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Intracellular diminazene aceturate content and adenosine incorporation in diminazene aceturate-resistant *Babesia gibsoni* isolate *in vitro*

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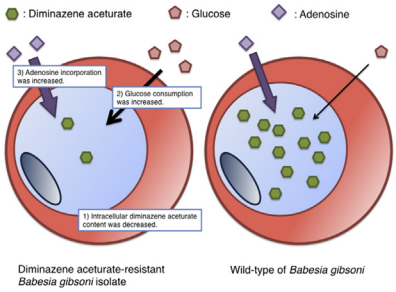
HIGHLIGHTS

- DA content in the DA-resistant *B. gibsoni* isolate was decreased.
- Glucose consumption of the DA-resistant isolate was increased.
- Adenosine incorporation in the DA-resistant isolate was enhanced.
- Adenosine incorporation in the wild-type was not inhibited by DA.

GRAPHICAL ABSTRACT



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ABSTRACT

The mechanism of the development of diminazene aceturate (DA) resistance in *Babesia gibsoni* is still unknown even though DA-resistant *B. gibsoni* isolate was previously developed *in vitro*. To clarify the mechanisms of DA-resistance in *B. gibsoni*, we initially examined the intracellular DA content in the DA-resistant isolate using high-performance liquid chromatography, and compared it with that in the wild-type. As a result, the intracellular DA content in the DA-resistant isolate was significantly lower than that in the wild-type, suggesting that the decreased DA content may contribute to DA-resistance. Additionally, the glucose consumption of the DA-resistant isolate was significantly higher than that of the wild-type, indicating that a large amount of glucose is utilized to maintain DA-resistance. It is possible that a large amount of energy is utilized to maintain the mechanisms of DA-resistance. It was reported that as the structure of DA is similar with that of adenosine, DA may be taken up by the P2 transporter, which

Abbreviations: DA, diminazene aceturate; HSP70, heat shock protein 70; ATP, adenosine triphosphate; EDTA-2Na, ethylenediaminetetraacetic acid disodium salt; HPLC, high-performance liquid chromatography.

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Adenosine incorporation
Glucose consumption

contributes to the uptake of adenosine, in *Trypanosoma brucei brucei*, and that the uptake of adenosine is decreased in DA-resistant *T. brucei brucei*. In the present study, the adenosine incorporation in the DA-resistant *B. gibsoni* isolate was higher than in the wild-type. Moreover, the adenosine incorporation in the wild-type was not inhibited by the presence of DA. These results suggest that adenosine transport in *B. gibsoni* is not affected by DA and may not mediate DA-resistance. To clarify the mechanism of the development of DA resistance in *B. gibsoni*, we should investigate the cause of the decreased DA content in the DA-resistant isolate in the future.

1. Introduction

Babesia gibsoni is a blood protozoan of dogs and a causative pathogen of canine babesiosis (Yamasaki et al., 2011). Diminazene aceturate (DA), an aromatic diamidine derivative, has been used as a first-line agent for the treatment of *B. gibsoni* infection in dogs (Sakuma et al., 2009); however, DA cannot eliminate *B. gibsoni* from infected dogs, and relapses often occur (Farwell et al., 1982; Hwang et al., 2010a; Matsuu et al., 2008; Wulansari et al., 2003). In addition, the development of drug-resistant isolates is a concern, and a DA-resistant *B. gibsoni* isolate was previously developed *in vitro* (Hwang et al., 2010a). However, the mechanism of action of DA on *B. gibsoni* has not been elucidated, and the mechanism of the development of DA resistance in *B. gibsoni* is unknown. To treat canine babesiosis with DA effectively, these problems have to be clarified. Therefore, we analyzed some characteristics of the DA-resistant *B. gibsoni* isolate. This DA-resistant *B. gibsoni* isolate was more resistant to pentamidine, clindamycin and doxycycline than the wild-type (Hwang et al., 2010a). The transcription levels of the heat shock protein 70 (HSP70) gene in this isolate decreased during the development of DA-resistance (Hwang et al., 2010b). The nucleotide sequences of mitochondrial genes, such as *COXI*, *COXIII*, and *CYTb*, in DA-resistant isolates were not altered nor were their transcription levels, suggesting that DA did not affect mitochondrial DNA (Wickramasekara Rajapakshage et al., 2012a). Furthermore, the proliferation potential of the DA-resistant isolate is comparatively lower than that of the wild-type (Wickramasekara Rajapakshage et al., 2012a). The DA-resistant isolate contained higher adenosine triphosphate (ATP) and glucose concentrations than the wild-type, but the activity of the glycolysis pathway was not altered in the DA-resistant isolate (Wickramasekara Rajapakshage et al., 2012b). However, we could not clarify the mechanism of DA-resistance in the DA-resistant *B. gibsoni* isolate.

