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Rickettsia sp. in *Ixodes granulatus* Ticks, Japan

To the Editor: The genus *Rickettsia* consists of obligate intracellular bacteria that cause spotted fever and typhus fever; these bacteria are usually transmitted by an arthropod vector. We report isolation of a *Rickettsia honei*–like organism from the *Ixodes granulatus* tick; this organism may be a causative agent of rickettsiosis in Japan. Serotyping and DNA-sequencing analysis distinguished this *I. granulatus* isolate from previously reported *Rickettsia* spp.

During 2004–2005, an investigation of rickettsiosis was conducted in Okinawa Prefecture in the southernmost part of Japan, an area known to be inhabited by *I. granulatus*, a parasitic tick commonly found on small mammals. A total of 43 *I. granulatus* ticks (3 larvae, 27 nymphs, 8 adult females, and 5 adult males) were collected from small mammals (*Rattus rattus*, *R. norvegicus*, *Suncus murinus*, *Mus calori*, and *Crocidura watasei*) for the present study. For the isolation of *Rickettsia* spp., the cell line L929 was used as previously described (*1*). A total of 13 isolates, designated as strains GRA-1 to GRA-13, were obtained from 11 ticks (1 fed larva, 5 fed nymph, 1 fed adult female, 1 fed adult male, 1 unfed nymph molted from engorged larva, 2 unfed adult females molted from engorged nymphs) and from 1 pool of eggs and 1 larva derived from the engorged female tick.

Serotyping was performed by using a microimmunoperoxidase approach according to the method described by Philip et al. (*2*); we used anti-*Rickettsia* mouse serum and several spotted fever group *Rickettsia* antigens: 2 of the present isolates (GRA-1 and GRA-2) and 6 known members of the Asian *Rickettsia* spp. (*R. honei*, *R. japonica*, *R. asiatica*, *R. tamurae*, *R. sibirica*, and *R. conorii*). Differences among antigen reaction titers were calculated, and the results are given as the specificity difference (SPD) value. The SPD value between the present isolates and *R. honei* was 0 or 1, whereas the SPD values were $\geq$3 for the other spotted fever group *Rickettsia* spp. (Table). According to the criteria for serotyping (*2*), we assumed the isolates to be of the same serotype when the SPD value was $\leq$2. In addition to serotyping, a sequencing analysis was performed to genetically characterize the isolates. The archive of DNA sequences has been mostly established for the outer membrane protein A gene (*ompA*), citrate synthesis gene (*gltA*), and 17-kDa antigen gene. Thus, we determined these DNA sequences in the isolates and compared the results with those of representative *Rickettsia* spp. The *ompA* sequencing analysis showed a DNA sequence of 491 bp in the 6 isolates from *I. granulatus* (GenBank accession nos. AB444090–AB44095), which yielded the following similarity values: *R. slovaca* (98.0%), *R. honei* and Thai tick typhus *Rickettsia* (97.8%), and *R. honei* subsp. *marmionii* (97.6%). Sequencing of the 1,250-bp fragment of *gltA* of the strain GRA-1 (accession no. AB444098) showed >99% DNA similarity with that of *R. sibirica* (99.3%), *R. slovaca* (99.2%), *R. conorii* (99.2%), *R. honei* (99.1%), and certain types of *Rickettsia* spp. Moreover,17-kDa antigen gene sequencing analysis of a 392-bp fragment of the strain GRA-1 (accession no. AB444097) showed the highest levels of sequencing similar-

Table. Serotype results for *Rickettsia* sp. strains GRA-1 and GRA-2 from *Ixodes granulatus* tick, Okinawa Prefecture, Japan

Mouse antiserum to	Results*							
	GRA-1	GRA-2	TT-118	Aoki	IO-1	AT-1	246	Moroccan
Strains from this study								
Rickettsia sp., GRA-1	**320**†	**320** (0)‡	160 (1)	80 (3)	40 (6)	40 (6)	80 (4)	80 (3)
Rickettsia sp., GRA-2	**320** (0)	**320**	**320** (0)	40 (4)	20 (7)	40 (7)	40 (5)	40 (5)
Reference strains								
R. honei, TT-118	**5,120** (1)	**5,120** (0)	**5,120**	80 (7)	320 (7)	80 (9)	160 (7)	80 (7)
R. japonica, Aoki	2,560 (3)	2,560 (4)	1,280 (7)	**5,120**	320 (9)	320 (10)	320 (9)	32 0 (8)
R. asiatica, IO-1	640 (6)	640 (7)	80 (7)	160 (9)	**5,120**	80 (12)	80 (11)	160 (11)
R. tamurae, AT-1	640 (6)	320 (7)	80 (9)	80 (10)	80 (12)	**5,120**	160 (11)	40 (13)
R. sibirica, 246	1,280 (4)	1,280 (5)	320 (7)	160 (9)	160 (11)	80 (11)	**5,120**	320 (7)
R. conorii, Moroccan	640 (3)	640 (5)	80 (7)	80 (8)	20 (11)	20 (13)	160 (7)	**1,280**

*Highest serum dilutions against each *Rickettsia* antigen (specificity difference between each pair of strains), determined by microimmunoperoxidase method. **Boldface** indicates equivocal titer to homologous antigen.
†Highest serum dilution showing a positive reaction with antigen.
‡Specificity difference between each pair of strains.

ity value with *R. honei* and Thai tick typhus *Rickettsia* (99.5%) compared with those of the sequences of other deposited *Rickettsia* spp. Comprehensive analyses led us to presume that the isolate GRA-1 from *I. granulatus* was a genetic variant of *R. honei*, although further studies are necessary to better define the taxonomic position of our isolates.

The vector for *R. honei* was presumed to be ixodid ticks: *I. granulatus* in Thailand; *Amblyomma cajennense* in Texas, USA; and *Aponomma hydrosauri* in Australia (*3–5*). Lane et al. reported that a *Rickettsia* organism from a *Haemaphysalis* tick was closely related to *R. honei* in Australia (*6*). In the present study, we observed that the *Rickettsia* organism was maintained in the tick after molting. Moreover, *Rickettsia* organisms were also isolated from egg and unfed larva. These preliminary findings may suggest that *I. granulatus* is a possible vector for the *R. honei*–like bacterium in Japan.

Recently, a *Rickettsia* sp. was found in *I. granulatus* ticks; its proposed designation was unclassified *Rickettsia* IG-1, according to DNA sequencing from specimens obtained in Taiwan (*7*). With respect to the DNA sequences of *gltA* and *ompA*, our isolates from *I. granulatus* were identical to the *Rickettsia* IG-1.

R. honei, a member of the spotted fever group *Rickettsia,* has been reported as the etiologic agent of Flinders Island spotted fever in Australia (*8*) and also of Thai tick typhus (*3*). *R. honei* is a public health threat for rickettsiosis in these countries. Although the human health implications of the *Rickettsia* sp. found in this study are not yet known, knowledge from this study will be useful in epidemiologic investigation for rickettsiosis in Japan.

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Sin Nombre Virus Infection in Deer Mice, Channel Islands, California

To the Editor: Sin Nombre virus (SNV) is a highly virulent strain of hantavirus associated with rodent hosts in North America (*1,2*). Documenting the prevalence of SNV in wild rodent populations is an important component of determining risk for exposure and ultimately providing sound recommendations for epidemiologic management (*3*). Prevalence of SNV is highly variable. Deer mice (*Peromyscus maniculatus*) that inhabit the Channel Islands off the California coast often have rates of SNV that greatly exceed values on the mainland (*2*). Even though these islands have high rates of SNV prevalence and are recreational areas for humans, no surveys of the Channel Islands have been performed to document the dynamics of prevalence since 1994–1996 (*2,4*). We visited 4 of the Channel Islands in 2007 to document rates of SNV prevalence in *P. maniculatus*.

From May 3–15, 2007, we visited 4 of the Channel Islands off the California coast: East Anacapa Island, Santa Barbara Island, San Miguel Island, and Santa Rosa Island. On each island, mice were captured by using Sherman live traps from habitats characterized by giant coreopsis (*Coreopsis gigantea*), a shrub native to California, to provide a standardized habitat for comparisons across islands. The number of sampling areas depended largely upon the distribution of *C. gigantea* habitat and logistical considerations during each island visit (Table). Upon capture of the mice, blood samples were taken from the submandibular vein by using Medi-Point animal lancets (Medi-Point International, Inc., Mineola, NY, USA) and stored in sterile micropipette tubes. Samples were stored on ice until shipment to the California Department of Health Services' Viral and Rickettsial Disease Laboratory for processing. *P. maniculatus* serum samples were examined for immunoglobulin (Ig) G antibodies to the SNV nucleocapsid protein by ELISA with Centers for Disease Control and Prevention reagents (*5*).

