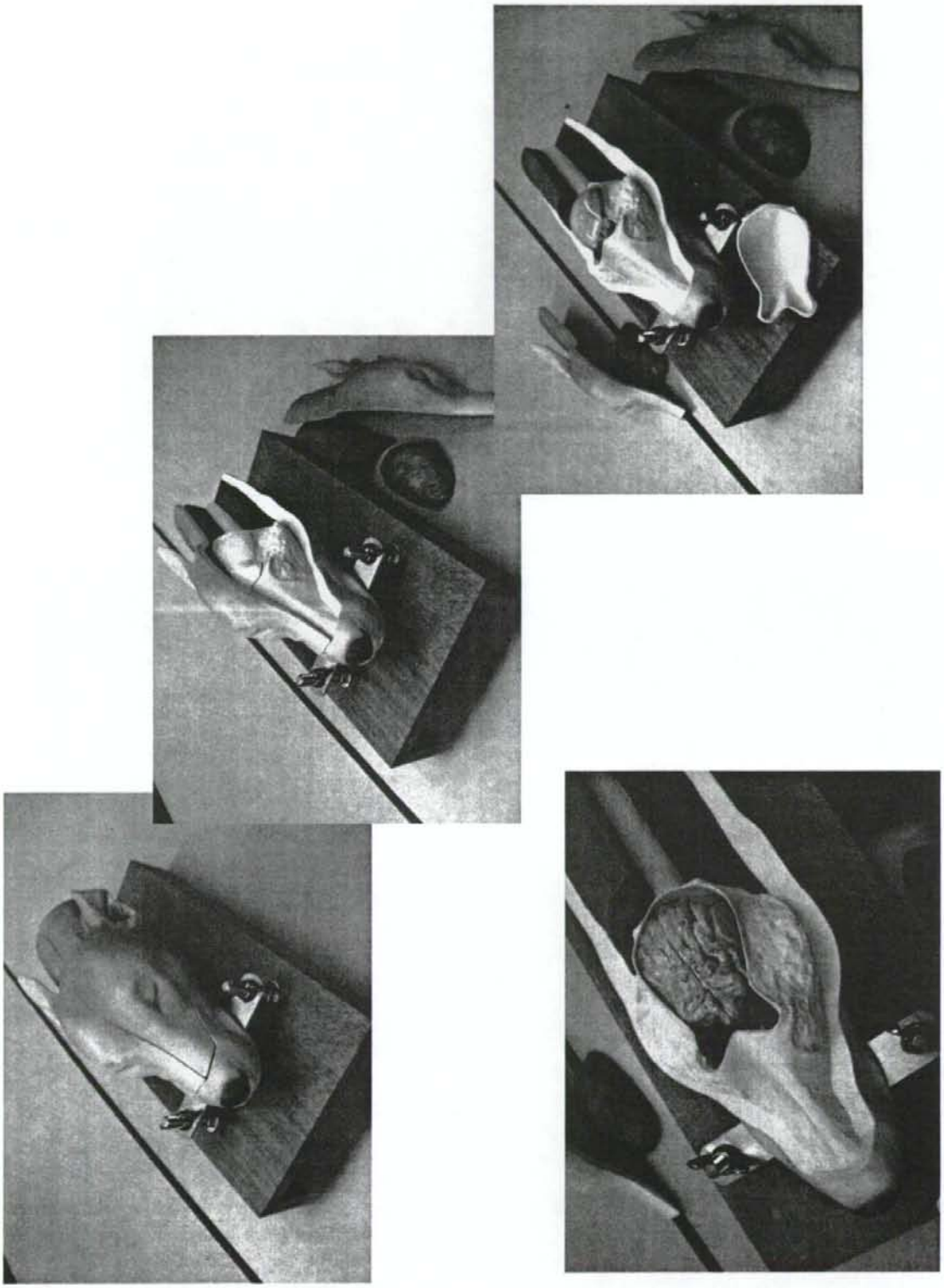

Ⅲ. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
川端寛樹	ボレリア感染症	山口徹，他	今日の治療指針	医学書院	東京	2009	142-143
川端寛樹	野兎病菌	バイオメデゥカルサイエンス研究会	バイオセーフティの辞典-病原微生物とハザード対策の実際-	みみずく舎/医学評論社	東京	2008	181-182
川端寛樹	ボレリア・ブルクドルフェリ抗体	櫻林郁之助，熊坂一成	新・臨床検査項辞典	医歯薬出版	東京	2008	664-665
今岡浩一	ブルセラ病とその検査	日本獣医師会	感染症検査実習マニュアル	日本獣医師会	東京	2008	95-108
今岡浩一	ブルセラ	バイオメディカルサイエンス研究会	バイオセーフティの事典	みみずく舎・医学評論社	東京	2008	169-171
井上 智、佐藤 克、梅田浩史、衛藤真理子	狂犬病 (Rabies)		JRA特別振興事業（ウエストナイルウイルス感染症等特別対策事業）	社団法人全国家畜畜産物衛生指導協会		2008	
井上 智	人獣共通感染症	和田 攻	産業保健ハンドブック	財団法人産業医学振興財団		2008	p32
井上 智	動物由来感染症	和田 攻	産業保健ハンドブック	財団法人産業医学振興財団		2008	p32-33
井上 智	狂犬病ウイルス	バイオメディカルサイエンス研究会	バイオセーフティの辞典	医学評論社		2008	p258-259

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Seto Y, Komiya T, Iwaki M,Kohda T, Mukamoto M, Takahashi M, Kozaki S	Properties of Corynephage attachment site and molecular epidemiology of Corynebacterium ulcerans isolated from humans and animals in Japan	Jpn. J. Infectious Diseases	61	116-122	2008
Hanaoka N, Sakata A, Takano A, Kawabata H, Watanabe H, Kurane I, Kishimoto T, Ando S.	Development of a pUC19-based recombinant plasmid to serve as a positive control in PCR for *Orientia tsutsugamushi*.	Microbiology and Immunology			Accepted
Takada N, Fujita H, Kawabata H, Ando S, Sakata A, Takano A, Chaithong U	*Rickttsia japonica* in Thailand.	Emerging Infectious Diseases			In press
Takano A, Ando S, Kishimoto T, Fujita H, Kadosaka T, Nitta Y, Kawabata H, Watanabe H	Novel *Ehrlichia* sp. found in *Ixodes granulatus* infested to rodents in Okinawa, Japan.	Microbiology and Immunology			In press
Fujita H, Kadosaka T, Nitta Y, Ando S, Takano A, Watanabe H, Kawabata H	*Rickettsia* sp. in *Ixodes granulatus* Ticks in Japan.	Emerging Infectious Diseases	14	1963-1965	2008
Kimura, M., Imaoka, K., Suzuki, M., Kamiyama, T. and Yamada, A.	Evaluation of a Microplate Agglutination Test (MAT) for Serological Diagnosis of Canine Brucellosis.	J. Vet. Med. Sci.	70	707-709	2008
Kubo M, Uni S, Agatsuma T, Nagataki M, Panciera R,Tsubota T, Nakamura S,Sakai H, Masegi T, Yanai T	*Hepatozoon ursi* n. sp. (Apicomplexa: Hepatozoidae) in Japanese black bear (*Ursus thibetanus japonicus*)	Parasitology International	57	287-294	2008
井上　智	アジアの狂犬病の現状を知る	JVM	61	184-187	2008
井上　智	狂犬病の診断技術向上のためのイヌの頭部解剖手技の習得モデルと教材開発の紹介	LABIO 21	34	33-35	2008
井上　智、野口　章	リッサウイルス感染症	検査と技術	36	1465-1467	2008
井上　智	人獣共通感染症が侵入・発生した場合の動物側の対応	JVM	61	901-907	2008
井上　智	世界・日本の現状と獣医師の役割	MVM	110	6-7	2008

