# Development of unstable resistance to diminazene aceturate in *Babesia bovis*

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**ABSTRACT**

Diminazene aceturate (DA) is commonly used in the treatment of bovine babesiosis caused by *Babesia bovis*. In this study, we attempted to develop resistance in *B. bovis in vitro* to DA and clofazimine (CF, a novel antibabesial agent) using short- and long-term drug pressures. In the short term, we found that 6.7 ± 2 (0.54 ± 0.16 µM)-, 12.9 ± 8.6 (1.05 ± 0.7 µM)-, and 14 ± 5.9 (1.14 ± 0.48 µM)-fold increases in the half-maximal inhibitory concentration ($IC_{50}$) of DA were demonstrated on *B. bovis* cultivated with 0.04 µM of DA pressure for 4, 8, and 12 days, respectively, as compared to that on parental culture (0.08 ± 0.0065 µM) before drug pressure was initiated. However, in *B. bovis* cultivated with 0.04 µM of DA pressure after 16 days, the parasites could not tolerate 0.8 µM of DA. In the long term, 7.6 ± 3.5-, 20.5 ± 0.1-, and 26.8 ± 5.5-fold increases in the $IC_{50}$ of DA were demonstrated on parasites from subcultures at days 8, 3, and 5 post-cultivation, respectively, in a drug-free medium, where these subcultures were obtained from *B. bovis* cultivated with DA pressure with changing doses for 30, 60, and 90 days, respectively. However, the second and third times, no increase was demonstrated on *B. bovis* from these subcultures at days 15 and 30 post-cultivation in a drug-free medium. In addition, in *B. bovis* cultivated with drug pressure after 90 days, the parasites tolerate up to 0.64 µM DA. All findings demonstrated that DA resistance in *B. bovis* is unstable and lost within 15 days of drug withdrawal. However, treatment with subtherapeutic doses of DA in cattle might result in the development of resistance in *B. bovis*, which may not even respond to subsequent treatments with high doses of DA. Thus, if the bovine babesiosis caused by *B. bovis* is unresponsive to DA, treatment with other antibabesial agents might be recommended.

Keywords: *Babesia bovis*, unstable drug resistance, diminazene aceturate, *in vitro*

## 1. Introduction

The emergence of drug resistance in pathogens which include bacteria, protozoa, and fungi is a major challenge for the successful treatment of infectious diseases (Fairlamb et al., 2016; Yoshida et al., 2017). During last few decades, protozoan parasites have developed resistance to several antiprotozoal agents through various mechanisms, such as genetic changes in the target sites, the reduced uptake and increased efflux of drugs, and metabolic regulations (Fairlamb et al., 2016; Lemieux et al., 2016). The emergence of drug-resistant parasites has serious implications for treating clinical cases, leading to increased morbidity and mortality (Fairlamb et al., 2016). For instance, the emergence of *P. falciparum* strains resistant to almost all of the antimalarial agents has severely undermined malaria control programs (Corey et al., 2016).

The development of drug resistance in *Babesia* species, which are intraerythrocytic apicomplexan parasites, has been well documented (Hwang et al., 2010; Krause et al., 2008; Lemieux et al., 2016; Matsuu et al., 2006). A combination therapy of atovaquone with azithromycin is recommended for the treatment of human babesiosis caused by *Babesia microti* (Krause et al., 2000), but *B. microti* strains resistant to both drugs have emerged recently (Krause et al., 2008; Lemieux et al., 2016; Wormser et al., 2010). In addition, a previous study found that treatment with atovaquone induces the development of resistance in *Babesia gibsoni* in dogs (Matsuu et al., 2006). The possible mechanism of atovaquone resistance is suggested to be point mutations that result in amino acid substitution in cytochrome *b*, which is the molecular target of atovaquone (Lemieux et al., 2016; Matsuu et al., 2006).

Diminazene aceturate (DA) is an antibabesial agent commonly used for the treatment of animal babesiosis, which is caused by a species of *Babesia* and has worldwide distribution (Bock et al., 2004; Mosqueda et al., 2012). Although different species of *Babesia* infect cattle, only three *Babesia* species including *B. bovis*, *B. bigemina*, and *B. divergens* are known to cause

severe clinical babesiosis (Bock et al., 2004). In particular, *B. bovis* causes the most severe form of bovine babesiosis, as the parasite-infected erythrocytes adhere to the endothelial cells in the capillaries in internal organs, such as the brain and lungs, leading to neurological and respiratory signs (Everitt et al., 1986). Control strategies against *B. bovis* include tick control, vaccination, and chemotherapy (Bock et al., 2004; Mosqueda et al., 2012). Tick-control strategies are often ineffective because of the development of acaricide resistance (Kunz and Kemp, 1994). Although live vaccines are used in several endemic countries, the wide use of such a vaccine is prevented by various factors, such as the risk of contamination with other blood pathogens, lack of protection due to strain variations, and time-consuming production procedures (Bock et al., 2004; Brown et al., 2006). Therefore, chemotherapy including DA is vital for minimizing the economic damage caused by *Babesia* parasites, including *B. bovis*. Unfortunately, however, recent studies have suggested the possible development of DA resistance in *Babesia* parasites (Hwang et al., 2010; Yamasaki et al., 2017). For instance, *in vitro* treatment with low to high doses of DA resulted in the development of DA-resistant *B. gibsoni* (Hwang et al., 2010). In addition, the low efficacy of DA against *B. bovis* in cattle has sometimes been documented, suggesting the possibility of the emergence of drug-resistant *B. bovis* in these cases (Mosqueda et al., 2012; Vial and Gorenflot, 2006). However, the development of DA-resistant *B. bovis* has never been investigated. Therefore, in the present study, we investigated whether continuous drug pressure could induce the emergence of DA-resistance in *B. bovis in vitro*. In parallel, we also investigated whether such drug pressure could result in *B. bovis* strains resistant to clofazimine (CF), an antibiotic that has recently been characterized as a potent antibabesial agent.

