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FULL PAPER

Analysis of energy generation and glycolysis pathway in diminazene aceturate-resistant *Babesia gibsoni* isolate *in vitro*

Bandula Kumara Wickramasekara Rajapakshage, Masahiro Yamasaki*, Masahiro Murakami, Yu Tamura, Sue Yee Lim, Tatsuyuki Osuga, Noboru Sasaki, Kensuke Nakamura, Hiroshi Ohta and Mitsuyoshi Takiguchi

Laboratory of Veterinary Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

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Abstract

In our previous study, the level of parasitemia of the diminazene aceturate (DA)-resistant *B. gibsoni* isolate was continuously lower than that of the wild-type, indicating the possible alteration of energy metabolism in that isolate. Therefore, in the present study, the concentrations of ATP, glucose, lactate, and pyruvate, and the activities of lactate dehydrogenase and pyruvate kinase in the wild-type and DA-resistant isolate of *B. gibsoni* were measured and compared to investigate the amount of energy generation and the activity of the glycolysis pathway. As a result, the intracellular ATP and glucose concentrations in the DA-resistant *B. gibsoni* isolate were significantly higher than those in the wild-type. Meanwhile, the concentrations of lactate and pyruvate and the activities of lactate dehydrogenase and pyruvate kinase in the DA-resistant *B. gibsoni* isolate were not different from those in the wild-type. These results indicated that the DA-resistant *B. gibsoni* isolate contained a higher ATP concentration than the wild-type, but the activity of the glycolysis pathway was not altered in the DA-resistant *B. gibsoni* isolate. However, we could not determine the mechanism of the high energy production of the DA-resistant *B. gibsoni* isolate. Further studies on the energy metabolism of *B. gibsoni* are necessary to clarify the mechanism of the high energy production in the DA-resistant *B. gibsoni* isolate.

Key words: *Babesia gibsoni*, diminazene aceturate, resistance, energy metabolism, glycolysis pathway

Introduction

Babesia gibsoni is a blood protozoan of dogs and a causative pathogen of canine babesiosis[37]. Although a number of drugs, such as diminazene aceturate (DA) and atovaquone, are used for treatment of the disease, it is difficult to eliminate this parasite from infected dogs, and

*Corresponding author: M. Yamasaki, Laboratory of Veterinary Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan
Phone: +81–11–706–5224. E-mail: masayama@vetmed.hokudai.ac.jp

relapses often occur[14,31]. It is clinically reported that one of the reasons for the relapse of canine babesiosis might be the development of drug resistance in *Babesia* parasites[10,25,31]. Indeed, DA resistance of *B. gibsoni* has also been reported *in vitro*[17,18].

To investigate the mechanisms of DA resistance of *B. gibsoni*, a DA-resistant *B. gibsoni* isolate was previously developed *in vitro*[17,34]. In African trypanosomes, as mitochondria are considered one of the common targets for diamidines, analogs of DA, the possibility of specific mitochondrial changes being involved in DA resistance was anticipated[21,24,33]. In apicomplexa, mitochondria are an attractive target of therapeutic drugs[11,26]. The alteration of mitochondrial DNA (mtDNA), such as *cytochrome c oxidase subunit I* (*COXI*), *cytochrome c oxidase subunit III* (*COXIII*), and *cytochrome b* (*CYTb*) genes, has been identified in the development of drug resistance in parasites. In particular, in *B. gibsoni*, mutation of the *CYTb* gene has been described in an atovaquone-resistant isolate[25,31]. In our previous report, accordingly, we focused on the mitochondria of *B. gibsoni* as a target of DA. In the results, there were no significant nucleotide and amino acid substitutions in *COXI*, *COXIII*, and *CYTb* genes, and gene transcription of those genes was not altered in the DA-resistant *B. gibsoni* isolate[34]. Thus, it is suggested that these genes are not closely involved in the DA resistance of *B. gibsoni*[34]. The mechanisms of DA resistance are still not clear. Meanwhile, in the previous study, the proliferation potential of the DA-resistant *B. gibsoni* isolate is comparatively lower than that of the wild-type[34]. A similar situation was described for pentamidine-resistant *Leishmania,* and 5-fluoroorotate and atovaquone-resistant *P. falciparum*[3,16]. These drug-resistant parasites grow slower than their parental wild-type clones *in vitro*[3,16]. Glibenclamide-resistant *L. mexicana* exhibit decreased activity of functional key enzymes such as acid phosphatase and pyruvate kinase that subsequently led to parasite growth retardation and lower proliferation potential[15]. Thus, the reason for the low proliferation potential of these drug-resistant parasites seems to be the alteration of energy metabolism[3,15]. In Babesia parasites, although the interaction between the proliferation of the parasites and energy metabolism is unclear, it is supposed that the energy metabolism of the DA-resistant *B. gibsoni* isolate is altered.