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Original Article

Properties of Corynephage Attachment Site and Molecular Epidemiology of *Corynebacterium ulcerans* Isolated from Humans and Animals in Japan

Yukiji Seto[1,2], Takako Komiya[1], Masaaki Iwaki[1], Tomoko Kohda[2], Masafumi Mukamoto[2], Motohide Takahashi[1] and Shunji Kozaki[2]*

[1]*Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo 208-0011, and* [2]*Department of Veterinary Science, Graduate School of Life and Environmental Science, Osaka Prefecture University, Osaka 599-8531, Japan*

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SUMMARY: Sporadic reports of *Corynebacterium ulcerans* infection in humans and animals have become increasingly common throughout the world. Between 2001 and 2006, five human cases, in addition to isolation of the bacterium from the carcasses of *Orcinus orca* and *Panthera leo*, were reported in Japan. While an isolate from *P. leo* generated only phospholipase D (PLD), the other isolates produced both PLD and diphtheria-like toxin (DLT). Pulsed-field gel electrophoresis analysis showed that isolates from *P. leo* and humans were genetically homologous. Southern blotting found that a human isolate was lysogenized by two corynephages coding DLT. Sequence analysis of the region of the DLT gene revealed that the integration in *C. ulcerans* occurred in the same manner as that in *C. diphtheriae*.

INTRODUCTION

Corynebacterium ulcerans was first isolated from human throat lesions in 1926 (1), and has recently been recognized as a distinct species within the genus *Corynebacterium* by DNA-DNA hybridization (2). *C. ulcerans* can carry the corynephage that codes for diphtheria toxin gene (*tox*), and toxigenic strains produce diphtheria-like toxin (DLT), which may be associated with classical pharyngeal diphtheria in humans. The organism also causes respiratory symptoms in macaques (3), mastitis in cattle (4), and caseous lymphadenitis in sheep and goats (5). Since there have been some cases in pets, such as chronic labial ulceration and rhinorrhea in dogs (6) and bilateral nasal discharge in cats (7,8), it has been suggested that clinical cases in humans are usually derived from infections in household pets (9). Sporadic cases in humans and animals have recently been reported throughout the world (10-17), and many reports have originated in the United Kingdom (7,19). In Japan, 5 cases in humans were reported between 2001 and 2006 (12,14). During the same period, *C. ulcerans* was identified from the carcass of *Orcinus orca* (killer whale) and *Panthera leo* (lion).

C. ulcerans is a member of the so-called "diphtheria group", which includes *C. diphtheriae* and *C. pseudotuberculosis* (4). Toxigenic strains of these bacteria can produce diphtheria toxin (DT) (*C. diphtheriae*) or DLT (*C. ulcerans* and *C. pseudotuberculosis*). The production of urease and a failure to reduce nitrates are characteristics of *C. ulcerans* that are not shared with other members of the diphtheria group (4). The characteristics of members of the group have been of special interest to those studying the epidemiology of diphtheria, since it was reported that diphtheria-like organisms had been isolated from cases of acute sore throat and from healthy carriers (4). Diphtheria is an acute infectious disease caused by the toxin-producing *C. diphtheriae*. The disease is typically characterized by local infection of the upper respiratory tract and occasionally the skin. Systemic manifestations can affect, in particular, the heart, kidneys, and peripheral nerves because of DT, which is a cytotoxic protein that inhibits cellular protein synthesis in eukaryotes by inactivating elongation factor 2 through ADP ribosylation (19). *C. ulcerans* also produces phospholipase D (PLD), which is a known virulence factor in *C. pseudotuberculosis* (4,20). Lipsky et al. have shown that there are three toxigenic groups in *C. ulcerans* based on the production of DLT and PLD, in which the three groups respectively produce DLT, PLD, or both (4). Another report indicated that PLD production was a feasible marker for distinguishing between *C. ulcerans* and *C. diphtheriae* (21). However, little recent attention has been directed at PLD production by *C. ulcerans*.

Toxin production by *C. diphtheriae* depends on infection of an organism with a beta corynephage encoding *tox*. In strains of *C. ulcerans* and *C. pseudotuberculosis*, phages are also able to induce the production of DLT. In toxigenic *C. diphtheriae*, site-specific recombination occurs between a phage attachment (*attP*) site and a bacterial attachment (*attB*) site (22). Certain strains of *C. diphtheriae* contain two *attB* sites (*attB*1 and *attB*2) in their chromosome, and the corynephage can integrate into either *attB*1 or *attB*2 with equal frequency. Lysogens containing two phage copies have also been isolated and were shown to contain either two phages tandemly integrated at the same *attB* site (unstable conformation) or one phage integrated in each of the two *attB* sites (stable conformation). The production of DT by a given lysogen is proportional to the number of prophages integrated into its chromosome (22,23). The sequence homologous to the beta corynephage integration site in *C. diphtheriae* is conserved in members of the genus *Corynebacterium*. The sequence of the *attB* site was also found in *C. ulcerans* (24,25). Maximescu et al. showed that *C. ulcerans* (strains 40c, A238, 298G, and 9304) could be lysogenized and converted with phage W from *C. diphtheriae* PW8; moreover, *C. ulcerans*

*Corresponding author: Mailing address: Department of Veterinary Science, Graduate School of Life and Environmental Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan. Tel: +81-72-254-9504, Fax: +81-72-254-9499, E-mail: kozaki@center.osakafu-u.ac.jp

phage "h" induced the production of DLT in *C. ulcerans* (strains 40 and A238) (26). Although the occurrence of phage conversion and the presence of the *attB* site in *C. ulcerans* have been demonstrated, the details of the relationship between the *attB* site and *tox* in the bacterium are not well understood in comparison with those of *C. diphtheriae*.

