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NOTE

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Further epidemiological survey for atovaquone resistant related gene of *Babesia gibsoni* in Japan during 2015–2018

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ABSTRACT. A single-nucleotide polymorphism causing the replacement of methionine with isoleucine (M121I) in cytochrome *b* of *Babesia gibsoni* has been reported to reduce the susceptibility to atovaquone (ATV) in *B. gibsoni* infection. In our previous study, *B. gibsoni* with M121I was suggested to exist in nature. Thus, further examinations were performed. In total, 105 genomic DNA samples from *B. gibsoni*-infected dogs were collected from western (98 samples from 15 prefectures) and eastern areas (7 samples from 4 prefectures) in Japan. The M121I variant population was identified using allele-specific real-time PCR: it was then detected in nine samples (8.57%), which was higher than that in the previous study (4.11%). Although there are unclear points, such as the history of ATV usage, careful attention should be given to emerging ATV resistance.

KEY WORDS: allele-specific real-time PCR, *Babesia gibsoni*, drug resistance, M121I variant population

Babesia gibsoni infects the red blood cells of dogs; this typically causes hemolytic anemia, which is often accompanied by fever, jaundice, hemoglobinuria, and an enlarged spleen [1, 3]. *B. gibsoni* has been considered endemic in western Japan [7].

A definitive strategy to eliminate *B. gibsoni* is yet to be established. However, atovaquone (ATV) has already been identified as a well-tolerated, metabolically stable, and effective agent with broad-spectrum anti-parasitic activity. In a previous study, ATV monotherapy was found effective against acute canine *B. gibsoni* infection; however, it resulted in relapse and did not respond to the second dose of ATV [8]. ATV-resistant parasites had a single-nucleotide polymorphism at the nucleotide (nt) position 363 in cytochrome *b* (cyt *b*), which caused the replacement of methionine with isoleucine (M121I). *B. gibsoni* with M121I showed reduced susceptibility to ATV *in vitro* [5]. Thus, the presence of M121I was suggested to affect therapeutic efficacy when using ATV for canine *B. gibsoni* infection.

In our previous study, the M121I variant population was measured using allele-specific real-time PCR in 73 genomic DNA (gDNA) samples from *B. gibsoni*-infected dogs in the period of 2011–2014. The M121I variant was detected in three dogs from Yamaguchi, Osaka, and Hiroshima prefectures (overall prevalence, 4.11%), with detected proportions of 8.55%, 14.04%, and 97.03%, respectively (Fig. 1A) [6]. The possibility that *B. gibsoni* with M121I exists in nature was then suggested. Thus, this present study was conducted to detect M121I variant populations in the field during another period 2015–2018.

In total, 105 gDNA samples were collected from dogs naturally infected with *B. gibsoni* from 2015 to 2018. All samples were provided by a commercial laboratory (Marupi Lifetech Co., Ltd., Osaka, Japan). These samples were collected from clinically suspicious cases and were confirmed to be positive for *B. gibsoni* infection using *B. gibsoni* P18 gene PCR [2]. Ninety-eight samples were collected from dogs in western areas including Shikoku area and Kyushu area (Okinawa, Kagoshima, Fukuoka, Oita, Yamaguchi, Hiroshima, Okayama, Hyogo, Ehime, Kochi, Kagawa, Tokushima, Osaka, Shiga, Wakayama, and Aichi prefectures), and 7 samples were from eastern areas (Kanagawa, Ibaraki, Fukushima and Hokkaido prefectures; Table 1 and Fig. 1A).

An allele-specific SYBR green real-time PCR assay was used in quantifying the M121I variant population [5]. To evaluate the M121I variant population, copy numbers were calculated for the wild-type allele (363G) and mutated allele (363T) in the cyt *b*. Allele-specific forward primers were designed to specifically detect and quantify cyt *b* 363G and 363T alleles. The reverse primer was common. Plasmid clones containing the target region, either 363G or 363T, were prepared and serially diluted tenfold to obtain 10^8–10^0 molecules/μl. A standard curve was created by plotting the log of the initial copy number of input plasmid DNA

