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Citation	Japanese Journal of Veterinary Research, 58(3&4), 155-164
Issue Date	2010-11
DOI	https://doi.org/10.14943/jjvr.58.3-4.155
Doc URL	https://hdl.handle.net/2115/44137
Type	departmental bulletin paper
File Information	JJVR58-3&4_p155-164.pdf



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

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Received for publication, May 12, 2010; accepted, July 5, 2010

Abstract

In our previous report, we developed a diminazene aceturate (DA)-resistant *Babesia gibsoni* strain that was maintained in culture with 200 ng/ml DA. While developing this strain, we also obtained DA-resistant *B. gibsoni* variants, which were maintained in culture with DA from 1 to 175 ng/ml for more than 8 weeks. Because heat shock protein 70 (Hsp70) seems to play important roles in adaptation to a stress environment in protozoan parasites, in the present study, we examined the copy number of *B. gibsoni* Hsp70 (*BgHsp70*) transcripts of those DA-resistant variants using quantitative real-time reverse transcription-polymerase chain reaction. We found that when wild-type *B. gibsoni* was exposed to 1 ng/ml DA, the level of *BgHsp70* transcripts was decreased at day 14. The copy number of *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 transcript levels of the *BgHsp70* gene might be reduced when the parasites are exposed to a low concentration of DA, and then might recover to the normal level after achieving resistance against DA. We expect that further study of the function of *BgHsp70* will elucidate the mechanism of drug resistance against DA in *B. gibsoni*.

Key words: *Babesia gibsoni*, drug resistance, heat shock protein 70, qRT-PCR, selection

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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 treatment of the disease^{4,6,27}. Recently, a new treatment strategy using atovaquone and azithromycin has been proposed^{15,16,18,21}; 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 our previous report, a DA-resistant *B. gibsoni* strain was developed *in vitro*¹⁰. During the development of the DA-resistant strain, the parasites were initially cultured with 1 ng/ml DA. After these parasites proliferated normally, the concentration of DA in the culture medium was increased approximately every 2 weeks and was adjusted based on the proliferation condition of the parasites. While developing the DA-resistant *B. gibsoni* strain, we also obtained DA-resistant *B. gibsoni* variants, which were maintained in culture with DA from 1 to 175 ng/ml for more than 8 weeks. Finally, the parasites cultured with 200 ng/ml DA were determined to be a DA-resistant *B. gibsoni* strain. This DA-resistant strain could survive direct exposure to 200 ng/ml 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 chaperone⁹ and plays important roles in cell proliferation and the control of cellular functions^{5,14,20}. Previously, the sequence of the *Hsp70* gene of *B. gibsoni* (*BgHsp70*) was determined^{24,30,31}. *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 increased³². 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 within the host¹⁴. 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 temperature¹², suggesting that Hsp70 also contributes to the development of drug resistance. Indeed, Witkowski *et al.* showed the overexpression of Hsp70 in an artemisinin-tolerant *P. falciparum* strain²⁶. Previous studies also suggested that Hsp70 was involved in drug resistance in breast cancer cells^{7,25}. 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 treatment¹⁹. In *Escherichia coli*, the bactericidal action of fluoroquinolone is moderately affected by DnaK and GroEL (Hsp60) chaperones²⁸. Considering these reports, we expect that *BgHsp70* is related to the development of resistance against DA in *B. gibsoni*. In the present study, we examined changes in the transcription of the *BgHsp70* gene during the development of the DA-resistant *B. gibsoni* strain, and found that the expression of the gene might be reduced when the parasites are exposed to a low concentration of DA.

Materials and Methods

In vitro cultivation of wild-type *B. gibsoni*: The strain of *B. gibsoni* used in the present study was originally obtained from a dog naturally