## 2. Materials and methods

87 *2.1. Parasite and cultivation*

88 The Texas strain of *B. bovis* was maintained in fresh cattle erythrocytes (from a mature
89 Holstein cow) and serum-free GIT culture medium supplemented with 1 × antibiotic-
90 antimycotic solution containing 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25
91 μg/ml of amphotericin B, as previously described (Bork et al., 2005). Cultures were maintained
92 in a multi-gas water-jacketed incubator in an atmosphere of 5% O2 and 5% CO2 at 37ºC. The
93 culture medium was changed every day. All chemicals were purchased from Sigma-Aldrich
94 (Tokyo, Japan).

95

96 *2.2. Drugs*

97 DA was purchased from Ciba-Geigy Japan Ltd. (Tokyo, Japan). CF was purchased from
98 Sigma-Aldrich (Tokyo, Japan). DA and CF were dissolved in Milli-Q water (MQW) and
99 dimethyl sulfoxide (DMSO), respectively, to prepare 10 mM stocks.

100

101 *2.3. Determination of the $IC_{50}$ of DA and CF*

102 The half-maximal inhibitory concentrations ($IC_{50}$s) of DA and CF were calculated on
103 *in vitro* growth of *B. bovis* in serum-free GIT culture media using a fluorescence-based growth-
104 inhibition assay as previously described (Rizk et al., 2015). Briefly, 97.5 μL of 0.01, 0.1, 0.5,
105 2.5, 5, 10, and 20 μM of DA and 0.025, 0.25, 1.25, 6.25, 12.5, 25, and 50 μM of CF prepared
106 in a culture medium was added in triplicate to a 96-well culture plate. To examine whether these
107 solvents affect the growth of parasites, media containing 0.2% MQW and 0.5% DMSO
108 (calculated based on their concentration in the highest dose of DA and CF, respectively) were
109 also added in triplicate as untreated controls. When the percentage of parasitized erythrocytes
110 (%PE) was calculated to be over 4% in previously maintained *B. bovis in vitro* cultures, the 4%
111 PE were further diluted with fresh erythrocytes to achieve 1% PE, and then 2.5 μL of the 1%

PE was added to each well. These 100 µl of cultures with 2.5% hematocrit concentration (HTC) and 1% initial PE were incubated as described in section 2.1 for 3 days without changing media. On day 3, 100 µL lysis buffer containing 2 × SYBR Green I (Lonza Rockland, Inc., Rockland, ME, USA) was added to each well, and then incubated at room temperature in the dark for 6 hours. The fluorescence values were determined at 485 nm excitation and 518 nm emission wavelengths. Fluorescence values were corrected for background signals, converted to growth rates relative to untreated controls, and then the $IC_{50}$s of DA and CF were calculated by a curve-fitting method. The calculated $IC_{50}$s of DA and CF against *B. bovis* were 0.08 ± 0.0065 µM and 1.55 ± 0.045 µM, respectively. These $IC_{50}$s of drugs were compared with the $IC_{50}$s of DA and CF on parasites subjected to drug pressure, as described in sections 2.4 and 2.5.

### *2.4. Short-term DA and CF pressure with ½ × $IC_{50}$ doses*

*B. bovis in vitro* cultures were subjected to DA and CF pressure for 24 days (Suppl. Fig. 1). Briefly, in 24-well culture plates, 100 µl of bovine erythrocytes containing 1% PE was added to each well together with 900 µl of culture medium (10% HCT) containing ½ × $IC_{50}$ doses of DA (0.04 µM) and CF (0.8 µM) and incubated as described in section 2.1. The culture medium was replaced every 24 hours with fresh medium containing 0.04 µM of DA or 0.8 µM of CF. In addition, 0.2% MQW and 0.5% DMSO solvents of DA and CF, respectively, were used as respective untreated controls. On days 4, 8, 12, and 16 (every 4 days), Giemsa-stained erythrocyte smears were prepared to calculate the %PE, and then parasite-infected erythrocytes from each culture were used to prepare subcultures with 1% initial PE. The drug pressure with the ½ × $IC_{50}$ dose was continued on the subcultures (1% initial PE) prepared on days 0, 4, 8, and 12, while the subcultures (1% or 4% initial PE) prepared from MQW-, DA-, DMSO-, and CF-treated cultures on day 16 were treated with 10 × $IC_{50}$ doses of DA (0.8 µM) and CF (16 µM), respectively, for the next 8 days. On days 4, 8, and 12, parasites from the treated cultures

were also used to determine the $IC_{50}$s of both DA and CF in order to assess the potential development of drug resistance in *B. bovis*. The $IC_{50}$s of DA and CF on *B. bovis* cultivated with drug pressure were calculated, essentially as described in section 2.3, using the fluorescence-based method. These $IC_{50}$s were then compared with the $IC_{50}$s of DA and CF as determined on previously untreated *B. bovis* cultures in section 2.3. All experiments were repeated 4 times.