The aims of this study were to determine the toxigenicity and genetic relatedness among isolates in Japan using restriction fragment length polymorphisms of rRNA genes (ribotyping) with *Bst*EII and pulsed-field gel electrophoresis (PFGE) with *Sfi*I. We found a correlation between the *attB* site and *tox* in *C. ulcerans* using Southern blot analysis with specific probes for *tox*, the *attB* site, and the PLD gene (*pld*).

MATERIALS AND METHODS

Bacterial strains and DNAs: We used seven strains isolated from humans and animals (Table 1). Strain 0509 was obtained from a swab from a 57-year-old man, whose chief complaint appeared to develop after the death of his dog. Another strain, 0607, was isolated from swab used to obtain a sample from a 58-year-old woman, who also kept a dog. The immunization status of these persons with diphtheria toxiod was not known. We encountered an isolate of the organism, strain Ran, from a female *O. orca* that had manifestations of decreased appetite and fever at day 4 post-partum, and the animal died the next day. The strain designated O-9 was obtained from the carcass of a male killer whale that had been one-half of a pair of *O. orca* from the same pool. The strain lion was isolated from a blood sample from a female *P. leo*, and the sample was simultaneously contaminated with two *Staphylococcus* spp. and a *Streptococcus* sp. *C. ulcerans* ATCC®51799 (identical to CCUG 2708, NCTC 7910, and DSM 46325), and *C. diphtheriae* PW8 and ATCC 700971 were used as reference strains in this study. The total DNA was isolated as described previously by Michel et al. (18) and was used for ribotyping, Southern blotting, and sequence analysis of 16S rRNA, *tox*, *pld* genes, and the *attB* site. For polymerase chain reaction (PCR) diagnosis to detect *tox* and *pld*, DNA was purified using a QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan).

Biotyping, toxigenicity testing, and sequencing: Biotyping of isolates was performed using API *Coryne* (BioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Sequencing of 16S rRNA gene of the isolates was carried out with purified DNA and the following primers: 16S-fD1 (5′-AGAGTTTGATCCTGGCTGAG-3′) and 16S-rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (27). Using a modified Elek test, we also examined the production of DT by the isolates (28) and an in vitro cytotoxicity assay (29,30) was carried out using the culture supernatants of the Elek broth (2% [wt/vol] proteose peptone [Oxoid, Basingstoke, UK], 0.1% [wt/vol] yeast extract [Oriental Yeast Co., Ltd., Tokyo, Japan], 0.26% NaCl, pH 7.4) for 24 h at 37°C with shaking (19). The culture supernatants were prepared by removing bacterial cells by centrifugation at 4,000 × *g* for 10 min. PLD activity was assayed a modified Zaki method (20,31). Briefly, isolates were grown in Brain heart infusion broth (Oxoid) for 24 h at 37°C with shaking. Bacterial cells were eliminated by centrifugation at 4,000 × *g* for 10 min. Sheep erythrocytes in Alsevers solution were washed three times in 10 volumes of buffer-saline (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.85% [wt/vol] NaCl, 0.1% [wt/vol] bovine serum albumin [Sigma-Aldrich Japan K.K., Tokyo, Japan], pH 7.3). Dilutions of the culture supernatants in buffer-saline (100 μl) were incubated with 1.5% washed sheep erythrocytes (100 μl) at 37°C for 45 min in a 96-well microtiter plate (Asahi Glass, Co., Ltd., Tokyo, Japan). *Staphylococcal* sphingomyelinase C (Biomol. International, L. P., Pa., USA) (0.01 U/50 μl) was added to each well. After incubation of the plate at 37°C for 60 min, it was allowed to stand on ice for 60 min. Zaki unit values of the samples were calculated from the dilution in which sheep blood cells were no longer protected from lysis by sphingomyelinase C. PCR was performed using TaKaRa *ExTaq* polymerase (Takara, Kyoto, Japan) according to the manufacturer's instructions. For PCR diagnosis, a fragment (248 bps) of *tox* in the isolates was detected by the method of Nakao et al. using the primers Tox1 and Tox2 (32). *C. diphtheriae* PW8 was used as a positive control. As a negative control, no templates were added to the reaction mixture. PCR diagnosis of *pld* was performed with the following primers based on published *pld* sequences (pldFw [5′-AACATCTCTCCGTGAAAAAT-3′; position, -157 to -137 base] and pldRv [5′-AGCTCAATCGCACCGTT GTC-3′; position, 1,165 to 1,180 base]). For the sequencing of *tox*, *pld*, and *attB* site-related genes, we carried out amplification using toxFw (5′-CCATGTAACCAATCTATCAA-3′; position, -179 to -159 base) and toxRv (5′-GTGCGCCCG GAGGGA-3′; position, 1,720 to 1,735 base) based on our sequence data, pldFw and pldRv, and CU-Rfw (5′-CGCGGA TCCGCGTACTCTCCGGGGCA-3′; position, 833 to 851 base on DLT) and attBRv (5′-CCGCAAGCACGAAGAAAC

Table 1. Clinical isolates of *C. ulcerans* from Japan

Strain	Date of isolation	Age (years)/sex	Source	Clinical details	Reference
Isolated from human					
0102	2001/Feb	52/F	Throat swab	Sore throat	13
0509	2005/Sep	57/M	Throat swab	Fever, partitas	This study
0510	2005/Oct	51/M	Lung	Lung lesion	25
0607	2005/Nov	58/F	Throat swab	Pseudomembrane	This study
Isolated from killer whale					
Ran	2004/Aug	16/F	Blood, Lung	Bacteremia, Purulent pneumonia	This study
O-9	2004/Sep	15 - 16/M	Blood, Lung	Bacteremia, Purulent pneumonia	This study
Isolated from lion					
lion	2005/Dec	6/M	Blood	Sepsis	This study

F, female; M, male.

CCCAGCCCACA-3′), respectively. The sequence data were analyzed by GENETYX-MAC 10.0 and DNASIS-Mac v3.2.