It has been generally accepted that protozoan parasites such as *B. gibsoni*[35], *B. bovis*[1,6], *B. rodhaini*[13,30], *Plasmodium* spp.[12,27,32] and an early blood stream form of *Trypanosomes*[8] derive most of their energy requirements from their own glycolytic system under normal physiological conditions. Thus, glycolysis plays a significant role in these parasites. Additionally, adaptation of glycolysis has been observed in many drug-resistant parasites such as pentavalent antimonials and glibenclamide-resistant *Leishmania* spp. and chloroquine-resistant *P. falciparum*[5,15,22,23]; however, in the DA-resistant *B. gibsoni* isolate, energy metabolism has not been investigated. Therefore, in the present study, the amount of energy generation and the activity of the glycolysis pathway of the DA-resistant *B. gibsoni* isolate were investigated.

Materials and Methods

Isolates of Babesia gibsoni: The wild-type *B. gibsoni* used in the present study originated from a naturally infected dog in the city of Nagasaki, Japan in 1973. The infected dog was not treated with any drugs when *B. gibsoni* was isolated. Since then, this wild-type has been maintained in cultures in our laboratory[36]. The DA-resistant *B. gibsoni* isolate was developed from this wild-type in the previous study[34]. Briefly, the wild-type was cultured in culture medium containing 1 ng/ml DA initially, and the concentration of DA gradually increased from 1 to 200 ng/ml after 252 days. Thereafter, the DA-resistant *B. gibsoni* isolate was maintained in culture medium containing 200 ng/ml DA for more than six months.

In vitro culture of Babesia gibsoni: The wild-type and the DA-resistant *B. gibsoni* isolate have been maintained in cultures in our laboratory according to the method of Yamasaki *et al.*[36]. In brief, 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, Carlsbad, CA, U.S.A.), 20% dog serum and canine HK red blood cells (RBCs) that contain a high concentration of potassium[19], 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. 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. The wild-type and the DA-resistant *B. gibsoni* isolate were maintained in culture media without DA and with 200 ng/ml DA, respectively. Thin smears were prepared and the level of parasitemia was determined by counting the number of parasitized erythrocytes per 1,000 erythrocytes.

Preparation of perchloric acid extract: To measure the concentrations of glucose, ATP, lactate, and pyruvate in *B. gibsoni* cultured using the methods described above, a perchloric acid (PCA) extract of the parasites was prepared. Since a large amount of erythrocyte-free parasites was required to prepare the PCA extract, culture volumes of the wild-type and the DA-resistant *B. gibsoni* isolate were increased up to 128 ml. A 500 μl culture sample of the parasites was doubled in volume with fresh uninfected erythrocyte suspension every other day for 2 weeks to reach 128 ml. To obtain erythrocyte-free parasites, the cultured parasites harbored in erythrocytes were harvested and the parasites were isolated from host cells by the method of Yamasaki *et al.* with some modifications[35]. Briefly, 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 0.075% saponine (Yoneyama Chemical Industries, Co., Osaka, Japan) in phosphate-buffered saline (PBS, pH 7.4) was added and incubated at room temperature for 10 min. After incubation, hemolyzed samples were centrifuged at 10,000 × g for 5 min at 4°C. After centrifugation, the supernatant was discarded, and the gray pellet of parasites was retained. The retained pellet was washed twice with PBS. The washed pellet was mixed with PBS and adjusted to a volume of 1,000 μl, named the parasite suspension. The protein concentration in the parasite suspension was estimated by the method of Bradford[7]. Bovine serum albumin (Sigma Chemical, Germany) was used to make the standard curve.

