

## Evaluation of Efficacy of Bruceine A, a Natural Quassinoid Compound Extracted from a Medicinal Plant, *Brucea javanica*, for Canine Babesiosis

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(Received 30 June 2008/Accepted 25 August 2008)

**ABSTRACT.** Bruceine A, a natural quassinoid compound extracted from the dried fruits of *Brucea javanica* (L.) Merr., was evaluated for its antibabesial activity *in vitro* and *in vivo*. Bruceine A inhibited the *in vitro* growth of *Babesia gibsoni* in canine erythrocytes at lower concentration compared with the standard antibabesial drug diminazene aceturate and killed the parasites within 24 hr at a concentration of 25 nM. Oral administration of bruceine A at a dosage of 6.4 mg/kg/day for 5 days resulted in no clinical findings in a dog with normal ranges of hematological and biochemical values in the blood. Three dogs were infected with *B. gibsoni* and two of them were treated with bruceine A at a dosage of 6.4 mg/kg/day for 6 days from day 5 post-infection. An untreated dog developed typical acute babesiosis symptoms including severe anemia, high fever, and complete loss of appetite and movement. However, the two bruceine A-treated dogs maintained their healthy conditions throughout the experimental period of 4 weeks although complete elimination of parasites from the peripheral blood was not achieved and decreases in the packed cell volume and the erythrocyte and platelet counts were observed. Since natural quassinoid compounds have been used as traditional medicines for the treatment of various ailments including cancer and malaria, the present results suggest that bruceine A or other related compounds are potential candidates for the treatment of canine babesiosis.

**KEY WORDS:** *Babesia gibsoni*, *Brucea javanica*, Bruceine A, Chemotherapy, Medicinal plant.

*J. Vet. Med. Sci.* 71(1): 33–41, 2009

Canine babesiosis is a tick-borne disease caused by the intraerythrocytic apicomplexan parasites, *Babesia gibsoni* and *B. canis*. Clinical signs of *B. gibsoni* infection are anemia, fever, thrombocytopenia, splenomegaly, lymphadenopathy, and lethargy [19]. During the acute phase of infection, infected dogs develop severe anemia and occasionally die if adequate treatment is not provided. However, most dogs that recovered from the acute phase become carriers of the parasites and may suffer from disease relapses for the rest of their life. *B. gibsoni* infection is endemic in many regions in Asia, Africa, Europe, Australia, Brazil, and North America [4, 10]. In Japan, *B. gibsoni* infection has long been problematic especially in western regions, but recently the distribution appears to be expanding to the eastern parts of Japan [21].

Diminazene aceturate and imidocarb dipropionate are the major drugs for the treatment of *B. gibsoni* infection [16], but these drugs are unable to eliminate the parasites completely from infected dogs [30]. These drugs also have some disadvantages. The toxicity of diminazene aceturate to kidney, brain, and liver can result in serious side-effects such as weakness, irritability, paralysis, lack of responsiveness to stimuli, and fatal hemorrhage in the central nervous

system [20, 26]. Due to these side-effects, the practical use of diminazene aceturate is not approved by the Food and Drug Administration (FDA) in the U.S.A. [5], and this drug was recently withdrawn from the market in Japan. The limited use of imidocarb dipropionate may be due to its high cost and systemic side-effects such as acute hepatic and renal failure especially in debilitated animals [1]. Therefore, an alternative chemotherapeutic agent with better activity and fewer side-effects is needed urgently. One possible source of such affordable treatment lies in the use of medicinal plants.

In the preceding study [24], Indonesian medicinal plants were screened for antibabesial activity *in vitro* and active quassinoid compounds were extracted from the fruit of *Brucea javanica* (L.) Merr., a plant species of the family Simaroubaceae. This plant contained a number of quassinoids as the bitter principles. Among these quassinoids, bruceine A, bruceantinol, and bruceine C showed sufficient antibabesial activities. The 50% growth-inhibitory concentration (IC<sub>50</sub>) values were 4 ng/ml (7.7 nM), 12 ng/ml (19.8 nM), and 107 ng/ml (189.7 nM), respectively, which compared well with the standard drug, diminazene aceturate, having an IC<sub>50</sub> value of 103 ng/ml (172.6 nM).

