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Prevalence of *Bartonella henselae, Bartonella clarridgeiae* and the 16S rRNA Gene Types of *Bartonella henselae* among Pet Cats in Japan

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ABSTRACT. The authors investigated bacteriologically the prevalence of *Bartonella* infection among 690 pet cats derived from 10 private animal hospitals in six cities (Sapporo, Hokkaido Prefecture; Sendai, Miyagi Prefecture; Joetsu, Niigata Prefecture; Fujisawa, Kanagawa Prefecutre; Kyoto, Kyoto Prefecture; Sanda, Hyogo Prefecutre) and 4 counties (Mishima, Osaka Prefecture; Hikawa, Shimane Prefecture; Aira, Kagoshima Prefecture; Shimajiri, Okinawa Prefecture) located from the north to the south of Japan. *Bartonella* species were isolated from 7.2% (50/690) of all the cats examined. No *Bartonella* species were isolated from the cats in Sapporo or Sendai. The isolation rate varied from 2% in Joetsu and Sanda to 20% in Shimajiri. *Bartonella clarridgeiae* was isolated from two of 50 cats in Kyoto, three of 50 in Mishima and one of 50 in Shimajiri, but not in cats from the other cities or counties. Though the cats of Joetsu, Fujisawa, Kyoto, Sanda, Aira and Shimajiri were infected with either *B. henselae* or *B. clarridgeiae*, one of eight infected cats in Mishima was harboring both *Bartonella* species. Type I of 16S rRNA gene was the predominant type among the isolates of *B. henselae*, but only one isolate derived from Shimajiri was found to be of type II. Prevalence of *B. clarridgeiae* and the 16S rRNA gene type of *B. henselae* among cats in Japan was demonstrated for the first time in this investigation.—KEY WORDS: *Bartonella clarridgeiae*, *Bartonella henselae*, feline, Japan, 16S rRNA type of *B. henselae*

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Cat scratch disease (CSD) is an emerging zoonosis caused by *Bartonella henselae* [10, 21] or *B. clarridgeiae* [16]. Although CSD patients develop mainly pyrexia, papules on the site of cat scratch or bite, and unilateral lymphadenopathy [10, 17], cats are themselves infected asymptomatically, showing long-term bacteremia with antibody formation.

The prevalence of bacteremia of *B. henselae* in cats has been reported to vary from 4 to 89% [3–5, 8, 14, 15, 19]. A recent investigation using polymerase chain reaction (PCR) showed that *B. henselae* is differentiated into two 16S rRNA gene types, types I and II [1]. Furthermore, a new *Bartonella* species, *B. clarridgeiae*, was isolated from a cat kept by an HIV-positive patient who manifested bacillary angiomatosis [7]. *B. clarridgeiae* was found to cause CSD in a veterinarian bitten by a cat infected with the organism [16] and has been isolated from pet and stray cats in France, the Netherlands and Indonesia [2, 11, 12, 18]. However, only one report is available for the isolation of *Bartonella henselae* from cats in a limited area in Japan [19]. Furthermore, there are no reports on the survey for the prevalence of not only *Bartonella* species but also the 16S rRNA gene types of *B. henselae* in cats all over Japan.

In this study, the authors investigated bacteriologically the prevalence of *B. henselae* and *B. clarridgeiae* infection among cats raised in six cities and four counties of 10 prefectures located from the north to the south of Japan. We conducted also 16S rRNA gene typing of feline isolates of *B. henselae.*

MATERIALS AND METHODS

Cat samples: During the investigation period from January 1995 to July 1998, a total of 690 blood samples were collected from cats at 10 private veterinary hospitals located in Sapporo City (Hokkaido Prefecture), Sendai City (Miyagi Prefecture), Joetsu City (Niigata Prefecture), Fujisawa City (Kanagawa Prefecture), Kyoto City (Kyoto Prefecture), Mishima County (Osaka Prefecture), Sanda City (Hyogo Prefecture), Hikawa County (Shimane Prefecture), Aira County (Kagoshima Prefecture) and Shimajiri County (Okinawa Prefecture). The veterinarians checked for cats' general body conditions and flea infestation or obtained as much information as possible from the owners. Blood was aseptically taken from cats and dispensed into 2-m*l* EDTA tubes (Venoject II, Termo, Japan). The samples were sent to our laboratory under frozen conditions and stored at −85°C until examined.

