NOTE *Parasitology*

Molecular Survey of *Babesia gibsoni* Using *Haemaphysalis longicornis* Collected from Dogs and Cats in Japan

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ABSTRACT. A nationwide survey of *Babesia gibsoni* using *Haemaphysalis longicornis* collected from dogs and cats in Japan was conducted using molecular methods. A total of 1,341 *H. longicornis,* including 305 females, 14 males, 332 nymphs and 690 larvae (153 pools) from 44 prefectures, were examined by *B. gibsoni*-targeted PCR. Partial sequence analysis revealed that 12 of 13 positive samples sequenced, including samples from Tottori, Hiroshima, Yamaguchi, Tokushima, Ehime and Oita prefectures (all in western Japan), were identical to *B. gibsoni*, and 1 sample from Kyoto Prefecture was most closely related to a *Babesia* species recently detected from feral raccoons in Hokkaido. *H. longicornis* is a candidate for transmission vector tick of the new *Babesia* species.

KEY WORDS: *Babesia gibsoni*, distribution, *Haemaphysalis longicornis*

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Babesia infection causes severe hemolytic anemia and sometimes death, in canine hosts [1]. *B. gibsoni* is the major *Babesia* species infecting dogs in mainland Japan [6, 7]. Most clinical cases have been reported in the western part of Japan [6]. Although some studies suggest the possibility of *B. gibsoni* establishment in eastern Japan [6, 7, 10, 11], there have been few studies on the distribution of canine *Babesia* species in Japan that were based on scientific evidence. Thus, the distribution of *B. gibsoni* infection in Japan has not been accurately described.

Dogs and cats are often exposed to a large number of tick species that reflects the distribution of tick vectors in the environment [15, 16]. Blood-sucking vectors can reliably demonstrate the existence of pathogens in a specific area, because they contain both infected host blood and the pathogen itself [15]. Thus, ticks collected from animals provide useful information for epidemiological studies of tick-borne diseases, which can be analyzed using powerful molecular techniques, such as polymerase chain reaction (PCR) and sequence analysis [2, 3, 6, 14, 17]. *H. longicornis,* the predominant tick in dogs and cats [15, 16], is thought to be the main vector of *B. gibsoni* and has a wide geographic distribution in Japan [7, 15, 16]. In this study, *H. longicornis* samples collected from dogs and cats were subjected to molecular analysis to obtain information on *B. gibsoni* distribution in Japan.

Between May and December 2011, a total of 4,237 and 298 ticks were recovered from 1,162 dogs and 136 cats that were under examination or treatment at selected veterinary clinics in Japan. Among these, *H. longicornis* samples were collected from 741 dogs and 52 cats in 44 prefectures (except for Toyama, Nara and Okinawa) in Japan. Each clinic was asked to collect ticks from respective animals during normal clinical examinations. Collected ticks were stored in 70% ethanol and morphologically identified [15]. A total of 1,341 *H. longicornis,* including 305 females, 14 males, 332 nymphs and 690 larvae, were identified and used in this study. Other tick species collected in this study include *H. flava*, *H. hystricis, H. japonica, H. megaspinosa, H. formosensis, H. campanulata, H. ias, Ixodes ovatus*, *I. nipponensis, I. persulcatu*s, *I. granulatus, Rhipicephalus sanguineus* and *Amblyomma testudinarium.* All analyzed female, nymphs and larval ticks were semi- or fully-engorged. Blood sucking status of male ticks was not clearly determined. For adults and nymphs, 1 tick per dog was selected for analysis. When ticks recovered from 1 animal were all in the larval stage, several (2 to 5) larvae were pooled into a tube. A total of 153 larval pools were prepared. Total DNA was extracted from each tick (adult or nymph) or larval pool using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), adjusted in 200 μl of TE buffer and stored at −20°C until further use [7].

This study utilized a *B. gibsoni*-targeted nested PCR based on the 18S rRNA gene. The first PCR was genus specific with primers Babesia-F (5′ GTG AAA CTG CGA ATG GCT CA 3′) and Babesia-R (5′ CCA TGC TGA AGT ATT CAA GAC 3′) in a total reaction volume of 25 μl as previously reported [7]. The second PCR was a 25-μl reaction containing 5 μl of the first amplicon diluted 1:100 in distilled water using primers Gibsoni-172F (5′ CGT TTA TTA GTT CTA AAC CTC C 3′) and Gibsoni-617R (5′ GAC AAG GCA AGT AGC CGA G 3′) that were newly designed for this study based on the 18S rRNA gene sequence of *B. canis* and