The present study was examined on the effect of bruceine A against *B. gibsoni* *in vitro* in more detail and evaluated the efficacy of bruceine A for dogs at an early stage of infection with *B. gibsoni* by investigating their clinical signs, the level of parasitemia, and hematological and biochemical values in

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the blood. The amount of parasite DNA in the peripheral blood was also monitored using a real-time polymerase chain reaction (PCR) method as a new assessment of antibabesial chemotherapeutics.

## MATERIALS AND METHODS

**Preparation of bruceine A:** The dried fruits of *Brucea javanica* were purchased from Bandar Jaya traditional market, Lampung, Indonesia, in April 2005. The plant species was identified by Mr. Aris Winarso at the Herbal Medicine Research and Education Centre, Lampung, Indonesia. Extraction and purification of bruceine A was described in the previous paper [24]. Briefly, air dried fruits (1 kg) were boiled with 5 l of water for 30 min twice. The boiling water was filtered and extracted with ethyl acetate (EtOAc) to give aqueous and EtOAc fractions. The EtOAc fraction was filtered, evaporated, and chromatographed on a silica gel column with chloroform, methanol (MeOH)-chloroform (3:97, 2l), MeOH-chloroform (1:4, 2l), MeOH-chloroform (7:3, 2l), and MeOH, successively. Each fraction was tested for its antibabesial activity against *B. gibsoni*. The active fraction was then chromatographed on a silica gel column with hexane-EtOAc (1:1) to give ten fractions. Bruceine A was detected in the fifth fraction and crystallized using MeOH. The structure was determined by means of NMR and mass spectra.

**Parasites:** The strain of *B. gibsoni* used was originally isolated from a naturally infected dog in Nagasaki Pref. in 1973 and has been maintained in dogs at Hokkaido University since then. The parasites were also maintained in 24-well plates (Corning, Corning, NY, U.S.A.) at 37°C with a gas flow mixture composed of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub>. Approximately 60% of the culture supernatant was replaced daily with an equal volume of fresh medium. Every 7 days, a half volume of erythrocyte suspension was replaced with the same volume of uninfected erythrocyte suspension.

**Preparation of canine erythrocyte suspension:** Canine erythrocytes were prepared by the method of Yamasaki *et al.* [34] with some modifications. Venous blood was collected from the jugular vein of healthy adult dogs. The erythrocytes were washed three times in a modified Vega y Martinez phosphate-buffered saline solution (mVYM) [29] by centrifugation at  $1,200 \times g$  for 5 min at 4°C. After two additional washes with RPMI-1640 (Gibco, Invitrogen Co., CA, U.S.A.) medium, the cells were resuspended in RPMI-1640 supplemented with 0.1 mg/ml sodium pyruvate (Wako, Osaka, Japan), 0.3 mg/ml L-glutamine (Wako, Osaka, Japan), 2 mg/ml sodium bicarbonate (Wako, Osaka, Japan), 100 units/ml penicillin G potassium (Meiji Seika, Tokyo, Japan), and 20% (v/v) heat-inactivated dog serum to yield a packed cell volume (PCV) of 5%.

**Assay for in vitro antibabesial activity of bruceine A:** Bruceine A and diminazene aceturate (Hoechst AG, Germany) were dissolved in DMSO and kept in the dark at 4°C until use. The stock solution was diluted with culture medium containing 0.1% DMSO. *B. gibsoni*-infected eryth-

rocytes (approximately 1% parasitemia) were obtained by diluting the cultures (5.0–6.5% parasitemia) with non-infected erythrocyte suspension. To each well of a 24-well culture plate a volume of 500  $\mu$ l infected erythrocyte suspension was added. After settling, a 50  $\mu$ l aliquot of the supernatant was removed and 50  $\mu$ l of bruceine A solution was added to give final concentrations of 0, 6.3, 12.5, 25, 50, and 100 nM. The plate was incubated at 37°C with a gas flow mixture composed of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> for 7 days. Approximately 60% of the supernatant was removed and replaced every 24 hr with fresh medium containing bruceine A at the appropriate concentration. Diminazene aceturate was used at concentrations of 0, 50, 100, 400, and 1,600 nM. Every 24 hr, 10  $\mu$ l of the erythrocyte suspension was sampled from each well and mixed with 90  $\mu$ l of phosphate buffered saline (PBS). The mixture was centrifuged and attached to a slide using a cytocentrifuge (Shandon Cytospin 2; Shandon, Cheshire, England) for 5 min at 450 rpm. The slide was fixed with methanol and stained with Giemsa solution (pH 7.4). The parasitemia level was determined by counting the number of parasitized erythrocytes in 2,000 erythrocytes. When the parasitemia level was less than 1%, an additional 3,000 erythrocytes were examined. The experiment was carried out in triplicate. Statistical analysis was carried out using Student's *t*-test.