Ribotyping and PFGE: For the detection of genetic correlations, isolates were analyzed by ribotyping (7,33) and PFGE (34,35). For ribotyping, 15 μg of total DNA was cleaved with 40 units of *Bst*EII (New England Biolabs Inc., Beverly, Mass., USA). The digested fragments were subjected to 1% agarose gel electrophoresis for 15 h and the samples were then blotted onto Hybond-N$^+$ membranes (GE Healthcare, Buckinghamshire, UK) with a Capillary Blotting Unit (Scotlab, Ltd., Strathclyde, UK). The efficiency of DNA transfer was confirmed by staining the gels with ethidium bromide, and the DNA fragments on the membranes were fixed with 0.4 N NaOH. Each membrane was further incubated at 37°C for 30 min with DIG Easy Hyb (Roche Diagnostics K.K., Tokyo, Japan), and hybridization was performed for 15 h at 37°C with fresh DIG-Easy Hyb containing DIG-labeled Oligo5Mix (100 pmol each) (33), 25 mg/ml fish DNA (Roche Diagnostics), 10 mg/ml Poly (A) (Roche Diagnostics), and 5 mg/ml Poly d (A) (Roche Diagnostics). After the samples were rigorously washed twice with 2 × SSC-0.1% SDS for 5 min at room temperature and treated twice with 0.5 × SSC-0.1% SDS for 15 min at 37°C, the bands were visualized with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) and detected using CDP-Star (GE Healthcare) and Hyperfilm ECL (GE Healthcare) in a cassette with an enhancing screen. For PFGE, each strain was grown on a sheep-blood agar plate for 24 h at 37°C. The cells were harvested and suspended in 0.5 ml PIV buffer (1 M NaCl, 10 mM Tris-HCl, [pH 7.5]). A portion (0.25 ml) of the suspension was mixed with 0.25 ml 1.6% Seakem® Gold Agarose (Cambrex Bio Science, Rockland, Maine, USA) at 50°C and then pipetted into a plug mold (Bio-Rad, Hertfordshire, UK). The agarose blocks were incubated overnight at 37°C in lysis buffer (6 mM Tris-HCl [pH 7.5], 1 M NaCl, 0.1 M EDTA, 0.5% Briji 58 [Sigma-Aldrich Japan], 0.2% deoxycholate [Wako Pure Chemical Industries, Ltd., Kyoto, Japan], 0.5% sodium lauroyl sarcosine, 1 mg/ml lysozyme [Nacalai Tesque, Kyoto, Japan]), followed by deproteination in proteolysis buffer (0.5 M EDTA [pH 8.0], 1% sodium lauroyl sarcosine [Sigma-Aldrich Japan], 2 mg/ml Proteinase K [Nacalai Tesque]) for 48 h (35). The DNA in the plug was cleaved with *Sfi*I (New England BioLabs), and PFGE was carried out in 1% agarose gel with 0.5 × Tris-borate-EDTA buffer at 14°C using CHEF Mapper (Bio-Rad). PFGE profiles were analyzed using Fingerprinting™ II software (Bio-Rad).

Southern blotting: *Bam*HI-digested DNA fragments were applied directly to 1.0% agarose gel and electrophoresis was performed with 1 × Tris-acetate-EDTA buffer for 14 h. The fractionated DNA was then transferred to a Hybond-N$^+$ membrane as described above. The membrane was incubated at 37°C for 30 min with DIG Easy Hyb, and hybridization was performed at 37°C for 15 h with fresh DIG-Easy Hyb (Roche Diagnostics) containing 20 ng/ml DIG-labeled probes. After the membrane was washed, bands were detected with alkaline phosphatase-conjugated anti-DIG antibody. The *tox*, *pld*, and *attB* site probes were obtained from a DIG PCR synthesis kit (Roche Diagnostics) using *C. diphtheriae* PW8 with Tox1 and Tox2 primers, strain ATCC®51799 with pldFw and pldRv primers, and strain ATCC®51799 with attBFw (5′-GCGCC CGTAGCTCAACGGATAGAGCA-3′) and attBRv primers, respectively.

RESULTS

Biotyping, sequencing of DLT and PLD, and toxigenicity: Isolates from Japan were identified as *C. ulcerans* using the API *Coryne* system (API code 0111326). Biochemical characteristics of the isolates were also confirmed by partial sequencing of the 16S rRNA gene, which was almost identical (99.9%) to that of strain ATCC®51799 (GenBank accession no. X84256) (27). To determine the toxigenicity of the isolates, we performed a diagnostic PCR using primers Tox1 and Tox2 for the detection of *tox*. Strains ATCC®51799 and lion did not harbor *tox* (nontoxigenic strains); however, strains 0102, Ran, O-9, 0509, 0510, and 0607 retained *tox* (toxigenic strains). We also determined the DLT production of the isolates using an Elek test and a cytotoxicity assay using Vero cells, and the results were consistent with the PCR findings (Table 2). Diphtheria antitoxin completely neutralized the culture supernatants of strains 0102, Ran, O-9, 0509, 0510, and 0607 (data not shown). We then sequenced the complete *tox* genes (1,683 bp) of strains Ran, O-9, 0509, 0510, and 0607, and compared these sequences with that of *tox* from strains 0102 (GenBank accession no. AB304278), A6361 (GenBank accession no. AY141014) (37), and *C. diphtheriae* (GenBank accession no. K01722) (36) (Table 3). Strains Ran and O-9 possessed identical *tox* sites (GenBank accession no. AB304279). The *tox* genes of strains 0509 and 0606 were

Table 2. Toxigenicty of *C. ulcerans* and *C. diphtheriae*

Strain	DLT			PLD	
	PCR	Cytotoxicity[1] (CD_{50}/25 μl)	Elek test	PCR	Zaki assay[2] (U/ml)
C. ulcerans					
ATCC®51799	−	ND	−	+	20
0102	+	362	+	+	40
Ran	+	256	+	+	20
O-9	+	362	+	+	20
lion	−	ND	−	+	160
0509	+	512	+	+	40
0510	+	512	+	+	80
0607	+	256	+	+	320
C. diphtheriae					
PW8	+	3,200	+	−	ND
ATCC700971	+	128	+	−	ND

[1]: Cytotoxicities of culture supernatants measured by pH color change methods.
[2]: *Staphylococcal* sphingomyelinase C inhibition activity in cultured supernatants.
+, positive; −, negative; ND, not detected.

Table 3. Nucleotide sequence and deduced amino acid residue homology of diphtheria-like toxin produced from *C. ulcerans*

Isolate	% Homology for nucleotide (roman) and deduced amino acid (*italic*) sequence[1]				
	C. ulcerans				*C. diphtheriae*
	0102	O-9	0510	A6361	DT
0102		*100.0*	*99.10*	*100.0*	*95.00*
O-9	99.70		*99.10*	*100.0*	*95.00*
0510	98.57	98.51		*99.10*	*94.82*
A6361	100.0	99.70	98.57		*95.00*
DT	95.18	95.24	95.18	95.18	

[1]: Sequences were aligned using GENETYX-MAC 10.0, and percent identities were determined by DNASIS-Mac v3.2 without gaps as the denominator.