infected with *B. gibsoni* in Nagasaki, Japan, in 1973 and has been maintained in dogs at Hokkaido University since then. The cultured *B. gibsoni* strain has been maintained in culture in our laboratory since 2000²⁹⁾ and was used as wild-type *B. gibsoni* in the present study. 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, CA, U.S.A.), 20% dog serum and canine HK red blood cells (RBCs) that contain a high concentration of potassium¹¹⁾, sufficient to yield a packed cell volume (PCV) of 5%. Every 24 hours, 60% of the culture supernatant was removed and replaced with an equal volume of fresh culture medium. Every 7 days, half of the erythrocyte suspension was removed and replaced with an equal volume of uninfected fresh erythrocyte suspension as a subculture³³⁾. Wild-type *B. gibsoni* were cultured in culture medium containing 1 ng/ml 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* variants: The DA-resistant *B. gibsoni* strain was developed in our previous study. Briefly, the wild-type *B. gibsoni* strain was cultured in culture medium containing 1 ng/ml DA, and then the concentration of DA was gradually increased from 1 to 200 ng/ml¹⁰⁾. 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 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 *BgHsp70* transcription against a 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 gene transcription of BgHsp70 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 *BgHsp70* transcripts in a cDNA sample was measured by qRT-PCR according to the method of Yamasaki *et al.* with some modifications³³⁾.

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 an 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 re-annealing 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'-TCGTATTT AACTGTCAGAGG-3'; Bg18S-2, 5'-ACGGTATCT GATCGTCTTCG-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.*³²⁾. 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. 1). 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. 2a). 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. 2b). The relative number of *BgHsp70* transcripts was also decreased at day 14 (Fig. 2c).

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 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. 3 and 4, respectively). The

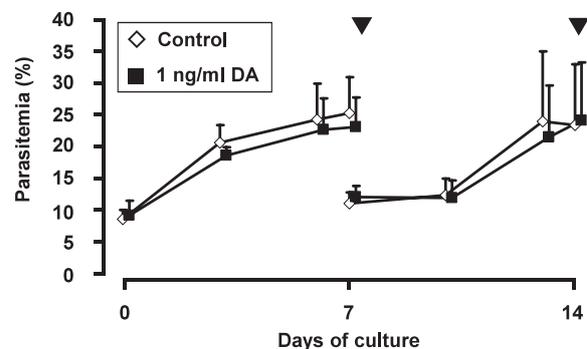


Fig. 1. Levels of parasitemia of wild-type *B. gibsoni* cultured with 1 ng/ml DA (closed square) and without DA (control, open diamond) for 14 days. Arrowheads indicate the points of subculture and sampling. Data are expressed as the means \pm SD ($n = 3$).

amounts of 18S rRNA in those variants cultured with various concentrations of DA were almost the same as in wild-type *B. gibsoni* (Figs. 3a and 4a).

The copy number of *BgHsp70* transcripts in the DA1 variant was significantly ($P < 0.05$) lower than in wild-type *B. gibsoni* (Fig. 3b). The copy numbers of *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 *BgHsp70* transcripts in these variants were lower than in wild-type *B. gibsoni*. The relative numbers of *BgHsp70* transcripts in DA1 and DA30 variants were significantly ($P < 0.05$) lower than in wild-type

B. gibsoni (Fig. 3c). 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. 4b); therefore, the relative amounts of *BgHsp70* transcripts in those variants were almost the same as in wild-type *B. gibsoni* (Fig. 4c).

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 hr: To examine the rapid responses of *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