**Assay for antibabesial activity of bruceine A in dogs:** Four beagle dogs (10-month-old, male) were used in this study. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University. One dog (dog A) was used to examine the side-effects of bruceine A and received a gelatin capsule containing bruceine A powder orally at dosages of 0.4, 0.8, 1.6, 3.2, and 6.4 mg/kg at 24 hr intervals. Since no acute toxicity was observed at any dosage, the same dog was administered bruceine A at an oral dosage of 6.4 mg/kg for 5 days at 24 hr intervals. Thus, dog A received bruceine A at a total dosage of 44.4 mg/kg. Clinical findings, body weight, and body temperature were monitored. Peripheral blood (5 ml) was collected and subjected to hematological and biochemical examinations. Packed cell volume (PCV), red blood cell counts, and platelet counts were measured using an automatic cell counter (Celltac- $\alpha$ , MEK-6258; Nihon Kohden, Tokyo, Japan). Serum biochemical parameters such as alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (CRE), gamma-glutamyl transpeptidase (GGT), glucose (GLU), and total cholesterol (TCHO) were measured using an analyzer (DRI-CHEM 7000V; Fuji Mechanical Industry Co., Ltd., Tokyo, Japan), and serum levels of electrolytes (Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup>) were measured with an ion-selective electrode (Dri-Chem Slide Na-K-Cl; Dri-Chem 800 V; Fuji Film Co., Ltd., Tokyo, Japan).

Three dogs (dogs B, C, and D) were inoculated intravenously with  $1.2 \times 10^9$  *B. gibsoni*-parasitized erythrocytes in a volume of 9 ml, which were harvested from a dog chroni-

cally infected with *B. gibsoni*. Bruceine A in a gelatin capsule was administered to two dogs (dogs B and C) at an oral dosage of 6.4 mg/kg/day for 6 days (a total dosage of 38.4 mg/kg) starting from day 5 post-infection. Dog D was served as the control and administered a gelatin capsule containing glucose powder. Clinical findings and body temperature were monitored for the experimental period of 28 days. Peripheral blood (500  $\mu$ l) was collected daily and a 200  $\mu$ l volume of each blood sample was subjected to hematological, biochemical, and microscopic examinations as described above. The rest of the blood sample was stored at  $-20^{\circ}\text{C}$  until use for real-time PCR assays. Hematological examinations were conducted daily, while serum biochemical examinations were performed on days 0, 4, 11, 18, and 25 post-infection. Blood smears were made from EDTA-anticoagulated peripheral blood samples and stained with Giemsa solution. The level of parasitemia was determined by counting 10,000 erythrocytes.

**Quantification of *B. gibsoni* p18 gene in the blood by real-time PCR:** The copy number of the *B. gibsoni* p18 gene in the peripheral blood was estimated using the real-time PCR method by Matsuu *et al.* [18]. DNA was extracted from 200  $\mu$ l of EDTA-anticoagulated whole blood samples using a commercial kit (QIAgen DNA Blood Mini Kit; Qiagen, Tokyo, Japan). Real-time PCR assays were performed with a reaction mixture (25  $\mu$ l) containing each primer (500 nM) and template DNA extract (0.5  $\mu$ l) using an Applied Biosystems 7300 real-time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) and Power SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan). The mixture was incubated at  $50^{\circ}\text{C}$  for 2 min and  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 60 s. The relative copy number of the p18 gene in the blood samples was estimated from a standard curve created by plotting the log initial copy number of input plasmid, which contains a 182-base pair fragment of the p18 target gene, against the threshold cycle (Ct) value. Each sample was measured in triplicate and the results were expressed as the copy number per 100 erythrocytes (mean  $\pm$  SD).

