Studies on the Application of Atovaquone against *Babesia gibsoni* Infection and Emergence of Drug-Resistant Variants

Babesia gibsoni 感染症におけるアトバコンの有効性と薬剤耐性に関する研究

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CONTENTS

General Introduction

Canine babesiosis is one of tick-borne protozoan diseases and affected dogs show severe anemia and thrombocytopenia [5, 6, 8]. In Japan, two species of babesia, *Babesia gibsoni* (*B. gibsoni*) and *B. canis*, are distributing especially in western part of Japan and Okinawa island, respectively [1, 14, 15, 19, 25]. However, there is a concern about the expansion of distribution area [15, 19, 25]. The problematic babesiosis is caused by highly pathogenic *B. gibsoni* rather than *B. canis* in canine practice [5, 8]. Affected dogs show various clinical signs including fever, anorexia and exercise intolerance [5, 6, 8]. Pale and icterus mucus membrane, splenomegaly and hepatomegaly are detected at the physical examination. Laboratory tests reveal the existence of anemia, thrombocytopenia and icterus. In severe cases, concurrent acute kidney failure, disseminated intravascular coagulation and immune-mediated hemolytic anemia are observed, and it becomes lethal in some cases [5, 6, 8]. In addition, some clinical stresses, such as administration of immunosuppressive drugs and concurrent infectious or other diseases, become a trigger of the acute onset of babesiosis in subclinically infected dogs [5, 6, 8, 31].

Diminazene aceturate has been used as a first line agent for the treatment against both *B. gibsoni* and/or *B. canis* infections in Japan for a long time [5, 8, 16]. Although diminazene aceturate shows anti-*Babesia* activity and a therapeutic potential against *B. canis* infection, it often fails to eliminate *B. gibsoni* from

affected dogs and a relapse often occurs [5, 8, 16]. Furthermore, diminazene aceturate has a narrow clinical safety margin and occasionally induces severe side effects such as cerebellar hemorrhage, hepatotoxicity, and necrosis at the injection site [5, 8, 16, 40]. As an alternative treatment strategy, a previous study demonstrated the possible efficacy of the three-drug combination therapy using clindamycin (CLDM), metronidazole (MNZ) and doxycycline (DOXY) for *B. gibsoni* infection [33]. In this study, a successful treatment was obtained in three of four *B. gibsoni* experimentally infected dogs with no adverse effects, and these three cases remained relapse-free after more than 3 years. However, this treatment was suggested to take a relatively long time to show its therapeutic effect [33]. Therefore, it had been still necessary to find an alternative fast-acting reagent to treat the cases with acute onset of babesiosis.

Recent studies showed that atovaquone (ATV), an analog of ubiquinone, would be an up-and-coming candidate as a new therapeutic reagent against babesiosis [2, 10, 12, 26, 37, 39]. ATV has been mainly used as an anti-protozoal drug or prophylactic agent against toxoplasmosis, malaria and pneumocystis pneumonia [10, 12, 26, 39]. The mechanism of action of ATV is presumed to obstruct the respiration of protozoa through the inhibition of cytochrome bc complex [3, 11, 20, 24, 32, 34, 36]. Inhibitory effect of ATV has also been reported on various *Babesia* spp. such as *B.*

divergence, *B. microti*, and *B. conradae* [7, 10, 12, 26, 37, 39]. Another candidate is a macrolide antibiotic, azithromycin (AZM), however, its mechanism of action against *Babesia* spp. is still unknown [11, 30]. In addition, it has been reported that the simultaneous use of ATV and AZM produces an additive/synergistic therapeutic effect on human and rodents babesiosis, while single use of each drug tends to result in a relapse of symptoms [10, 12, 21, 26, 30, 38, 39].

In 2004, Birkenheuer *et al.* found that the combination therapy with ATV and AZM showed a great effect on *B. gibsoni* infected dogs which revealed resistance to diminazene aceturate or imidocarb treatment [4]. However, the other report described that this treatment was not able to achieve a complete elimination of *Babesia* from host even though a great therapeutic effect was obtained [17]. Furthermore, recent reports suggested that drug-resistant variants against ATV would be possibly emerged, and that mutations in the *cytochrome b* (*cytb*) gene, resulting in amino acid substitutions at the putative ATV binding site, were strongly suggested to be concerned to the development of ATV-resistant phenotype [13, 22].

The clinical cases with the acute onset of *B. gibsoni* infection were often experienced and this disorder becomes problematic in canine practice. Therefore, it is essential to establish and characterize a new therapeutic strategy against *B. gibsoni* infection. In Chapter I, I studied the possible application of combination therapy

with ATV and AZM as a "first-line" in clinical cases with *B. gibsoni* infection. In addition, I also analyzed the possible emergence of drug-resistant variants in clinical cases after the ATV-based treatment. In Chapter II, I performed an epidemiological survey to search for drug-resistant variants of *B. gibsoni* in nature focused on the *cytb* gene. A series of these studies were carried out to provide novel insights for the treatment strategy of babesiosis and for understanding its underlying disadvantages.

Chapter I

Possible Emergence of Drug-Resistant Variants of *Babesia gibsoni* in Clinical Cases Treated with Atovaquone and Azithromycin

Abstract

There is no well-established treatment strategy for *Babesia gibsoni* infection. A new therapeutic protocol using atovaquone (ATV) and azithromycin (AZM) has been proposed, but there is concern about the possible induction of relapse and the emergence of ATV-resistant variants following treatment. The clinical use of combination therapy with ATV and AZM was evaluated as a first line treatment of clinical *B. gibsoni* infection in dogs, and the emergence of ATV-resistant variants were also investigate in this study.

Eight *B. gibsoni* naturally infected dogs showing signs of acute onset of disease were admitted to Kagoshima University Veterinary Teaching Hospital. These dogs received combination therapy with ATV and AZM, and their clinical courses and clinicopathological parameters were evaluated. In addition, alterations in the *cytochrome b* (*cytb*) gene of *B. gibsoni* were analyzed by polymerase chain reaction and DNA sequencing techniques.

All of the dogs with the exception in one case responded well to the treatment, with rapid improvement in their clinical condition and hematological parameters. However, 5 of the 8 dogs relapsed after treatment. Analysis of the *cytb* gene strongly suggested the emergence of ATV-resistant variants after treatment.

The results of this study indicated that the combination of ATV and AZM can be used as a first line treatment for dogs with babesiosis, but relapses occur. Attention should be paid to the possible *in vivo* selection of drug-resistant variants.

Introduction

Diminazene aceturate has been used widely as a first line agent for the treatment of *Babesia gibsoni* infection of dogs in Japan. Although diminazene aceturate has anti-*Babesia* activity, it often fails to eliminate *B. gibsoni* from affected dogs and a relapse may occur. Furthermore, diminazene aceturate has a narrow clinical safety margin and can induce severe adverse effects such as cerebellar hemorrhage, hepatotoxicosis, and necrosis at the injection site [5, 8, 16, 40]. Combination therapy of clindamycin (CLDM), metronidazole (MNZ) and doxycycline (DOXY) as an efficacious alternative treatment strategy for *B. gibsoni* infection [33]. Three of four *B. gibsoni* infected dogs were successfully treated with no adverse effects, and these 3 dogs remained relapse-free after more than 3 years. However, this treatment takes a relatively long time to show its therapeutic effect [33].

