SHORT REPORT: CLONING OF THE BABESIA GIBSONI CYTOCHROME B GENE AND ISOLATION OF THREE SINGLE NUCLEOTIDE POLYMORPHISMS FROM PARASITES PRESENT AFTER ATOVAQUONE TREATMENT

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Abstract. We determined the nucleotide sequence of the Babesia gibsoni cytochrome b (cytb) gene. DNA was extracted from B. gibsoni isolated from Aomori Prefecture, Japan, and 1,288 basepairs of the cytb gene, including 1,071 basepairs of the open reading frame, were sequenced. The cytb gene of B. gibsoni obtained from three dogs that had been experimentally infected with B. gibsoni and treated with atovaquone was also sequenced. The B. gibsoni cytb gene obtained from all three atovaquone-treated dogs contained a single polymorphism resulting in an amino acid change in one of the putative ubiquinone-binding sites of Plasmodium falciparum. This polymorphism was homologous to mutations in other apicomplexan protozoa that exhibit resistance to atovaquone. Two other single polymorphisms were identified in parasites isolated from two of the dogs. These results indicate that single nucleotide polymorphisms in the sequence for mitochondrial cytb gene may be associated with decreased susceptibility of Babesia species to atovaquone.

Babesia species are intra-erythrocytic apicomplexan parasites. They are transmitted by ticks and parasitize a wide range of vertebrate hosts. Babesia gibsoni causes canine babesiosis, which induces remittent fever, progressive anemia, thrombocytopenia, marked splenomegaly and hepatomegaly, and in some cases, the death of infected animals. Infection with B. gibsoni have been identified worldwide, and it is now recognized as a serious emergent disease in small animal medicine. Several chemotherapeutics, including diminazene aceturate, pentamidine isethionate, phenamidine isethionate, and clindamycin, have been used for treatment of B. gibsoni infections. However, these chemotherapeutics fail to eliminate B. gibsoni from host dogs completely. Therefore, an effective therapy is urgently needed.

Atovaquone is a novel antiprotozoal compound that has broad-spectrum activity against human protozoan pathogens, including Plasmodium spp., Toxoplasma gondii, and Babesia spp. This compound is an analog of ubiquinone, and its mechanism of action is by inhibition of mitochondrial electron transport. However, recurrence of disease and decreased sensitivity of protozoa to therapy have been reported when atovaquone alone was used. Mutation of the cytochrome b (cytb) gene, which is located in the mitochondrial genome, has been described in atovaquone-resistant isolates of Plasmodium spp., T. gondii, and Pneumocystis carinii.

A combination of atovaquone and azithromycin was reported to be effective for treatment of dogs that were naturally infected with B. gibsoni. In our previous study, we described the therapeutic efficacy of atovaquone against B. gibsoni using experimentally infected dogs, and suggested that the atovaquone allows recrudescence of parasites with decreased susceptibility to this drug. However, the mechanisms responsible have not been investigated. Genetic changes associated with decreased sensitivity of B. gibsoni to atovaquone may be valuable as markers for clinical application and for selection of drugs for use in combination with atovaquone. In this study, we identified a nucleotide coding sequence for B. gibsoni cytb, which might be the atovaquone target. Subsequently, the sequence of the cytb gene from atovaquone-treated animals with recurring parasitemia was determined and compared with that of the pretreatment parasite.

The Animal Care and Ethics Committee of Kitasato University approved the use of the animals in this study. The original parasite used in this study was isolated from a naturally infected Tosa dog in Aomori Prefecture, Japan, and was identified as an Asian genotype. Parasite was maintained in our laboratory by passage through a beagle (dog A) that was not exposed to drug treatment. Whole blood samples were collected from this dog, and EDTA was used as anticoagulant. Blood samples that had been stored in our laboratory were used to compare the nucleotide sequence of B. gibsoni cytb before and after atovaquone treatment. The blood samples were collected during our previous study from three experimentally infected dogs (B, C, and D) before atovaquone treatment and during the recurrence of B. gibsoni infection after atovaquone treatment (30 mg/kg twice a day for seven days). The recurrent parasites showed less sensitivity to atovaquone than those obtained before treatment.

Babesia gibsoni DNA was isolated from blood samples using a genomic DNA extraction kit (GFX Genomic Blood Purification Kit; Amersham, Buckinghamshire, United Kingdom). Total RNA from B. gibsoni was isolated using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer’s instructions. Reverse transcription to obtain cDNA was performed as follows: 10 µg of total RNA denatured at 65°C was reverse transcribed in a total volume of 40 µL using 2 µg of oligo-dT primer and 200 units of Superscript II reverse transcriptase (TaKaRa, Shiga, Japan) in a solution containing 50 mM Tris-HCl, pH 8.3, 3 mM MgCl₂, and 75 mM KCl (RT buffer) with 100 µM of each dNTP at 42°C for 1 hr.