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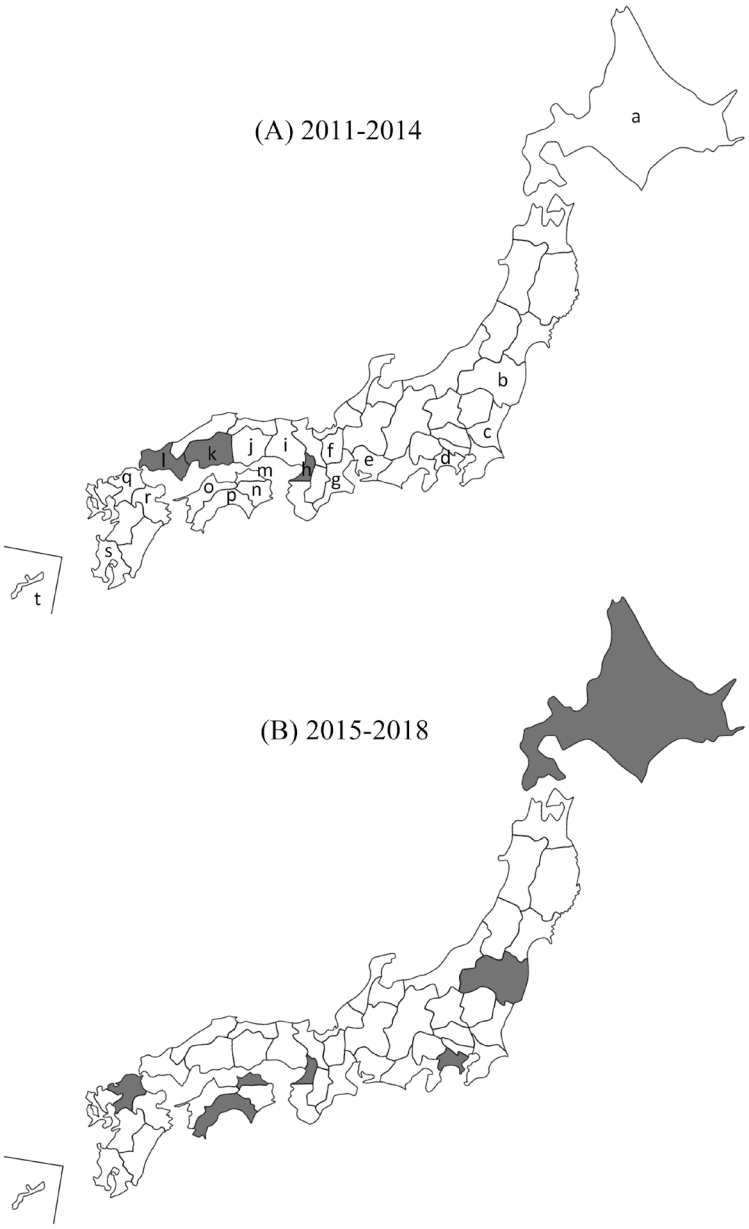

Fig. 1. Prefectures with dogs positive for M121I variant were shown in gray during 2011–2014 (A) and 2015–2018 (B). The alphabet corresponds to Table 1 and indicates the position of each prefecture (A).

against the threshold cycle value [5]. gDNAs from wild-type and ATV-resistant *B. gibsoni in vitro* cultures were used as negative and positive controls, respectively. Samples with 1% or more of M121I variant population were considered positive.

We measured the M121I variant population in 105 samples. The age, sex, breeds of dogs, and prefectures in these samples are presented in Table 1. The M121I variant population in 96 samples (91.42%) was under 1%. An M121I variant population >1% was detected in 9 samples, namely, 1.20% (breed unknown, male, 3 y 0 m, Kagawa), 1.66% (Tosa, male, 2 y 2 m, Hokkaido), 1.94% (Tosa, male, 3 y 0 m, Fukushima), 3.02% (mix, female, 13 y 6 m, Fukuoka), 7.99% (American Pit Bull terrier, sex unknown, 1 y 8 m, Kanagawa), 12.01% (mix, male, 8 y 0 m, Osaka), 17.08% (mix, male, 10 y 0 m, Kagawa), 14.75% (mix, male, 12 y 11 m, Kagawa), 21.06% (mix, male, 7 y 5 m, Kochi) (Table 2 and Fig. 1B).