Recently, a new treatment strategy has been proposed that uses a combination of two drugs, an analog of ubiquinone, atovaquone (ATV), and a macrolide antibiotic, azithromycin (AZM) [2, 10, 12, 26, 37, 39]. The use of ATV alone inhibits the growth of *Babesia* sp., and ATV is used for the treatment of protozoal diseases caused by *Plasmodium* and *Pneumocystis* infections [10, 12, 26, 39]. ATV is presumed to act through the inhibition of the cytochrome bc complex in protozoa [2,

11, 20, 24, 32, 34, 36]. AZM also demonstrates an anti-*Babesia* effect, but its mechanism of action against *Babesia* spp. is unknown [11, 30]. In addition, the simultaneous use of ATV and AZM produces an additive or synergistic therapeutic effect, while the single use of each drug tends to result in a relapse of signs [26, 38, 39].

Combination therapy with ATV and AZM is very effective in eliminating *B. gibsoni* infection, but results are not consistent based on molecular analysis [4, 17]. Furthermore, the emergence of drug-resistant variants can occur after the use of ATV alone, and these variants might be caused by mutations in the *cytochrome b* (*cytb*) gene, resulting in amino acid substitutions at the putative ATV binding site [22].

I used combination therapy with ATV and AZM in this study as a first line therapy in *B. gibsoni* infected dogs. My results indicate that this drug combination relieved the acute crisis of babesiosis rapidly, but was associated with a high relapse rate. I provide an overview of the clinical cases treated with ATV and AZM, and present the results of molecular analyses of possible ATV-resistant variants, especially those from relapsed cases.

Materials and Methods

Dogs

Eight dogs with acute onset of babesiosis, exhibiting anemia, thrombocytopenia or both, and with microscopic or molecular evidence of parasitemia, were enrolled in this study. The 8 dogs represent all the cases of babesiosis at Kagoshima University Veterinary Teaching Hospital (KUVTH) during 2007–2008. The dogs included were 2 Golden Retrievers, 2 Toy Poodles, 1 Miniature Dachshund, 1 Miniature Schnauzer, 1 Labrador Retriever and 1 mixed breed dog. There were five males (1 neutered) and three females. The dogs' ages ranged from 1.5–14 years old.

The 8 dogs were initially examined for diagnosis and treatment, except for one dog (Dog 5), which was treated with diminazene aceturate at the referral hospital, 5 days prior to its admission to KUVTH. The other dogs had not received any anti-protozoal drugs prior to their admission to KUVTH. All dogs had anorexia, depression, pale mucus membranes, and hepatomegaly, splenomegaly or both at the time of their first visit (Table 1). In addition, all dogs had mild to severe regenerative anemia and severe thrombocytopenia on complete blood count (CBC). Blood smear specimens stained with modified Wright-Giemsa staining were prepared for all dogs at the first visit. *B. gibsoni* parasitemia was detected in 7 dogs by examination of

blood smears. The remaining dog (Dog 3) could not be diagnosed on the basis of examination of a blood smear, because of a low parasitemia. In this dog, two types of polymerase chain reaction (PCR) techniques, which amplify the *B. gibsoni*-derived *p18* gene and detect the 18S ribosomal RNA gene derived from *Babesia* spp., were used to make a diagnosis [3, 9]. After diagnosis, the 8 dogs received combination therapy with ATV and AZM, and the changes in clinical parameters were evaluated.

Treatment protocol

Combination therapy with ATV (Mepron, Glaxo SmithKline, Middlesex, UK) and AZM (Zithromac, Pfizer Japan Inc., Tokyo, Japan) was initiated in all 8 dogs from the day of diagnosis. The dosages and duration of the combination therapy were based on previous report [4] (ATV, 13.3 mg/kg, PO, q8h; AZM, 10 mg/kg, PO, q24h). ATV was administered for 10 days to all dogs, but some received AZM for more than 10 days, if PCV did not increase to 35% during this period.

Relapse of babesiosis was defined in this study as the re-appearance of *B. gibsoni* in blood smear specimens or the progression of anemia, thrombocytopenia or both after ATV and AZM treatment. At the time of relapse, combination therapy with ATV and AZM, at the same dosages mentioned above, was reinitiated, but the

treatment period was extended, depending on the clinical signs and laboratory test results. In case of a second relapse during or after the second therapy with ATV and AZM, the treatment protocol was changed to a 3-drug combination therapy with CLDM (Dalacin, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) (25 mg/kg, PO, q12h), MNZ (Flagyl, Shionogi & Co., Ltd., Osaka, Japan) (15 mg/kg, PO, q12h) and DOXY (Vibramycin, Pfizer Japan Inc., Tokyo, Japan) (5 mg/kg, PO, q12h). The owners were instructed to continue this 3-drug combination therapy for at least 3 months [33].

Evaluation of clinical parameters

After diagnosis and initiation of therapy, each animal's clinical signs, hematological parameters, and *B. gibsoni* infection status were monitored. Blood samples were collected at initial examination. Half of each blood sample was anti-coagulated with EDTA and used for CBC, determination of the percent parasitemia from blood smear, and PCR analysis. The other half of the samples were treated with heparin and the plasma was used for biochemical analysis. CBC was performed using an automatic blood calculator (pocH-100*i*, Sysmex, Kobe, Japan). Blood smear specimens were stained with modified Wright-Giemsa staining. The parasitemia of *B. gibsoni* was calculated in blood smears as the percentage of

parasite infected erythrocytes/1000 erythrocytes. Detection of *B. gibsoni*-derived genomic DNA by PCR was performed as mentioned above [3, 9]. Biochemical parameters were analyzed using a FUJI DRICHEM 3500V (FUJIFILM Medical Co., Ltd., Tokyo, Japan). Plasma C-reactive protein (CRP) levels were determined using Arrows Laser CRP-2 (Arrows, Osaka, Japan).

