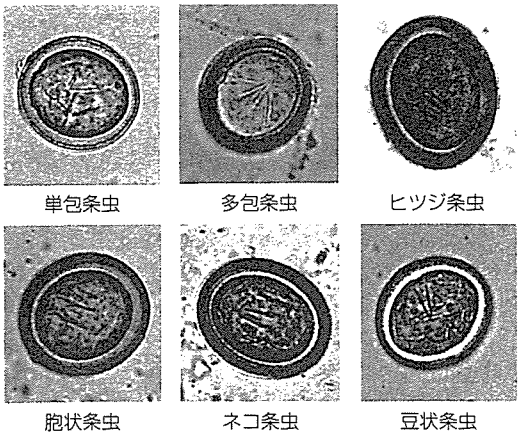

図 2　テニア科条虫卵の比較
大きさはいずれも 30～40 μm.

である.

イヌのエキノコックス検査法

エキノコックスの検査が必要となるイヌは，通常であれば有病地（国内であれば北海道）で飼育歴のあるものに限られる.検査材料は糞便であり，虫体や虫卵の検出・同定を試みるか，それらに由来する抗原や遺伝子の検出を図ることによって行われる．虫卵検査は寄生虫の検査法としてもっとも一般的な方法であるが，エキノコックス（単包条虫，多包条虫）の場合は，虫卵が検出されても形態的にエキノコックス卵であるか，他のテニア科条虫卵であるかの区別ができない（図 2）．このために，虫卵などの虫体由来物を対象とした PCR 法による遺伝子検査が実施されている．冒頭で述べた埼玉県の捕獲犬から見出された虫卵は，感染研でこの方法を用いて多包条虫（北海道タイプ）のものと判定された.

エキノコックス成虫は 1 匹ごとの虫卵排出数が少なく，また感染初期には虫卵の排出そのものがないことなどから，寄生があるにもかかわらず虫卵が検出できない場合がある．そこで，腸管内に寄生している虫体の分泌排泄物を抗原として検出するためのサンドイッチ ELISA 法が開発されている.この方法は,現在のところ検出感度のもっとも高い方法と考えられているが，偽の陽性反応を識別するために，陽性の結果が得られたならば駆虫剤投与後にも検査を実施し，陰性に転ずるかどうかをかならず確認する必要がある.

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Predominant T Helper 2 Immune Responses against *Bartonella henselae* in Naturally Infected Cats

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Abstract: **This study was conducted to explicate the mechanism of long-term bacteremia in *Bartonella henselae*-infected cats by the examining host immune responses. Blood samples were collected from three naturally infected cats and the IgG antibody titers and the cytokine responses in peripheral blood mononuclear cells (PBMC) were examined by quantitative reverse transcriptase-polymerase chain reactions (RT-PCR). Relapsing bacteremia was found in two of the three cats during the examination period. The quantitative RT-PCR analyses demonstrated that increases of the mRNA expressions in interleukin-4 (IL-4) but not in gamma-interferon (IFN-γ) were observed in PBMC from these infected cats after the bacteremia had peaked, showing that the T helper 2 (Th2) responses were specifically induced in the cats. Furthermore, the specific antibody titer increased, resulting in a decrease in the number of *B. henselae* to undetectable levels in these cats. However, the number of bacteria increased again in two of these cats at 90 and 45 days after the previous bacteremia, respectively. These results suggest that *B. henselae* predominantly induced IL-4 production from PBMC and resulted in stimulation of the humoral immune responses, including the secretion of specific antibodies in the cats. Furthermore, the specific antibody may play a role in eliminating the bacteria from cats partially but not completely, because relapsing bacteremia was found in these two cats.**

Key words: ***Bartonella henselae*, Cat-scratch disease, Cytokine, Th2**

Accumulating studies have demonstrated that *Bartonella henselae* is associated with a spectrum of human diseases including cat-scratch disease (CSD), bacillary angiomatosis, peliosis hepatis, endocarditis, relapsing fever and bacteremia in both immunocompetent and immunocompromised persons (6, 18, 25, 27–29). It has been found that bacteremic cats play an important role as a reservoir of *B. henselae* (5, 13, 22). Despite high levels of bacteria in cats for many months, most bacteremic cats are asymptomatic, and the pathogenesis and mechanisms of the establishment of persistent infection by *B. henselae* in cats are not fully understood (9, 17).