Compared with our previous study, with the sampling carried out in 2011–2014 [6], the number of *Babesia*-positive samples had increased. The frequency identified it the Shikoku area (Kagawa, Tokushima, Kochi, and Ehime prefectures) had significantly increased; specifically, samples from Kagawa prefecture were identified to have increased the most (from 8 to 27). In another report in 2012, 92.7% of animal hospitals in Kagawa prefecture had confirmed or were suspected cases of canine babesiosis [7]. Shikoku area is considered highly endemic of *B. gibsoni* infection. The sample number in eastern areas also increased, and in this study, we collected samples from Fukushima and Kanagawa prefectures for the first time. In this report, all eight dogs from eastern Japan were breeds as fighting dogs; this information may be critical in hypothesizing the rise in canine babesiosis cases in eastern Japan.

Table 1. The signalments of *Babesia gibsoni* positive dogs (Age, Sex, Breed, and Prefecture)

	Signalments	2011–2014	2015–2018
Age	0–1	13	21
	2–3	6	15
	4–5	9	6
	6–7	13	17
	8–9	9	9
	10–11	9	13
	12–13	2	13
	Over 14	0	3
	Unknown	12	8
Sex	Male	39	55
	Female	14	27
	Castrated male	8	6
	Spayed female	8	10
	Unknown	4	7
Breed	Mix	15	33
	Shih-tzu	3	1
	Shetland	2	4
	Shiba	5	2
	Toy Poodle	3	2
	Tosa	4	5
	Plott Hound	2	0
	Border Collie	3	1
	Pomeranian	2	3
	M. Duchshund	5	4
	Yorkshire Terrier	3	3
	Chihuahua	3	3
	Unknown	12	20
	Others[a)]	11	24
Prefecture			
Eastern area	Hokkaido [a]	1	1
	Fukushima [b]	0	2
	Ibaraki [c]	1	1
	Kanagawa [d]	0	3
Western area	Aichi [e]	1	1
	Shiga [f]	1	1
	Wakayama [g]	10	12
	Osaka [h]	16	9
	Hyogo [i]	9	4
	Okayama [j]	3	7
	Hiroshima [k]	4	3
	Yamaguchi [l]	3	9
(Shikoku area)	Kagawa [m]	8	27
	Tokushima [n]	0	6
	Ehime [o]	1	6
	Kochi [p]	6	2
(Kyushu area)	Fukuoka [q]	4	4
	Oita [r]	1	1
	Kagoshima [s]	0	1
	Okinawa [t]	0	1

a) Others in 2015–2018; N=3; Pagu, Labrador Retriever. N=2; Golden Retriever, American Pit Bull Terrier, Bernese Mountain Dog. N=1; Irish Terrier, Kishu, German Shepherd dog, French Bulldog, Standard Poodle, Papillon, Siberian Husky, Beagle, Brittany Spaniel, Maltese, M. Schnauzer, and Rottweiler.

As the number of samples increased, the number of samples at almost all ages has also increased, especially those from young dogs <3 years old. The severity of canine babesiosis is likely affected by the dog's immune status, and the disease has been reported to be severe in puppies <8 months of age [4]. Early diagnosis and early treatment are therefore required for *B. gibsoni* infection at a young age. The number of samples from male dogs was high in both periods. To our knowledge, there is no report on gender difference differences among cases of canine babesiosis, thus requiring further investigations. Sample numbers of both mixed and other breeds increased as compared with our previous study (2011–2014). Among the breeds included in "other breeds", general domestic dogs were common, and no limitation in breed was observed. Although further investigation is necessary to determine whether age, sex, and breed affect the epidemiology and clinical manifestation of canine babesiosis, dogs at any age and sex may have a risk of infection, regardless of breed.