Detailed information regarding SNV prevalence, sampling location, and sampling effort is presented in the Table. We compare our 2007 data with data collected in 1994 by Jay et al. (*2*) because 1994 was the only other year when all 4 islands used in our study were sampled. Graham and Chomel (*4*) also collected data from San Miguel Island and Santa Rosa Island in 1995 and 1996 (the use of the average prevalence from 1995 and 1996 for these 2 islands does not change any of our results).

There was no significant difference in prevalence of SNV antibodies between our 2007 results and the prevalence found by Jay et al. (*2*) in 1993–1994 (paired t test $t = 0.13, 3$; df = 3; p = 0.91). Overall, 36 male and 42 female mice were captured; the sex of captured animals was independent of SNV infection (9 males and 6 females positive for SNV; test of independence $\chi^2 = 0.28$, 1 df, p = 0.59). We captured only 2 subadult mice on islands where we also detected antibodies to SNV; 1 mouse tested positive, the other tested negative. Although our sample sizes precluded detecting very low rates of SNV infection with confidence on Santa Barbara and East Anacapa Islands, the consistency of our results with those of Jay et al. (*2*) suggests that our sampling was sufficient for comparative purposes.

Several studies now indicate the importance of long-term surveillance of SNV prevalence in wild rodent populations for understanding the factors that may contribute to outbreaks of human disease, e.g. (*6*). These studies often document the generally positive, though often temporally delayed, relationship between population density of *P. maniculatus* and seroprevalence for SNV (*7*). Our results suggest a high degree of temporal stability in prevalence of antibodies to SNV in *P. maniculatus* on the Channel Islands, despite considerable variation in host population density between earlier studies and ours (*4,8*). Although we cannot know the prevalence of SNV among *P. maniculatus* on the Channel Islands in periods between the studies by Jay et al. (*2*), Graham and Chomel (*4*), and our own, SNV prevalence on these islands is quite similar to levels previously recorded both for islands with relatively low prevalence

Table. Sin Nombre virus in *Peromyscus maniculatus* mice on 4 Channel Islands, California, May 3–15, 2007*

Island*	No. trap nights	Prevalence, %	
		2007	1994
East Anacapa†	180	0	0
San Miguel‡	104	26.3	17.9
Santa Barbara§	216	0	0
Santa Rosa¶	216	47.6	58

*The number of captured mice that were sampled for Sin Nombre virus (SNV) was 23 on East Anacapa, 19 on San Miguel Island, 15 on Santa Barbara Island, and 21 on Santa Rosa Island. The 1994 data in the table are from a study by Jay et al. (2) and are included for comparison puposes.
†East Anacapa: 34°00'56"N/119°21'49"W.
‡San Miguel: 34°02'18"N/120°20'54"W.
§Santa Barbara: 33°28'30"N/119°02'12"W.
¶Santa Rosa: 34°00'03"N/120°03'30"W.

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Hepatozoon ursi n. sp. (Apicomplexa: Hepatozoidae) in Japanese black bear (*Ursus thibetanus japonicus*)

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Abstract

Morphological and genetic features of a new *Hepatozoon* species, *Hepatozoon ursi* n. sp., in Japanese black bear (*Ursus thibetanus japonicus*) were studied. Schizogonic developmental stages were observed in the lungs of Japanese black bears. The schizonts were sub-spherical in shape and 45.7±4.6×42.7±4.5 μm in size. Each mature schizont contained approximately 80–130 merozoites and 0–5 residual bodies. The merozoites were 7.0±0.7×1.8±0.3 μm in size. Intraleukocytic gametocytes were slightly curved, cigar-like in shape and had a beak-like protrusion at one end. The size of the gametocytes was 10.9±0.3×3.3±0.2 μm. The analyses of the18S rRNA gene sequences supported the hypothesis that *H. ursi* n. sp. is different from other *Hepatozoon* species. Mature *Hepatozoon* oocysts were detected in two species of ticks (*Haemaphysalis japonica* and *Haemaphysalis flava*) collected on the bears infected with *H. ursi* n. sp. Two measured oocysts were 263.2×234.0 μm and 331.8×231.7 μm, respectively. The oocysts contained approximately 40 and 50 sporocysts, respectively. The sporocysts were sub-spherical in shape and 31.2±2.5×27.0±2.9 μm in size. Each sporocyst contained at least 8–16 sporozoites, with the sporozoites being 12.2±1.4×3.5±0.5 μm in size. *H. ursi* n. sp. is the first *Hepatozoon* species recorded from the family Ursidae.

Keywords: *Hepatozoon ursi* n. sp.; Japanese black bear (*Ursus thibetanus japonicus*); Lung

1. Introduction

Hepatozoon, a genus belonging to the phylum Apicomplexa, is one of the parasitic protozoa that infect various species of domestic and wild animals [1]. Vertebrates, intermediate hosts of *Hepatozoon*, become infected with this protozoal parasite through ingestion of hematophagous arthropods, such as ticks, which are its definitive hosts [1]. Schizogonic development occurs in various organs of the intermediate hosts, with the ultimate invasion of blood cells, commonly leukocytes in mammals, by merozoites which then become gametocytes [1]. In mammals, most *Hepatozoon* species have been recorded from rodents and carnivores [1].

There has been only one report on *Hepatozoon* infection in the family Ursidae [2]. Uni et al. reported the histopathological features of hepatozoonosis in Japanese black bear (*Ursus thibetanus japonicus*) from central Japan [2]. All of the 18 bears examined, collected in Fukui, Shiga and Gifu Prefectures, were

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Fig. 1. A map of Gifu Prefecture. This map indicates the origin of the examined bears. Bar: 50 km.

infected with *Hepatozoon* sp., with only the lungs being affected [2]. The mean sizes of schizonts and merozoites were 52.9×40.1 μm and 4.9×2.0 μm, respectively [2]. Infiltrations of eosinophils and neutrophils were not obvious around immature and mature schizonts of normal appearance; however, such infiltrations were found around collapsed schizonts [2]. Nodules consisting of macrophages were also observed; each macrophage included a zoite [2]. A test aimed at determining the definitive host of the *Hepatozoon* sp. ended in failure, and no blood smear examination was performed [2].

Although the host specificities of *Hepatozoon* spp. have not been clear yet, the *Hepatozoon* sp. in Japanese black bears seemed to be different from other recorded species based on some criteria such as a difference in pathogenicity [2]. In the present report, we describe detailed information, including genetic analyses and hematological studies, pertaining to *Hepatozoon* species in Japanese black bear and propose the name *Hepatozoon ursi* n. sp. Additionally, we describe the suspected definitive hosts of this new *Hepatozoon* species.

2. Materials and methods

2.1. Animals

Thirty-five Japanese black bears were killed under a pest animal control program in Gifu Prefecture (Fig. 1), between July and October 2006. Seven of the bears (T1–T7) were from Takayama, six (Ib1–Ib6) from Ibigawa, five (N1–N5) from Neo, Motosu, five (E1–E5) from Ena, four (Gu1–Gu4) from Gujo, four (Se1–Se4) from Sekigahara, three (Ge1–Ge3) from Gero and one (It1) from Itadori, Seki. Twenty-two bears were male and 13 were female. Twenty-seven bears were young adult to adult and eight were juvenile (born in the last winter). Blood samples were obtained from the hearts as soon after death as possible. The lungs were collected at necropsy.

Nine Japanese black bears (Sh1–Sh9) were captured in Shirakawa, Gifu (Fig. 1), between June and August 2006, for scientific research which aimed to study the ecology and physiology of Japanese black bears. Six bears were male and three were female. All of them were young adult to adult. These bears were captured using barrel-type traps and were immobilized with tiletamine-zolazepam mixture (Zoletil®, Virbac, Carros, France; 3 mg/kg of the estimated body weight) and medetomidine (Domitor®, Meiji Seika Kaisha Ltd., Tokyo, Japan; 40 μg/kg) by means of intramuscular administration using a dart-blowgun. Extra doses were administered as necessary. Peripheral blood samples were obtained from the jugular veins. After being weighed and having their physical condition monitored, the bears were prescribed atipamezole (Antisedan®, Meiji; 5 times of the final dose of medetomidine) and were released at the site of capture.