Table 4. Nucleotide sequence and deduced amino acid residue homology of phospholipase D produced from *C. ulcerans*

Isolate	% Homology for nucleotide (roman) and deduced amino acid (*italic*) sequence[1]						
	ATCC*51799	0102	O-9	lion	0510	0607	ATCC739
ATCC*51799		*99.69*	*97.39*	*99.02*	*97.72*	*99.02*	*98.04*
0102	98.05		*96.74*	*99.67*	*97.72*	*99.67*	*98.04*
O-9	96.75	96.96		*97.06*	*98.37*	*97.06*	*96.74*
lion	98.16	99.89	97.07		*98.74*	*100.0*	*98.37*
0510	96.96	97.18	98.18	97.29		*98.74*	*98.37*
0607	99.05	99.78	96.96	99.89	97.18		*98.37*
ATCC739	97.40	96.86	96.10	96.96	98.26	96.86	

[1]: Sequences were aligned using GENETYX-MAC 10.0, and percent identities were determined by DNASIS-Mac v3.2 without gaps as the denominator.
GenBank accession nos. ATCC*51799, AB304281; 0102, AB304282; O-9, AB304283; lion, AB304284; 0510, AB304285; 0607, AB304286.

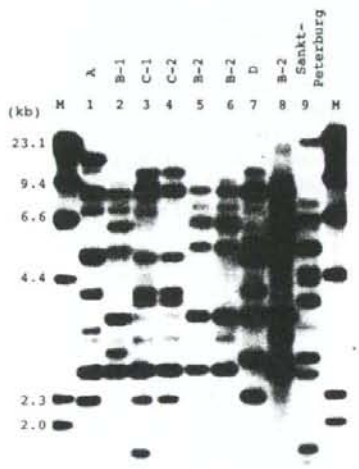

Fig. 1. *Bst*EII rRNA gene profiles of isolates and ATCC strains. M, molecular size standard (sizes are indicated on the left); lane 1, ATCC*51799; lane 2, 0102; lane 3, Ran; lane 4, O-9; lane 5, lion; lane 6, 0509; lane 7, 0510; lane 8, 0607; lane 9, *C. diphtheriae* ATCC 700971.

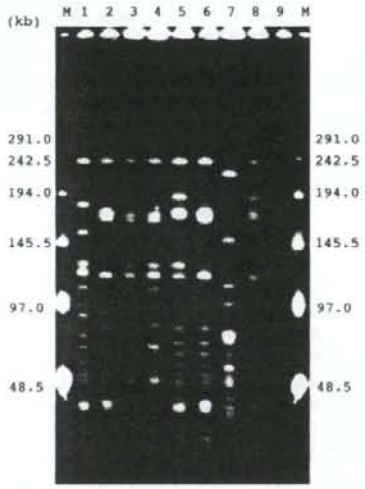

Fig. 2. *Sfi*I PFGE profiles of *C. ulcerans* isolates from Japan and ATCC strains. M, lambda concatemer as a size standard (sizes are indicated on the left); lane 1, ATCC*51799; lane 2, 0102; lane 3, Ran; lane 4, O-9; lane 5, lion; lane 6, 0509; lane 7, 0510; lane 8, 0607; lane 9, *C. diphtheriae* ATCC 700971.

indistinguishable from that of strain 0102; however, that of strain 0510 (GenBank accession no. AB304280) differed by 5 amino acid residues from that of strain 0102. Although the majority of *C. ulcerans* strains possess the PLD gene, confinement data on the PLD production of isolates were not available. When we determined by PCR analysis that the isolates in Japan had *pld* and that they yielded PLD in the Zaki assay (Table 2), strains 0102, Ran, O-9, 0509, 0510, and 0607 were found to produce both DLT and PLD, whereas strain lion generated PLD only, as seen in strain ATCC®51799. We sequenced the *pld* gene of the isolates in order to examine their similarity, because they could potentially be of use as a distinctive marker within the genus *Corynebacterium*. The sequences closely resembled (over 95% similarity) that of *C. ulcerans* ATCC 739 (GenBank accession no. L16585) (20) (Table 4).

Ribotyping and PFGE analysis: We determined the genomic relationship of isolates using ribotyping and PFGE. Figure 1 shows that six ribotype patterns (ribotype A, B-1, B-2, C-1, C-2, and D) were identified among the nine strains; ribotype B-1 was identical to ribotype B-2, with the exception of one band, and ribotype C-1 contained one band more than ribotype C-2. Ribotype groups B (B-1 and B-2) and C (C-1 and C-2) exhibited high similarity, despite their different sources. There were two unique ribotypes in the reference strain ATCC®51799 (ribotype A) and strain 0510 (ribotype D). PFGE using *Sfi*I was performed in order to further discriminate between ribotypes (Fig. 2). Four types (P1, P2, P3, and P4) of PFGE pattern were found among the nine strains using cluster analysis (Fig. 3). P2 consisted of a cluster and was classified as ribotype group B. There was high (97.5%) relatedness between strains Ran and O-9 (P3). The PFGE patterns of strains ATCC®51799 (P1) and 0510 (P4) were distinct from each other, as were the ribotype patterns. Ribotype and PFGE type had the same groupings as those of the nine strains.

Southern hybridization and sequencing of the *attB* site of isolates: To determine the location of *tox* and the *attB* site in the isolates, we performed a Southern blot analysis with *tox* and *attB* sites probes. *Bam*HI-digested DNA was used because the *tox* genes did not contain any *Bam*HI sites. Of the eight strains tested, only strain 0102 contained two frag-

ments hybridizing with *tox*-probe (*tox* fragment). The other toxigenic strains had one *tox* fragment, and, as expected, no reactive bands were observed in strains ATCC®51799 and lion (Fig. 4A). One *attB* homologous site was present in the nontoxigenic strains; however, 2 or 3 *attB* homologous sites were present in toxigenic strains (Fig. 4B). Southern blotting was also carried out with a *pld*-specific probe, which demonstrated that each strain possessed a single *pld* copy (Fig. 4C). The toxigenic strains had one or two bands that reacted with both the *tox* and *attB* site probes; however, no *pld* genes were colocated with any *attB* homologous regions.