Molecular analysis of B. gibsoni cytb gene

Nucleotide and deduced amino acid sequences of the *cytb* gene were analyzed using the blood samples collected before and after treatment or at relapse. In order to amplify and determine the nucleotide sequence efficiently, I divided the *cytb* gene to three parts and constructed oligonucleotide primers for nested-PCR analysis based on the reported *cytb* gene sequence (DDBJ/GenBank/EMBL accession number, AB215096) [22]. In the first round PCR, the primers 5'-TTT AGT GAA GGA ACT TGA CAG GTA-3' (cytb-F, nt (-119– -96) and 5'-ATA TGC AAA CTT CCC GGC TAA AC-3' (cytb-R, nt 1105–1086) were used. The second round PCR was carried out using three sets of primers: 5'-GGA AAC AGG GCT TTA ACC AA-3' (cytb-1F, nt -35– -57) and 5'-CCG GAA TCC AAT AAA ACA GG-3' (cytb-1R, nt 412–393), 5'-CCT TGG TCA TGG TAT TCT GGA-3' (cytb-2F, nt 277–297) and 5'-AAC ATC TCC CTG AAA CAA TGG TA-3' (cytb-2R, nt 702–680), and 5'-ATT TGC TGC

TTT GGG TGT TC-3' (cytb-3F, nt 642–661) and 5'-AAA CTT CCC GGC TAA ACT CC-3' (cytb-3R, nt 1099–1080). The first round PCR product was used as template DNA for all second round reactions. PCR amplification was performed under the following conditions for both first and second reactions: 1 cycle of pre-denaturation (5 min, 95°C); 35 cycles of denaturation (30 sec at 95°C); annealing (1 min at 54°C), and polymerization (1 min at 72°C); and 1 cycle of complete elongation (10 min at 72°C). The nucleotide sequence of the amplified DNA fragments was determined by direct sequencing using the dideoxy chain termination method (ABI Prism Big Dye Primer Cycle Sequencing Kit, Applied Biosystems, Foster City, CA). GENETYX Version 8.0 software (Software Development Co., Ltd., Tokyo, Japan) was used to characterize the obtained nucleotide sequence data.

Results

Clinical courses of cases

The clinical courses and laboratory parameters of all 8 dogs are summarized in Figure 1 and Table 2, respectively. After diagnosis, all dogs received combination therapy with ATV and AZM, and had rapid recovery of clinical signs and laboratory parameters. Dogs 1 and 2 showed ideal responses to the treatment and experienced no relapses during the observation period. Multiple mast cell tumors were found in the skin and spleen in Dog 1 at day 333. These were treated by surgical resection of the skin lesions and by splenectomy, followed by chemotherapy with vinblastine (Exal, Nippon Kayaku Co., Ltd., Tokyo, Japan) and prednisolone (Predonine, Shionogi & Co., Ltd., Osaka, Japan) (Fig.1A). Despite immunosuppression due to splenectomy and chemotherapy, the animal had no signs of relapse of babesiosis. Dogs 1 and 2 remained alive, with no clinical signs, for the duration of the study (Table 2).

Five dogs (Dogs 3, 4, 6, 7 and 8) relapsed after cessation of the first 10-day ATV/AZM treatment (Fig.1C, D, F, G, and H). These dogs had continuous positive results on PCR analysis, even after the 10-day ATV/AZM treatment. These dogs received a second ATV/AZM treatment, and the duration of administration was

extended to 20, 40 and 24 days in Dogs 3, 4 and 7, respectively (Fig.1C, D, and G). Three (Dogs 3, 6 and 8) of the 5 relapsed dogs responded well to the second ATV/AZM treatment, with improved clinical signs and laboratory test results (Fig.1C, F, and G). The treatment protocol in Dog 8 was changed to 3-drug combination therapy with CLDM, MNZ and DOXY on day 72, because the owner said that the dog would not accept a liquid drug.

The remaining 2 dogs (Dogs 4 and 7) did not respond to the second ATV/AZM treatment and had a second relapse. Notably, Dog 4 had a progression of anemia and parasitemia, even during the ATV and AZM administration. ATV/AZM treatment was replaced by 3-drug combination therapy in these dogs, with good responses and no signs of subsequent relapse (Fig.1D). The 3-drug combination therapy was applied to Dog 4 and Dog 7 for 106 and 42 days, respectively (Fig.1D and G). I recommended continuing this therapy to the owner of Case 7, however, the owner decided to stop treatment halfway through the recommend treatment period.

In total, 5 of the 8 dogs experienced either 1 or 2 relapses, and 2 of the 5 dogs demonstrated resistance to the ATV and AZM treatment (Table 2). However, no adverse effects due to ATV and AZM were detected in any animals, including those with relapses. Two dogs (Dogs 5 and 8) died during the observation period, but neither the ATV/AZM treatment nor the *Babesia* infection itself seemed to be the

cause of death. Dog 5 had been treated with diminazene aceturate just before admission to KUVTH, and died on day 7 after the sudden development of neurological signs. Furthermore, this dog had a tendency towards recovery based on packed cell volume (PCV) and platelet counts, although these could only be evaluated at two points (Fig.1E). Death in this case was possibly due to an adverse effect of diminazene. Dog 8 had an uncontrollable extramedullary plasma cell tumor, and died on day 170. The total plasma protein level was very high at the time of death, and, therefore, hyperviscosity syndrome was thought to be the likely cause of death.

Characterization of B. gibsoni cytb gene

This study found 5 and 2 dogs demonstrating relapse and ATV resistance, respectively. Accordingly, I evaluated the possible emergence of ATV-resistant strains of *B. gibsoni* by determining the nucleotide sequence of the *cytb* gene [4, 8, 21].

Compared with the standard sequence of the *B. gibsoni cytb* gene, single nucleotide substitutions were detected at 7 locations (nt 51, nt 322, nt 361, nt 363, nt 492, nt 558 and nt 676) in cases. Four of these substitutions could give rise to non-synonymous amino acid substitutions, including A108T, M121I, M121V and

I226V (Table 3). The *cytb* gene sequences derived from Dog 1, which did not experience a relapse, were identical to the standard sequences throughout the observation period. The *cytb* gene from the other dog without a relapse (Dog 2) had a substitution at nt 676 resulting in an amino acid substitution at position 226, from isoleucine to valine (I226V). This type of substitution was also observed in other dogs, both with and without relapses. A108T was observed in Dog 7, and the *cytb* gene associated with this amino acid substitution was detectable even at the first visit. Therefore, I226V and A108T do not appear to be directly related to the drug-resistance phenotype (Table 3). The M121I and M121V substitutions, however, were suspected to be related to the drug-resistance phenotype, because all dogs possessed *B. gibsoni* with M121I or M121V amino acid substitutions at the time of relapse (Table 3). *B. gibsoni* with the M121I substitution was dominant in Dog 3, even at the time of the primary onset of the disease.

Discussion

I used combination therapy with ATV and AZM in this study as first line treatment in 8 dogs that were naturally infected with *B. gibsoni,* and showed clinical signs of the acute onset of babesiosis. Clinical signs and hematological parameters improved soon after the initiation of the treatment in all cases. No obvious adverse effects related to this treatment protocol were detected in any dogs. To date, diminazene aceturate or CLDM-based treatment protocols have been widely used to treat *B. gibsoni* infection in Japan, but they are associated with adverse effects and are slow to have an effect [5, 8, 16, 30]. In a previous study using a 3-drug combination therapy, it took approximately 2 weeks to end the acute crisis of babesiosis, and supportive therapies, including blood transfusion, were required [33]. However, in the current study, clinical signs in 8 dogs were well controlled by the administration of ATV and AZM, without the need for any special supportive therapies. Therefore, this combination therapy with ATV and AZM seems to be a potent, rapid-acting, and clinically useful strategy for treating canine babesiosis.