Isolation of Bartonella species: The blood in the EDTA tubes was thawed at room temperature and centrifuged at 3,800 rpm for 70 min. After centrifugation, the supernatant was removed and 120 *μl* of Medium 199 (GIBCO, U.S.A.) was added to the sediment, which was mixed well. The mixture was inoculated to two 7% rabbit blood-agar plates, which were incubated at 35°C in a 5% CO_2 atmosphere for 4 weeks.

Identification of Bartonella species: Three to five colonies suspected to be *Bartonella* were selected and subjected to identification of *Bartonella* species by PCR of citrate synthase gene (*glt*A gene) with the primers BhCS.781p (5'-

Table 1. The PFGE conditions for differentiation of *B. henselae* and *B. clarridgeiae* with each restriction enzyme

Enzyme	Digestion condition	Voltage	PFGE running conditions		
			Pulse time	Run time	Agarose concentration
Asc I	20 IU/plug, 37°C, overnight	4.5 V/cm	5–120S	33 hr	1.0%
Sma I	20 IU/plug, 30°C, overnight	5.7 V/cm	3–10S	26 hr	1.5%

GGG GAC CAG CTC ATG GTG G-3') and BhCS.1137n (5'-AAT CGA AAA AGA ACA GTA AAC A-3') and restriction fragment length polymorphism (RFLP) analysis by the digestion of the amplified *glt*A gene with *Taq* I and *Hha* I (Takara Biochemicals, Japan) [19]. Furthermore, *B. clarridgeiae* was identified by pulsed-field gel electrophoresis (PFGE) by digestion with *Sma* I and *Asc* I and comparing the results with the profiles of a reference strain of *B. clarridgeiae* (ATCC 51734). The conditions of PFGE are given in Table 1.

After identification of *B. clarridgeiae* by RFLP and PFGE, two representative strains isolated from the cats in Mishima and Shimajiri were subjected to DNA sequencing of the *glt*A gene for confirmation of the species. Sequencing was performed with a DSQ-2000L DNA sequencer (SHIMADZU, Kyoto, Japan) by using primer BhCS.781p labeled with fluorescein isothiocyanate. The DNA sequences of the *glt*A gene from two *B. clarridgeiae* strains were compared with that of a reference contained in the data base of the DNA Data Bank of Japan by using Genetyx-Mac software.

16S rRNA gene typing: The 16S rRNA gene typing of *B. henselae* was performed by PCR following the method of Bergmans *et al.* [1] with a minor modification. Briefly, 41 μl of super *Taq* premix kit (Sawady Technol. Co., Ltd., Japan), 2 μl of each set of 16SF and BH1 or 16SF and BH2 primers, 5 μl of the extracted DNA sample and 50 μl of sterile mineral oil were dispensed into a 500-μl Eppendorf tube. DNA amplification was performed with Zymoreactor II AB-1820 (Atto Corp., Japan) with initial denaturation (95°C, 3 min), followed by 30 cycles of denaturation (95°C, 20 sec), annealing (56°C, 30 sec) and extension (73°C, 1 min), with a single final extension step (73°C, 5 min). The amplified PCR product was subjected to electrophoresis in a 4% agarose (NuSieve GTG agarose, FMC BioProducts, Rockland, ME, U.S.A.). When a specific band of 185 bp was detected with primers 16SF and BH1, the strain was identified as type I. When the specific band of 185 bp was observed with primers 16SF and BH2, the strain was regarded as type II.

Statistical analysis: The results obtained were analyzed by the χ^2 test.

RESULTS

Prevalence of Bartonella infection among pet cats in Japan: The overall prevalence of *Bartonella* species among pet cats in Japan was found to be 7.2% (50/690). No *Bartonella* species were isolated from those in either Sapporo or Sendai. The isolation rate varied from 2.0% (1/49 and 1/50) in Joetsu and Sanda to 20% (10/50) in Shimajiri (Fig. 1). The positive rates in Kyoto, Mishima and Shimajiri were significantly higher than those in Joetsu, Fujisawa and Sanda ($p<0.01$).