2.2. Histopathological examinations of the lungs

The lungs were fixed in 10% neutral-buffered formalin solution, dehydrated, embedded in paraffin and sectioned. Sections were deparaffinized and stained with hematoxylin and eosin (H&E). The sizes of schizonts and merozoites were measured on a computer display using Photoshop® 6.0 (Adobe Systems Inc., San Jose, CA, USA) and were given as the mean±standard deviation.

2.3. Hematological examinations

A total of 14 blood samples (bears T3, T4, T7, N5, E2 and Sh1–Sh9) were examined. Blood samples were anticoagulated with EDTA and kept at 4 °C until used. Red blood cell counts and white blood cell counts were examined using the F-520 automated blood cell counter (Sysmex Co., Kobe, Hyogo, Japan). The packed cell volume was also examined. Thin blood smears were prepared, air-dried, fixed with methanol and stained with Wright Giemsa. Five thousand or 10,000 leukocytes were counted in order to calculate the parasitism rate of gametocytes (number of gametocytes per 1000 leukocytes). The size of the gametocytes was measured as described above.

2.4. Examinations of ticks

A total of 49 ticks collected on the body surface of the bears were examined. These ticks were identified as *Haemaphysalis japonica* (n=32), *Haemaphysalis flava* (n=3), *Dermacentor taiwanensis* (n=10) and *Amblyomma testudinarium* (n=4). The ticks were fixed in 70% ethanol, cut along the sagittal plane and subjected to the routine histological method as described above for lungs. The sizes of oocysts, sporocysts and sporozoites were measured as described above.

Table 1
Primers used in the present study

Name	Sequence (5′→3′)	Reference
HepF	ATA CAT GAG CAA AAT CTC AAC	Inokuma et al. [4]
HepR	CTT ATT ATT CCA TGC TGC AG	
BmF1	GCG ATG TAT CAT TCA AGT TTC TG	Simpson et al., [5]
BmR1	TGT TAT TGC CTT ACA CTT CCT TGC	

2.5. Immunohistochemical analyses

Paraffin-embedded sections of the lungs (bears N4 and E3) and of a male *H. flava* (collected on bear Sh6) were subjected to immunohistochemistry using the EnVision™+system (Dako, Glostrup, Denmark). Epitope retrieval was performed by autoclaving (121 °C, 15 min) with the Target Retrieval Solution (Dako). Endogenous peroxidase was inactivated with 0.3% H_2O_2-methanol. Rabbit anti-*Hepatozoon americanum* antiserum was used as the primary antibody [3]. Positive reactions were colored with 3,3′-diaminobenzidine tetrahydrochloride. Sections were counterstained with Meyer's hematoxylin.

2.6. Ultrastructural analyses

Formalin-fixed samples of the lung (bear N4) were postfixed with 1% osmium tetroxide, dehydrated and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then observed using the H-8100 transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

2.7. DNA extraction, PCR amplification and sequencing

Fresh samples of the lungs and of buffy coat were kept at −80 °C until used. DNA extracts were prepared from seven samples of the lungs (bears T1, T3, N2, E1, E3, Ge1 and Ge2) and two samples of buffy coat (bears Sh2 and Sh7) using an Easy-DNA™ kit (Invitrogen Co., Carlsbad, CA, USA). Two primer sets (Table 1), which were designed by Inokuma et al. [4] and by Simpson et al. [5], were used for amplification of the partial 18S rRNA gene of *Hepatozoon* species. The PCR mixture contained 2.5 μl of 10×Ex Taq™ buffer (Takara Bio Inc., Otsu, Shiga, Japan), 0.2 mM of each dNTP (Takara), 15 pmol of each primer, 1 U of Ex Taq™ polymerase (Takara) and 1 μl of DNA extract and Milli-Q® water in a total volume of 25 μl. PCR conditions were 94 °C for 3 min followed by 31 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s. A final extension step at 72 °C for 5 min was also used. All amplifications were performed using the MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were run on 0.8% agarose gel and were purified using a GENECLEAN® II kit (Qbiogene, Inc., Morgan Irvine, CA, USA). Purified PCR products were directly sequenced in both the forward and reverse directions using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and the automated sequencer, ABI PRISM® 3130-Avant Genetic Analyzer (Applied Biosystems).

2.8. Phylogenetic analyses

The 18S rRNA gene sequences of *Hepatozoon* obtained from the bears and of some related protozoa, deposited in GenBank, were used. Analyzed species (or isolates) and their GenBank accession numbers are shown in Table 2. Multiple sequence alignment was performed using the CLUSTALW 1.83 program [6]. Calculation of the distance matrices, using the Kimura's two-parameter method [7], and construction of the phylogenetic tree, using the neighbor-joining method [8], were performed using the MEGA 3.1 program [9]. Confidence values for each branch of the tree were estimated by means of bootstrap analysis for 1000 replications [10].

Table 2
Protozoa used for phylogenetic analyses

Species or isolate	GenBank accession number
Hepatozoon ursi (Gifu 1)	EU041717[a]
Hepatozoon ursi (Gifu 2)	EU041718[a]
Hepatozoon americanum	AF176836
Hepatozoon canis	AF176835
Hepatozoon canis (Curupira 1)	AY461376
Hepatozoon canis (Spain 1)	AY150067
Hepatozoon catesbianae	AF176837
Hepatozoon felis (Spain 1)	AY620232
Hepatozoon felis (Spain 2)	AY628681
Hepatozoon sp. (Curupira 2)	AY461377
Hepatozoon sp. (Boiga)	AF297085
Hepatozoon sp. (BV1)	AY600626
Hepatozoon sp. (BV2)	AY600625
Hepatozoon sp. (HepBiCM001)	AB181504
Cryptosporidium muris	L19069
Eimeria gruis	AB243081
Isospora felis	L76471
Neospora caninum	U17346
Plasmodium vivax	U93234
Sarcocystis cruzi	AF017120
Toxoplasma gondii	L24381
Voromonas pontica	AF280076

[a] New sequences reported in the present paper.

3. Results

3.1. Description of H. ursi n. sp

Intermediate host: Japanese black bear (*U. thibetanus japonicus*).

Location in intermediate host: Schizonts in the lungs (the alveolar wall), gametocytes in leukocytes (probably neutrophils) in peripheral blood.

Prevalence and density in intermediate host: Prevalence of infection 100%. Parasitism rate of gametocytes 1.3–42.6 per 1000 leukocytes.

Suspected definitive host: *H. japonica* and *H. flava*.

Locality: Gifu, Japan.

Type specimens: The H&E stained section of the lung of bear N4 containing trophozoites and schizonts and the Wright Giemsa stained blood smear of bear Sh2 containing intraleukocytic gametocytes are deposited in the National Museum of Nature and Science, Tokyo, Japan, under the accession numbers NSMT-Pr 222a–b.

Etymology: This species is named after its intermediate host, *U. thibetanus japonicus*.

3.2. Histopathological and hematological findings

Schizogonic developmental stages of *H. ursi* and nodules of merozoite/gametocyte-laden macrophages were observed in all 35 bear lungs that were histopathologically examined (Fig. 2).