Some concerns were raised by this protocol. Although 2 dogs showed rapid clinical improvements after the 10 day treatment and did not experience any relapse during the observation period, 5 dogs did relapse at approximately 1 month after the

cessation of treatment. Possible reasons for the relapses include inadequate drug dosages or medication periods. The 10-day treatment protocol was proposed, but a recent report revealed that this 10-day treatment protocol may be inadequate for controlling the parasitemia in the experimentally infected dogs [4, 17]. My findings support this latter report. The appropriate dosages and duration of treatment must be determined. I did not evaluate the immunological status of the animals in this study. The future use of convenient and sensitive methods to evaluate immunological function (*e.g.* evaluation of antibody titer using enzyme-linked immunosorbent assays or immunochromatography) or levels of parasitemia (*e.g.* quantification of *Babesia*-derived DNA by real time PCR) in the hosts may help to identify suitable dosages and treatment durations [18, 23, 24, 35]. On the other hand, the results from longitudinal PCR analyses in this study did not always match the clinical course. This might have been due to the sensitivity of PCR itself and the possible detection of dead parasite-derived DNA. Thus, establishing a simple method to distinguish re-infection from relapse in dogs with positive PCR results continuously or intermittently is required.

The results of this study raise some possible doubts concerning the therapeutic efficacy of AZM, as some of the relapsed dogs had been continuously treated with AZM alone, after the withdrawal of ATV administration. Two relapsed dogs (Dogs 3

and 4) responded after the resumption of ATV administration. This suggests that the anti-*B. gibsoni* activity of the ATV/AZM combination therapy was due mainly to the action of ATV. However, previous reports demonstrated an additive effect of ATV and AZM against *B. microti* and *B. divergens* infections, while the relapse rate was high in cases treated with either ATV or AZM alone, suggesting that AZM was required for efficient control of the disease [26, 38, 39]. The mechanism of AZM action against *Babesia* spp. is unknown, and further studies are required to clarify this point.

Two of the 5 cases in this study (Cases 4 and 7) experienced two relapses, and thrombocytopenia, progression of anemia, and reappearance of parasitemia were detected in these animals, even after the second treatment or during the period of treatment with ATV and AZM. Previous reports have also suggested that ATV sensitivity was reduced in experimentally infected dogs that relapsed after receiving ATV treatment [17, 21, 22]. Furthermore, drug-resistant variants were suspected to occur in naturally infected, as well as in experimentally infected dogs [17, 22]. Therefore, I performed molecular analysis of the *cytb* gene, which has previously been reported as being potentially responsible for the development of the ATV-resistant phenotype in *B. gibsoni* [17, 22]. Their results demonstrated that ATV-resistant strains had three patterns of amino acid substitutions in the *cytb* gene,

one of which, M121I, was located at a putative ATV-binding site. Some nucleotide and amino acid substitutions were detected in the *cytb* gene in my cases, most of which did not appear to be related to the ATV-resistant phenotype. However, nucleotide substitutions resulting in the substitution of the amino acid residue at 121 were also detected in my study. My results, in common with previous report, also suggested that these substitutions at residue 121 may be responsible for the development of ATV-resistance, because this substitution was only observed in the animals that experienced relapses [22]. However, these *B. gibsoni* strains have not yet been tested for *in vitro* ATV resistance [11, 17, 20, 22, 24, 34, 36]. Findings in this study also suggest that ATV-resistance in naturally infected dogs could have been induced by the same mechanism as that acting in experimentally infected cases [4, 8, 21].

Surprisingly, the possibly drug-resistant M121I variant was dominant in one dog in this study, even at the onset of the disease. This suggests that ATV-resistant variants may exist in nature, and that these ATV-resistant variants have emerged mainly through *in vivo* selection, although mutation of the *cytb* gene during ATV treatment cannot be ruled out. Epidemiological surveys of *B. gibsoni cytb* genotypes are required to determine the distribution of *B. gibsoni* variants in nature, and to establish appropriate treatment strategies based on *cytb* genotype.

Chapter II

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

Abstract

In this study, I 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.

Introduction

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* [5, 6, 8]. 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 [5, 8]. 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 [5, 8]. In Japan, *B. gibsoni* infected dogs are mainly observed in the western part of Japan although affected areas seem to expand towards the northeast [14, 15, 25]. The definitive treatment strategy for *B. gibsoni* infection has not yet been established [5, 8, 33, 40]. Diminazene aceturate is used as a first-line drug for *B. gibsoni* infection in Japan [16]. But it often fails to eliminate babesia from the host and a relapse of the disease is frequently observed [16, 33]. Furthermore, diminazene aceturate has a narrow safety margin and occasionally induces severe side effects [16]. For these reasons, there have been efforts in canine practice to establish an alternative therapeutic strategy against *B. gibsoni* infection.

Recent studies 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 [4, 21, 27]. However, I found that approximately 60% of ATV-treated dogs experienced a relapse of the disease [27]. 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 [27]. One of them will be a substitution for ATV-resistance, resulting in M121I amino acid (AA) substitution as previously reported [22, 27]. Surprisingly, *B. gibsoni* with M121I was dominant in one case, even at the primary onset of disease [27]. 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.

Materials and Methods

Blood samples from B. gibsoni infected dogs

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 samples were collected from clinically suspicious cases and were confirmed to be positive for *B. gibsoni* infection by following *B. gibsoni* 18S ribosomal RNA gene PCR [9]. Remaining 14 samples were collected from clinical cases diagnosed at the Kagoshima University Veterinary Teaching Hospital (KUVTH) by blood examination and 18S ribosomal RNA 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 collected at KUVTH were obtained from dogs with no treatment history during the first visit.

Amplification and DNA sequencing of B. gibsoni cytb gene

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* 18S ribosomal RNA gene by PCR analyses [3, 9]. 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 [27]. 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.

Results

Compared with the standard sequence of the *B. gibsoni cytb* gene (DDBJ/GenBank/EMBL accession number, AB215096), 40 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 4). Nucleotide substitutions resulting in AA substitutions A108T, M121I, I226V, V220I, 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%) [13, 22]. 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 [22].

Figure 2 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.

Discussion

To my 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 [22]. However, it is noteworthy that the ATV-resistant variants with M121I, V220I or I303V were dominant in some cases. These 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 multiple cases infected with *B. gibsoni* possessing V220I, I226V, I303V or P310S *cytb* were found, these AA substitutions might not contribute to the development of an ATV-resistant phenotype in clinical cases. My 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 were dominant [27]. Furthermore, recent study showed that the major candidate

responsible for ATV-resistance is M121I [13]. However, 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 [13, 22, 27].

I found three cases whose dominant *B. gibsoni* were M121I type *cytb*. As mentioned above, the prevalence was low (3.3%). However, I have to allow for the limitations of this study and a possible underestimation of the true prevalence. I simply retrospectively evaluated the *cytb* genotypes of *B. gibsoni* in infected dogs. Therefore, I do not know whether the dogs infected with variant *cytb* types of *B. gibsoni* actually show resistance against ATV treatment or not. In addition, I have to consider the possibility that a minor population of such genotypes might have been missed in this study 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 [27].

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.