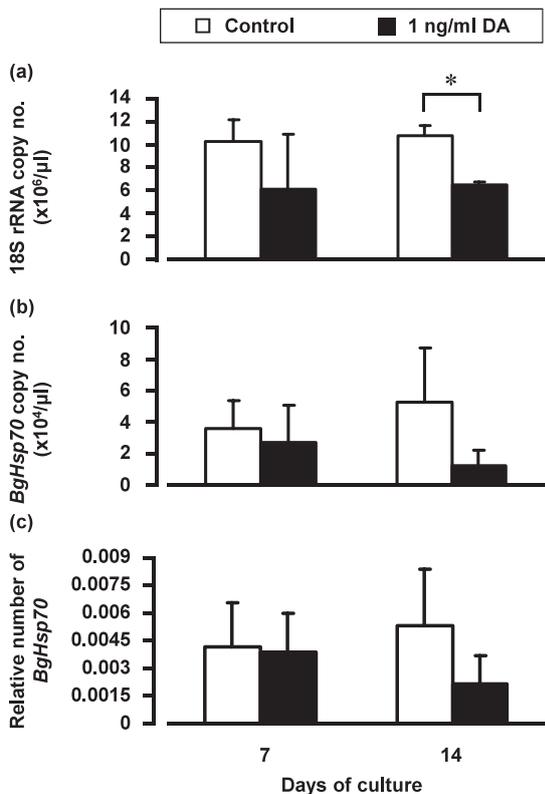


Fig. 2. Changes of the amount of *B. gibsoni* 18S rRNA (a), the copy number of *BgHsp70* transcripts (b) and the relative numbers of *BgHsp70* transcripts (c) in wild-type *B. gibsoni* cultured in culture medium containing 1 ng/ml DA (closed bar) and without DA (control, open bar) for 14 days. Data are expressed as the means \pm SD ($n = 3$). *Significantly ($P < 0.05$) different from the control group.

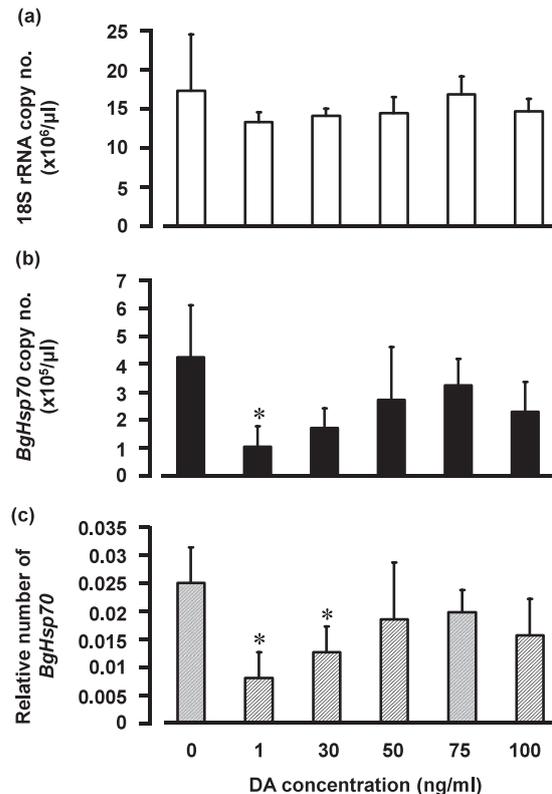


Fig. 3. The amounts of *B. gibsoni* 18S rRNA (a), copy numbers of *BgHsp70* transcripts (b) and the relative numbers of *BgHsp70* transcripts (c) in DA0 (wild-type *B. gibsoni*), DA1, DA30, DA50, DA75 and DA100 variants of *B. gibsoni*, which were maintained in culture medium containing 0, 1, 30, 50,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

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. 5a and 5b, respectively). In wild-type *B. gibsoni*, the amount of 18S rRNA was almost constant throughout the incubation period (Fig. 6a). The copy number of *BgHsp70* transcripts appeared to have slightly decreased at hour 24, although it was nearly constant until hour 12 (Fig. 6b); therefore, the relative numbers of *BgHsp70* transcripts were slightly, but not significantly, decreased at hour 24 (Fig. 6c).

In the DA-resistant *B. gibsoni* strain, the amount of 18S rRNA was also almost constant throughout the incubation period (Fig. 7a). There was a significant ($P < 0.05$) difference in the