2.5. *Long-term DA and CF pressure with changing doses*

Similar to short-term drug pressure, the cultures were initiated with 1% PE and 10% HTC, and the culture medium containing DA and CF was replaced every day (Suppl. Fig. 2). However, instead of preparing subcultures, fresh erythrocytes were added to the cultures, based on the HTC reductions, to maintain 10% HCT throughout the experiment. The drug pressure was initiated with a ½ × $IC_{50}$ dose for DA (0.04 µM) or CF (0.8 µM). Monitoring of the %PE was performed by using Giemsa-stained erythrocyte smears. The drug doses were increased or decreased based on the levels of %PE. Subcultures were prepared and maintained in a drug-free medium after 30, 60, and 90 days of drug pressure. When over 4% PE were calculated in these subcultures (on days 8, 3, and 5 for subcultures prepared from *B. bovis* cultivated with drug pressure for 30, 60, and 90 days, respectively), parasites from these subcultures were used to determine the $IC_{50}$s of both DA and CF for the first time. The second and third times, the $IC_{50}$s of drugs on subcultures maintained in a drug-free medium were determined on days 15 and 30, respectively. The experiment was repeated twice.

*2.6. DA and CF pressure with constant and increasing doses*

After 90 days of drug pressure, as described in section 2.5, the cultures were treated with $IC_{50}$ doses of DA and CF for another four days (Suppl. Fig. 2). After the %PE was calculated at day 94, subcultures (1% initial PE and 10% HTC) were prepared and subjected to

treatment with the $IC_{50}$ doses of DA and CF for three days. After this 3-day treatment, subcultures were prepared at day 97 from these cultures and treated with 2 × $IC_{50}$ doses of DA (0.16 µM) and CF (3.2 µM) for 4 days. Subsequent subcultures prepared from the DA-treated cultures on days 101, 105, 109, 113, 117, and 121 were each treated for 4 days with 4 ×, 5 ×, 6 ×, 7 ×, 8 ×, and 9 × $IC_{50}$ doses of DA (0.32, 0.4, 0.48, 0.56, 0.64, and 0.72 µM, respectively). On the other hand, subcultures from CF-treated cultures prepared on days 97, 101, 105, 109, 113, and 117 were each treated for 4 days with a 2 × $IC_{50}$ dose of CF (3.2 µM), as the %PE in treated cultures did not increase. The experiment was repeated twice.

### *2.7 Statistical analysis*

An unpaired Student's *t*-test with two-tailed method in Microsoft Excel 2016 was used to calculate the *P* values. A *P* value < 0.05 was considered to indicate significant differences between the $IC_{50}$s of drugs or the %PE in drug-treated cultures and their untreated controls.

## 3. Results

### *3. 1 Short-term DA and CF pressure with ½ × $IC_{50}$ doses*

As described in section 2.3, the calculated $IC_{50}$s of DA and CF on *B. bovis* in the parental culture were 0.08 ± 0.0065 µM and 1.55 ± 0.045 µM, respectively. We used ½ × $IC_{50}$s of DA (0.04 µM) and CF (0.8 µM) as drug pressure for 16 days because *B. bovis* was able to tolerate a ½ × $IC_{50}$ dose of the drugs (Suppl. Fig. 3A and B). On every $4^{th}$ day of cultures, the $IC_{50}$s of DA were 0.54 ± 0.16, 1.05 ± 0.7, and 1.14 ± 0.48 µM in *B. bovis* cultivated with 0.04 µM DA pressure for 4, 8, and 12 days, respectively, demonstrating that the $IC_{50}$ of DA on the parental culture (0.08 ± 0.0065 µM) was increased by 6.7 ± 2-, 12.9 ± 8.6-, and 14 ± 5.9-folds, respectively (Fig. 1A, DA-line in the development of resistance). In the case of the untreated

control, the determined $IC_{50}$s of DA were similar against parasites from parental and 0.2% MQW-treated cultures (Fig. 1A, MQW-line in the development of resistance). On day 16, 0.04 µM DA- and MQW-treated parasites in subcultures (1% or 4% initial PE) were subjected to treatment with 10 × $IC_{50}$ dose (0.8 µM) of DA for 8 days. Although the %PE in subcultures was significantly higher than that in the untreated MQW control during treatment with 0.8 µM DA, parasites cultivated with 0.04 µM DA pressure could not tolerate the 10 × $IC_{50}$ dose (0.8 µM) of DA for more than 8 days (Fig. 1B). On the other hand, the $IC_{50}$ of CF (1.55 ± 0.045 µM) increase of less than 5-fold was demonstrated against parasites cultivated with CF pressure for 4, 8, and 12 days and the untreated DMSO control as well (Fig. 1A, DMSO- and CF-lines in the development of drug resistance). Additionally, in *B. bovis* cultivated with CF pressure with a ½ × $IC_{50}$ dose of CF after 16 days, the parasites died on day 4 after treatment with CF at a 10 × $IC_{50}$ dose, as this result was similar to that of DMSO control (Fig. 1C). In addition, we found that parasites cultivated with CF and DA pressures were still sensitive to DA and CF, respectively, and the determined $IC_{50}$s of DA and CF were comparable to those against their untreated DMSO and MQW controls (Fig. 1A, DMSO-, CF-, MQW-, and DA-lines in cross-resistance).