Conclusion

In Japan, *B. gibsoni* infected dogs are mainly observed in the western part of Japan although affected areas are expanding towards the northeastern part of Japan [14, 15, 25]. At present, vaccine strategy is unavailable for the prophylactic purpose, thus a control of tick infestation is the best way to prevent babesia infection in dogs [28, 29]. However, the complete prevention of tick infestation seems to be difficult for dog owners because of the lack of information and economical reasons. Accordingly, babesia infected dogs are still experienced clinically and a part of them develops acute onset of babesiosis. These facts indicate that we still have to discuss about a treatment protocol for canine babesiosis.

Canine babesiosis caused by *B. gibsoni* become often fatal if the dogs were not appropriately treated [5, 6, 8]. Several treatment strategies using anti-protozoan and/or antibiotic agents, such as diminazene aceturate, imidocarb and CDLM, are currently applied to canine babesiosis cased by *B. canis* or *B. gibsoni*, however, no definitive starategies have not been established yet [5, 8, 16, 33, 40]. The relapse and the adverse effect are major problems in current treatment protocols especially against *B. gibsoni* infection [16, 33, 40]. Therefore, the establishment of definitive treatment startegy is a very important subject in babesiosis.

In Chapter I, a 10-day treatment protocol with ATV and AZM was applied to eight cases naturally infected with *B. gibsoni*. After the treatment, ideal clinical

effects including rapid responses and no adverse effects were obtained. From these findings, it was highly suggested that ATV and AZM, especially ATV, would be a potent and rapid-acting anti-babesia agent(s) and be available as a first line treatment protocol. However, more than half of treated dogs unexpectedly experienced relapse of the disease. Therefore, this treatment protocol has not become a completely satisfied one yet. A reconsideration of the treatment period and drug dosages will be required for ATV and AZM based treatment to make it more suitable. In addition, DNA sequencing analyses of *B. gibsoni cytb* gene revealed that the mutation of this gene would be closely related to the relapse of disease and be easily induced or selected in dogs treated with ATV. Furthermore, this study clarified that the M121I substitution in CYTB, which was recognized as a most important mutation to ATV resistance *in vitro*, could be observed even in clinical cases [27]. In this chapter, I found out both advantages and disadvantages of ATV-based treatment protocol. These findings would be very valuable for veterinary clinicians because they obtained an additional treatment option against babesiosis, but on the other hand, might be suggesting the warning for the emergence of drug-resistant variants through the unplanned overuse of ATV.

One additional important point was found in Chapter I. It was the fact that a possibly drug-resistant M121I variant was dominant in one of cases, even at the

primary onset of the disease. This finding suggests that ATV-resistant variants might already exist in nature and *B. gibsoni* would be heterogeneous on *cytb* gene. If such strains of *B. gibsoni* are observed frequently, ATV-based treatment will become harmful rather than useful. Thus it would be important to know the prevalence of possibly ATV-resistant variants of *B. gibsoni* in the field. From these backgrounds, I performed molecular epidemiological survey of the *B. gibsoni cytb* gene in Chapter II. This survey clarified that there have already been several variants on *cytb* gene. Noteworthy is that possible drug-resistant variants with M121I, V220I or I303V, which were previously reported by Matsuu *et al.* as a responsible AA substitutions in CYTB, were dominant in some cases [22]. Although its frequency was not high, it would be a threat in the future where we use ATV for the treatment of babesiosis. The results obtained in this survey might be a warning to the future use of ATV. Therefore, a large scale molecular epidemiological survey based on *B. gibsoni cytb* gene with larger number of samples might be required. In addition, it will be necessary to establish the easy way which makes it possible to distinguish ATV susceptible *B. gibsoni* strains from resistant ones in hospital.

In conclusion, I investigated a treatment protocol for the acute onset of *B. gibsoni* infection and the prevalence of possible drug-resistant variants in the field. In Chapter I, I pointed out both advantages and disadvantages of ATV-based

treatment strategy. The findings in Chapter I are very important to urge a improvement of treatment protocols and to draw the attention to the emergence of drug resistant variants. The findings in Chapter II offer fundamental information about the prevalence of drug-resistant variants in Japan. Annual surveys might have a great significance to avoid the unnecessary spread of such strains in the future. Infectious diseases, including not only babesiosis but also other viral, bacterial and rickettsial infections, still remain as a major cause of disease in small animal practice in Japan. I hope a series of this study will contribute to overcome serious infectious disease, babesia infections in dogs.

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Tables

Table 1. Clinical profile of eight cases in this study.

Case number	Breed	Sex	Age (years)	Chief complaints	Physical examination findings
1	Golden Retriever	Male	9.5	Depression, Anorexia Vomition	Pale mucus membrane Splenohepatomegaly
2	Golden Retriever	Female	5	Depression, Anorexia	Pale mucus membrane Splenomegaly
3	Toy Poodle	Male	1.5	Depression, Hematouria	Pale mucus membrane Splenohepatomegaly
4	Minuature Dachshund	Female	3.5	Depression, Diarrhea Vomition	Pale mucus membrane Splenomegaly
5	Minuature Schnauzer	Female	2.5	Depression, Anorexia Hematouria	Pale mucus membrane
6	Labrador Retriever	Male	3	Depression, Anorexia Rapid breathing	Pale mucus membrane Splenomegaly
7	Toy Poodle	Male	2.5	Depression , Anorexia Weight loss	Pale mucus membrane
8	Mixed Breed	Neutered male	14	Depression, Anorexia	Pale mucus membrane

Table 2. Summary of clinical outcomes of 8 cases after the first 10-day treatment.

Case number	Relapse after 10-day Rx	Rx after the 1st relapse	Rx after the 2nd relapse	Current status (Days after Dx)
1	No	N/A*	N/A	Alive (671+)
2	No	N/A	N/A	Alive (344+)
3	Yes (Once)	ATV+AZM for 20 days	N/A	Alive (469+)
4	Yes (Twice)	ATV+AZM for 40 days	CLDM+MNZ +DOXY	Alive (419+)
5	N/A	N/A	N/A	Died (7)
6	Yes (Once)	ATV+AZM for 10 days	N/A	Alive (299+)
7	Yes (Twice)	ATV+AZM for 24 days	CLDM+MNZ +DOXY	Alive (254+)
8	Yes (Once)	ATV+AZM for 10 days	(CLDM+MNZ +DOXY)	Died (170)

*N/A, not applicable; ATV, atovaquone; AZM, azithromycin; CLDM, clindamycin; MNZ, metronidazole; DOXY, doxicycline

Table 3. Changes in *B. gibsoni cytb* gene before and after treatment with ATV and AZM.