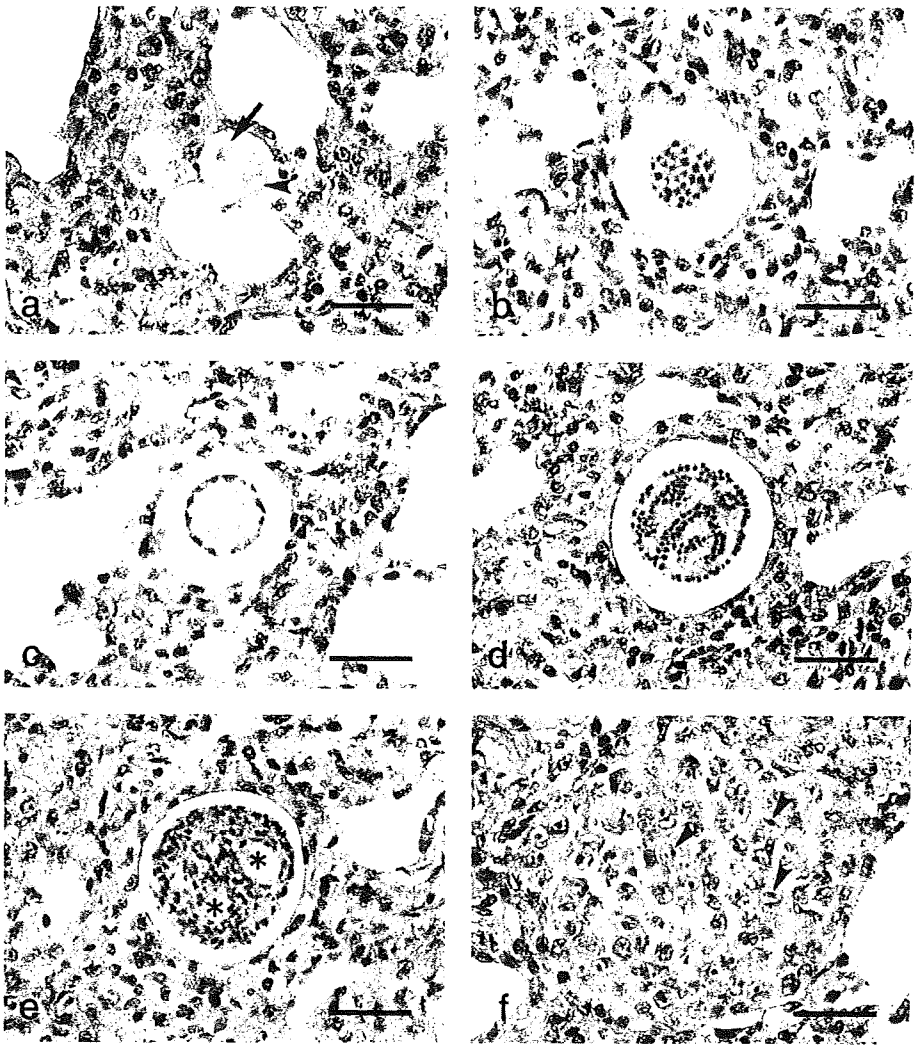

Fig. 2. Various developmental stages of *Hepatozoon ursi* n. sp. detected in the lung of bear N4. a) A trophozoite (arrow) within the cytoplasm of the host cell. Arrowhead indicates the nucleus of the host cell. b–d) Immature schizonts. e) A mature schizont consisting of numerous merozoites and two residual bodies (asterisks). f) A nodule consisting of the accumulation of macrophages. Each macrophage contains a merozoite or a gametocyte (arrowheads). H&E stain. All bars: 30 μm.

In the lungs, various degrees of thickening of alveolar wall and small foci of pyogranulomatous inflammation with infiltrations of neutrophils and eosinophils were observed.

All 14 examined blood samples of bears had *H. ursi* gametocytes (Fig. 3), but obvious hematological changes related to *H. ursi* infection were not observed. Some hematological values with the parasitism rate of gametocytes are shown in Table 3. In two bears (N5 and E2), the parasitism rate was not calculated because of the poor condition of the blood smear.

3.3. Morphological features

3.3.1. Trophozoites, schizonts and merozoites

Trophozoites were found in the parasitophorous vacuoles of the host cells, which had characteristic foamy cytoplasm and resembled swollen macrophages (Fig. 2a). Schizonts were located in the centers of the enlarged parasitophorous vacuoles of the host cells (Fig. 2b–e). Mature and premature schizonts were subspherical in shape and 45.7±4.6×42.7±4.5 μm (37.4–55.4×34.0–52.4 μm, $n=18$) in size (Fig. 2d–e). Each mature schizont contained approximately 80–130 merozoites and had 0–5 residual bodies (Fig. 2e). The merozoites were 7.0±0.7×1.8±0.3 μm (5.7–7.8×1.5–2.4 μm, $n=8$) in size.

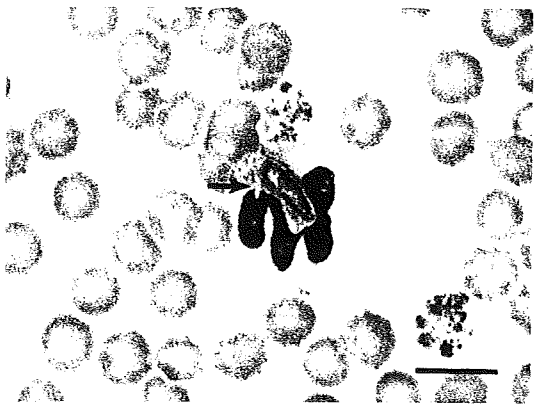

Fig. 3. A *Hepatozoon ursi* n. sp. gametocyte in the cytoplasm of the neutrophil detected in the peripheral blood smear of bear Sh2. Note the beak-like protrusion (arrow). Wright Giemsa stain. Bar: 10 μm.

Table 3
Hematological values of the examined bears

	N	Mean	SD[a]	SE[b]	Minimum	Maximum
Red blood cells ($\times 10^6$/μl)	13	10.385	3.258	0.904	6.18	18.87
Packed cell volume (%)	14	40.3	6.4	1.7	21	50
White blood cells (/μl)	14	9771.4	5753.6	1537.7	4800	28600
Neutrophil segmented (/μl)	12	6675.1	2482.9	716.8	3240	12126
Band (/μl)	12	46.8	30.3	8.7	22	130
Lymphocyte (/μl)	12	1214.8	369.2	106.6	504	1841
Monocyte (/μl)	12	292.0	140.8	40.6	132	657
Eosinophil (/μl)	12	342.5	370.4	106.9	2	1277
Basophil (/μl)	12	45.5	47.4	13.7	3	159
Hepatozoon ursi gametocytes (/10^3 WBCs)	12	13.18	11.87	3.43	1.3	42.6

[a] SD: Standard deviation.
[b] SE: Standard error.

3.3.2. Gametocytes

Gametocytes were slightly curved, cigar-like in shape and had a beak-like protrusion at one end (Fig. 3). The size of the gametocytes, excluding the protrusion, was 10.9±0.3×3.3±0.2 μm (10.5–11.5×2.9–3.6 μm, n=18). Occasionally, only unstained capsule-like structures were observed in the cytoplasm of the leukocytes. The morphological appearance of the host leukocytes resembled that of neutrophils.

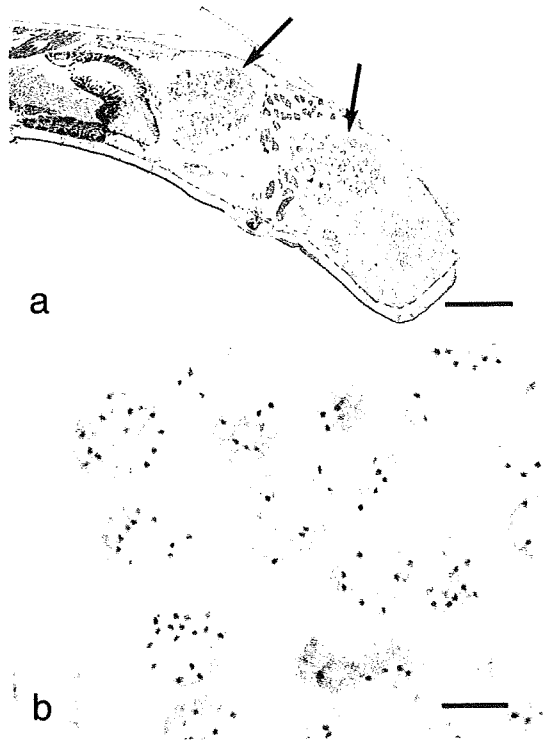

Fig. 4. H&E stained section of the male *Haemaphysalis flava* collected on bear Sh6. a) Two mature *Hepatozoon* oocysts (arrows) within the hemocoel. Bar: 200 μm. b) Higher magnification of the oocyst. The shapes of some sporocysts have been changed by the process of fixation and following histopathological methods. Bar: 20 μm.