A 700 μl sample of parasite suspension was mixed with 350 μl ice-cold PCA and centrifuged at 800 × g for 10 min at 4°C. After centrifugation, a drop of 0.05% methyl orange was added to 1 ml of supernatant and neutralized with 3 M K_2CO_3 on ice. The resultant supernatant was the PCA extract, and which was stored on ice until use.

The PCA extract of uninfected erythrocytes was also prepared according to the same procedure described above. The stored uninfected erythrocyte suspension for subculture was utilized to prepare the PCA extract of uninfected erythrocytes.

Preparation of parasite lysate: To measure the activities of lactate dehydrogenase (LDH) and pyruvate kinase (PK), the parasite lysate was prepared. Parasite suspensions of the wild-type and the DA-resistant *B. gibsoni* isolate were prepared according to the procedure described above. A 200 μl sample of parasite suspension was added to 1,800 μl beta-mercaptoethanol-EDTA stabilizing solution (0.00005% beta-mercaptoethanol, 0.1% EDTA, pH 7.0) and completely frozen at −80°C as a parasite lysate. When enzyme activities were measured, the frozen parasite lysate was thawed in water at room temperature and then maintained at 0°C. The activities of enzymes were measured within 2 hrs after thawing the parasite lysate.

The erythrocyte lysate of uninfected erythrocytes was also prepared according to the

same procedure as described above. The stored uninfected erythrocyte suspension for a subculture was utilized to prepare the erythrocyte lysate of uninfected erythrocytes.

Reagents: The concentrations of substrates and activities of enzymes were measured according to the method of Beutler with some modifications[4]. The reagents for measurements were prepared by the method of Beutler[4]. Tris/EDTA buffer consisted of 1 M Tris-HCl and 5 mM EDTA, pH 8.0. Potassium phosphate buffer was prepared by mixing 1 M K_2HPO_4 and 1 M KH_2PO_4 to adjust the pH to 8.0. Glycine hydrazine buffer consisted of 2 M glycine, 0.8 M hydrazine sulfate, and 0.01 M EDTA. Magnesium (Mg) reagent included 0.1 M $MgCl_2$, NADP reagent included 2 mM NADP, NADH reagent included 2 mM NADH, NAD reagent included 5 mM NAD, glucose (Glu) solution included 20 mM glucose, ATP solution included 20 mM ATP, glucose-6-phosphate dehydrogenase (G6PD) solution included 60 U/ml G6PD, and hexokinase (Hx) solution included 400 U/ml hexokinase.

Measurements of intracellular ATP concentration: A 100 µl sample of Tris/EDTA buffer, 20 µl Mg reagent, 200 µl NADP reagent, and 50 µl Glu solution were mixed in two quartz cuvettes on ice. For the sample, 200 µl of the PCA extract and 415 µl distilled water (DW) were added to one cuvette. For the blank, 615 µl DW was added to the other cuvette. Then, 5 µl G6PD solution was added to both cuvettes, which were incubated at 37°C for 10 min. After incubation, 10 µl Hx solution was added to both cuvettes and the changes in optical density (OD) at 340 nm were measured using a spectrophotometer (U-3210; Hitachi, Japan).