Case	Time of isolation	Substitutions		
		Site	Nucleotide	Amino acid
1	Pre Rx (day1)	None	--	--
	Post Rx (day22)	None	--	--
2	Pre Rx (day1)	nt 676	ATA → GTA	I226V
	Post Rx (day7)	nt 676	ATA → GTA	I226V
3	Pre Rx (day1)	nt 363	ATG → GTG	M121I
		nt 676	ATA → GTA	I226V
	Relapse (day41)	nt 363	ATG → GTG	M121I
		nt 676	ATA → GTA	I226V
4	Pre Rx (day1)	nt 676	ATA → GTA	I226V
	1st relapse (day54)	nt 363	ATG → GTG	M121I
		nt 676	ATA → GTA	I226V
	2nd relapse (day74)	nt 361	ATG → ATT	M121V
		nt 676	ATA → GTA	I226V
5	Pre Rx (day1)	None	--	--
6	Pre Rx (day1)	nt 676	ATA → GTA	I226V
	Relapse (day64)	nt 363	ATG → GTG	M121I
		nt 676	ATA → GTA	I226V
7	Pre Rx (day1)	nt 322	GCT → ACT	A108T
		nt 676	ATA → GTA	I226V
	1st relapse (day47)	nt 322	GCT → ACT	A108T
		nt 363	ATG → GTG	M121I
		nt 676	ATA → GTA	I226V
	2nd relapse (day88)	nt 322	GCT → ACT	A108T
		nt 363	ATG → GTG	M121I
		nt 676	ATA → GTA	I226V
8	Pre Rx (day1)	None	--	--
	Relapse (day55)	nt 363	ATG → GTG	M121I

Table 4. 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 %)

Figure Legends

Fig. 1 Clinical course and change in hematological parameters of naturally *B. gibsoni*-infected dogs treated with ATV and AZM. (A) to (F) are corresponding to data from Cases 1 to 8, respectively.

Fig. 2 Geographical distribution of *B. gibsoni* based on CYTB AA sequences. Each dot represents one case.

Figures

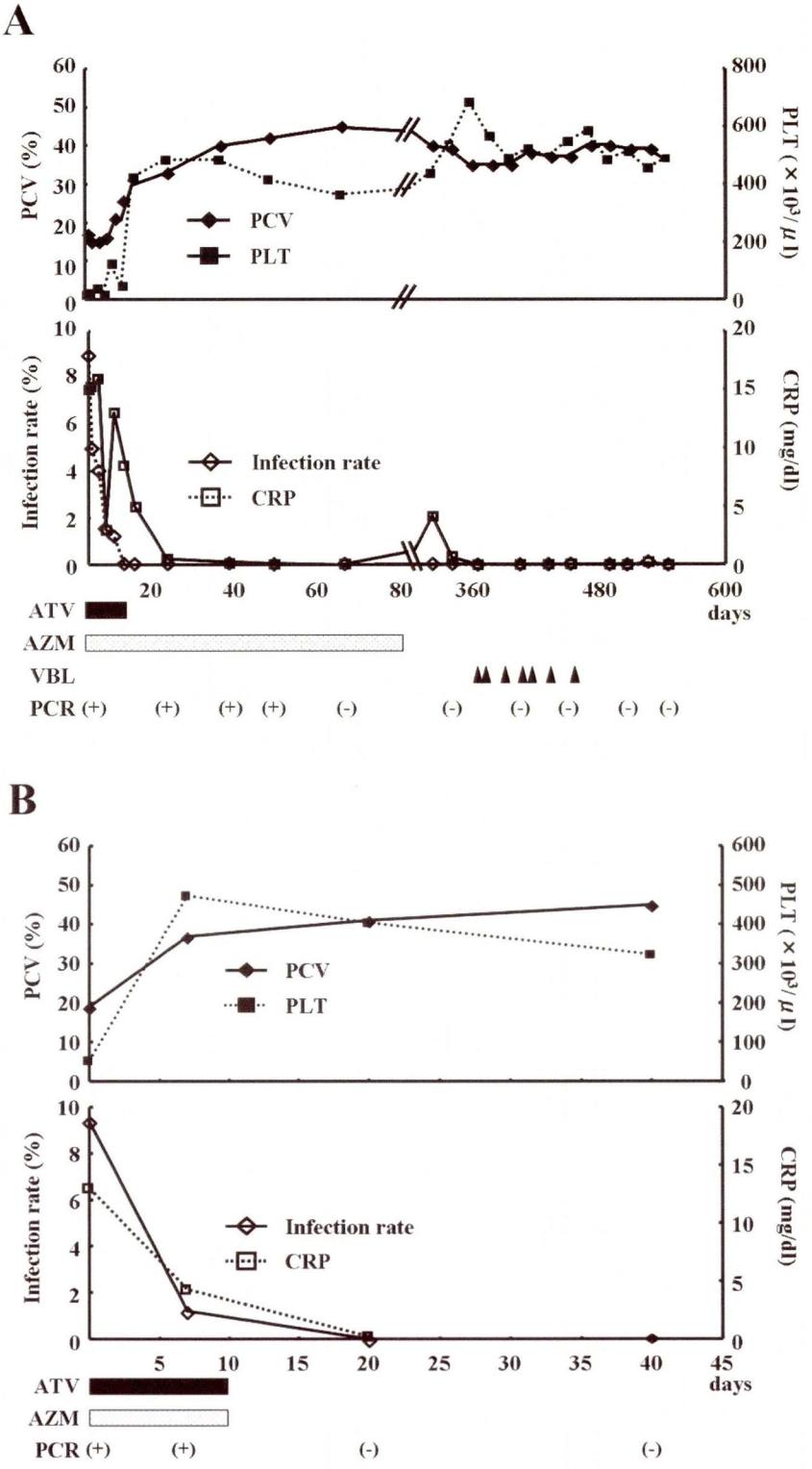
A
PCV (%)
PLT (×10³/μl)
PCV
PLT
Infection rate (%)
CRP (mg/dl)
Infection rate
CRP
days
ATV
AZM
VBL
PCR (+) (+) (+) (+) (-) (-) (-) (-) (-) (-)
B
PCV (%)
PLT (×10³/μl)
PCV
PLT
Infection rate (%)
CRP (mg/dl)
Infection rate
CRP
days
ATV
AZM
PCR (+) (+) (-) (-)

