**NOTE** *Wildlife Science*

# Epidemiological Survey of Tick-Borne Protozoal Infection in Iriomote Cats and Tsushima Leopard Cats in Japan

Morihiro TATENO[1)], Takuma NISHIO[1)], Tomohide MATSUO[2)], Masato SAKUMA[1)], Nozomi NAKANISHI[3)], Masako IZAWA[3)], Yumiko ASARI[4)], Maki OKAMURA[4)], Takako SHIMOKAWA MIYAMA[5)], 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)]*Laboratory of Parasitology, Joint Faculty of Veterinary Medicine, Kagoshima University, 1–21–24 Korimoto, Kagoshima, Kagoshima 890–0065, Japan*
[3)]*Laboratory of Ecology and Systematics, Faculty of Science, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903–0213, Japan*
[4)]*Iriomote Wildlife Conservation Center, Ministry of the Environment, Komi, Taketomi, Yaeyama-gun, Okinawa 907–1432, Japan*
[5)]*Veterinary Teaching Hospital, Kagoshima University, 1–21–24 Korimoto, Kagoshima, Kagoshima 890–0065, Japan*

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ABSTRACT. This epidemiological survey was conducted to determine the prevalence of *Hepatozoon*, *Babesia* and *Theileria* infection in the Iriomote cat (IC) and the Tsushima leopard cat (TLC). Blood samples from 43 ICs and 14 TLCs were collected between November 2002 and January 2012. Polymerase chain reaction and DNA sequencing analyses detected a *Hepatozoon felis* infection prevalence of 72.0% (31/43 cats) and 100% (14/14 cats) in ICs and TLCs, respectively. The degree of *Hepatozoon* parasitemia observed on blood smears ranged from 0.1 to 4.7%. However, no cases had obvious clinical signs of hepatozoonosis. Neither *Babesia*- nor *Theileria*-infected wildcats were detected in this study.

KEY WORDS: *Babesia*, *Hepatozoon*, Iriomote cat, *Theileria*, Tsushima leopard cat.

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The Iriomote cat (IC), *Prionailurus bengalensis iriomotensis*, and the Tsushima leopard cat (TLC), *Prionailurus bengalensis euptilura*, are the only two subspecies of leopard cats in Japan [15]. The current populations of each are estimated to be approximately 100 animals; therefore, their conservation is a great concern [13]. One of the most important factors threatening their survival is the expansion of human activities in wildcat habitats. Infectious diseases may be another potential factor limiting these wildcat populations [9, 11, 17, 18]. Ectoparasites, such as ticks and lice, are potential disease vectors and have frequently been observed in ICs and TLCs. Thus, both ICs and TLCs are at risk of infection with arthropod-borne bacterial, protozoan and rickettsial diseases. In this study, we conducted a molecular epidemiological survey to determine the prevalence of the tick-borne protozoan pathogens *Hepatozoon* spp., *Babesia* spp. and *Theileria* spp. in these two endangered Japanese wildcat species as a part of ongoing conservation activities.

Capture and sample collection from both ICs and TLCs were done with permission from the Ministry of Environment and the Agency for Cultural Affairs in Japan. Capture procedures were conducted in cooperation with the Iriomote Wildlife Conservation Center and the Tsushima Wildlife Conservation Center of the Ministry of Environment. A series of ecological surveys were performed between August 2002 and January 2012. During this period, 43 ICs and 14 TLCs were either captured or found dead. Their degree of maturation was estimated from their physical appearance and dental growth. Almost all of the Japanese wildcats examined over the study period were infested with ticks. At least three tick species (larvae [L] of *Amblyomma testudinarium*, nymphs [N] of *Haemaphysalis longicornis* and L and N of *H. hystricis*) were found on the ICs, and four tick species (N of *A. testudinarium*, adults [Ad] of *I. tanuki*, Ad and L of *H. megaspinosa* and Ad and N of *H. campanulata*) were found on the TLCs. A total of 61 blood samples were collected from ICs and 29 from TLCs (Tables 1 and 2). Ten ICs and seven TLCs were trapped on multiple occasions, and thus, several blood samples were collected from the same cat. Blood samples were anti-coagulated with EDTA, and complete blood counts were performed using both an automated calculator (pocH-100iV, Sysmex, Kobe, Japan) and microscopic examination of Wright-Giemsa stained blood smears. The degree of parasitemia was estimated based on examination of 1,000 neutrophils and/or erythrocytes. The remaining blood samples were stored at −80°C until PCR analysis.