Determination of intracellular glucose concentration: A 100 µl sample of Tris/EDTA buffer, 50 µl Mg reagent, 200 µl NADP reagent, and 250 µl ATP solution were mixed in two quartz cuvettes on ice. For the sample, 200 µl PCA extract and 185 µl DW were added to one cuvette. For the blank, 385 µl DW was added to the other cuvette. Then, 5 µl G6PD solution was added to both cuvettes, which were incubated at 37°C for 10 min. After incubation, 10 µl Hx solution was added to both cuvettes and the changes in OD at 340 nm were measured.

Determination of intracellular lactate concentration: A 500 µl sample of glycine hydrazine buffer and 200 µl NAD reagent were mixed in two quartz cuvettes on ice. For the sample, 100 µl PCA extract and 190 µl DW were added to one cuvette. For the blank, 290 µl DW was added to the other cuvette. Then, both cuvettes were incubated at 37°C for 10 min. After incubation, 10 µl of 2,500 U/ml LDH solution was added to both cuvettes and the changes in OD at 340 nm were measured.

Determination of lactate dehydrogenase activity: A 100 µl sample of Tris/EDTA buffer, 100 µl NADH reagent, and 200 µl parasite lysate were mixed in two quartz cuvettes on ice. For the sample, 500 µl DW was added to one cuvette. For the blank, 600 µl DW was added to the other cuvette. Then, both cuvettes were incubated at 37°C for 10 min. After incubation, 100 µl of 10 mM sodium pyruvate solution was added only to the sample, and the changes in OD at 340 nm were measured.

Determination of intracellular pyruvate concentration: A 200 µl sample of potassium phosphate buffer and 100 µl NADH reagent were mixed in two quartz cuvettes on ice. For the sample, 200 µl PCA extract and 490 µl DW were added to one cuvette. For the blank, 690 µl DW was added to the other cuvette. Then, both cuvettes were incubated at 37°C for 10 min. After incubation, 10 µl of 100 U/ml LDH solution was added to both cuvettes and the changes in OD at 340 nm were measured.

Determination of pyruvate kinase activity: A

100 μl sample of Tris/EDTA buffer, 100 μl NADH reagent, 100 μl of 1 M KCl, 100 μl Mg reagent, 100 μl of 60 U/ml LDH and 200 μl parasite lysate were mixed in two quartz cuvettes on ice. For the sample, 50 μl of 30 mM ADP and 150 μl DW were added to one cuvette. For the blank, 200 μl DW was added to the other cuvette. Then, both cuvettes were incubated at 37°C for 10 min. After incubation, 100 μl of 50 mM phosphoenolpyruvate was added to both cuvettes, and the changes in OD at 340 nm were measured.

Calculation: The concentrations of ATP, glucose, and pyruvate (Eq. 1), the concentration of lactate (Eq. 2), and the activities of LDH and PK (Eq. 3) were calculated from ΔOD by the formula given below. The protein concentration in the parasite suspension was used for the calculation. These experiments were repeated three times.

Eq. 1:
Substrate (μmols/mg protein)
$= (\Delta OD_{340} \times 1.5675)$ / protein concentration (mg/ml)
Eq. 2:
Lactate (μmols/mg protein)
$= (\Delta OD_{340} \times 1.5675)$ / protein concentration (mg/ml) $\times 2$

Eq. 3:
Enzyme activity (IU/mg protein)
$= (10 \times OD_{340} \times 200)$
/ (protein concentration (mg/ml) $\times 6.22 \times 200$)

Statistical analysis: The data was expressed as the means ± SD. Two-sample *t*-tests were used to compare statistical differences between the DA-resistant and wild-type *B. gibsoni*. $P < 0.05$ was considered significant.

