 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. gibboni cultured in 200 ng/ml DA were isolated from infected host cells by the method of Sugimoto et al. [26] with some modifications. A suspension of infected erythrocytes was centrifuged at 800 × g for 5 min at 4°C. After 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. Hemolysin was then added to a 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 a final concentration of 5 mM. The samples were then centrifuged at 5,000 × 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 × 10^7/ml 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 hr, and the level of parasitemia was determined by counting the number of parasitized erythrocytes per 1,000 erythrocytes. In addition, B. gibboni 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.

Effects of other anti-babesial drugs against the diminazene aceturate-resistant B. gibboni strain in vitro: The growth-inhibitory effects of four drugs, clindamycin hydrochloride (Pfizer, Tokyo, Japan), doxycycline hydrochloride (MP Biomedicals, LLC, Santa Ana, CA, U.S.A.), metronidazole (Sigma-Aldrich, Tokyo, Japan) and pentamidine isothionate salt (Sigma-Aldrich), against the DA-resistant B. gibboni strain were compared with those against a DA-sensitive strain. These drugs were diluted in a small quantity of dimethyl sulfoxide (DMSO) and further diluted in the culture medium. Cultured erythrocytes infected with either the DA-resistant or DA-sensitive B. gibboni strain were collected and washed twice with a modified Vega y Martinez phosphate-buffered saline solution (mVYM solution) [29] and once with RPMI-1640. After washing, the infected erythrocytes were resuspended to a final packed cell volume of 5% in culture medium containing one of the drugs. The 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 isothionate 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 hr, 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 hr, 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. gibboni were calculated from the level of parasitemia by using the formula given below (Eq. 1). The 50% inhibitory concentration (IC50) on day 7 of culture was calculated using probit analysis. We performed one experiment in duplicate and repeated the same experiment 3 times. In the analysis, all data from the 3 experiments were used in one analysis.

\[
\text{GIR} = \frac{(\text{Parasitemia of control group} - \text{Parasitemia of DA group})}{\text{Parasitemia of control group}} \\
\times 100\%
\]

Statistical analysis: Statistical analysis was performed by using a paired t-test to compare the levels of parasitemia among different concentrations of each drug. Two-sample t-tests were used to compare the levels of GIRs between the DA-resistant and DA-sensitive B. gibboni strains.

RESULTS

Development of a strain of B. gibboni resistant to diminazene aceturate: To develop a DA-resistant B. gibboni strain, B. gibboni was cultured in culture media containing various concentrations of DA. When the DA-sensitive B. gibboni strain 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, we 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 MATERIALS AND METHODS (Fig. 2). When the concentration of DA was increased from 1 to 5 ng/ml at week 1 (Fig. 2B), the parasites could not proliferate during weeks 2 to 3 (Fig. 2A). 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 were unable to proliferate in week 10. Decreasing the
The DA concentration to 10 ng/ml resulted in proliferation of the 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 were able to 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 DA-sensitive parasites removed from erythrocytes were designated DA-sensitive erythrocyte-free parasites. When the DA-sensitive erythrocyte-free parasites were cultured with fresh uninfected erythrocytes without DA as a positive control, the parasites were able to invade erythrocytes and proliferate well after day 2 of 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 observed 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 observed on day 4 of culture, and the level of parasitemia then increased to 1.9 ± 0.81% on day 10 of culture (Fig. 3). Because these parasites maintained in culture medium containing 200 ng/ml DA were able to survive direct exposure to DA, they were considered to be a DA-resistant *B. gibsoni* strain.

**Effects of other anti-babesial drugs on diminazene aceturate-resistant *B. gibsoni* strain in vitro:** To investigate the characteristics of the DA-resistant *B. gibsoni* strain, the effects of other anti-babesial drugs on it were observed. In the present study, we calculated the GIRs from the number of parasitized erythrocytes and compared those of the DA-resistant and DA-sensitive *B. gibsoni* strains. When the
DA-resistant and DA-sensitive *B. gibsoni* strains were cultured in medium containing clindamycin, the GIRs gradually increased throughout the culture period (Fig. 4). The GIRs for the DA-resistant *B. gibsoni* strain were slightly lower than those for the DA-sensitive strain at 150, 200 and 250 µg/ml clindamycin (Fig. 4). The GIR for the DA-resistant strain at 250 µg/ml clindamycin on day 7 (43.1 ± 8.96%) was significantly lower than that for the DA-sensitive strain (68.0 ± 6.12%). The GIRs for the DA-resistant *B. gibsoni* strain at 150 µg/ml clindamycin on days 4 and 6 and those at 200 µg/ml clindamycin on days 6 and 7 were also significantly lower (*P*<0.05) than those for the DA-sensitive *B. gibsoni* strain (Fig. 4). Moreover, the IC$_{50}$ of clindamycin for the DA-resistant *B. gibsoni* strain on day 7 was higher than that for the DA-sensitive *B. gibsoni* strain (Table 1). The values in the brackets in Table 1 are the calculated IC$_{50}$ values of the drugs. Since the values were higher or lower than the used concentration of each drug, we believed that they were probably not accurate.