B. henselae invades feline erythrocytes and endothelial cells, suggesting that the agent belongs to an intracellular pathogen (15). Cell-mediated immunity (CMI) is believed to play significant roles in excluding intracellular pathogens. To evaluate the functions of CMI and humoral immunity (HI), examining the cytokine expression levels in T-lymphocytes is considered important. Because it has not been possible to assess the feline cytokine expression, there has been no information about the effectiveness of CMI against *B. henselae* infection in cats.

In the present study, we used a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to evaluate the induction of feline gamma-interferon (IFN-γ) and feline interleukin 4 (IL-4), which are representatives of activations of T helper 1 (Th1) and T helper 2 (Th2), respectively. Furthermore, the mRNA expressions of tumor necrosis factor alpha (TNF-α), which is a key cytokine inducing the Th1 responses, were also examined by quantitative RT-PCR (32). The relationship between the levels of bacteremia and the Th1/Th2 cytokines were examined by using three naturally *B. henselae* infected cats showing relapsing bacteremia.

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Abbreviations: CMI, cell mediated immunity; CSD, cat-scratch disease; PBMC, peripheral blood mononuclear cells.

Materials and Methods

Experimental animals. One female (Cat A) and two male (Cats B and C) cats were used in this study. Before the examinations, these three cats were examined for the *B. henselae* infection by serological and bacteriological examinations and were confirmed to be naturally infected with the organisms as shown by IgG antibody positives for the bacteria. They were housed individually from the age of 1 month, and blood samples were collected during the 318-day experimental period of once or twice a month. Animal care was carried out in accordance with the guidelines for the care and use of laboratory animals by the College of Bioresource Sciences at Nihon University.

Isolation and viable colony count of Bartonella *from cat blood.* One milliliter aliquot of the blood collected in an EDTA-tube (Terumo, Tokyo) was centrifuged at 1,000×*g* for 10 min, and the plasma was separated and used for serological analysis. The pellet was resuspended in 500 µl of supplemented Medium 199 (Gibco BRL, Md., U.S.A.) for the isolation of *B. henselae* and stored at −80 C for more than 8 hr (14). The aliquot was thawed at room temperature and plated onto two heart infusion agar (HIA) plates (DIFCO, Mich., U.S.A.) containing 7% rabbit blood. The plates were incubated at 35 C in an atmosphere containing 5% CO_2 for 4 weeks. The numbers of colonies formed on the plates were counted and the colony forming units (CFU) per ml of blood were calculated. Then, two to nine colonies were picked and subcultured on HIA plates containing 5% rabbit blood and submitted for the identification of *Bartonella* species.

For typing of the 16S rRNA gene of the isolates, PCR was performed as described previously with some modifications (3). The genomic DNA of the isolates was extracted by using a commercial kit (InstaGene Matrix, BioRad, Calif., U.S.A.). The template DNA (10 ng) was mixed with the reaction solution (10 mM Tris, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) containing 0.8 mM of dNTPs, 20 pmol each of sense/antisense primers and 2.5 U of *Taq* polymerase (Promega, Wis., U.S.A.), and adjusted to a final volume of 50 µl. The PCR reaction was performed by using a thermal cycler (iCycler, BioRad). The sequences of primer pairs used and condition of the PCR were the same as described in a previous report (3). The amplified products were subjected to electrophoresis in a 2% agarose gel and stained with 0.6 µg/ml ethidium bromide solution.

Analysis of antibody responses of cats to B. henselae *Houston-1.* Titers of IgG antibodies against *B. henselae* were determined by an indirect immunofluorescence antibody test (IFA) as described previously (19, 23). Briefly, plasma was serially diluted with phosphate-buffered saline ranging from 1:32 to 1:1,024 by twofold dilutions and incubated on slides containing *Felis catus* whole-fetus (FCWF) cells infected with *B. henselae* Houston-1 at 37 C for 1 hr. The binding antibodies were probed with fluorescein isothiocyanate-labelled goat anti-cat IgG (ICN Pharmaceuticals, Inc.-Cappel Products, N.C., U.S.A.). More than three readers evaluated the results subjectively. Plasma samples with a titer of ≧1:64 were provisionally considered as positive in accordance with the previous reports (19, 23).