Total DNA was extracted from 200 $\mu l$ of each blood sample using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) with a final elution volume of 200 $\mu l$. As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was amplified in each sample of extracted DNA as described elsewhere [11, 26]. If the G3PDH gene

*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. Profile of examined ICs and results from PCR and blood smear analysis

| Cat ID | Gender[a)] | Age[b)] | Sampling year | Alive/ Dead[c)] | PCR (*Hepatozoon*) | Parasitemia (%) (*H. felis*)[d)] |
|---|---|---|---|---|---|---|
| E-61 | F | SA | 2003 | D | (−) | N/T |
| E-83 | F | A | 2008 | AL | (+) | N/T |
| | | | 2011 | AL | (+) | 0.0 |
| W-87 | F | A | 2007 | AL | (+) | 0.0 |
| W-119 | F | J | 2006 | CRD | (−) | N/T |
| W-127 | F | A | 2010 | AL | (+) | 0.0 |
| | | | 2011 | AL | (+) | 0.0 |
| | | | 2011 | AL | (+) | 0.3 |
| W-130 | F | A | 2011 | AL | (+) | 4.7 |
| W-134 | F | A | 2010 | AL | (+) | 0.0 |
| | | | 2010 | AL | (+) | 0.1 |
| | | | 2011 | CRD | (+) | N/T |
| W-135 | F | A | 2010 | CRD | (+) | N/T |
| W-137 | F | J | 2010 | CRD | (−) | N/T |
| W-148 | F | A | 2011 | AL | (−) | N/T |
| E-30 | M | A | 2003 | AL | (+) | N/T |
| E-33 | M | A | 2004 | AL | (+) | N/T |
| E-60 | M | A | 2004 | AL | (+) | N/T |
| | | | 2005 | AL | (+) | 0.1 |
| | | | 2007 | AL | (+) | 0.5 |
| | | | 2010 | DY>D | (+) | N/T |
| E-67 | M | A | 2005 | AL | (+) | N/T |
| | | | 2006 | AL | (+) | N/T |
| | | | 2006 | AL | (+) | N/T |
| | | | 2008 | AL | (+) | 0.0 |
| E-70 | M | A | 2006 | AL | (+) | 0.1 |
| E-71 | M | A | 2006 | AL | (+) | 0.0 |
| E-72 | M | A | 2006 | AL | (+) | 4.3 |
| E-82 | M | A | 2012 | AL | (+) | 0.5 |
| E-84 | M | J | 2008 | D | (−) | N/T |
| E-89 | M | J | 2010 | CRD | (−) | N/T |
| E-91 | M | SA | 2010 | CRDY | (+) | N/T |
| | | | 2010 | D | (+) | N/T |
| E-92 | M | J | 2010 | AL | (−) | N/T |
| E-98 | M | A | 2011 | AL | (+) | 0.0 |
| E-100 | M | A | 2012 | AL | (+) | 0.0 |
| E-102 | M | SA | 2012 | AL | (−) | N/T |
| W-48 | M | A | 2006 | AL | (−) | N/T |
| | | | 2006 | AL | (−) | N/T |
| W-99 | M | A | 2008 | CRD | (+) | N/T |
| W-101 | M | A | 2007 | AL | (+) | 1.3 |
| W-106 | M | A | 2005 | AL | (+) | 1.9 |
| | | | 2007 | AL | (+) | 0.3 |
| W-108 | M | A | 2007 | AL | (+) | 1.3 |
| W-113 | M | A | 2006 | AL | (+) | 0.1 |
| W-118 | M | A | 2006 | AL | (+) | 1.4 |
| W-120 | M | A | 2006 | AL | (+) | 0.3 |
| W-121 | M | A | 2006 | AL | (−) | N/T |
| W-126 | M | A | 2011 | AL | (+) | 0.5 |
| W-129 | M | A | 2010 | AL | (+) | 0.7 |
| | | | 2010 | AL | (+) | 0.8 |
| | | | 2011 | AL | (+) | 3.1 |
| | | | 2011 | AL | (+) | 1.2 |
| W-131 | M | J | 2009 | CRD | (−) | N/T |
| W-140 | M | A | 2011 | AL | (+) | 0.0 |
| | | | 2011 | AL | (+) | 0.2 |
| W-143 | M | A | 2011 | AL | (+) | 0.1 |
| W-145 | M | A | 2012 | AL | (+) | 0.2 |
| W-146 | M | A | 2011 | AL | (+) | 0.0 |
| W-149 | M | A | 2011 | AL | (+) | 1.5 |
| D-043 | M | J | 2009 | CRD | (−) | N/T |