When the DA-resistant and DA-sensitive *B. gibsoni* strains were cultured in medium containing doxycycline, the GIRs also gradually increased over the culture period (Fig. 5). The GIRs for the DA-resistant *B. gibsoni* strain at both 5 and 10 µg/ml doxycycline were almost the same as those for the DA-sensitive strain. On the other hand, the GIRs for the DA-resistant *B. gibsoni* strain at both 20 and 40 µg/ml doxycycline on days 6 and 7 were significantly lower (*P*<0.05) than those for the DA-sensitive strain. The GIR for the DA-resistant strain at 40 µg/ml doxycycline on day 7 (52.4 ± 2.24%) was significantly lower than that for the DA-sensitive strain (80.3 ± 7.02%). The IC$_{50}$ of doxycycline for the DA-resistant *B. gibsoni* strain on day 7 was higher than that for the DA-sensitive *B. gibsoni* strain (Table 1). When the DA-sensitive *B. gibsoni* strain was cultured in medium containing pentamidine, the GIRs also gradually increased throughout the culture period (Fig. 6).
taining pentamidine, the GIRs for the DA-resistant strain were obviously lower than those for the DA-sensitive strain (Fig. 6). Although the GIRs for the DA-resistant strain at 200, 300 or 400 ng/ml pentamidine also gradually increased throughout the culture period, that at 100 ng/ml pentamidine hardly increased. The GIRs for the DA-resistant strain on days 4 to 7 at 100 ng/ml pentamidine, those on days 5 to 7 at 200 ng/ml pentamidine, and those on days 3 to 7 at both 300 and 400 ng/ml pentamidine were significantly lower (P<0.05) than those for the DA-sensitive strain (Fig. 6). The GIR for the DA-resistant strain at 400 ng/ml pentamidine on day 7 (43.5 ± 7.16%) was significantly lower than that for the DA-sensitive strain (84.7 ± 2.21%). The IC50 of pentamidine for the DA-resistant strain was over 400 μg/ml, which was higher than that for the DA-sensitive strain (Table 1).

In the present study, even 800 μg/ml metronidazole did not affect the proliferation of the DA-sensitive strain (data not shown). Furthermore, erythrocytes were lysed in the culture medium containing 800 μg/ml metronidazole. Therefore, the IC50 of metronidazole for both the DA-sensitive and DA-resistant strains were not determined.

**DISCUSSION**

In the present study, we succeeded in developing a DA-resistant strain. First, a DA-sensitive strain was cultured in culture medium with 1 ng/ml DA, and the DA concentration was gradually raised. Finally, the parasites were able to proliferate in culture medium containing 200 ng/ml DA after day 420 of culture. These parasites were maintained in 200 ng/ml DA thereafter. Since the previously reported IC50 values of DA for B. gibsoni include 88.43 ± 10.94 nM, which corresponds to 45.59 ng/ml [20], 89.02 ± 17.29 nM, which corresponds to 45.89 ng/ml [18], and 103 ± 12 ng/ml [25], respectively, these parasites, which have IC50 values of DA that are higher than for the normal B. gibsoni strain, are considered to have resistance against DA. The 200 ng/ml DA in the culture medium in this study was much higher than the reported IC50 values. Thus, this B. gibsoni strain maintained in 200 ng/ml DA was able to survive and proliferate in a concentration of DA adequate to inhibit proliferation of the DA-sensitive strain 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 come into direct contact with DA 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 DA-sensitive 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

### Table 1. The 50% inhibitory concentrations (IC50) of clindamycin, doxycycline, metronidazole and pentamidine for the DA-resistant and DA-sensitive B. gibsoni strains on day 7 of culture