In trypanosomiasis and leishmaniasis, DA-resistant parasites have been reported, and a few mechanisms of DA resistance were also reported (Bitonti et al., 1986; Carter et al., 1995; Leon et al., 1977). For example, DA can inhibit the DNA replication and mitochondrial respiratory activity of these pathogens (Bitonti et al., 1986; Leon et al., 1977). Additionally, decrease in DA concentration within *Trypanosoma equiperdum* and *T. brucei brucei* can result in DA resistance (Barrett et al., 1995; Carter et al., 1995; Matovu et al., 2003). The loss of P2 transporter function, which usually contributes to the uptake of adenosine, in *T. brucei brucei* has been implicated in resistance to DA (Carter et al., 1995). Moreover, as the structure of DA is similar with that of adenosine, DA is speculated to be taken up by the P2 transporter. Therefore, the decrease in P2 transporter may result in a decrease in DA concentration within these parasites (Carter et al., 1995). It is possible that *B. gibsoni* also has a similar mechanism of DA resistance, although there is no report on the P2 transporter of *B. gibsoni*.

In the present study, to clarify the mechanism of the development of DA resistance in *B. gibsoni*, we initially determined the DA concentration within the cells of both DA-resistant *B. gibsoni* isolates and wild-type *B. gibsoni*. Then, we compared the adenosine uptake in the DA-resistant isolate with that in wild-type of *B. gibsoni*.

2. Materials and methods

2.1. Preparation of canine erythrocytes

Canine erythrocytes containing a high concentration of potassium (high kalium, HK) and a low concentration of sodium as a result of inherited high Na,K-ATPase activity (Inaba and Maede, 1984) were obtained from three mongrel male dogs that had inherited high kalium (HK) erythrocytes. Dogs with HK erythrocytes have been maintained since 1986 in our laboratory. Canine HK erythrocytes were identified by measuring the intracellular concentrations of potassium and sodium (Yamasaki et al., 2005). The dogs used had body weights of 8–12 kg and were 2–3 years old. In the experimental protocols for animal care and handling, the investigators adhered to the guidelines of Hokkaido University, which conform to those of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The present study was approved by the Committee for Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University (approval number: 12-0010).

2.2. 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 drug when *B. gibsoni* was isolated. Since then, this wild-type strain has been cultured in our laboratory (Yamasaki et al., 2003). DA-resistant *B. gibsoni* isolate was previously developed from this wild-type *in vitro* by Wickramasekara Rajapakshage et al. (2012a). The DA-resistant *B. gibsoni* isolate was maintained in culture medium containing 200 ng/mL DA for more than six months.

2.3. In vitro culture of Babesia gibsoni

The wild-type *B. gibsoni* and DA-resistant *B. gibsoni* isolate have been cultured in our laboratory according to the method of Yamasaki et al. (2003). In brief, the parasites were incubated at 38 °C in a humidified atmosphere of 5% (v/v) CO_2, 5% (v/v) O_2, and 90% (v/v) N_2 in a culture medium consisting of RPMI-1640 with L-glutamine and 25 mM HEPES (Invitrogen, Carlsbad, CA, U.S.A.), 20% (v/v) dog serum, and canine HK erythrocytes sufficient to yield a packed cell volume (PCV) of 5% (v/v). Every 24 h, 60% (v/v) of the culture supernatant was removed and replaced with an equal volume of fresh culture medium (Yamasaki et al., 2005). 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 DA-resistant isolate