NOTE *Internal Medicine*

Molecular Epidemiological Survey of the *Babesia gibsoni cytochrome b* Gene in Western Japan

Masato SAKUMA[1)], Kenta FUKUDA[2)], Katsuyoshi TAKAYAMA[2)], Yukuharu KOBAYASHI[2)], Takako SHIMOKAWA MIYAMA[3)], Asuka SETOGUCHI[1)] and Yasuyuki ENDO[1)]*

[1)]*Laboratory of Small Animal Internal Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, 1–21–24 Korimoto, Kagoshima, Kagoshima 890–0065, Japan*
[2)]*ADTEC Co., Ltd., 1693–6 Yokkaichi, Usa, Oita 879–0471, Japan*
[3)]*Veterinary Teaching Hospital, Kagoshima University, 1–21–24 Korimoto, Kagoshima, Kagoshima 890–0065, Japan*

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ABSTRACT. In this study, we conducted a survey of the *cytochrome b* (*cytb*) gene of *Babesia gibsoni* (*B. gibsoni*) isolated from clinical cases to determine the prevalence of potential atovaquone (ATV)-resistant variants. Ninety-two blood samples were collected from naturally *B. gibsoni* infected dogs. The *cytb* nucleotide sequence was determined by direct sequencing. Twelve non-synonymous amino acid substitutions were identified in *cytb*. The principal ATV-resistant substitution, M121I, was detected in three cases. This survey determined that potentially ATV-resistant *B. gibsoni* strains are present in dogs in Japan.

KEY WORDS: *Babesia gibsoni*, *cytochrome b* gene, drug-resistance, epidemiological survey.

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Babesia is a tick-borne protozoan pathogen. Dogs are known to be susceptible to two species of Babesia, *Babesia gibsoni* (*B. gibsoni*) and *B. canis* [3–5]. In canine practice, *B. gibsoni* infection is more problematic than *B. canis* infection in Japan, because of its virulence and difficulty of treatment. Severe hemolytic anemia and thrombocytopenia are observed in dogs showing an acute onset of disease [3, 5]. Concurrent clinical symptoms such as acute kidney failure, disseminated intravascular coagulation and secondary immune-mediated hemolytic anemia can be also observed and are often fatal [3, 5]. In Japan, *B. gibsoni* infected dogs are mainly observed in the western part of Japan, although affected areas seem to expand towards the northeast [8, 9, 13]. The definitive treatment strategy for *B. gibsoni* infection has not yet been established [3, 5, 10, 15, 16]. Diminazene aceturate is used as a first-line drug for *B. gibsoni* infection in Japan [10]. But, it often fails to eliminate babesia from the host, and a relapse of the disease is frequently observed [10, 15]. Furthermore, diminazene aceturate has a narrow safety margin, and occasionally induces severe side effects [10]. For these reasons, there have been efforts in canine practice to establish an alternative therapeutic strategy against *B. gibsoni* infection.

Recent studies by us and others have shown that atovaquone (ATV), an analogue of ubiquinone and one of the anti-plasmodium drugs, can be useful and effective for dogs showing an acute onset of babesiosis caused by *B. gibsoni*, and results in rapid improvements in clinical symptoms without any adverse effects [2, 11, 14]. However, we found that approximately 60% of ATV-treated dogs experienced a relapse of the disease [14]. Furthermore, DNA sequencing of the *B. gibsoni cytochrome b* (*cytb*) gene, presumed as an ATV binding site, revealed that the gene from the relapsed cases had different nucleotide sequences [14]. One of them will be a substitution for ATV-resistance, resulting in M121I amino acid (AA) substitution as previously reported [7, 12]. Surprisingly, *B. gibsoni* with M121I was dominant in one case, even at the primary onset of disease [14]. At present, many veterinarians have started to use ATV for clinical cases of babesiosis, and ATV has become commercially available, even in Japan. Therefore, it is important to know the prevalence of possible drug-resistant variants in the environment. In the present study, a molecular epidemiological survey of the *B. gibsoni cytb* gene was carried out to investigate its polymorphism and the prevalence of possibly ATV-resistant *B. gibsoni* variants in Japan.