The number of samples from eastern areas had greatly increased from 2 to 7. Interestingly, the breeds were Tosa (Hokkaido [n=1], Fukushima [n=2], and Ibaraki [n=1] prefectures), Kishu (n=1) and American Pit Bull Terrier (Kanagawa prefecture [n=2]). These breeds are sometimes used as fighting dogs. In a previous study, Tosa dogs from Aomori prefecture widely subject to subclinical infection by *B. gibsoni*, which may have been transmitted during dog fighting [9]. Although it remains unclear whether the dogs in this report were fighting dogs, being a fighting dog may be a risk factor for canine babesiosis in eastern Japan. In eastern Japan, few natural infections have been confirmed in breeds other than fighting dogs [7]. In this study, *B. gibsoni* was found in a Brittany spaniel, which is not a fighting dog breed, in Kanagawa prefecture. Babesiosis was also previously confirmed in dogs from six areas (Ibaraki, Gunma, Saitama, Tokyo, and Nagano prefectures); these dogs had no history of travel, specially travel to western Japan, and they were not fighting dog breeds [7]. Although it is unknown if the dog in the present study had a history of travel to western Japan, our finding may suggest that dogs other than fighting dogs in eastern Japan could have canine babesiosis.

The M121I variant population was detected in 9 samples (8.57%) and had thereby increased compared with the period of 2011–2014 (4.1%) [6]. ATV was previously an imported drug and difficult to obtain until it was approved in Japan in January 2012 as a preventive/therapeutic drug for human malaria. Subsequently, Malarone® (GlaxoSmithKline, Tokyo, Japan), which is a combination drug of ATV and proguanil was approved as a therapeutic/preventive drug for *Pneumocystis carinii* pneumonia. It might therefore become easier for veterinarians in Japan to use ATV for *B. gibsoni* infection, and that might induce increased frequency of ATV use as well as post-use recurrence. Osaka prefecture was also reported to have an M121I variant population during 2011–2014 [6], whereas, no M121I variant population was observed in this study in Yamaguchi and Hiroshima prefectures, which both had M121I variant populations during 2011–2014. The M121I variant population was detected in several new areas (Hokkaido, Fukushima, Kanagawa, Kagawa, Kochi, and Fukuoka prefectures). It was the first time that multiple positive samples were observed from a prefecture (three cases in Kagawa

Table 2. Summary of dogs positive for M121I variant (Prefecture, Population, Breed, Sex, and Age)

Dog ID	Prefecture	Population of M121I (%)	Breed	Sex	Age
1	Hokkaido	1.66	Tosa	Male	2 y 2 m
2	Fukushima	1.94	Tosa	Male	3 y 0 m
3	Kanagawa	7.99	American Pit Bull Terrier	Unknown	1 y 8 m
4	Osaka	12.01	Mix	Male	8 y 0 m
5	Kagawa	17.08	Mix	Male	10 y 0 m
6	Kagawa	14.75	Mix	Male	12 y 11 m
7	Kagawa	1.20	Unknown	Male	3 y
8	Kochi	21.06	Mix	Male	7 y 5 m
9	Fukuoka	3.02	Mix	Female	13 y 6 m

prefecture). It has been suggested that *B. gibsoni* with M121I may be relatively prevalent in those prefectures where they have been detected consecutively over the two periods and where multiple samples have been detected. Meanwhile, since there were areas, in which the M121I variant population went undetected despite being previously detected and new areas were identified as areas with circulating M121I populations, it is suggested that the area endemic of *B. gibsoni* with M121I may be expanding. Since data on the use of ATV or and on whether the infections observed here were old or new infections was not clear, further research including the background of the samples is required.

Of the population samples positive for M121I variant, three samples may have been from fighting dogs in Hokkaido, Fukushima, and Kanagawa prefectures. Further, it remains unclear whether *B. gibsoni* with M121I can be transmitted in a way similar to the wild-type strain. If these dogs were indeed fighting dogs, they might provide clues as to the mode of transmission of *B. gibsoni* with M121I. There were many males of the population samples positive for M121I variant. Since there were more samples from males than females in this study, there may be an association between the growth and/or transmission of *B. gibsoni* M121I and dog sex.

Conclusively, the positive rate of M121I variant population was increasing, and the area in which it had been recognized was expanding. Measures of prevent and control potentially emerging *B. gibsoni* with M121I are required.

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