Regarding the age of the cats, the positive rate of *Bartonella* species was found to be 9.5% (12/126) in cats under 1 year old, 11.8% (14/119) in those 1 to <2 years old, 14.8% (9/61) in those 2 to <3 years old, 3.4% (11/325) in those over 3 years old and 6.8% (4/59) in age-unknown cats. The positive rates in cats under 1 year old, 1 to <2 years old and 2 to <3 years old were significantly higher than those in cats over 3 years old ($p<0.01$). The rate of flea infestation ranged from 4.0% in Sapporo to 58% in Shimajiri (not examined in Joetsu or Hikawa) (Table 2).

Identification of Bartonella species: In PCR for the citrate synthase gene, *B. henselae* showed a single band of 380 bp, and *B. clarridgeiae* had an extra faint band of around 510 bp. All the amplified *glt*A genes of *B. henselae* digested with *Taq*I showed three fragments. On the other hand, those of *B. clarridgeiae* revealed the same profiles as undigested ones. Two fragments were observed in the digestion of amplified *glt*A gene of *B. henselae* with *Hha*I. Three fragments were obtained by digestion of the amplified *B. clarridgeiae glt*A gene. The RFLP profiles of the PCR products digested with *Taq*I and *Hha*I allowed differentiation of *B. henselae* from *B. clarridgeiae* (Fig. 2). Although the PFGE profiles of the digestion with *Sma*I and *Asc*I of *B. henselae* genomic DNA showed various profiles depending upon the strains (data not shown), those of *B. clarridgeiae* revealed specific profiles after digestion with both enzymes (Fig. 3).

Two representative cat isolates of *B. clarridgeiae* had a sequence identical to the reference *glt*A gene by Genetyx-Mac software.

B. henselae type I showed a specific single band of 185 bp as a result of PCR of the 16S rRNA gene with the primer set of 16SF and BH1. *B. henselae* type II revealed a specific band of 185 bp and one extra band of around 700 bp under the PCR conditions with the primers of 16SF and BH2 (Fig. 4).

Distribution of the 16S rRNA gene types and B. clarridgeiae: *B. clarridgeiae* was isolated from a cat in Shimajiri, two cats in Kyoto and three cats in Mishima. Although most positive cats harbored one species, either *B.*