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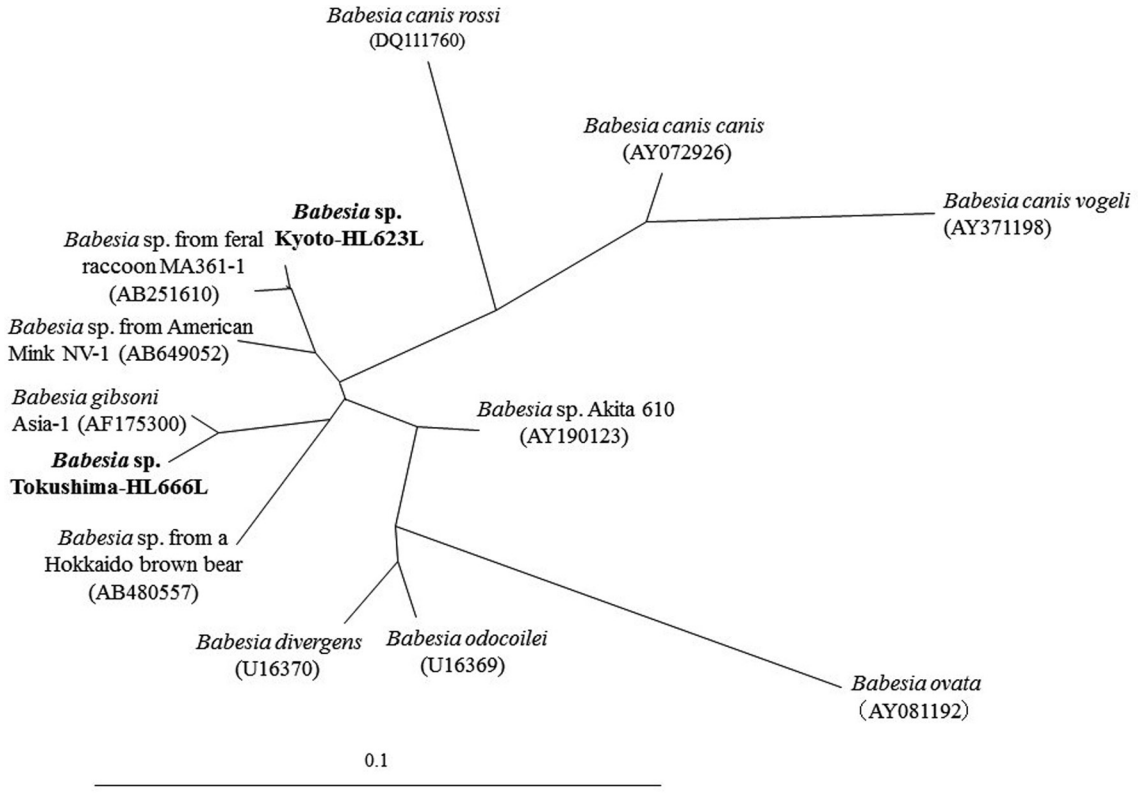

Fig. 1. Phylogenetic relationship of *Babesia* spp. detected from 2 canine ticks (Tokushima-HL666L and Kyoto-HL623L) and the other *Babesia* spp. based on 18S rRNA gene using an unrooted maximum-likelihood method (HKY model). *B. gibsoni* Asia-1 (AF175300), *B. canis rossi* (DQ111760), *B. canis canis* (AY072926), *B. canis vogeli* (AY371198), *B. divergens* (U16370), *B. odocoilei* (U16369), *B. ovate* (AY081192), *Babesia* sp. from a Hokkaido brown bear (AB480557), *Babesia* sp. from feral raccoon MA361-1 (AB251610), *Babesia* sp. from American mink NV-1 (AB649052) and *Babesia* sp. Akita 610 (AY190123) are shown. Scale bar represents 10% divergent.

B. gibsoni. Conditions of the second PCR were as previously described, except the annealing temperature was changed to 58°C. To confirm the PCR results, an approximately 446 bp PCR product from positive reactions was purified using the QIAquick PCR purification kit (QIAGEN), and partial sequence was determined through direct sequencing by Big-Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Bio Science, Carlsbad, CA, U.S.A.).

Sequence data of the PCR products were analyzed for homology with registered sequences using the standard nucleotide BLAST program (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The partial nucleotide sequences of the 18S rRNA gene of the *Babesia* species detected from ticks determined in the present study have been deposited in GenBank under accession numbers from KJ733839 to KJ733851. Phylogenetic analyses were performed by using an unrooted maximum-likelihood method (HKY model) with ClustalW2 (EMBL-EBI, https://www.ebi.ac.uk/Tools/msa/ clustalw2/) and the PhyML version 3.0 program [4] (http://www.atgc-montpellier.fr/ phyml/). The tree result was viewed using TreeView version 1.6.6 [12].

A total of 13 tick samples removed from dogs in seven prefectures showed a single band of the appropriate size in the *B. gibsoni*-targeted PCR. None of the ticks collected from cats gave positive results. Analysis of the partial sequences of the 13 PCR products (excluding the primer region) showed that 12 samples from Tottori, Hiroshima, Yamaguchi, Tokushima, Ehime and Oita prefectures were 100% identical to the registered sequences of *B. gibsoni* isolates from Japan, such as *B. gibsoni* Asia-1 (AF175300) (Fig. 1). These 6 prefectures are all in western Japan, consistent with the distribution of most clinical cases of *B. gibsoni* [6].

