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Development of in vitro atovaquone-resistant *Babesia gibsoni* with a single-nucleotide polymorphism in *cytb*

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ABSTRACT

An atovaquone (ATV)-resistant *Babesia gibsoni* was developed by in vitro exposure of uncloned wild type (WT) *B. gibsoni* to 800 nM ATV for 6 days. Sequence analysis of mitochondrial genes showed a single-nucleotide polymorphism (SNP) at *cytb* nt363 (G to T) that resulted in the substitution of methionine with isoleucine (M121I), which is one of the SNPs reported in a previous in vivo study. 363T or 363G allele-specific real-time polymerase chain reaction (PCR) revealed that an M121I variant was present in over 99% of the ATV-resistant population. As neither ATV resistance nor gene polymorphisms appeared in the *B. gibsoni* WT sibling clones, the expression of ATV resistance in this study was suspected to be because of selective multiplication of the *B. gibsoni* M121I variant. This ATV-resistant *B. gibsoni* displayed the same sensitivity as the WT *B. gibsoni* against 5 other drugs, including diminazene aceturate, azithromycin, doxycycline, clindamycin, and proguanil. This is the first report on the in vitro establishment of an ATV-resistant *B. gibsoni* with gene polymorphisms.

1. Introduction

Babesia gibsoni is an intraerythrocytic apicomplexan parasite in dogs. This parasite has been reported to occur endemically in Asia, Africa, Europe, North America (Kjemtrump et al., 2000), and Australia (Muhlninckel et al., 2002). This parasite induces severe hemolytic anemia in the acute stage, which is often accompanied by fever, cholopla-nia, hemoglobinuria, and enlarged spleen (Farwell et al., 1982; Conrad et al., 1991).

Early treatment is required for acute *B. gibsoni* infections, but this has a number of drawbacks. Although diminazene aceturate (DA) has been used to treat acute *B. gibsoni* infections in Japan, it often fails to eliminate the parasite from the affected dogs and causes severe adverse effects such as pain at the injection site, and nervous symptoms due to cerebral hemorrhage (Boozer and Macintire, 2003; Wulansari et al., 2003). Some studies have reported the effectiveness of new combination therapies such as clindamycin (CLDM)–metronidazole–doxycycline (DOXY), or DOXY–enrofloxacin–metronidazole, on experimentally infected dogs or clinical patients, but these therapies take a long time to show clinical effectiveness (Suzuki et al., 2007; Lin and Huang, 2010). Atovaquone (ATV) is a novel antiprotozoal compound that has a broad-spectrum activity against human protozoan pathogens, including *Plasmodium* spp., *Toxoplasma gondii* (Baggish and Hill, 2002), and *Babesia* spp. (Hughes and Oz, 1995; Pudney and Gray, 1997; Gray and Pudney, 1995). This compound is an analog of ubiquinone, and its mechanism of action is mediated by the inhibition of mitochondrial electron transport (Hudson et al., 1991; Baggish and Hill, 2002). ATV is effective against acute *B. gibsoni* infections, but ATV monotherapy results in relapse as well as the emergence of drug-resistant

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variants with some single-nucleotide polymorphisms (SNPs) in the cytochrome b gene (*cytb*) (Matsuu et al., 2004, 2006). Combination therapy with ATV and azithromycin (AZM) has been reported to be more effective than ATV monotherapy for *B. microti* in hamsters (Wittner et al., 1996), and this combination therapy is also recommended for human *Babesia* spp. infections (Krause et al., 2000). This combination therapy has also been used in *B. gibsoni* infections (Birkenheuer et al., 2004). However, this therapeutic modality could not completely eliminate parasites in acute *B. gibsoni* infections, and an ATV-resistant parasite possessing plural SNPs in *cytb* has emerged (Jefferies et al., 2007; Sakuma et al., 2009). The additive or synergistic effects of these drugs against *B. gibsoni* have not been fully evaluated. Therefore, it is necessary to conduct detailed in vitro studies on ATV-based treatment strategies that can work against emergence of resistance.

In order to achieve this, it is necessary to elucidate the relationship between the SNPs in *cytb* and ATV resistance, and to develop a genetic marker for ATV-resistant *B. gibsoni*. In previous in vivo studies, single-nucleotide substitution at nt363 in *cytb* resulting in the replacement of methionine with isoleucine (M121I) seemed to be the factor responsible for ATV resistance, because M121I was localized in the region thought to be the ATV-binding site in *P. falciparum* (Korsinczky et al., 2000); this substitution was frequently found (Matsuu et al., 2006; Sakuma et al., 2009). However, the relationship between the SNPs including M121I and resistance has not been demonstrated in vitro, and the mechanism underlying ATV treatment-induced resistance development in the parasite is unclear. It is suggested that mutation in *cytb* following ATV treatment or selective multiplication of parasites with *cytb* SNPs may have taken place.