3.4. Oocysts detected in ticks

Mature *Hepatozoon* oocysts were detected in a male *H. flava* (collected on bear Sh6) and a male *H. japonica* (collected on bear Sh5). In the *H. flava*, two oocysts were observed within the hemocoel (Fig. 4). It was not possible to observe details of an oocyst detected in the *H. japonica* because of the poor condition of the specimen. Only two oocysts found in the *H. flava* were measured, with the sizes being 263.2×234.0 μm and 331.8×231.7 μm, respectively (Fig. 4a). The oocysts contained approximately 40 and 50 sporocysts, respectively (Fig. 4a). The sporocysts were sub-spherical in shape and 31.2±2.5×27.0±2.9 μm (28.0–34.6×23.7–32.0 μm, n=5) in size (Fig. 4b). Each sporocyst contained at least 8–16 sporozoites (Fig. 4b). The sporozoites were 12.2±1.4×3.5±0.5 μm (10.0–14.0×2.9–4.2 μm, n=4) in size. The H&E stained section of *H. flava* containing *Hepatozoon* oocysts is deposited in the National Museum of Nature and Science under the accession numbers NSMT-Pr 240.

3.5. Immunohistochemistry

Trophozoites, schizonts, merozoites/gametocytes within macrophage nodules and oocysts showed strongly positive reaction with anti-*H. americanum* antiserum (Fig. 5).

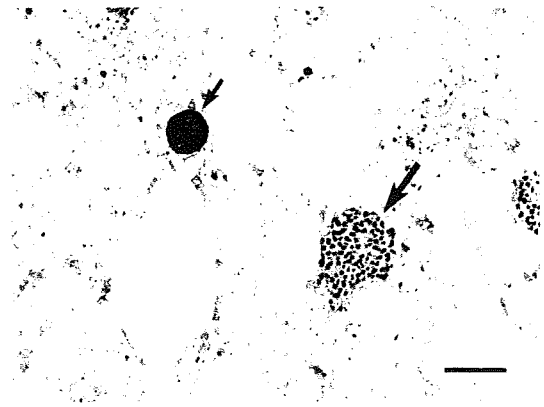

Fig. 5. The lung of bear E3. A schizont (small arrow) and merozoites/gametocytes within a macrophage nodule (large arrow) are positively stained with anti-*Hepatozoon americanum* antiserum. Immunohistochemistry, counterstained with Meyer's hematoxylin. Bar: 75 μm.

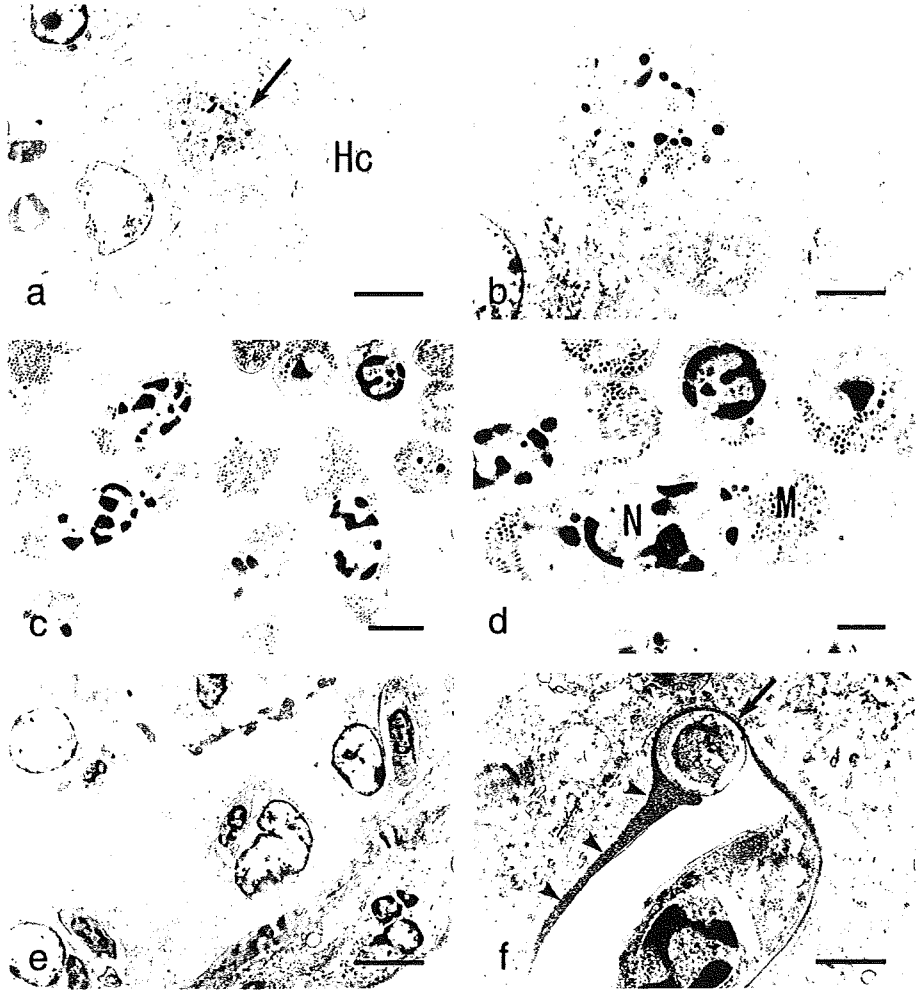

Fig. 6. Transmission electron micrograph of *Hepatozoon ursi* n. sp. detected in the lung of bear N4. a) Trophozoite (arrow). Note the swollen cytoplasm of the host cell (Hc). Bar: 4 μm. b) Higher magnification of the trophozoite Bar: 2 μm. c) Merozoites within mature schizont. Bar: 2 μm. d) Higher magnification of merozoites. Merozoite has an elongated nucleus (N) and numerous micronemes (M). Bar: 1 μm. e) Nodule of gametocyte-laden macrophages. Five gametocytes are shown. Bar: 5 μm. f) Higher magnification of gametocyte. Note the thick electron dense layer (arrowheads) and a beak-like protrusion (arrow). Bar: 1 μm.

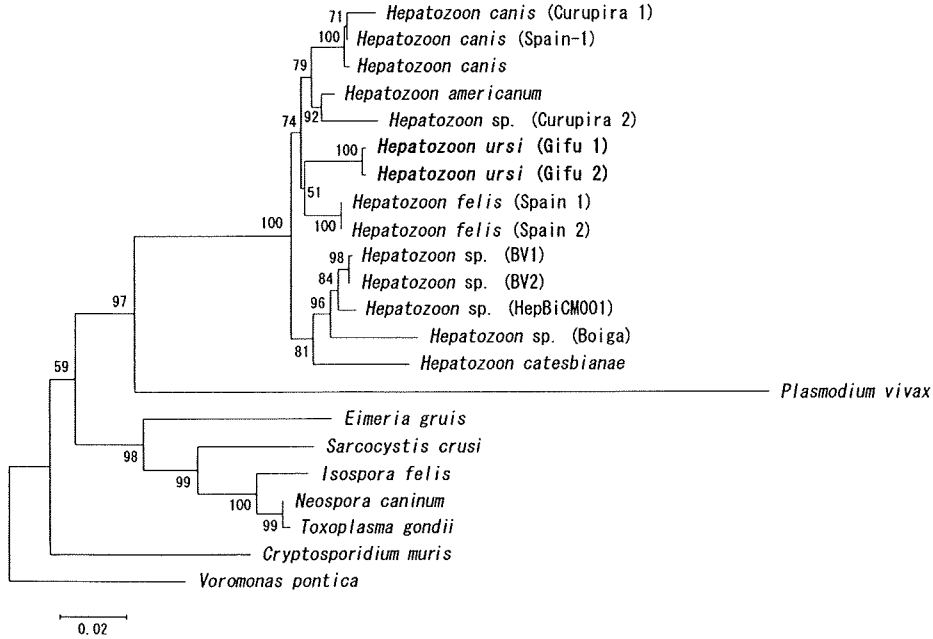

Fig. 7. The phylogenetic tree based on the 18S rRNA gene sequences of *Hepatozoon* species and some related protozoa, constructed using the neighbor-joining method. Numbers on branches are bootstrap values. Scale bar indicates an evolutionary distance of 0.02 nucleotide substitutions per nucleotide site.

3.6. Ultrastructural features

Trophozoites were oval in shape and located in the parasitophorous vacuoles of the host cells (Fig. 6a–b). Merozoites within mature schizonts had an elongated nucleus, a few rhoptries, numerous micronemes and a number of other organelles (Fig. 6c–d). Gametocytes within macrophage nodules were enclosed by an electron dense layer and had an elongated nucleus (Fig. 6e–f). The gametocytes, in contrast to the merozoites within schizonts, had few micronemes.