*3.2 Long-term DA and CF pressure with changing doses*

After 30, 60, and 90 days of drug pressure with changing doses, three subcultures were obtained in drug-free medium (Suppl. Fig. 4A and B). Parasites from these 3 subcultures were then used to determine the $IC_{50}$s of drugs for the first time on days 8, 3, and 5, and then for a second and third time on days 15 and 30, respectively (Suppl. Fig 5A, B, and C). The first time, 7.6 ± 3.5-, 20.5 ± 0.1-, and 26.8 ± 5.5-fold higher $IC_{50}$s of DA against *B. bovis* sourced from these 3 subcultures at 8, 3, and 5 post-cultivation in drug-free medium, respectively, as compared to the $IC_{50}$ of DA (0.08 ± 0.0065 µM) determined before the drug pressure was initiated (Fig. 2A,

DA-line in the development of drug resistance). However, the second and third times, no increase in the $IC_{50}$ of DA was demonstrated on parasites from subcultures at days 15 and 30 post-cultivation in drug-free medium. In contrast, all $IC_{50}$ values of CF determined on subcultures obtained from *B. bovis* cultivated with 30, 60, and 90 days of CF pressure were comparable to the $IC_{50}$ of CF determined before the initiation of drug pressure, except for the first $IC_{50}$ of CF (increased 6-fold) on the subcultures obtained from parasites cultivated with 90 days of CF pressure (Fig. 2A, CF-line in the development of drug resistance, and Suppl. Fig. 6). In addition, all $IC_{50}$s of DA and CF on subculture-obtained parasites cultivated with CF and DA over the long term, respectively, were similar to the $IC_{50}$s of drugs determined on parental cultures before the initiation of drug pressure, further confirming that unstable resistance induced by DA is specific to DA (Fig. 2A, CF- and DA-lines in cross-resistance, and Suppl. Fig. 6).

*3.3 DA and CF pressure with constant and increasing doses*

After 90 days of drug pressure, we tested the effect of increasing doses of DA and CF on the development of drug resistance. After treating with fixed doses for 4 days, subcultures were prepared, and treatment was continued for several days with increased doses of DA and CF. The drug doses were increased until the parasites died. The parasites grew in an $8 \times IC_{50}$ dose (0.64 µM) of DA, but all parasites died when treated with $9 \times IC_{50}$ dose (0.72 µM) of DA (Fig. 2B). In contrast, all CF-treated parasites died when treated with CF at $2 \times IC_{50}$ dose (3.2 µM) at day 121 (Fig. 2C).

## 4. Discussion

The emergence of drug-resistant parasites is one stumbling block for the effective treatment of babesiosis (Krause et al., 2008; Matsuu et al., 2006; Vial and Gorenflot, 2006; Wormser et al.,

2010; Yeruham et al., 1985). In general, parasite lines are considered to be resistant to a given drug if the $IC_{50}$ of the drug increased by more than 10-fold as compared to the parent line (Nzila and Mwai, 2010). In the present study, we also found that a more than 10-fold increase in the $IC_{50}$ of DA was determined on *B. bovis* cultivated with a ½ × $IC_{50}$ dose (0.04 μM) of DA for 8 and 12 days (Fig. 1A). Therefore, the present findings suggest that short-term pressure with DA induces resistance in *B. bovis*. However, when we attempted to expose drug resistance from the culture with 16 days of pressure using a treatment with a 10 × $IC_{50}$ dose (0.8 μM), parasites did not grow for more than 8 days (Fig. 1B). These observations suggest that DA-resistant parasites emerging after short-term drug pressure cannot tolerate high doses of DA. On the other hand, no more than a 5-fold increase in the $IC_{50}$ of CF was demonstrated on *B. bovis* cultivated with a ½ × $IC_{50}$ dose (0.8 μM) of CF. These slight changes in the $IC_{50}$ of drugs may not indicate the development of resistance, as the fold changes were less than 10 (< 5-fold) (Corey et al., 2016; Nzila and Mwai, 2010). Therefore, this result suggests that the development of resistance to CF in *B. bovis* is unlikely with a short-term treatment. A previous study found a low efficacy of antibabesial agents, including clindamycin, doxycycline, and pentamidine, against DA-resistant *B. gibsoni* (Hwang et al., 2010). In addition, our previous study found that both DA and CF may bind AT-rich DNA regions in mitochondrial and plastid (apicoplast) genomes of *B. bovis* (Tuvshintulga et al., 2017). However, in the present study, DA-resistant *B. bovis* was not resistant to CF, indicating that resistance in *B. bovis* induced by DA pressure is specific to DA.