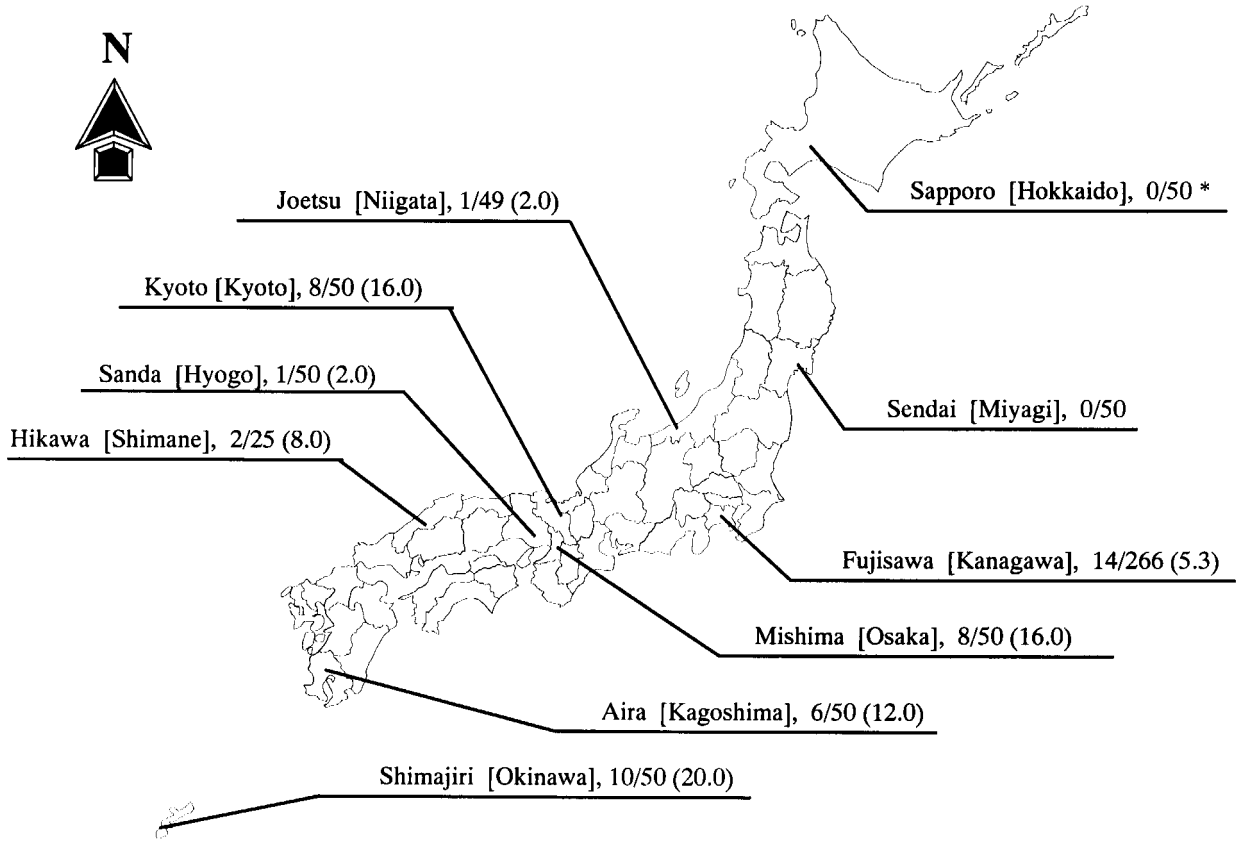

Fig. 1. Prevalence of *Bartonella* species among pet cats in Japan. * City or County [Prefecture], No. positive/No. examined (%)

Table 2. Prevalence of *Bartonella* species in relation to cat age groups and flea infestation of cats

City or County (Prefecture)	Cat age group				flea infestaiton (%)
	1>	1–<2	2–<3	3≤	
Sapporo (Hokkaido)	0/10[a)]	0/7	0/2	0/31	2/50 (4.0)
Sendai (Miyagi)	0/9	0/7	0/3	0/31	20/50 (40.0)
Joetsu (Niigata)	0/3	0/10	0/5	1/13	not examined
Fujisawa (Kanagawa)	5/50	3/45	2/18	2/119	82/258 (31.8)[c)]
Kyoto (Kyoto)	0/13	3/8	2/3	2/25	5/50 (10.0)
Mishima (Osaka)	4/4	2/11	1/7	0/22	21/50 (42.0)
Sanda (Hyogo)	0/18	0/8	0/3	1/21	14/50 (28.0)
Hikawa (Shimane)	0/1	0/2	2/8	0/14	not examined
Aira (Kagoshima)	0/8	2/11	1/4	3/27	23/50 (46.0)
Shimajiri (Okinawa)	3/10	4/10	1/8	2/22	29/50 (58.0)
Total (%)	12/126 (9.5)[b)]	14/119 (11.8)[b)]	9/61 (14.8)[b)]	11/325 (3.4)	

a) No. positive/No. examined.
b) Statistically significant from the cat group of over 3 years old ($p<0.01$).
c) The flea infestation was not examined in eight cats.

henselae or *B. clarridgeiae*, a cat in Mishima was infected with both species. Most isolates of *B. henselae* were identified as 16S rRNA gene type I, with one isolate of type II detected in Shimajiri (Table 3).

DISCUSSION

In this study, it was found that 7.2% of cats examined were positive for *Bartonella* species. Although no *Bartonella* species were isolated from the northern areas, Sapporo (Hokkaido Prefecture) or Sendai (Miyagi