Results

Intracellular ATP and glucose concentration in B. gibsoni: As reported previously[34], the level of parasitemia in the DA-resistant *B. gibsoni* isolate was lower than in the wild-type (Fig. 1); therefore, the concentrations of ATP and glucose in the wild-type and the DA-resistant isolate were measured and compared. As shown in Fig. 2, the intracellular ATP concentration in the DA-resistant *B. gibsoni* isolate was significantly ($P < 0.05$) higher than in the wild-type. Furthermore, the intracellular glucose concentration in the DA-resistant *B. gibsoni* isolate was also significantly ($P < 0.05$) higher than in the wild-

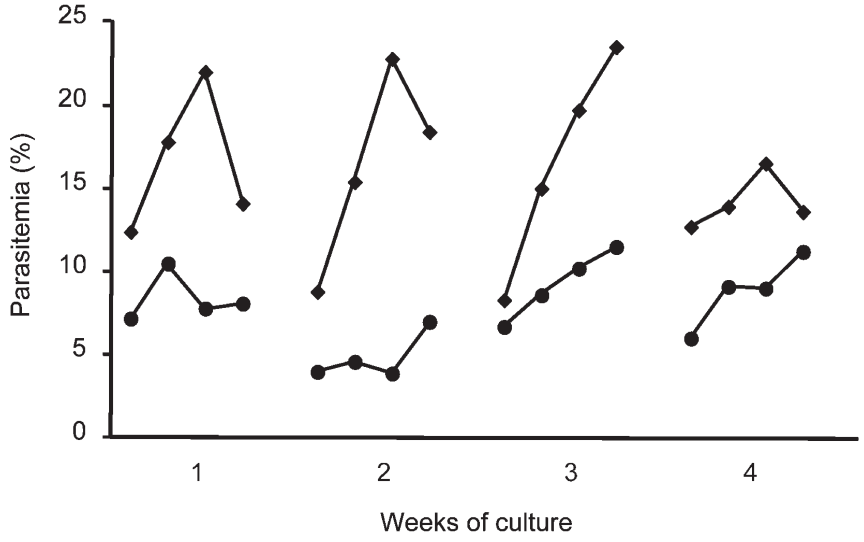

Fig. 1. The levels of parasitemia in the wild-type (square) and the DA-resistant *B. gibsoni* isolate (circle) *in vitro* for 4 weeks. The levels of parasitemia were measured every other day using Giemsa staining of blood smears.

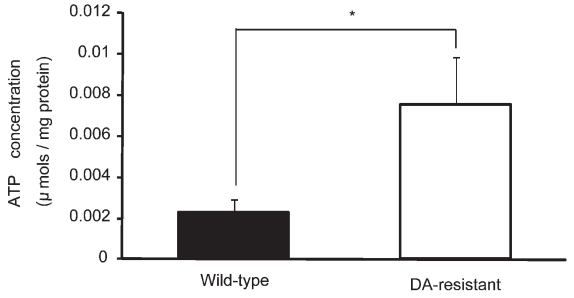

Fig. 2. ATP concentration of the wild-type (closed bar) and the DA-resistant *B. gibsoni* isolate (open bar). Data are expressed as the means ± SD (n=3).* Significantly ($P < 0.05$) different from the value in the wild-type.

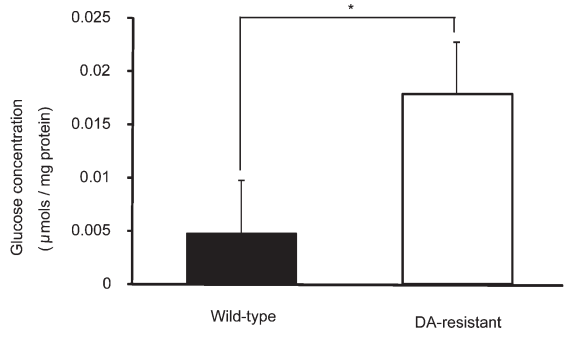

Fig. 3. Glucose concentration of the wild-type (closed bar) and the DA-resistant *B. gibsoni* isolate (open bar). Data are expressed as the means ± SD (n = 3).* Significantly ($P < 0.05$) different from the value in the wild-type.

type (Fig. 3). Additionally, intracellular concentrations of ATP and glucose in uninfected erythrocytes were 0.009 ± 0.003 μmols/mg protein and 0.007 ± 0.003 μmols/mg protein, respectively.