a) F, female; M, male.
b) A, adult; SA, subadult; J, juvenile.
c) AL, alive; DY, dying; CRDY, car-related dying; CRD, car-related death; D, dead.
d) N/T, not tested.

was successfully amplified, the samples were then subjected to a screening PCR analysis for pathogens.

As a screening PCR, nested PCR was performed to amplify a partial 18S rRNA sequence derived from *Hepatozoon*, *Babesia* and/or *Theileria* species using the primers F1 (5′-AGT CAT ATG CTT GTC TTA-3′) and R1 (5′-CCA TCA TTC CAA TTA CAA-3′) for the first round, and then F2 (5′-GAA ACT GCG AAT GGC TCA TTA-3′) and R2 (5′-CGG TAG GCC AAT ACC CTA CCG TC-3′) [3, 6]. These primer sets are universal and recognize 18S rRNA genes from *Hepatozoon* spp., *Babesia* spp. and *Theileria* spp. The three different pathogens were differentiated from each other based on the size of the amplified DNA fragments (*Hepatozoon* spp., 267 bp; *Babesia* spp., 230 bp; *Theileria* spp., 242 bp). The sensitivity of the screening PCR was determined for each pathogen as reported previously [11, 26], and as little as one copy of DNA in the reaction mixture was successfully detected (data not shown).

The G3PDH gene was successfully amplified in all samples; thus, all samples underwent screening PCR. In the screening PCR, relevant bands of DNA were seen after amplification in 48 samples from 31 ICs and 28 samples from 14 TLCs (data not shown). Since all of the amplified DNA fragments were 267 bp long, we concluded that they were derived from *Hepatozoon* spp. Most of the adult cats were PCR-positive (Tables 1 and 2). Negative PCR results were mainly observed in immature cats, including ICs E-61, E-84, E-89, E-92, E-102, W-119, W131, W-137 and D-043, and TLC CMT-33. CMT-33 had been negative in 2010; however, he was PCR positive at the time of recapture in 2011 (Table 2).

The seventy-six samples that were positive on initial screening PCR were then analyzed by nested PCR with the primer sets LS1 (5′-GGT TGA TCC TGC CAG TAG T-3′) and LR1 (5′-GAC TTC TCC TTC TTT AAG TGA TAA G-3′) for the first round, and LS2 (5′-ATA CAT GAG CAA AAT CTC AAC-3′) and LR2 (5′-TCT TCG ATC CCC TAA CTT TC-3′) for the second round. This enabled us to analyze