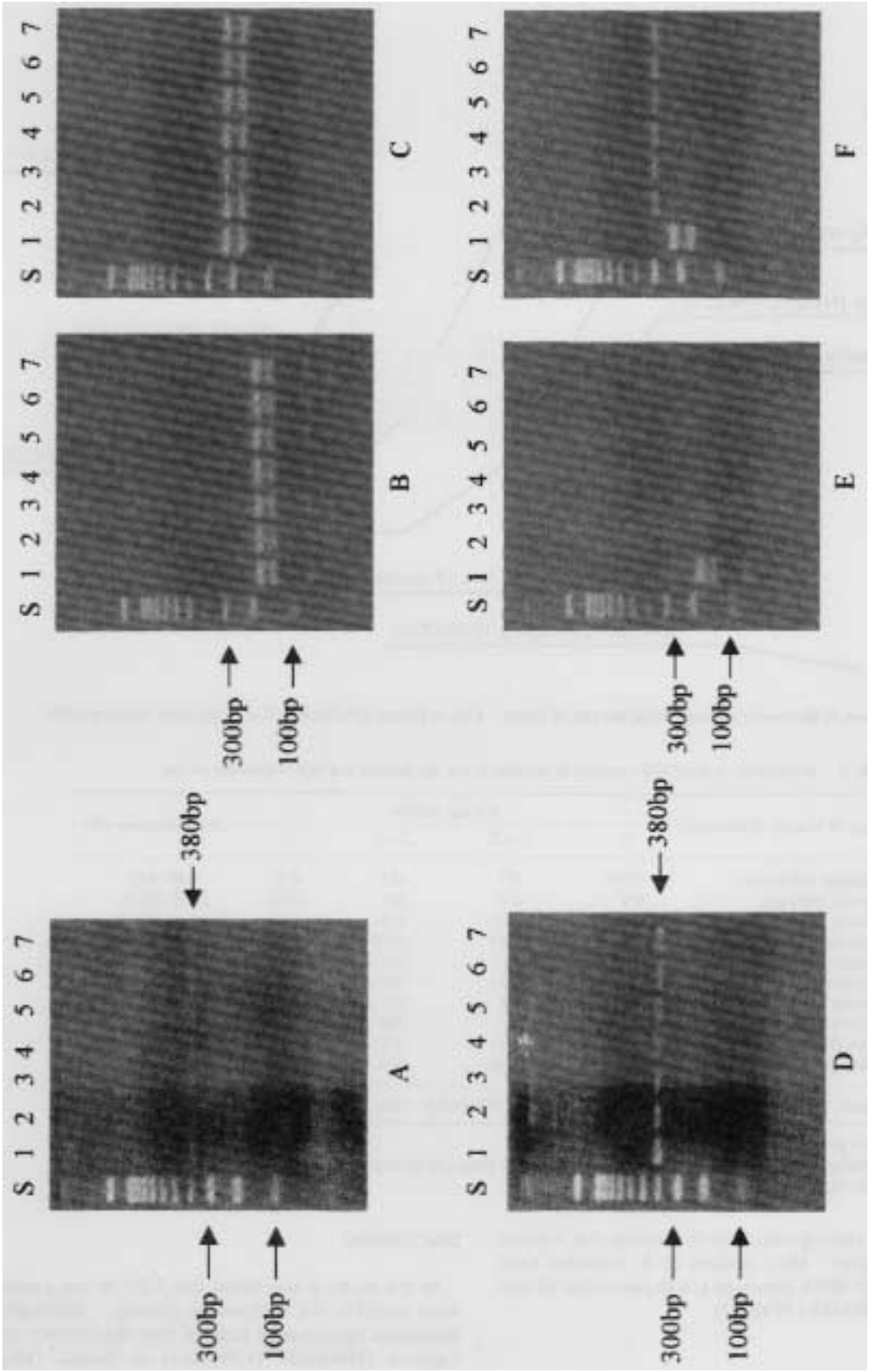

Fig. 2. Identification of *Bartonella* species by PCR-amplified citrate synthae gene (A and D) and the RFLP profiles after digestion with *Taq* I (B and E) and *Hha* I digestions (C and F). A-C; size standard (S), *B. henselae* ATCC 49882 (lane 1), *B. henselae* strains isolated from two different cats in Mishima (lanes 2–7), D-F; Size standard (S), *B. henselae* ATCC49882 (lane 1), *B. clarridgeiae* ATCC 51734 (lane 2), *B. clarridgeiae* strains isolated from two different cats in Shimajiri (lanes 3–5) and Kyoto (lanes 6 and 7).