Ninety-two blood samples were collected from dogs naturally infected with *B. gibsoni* during 2007 to 2011. Of them, 78 cases were kindly provided by a commercial laboratory (ADTEC Co., Ltd., Oita, Japan). These 78 samples were collected from clinically suspicious cases and were confirmed to be positive for *B. gibsoni* infection by following *B. gibsoni p18* rRNA gene PCR. Remaining 14 samples were collected from clinical cases diagnosed at the Kagoshima University Veterinary Teaching Hospital (KUVTH) by blood examination and *p18* rRNA gene PCR. Samples from the commercial laboratory were collected from dogs in areas west of the Kanto plain of Japan (Tokyo, Shizuoka, Kyoto, Nara, Osaka, Hyogo, Okayama, Hiroshima, Kagawa, Kochi, Yamaguchi, Fukuoka, Saga, Nagasaki, Kumamoto, Miyazaki, Kagoshima and Okinawa Prefectures) that had not been treated with any anti-protozoal drugs including ATV. Samples obtained at KUVTH

*CORRESPONDENCE TO: ENDO, Y., Laboratory of Small Animal Internal Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, 1–21–24 Korimoto, Kagoshima 890-0065, Japan.
e-mail: k5155981@kadai.jp

Table 1. The frequency of nucleotide and deduced AA substitutions in the *B. gibsoni cytb* gene

Substitutions			Frequency (%)
Site	Nucleotide	Amino acid	
nt 94	CTG>CTA	-	16/92 (18.0%)
nt 102	CTA>CTG	-	4/92 (4.5%)
nt 127	ATG>GTG	M43V	1/92 (1.1%)
nt 129	ATG>ATT	M43I	1/92 (1.1%)
nt 201	GCT>GCC	-	7/92 (7.9%)
nt 207	TGT>TGC	-	4/92 (4.5%)
nt 240	CAT>CAC	-	1/92 (1.1%)
nt 267	AGT>AGC	-	1/92 (1.1%)
nt 322	GCT>ACT	A108T	2/92 (2.2%)
nt 363	ATG>ATA	M121I	3/92 (3.3%)
nt 378	GCA>GCG	-	4/92 (4.5%)
nt 393	AAC>AAT	-	4/92 (4.5%)
nt 406	GGA>GGG	I136V	1/92 (1.1%)
nt 423	ATT>ATC	-	4/92 (4.5%)
nt 430	TAC>TAT	-	2/92 (2.2%)
nt 456	CCA>CCG	-	11/92 (12.4%)
nt 492	TTA>TTG	-	5/92 (5.6%)
nt 535	ATC>ATT	-	5/92 (5.6%)
nt 558	AAT>AAC	-	14/92 (15.7%)
nt 588	GTA>GTG	-	4/92 (4.5%)
nt 640	TGC>TGT	-	4/92 (4.5%)
nt 648	GCT>ACT	A216T	1/92 (1.1%)
nt 658	GTT>ATT	V220I	6/92 (6.5%)
nt 676	ATA>GTA	I226V	11/92 (12.0%)
nt 726	CCA>CCG	-	8/92 (9.0%)
nt 810	GCA>GCT	-	4/92 (4.5%)
nt 827	GGC>GGT	A276V	1/92 (1.1%)
nt 829	GCA>CCA	-	1/92 (1.1%)
nt 832	CAT>CAC	-	1/92 (1.1%)
nt 835	CAT>CAC	-	5/92 (5.6%)
nt 869	AGC>AGT	A290V	1/92 (1.1%)
nt 907	ATA>GTA	I303V	7/92 (7.6%)
nt 928	CCT>TCT	P310S	15/92 (16.3%)
nt 948	TAT>TAC	-	1/92 (1.1%)
nt 952	TAC>TAT	-	4/92 (4.5%)
nt 984	GAT>GAC	-	4/92 (4.5%)
nt 1002	GGT>GGC	-	1/92 (1.1%)
nt 1042	CTA>TTA	-	1/92 (1.1%)
nt 1068	TTA>TTG	-	2/92 (2.2%)

were collected from dogs with no treatment history during the first visit. Total DNA was isolated from whole blood using a DNA extraction kit (DNA blood mini kit, QIAGEN, Hilden, Germany) and used for the following PCR analyses as a template. These samples were confirmed to be positive for GAPDH gene amplification as an internal control and for *B. gibsoni p18* rRNA gene by PCR analyses [1, 6]. Nested PCRs were carried out for amplification of the *B. gibsoni cytb* gene, and the nucleotide sequence was determined by direct sequencing by the methods previously described [14]. GENETYX Version 11.0 software (Software Development Co., Ltd., Tokyo, Japan) was used to characterize the obtained nucleotide sequence data. Then, the prevalence of nucleotide and amino acid (AA) substitutions was evaluated.

Compared with the standard sequence of the *B. gibsoni cytb* gene (DDBJ/GenBank/EMBL accession number, AB215096), 39 sites with nucleotide substitutions were detected in *B. gibsoni* isolated from 92 cases. Most were induced synonymous AA substitutions; however, 12 types were induced non-synonymous AA substitutions (Table 1). Nucleotide substitutions resulting in AA substitutions A108T, M121I, V220I, I226V, I303V and P310S were observed in multiple cases, while other types were detected in single case each. *B. gibsoni* possessing CYTB with M121I substitution, a major candidate responsible for the ATV-resistant phenotype, was found in 3 cases (3.3%) [7, 12]. The other types of substitutions, V220I and I303V, related to ATV-resistance as reported by Matsuu *et al.* were also detected in 6 (6.5%) and 7 cases (7.6%), respectively [12].