The profile of the 12 ticks positive for *B. gibsoni* and their hosts is summarized in Table 1. Concerning about tick stages, 7, 0, 1 and 4 ticks among 305 females, 14 males, 332 nymphs and 153 larval pools showed positive for *B.gibsoni*, respectively. Mann-Whitney U-test analysis (http://aoki2.si.gunma-u.ac.jp/exact/docs/When2Use. html) revealed that this result was not statistically significant (P=0.6452). Eleven of the 12 dogs showed some clinical sign or abnormal condition, including babesiosis, renal failure, heart failure, otitis externa, anorexia and lameness. Furthermore, 3 dogs in Hiroshima, Tokushima and Ehime prefectures were clinically

Table 1. Profile of *B. gibsoni*-positive *H. longicornis* samples from dogs

Tick				Host	
No.	Stage	Numbers	Month	Prefecture	Diagnosis/condition
1	Larva	1	July	Tottori	Clinically healthy
2	Female	1	July	Hiroshima	Babesiosis
3	Female	1	July	Hiroshima	Heart failure
4	Female	1	June	Yamaguchi	Renal failure
5	Female	1	June	Yamaguchi	Pneumonia
6	Female	1	June	Yamaguchi	Lameness
7	Larva	4	June	Yamaguchi	Otitis externa
8	Female	1	June	Tokushima	Tick infestation
9	Nymph	1	Sep.	Tokushima	Babesiosis
10	Larva	5	Sep.	Tokushima	Anorexia
11	Female	1	May	Ehime	Babesiosis
12	Larva	1	Sep.	Oita	Anorexia

diagnosed with babesiosis. As the most of tick samples used in this study were fully- or semi-engorged, they contained peripheral blood of the host animals. A positive PCR result from an engorged tick may reflect infection of either the tick or the dog, which both provide evidence of *B. gibsoni* in the specific region where the tick was collected. The relationship between the positive ticks and the clinical signs of the other nine dogs is not clear.

There were no *B. gibsoni*-positive ticks recovered in eastern Japan (Hokkaido, Aomori, Iwate, Akita, Miyagi, Yamagata, Fukushima, Tochigi, Gunma, Ibaraki, Saitama, Chiba, Tokyo, Kanagawa, Yamanashi, Nagano, Shizuoka, Niigata, Ishikawa, Fukui, Gifu and Aichi), however, this does not exclude the possibility of *B. gibsoni* occurrence in the area, as the results only suggest that the density of *B. gibsoni* in eastern Japan is much lower than in western Japan (Shiga, Mie, Kyoto, Osaka, Wakayama, Hyogo, Tottori, Okayama, Shimane, Hiroshima, Yamaguchi, Kagawa, Tokushima, Kochi, Ehime, Fukuoka, Saga, Nagasaki, Oita, Kumamoto, Miyazaki and Kagoshima). Indeed, some studies suggest the possibility of *B. gibsoni* establishment in eastern prefectures [6, 7, 10, 11]. More tick samples should be examined to evaluate the area-specific distribution of *B. gibsoni*.

Another tick sample (DNA extracted from a pool of 3 larvae) from Kyoto Prefecture was most closely related to *Babesia* sp. detected from feral raccoon (*Procyon lotor*) in Hokkaido (AB251610) with 99.8% identity [7] (Fig. 1). This was also similar to *Babesia* sp. detected from a Hokkaido brown bear (*Ursus arctos* yesoensis) in Japan (AB480557), *Babesia* sp. detected from a wild American mink (*Neovison vison*) in Hokkaido (AB649052) and *B. odocoilei* (U16369) detected from an elk (*Cervus elaphus* cancadensis) in Canada with 99.0, 98.5 and 98.0% identities, respectively [5, 9, 13].

Although a *B. gibsoni*-targeted nested PCR was designed for the present study, sequence analysis of the PCR products revealed that 1 of the 13 positive samples was another *Babesia* species. This result revealed a lack of specificity of the newly designed PCR and also suggests the existence of a *Babesia* sp. closely related to *B. gibsoni* in Japan. This sample was detected from a pool of larvae collected from a dog in Kyoto Prefecture and showed highest homology (99.8% identity) with a *Babesia* species recently detected from feral raccoons in Hokkaido, Japan. The *Babesia* sp. detected from raccoon is thought to be established in the Japanese raccoon population, which is originally native to North America [8]. The positive dog was presented to the local veterinarian with an external injury without any signs of *Babesia* infection, such as fever or anemia. The pathogenicity of the new *Babesia* sp. to human and animals is still unknown. Although it is difficult to determine whether the present positive result came from the tick itself or the canine blood, *H. longicornis* is a possible vector of the new *Babesia* species. More epidemiological studies are needed to clarify the vectors and the distribution of this new *Babesia* in Japan.

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