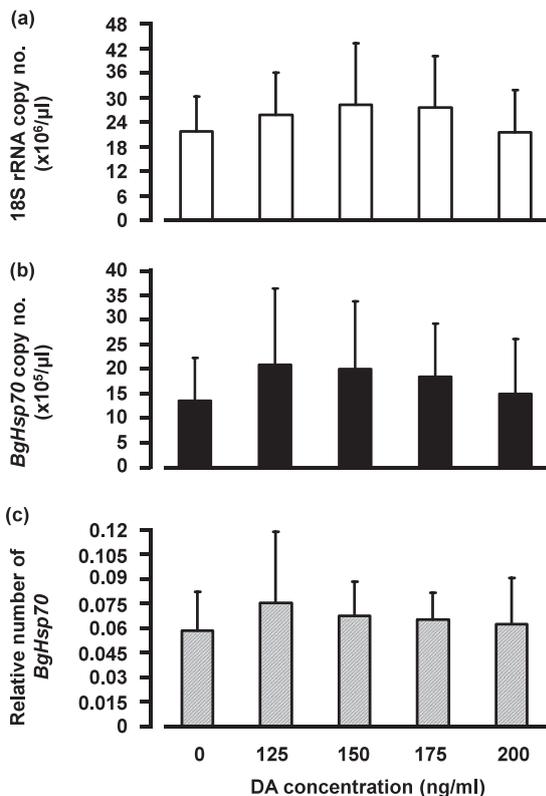


Fig. 4. The amounts of *B. gibsoni* 18S rRNA (a), copy numbers of the *BgHsp70* transcripts (b) and relative numbers of *BgHsp70* transcripts (c) in DA0 (wild-type *B. gibsoni*), DA125, DA150, DA175 and DA200 (DA-resistant strain) variants of *B. gibsoni*, which were maintained in culture medium containing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

copy number of *BgHsp70* transcripts at hour 6 between parasites cultured with 500 ng/ml DA and without DA (Fig. 7b). Otherwise, the copy number of *BgHsp70* transcripts in the DA-resistant strain incubated with 500 ng/ml DA was nearly constant throughout the incubation period (Fig. 7b); therefore, the relative numbers of *BgHsp70* transcripts were also almost constant (Fig. 7c).

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 stress¹⁴. 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^{3,7,25}; however, in the present study, the transcription of the *BgHsp70* gene of wild-type *B. gibsoni* was

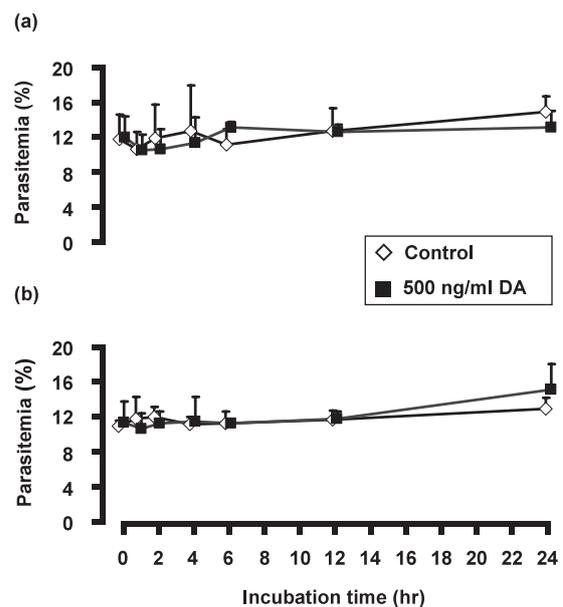


Fig. 5. The levels of parasitemia of wild-type *B. gibsoni* (a) and DA-resistant (b) *B. gibsoni* strains cultured with 500 ng/ml DA (closed square) and without DA (control, open diamond) for 24 hr. Data are expressed as the means \pm SD ($n = 3$).

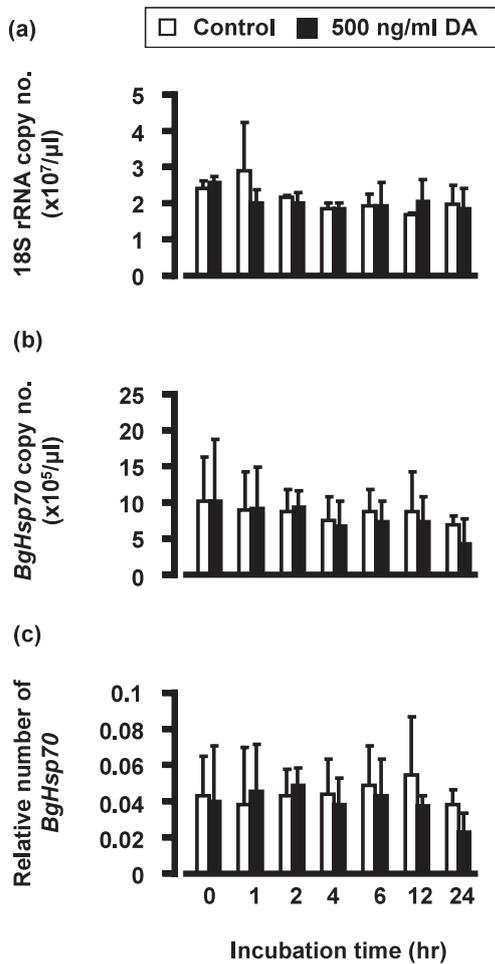


Fig. 6. The amounts of *B. gibsoni* 18S rRNA (a), copy numbers of *BgHsp70* transcripts (b) and the relative numbers of *BgHsp70* transcripts (c) in wild-type *B. gibsoni* cultured with 500 ng/ml DA (closed bar) and without DA (control, open bar) were observed for 24 hr. Data are expressed as the means \pm SD (n = 3).