Table 2. Profile of examined TLCs and results from PCR and blood smear analysis

| Cat ID | Gender[a)] | Age[b)] | Sampling year | Alive/ Dead[c)] | PCR (*Hepatozoon*) | Parasitemia (%) (*H. felis*)[d)] |
|---|---|---|---|---|---|---|
| CFM-20 | F | A | 2006 | AL | (+) | N/T |
| CFS-18 | F | A | 2002 | AL | (+) | 0.0 |
| CFS-26 | F | A | 2006 | AL | (+) | 0.0 |
| CFT-17 | F | A | 2002 | AL | (+) | N/T |
| CFT-24 | F | A | 2008 | AL | (+) | 0.0 |
| | | | 2009 | AL | (+) | N/T |
| | | | 2010 | AL | (+) | N/T |
| | | | 2011 | AL | (+) | N/T |
| CFT-25 | F | A | 2006 | AL | (+) | 0.0 |
| | | | 2009 | AL | (+) | N/T |
| | | | 2011 | AL | (+) | N/T |
| CFT-27 | F | A | 2008 | AL | (+) | 0.0 |
| | | | 2009 | AL | (+) | N/T |
| | | | 2010 | AL | (+) | N/T |
| | | | 2011 | AL | (+) | N/T |
| CFT-28 | F | A | 2009 | AL | (+) | N/T |
| | | | 2011 | AL | (+) | N/T |
| CMM-19 | M | A | 2002 | AL | (+) | N/T |
| | | | 2005 | AL | (+) | 0.0 |
| | | | 2006 | AL | (+) | 0.0 |
| CMS-29 | M | A | 2008 | AL | (+) | 0.0 |
| | | | 2009 | AL | (+) | N/T |
| | | | 2010 | AL | (+) | N/T |
| | | | 2011 | AL | (+) | N/T |
| CMS-32 | M | SA | 2009 | AL | (+) | N/T |
| CMS-34 | M | A | 2011 | AL | (+) | N/T |
| CMT-33 | M | SA | 2010 | AL | (−) | N/T |
| | | A | 2011 | AL | (+) | N/T |
| MM-22 | M | A | 2008 | AL | (+) | 0.0 |

a) F, female; M, male.
b) A, adult; SA, subadult.
c) AL, alive.
d) N/T, not tested.

most of the 18S rRNA gene (809 bp) [1, 7, 8, 12]. Successful amplifications were obtained from all tested samples, and the nucleotide sequences of the amplicons were determined (GenBank/EMBL/DDBJ Accession number AB771501 to AB771577). BLAST analysis revealed that all of the DNA amplicons were highly similar (99.9%) to the 18S rRNA gene of *Hepatozoon felis*(*H. felis*) originally isolated from a wild Indian leopard (*Panthera pardus fusca*) in Asia (HQ829443) [19]. The prevalence of *H. felis* in this study was 72.0% (31/43 cats) and 100% (14/14 cats) in ICs and TLCs, respectively. $\chi^2$ test revealed no significant difference in *H. felis* prevalence between genders in ICs (95% confidence interval of odds ratio, 0.09–1.84; $P$=0.233). The odds ratio for TLCs was not calculated, because of the 100% infection rate in both genders. The *H. felis* prevalence in juvenile, subadult and adult ICs was 0% (0/7 cats), 33.3% (1/3 cats) and 90.9% (30/33 cats), respectively (Table 1). All adult TLCs were infected with *H. felis,* but its prevalence in subadult TLCs was 50% (1/2 cats) (Table 2). Neither *Babesia* nor *Theileria*-derived genes were detected in ICs and TLCs in this survey.

Thirty-five blood smears from 26 ICs and 9 smears from 8 TLCs were available (Tables 1 and 2). Although all of the 34 wildcats were PCR positive for *H. felis*, cytoplasmic gamonts of *H. felis* in neutrophils were microscopically detected in only 24 out of the 35 samples from ICs (68.6%), and none of the examined TLCs showed microscopic evidence of *H. felis* parasitemia. The degree of parasitemia in the 24 samples containing visible cytoplasmic gamonts ranged from 0.1% to 4.7% (Table 1). No piroplasms were detected in erythrocytes in any cases.