This study was conducted to develop an in vitro ATV-resistant *B. gibsoni* by exposing the parasite culture to ATV for 6 days, and to perform mitochondrial gene analysis. At this time, uncloned wild-type (WT) *B. gibsoni* and WT sibling clones were used for ATV exposure, to confirm how the ATV resistance emergences. Furthermore, this study evaluated the sensitivity of the ATV-resistant *B. gibsoni* to the anti-babesial drugs, including DA, AZM, DOXY, CLDM, and proguanil (PG), to examine whether the ATV-resistant parasite possessed resistance against those drugs.

2. Materials and methods

2.1. In vitro culture of B. gibsoni

B. gibsoni parasites were isolated from a naturally infected Tosa dog in Aomori Prefecture, Japan, in 2004. The parasites were maintained in vitro culture at our laboratory (Matsuu et al., 2008) and were used as the uncloned wild-type (WT) *B. gibsoni* in the present study. Four *B. gibsoni* WT sibling clones were obtained through a modified limiting dilution procedure, as described previously (Korsinczky et al., 2000).

In vitro culture of *B. gibsoni* was performed as reported as previously (Matsuu et al., 2008). Briefly, 200 μL of packed infected RBCs were dispensed into 1800 μL of culture medium to obtain a 10% packed cell volume in each well of a 12-well plate. Each parasite was incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 25 mM pyruvic acid, 2 mM L-glutamine, 100 units/mL penicillin G, 100 μg/mL streptomycin (Invitrogen), and 10% fetal bovine serum (FBS; Funakoshi, Tokyo, Japan). Half the volume of culture medium was replaced with fresh medium every day. Canine erythrocytes were obtained from a healthy beagle, and subculture was carried every 7 days.

2.2. In vitro cultivation of B. gibsoni with ATV and isolation of ATV-resistant parasite

For development of ATV-resistant *B. gibsoni*, different concentrations of ATV were exposed to WT *B. gibsoni* and 4 clones. ATV was obtained from Wako Chemicals (Osaka, Japan), and a stock solution was prepared with dimethyl sulfoxide (DMSO). ATV stock solution was diluted with culture medium to yield final concentrations of 100, 200, 400, 800, and 1600 nM. Twenty-hundred micro litter of each *B. gibsoni* culture suspensions were dispensed per well in 96-well plates in triplicate for each ATV concentration. Identical cultures containing only DMSO and without ATV were prepared to be used as controls. The final concentration of DMSO was adjusted to 0.1%. These plates were incubated at 5% CO_2 and 37 °C for 6 days. Every day, half of the medium was replaced with fresh medium, according to the concentration of ATV. The growth inhibition rate of the parasite was calculated by counting the number of parasitized erythrocytes from each of the wells containing drugs and that in the control wells without the drug every 2 days from blood smear (Matsuu et al., 2008). The half-maximal inhibitory concentration (IC_{50}) of ATV was determined after 6 days incubation (Matsuu et al., 2008).

After 6 days of ATV exposure, parasites from each ATV concentration were dispensed into new wells in 12-well culture plates containing ATV-free culture medium and normal dog erythrocytes, and viability was determined by culturing for the next 7 days. Of the parasite-positive wells identified through blood smear observation, the parasites that had been exposed to the highest concentration of ATV were isolated, and examined their ATV sensitivity. The methods were similar to the ATV-exposure step (final ATV concentration: 200–3200 nM), and the growth inhibition rate and IC_{50} was calculated as described above. If parasites demonstrated low sensitivity following exposure to ATV, they were isolated as ATV-resistant *B. gibsoni*.

2.3. Sequence analysis of WT and ATV-resistant B. gibsoni

The nucleotide and deduced amino acid sequences of the mitochondrial *cytb* in the each parasite were analyzed. *B. gibsoni* DNA was isolated from 200 μL of each culture by using a genomic DNA extraction kit (QIAamp DNA Mini Kit; Qiagen, USA). To amplify the open reading frame (ORF) region of *cytb*, 2 primer sets were designed on the basis of the reported gene sequence (accession