Fig. 1

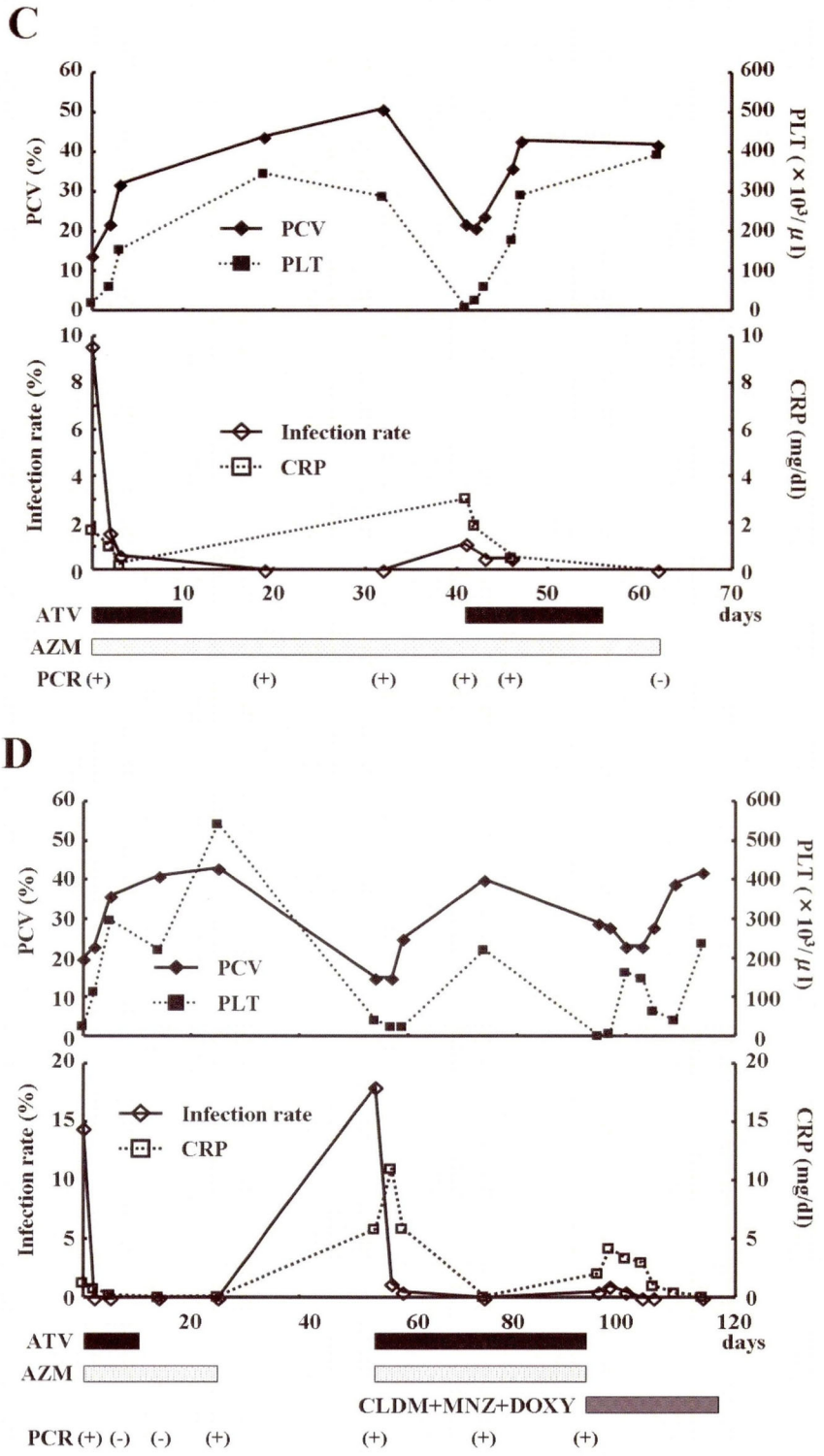

Fig. 1 (Continued)

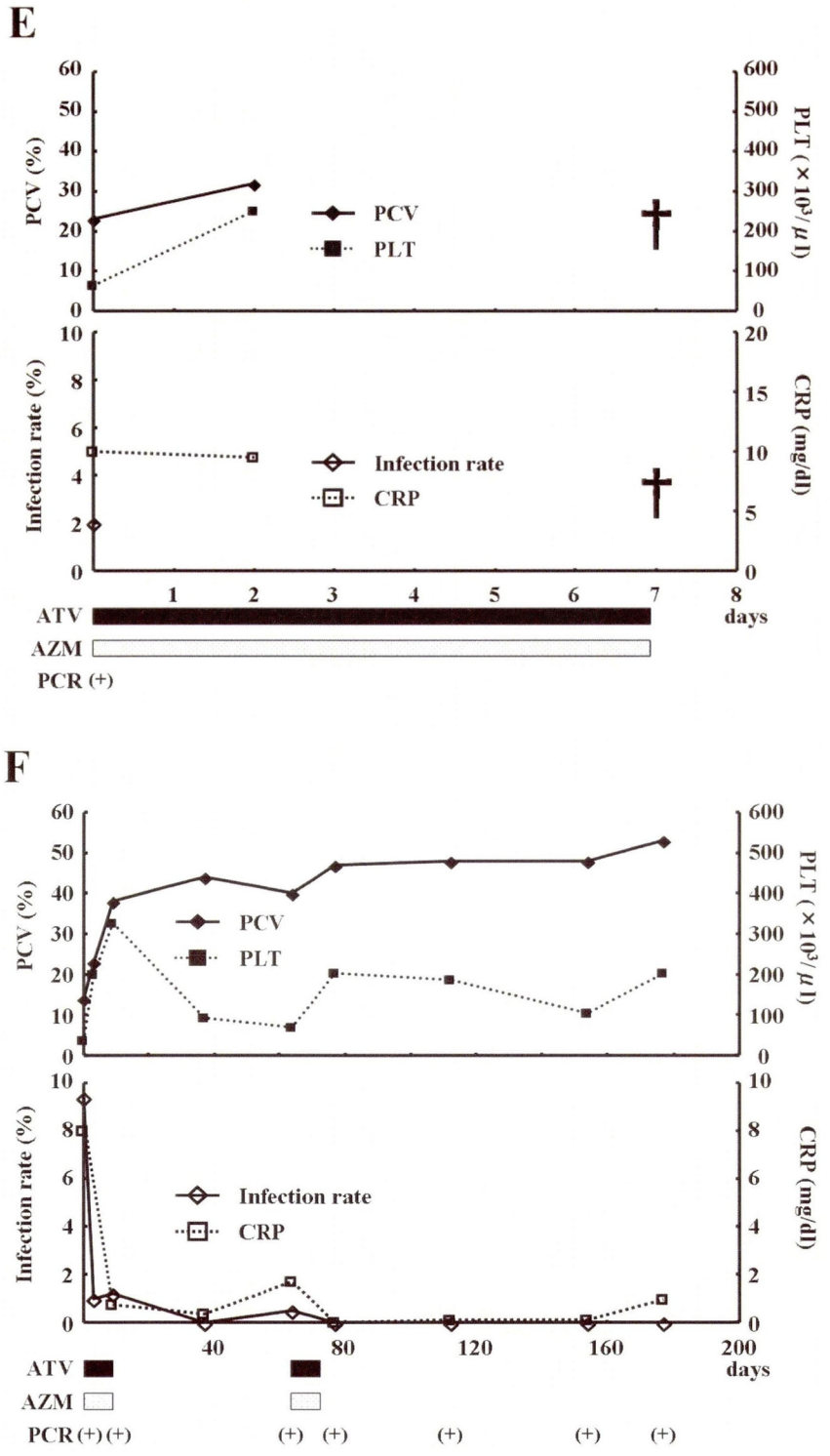

Fig. 1 (Continued)