This epidemiological survey investigated the tick-borne diseases, hepatozoonosis, babesiosis and theileriosis in Japanese wildcat populations. Our results demonstrate that ICs and TLCs are infected with *H. felis*, but not with *Babesia* or *Theileria*. Previous studies have shown a prevalence of *Hepatozoon* infection ranging from 4 to 100% in Brazilian wild felids (ocelot, little spotted cat, margay and jaguarondi), Indian wild felids (Asiatic lion, Indian tiger and Indian leopard) and African lions and cheetahs [2, 4, 16, 19]. The prevalence of *H. felis* in ICs and TLCs was 72.0% and 100%, respectively, which are higher rates of infection than those reported in other wildcat species in previous studies [2, 19]. This might be due to the different environment found on Iriomote island and Tsushima island compared with the habitat of other wildcat species. Evaluation of the prevalence of *H. felis* in ticks inhabiting both islands will further our understanding of why Japanese wildcats have such a high prevalence of infection with this pathogen. In addition, the reason underlying the high *H. felis* prevalence in both wildcat species might be clarified by comparing the tick infestation rate with the rate of infection with *H. felis* in each wildcat species and in the domestic cats inhabiting each island.

Our results suggest that *H. felis* infection likely occurred during the wildcats' adult life. Negative PCR results occurred mainly in young and juvenile cats, and one subadult TLC converted from non-infected to infected over the study period. It may be that these immature cats have less frequent opportunities for infestation and ingestion of ticks than adult cats, resulting in their lower prevalence of *H. felis*. This also suggests that *H. felis* is primarily transmitted via ticks and not through vertical transmission.

The majority of the wildcats in our survey tested positive for *H. felis*; however, we did not find any physical or hematologic abnormalities in *H. felis*-infected animals, except for the cats that were found dying or dead (mostly car-related deaths). *H. felis* infection in domestic cats rarely causes disease in the infected host, and a previous case report also suggests that *H. felis* is not very virulent in ICs [22, 25]. Previous histopathological analysis also suggests a low pathogenicity of *H. felis* in Japanese wildcats [14]. However, *H. felis*-infected domestic cats with parasitemia have been reported to have elevated serum levels of skeletal muscle-derived enzymes, such as creatine kinase and lactate dehydrogenase, which indicates damage to muscular tissue [5, 20]. Therefore, detailed evaluation of serum biomarkers of skeletal muscle might provide further information about the health status of *H. felis*-infected ICs and TLCs.

In this study, *H. felis* parasitemia was only visible in blood smears from ICs. The reason for this could not be determined from the small number of animals in our study. A previous histopathological study has shown that *Hepatozoon* spp. can be detected in multiple tissues in ICs, including the heart, tongue, masseter muscle and diaphragm, whereas they are only detected in the heart in TLCs [14]. Both wildcats inhabit islands, but the islands are geographically and climatically separated. Therefore, it is plausible that the virulence and/or tissue tropism of *H. felis* is subtly different between islands. In addition, there may be an influence of infectious diseases other than *H. felis* on host susceptibility. Acute onset of hepatozoonosis in domestic dogs is often observed in cases of co-infection with organisms, such as *Babesia*, internal parasites, *Dirofilaria* and others [10, 21, 23]. Since little is currently known about infectious diseases in Japanese wildcats, a wide range of potential pathogens should be evaluated to determine their contribution to the development of *Hepatozoon* parasitemia in these animals.

We detected neither *Babesia* nor *Theileria* infection in Japanese wildcats. Susceptibility to these pathogens is widespread among other felids [24, 27], and it is unlikely that Japanese wildcats are not susceptible to these pathogens. We consider it more likely that these pathogens, which have a tropism to felids, have not been introduced into the island habitats of these Japanese wildcats.

In conclusion, this molecular epidemiological study of two subspecies of leopard cat in Japan detected *H. felis* infection in most of the mature wildcats tested. In spite of the high prevalence of infection, no obvious pathogenicity of *H. felis* in Japanese wildcats was observed in this study; however, continued surveillance for infectious organisms, including *Hepatozoon*, is necessary for the conservation of this endangered species.

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