Next, the stability and tolerable dose of drug-resistant *B. bovis* in a drug-free medium were investigated after long-term drug pressure with changing doses based on the levels of %PE. We found that after 30, 60, and 90 days of drug pressure, approximately 7.6, 20, and 26-fold increases, respectively, in the $IC_{50}$ of DA were demonstrated on *B. bovis* in subcultures at days 8, 3, and 5 post-cultivation in a drug-free medium, respectively, suggesting that DA resistance becomes more prominent with the duration of drug pressure. On the other hand, a previous

study interpreted that a biphasic dose-response curve of drug indicates presence of two populations in a culture line such as drug-resistant and -sensitive parasites, whereas a monophasic dose-response curve indicates a single population (Nzila and Mwai, 2010). In the present study, we observed the monophasic dose-response curve of DA against parasites from parental culture and 0.04 μM DA-treated cultures in the short-term drug pressure, but the biphasic dose response curve of DA was only observed against *B. bovis* cultivated in a drug-free medium after the long-term drug pressure (Suppl. Fig. 7A and B). These results indicate that the DA resistance induced by drug pressure might be unstable when cultivated in a drug-free medium. Moreover, in the long-term drug pressure, no increase was demonstrated again on these parasites in a drug-free medium on days 15 and 30 post-cultivation, confirming that DA-resistant parasite is lost when cultivated in a drug-free medium for 15 days. The findings of present study collectively indicate that drug pressure with DA results in the development of unstable resistance in *B. bovis*. In previous studies, resistance to DA has been reported in *Trypanosoma* and *B. gibsoni* (de Koning et al., 2004; Hwang et al., 2010). The uptake of DA in *Trypanosoma brucei* and *T. equiperdium* is mediated by an adenosine transported known as P2, which is encoded by the TbAT1 and TevAT1 genes, respectively (de Koning et al., 2004; Witola et al., 2005). It has been proposed that the loss of activity of the P2 transporter in DA-resistant strains results in the low uptake of DA into *Trypanosoma,* leading to the reduced efficacy of DA (de Koning et al., 2004). In addition, upregulated mRNA expression of the TeDR40 gene contributes to increased resistance of *T. evansi* to DA (Witola et al., 2005). In a recent study, the reduced uptake of DA was observed in *B. gibsoni* resistant to DA, similar to DA-resistant *Trypanosoma* (Yamasaki et al., 2017). The same study found that the reduced update of DA is unlikely due to the loss of P2 transporter activity. Although the genes linked to DA-resistant *Babesia* are unknown, our previous study revealed that *B. bovis* mitochondrial (*cob* and *cox3*) and apicoplast (*tufA* and *clpC*) genes were upregulated during treatment with

DA, while these genes were downregulated in CF-treated *B. bovis* (Tuvshintulga et al., 2017). This may imply that temporally upregulated apicoplast and mitochondrial genes contribute to developing the unstable resistance in *B. bovis* to DA. Nevertheless, future investigation is necessary to clarify this assumption by using a quantitative analysis of mRNA in *B. bovis* withdrawal DA pressure. Although the mechanism of DA resistance in *B. bovis* is unclear, the present findings indicate that such mechanisms operate only during drug pressure, and parasites lose their resistance within 15 days of drug withdrawal. Therefore, the acquisition of DA-resistant *B. bovis* from cattle and its subsequent transmission to other cattle by tick vectors are highly unlikely. However, the unstable resistance of DA might potentially implicate the outcome of DA treatment in cattle with clinical babesiosis. Following intramuscular administration of a standard dose of DA (3.5 mg/kg dose), the half dose of this compound is eliminated from cattle's bodies within 7 days (Kellner et al., 1985). However, DA residue can still be found in urine and feces for more than 20 days. Therefore, if DA is administered at low doses, which are not sufficient to kill all parasites, surviving *B. bovis* in cattle might develop DA resistance, leading to treatment failures, even if cattle are subsequently treated with high doses of DA. Previously reported DA treatment failures in cattle with babesiosis caused by *B. bovis* might have been associated with the unstable development of DA resistance, although the details on doses of DA used in such cases are unknown (Mosqueda et al., 2012; Vial and Gorenflot, 2006).

In summary, the present study found that DA treatment results in the development of resistance in *B. bovis in vitro*. When the doses of DA were increased gradually, *B. bovis* was able to tolerate up to an 8-fold dose, as compared to the $IC_{50}$ dose of DA determined before the drug pressure. Resistance was stable for less than 15 days of drug withdrawal, indicating that DA resistance in *B. bovis* is unstable. However, unstable resistance to DA might lead to DA treatment failures in cattle with clinical babesiosis caused by *B. bovis*. On the other hand, our

findings showed that the development of resistance to CF is unlikely in *B. bovis*. We also found that DA-resistant *B. bovis* is still sensitive to CF, suggesting that resistance is specific to DA only. Thus, in situations where bovine babesiosis caused by *B. bovis* is unresponsive to DA, treatment with alternative antibabesial agents, such as CF, might be recommended.

## Authors' contributions

All authors conceived the present study. B.T. conducted all experiments in the present study and wrote the first draft of the manuscript, and all authors subsequently edited the manuscript.

## Conflict of interest statement

The authors declare that they have no competing interests.

## Acknowledgments

This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKEN, Grant Number 18 H02337.