The Southern blots indicated that both the *tox* gene and *attB* site were located together in single *Bam*HI-digested fragments. PCR analysis with the primers attBRv and CU-Rfw was performed to clarity the relevance of these sequences. These primers amplified a region of 635 bps in all toxigenic strains (data not shown). Figure 5 shows the alignment of regions downstream of the *tox* and *attB* sites of strains 0102, O-9, 0510, known *attB*-related sites of *C. ulcerans* and *C. diphtheriae*, and the *attP* site of corynephage. The regions of strains 0102 and O-9 were identical to those of strains 0509 and 0607, and that of strain Ran, respectively. There were major portions of the *attP*/*attB* common core, which was created by site-specific recombination between the *attP* and *attB* sites (24), located 83 bps after the *tox* stop codon. The regions were more similar to the *attP* site than to the *attB*-related site.

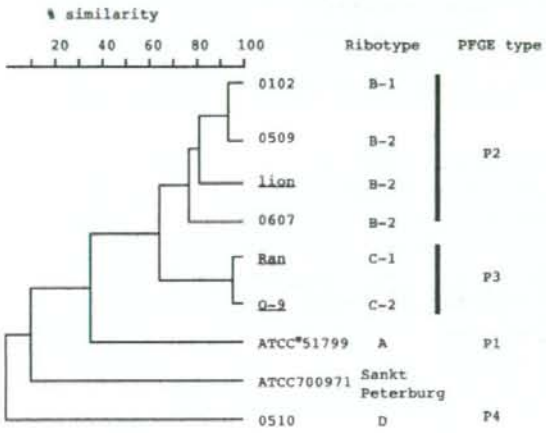

Fig. 3. Dendrogram showing the genetic relationships of *C. ulcerans* isolates in Japan. The isolates obtained from animals are underlined.

DISCUSSION

We described here the first cases of *C. ulcerans* isolated from *O. orca* and *P. leo*, the utility of ribotyping and PFGE for epidemiological surveillance, the similarity of *tox* and *pld* sequences between these strains, and the properties of the corynephage attachment site.

Although person-to-person spread of toxigenic *C. ulcerans* was not detected, it is thought that diseased animals were associated with the *C. ulcerans* infection of patients in some cases (12). To clarify the source of the bacterium, epidemiological surveillance is needed. The utility of subtyping of *C. ulcerans* has not been investigated in nearly as much detail as has that of *C. diphtheriae*. Subtyping methods include serotyping, phage typing, ribotyping, and PFGE (38-42). We carried out ribotyping and PFGE using the same protocols as those used for examination of *C. diphtheriae*. Interestingly, the lion strain and certain other clinical isolates (strains 0509 and 0607) shared similar ribotypes and PFGE type patterns. It is possible that the isolates were genetically homologous. The data indicated the possibility of the association of some diseases in animals with *C. ulcerans* infection in humans in Japan, which has also been observed in European countries. Moreover, *tox* and *pld* gene sequencing, Southern blot, ribotyping, and PFGE analyses demonstrated that two of the isolates from *O. orca* corresponded to a single strain. This finding pointed out the possibility of ease of transmission due to close contact with animals. Strains Ran, O-9, and lion were isolated from one facility. This finding suggested that there were both toxigenic and nontoxigenic bacteria present at the same facility. Because we were unable to use European strains in this study, there are no definite conclusions regarding differences between Japanese and European strains. However, these isolates in Japan are likely to be characteristic of those previously observed in Japan based on comparisons with

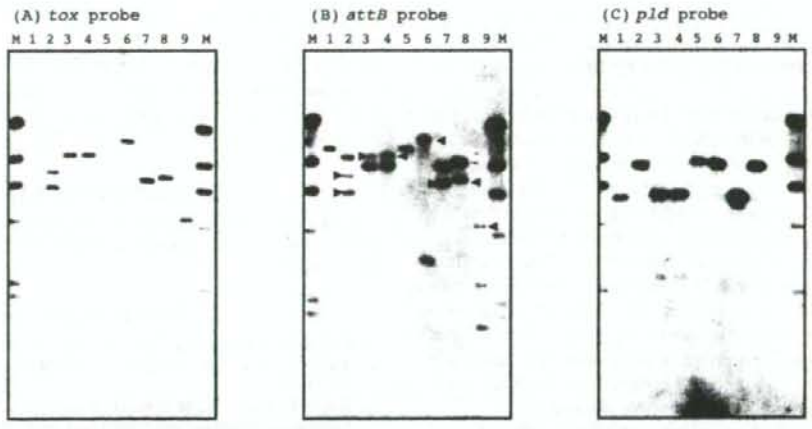

Fig. 4. Southern blot analysis of *C. ulcerans* and *C. diphtheriae* genomic DNA with DIG-labeled (A) *tox*, (B) *attB* site, and (C) *pld* specific probes. M, DIG-labeled molecular standard marker; lane 1, ATCC®51799; lane 2, 0102; lane 3, Ran; lane 4, O-9; lane 5, lion; lane 6, 0509; lane 7, 0510; lane 8, 0607; lane 9, *C. diphtheriae* ATCC 700971. Arrows indicate the bands that are the same in *tox* fragments.

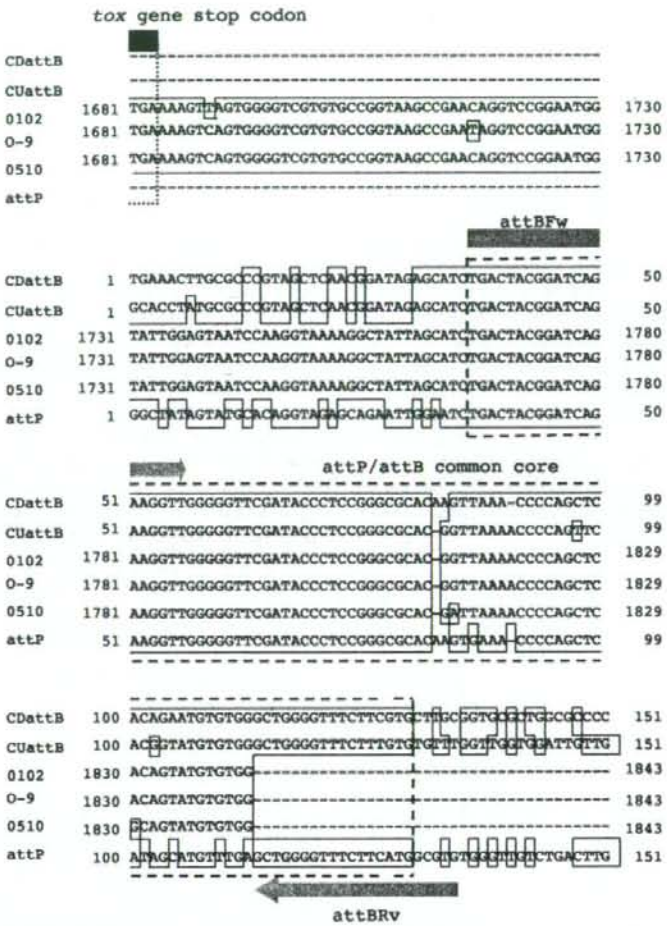

Fig. 5. Alignment of the deduced nucleotide sequences of attachment site homologous genes from *C. ulcerans* strains 0102, O-9, 0510, *C. ulcerans attB*-related site, *C. diphtheriae attB*-related site, and corynephage-gamma DNA for the *attP* site.

the ribotyping data from UK-derived isolates (7). The dendrogram derived from the PFGE pattern was same as that obtained from the ribotype grouping in our study. However, a larger number of isolates will be needed to determine the comparative usefulness of molecular epidemiological typing in *C. ulcerans*.