## RESULTS

**In vitro antibabesial activity of bruceine A:** The kinetics of parasitemia in the canine erythrocytes with *B. gibsoni* in culture for 7 days is shown in Fig. 1. While previous report showed that bruceine A had an  $\text{IC}_{50}$  value of 4 ng/ml (7.7 nM) against the parasites at day 3 of the culture [24], a similar result was obtained at day 3 in this study, in which the level of parasitemia for the untreated culture and the culture treated with 6.3 nM bruceine A was 3 and 1.6%, respectively (Fig. 1-A, inset). Thus, bruceine A at 6.3 nM inhibited parasite growth by 47% at day 3 of the culture. Although parasitemia reached 12% at day 7 in the drug-free culture, it remained around 3% in the culture in the presence of 6.3 nM bruceine A, indicating that complete growth inhibition was not obtained at this concentration of the drug. On

the other hand, parasitemia levels rapidly decreased within one day with 25 nM bruceine A, (Fig. 1-A, inset) and some morphological abnormalities of the parasites including a pyknosis-like and comma-shaped changes were observed (data not shown). Similar results of rapid decrease in parasitemia and morphological changes of the parasites were observed in the presence of 50 and 100 nM bruceine A (data not shown). However, no such rapid reduction in parasitemia was observed with the standard antibabesial drug, diminazene aceturate, even at the highest concentration of 1,600 nM (Fig. 1-B).

**In vivo effect of bruceine A on *B. gibsoni*-infected dogs:** One dog (dog A) was used to the examination for the side-effects of bruceine A and received this compound orally in a gelatin capsule at a dosage of 6.4 mg/kg/day for 5 days. No serious clinical findings were found in this dog. Hematological and serum biochemical values including PCV, red blood cell counts, platelet counts, ALP, ALT, AST, BUN, CRE, GGT, GLU, TCHO, and electrolytes ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ ) were all within respective reference range (data not shown).

Three dogs were then infected with *B. gibsoni*. Two of them (dogs B and C) were administered 6.4 mg/kg/day bruceine A orally for 6 days from day 5 post-infection, and the other one (dog D) was kept untreated. The kinetics of parasitemia and body temperature in these dogs is shown in Fig. 2. In the untreated dog D a gradual rise in parasitemia was observed and the value reached a peak of 2.3% on day 13 (Fig. 2, Dog D). This dog showed severe pallor in the mucous membranes from day 12 and its body temperature began to rise from day 8 and rose to  $40.4^{\circ}\text{C}$  on day 14 (Fig. 2, Dog D). The dog exhibited complete loss of appetite and movement on day 13 and reached the humane endpoint (f in Fig 2, Dog D) on day 14 before receiving a subcutaneous infusion to prevent lasting harm. Bruceine A-treated dogs (dogs B and C) also showed a gradual increase in parasitemia levels until day 11, but there were no obvious peaks and the levels were kept below 1% during the experimental period (Fig. 2, Dogs B and C). Dog B did not show any clinical signs. Dog C showed pallor in the mucous membranes from day 13 for 4 days and developed high fever sporadically on days 14 and 15. Anorexia and depression were, however, not observed in these bruceine A-treated dogs.

The kinetics of PCV and platelet counts is shown in Fig. 3. In dog B the PCV level decreased gradually but it was maintained at over 28% (Fig. 3, Dog B). The PCV in dog C decreased markedly and reached nadir values of 18% on day 15, and 15% (the humane endpoint in this experiment) in dog D on day 14, respectively (Fig. 3, Dogs C and D). However, in dog C the level recovered and was maintained at around 30 to 40%. The decrease in erythrocyte counts was associated with the decrease in PCV values in each dog (data not shown). A drastic decrease in platelet counts was observed in these dogs. Although the respective dogs had about 300,000/ $\mu$ l platelets in the blood before infection, they contained less than 10,000/ $\mu$ l on day 14 (Fig. 3, Dogs B, C, and D). Dog B maintained platelet counts at a lower