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**Figure legends**

Fig. 1. Short-term DA and CF pressure with a ½ × $IC_{50}$ dose. A) The $IC_{50}$s of drugs calculated after 4, 8, and 12 days of drug pressure are illustrated as fold changes compared with the $IC_{50}$s of drugs determined before the initiation of drug pressure. Note that the $IC_{50}$ of DA on *B. bovis* previously subjected to DA pressure significantly increased, while the $IC_{50}$ of CF on *B. bovis* cultivated with CF pressure increased only slightly. Also note that the $IC_{50}$s of CF and DA on *B. bovis* that had been subjected to DA and CF drug pressure, respectively, were comparable to the $IC_{50}$s of drugs on the parental line and those against their untreated controls. B) Monitoring of %PE in a 10 × $IC_{50}$ dose of DA (0.8 µM)-treated culture initiated with *B. bovis* that had been subjected to 16 days of drug pressure with DA. Note that *B. bovis* cultivated with drug pressure grew with high parasitemia as compared with that in the untreated MQW control. C) Monitoring of %PE in a 10 × $IC_{50}$ dose of CF (16 µM)-treated culture initiated with *B. bovis* that had been subjected to 16 days of drug pressure with CF. Note that *B. bovis* cultivated with drug pressure and the untreated DMSO control lines grew similarly with low parasitemia.

Fig. 2. A long-term drug pressure with changing doses of DA or CF. A) *B. bovis* cultures were subjected to 30, 60, and 90 days of DA and CF drug pressure with doses determined based on the parasitemia dynamics; they were then maintained in drug-free medium. Parasites from the cultures maintained in a drug-free medium were then used to determine the $IC_{50}$s of drugs for the first time on days 8, 3, and 5, and then for second and third times on days 15 and 30, respectively. The fold changes in the $IC_{50}$ of drugs as compared with the $IC_{50}$s of drugs on the parental lines were plotted. Note that the first $IC_{50}$s of DA on *B. bovis* that had been subjected

to drug pressure were significantly higher as compared to the $IC_{50}$s of DA on the parental line, and that second and third determinations of the $IC_{50}$s of DA were comparable to the $IC_{50}$s of DA on the parental line. Also note that fold changes in the $IC_{50}$s of CF on *B. bovis* cultivated with CF drug pressure were less pronounced. Additionally, the $IC_{50}$s of DA and CF on *B. bovis* that had been subjected to CF and DA drug pressure, respectively, were comparable to those against the parental line. B) Monitoring of %PE in culture treated with constant as well as increasing doses of DA, showing *B. bovis* was able to grow in an 8 × $IC_{50}$ dose of DA (0.64 µM). C) Monitoring of %PE in culture treated with constant as well as increasing doses of DA, showing *B. bovis* was able to grow only 2 × $IC_{50}$ of CF (3.2 µM).