Intracellular lactate concentration and lactate dehydrogenase activity: The concentration of lactate, the final product of glycolysis, was measured and compared between the wild-type and the DA-resistant isolate. As a result, the lactate concentration in the DA-resistant *B. gibsoni* isolate was not significantly different from that in the wild-type, although the average was higher in the DA-resistant isolate than in the wild-type (Fig. 4). In addition, the activity of LDH was measured and compared between the wild-type and the DA-resistant isolate. As a result, LDH activity in the DA-resistant *B.*

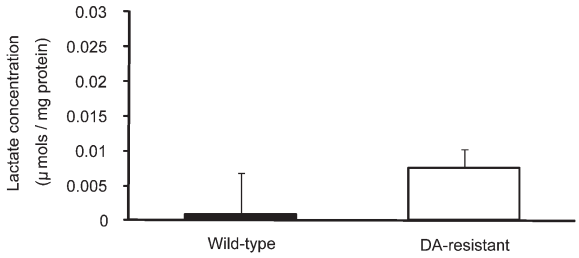

Fig. 4. Lactate concentration of the wild-type (closed bar) and the DA-resistant *B. gibsoni* isolate (open bar). Data are expressed as the means ± SD (n = 3).

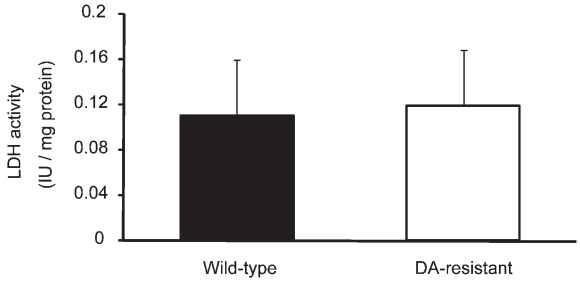

Fig. 5. Lactate dehydrogenase (LDH) activity of the wild-type (closed bar) and the DA-resistant *B. gibsoni* isolate (open bar). Data are expressed as the means ± SD (n = 3).

gibsoni isolate was almost the same as that in the wild-type (Fig. 5). Additionally, the lactate concentration and LDH activity in uninfected erythrocytes were 0.014 ± 0.010 μmols/mg protein and 0.004 ± 0.002 IU/mg protein, respectively.

Intracellular pyruvate concentration and pyruvate kinase activity: The concentration of pyruvate, the intermediate product of the glycolysis pathway, was assessed and compared between the wild-type and the DA-resistant isolate. As shown in Fig. 6, mean pyruvate concentration in DA-resistant *B. gibsoni* isolate was not different from that in the wild-type. Additionally, the activity of PK was measured and compared between the wild-type and the DA-resistant isolate. As shown in Fig. 7, the PK activity in the DA-resistant *B. gibsoni* isolate was almost the same as in the wild-type. Additionally, the pyruvate concentration and PK activity in uninfected erythrocytes were 0.007 ± 0.003 μmols/mg protein and 0.005 ± 0.004 IU/mg protein, respectively.

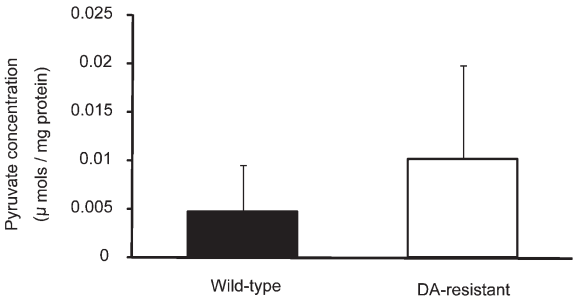

Fig. 6. Pyruvate concentration of the wild-type (closed bar) and the DA-resistant *B. gibsoni* isolate (open bar). Data are expressed as the means ± SD (n = 3).