The results of the Southern blot analysis revealed that the *tox* genes were close to the *attB* sites in these isolates. Strain 0102 was a tandem double lysogen (unstable conformation) and the other strains were single lysogens. The production of DT by *C. diphtheriae* was shown to be proportional to the number of prophages integrated into its chromosome. The culture supernatant of strain 0102 had equivalent cytotoxic activity to that of the single lysogen strains under the conditions used here. We also demonstrated that the *attP*/*attB* common core was located downstream of the *tox* gene. The present results suggest that the number of *attB* sites in *C. ulcerans* differs from that in *C. diphtheriae*; however, the integration system appears to function in the same manner.

There have been few investigations of the pathogenicity of PLD production by *C. ulcerans*. We cannot exclude the possibility that differences in toxin production may exert an influence on *C. ulcerans* infection. Due to PLD production and the high level of similarity between *pld* genes among the isolates, PLD may be useful as a marker of *C. ulcerans* (21). It was moreover noteworthy that the DLT sequences of four toxigenic strains were identical to that of a German isolate, A6361 (Table 3). The identity of these DLT sequences suggests that there is a high degree of conservation in DLT. We are currently investigating differences in the immunological and cytotoxic activities of DLT and DT.

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NOTE *Public Health*

Evaluation of a Microplate Agglutination Test (MAT) for Serological Diagnosis of Canine Brucellosis

Masanobu KIMURA[1], Koichi IMAOKA[1]*, Michio SUZUKI[1], Tsuneo KAMIYAMA[1] and Akio YAMADA[1]

[1]*Department of Veterinary Science, National Institute of Infectious Diseases, 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8640, Japan*

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ABSTRACT. A microplate agglutination test (MAT) was compared with the tube agglutinin test (TAT), a standard test for the diagnosis of *Brucella canis*, in terms of the sensitivity and specificity. The results showed that MAT was more sensitive, simpler to perform and easier to read the results than TAT. On top of that the MAT allows us to handle a larger number of samples at once. Using this method we conducted sero-surveillance of the prevalence of *B. canis* in dogs kept in an Animal Shelter located in Kanagawa Prefecture. Twelve of 485 (2.5%) showed seropositive against *B. canis*. These results indicate that *B. canis* infection in dogs is still occurring in Japan.
KEY WORDS: *Brucella canis*, canine brucellosis, microplate agglutination test.

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Brucellosis, one of the major zoonoses worldwide, is caused by a bacteria belonging to the genus *Brucella* [4]. Among many species of the genus *Brucella*, *B. melitensis*, *B. abortus*, *B. suis* and *B. canis* are known to result in human brucellosis. Although *Brucella* spp. with smooth-type lipopolysaccharides (LPS), such as *B. melitensis*, *B. abortus* and *B. suis*, are known to infect several domestic animals, such as cows, sheep, goats and pigs, *B. canis*, one of *Brucella* spp. with rough LPS, infects a limited host range, such as dogs and wild canidae. *B. canis* infection in dogs is usually asymptomatic but can sometimes cause contagious abortion, epididymitis, testicular atrophy and infertility [3]. Most canine infections occur by direct contact with lochia at the time of abortion or vaginal discharges in infected female dogs. Semen and urine from infected male dogs have also been implicated as sources of infection [7]. Drug therapy for *B. canis* infection requires an appropriate regimen of antibiotic combination, but relapse may ensue, because *B. canis* often persists within macrophages or other type of cells [3]. Humans are rarely infected with *B. canis*. Most human infections are asymptomatic; however, several clinical symptoms, which are milder than those observed with other *Brucella* spp., are sometimes noticed [12].

In Japan, *B. canis* infection was first reported in a breeding colony of beagles in 1972 [20]. Several epidemiological studies of canine brucellosis in Japan were conducted in the 1970s and 1980s [10, 11, 15–18], but there have only been a few reports since then. In 2003 and 2006, canine brucellosis emerged as outbreaks in large breeding colonies, suggesting that *B. canis* infection is still enzootic in Japan. To assess the possible risk of *B. canis* on human, determination of the prevalence of *B. canis* in the dog population in Japan seemed helpful.

Although tube agglutination test (TAT) is the most widely used laboratory test for the detection of *B. canis* antibodies in both humans and canines, it is time-consuming and cumbersome in terms of performance and measurement of results [2]. On the other hand, microplate agglutination test (MAT) described for *B. canis* [5] and *B. abortus* [1, 6] appeared advantageous, because a larger number of samples can be processed simultaneously by this method. In the present study, we attempted to evaluate whether the use of MAT with safranine-stained bacterial cells as antigens could serve as a substitute for TAT to conduct sero-epidemiologic investigations of canine brucellosis in Japan.

TAT was carried out by placing 0.5 m*l* of 2-fold serially diluted sera and an equal volume of *B. canis* antigen solution (OD_{600}=1) purchased from the Kitasato Institute (Tokyo, Japan) in glass tubes. After incubation at 50°C for 24 hr, the agglutination titer was determined and expressed as a reciprocal of final serum dilutions, which gave rise to agglutination as observed in the 50% control tube. Titers of 160 or higher were considered positive. Anti-*B. canis* antibody was prepared in our laboratory by immunizing a rabbit with inactivated *B. canis* whole antigen and was included as a reference.

MAT was performed as follows. First, serum samples, 2-fold serially diluted in phosphate-buffered saline, were prepared in a 96-well U-bottom microplate. Then, an equal volume (25 μl) of *B. canis* antigen solution (Kitasato Institute), which is same as used in TAT, containing 0.005% safranine solution (2% of Favor G®, Nissui Pharmaceutical Co., Tokyo, Japan) was added to each well. The sealed plates were mixed gently for 20 sec and incubated at 50°C for 24 hr in a humid atmosphere. The titers were expressed as a reciprocal of the highest dilution of sera showing agglutination. Safranine-stained antigens made it possible to judge the results more easily and objectively. An agglutination titer greater than 160 was considered positive.