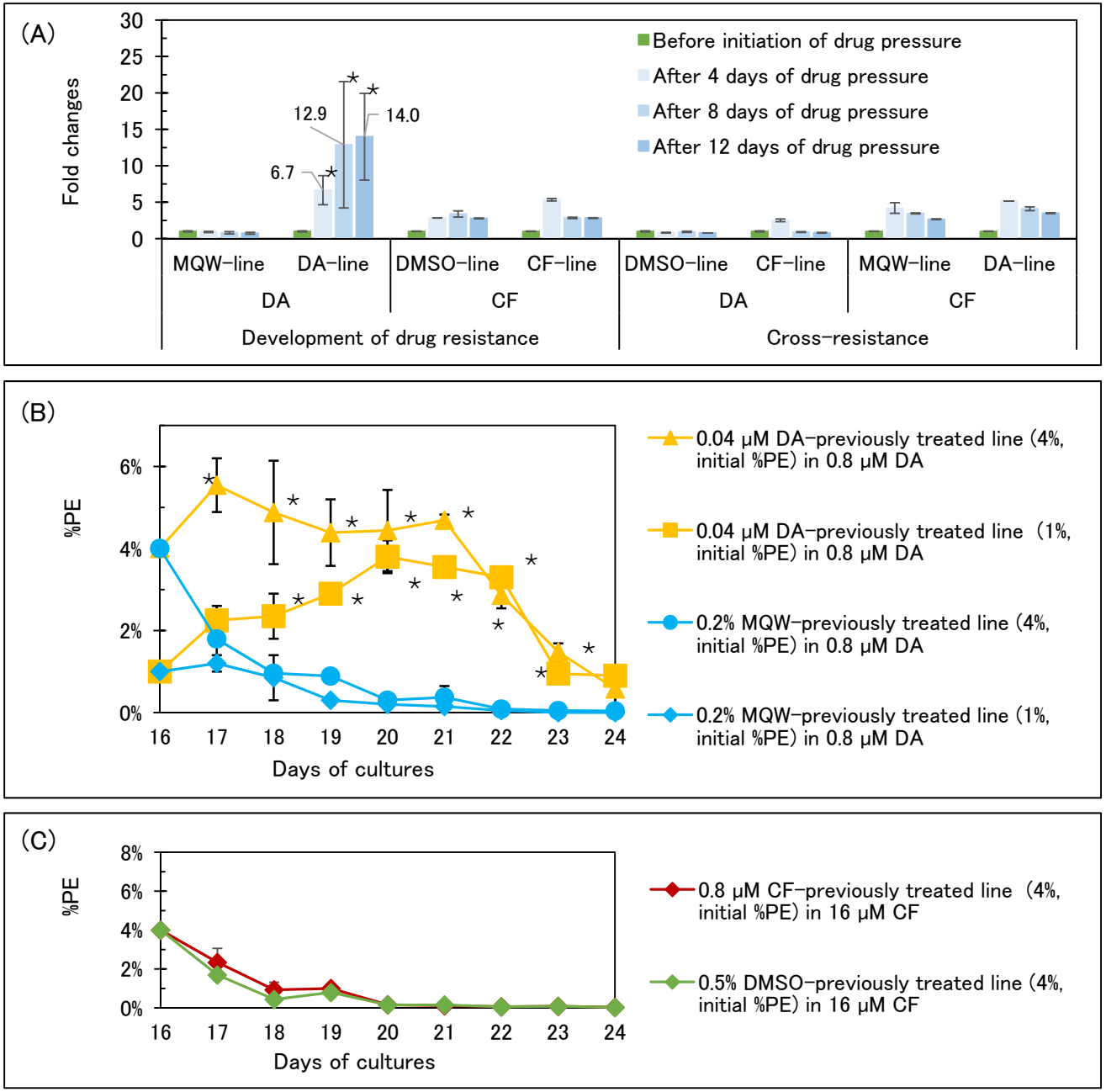

(A)
Fold changes
30
25
20
15
10
5
0
12.9
14.0
6.7
Before initiation of drug pressure
After 4 days of drug pressure
After 8 days of drug pressure
After 12 days of drug pressure
MQW-line
DA-line
DMSO-line
CF-line
DMSO-line
CF-line
MQW-line
DA-line
DA
CF
DA
CF
Development of drug resistance
Cross-resistance
(B)
%PE
6%
4%
2%
0%
16
17
18
19
20
21
22
23
24
Days of cultures
0.04 μM DA-previously treated line (4%, initial %PE) in 0.8 μM DA
0.04 μM DA-previously treated line (1%, initial %PE) in 0.8 μM DA
0.2% MQW-previously treated line (4%, initial %PE) in 0.8 μM DA
0.2% MQW-previously treated line (1%, initial %PE) in 0.8 μM DA
(C)
%PE
8%
6%
4%
2%
0%
16
17
18
19
20
21
22
23
24
Days of cultures
0.8 μM CF-previously treated line (4%, initial %PE) in 16 μM CF
0.5% DMSO-previously treated line (4%, initial %PE) in 16 μM CF