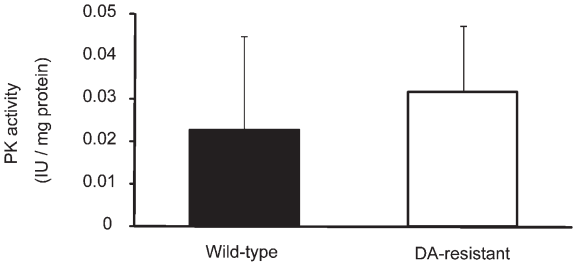

Fig. 7. Pyruvate kinase (PK) activity of the wild-type (closed bar) and the DA-resistant *B. gibsoni* isolate (open bar). Data are expressed as the means ± SD (n = 3).

Discussion

In general, parasite proliferation needs a lot of energy. In the previous report, the level of parasitemia of the DA-resistant *B. gibsoni* isolate was consistently lower than that of the wild-type[34]; therefore, we expected that the energy metabolism in the DA-resistant isolate might be suppressed. Accordingly, the concentrations of ATP and glucose were measured. ATP concentration reflects the level of total energy. The importance of glucose as an energy source for blood protozoa is well known[13]. In particular, it is suggested that *B. gibsoni* can easily use glucose as their main energy source[35]. Unexpectedly, in the present study, the concentrations of ATP and glucose in the DA-resistant *B. gibsoni* isolate were significantly higher than those in the wild-type. These results indicated that the DA-resistant isolate consumed more glucose and generated more ATP than the wild-type, although the proliferation potential of the DA-resistant isolate is lower than that of the wild-type. An increase in energy metabolism observed for drug-resistant parasites is associated with increased ATP delivery to essential ATP-consuming cell processes. For example, the upregulation of ion-motive ATPases and overexpression of vacuolar ATPases both modifying cytoplasmic pH; and upregulation of F_1F_O-ATPase for the regulation of drug efflux were reported in drug resistance of *Leishmania* spp.[5,9,23,28]. Thus, the drug-resistant protozoan could consume extra energy to survive under drug pressure. Similarly, it is possible that the DA-resistant *B. gibsoni* isolate may use extra energy to survive under stress by DA rather than for proliferation. Therefore, the energy metabolism in the DA-resistant isolate seemed to be up-regulated compared to that in the wild-type. It has been generally accepted that *Babesia* parasites derive most, if not all, of their energy requirement from the conversion of glucose to lactate via anaerobic glycolysis[29,35]. It was also suggested that *B. gibsoni* parasites derive their energy from glycolysis[35]. Accordingly, the enzyme reactions of the glycolysis pathway in the DA-resistant *B. gibsoni* isolate were investigated.

LDH activity is expected to be elevated when the glycolysis pathway is activated to generate a lot of energy in the DA-resistant *B. gibsoni* isolate. Therefore, the LDH activity and lactate concentration in the DA-resistant *B. gibsoni* isolate were compared with that in the wild-type. As a result, the LDH activity and lactate concentration did not differ between the wild-type and the DA-resistant *B. gibsoni* isolate. In general, the conversion of pyruvate to lactate via LDH is reversible and is regulated by the lactate concentration (suppressing feedback), indicating that the activity of this reaction represents the activity of the glycolysis pathway[2]. Similarly, Bork *et al.* reported that LDH plays important roles in regulating glycolysis in *Babesia* parasites[6]. From the present result and previous reports, the activity of the glycolysis pathway in the

DA-resistant *B. gibsoni* isolate was not considered to be enhanced.