The primer pair was designed according to the sequence of nucleotides from B. bovis (GenBank accession no. AF053002), B. bigemina (accession no. F109354), and Theileria annulata (accession no. M63015) as follows: JD279, 5′-TGG AA(C/T) TT(A/T) GG(T/T) TG(T/T) G(T/T) A TT(A/T) G(G/A) TG(T/T) A TTA CTC CAT AAG TTA-3′. A polymerase chain reaction (PCR) was performed on 20 µL of a mixture containing 1 µg of template genomic
DNA, 10 pmol of each primer, 200 μM deoxynucleoside triphosphate (dNTP), and 1.25 units Taq Gold DNA polymerase (Invitrogen, Carlsbad, CA). The PCR was repeated for 40 cycles with denaturation for 30 seconds at 94°C, annealing for 1 minute at 40°C, and extension for 1 minute at 72°C to obtain a 533-basepair fragment. The PCR product was ligated into the pCR2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen). The entire ligation reaction was used to transform Escherichia coli DH5α competent cells. Plasmid DNA from two positive transformants was used to sequence the DNA of the insert. Both strands of the plasmid insert DNA were sequenced using the Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The sequencing analysis was performed using GENETYX-MAC version 10 (Software Development, Co. Ltd., Tokyo, Japan). The similarities of this nucleotide sequence to those of B. bovis, B. bigemina, and T. annulata were 82.2%, 80.3%, and 64.5%, respectively.

Subsequently, 3′ rapid amplification of cDNA ends was performed to determine the sequence of the gene at the 3′ end.
end of cytb. A primer was designed as follows: Ky1, 5'-GAG TAT TAA CAG AAG TTA ATA TGG-3' and Ky2, 5'-CCC ATA ACT TCT GTT AAT ACT C-3'. A primer set for CYTb 1, 5'-TGT ATT ACT ATA CTG TGA GT-3' and CYTb 2, 5'-AAC TCC CCT CTG TTT TA-3', was designed corresponding to the nucleotide sequences obtained for the 3' and 5' ends (Figure 1). The PCR was conducted using primers CYTb 1 and CYTb 2 with B. gibsoni genomic DNA and the resulting fragment of 1,228 basepairs was cloned and sequenced as described earlier in this report.

We determined the complete sequence of the B. gibsoni cytb gene. This is the first such determination in the genus Babesia. Sequences of the B. gibsoni cytb open reading frame (ORF) were obtained from at least three independent PCR amplifications. The complete sequence of the B. gibsoni cytb gene (1,228 basepairs, including 1,071 basepairs of the ORF encoding a protein of 357 amino acids) was obtained (accession no. AB215096; Figure 1). The ORF shows a highly conserved protein with strong identity throughout to that of other apicomplexan parasite cytb amino acids. Comparison of the deduced amino acid sequence against those of B. bovis, B.
*B. gibsoni*, and *T. annulata*, which are partially defined, showed 79.7%, 82.5%, and 52.1% identity, respectively, within their defined partial sequences. Comparison of the deduced amino acid sequence against those of *Theileria parva* (accession no. Z23263), *P. falciparum* (accession no. AU086218), and *T. gondii* (accession no. AF023246) showed 53.8%, 41.4%, and 41.5% identity, respectively (Figure 2).

The nucleotide sequence of the cytb gene amplified from blood samples taken from two dogs in Okinawa Prefecture showed > 99% identity to that obtained from dogs in Aomori Prefecture. At amino acid 309, tyrosine was replaced by cysteine in *B. gibsoni* isolated in Okinawa Prefecture.

Pretreatment parasite DNA and recurrent parasite DNA extracted from three dogs was subjected to PCR using the primer pair CYTb 1 and CYTb 2. The 1,228-basepair fragment was then sequenced directly. In instances where no synonymous mutation was observed, a second PCR was conducted on the original template DNA, which was then sequenced to ensure that the change was not a PCR artifact.

*A G-to-T or G-to-A substitution at nucleotide 363, which resulted in methionine being replaced by isoleucine (M121I mutation), was observed in the cytb gene of *B. gibsoni* from all three dogs after atovaquone treatment. In the malaria parasite, atovaquone is believed to inhibit the cytochrome bc1 complex by competitive binding with coenzyme Q. The M121I mutation was identified in *B. gibsoni* from all three dogs in this study, and was localized in the region believed to be the target region responsible for the effect of atovaquone in *P. falciparum* (Figure 2). Moreover, the same substitution has been reported in atovaquone-resistant *Plasmodium* spp. Since the recurrent parasites in this study displayed less atovaquone sensitivity after atovaquone treatment than in our previous study, the M121I mutation identified in this study may be associated with the loss of sensitivity.

Two other single polymorphisms, a G-to-A substitution at
nucleotide 658, which resulted in valine being replaced by
isoleucine (V220I mutation), and an A-to-G substitution at
nucleotide 907, which resulted in isoleucine being replaced by
valine (I303V), were observed in the cytb gene of B. gibsoni
from two of three dogs after atovaquone treatment. The
V220I mutation was a mixed population with wild-type nucle-
otide in B. gibsoni from dog B and dog C, but was not de-
tected in B. gibsoni from dog D. The I303V mutation was a
complete substitution in B. gibsoni from dog B, a mixed popu-
lation with wild-type nucleotide in B. gibsoni from dog C, and
was not detected in dog D (Figure 3). Whether the polymor-
phisms induce less sensitivity against atovaquone and how
sensitivity is reduced in B. gibsoni are not known. Further
study is needed to establish a direct correlation between the
three single polymorphisms and atovaquone resistance.

Atovaquone is a major component of a new antibabesial
treatment in which azithromycin is used as a combination
drug for canine babesiosis.17 This information on polymor-
phisms of the atovaquone-binding site in B. gibsoni may be
useful not only for treatment of canine babesiosis, but also for
treatment of human babesiosis. These single polymorphisms
in the cytb gene may be useful as molecular markers for moni-
toring the development and spread of drug-resistant parasites
in the field. We are presently conducting an in vitro study to
establish whether atovaquone resistance is associated with
the three polymorphisms in the cytb gene.

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