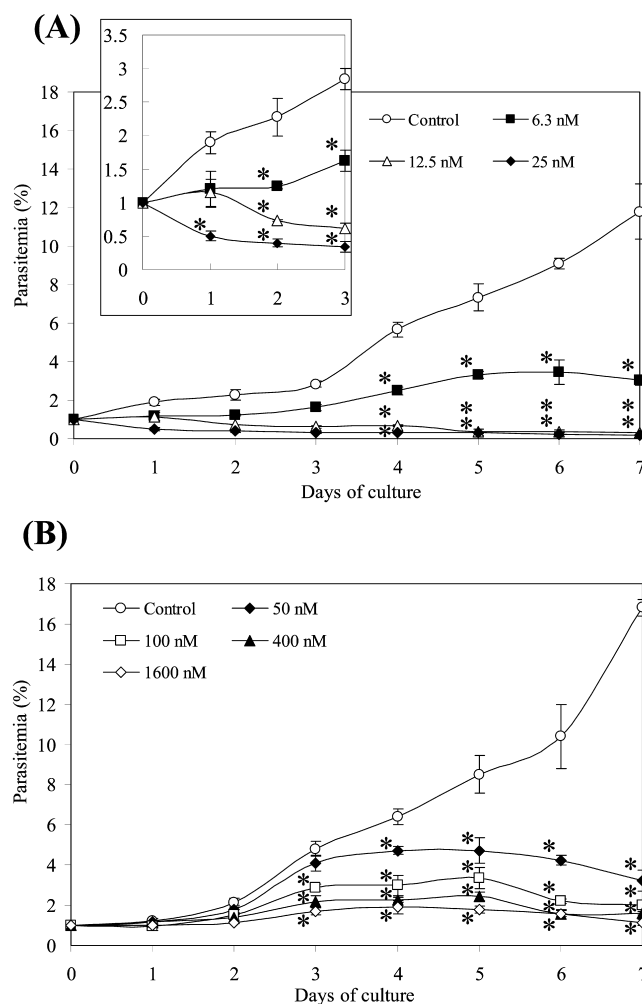


Fig. 1. Growth of *Babesia gibsoni* in the canine erythrocytes *in vitro* in the presence of bruceine A (A) and diminazene aceturate (B). Every 24 hr, each culture suspension was sampled and parasitemia was determined. Data show the means  $\pm$  SD of two separate experiments. Insert indicates an expanded view of the growth curves for the initial 3 days in the presence of bruceine A. The asterisk (\*) indicates statistically significant difference at  $p < 0.01$  from the control values by Student's *t*-test.

level of less than 55,000/ $\mu$ l during the experimental period. In dog C, the platelet counts increased to a normal level (240,000/ $\mu$ l) on day 21 but then decreased again.

With respect to serum biochemical values and serum levels of electrolytes, only the ALT value (136 U/l) in dog B on day 4 exceeded the reference value (17–78 U/l), whereas all other parameters were within each reference range (data not shown).

**Detection of *B. gibsoni* p18 gene in the blood:** The kinetics of the p18 gene copy number and parasitemia in the dogs is shown in Fig. 4. Parasite DNA was detected in the peripheral blood from day 1 and the p18 gene copy number reached a first peak on day 11, showing 4.3, 8.0, and 7.0

copies/100 erythrocytes in dogs B, C, and D, respectively (Fig. 4). The copy number decreased subsequently in all three dogs but then gradually increased again in dogs B and C.

## DISCUSSION

This study showed that bruceine A had potent antibabesial activities against *B. gibsoni* *in vitro* and *in vivo*. The *in vitro* results indicated that a rapid decrease in parasitemia was induced by bruceine A at a concentration of 25 nM, suggesting that the parasites were killed at this concentration. The oral administration of bruceine A to dogs at an early