Subsequently, the PK activity and pyruvate concentration in the DA-resistant *B. gibsoni* isolate were compared with that in the wild-type. Pyruvate produced by pyruvate kinase lies at the intersection of multiple metabolic pathways[38]; therefore, it was expected that PK activity would be elevated when some metabolic pathways, in which pyruvate is metabolized, were activated in DA-resistant *B. gibsoni* isolates. However, according to the present study, there was no difference in the PK activity and pyruvate concentration between the wild-type and the DA-resistant *B. gibsoni* isolate, indicating that the production of pyruvate in the DA-resistant *B. gibsoni* isolate was not enhanced. It is well known that pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase complex (PDC) and that acetyl-CoA is utilized in the tricarboxylic acid (TCA) cycle to generate energy. Yamasaki *et al.* reported that *B. gibsoni* has glutamate dehydrogenase activity, which plays an important role in the TCA cycle[35], suggesting that the parasites have a TCA cycle. Moreover, in *B. rodhaini,* it was demonstrated that glycolyticaly derived pyruvate can be converted to several products, such as oxaloacetate, malate, fumarate, acetyl-CoA, lactate, and alanine[29]. Additionally, *B. bovis* and *B. rodhaini* can convert a significant amount of pyruvate to alanine-like catabolic amino acids[1,30]. Similarly, it is possible that *B. gibsoni* can also convert pyruvate to these products; however, the pyruvate-mediated metabolic pathways of *B. gibsoni* were not activated according to the findings of this study.

According to the present study, the DA-resistant *B. gibsoni* isolate contained a higher ATP concentration than the wild-type and that the activity of the glycolysis pathway was not altered in the DA-resistant *B. gibsoni* isolate; however, we could not determine the mechanism of the high energy production of the DA-resistant *B. gibsoni* isolate. It is expected that *Babesia* parasites have several energy metabolic systems and that the DA-resistant *B. gibsoni* isolate generates extra energy via an energy metabolic system other than the glycolytic pathway, although currently available information on the energy metabolism of the *Babesia* parasite is limited. Recently, it was reported that TCA metabolism in the human malaria parasite *P. falciparum* is largely disconnected from glycolysis and is organized with fundamentally different architecture from the canonical textbook pathway[20]. Kellen *et al.*[20] reported that this pathway is not cyclic, but rather is a branched structure in which the major carbon sources are the amino acids glutamate and glutamine. In *Babesia* parasites, it is speculated that TCA metabolism also might be disconnected from glycolysis, and that pyruvate might not be necessary for TCA metabolism. According to our previous study, transcription levels of *COXI*, *COXIII*, and *CYTb* genes in mitochondrial DNA in the DA-resistant *B. gibsoni* isolate are not altered compared to those in the wild-type[34]; however, the activities of enzymes in TCA metabolism and the electron transport system in the parasites have yet to be assessed. It is possible that the DA-resistant *B. gibsoni* isolate could generate a lot of energy via TCA metabolism and the electron transport system. Accordingly, to elucidate the mechanisms of the DA resistance of *B. gibsoni*, TCA metabolism and the electron transport system in mitochondria of the DA-resistant *B. gibsoni* isolate should be investigated in further studies.

In the present study, we succeeded in measuring the concentrations of ATP, glucose, lactate, and pyruvate, and the activities of LDH and PK in cultured *B. gibsoni* using the method of Beutler[4] with some modifications, although this method was developed to measure those in erythrocytes. In the present study, the protein concentration in the parasite suspension was used to standardize the concentrations of substrates and the activities of enzymes. It was assumed that the level of parasitemia of cultured *B. gibsoni* would not affect the calculated result using this calculation method. In addition, the

amounts contaminating the erythrocyte membrane were fairly low and almost the same between the wild-type and the DA-resistant isolate. Thus, it was considered that the results obtained in the present study could be compared between the wild-type and the DA-resistant *B. gibsoni* isolate. In the future, other substrate concentrations and other enzyme activities in the energy metabolism of the parasites could be assessed using the method of Beutler with some modifications.

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