3.7. Genetic analyses

On PCR assay, both primer sets (HepF/R and BmF1/R1) produced positive results (the sizes of PCR products were approximately 625 bp and 1,110 bp, respectively) in all nine samples. After combining two sequences, which were amplified with these primer sets, the multiple sequence alignment and construction of phylogenetic tree were performed (of the consensus sequences, approximately 1240 bp, 1209 bp or 1207 bp fragments were used). These nine sequences were classified into two genotypes (Gifu 1 and Gifu 2), with the Gifu 2 genotype being obtained from only two bears (E3 and Ge2). These two sequences were 99% identical to each other. The sequences were deposited in GenBank under the accession numbers EU041717 and EU041718, respectively.

In comparison with the 18S rRNA gene sequences of other *Hepatozoon* species, *H. ursi* was most closely related to *H. felis* (96% homology). The phylogenetic tree indicated that the analyzed *Hepatozoon* species were classified into two clades (Fig. 7): the species infecting carnivores and the species infecting anuran, snake and rodents. *H. ursi* was included in the carnivore-related *Hepatozoon* group, and although it was supported only by a relatively low bootstrap value (51%), constituted a sub-group with *H. felis* (Fig. 7).

4. Discussion

The features of schizonts, the histopathological alterations in the lungs and the prevalence of infection of *H. ursi* closely resembled previous descriptions of *Hepatozoon* infection in Japanese black bears [2]. Considering the high prevalence (100%) and the fact that juvenile cubs were also infected, *H. ursi* may be one of the most common parasites in Japanese black bears in central Japan. In contrast, *Hepatozoon* species have not been reported in American black bears (*U. americanus*) in the United States [11] or in European brown bears (*U. arctos*) in Sweden [12], despite examinations of blood smears and/or histopathological examinations of lungs. Moreover, Gjerde et al. did not mention *Hepatozoon* sp. in their report of trypanosomes in polar bears (*U. maritimus*) in Svalbard, Norway [13].

Aside from *H. ursi*, a number of *Hepatozoon* species which prefer the lung for the site of schizogonic development have been reported in mammals: i.e. *H. griseisciuri* in grey squirrel (*Sciurus carolinensis*) [14,15] and unnamed *Hepatozoon* sp. in mink (*Mustela vison*) [16]. However, the size of the schizonts ($45.7\pm4.6\times42.7\pm4.5$ μm) and the number of merozoites in each schizont (approximately 80–130) of *H. ursi* are markedly larger than those of *H. griseisciuri*, being 8–24 μm in diameter with 6–19 merozoites [14], or those of *Hepatozoon* sp. in mink, 29–38×19–24 μm in size with 34–38 merozoites [16]. These morphometric features of *H. ursi* schizonts are relatively similar to those of *H. americanum* schizonts, being 48–$68\times$ 40–63 μm in size and having 61–117 merozoites [17]. However, this *Hepatozoon* species principally parasitizes the skeletal muscles of American canids [3,17,18]. In addition, the size of *H. ursi* merozoites, at $7.0\pm0.7\times1.8\pm0.3$ μm, is slightly smaller than that of *H. americanum*, $7.5\pm0.85\times2.7\pm0.67$ μm [17].

The shape (slightly curved and cigar-like, with a beak-like protrusion) and the size ($10.9\pm0.3\times3.3\pm0.2$ μm) of *H. ursi* gametocytes are considerably different from those of other *Hepatozoon* species gametocytes. *H. americanum* gametocytes were described as being elongated and measuring $8.8\pm0.57\times3.9\pm0.54$ μm in size [17]. *H. canis* gametocytes detected in a Japanese dog were oval to elliptical in shape and 8.9×5.2 μm in mean size [19]. The beak-like protrusion of gametocyte was one of the most characteristic morphological features of *H. ursi*. Such a structure has been reported for *H. procyonis* gametocyte [20–22]. Not only the shape but also the size of *H. ursi* gametocyte is relatively similar to that of *H. procyonis*, 10.9×5.4 μm recorded by Richards [20] or $10.43\pm0.68\times4.03\pm0.18$ μm and $10.20\pm0.66\times3.70\pm0.48$ μm recorded by Rodrigues et al. [22]. However, *H. procyonis* schizonts were principally detected in the myocardium of raccoons (*Procyon lotor*) and measured 50×85 μm [20] or $31.2\pm7.8\times$ 22.7 ± 5.5 μm [21] in size. Additionally, bears may not be infected with *H. procyonis*. In a study on endoparasites of American black bears in the southeastern United States, including Georgia, *Hepatozoon* species were not found in the peripheral blood despite the evidence that the bears were infected with some species of helminths which were common in raccoons [11]. On the other hand, *H. procyonis* was seen in 16.7–40% of raccoons in Georgia [20,23,24].

A number of mature *Hepatozoon* oocysts were detected in two tick species, *H. flava* and *H. japonica*. The morphological and morphometric features of these oocysts were relatively similar to those of *H. canis* oocysts detected in *Haemaphysalis longicornis* and *H. flava* which were collected from Japanese dogs [25]. Unfortunately, we did not perform the experimental transmission and the genetic analysis of oocyst in the present study. Therefore, these oocysts were strongly suspected, but not determined, as those of *H. ursi*.

The results of immunohistochemistry confirmed that *H. ursi* is a member of the genus *Hepatozoon*. Although Panciera et al. reported that the antiserum used in this study cross-reacted to some other apicomplexans, those reactions were weak [3].

Ultrastructurally, the gametocytes within macrophage nodules were quite distinct from merozoites within schizonts. Although we detected only gametocytes in macrophage nodules, Cummings et al. found both merozoites and developing gametocytes of *H. americanum* in similar granulomas [26]. *Hepatozoon* merozoites may develop into gametocytes in such nodules.

According to Smith [1], the classification of *Hepatozoon* with classical criteria, such as host species, is relatively difficult. Recently, the 18S rRNA gene sequence has been used as a useful criterion for identification of *Hepatozoon* species [4,27,28]. In the

present study, the analyses of the 18S rRNA gene sequences supported the conclusion that *H. ursi* was a novel species of carnivore-related *Hepatozoon*. The phylogenetic relationship within the genus *Hepatozoon* of the present study basically agreed with that reported by Criado-Fornelio et al. [27].

The pathogenicity of *H. ursi* against Japanese black bears has not yet been completely clarified. However, interstitial pneumonia, characterized by thickening of alveolar wall, and small foci of pyogranulomatous inflammation may cause respiratory disorders.

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Ⅳ. 平成21年度総括・分担研究報告書

厚生労働科学研究費補助金(インフルエンザ等新興・再興感染症研究事業)
総括研究報告書

動物由来感染症の生態学的アプローチによるリスク評価等に関する研究

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研究要旨　昨年度に引き続き国内では稀であったり、存在様式に不明な点の多い動物由来感染症を対象に研究を継続し以下の成績を得た。

① 5牧場のウシ431頭における、Q熱コクシエラ *Coxiella burnetii* (*C.burnetii*)に対する抗体の検出を試みた。平均 10.4%のウシが *C.burnetii* 抗体陽性と判定されたが、特定の牧場では陽性率が28.4%で、他の牧場に比べて明らかに高かった。322 検体について Real-time PCR 法での遺伝子検出を実施したが、全て陰性であった。

② 新規に見出した回帰熱病原体類縁のボレリアについて解析した結果、本ボレリアは北米で回帰熱病原体として見出される *Borrelia turicatae, B. parkeri* と類縁であること、ならびに国内棲息の鳥類寄生マダニであるサワイカズキダニが媒介ベクターである可能性が高いことを明らかとした。

③ 愛護センター収容の犬・猫および猟犬からジフテリア毒素産生性 *Corynebacterium ulcerans* (*C.ulcerans*)を分離するとともに、犬から犬または猫から猫への菌の拡散(不顕性感染)を確認した。また、ジフテリア様症状を呈した患者ならびに患者との接触が疑われた野良猫から本菌を分離し、両者が遺伝的に同一であることをPFGEおよび毒素遺伝子の解析から明らかにした。

④ 野生動物におけるブルセラ症の実態を野生イノシシおよびシカについて調査した。イノシシ 334 検体中、2 検体(0.6%)が家畜ブルセラ菌に対し、32 検体が(9.6%)がイヌブルセラ菌 *B. canis* に対し陽性だった。ニホンジカ 97 検体では、*B. canis* に対し 1 検体(1.0%)が抗体陽性だった。この検体はウェスタンブロッティング(WB)でも陽性であることが確認された。抗体陽性のイノシシ 34 頭中 21 頭からとニホンジカ 1 頭からブルセラ菌遺伝子が PCR 増幅されたが、いずれも *B. canis* 遺伝子と100%配列が一致した。