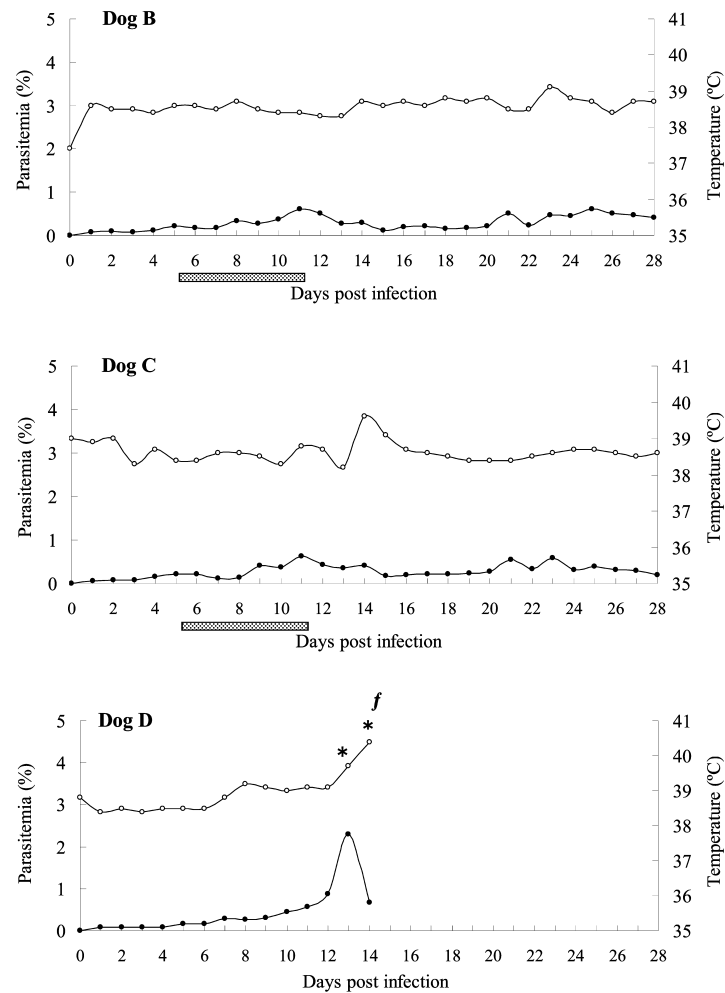


Fig. 2. Changes in parasitemia (●) and body temperature (○) in the dogs (B, C, and D) infected with *Babesia gibsoni*. Dogs B and C were administered bruceine A (6.4 mg/kg/day) orally from day 5 post-infection for 6 days. The dark grey bars indicate the period of bruceine A administration. Untreated dog D showed complete loss of appetite and movement (\*) and reached humane end-point (f).

stage of infection with *B. gibsoni* relieved some of the clinical signs of infected dogs. An untreated dog developed severe anemia, high fever, and complete loss of appetite and movement, whereas two bruceine A-treated dogs maintained healthy conditions. However, in these treated dogs decreases in PCV value and erythrocyte and platelet counts were observed and complete elimination of the parasites from the peripheral blood was not achievable. These results suggested that bruceine A inhibited the growth of parasites to a certain extent and prevented the manifestation of clinical signs associated with *B. gibsoni* infection in dogs.

It is reported that the degree of thrombocytopenia is more severe than that of anemia in *B. gibsoni*-infected dogs [9, 11, 17, 33]. This clinical finding was also observed in the present study. Dog C showed a temporal recovery from

thrombocytopenia from day 15, however, this may not be related to the drug treatment since dog B did not show a similar recovery. Treatment with atovaquone alone, atovaquone in combination with azithromycin, and clindamycin alone against *B. gibsoni*-infected dogs has been reported to show a rapid decrease in parasitemia and significant improvement of clinical findings including anemia and thrombocytopenia, in spite of unsuccessful in total elimination of circulating parasites [11, 17, 33]. Unlike *in vitro* results, a rapid elimination of the parasites in the peripheral erythrocytes was not observed in the infected dogs by treatment with bruceine A in the present study. Since sequestration of *B. gibsoni*-infected erythrocytes within the tissues such as the lymph node and spleen has been suggested [11, 17], bruceine A might inhibit the growth of parasites in the