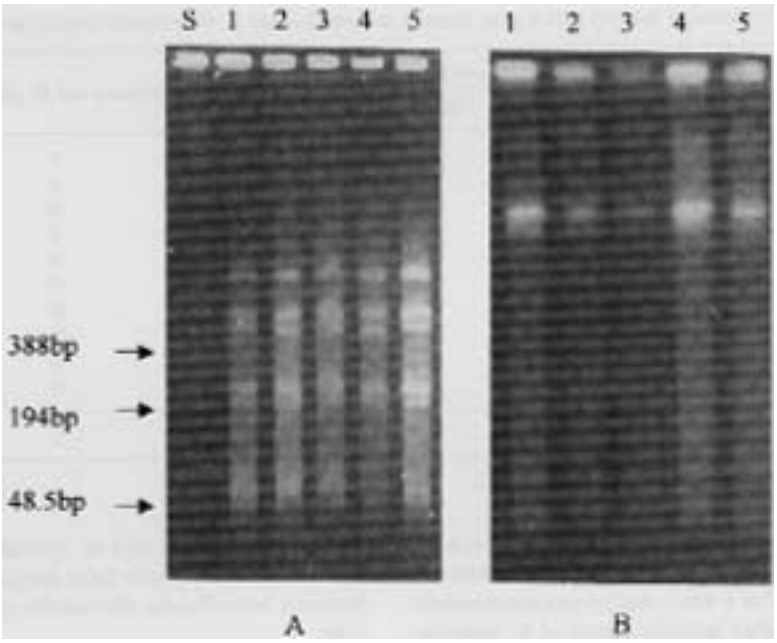

Fig. 3. Identification of *B. clarridgeiae* from PFGE profiles after digestion with *Asc* I (A) and *Sma* I (B). A; Size standard (S), *B. clarridgeiae* strains isolated from two different cats in Shimajiri (lanes 1–3) and Kyoto (lanes 4 and 5). B; *B. clarridgeiae* strains isolated from two different cats in Shimajiri (lanes 1–3) and Kyoto (lanes 4 and 5).

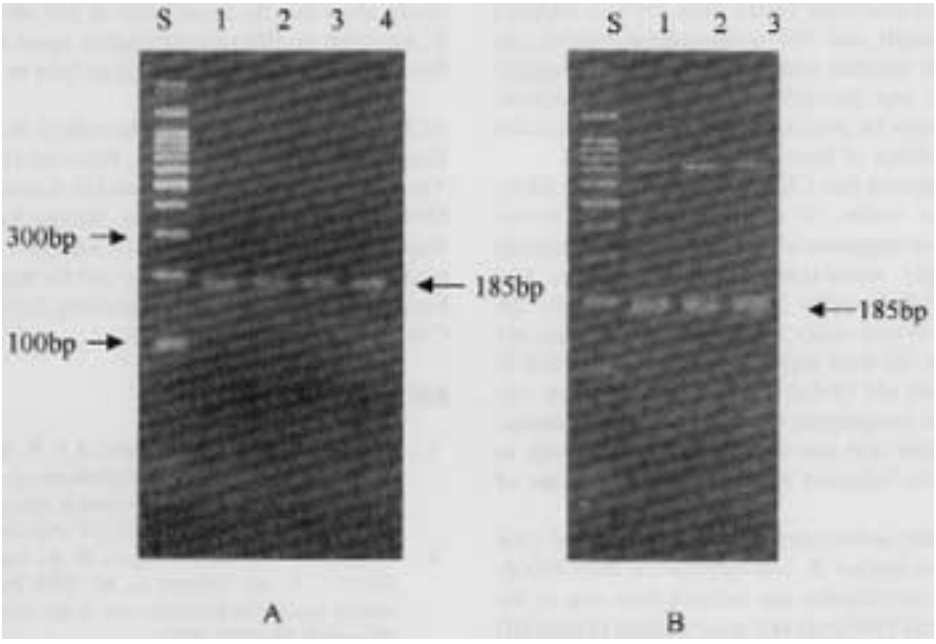

Fig. 4. Identification of the 16S rRNA gene types of *B. henselae* with 16SF and BH1 primers (A) and with 16SF and BH2 primers (B). A; Size standard (S), *B. henselae* ATCC 49882 (lane 1), *B. henselae* type I isolated from a cat in Mishima (lanes 2–4), B; *B. henselae* type II strains isolated from a cat in Shimajiri (lanes 1–3).