⑤ 野生動物における野兎病菌浸潤状況を野生動物血液 1,011 検体、臓器 152 個体分、体表付着ダニ 38 個体分について調査した。臓器および体表付着ダニから菌分離およびゲノム DNA の検出を試みたところ、斃死ノウサギの臓器からのみ菌分離とゲノム DNA が検出された。抗野兎病菌抗体のスクリーニングにて 6 種の動物種(ツキノワグマ、ノウサギ、ホンドタヌキ、ハクビシン、ハタネズミ、ノスリ)で陽性が認められ、ウエスタンブロット法及び間接蛍光抗体法ツキノワグマ由来 9 検体の陽性が確認された。

⑥ また、日本国内分離野兎病菌株のパルスフィールドゲル電気泳動(PFGE)解析を行い、国内株が非常に高い遺伝的多型を有していること、日本分離株は海外由来株と明確に異なるバンドパターンを示し、両者の鑑別にPFGEが有用な手法の一つであることを示した。

⑦ 猟犬計 155 例について日本紅斑熱、ツツガムシ病、ライム病、ジフテリア症、レプトスピラ症およびヘパトゾーン症の病原体に対する抗体保有状況について調査を実施した結果、日本紅斑熱は5/149例, ツツガムシ病は28/149例, ジフテリア症は13/153例, ライム病ボレリアは10/73例, レプトスピラ症は 12/153 例, ヘパトゾーン症は 18/155 例で陽性となる個体が認められた。猟犬が国内における動物由来感染症の浸潤状況を把握するための有用な指標となりうる可能性が示唆された。

⑧ イヌ頭部モデル開発に関しては、主に解剖手技習得モデルの改良と、本モデルを利用した技術研修の試行を行った。その結果、開発したモデルと教材が自治体現場での狂犬病啓発と技術研修等に有用であると期待された

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A. 研究目的

野兎病、ブルセラ症などは国内の環境の変化、衛生状態の向上などにより感染者の報告は極めて少なくなっている。しかし、これまでの我々や他のグループの調査から、依然として国内にこれらの病原体が存続していると考えられるにもかかわらず、その実態は不明な点が多く、リスクの正しい評価ができていない。また、Q 熱においても、典型的な患者報告が極めて少なかったが、最近典型的な患者の発生が報告された。従って、国内に存在することは間違いないが、その存在様式等はやはり不明な点が多い。ジフテリア毒素産生コリネバクテリウムウルセランス感染はジフテリアに極めて類似する病態を呈し、動物から感染する可能性が指摘されているが、その実態はやはり不明な点が多い。また、ライム病ボレリアに関してもその生態系における国内での存在様式を明らかにする必要がある。本研究はこれらの点を踏まえ、動物由来感染症の病原体の生態系における存在様式を精査し、そのリスク評価を改めて行うことを目的とする。一方、国内への侵入が憂慮される狂犬病について、国内侵入をいち早く察知するために不可欠な診断技術向上のため、実習用モデルの試作を行う。

B. 研究方法

病原体あるいは抗体の検出は個々の報告書に記載した方法による。

C. 研究結果

（1）Q 熱に関する研究

Q 熱コクシエラの生態系における感染リスク評価に関する研究の一環として、本年度は、ウシにおける、*C.burnetii* の抗体保有率および遺伝子検出率について検討し、感染リスクの評価のための基本状況の把握を行った。検体提供について協力が得られた北海道の 5 牧場で飼育されるウシ 431 頭を対象にした。血清抗体価は、全血と血清が同時に採取された 4 牧場(A,B,C,D)の 322 血清と、血清のみ採取された 1 牧場(E)の 109 検体を用い IFA を施行した。全体としては 10.4%が *C.burnetii* 抗体陽性と判定された。しかしながら、E 牧場においては、陽性が 28.4%と他の牧場に比べて非常に高い値を示した。E 牧場を除いた 4 牧場における陽性の割合は 4.3%であった。ウシ全血から抽出した DNA、計 322 検体について Real-time PCR 法にて遺伝子検出を実施したが、全検体陰性であった。抗体保有率が高かった E 牧場のウシでは DNA については検討できなかったため、採材時点での感染の有無は不明であるが、過去に流行等があった可能性は否定できず、地域によっては一定の感染リスクは存在する可能性が示唆された。今後感染リスクの評価を適切に行うためには、さらにウシの検体を増やして詳細な検討を行うとともに、未だ不明な野生動物やダニ等の生態系での存在様式についても検討することが必要と考えられた。

（2）ボレリアに関する研究

昨年度までの調査研究の過程で、関西地方の渡り鳥コロニーに棲息するマダニの一種サワイカズキダニ（学名：*Carios sawaii*）より回帰熱ボレリアの遺伝子断片を見出した。国内では統計が存在する 1956 年以降、回帰熱症例の報告はなく、野生動物やマダニなどの環境材料からの回帰熱病原体の検出もなされていない。そこで本ボレリアの遺伝学的同定を行うとともに、サワイカズキダニにおける保菌率、およびサワイカズキダニのボレリア媒介種としての評価を行った。その結果検出されたボレリアは北米で回帰熱病原体として見出される *Borrelia turicatae, B. parkeri* と類縁であること、ならびに国内棲息の鳥類寄生マダニであるサワイカズキダニが媒介ベクターである可能性が高いことを明らかとした.

（3）コリネバクテリウムに関する研究

昨年度までに国内ではジフテリア様症状を呈する 5 名の患者からジフテリア毒素産生性 *Corynebacterium ulcerans* が分離された。患者の環境調査は実施されずに、感染経路に愛玩動物があることが疑われていた。平成 21 年 2 月に東京都内で 6 例目患者発生が確認され、初めて患者自宅の詳細な環境調査を実施した。調査の結果、患者が発症する以前に自宅に集まる野良猫の 1 匹が風邪様症状を呈してクシャミ、鼻水を飛散していたことを確認し、その後、患者に同菌が感染した結果、咽頭炎等が発現したこと

が判明した。野良猫の咽頭等数カ所から患者と遺伝子型が一致する菌を分離し、さらに親猫と同居の子猫の鼻水からも同菌を分離した。この調査結果により発症野良猫から人が感染し、さらに猫から同居猫の感染も確認した。

地方自治体の動物愛護センターに搬入された犬または猫の咽頭スワブおよび屠畜場に搬入された牛または豚の咽頭スワブ等から菌分離調査を実施した結果、数カ所の自治体の愛護センターの猫と犬よりジフテリア毒素産生性 *C.ulcerans* が分離された。しかし、現在までに畜産動物からの菌分離は陰性であった。

さらに開業獣医師の協力を得て複数の地域で調査した結果、一般家庭で飼育している猫からも本菌を分離し、同居猫にはジフテリア抗毒素を保有し過去の感染既往も疑う例もあった。また、野生動物と接触機会の多い猟犬についてジフテリア抗毒素保有状況を調査した結果、複数の地域の猟犬において抗体陽性犬を確認した。これら猟犬で菌分離の追跡調査を実施した結果、同居犬の2頭からジフテリア毒素産生性 *C.ulcerans* を分離した。

現在までの調査結果を総括すると、野外活動時間の多い犬や猫は本菌を保菌または感染している可能性が高く、動物間では菌の伝播がおこり感染が成立する。感染動物では排菌量が多いために、免疫力が低下している人は感染を起こす危惧がある。

（4）ヒトの動物由来感染症への曝露の指標としての猟犬の応用に関する研究

猟犬は，山林を跋渉し野生鳥獣を捕獲することから，ダニ類への暴露や野生動物との接触の機会が多く，しばしば野生動物を生食することもある。そのため，ダニ媒介性感染症や野生動物由来人獣共通感染症のモニターのために有用であると考えられる。西日本を中心とした各県で飼育された猟犬計155例について，リケッチア(日本紅斑熱，ツツガムシ病)，ライム病，ジフテリア症，レプトスピラ症およびヘパトゾーン症の抗体保有状況について調査を実施した。日本紅斑熱は5/149例，ツツガムシ病は28/149例，ジフテリア症は13/153例，ライム病ボレリアは10/73例，レプトスピラ症は12/153例，ヘパトゾーン症は18/155例でそれぞれ陽性を示した。宮崎および熊本県など九州地方の猟犬が占める陽性割合は，日本紅斑熱は60%，ツツガムシ病は85.7%，ジフテリア感染症は84.6%，レプトスピラ感染症は66.7%，ヘパトゾーン感染症は88.9%であり，各感染症とも九州地方を中心に陽性個体が多くみられる傾向があった。このことは，野生動物を含めた他の動物における報告，媒介節足動物の分布，人でのこれらの疾病の発生状況とよく合致していた。したがって，本研究から猟犬は人獣共通感染症の有用な指標となりうると考える。今後さらに他の地域に調査を広げることで，全国的なこれらの野外を中心とした感染症の疫学情報の収集に貢献することが期待できると考える。