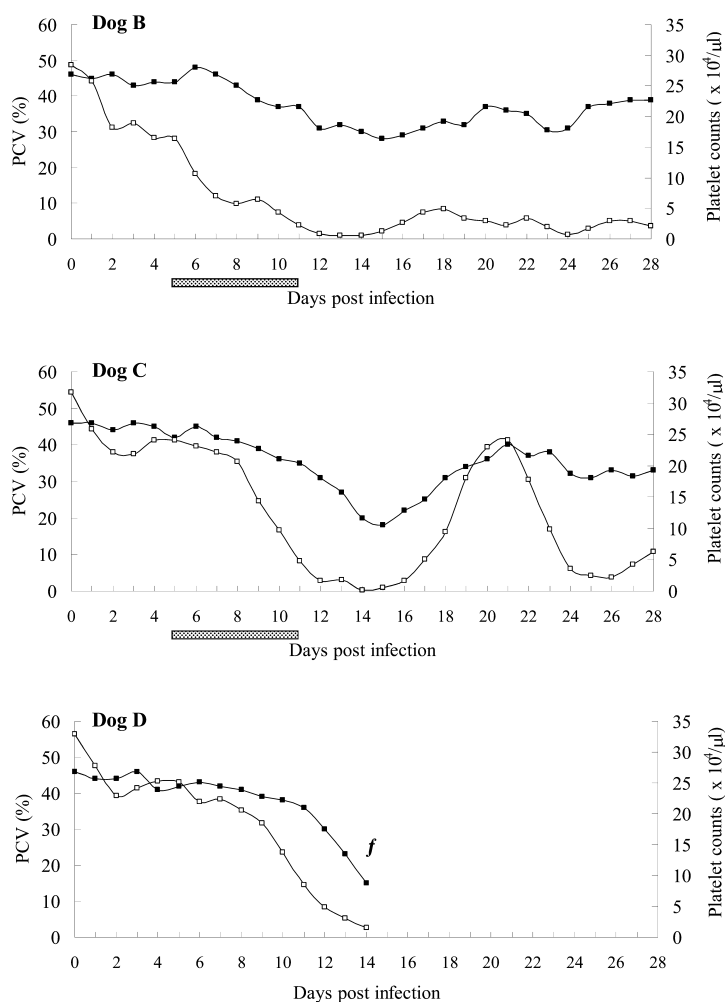


Fig. 3. Changes in packed cell volume (PCV) (—■—) and platelet counts (—□—) in the dogs (B, C, and D) infected with *Babesia gibsoni*. Dogs B and C were administered bruceine A (6.4 mg/kg/day) orally from day 5 post-infection for 6 days. The dark grey bars indicate the period of bruceine A administration. Humane endpoint is indicated by *f*.

tissues and prevent the manifestation of clinical signs. Another possible explanation is that metabolized-compounds derived from bruceine A exhibited reduced antiparasitic activities. In fact, a slight structural change and modification of quassinoid compounds have resulted in a variety of antibabesial activities [24]. Further studies including curative dosage, effective route, and period of administration of bruceine A as well as a rational design of combination therapy are required.

Bruceine A was found to have some toxic effects on treated animals [2, 23]. The risk of side-effects is likely to increase with higher dosages of the drug; therefore, reduction of the risk needs to be considered. In fact, liposomal encapsulation of another antibabesial drug, imidocarb, resulted in the reduction of its *in vivo* toxicity by 50-fold compared to the unencapsulated drug [27]. Hence, a similar approach may be useful for the development of more ratio-

nal treatment regimens with bruceine A.

*Brucea javanica*, a plant species of the family Simaroubaceae, is widely distributed throughout South East Asian countries and its fruits have been used as a source of traditional medicine against malaria, dysentery, and cancer. The bitter principles of this plant are quassinoids, some of which have been investigated for their biological properties including antitumor [6, 12, 15], antiamebic [31], antimalarial [7, 23], and antibabesial activities [24]. Kirby *et al.* [13] showed that seven quassinoids such as ailanthinone, bruceantin, bruceine B, glaucarubinone, holacanthone, chaparrin, and glaucarubol were proved to inhibit protein synthesis in malaria parasites. Inhibition of nucleic acid synthesis was also detected as a subsequent reaction. There are reports on the *in vitro* activity of bruceine A against protozoan parasites, including *Plasmodium falciparum*, *Entamoeba histolytica*, and *Giardia intestinalis* [23, 31, 32].