Prefecture), the highest prevalence of bacteremic cats was found in Shimajiri (Okinawa Prefecture) (20%) followed by Kyoto (Kyoto Prefecture) (16%) and Mishima (Osaka Prefecture) (16%). Shimajiri is located in Okinawa, the most southwestern prefecture of Japan (24° 20' to 26° 10' N/127° 40' to 128° 20' E), and the climate is subtropical

Table 3. Prevalence of the 16S rRNA gene types of *B. henselae* and *B. clarridgeiae* in pet cats

City or County (Prefecture)	*B. henselae*		*B. clarridgeiae*	*B. henselae* and *B. clarridgeiae*
	type I	type II		
Sapporo (Hokkaido)	0	0	0	0
Sendai (Miyagi)	0	0	0	0
Joetsu (Niigata)	1	0	0	0
Fujisawa (Kanagawa)	14	0	0	0
Kyoto (Kyoto)	6	0	2	0
Mishima (Osaka)	5	0	2	1[a)]
Sanda (Hyogo)	1	0	0	0
Hikawa (Shimane)	2	0	0	0
Aira (Kagoshima)	6	0	0	0
Shimajiri (Okinawa)	8	1	1	0
Total	43	1	5	1

a) *B. henselae* isolated was identified as type I.

(average temperature: 14.0°C in January to 31.2°C in July, humidity: 62% in January to 82% in August, 1997). Jameson *et al.* suggested that cats in a warm and humid environment were associated with higher seroprevalence of *B. henselae* than those in a cold and dry environment [13]. Ueno *et al.* also found that a higher seroprevalence of cats was observed in the central and southwestern areas than in the northeastern areas of Japan [24]. Furthermore, *B. henselae* bacteremia was experimentally transmitted from cat to cat by fleas (*Ctenocephalides felis*) [6]. In this study, the rate of flea infestation in cats examined varied from 4.0% in Sapporo to 58.0% in Shimajiri, and 70% of *Bartonella*-positive cats in Shimajiri were infested with fleas. These facts suggest that the climate and prevalence for arthropod vectors, especially fleas, may be associated with bacteriological and serological prevalence of *Bartonella* species in cats.

It has been reported that CSD patients were more likely to own a kitten under 12 months old [4]. Several investigations have suggested also that cats under 12 months old are strongly associated with bacteremia and seropositivity of *B. henselae* [2, 4, 8]. In this study, the bacteremic rates in cats under 1 year old, 1 to <2 years old and 2 to <3 years old were significantly higher than that in those over 3 years old ($p<0.01$), though the positive rate was rather low in comparison with that in other countries. These data indicate that cats in Japan are more likely to acquire *Bartonella* infection during their first 3 years of life.

In this study, the authors demonstrated for the first time that cats in Japan harbor *B. clarridgeiae* in their blood. Interestingly, *B. clarridgeiae* was isolated from cats in the western (Kyoto and Mishima) and southwestern (Shimajiri) areas, but not in the northern, northeastern, northwestern or central areas of Japan. Although most cats harbored a single species, *B. henselae* or *B. clarridgeiae* in their blood, one cat in Mishima was infected with both species. Gurfield *et al.* [11] also reported coinfection with several strains of *B. henselae* or both *Bartonella* species in French cats. Yamamoto *et al.* [25] showed the lack of cross-protection between *B. henselae* and *B. clarridgeiae* in experimentally inoculated cats. These facts suggest that cross-protection between heterologous *Bartonella* species may not occur in cats.

It has been reported that *B. henselae* type II was detected from 18% of the isolates from CSD patients in the Netherlands [2], 19% of cat isolates in France [12] and 94% of those isolates in Germany [23]. By contraries, the authors found that only one cat in Shimajiri harbored type II of *B. henselae* among the cats examined in this study. These results show that the distribution of 16S rRNA gene type of *B. henselae* is different depending upon the country and that type I is the predominant gene type in Japan.

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