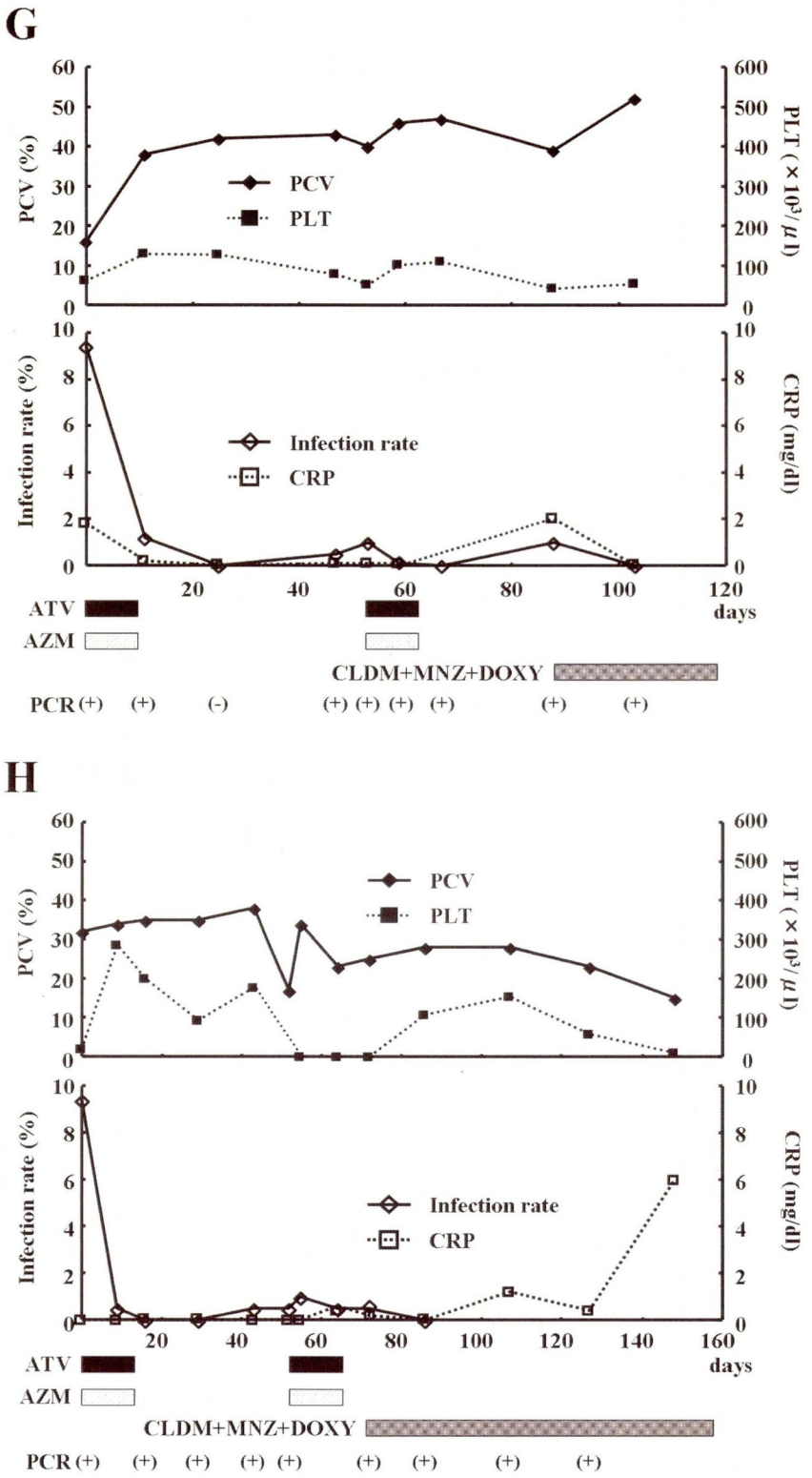

Fig. 1 (Continued)

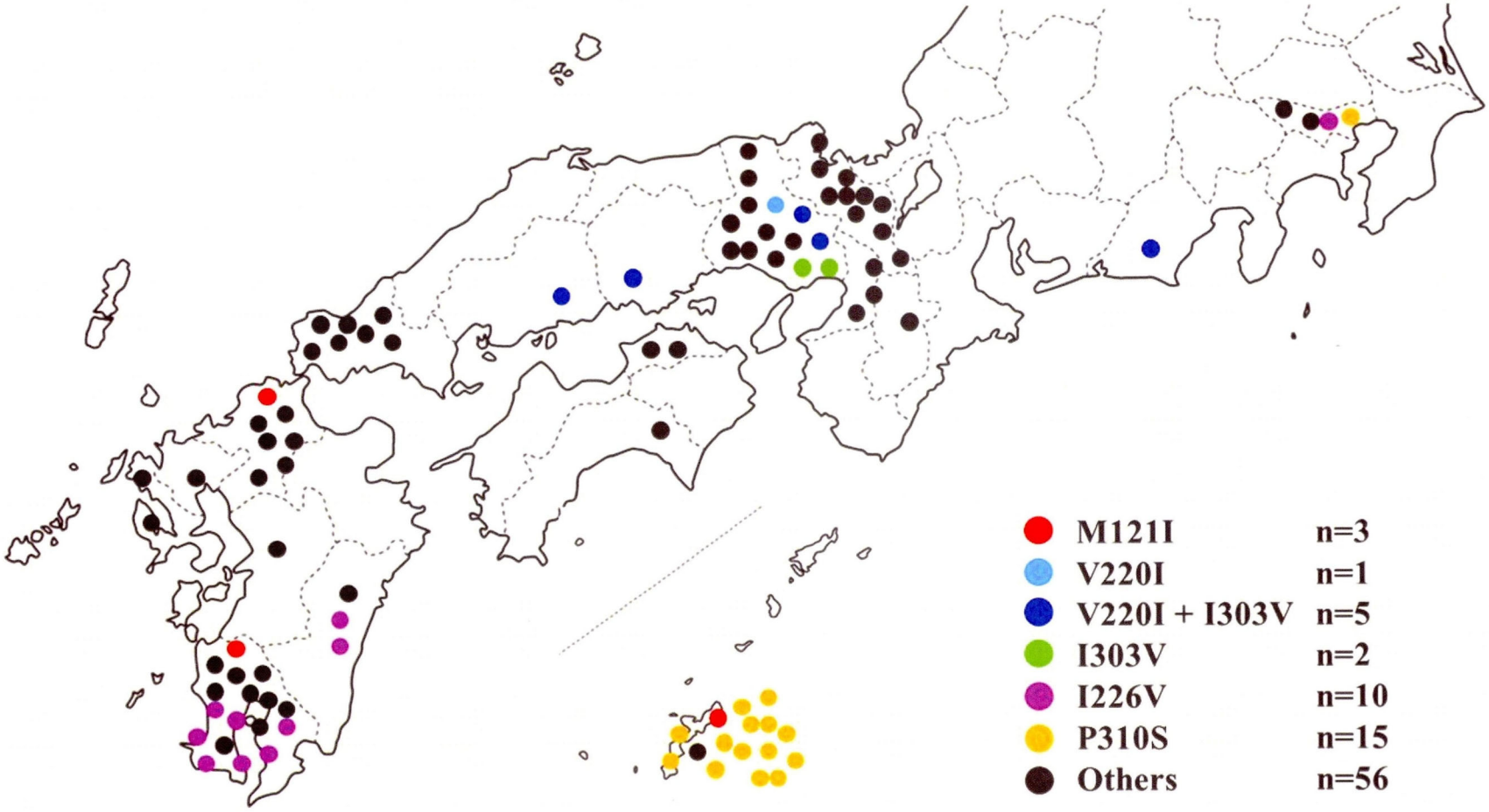
M121I n=3
V220I n=1
V220I + I303V n=5
I303V n=2
I226V n=10
P310S n=15
Others n=56

Fig. 2