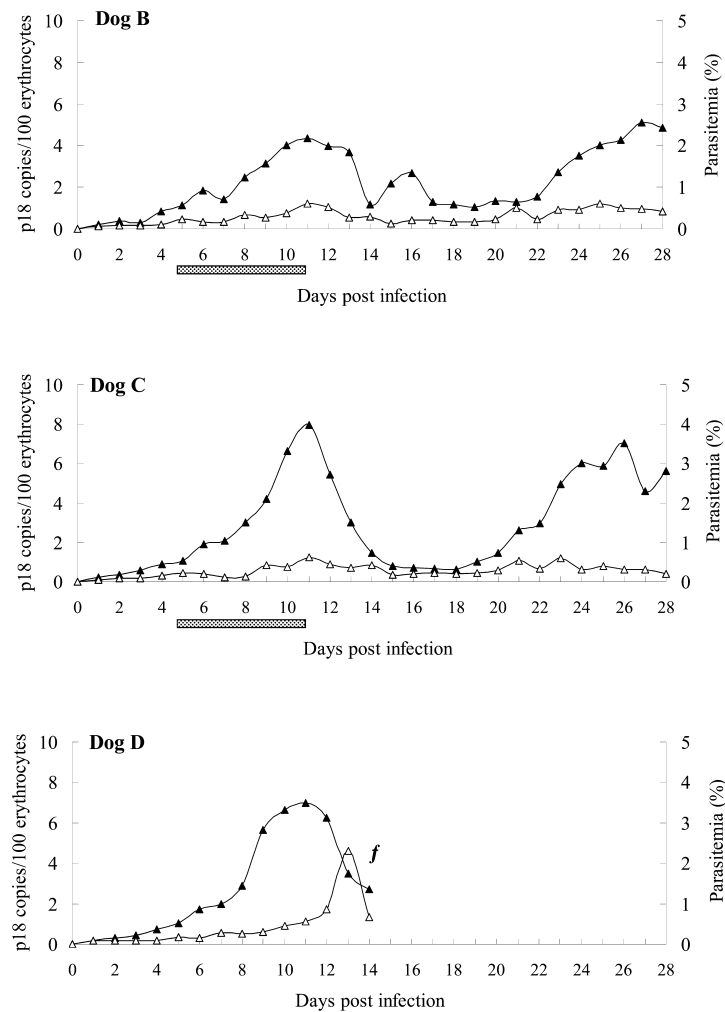


Fig. 4. Changes in copy number of *Babesia gibsoni* p18 gene ( $\blacktriangle$ ) and parasitemia ( $\triangle$ ) in the dogs (B, C, and D) infected with *Babesia gibsoni*. Dogs B and C were administered bruceine A (6.4 mg/kg/day) orally from day 5 post-infection for 6 days. The dark grey bars indicate the period of bruceine A administration. Humane endpoint is indicated by *f*.

Although the mode of action of bruceine A against *Babesia* species is unknown, it appears to be different from those of the currently available antibabesial drugs [25, 30]. Further studies are required to elucidate the mechanism by which bruceine A displays its potent antibabesial activity for the further development of novel combination chemotherapies with different antibabesial compounds.

Quassinoid compounds have anti-inflammatory activities *in vitro* and *in vivo* [8, 14, 28]. Hall *et al.* [8] reported that brusatol, another analogous quassinoid to bruceine A, reduced inflammation and arthritis in rodents. They found that the structures of 3-hydroxy- $\delta$ -3-2-oxo moiety, a C-15 ester-bearing  $\delta$ -lactone ring, and C-11 and C-12 free hydroxyl groups are important for the anti-inflammatory activity of brusatol. Bruceine A possesses a similar structure with potent active sites. Investigation of the anti-

inflammatory properties of bruceine A may explain the mechanisms of the reduction in disease severity in dogs infected with *B. gibsoni*.

The standard method for quantification of babesial parasites is microscopic examination of a blood smear specimen. However, it is often difficult to quantify the low levels of parasites in the peripheral blood during acute phase of infection, asymptomatic infection and chemical treatment of infected animals. In the present study a real-time PCR method using the *B. gibsoni* p18 gene was applied to monitor the growth of *B. gibsoni* *in vitro* and a good correlation was found between p18 gene copy numbers and parasitemia levels in the bruceine A-treated cultures (unpublished). However, in this *in vivo* study, the copy number was not parallel to the level of parasitemia. In particular, the copy numbers were smaller than those expected from the data of

parasitemia on days 11–14 in dog D, and higher than those on days 23–28 in dogs B and C. Since multi-divided forms of *B. gibsoni* were not commonly detected in the peripheral blood of infected dogs, extra-erythrocytic merozoites existing in the blood or circulating macrophages may take the parasite DNA. In fact, *Plasmodium chabaudi* DNA could be detected in mouse peripheral blood by PCR at least until 24 hr after the injection of dead parasites [22]. This real-time PCR method will be useful not only for antibabesial chemotherapeutic studies but also for understanding the mechanisms of circulation and hiding/sequestration of *B. gibsoni* parasites in the host.

In conclusion, bruceine A is a potent antibabesial compound. Further pharmacokinetic and pharmacodynamic studies could contribute for novel information on the efficacy of bruceine A for canine babesiosis and its rational administration schedule in dogs.

**ACKNOWLEDGMENTS.** We thank Dr. A. Matsuu at the University of Tottori for kindly providing the plasmid containing the p18 target gene. This work was supported in part by Grant-in-Aid 17–05213 from the Japan Society for the Promotion of Science, and 18380178 and the 21st Century COE Program, “Program of Excellence for Zoonosis Control” from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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