reduced by weak concentrations of DA, such as that of 1 ng/ml. Similarly, the reduction of Hsps was reported for various organisms. Sorensen *et al.* reported in a review that the direction of evolutionary adaption of Hsp levels seemed to be divergent²². 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 pollution⁸. In selection experiments with different species of *Drosophila*, the expression of Hsp70 was lower in lines that had previously been

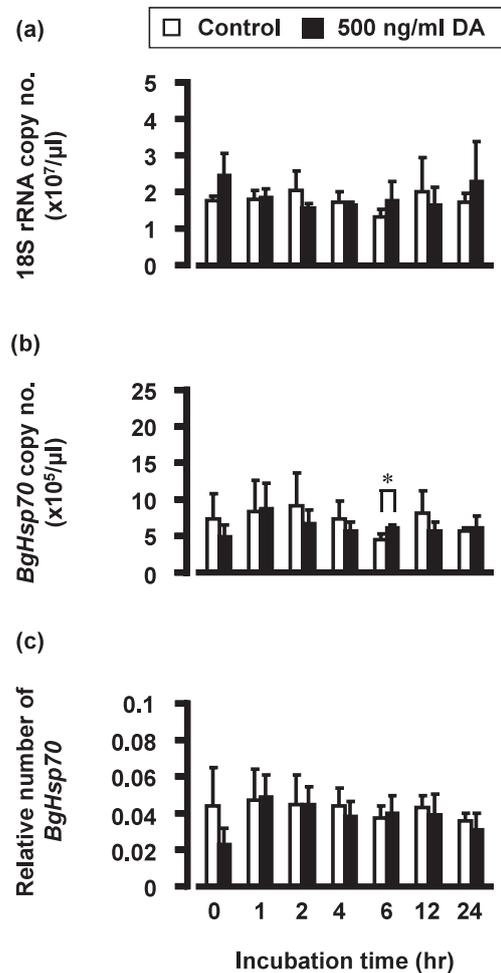


Fig. 7. The amounts of *B. gibsoni* 18S rRNA (a), copy numbers of the *BgHsp70* transcripts (b) and relative numbers of *BgHsp70* transcripts (c) in the DA-resistant *B. gibsoni* strain cultured with 500 ng/ml DA (closed bar) and without DA (control, open bar) were observed for 24 hr. Data are expressed as the means \pm SD (n = 3). *Significantly ($P < 0.05$) different from the control group.

exposed to stressful conditions for many generations^{13,23}. An experiment using North Sea mussels also showed that phenotypes displaying low Hsp levels appeared to be evolutionarily favored². 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 transcript levels of the *BgHsp70* gene would return to the normal level after achieving

resistance to DA; however, since the mechanism for the reduction of *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.* reported that the gene transcription and protein synthesis of *BgHsp70* were increased by a temperature shift for 1 hr³²⁾. Therefore, we expected to find a rapid response of the *BgHsp70* transcription against the strong pressure of DA; however, the present results suggested that there was no rapid response of *BgHsp70* transcription to this DA pressure in either wild-type *B. gibsoni* or the DA-resistant *B. gibsoni* strain. Since DA does not show antibabesial activity within 24 hr, 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 hr; therefore, the relative numbers of *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*³²⁾ and several genes in *Plasmodium falciparum*^{1,17,34)} by qRT-PCR. However, the amount of 18S rRNA was decreased when wild-type *B. gibsoni* were cultured with 1 ng/ml DA. Yamasaki *et al.* reported that the amount of 18S rRNA was also decreased at a lower temperature³²⁾. 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.

Acknowledgments

This work was supported in part by a grant (no. 18780223) from the Science Research Fund of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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