We have experienced an outbreak of *B. canis* infection in 2003 [8]. Sera obtained from dogs involved in the outbreak were examined for the presence of anti-*Brucella* antibody by TAT. Fifty-one of 110 sera tested positive for antibody against *B. canis*. These sera were subjected to MAT for

* CORRESPONDENCE TO: IMAOKA, K., Department of Veterinary Science, National Institute of Infectious Diseases, 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8640, Japan.
e-mail: imaoka@nih.go.jp

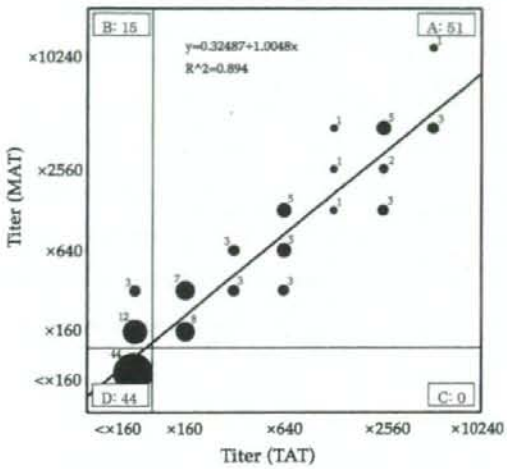

Fig. 1. Correlation of antibody titers determined by TAT and MAT. Sera were obtained from 110 dogs in the outbreak of *B. canis* infection in 2003. The titers are the reciprocals of the highest dilution of serum showing agglutination. Pearson's product-moment correlation coefficient (r) was determined to be 0.945, $p<0.01$ by regression analysis. A-D shows each area and the number of dogs of each area.

determining its specificity and sensitivity. As shown in Fig. 1, 15 sero-negative samples (1:80) by TAT became positive (1:160 and 1:320) when MAT was employed. Combinatorial polymerase chain reaction method [9] showed that *B. canis*-specific gene segments were present in the sera of those 15 dogs [8], which indicate that they were infected with *B. canis* (data not shown). Therefore, MAT appeared superior to TAT in terms of sensitivity as shown in the previous report by Dump *et al.* [5]. The titers determined by two methods correlated well ($R^2 = 0.894$) as shown in Fig. 1.

We have therefore decided to apply MAT in the sero-surveillance of *B. canis* in Japan. During the period from February 2003 to December 2006, 485 serum samples were obtained from dogs in an animal shelter in Kanagawa Prefecture (Table 1). The dogs were categorized into three groups according to their origin: pets, strays, and unknown.

The results are summarized in Table 1. Of 485 dogs, 12 (2.5%) tested positive for antibody against *B. canis*. Sero-prevalence in this study (2.5%) seemed slightly lower than those of 1970's (0.8%–21.7%) [10], but there was no apparent difference. This indicated that the disease was enzootic but not epizootic in Japan. Of the 12 sero-positive dogs, 3 were pets and 9 were strays. The reason why the apparent prevalence of infection was higher in strays (5.7%) than in pets (0.9%) was unknown, but it was likely that stray dogs had more opportunities to encounter other dogs, fomites or environments contaminated by bacteria. No differences attributed to the sex (Table 1) or breed (data not shown) of dogs were observed in the prevalence of infection.

Although symptomatic infections of *B. canis* in humans are rarely reported, Lucero *et al.* [12] pointed out that the possibility of *B. canis* infection in humans may be more widespread than speculated. Recently, an unusually severe form of human brucellosis caused by *B. canis* was reported [13]. Because human infections commonly occur after contact with the blood, semen, or placenta of infected dogs [19], an understanding of the prevalence of *B. canis* infection in dogs may help in inferring preventive measures for reducing human exposure to the bacteria. The results of the present sero-epidemiologic study showed that *B. canis* infection is still enzootic in Japan. Moreover, human brucellosis cases reported in 2002, 2005 and 2006 were probably caused by *B. canis* [14]. It therefore seems, prudent that individuals at high risk of infection such as veterinarians, breeders and pet owners, be advised of possible *B. canis* infection in dogs.

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Table 1. Sero-prevalence of antibodies against *B. canis* in dogs determined by MAT

Year	Male		Female		Total	
	Negative	Positive	Negative	Positive	Negative	Positive
2003	67	0	24	1	91	1
2004	82	3	38	2	120	5
2005	103	3	74	1	175	4
2006	52	2	29	0	81	2
Total	304	8 (2.6%)	165	4 (2.4%)	467	12 (2.5%)

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University of Berlin, Berlin (M. Lierz); and Charité, Berlin (T. Schneider)

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Address for correspondence: Andreas Jansen, Department of Infectious Diseases Epidemiology, Robert Koch Institute, Seestrasse 10, 13353 Berlin, Germany; email: jansena@rki.de

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–AB444095), 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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Hiromi Fujita, Teruki Kadosaka, Yoshiki Nitta, Shuji Ando, Ai Takano, Haruo Watanabe, and Hiroki Kawabata

Author affiliations: Ohara General Hospital, Fukushima, Japan (H. Fujita); Aichi Medical University School of Medicine, Aichi, Japan (T. Kadosaka); Okinawa Prefectural Institute of Animal Health, Okinawa, Japan (Y. Nitta); National Institute of infectious Diseases, Tokyo (S. Ando, A. Takano, H. Watanabe, H. Kawabata); and Gifu University, Gifu, Japan (A. Takano)

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Address for correspondence: Hiroki Kawabata, National Institute of infectious Diseases-Bacteriology, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan; email: kbata@nih.go.jp

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*)

Masahito Kubo [a,b], Shigehiko Uni [c], Takeshi Agatsuma [d], Mitsuru Nagataki [d], Roger J. Panciera [e], Toshio Tsubota [f], Sachiko Nakamura [a,g], Hiroki Sakai [b], Toshiaki Masegi [a,b], Tokuma Yanai [a,b,*]

[a] *United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*
[b] *Laboratory of Veterinary Pathology, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*
[c] *Department of Protozoal Diseases, Graduate School of Medicine, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan*
[d] *Department of Environmental Health Science, Kochi Medical School, Oko-cho Kohasu, Nankoku, Kochi 783-8505, Japan*
[e] *Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma 74078, USA*
[f] *Laboratory of Ecology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan*
[g] *Laboratory of Zoo and Wildlife Medicine, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*

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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

* Corresponding author. 1-1 Yanagido, Gifu 501-1193, Japan. Tel./fax: +81 58 293 2944.
E-mail address: yanai@gifu-u.ac.jp (T. Yanai).

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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).