<table>
<thead>
<tr>
<th>Anti-babesial drugs</th>
<th>DA-resistant B. gibsoni strain</th>
<th>DA-sensitive B. gibsoni strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clindamycin (μg/ml)</td>
<td>&gt; 250 (336.8)α)</td>
<td>120.42</td>
</tr>
<tr>
<td>Doxycycline (μg/ml)</td>
<td>24.49</td>
<td>10.01</td>
</tr>
<tr>
<td>Metronidazole (μg/ml)</td>
<td>NDβ)</td>
<td>NDβ)</td>
</tr>
<tr>
<td>Pentamidine (ng/ml)</td>
<td>&gt; 400 (487.08)α)</td>
<td>&lt; 100 (40.09)α)</td>
</tr>
</tbody>
</table>

a) Values were calculated using probit analysis, but were larger or smaller than the tested concentration of each drug.
b) Not determined. Experiments were not performed.
c) Not determined. Erythrocytes were lysed by metronidazole in the tested concentration.
could remain alive and infective. On the other hand, when the DA-sensitive erythrocyte-free parasites were cultured with DA, all of the parasites were destroyed, indicating that the DA-sensitive 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 and 200 ng/ml DA, they 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.

Although atovaquone, which is an anti-protozoal drug, combats B. gibsoni infection in dogs, the sensitivity of B. gibsoni to atovaquone is decreasing [18]. Moreover, Matsumoto et al. [19] and Sakuma et al. [24] 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 addition, there is no report proving the resistance of B. gibsoni to any anti-babesial drugs. Accordingly, this is the first report clearly demonstrating drug resistance of B. gibsoni in vitro. However, we could not determine whether all the parasites developed resistance to DA or the DA-resistant parasites were selected from a mixed population of parasites. Nevertheless, according to the results from the present study, we suspect that these parasites, which are strongly resistant to DA, might be selectable 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 a low concentration of DA. Consequently, the effect of DA on B. gibsoni in vitro would be decreasing if the DA concentration in the peripheral blood is maintained over 1 ng/ml for a few days in clinical cases. Further studies will be necessary to clarify this hypothesis.

To investigate the characteristics of the DA-resistant B. gibsoni strain, we compared the GIRs of clindamycin, doxycycline and pentamidine for the DA-resistant B. gibsoni strain with those for the sensitive strain. We found that the DA-resistant B. gibsoni strain showed strong resistance against pentamidine and weak resistance against clindamycin and doxycycline. These results suggested that pentamidine would have inhibitory effects against B. gibsoni similar to DA.

The mechanism of action of DA against B. gibsoni is currently unknown. In contrast, those against Trypanosoma spp. and Leishmania spp. were reported to be inhibition of DNA replication and mitochondrial respiratory activity [3, 16, 17, 21]. On the other hand, pentamidine inhibits the DNA replication and mitochondrial respiratory activity of Pneumocystis carinii [28], breaks the double-stranded DNA of Lewis lung carcinoma in a mouse tumor model in vivo [7], inhibits protein biosynthesis in a cell-free rat liver system in vitro [2] and alters lipicid metabolism in Leishmania donovani and L. amazonensis [1]. The present results and those of previous reports suggest that DA and pentamidine might affect B. gibsoni via mechanisms of action such as inhibition of DNA replication and via mitochondrial respiration activity. Therefore, observation and comparison of metabolic pathways such as DNA replication, mitochondrial respiration and protein biosynthesis of the DA-resistant and DA-sensitive B. gibsoni strains should result in elucidation of the mechanism of DA resistance of the parasites. In addition, in the present study, we could not determine whether or not the resistance of B. gibsoni against DA is reversible. However, it has been reported that mutations of ATP-Binding Cassette (ABC) transporter superfamily gene would relate to the pentamidine resistance of Leishmania major [5]. The mutations of certain genes seem to be irreversible for the pathogens. Therefore, analysis of some genes of the DA-resistant B. gibsoni strain will lead us to elucidation of both the mechanism and reversibility of the DA resistance of the parasites. Moreover, clindamycin and doxycycline inhibit protein synthesis in bacteria [22, 23] and target the apicoplast of some Apicomplexan parasites [10, 30]. 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. For example, resistance of Plasmodium falciparum to chloroquine is associated with increased drug efflux, and drug efflux is mediated by an ATP-dependent efflux pump [15]. 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 [12, 31]. Therefore, it is possible that a certain transporter that transports DA is upregulated or downregulated in the DA-resistant B. gibsoni strain. In this case, since pentamidine shares a similar structure with the DA, it would 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, they would be transported slightly. In the future, discovery of the characteristics, alternations and gene mutations of the DA-resistant strains will result in development of novel and easy techniques for detecting DA-resistant B. gibsoni strains. This will greatly contribute to determining treatment strategies in clinical practice.

In the present study, we succeeded in developing a DA-resistant B. gibsoni strain in vitro. This resistant strain was continuously maintained in culture medium containing 200 ng/ml DA. Since it 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 anti-babesial drugs.

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