Figure 1 shows the geographical distribution of dogs with *B. gibsoni* that showed nucleotide substitutions in *cytb*, resulting in AA substitutions. The isolates with I226V and P310S were mainly distributed in south Kyushu and Okinawa, respectively. Three cases with M121I were found in Fukuoka, Kagoshima and Okinawa; however, no specific distribution pattern was observed in this type of *B. gibsoni*. Other candidates related to ATV-resistance, *B. gibsoni* with V220I and/or I303V, were mainly detected in the Kansai area.

To our knowledge, this is the first molecular epidemiological report concerning the *cytb* gene of *B. gibsoni*. This survey clarified that there have already been several variants of the *B. gibsoni cytb* gene, and suggested that some genotypes were related to regionality. Most of the polymorphisms were different from substitutions including M121I, V220I or I303V, which have been suggested to be correlated with ATV-resistance [12]. However, it is noteworthy that the ATV-resistant variants with M121I, V220I or I303V were dominant in some cases. Our findings suggest that *B. gibsoni* with ATV-resistance has already been existing in nature. Although its prevalence is not high, it would be a threat in the future with use of ATV for canine babesiosis. The results obtained in this survey might be a contraindication for future use of ATV.

In this study, although we found multiple cases infected with *B. gibsoni* possessing V220I, I226V, I303V or P310S *cytb*, these AA substitutions might not contribute to the development of an ATV-resistant phenotype in clinical cases. Our previous study revealed that clinical cases that were treated with ATV showed a good response against the treatment, even though *B. gibsoni* with those AA substitutions was dominant [14]. Furthermore, a recent study showed that the major candidate responsible for ATV-resistance is M121I [7]. However, a further evaluation, especially of V220I and I303V, is necessary to clarify the relationship between substitutions in *cytb* and ATV-resistance. At present, it is likely that M121I is the most important AA substitution in *cytb* for the development of ATV-resistance [7, 12, 14].

We found three cases whose dominant *B. gibsoni* were M121I type *cytb*. As mentioned above, the prevalence was low (3.3%). However, we have to allow for the limitations

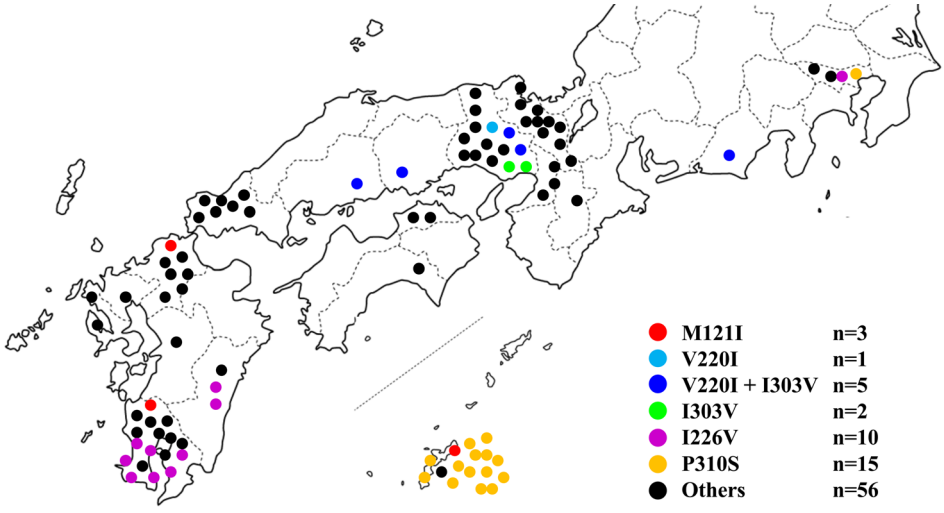

Fig. 1. Geographical distribution of *B. gibsoni* based on CYTB AA sequences. Each dot represents one case.

of this study and a possible underestimation of the true prevalence. We simply retrospectively evaluated the *cytb* genotypes of *B. gibsoni* in infected dogs. Therefore, we do not know whether the dogs infected with variant *cytb* types of *B. gibsoni* actually show resistance against ATV treatment or not. In addition, we have to consider the possibility that a minor population of such genotypes might have been missed in our analysis, because the population of *B. gibsoni* may have been too crude, and a dominant genotype of *B. gibsoni* should have been selectively amplified in the PCR analysis [14].

The findings obtained in this study provide useful baseline data concerning the prevalence of several genotypes of *B. gibsoni* in Japan. An additional large-scale epidemiological survey of the *B. gibsoni cytb* gene with a larger number of samples might be required in the future. Furthermore, studies on the population of ticks with *B. gibsoni* and the relationships between *cytb* genotypes and clinical outcomes would be also necessary.

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