（5）ブルセラ症に関する研究

ブルセラ症(brucellosis)はブルセラ属菌(genus *Brucella*)による人獣共通感染症である。野生動物におけるブルセラ症の存在を確認するために、日本各地から野生イノシシおよびニホンジカの血液サンプルを入手し、家畜ブルセラ菌(*B. melitensis, B. abortus, B. suis*)とイヌブルセラ菌(*B. canis*)に対する抗体保有状況をマイクロプレート凝集反応（MAT）により検討した。これまでに入手したイノシシ334サンプル中2サンプル（0.6%）が家畜ブルセラ菌に対し、32サンプル（9.6%）が *B. canis* に対し抗体陽性を示した。ニホンジカ97サンプルについては、*B. canis* に対し1サンプル（1.0%）が抗体陽性を示した。抗体陽性サンプルは、ウェスタンブロッティング（WB）でも陽性を示した。さらに、血液サンプルからDNAを抽出し、標的遺伝子の異なる4セットのブルセラ特異的プライマーを用いたPCRを実施した。*B. canis* に対し抗体陽性サンプルでは、イノシシで21/34、ニホンジカで1/1が4セットのプライマーのいずれかで陽性となった。増幅産物のシーケンスを確認したところ *B. canis* 遺伝子と100%配列が一致した。家畜ブルセラ菌抗体陽性サンプルからの遺伝子検出はできなかった。

国内のイノシシとニホンジカのブルセラ属菌感染が示唆されるが、確定には、他菌との交差反応の確認も含め、さらに検証が必要である。

（6）野兎病に関する研究

本研究期間中に数例の野兎病患者発生が報告されるとともに、猟友会により野兎病菌感染ノウサギが発見された。そこで周辺地域における小型野生哺乳類、ダニ、ならびに水、土壌からの野兎病菌の分離、ゲノムDNAの検出を試み、小型哺乳類では血清学的検索も行った。小型哺乳類は菌分離、ゲノムDNA検出とも陰性で、野兎病菌に対する抗体も検出されなかったが、周辺の土壌から野兎病菌ゲノムDNAが検出された。また、水1検体からDNAが検出された。今後も更なる調査により自然環境での野兎病菌

維持様式を検討する必要があると考えられた。

（7）狂犬病に関する研究

昨年度に引き続き狂犬病発症が疑われるイヌの解剖手技習得に必要なモデル・教材として（1）解剖手順習得モデル、（2）実技取得モデル、（3）脳モデルのプロトタイプを作成した。これらのモデルについて自治体等関係機関の現場担当者等とともに、改良点や課題点について検討を行った。

D. 考察

精度の向上した検査法を用い、ウシ血清中のQ熱コクシエラに対するIgG抗体の保有調査をしたところ、平均 10.4%が陽性だった。しかしいずれの検体からもコクシエラ遺伝子は検出されなかったことから、これらの抗体は過去の感染を示していると考えられた。特定の牧場において他よりも抗体陽性率が高い傾向が認められたことは、この牧場においてかつて流行があったことを示唆している。ヒトへのリスクをより正確に評価するためには、今後IgMの測定により、急性感染や最近の感染との関連も検討することに加えて、ウシの疾病との関連性の調査検討が重要である。

国内で記録が存在する 1956 年以降、初めて回帰熱ボレリアの存在が見出された。今後，健康被害の有無など疫学調査，さらには病原体の分離とその病原性についての解析が必要である。また，本ボレリアは渡り鳥類のコロニーから見出されたことから、生態系の中でのボレリアの存在様式に鳥類が関与している可能性も示唆された。

コリネバクテリウムウルセランスについては、PFGEおよび毒素遺伝子の解析結果でヒト分離株とイヌ分離株が一致したことから、ヒトの感染にイヌの介在が疑われる。一般的にコリネ属菌は皮膚等の一般細菌叢として分離され、*C. ulceras* もこれら細菌叢の一部として存在している可能性もある。またネコ白血病ウイルスに感染しているネコでは免疫力が低下していることを考え合わせると、ネコ白血病等に罹患したネコが感染源になる可能性もあり、注意を要すると考えられた。

イノシシとニホンジカの血液サンプルよりMAT、WBにより抗 *B. canis* 抗体を検出し、さらに PCR と産物のシーケンスにより *B. canis* 特異的遺伝子を検出した。イノシシ2サンプルからはMATとWBにより抗家畜ブルセラ菌抗体も検出されたが、ブルセラ特異的遺伝子は検出されなかったことから、交差反応の可能性もあり、さらに検証が必要である。今後は、菌分離も視野に入れIFA、ELISAなどの手法も用いて国内の野生イノシシ、ニホンジカのブルセラ属菌感染について、検討をすすめることが必要であると考える。また、より特異的な抗体検査法の開発も必要である。

各種野生動物の抗野兎病菌抗体調査では、今回、初めて国内生息の野生動物から初めて野兎病菌に対する抗体を検出した。特にツキノワグマ由来9検体は3種の検査（MA、ELISAおよびIFA）にて陽性であったため、その由来動物は過去に野兎病菌に感染したと強く示唆された。抗体陽性検体由来動物は全て東北地方にて捕獲された動物であったことからヒトの症例同様、野兎病菌感染野生動物が東北地方に存在すると考えられた。野兎病菌感染が致死的となる野生げっ歯類やノウサギからの病原体検出では分離および核酸検出において斃死ノウサギのみが陽性となり、新しい野兎病菌分離株NVF1を得た。また、ノウサギや野ネズミなど野兎病菌に高感受性の動物種では健常個体からの野兎病菌や抗野兎病菌抗体の検出は困難と考えられた。

日本国内で分離された野兎病菌のPFGEによる型別を行うにあたり、まずこれら菌株に適した制限酵素の選択を行った。その結果、現在まで用いられていない新たな酵素を含む計7種の制限酵素が国内分離菌株の型別には有用であることが判明した。この全てが海外由来株との鑑別に有用である可能性が示唆された。これら7種の制限酵素を用いて多くの株でPFGE解析を実施し、各制限酵素の型別および海外株との鑑別診断に有用であるか検討が必要と考えられた。今回対象とした重要な感染症は，猟犬でもしばしば陽性個体が認められ，明らかな地域差が認められた。その多くはヒトでの患者の発症と比較的類似していた。特に日本紅斑熱，ツツガムシ病，ジフテリア症，ライム病ボレリア，およ

びレプトスピラ症にいずれにおいても明らかに地域差があった。特に陽性個体の発生率は，宮崎県および熊本県など九州地方の猟犬が占める割合が高く，日本紅斑熱は60%，ツツガムシ病は85.7%，ジフテリア感染症は84.6%，レプトスピラ感染症は66.7%，ヘパトゾーン感染症は88.9%であった。このことから猟犬は野外に由来する動物由来感染症の有用な指標となりうると考える。今後さらに他の地域に調査を広げることで，全国的なこれらの野外を中心とした感染症の疫学情報の収集に貢献することが期待される。

今般開発した狂犬病診断のためのイヌ頭部モデルと教材は、自治体の現場での狂犬病啓発と技術研修等に対して効果があると期待され、本研究の目的である「狂犬病の診断技術向上のために必要となる解剖手技習得モデル・教材の開発」は自治体等における担当者への実技伝達のみならず、発生時を想定した意識啓発と動物由来感染症である狂犬病の感染源対策に対する危機管理意識の向上にも大いに貢献することが示唆された。

E. 結論

国内での存在は明らかにされているがその存在様式が不明な動物由来感染症について実態調査を昨年度に引き続き実施し、今後も同様な地道な調査研究が動物由来感染症対策における科学的根拠を提供するために極めて重要であることを示すことができた。

F. 健康危機情報

特になし

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H．知的財産権の出願・登録状況
（予定を含む。）
なし