Figure 1

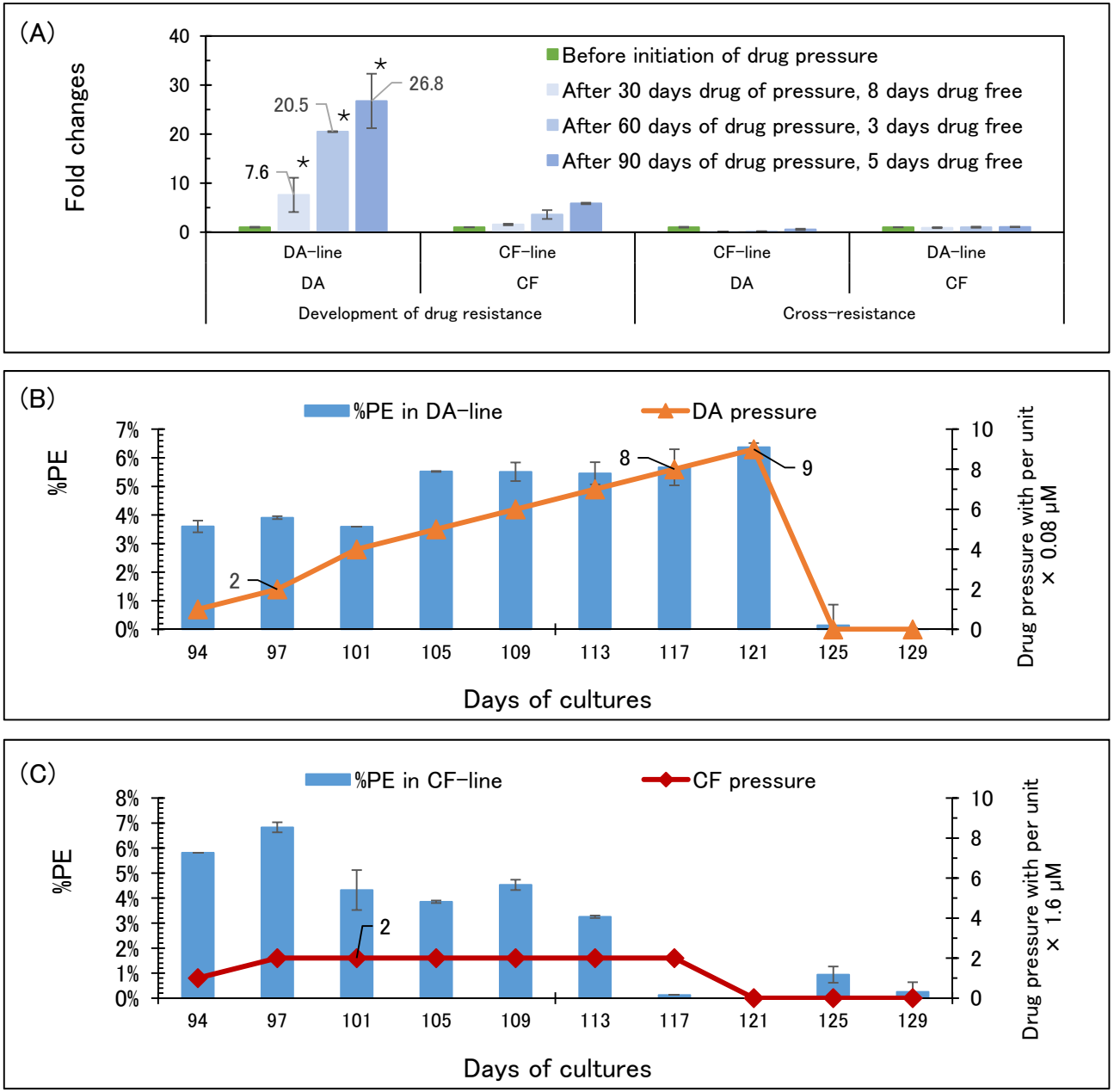
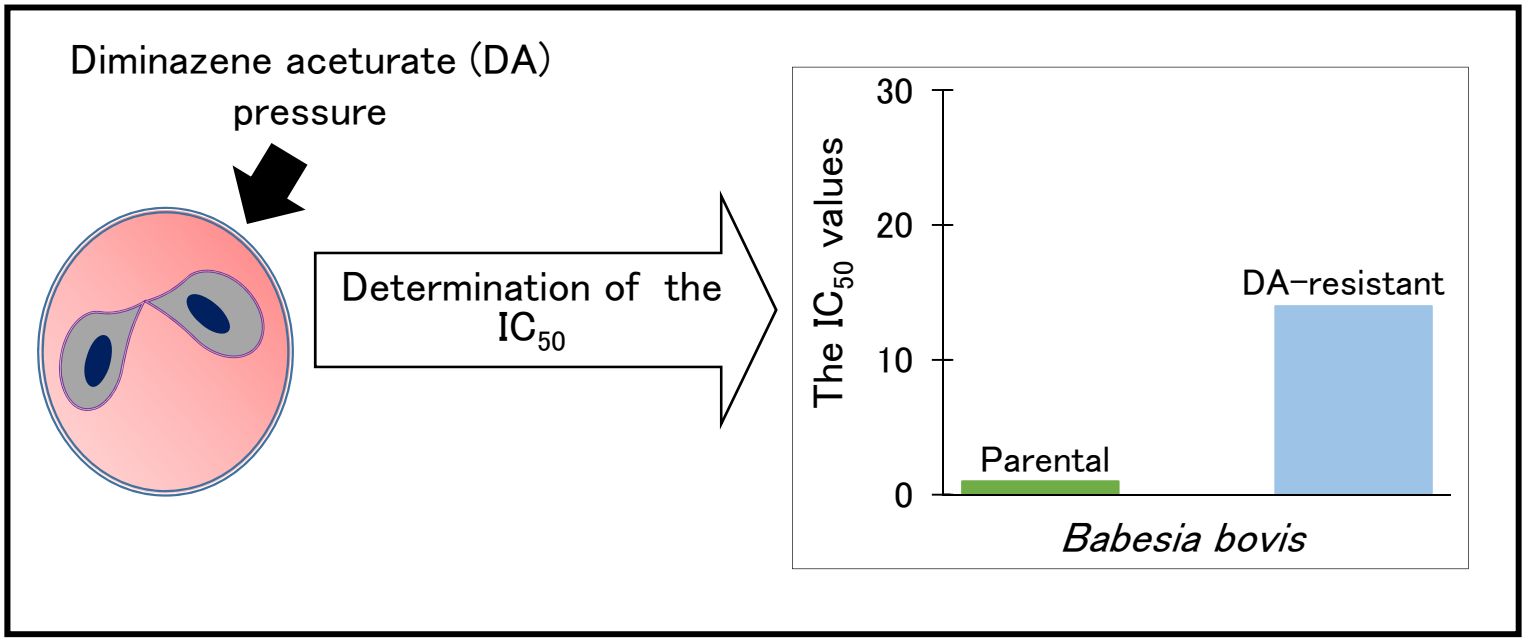

(A)
Fold changes
40
30
20
10
0
Before initiation of drug pressure
After 30 days drug of pressure, 8 days drug free
After 60 days of drug pressure, 3 days drug free
After 90 days of drug pressure, 5 days drug free
7.6
20.5
26.8
*
*
*
DA-line
DA
CF-line
CF
CF-line
DA
DA-line
CF
Development of drug resistance
Cross-resistance
(B)
%PE in DA-line
DA pressure
%PE
7%
6%
5%
4%
3%
2%
1%
0%
10
8
6
4
2
0
2
8
9
94
97
101
105
109
113
117
121
125
129
Drug pressure with per unit × 0.08 μM
Days of cultures
(C)
%PE in CF-line
CF pressure
%PE
8%
7%
6%
5%
4%
3%
2%
1%
0%
10
8
6
4
2
0
2
94
97
101
105
109
113
117
121
125
129
Drug pressure with per unit × 1.6 μM
Days of cultures

Figure 2

# *Graphical abstract*



# *Highlights*

- The present study found that low doses of diminazene aceturate induced the development of resistance in *B. bovis in vitro.*
- When the doses of DA were increased gradually, *B. bovis* was able to tolerate up to an 8–fold dose, as compared with the $IC_{50}$ dose of DA determined before the drug pressure.
- Resistance was stable for less than 15 days of drug withdrawal, indicating that DA resistance in *B. bovis* is unstable.
- The present study suggested that if bovine babesiosis caused by *B. bovis* is unresponsive to DA, subsequent treatment should be altered by another antibabesial agent.