Induction of cytokine mRNA expressions and RNA preparation. Peripheral blood mononuclear cells (PBMC) collected from days 0 to 121 of the examinations were assessed for the cytokine mRNA expressions. The PBMC were prepared from the cats by using the Lymphocyte separation medium (ICN Pharmaceuticals, Inc.-Cappel Products), and suspended in complete RPMI medium consisting of RPMI1640 (Invitrogen, Calif., U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum, 200 U/ml penicillin and 200 µg/ml streptomycin. The PBMC in the wells (5×10^6 cells/well) of a 24-well microplate were stimulated with 0.5 µg/ml of concanavalin A (SIGMA, Mo., U.S.A.) for 4 hr at 37 C. Following the stimulation, the PBMC were harvested and the total RNA were extracted by using Trizol reagent (Invitrogen). For synthesis of the first strand cDNA, all of the extracted total RNA was added to a 20 µl reaction mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) containing 1.3 µM of the oligo d(T) primer (15 mer), 2 mM of dNTP mix, 40 U of RNasin (Promega) and 11 U of AMV reverse transcriptase (Promega), and this was incubated at 45 C for 1 hr. The cDNA was diluted fivefold with distilled water and used as template cDNA for the quantitative PCR.

Quantitative PCR for cDNA of IFN-γ, IL-4 and TNF-α in PBMC from cats. For the quantitative real-time PCR analysis of the cDNA levels, the LightCycler system (Roche Molecular Biochemicals, Ind., U.S.A.) and a LightCycler-FastStart DNA Master SYBR Green I containing the FastStart *Taq* DNA polymerase were used. Each reaction (20 µl) contained 2 µl of the respective cDNA primers at 0.2 mM, and Mg^{2+} at 2 mM. The sequences of the primers used in this study are summarized in Table 1. Following the activation of the FastStart *Taq* DNA polymerase (95 C for 15 min), 35 cycles of the amplification programs were performed. The conditions of the PCR for each target DNA are shown in Table 2. Quantitative results were expressed by determining the detection threshold or the crossing point (Cp), which marked the cycle when the fluores-

Table 1. Primer sequences used for the quantitative PCR by LightCycler

Primer	Sequence	Accession No.[a]
Feline IFN-γ 5′	GGA CAC CAT CAA GGA AGA CA	D30619
Feline IFN-γ 3′	AAC AGA TTC TGG CTC CTT TT	
Feline IL-4 5′	CCA CGG CCA GAA CTT CAA T	X87408
Feline IL-4 3′	GGT CCT GTT TGC CAT GCT	
Feline TNF-α 5′	CAT GTA GTA GCA AAC CCC GA	M92061
Feline TNF-α 5′	GAC CCT GGT CTG GTA GGA AA	
Feline GAPDH 5′	GAG AAA GCT GCC AAA TAC	AB038241
Feline GAPDH 3′	ATA CCA GGA AAT GAG CTT G	

[a] The references of the mRNA sequence of the genes in DNA data bank.

Table 2. PCR conditions for each target cDNA by LightCycler

Step	GAPDH	IFN-γ	IL-4	TNF-α
Denaturing	95 C, 15 sec	95 C, 15 sec	95 C, 15 sec	95 C, 15 sec
Annealing	53 C, 5 sec	54 C, 5 sec	60 C, 5 sec	58 C, 5 sec
Extension	72 C, 13 sec	72 C, 13 sec	72 C, 13 sec	72 C, 13 sec
Fluorescence acquisition	86 C	82 C	86 C	87 C

cence of a given sample significantly exceeded the baseline signal. They were expressed as a fractional cycle number. Then the Cps were plotted against the known concentration of the plasmid DNA (pGEM-T vector, Promega) containing each of the cloned target DNAs to obtain the standard curve. The expression level was determined as a ratio between the target gene and the housekeeping gene, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

Results

Numbers of B. henselae *and the IgG Antibody Titers to the Organisms in Cats*

Two (Cats A and B) of the three infected cats showed bacteremia during the examination periods (Fig. 1). All the isolates from these cats were identified as 16S rRNA type I by a type-specific PCR (data not shown).

In Cat A, the first bacteremia (5,836.8 CFU/ml) was detected on day 12 of the examination, and fell below the detectable level on day 43. Following the decrease in the bacterial number, the IgG antibody titer increased to 1:256 until day 61, and then decreased from day 89, when it fell below the detectable level. Ninety days after the end of the first bout of bacteremia (day 133), the second bacteremia infection (170.3 CFU/ml) was detected, and the IgG antibody titer increased again.

In Cat B, the first peak of bacteremia (175.5 CFU/ml) appeared on day 12, and the bacterial number decreased to 7.7 CFU/ml on day 30, when the IgG antibody titer reached 1:128. Until day 43, the levels of both the bacteremia and the IgG antibody titer decreased. The second bacteremia was observed on day 75 (1.7 CFU/ml) and the IgG antibody titer increased to 1:128. After this, the IgG antibody titers repeated their up and down pattern (1:32 to 1:64), though no bacteremia was observed during the following experimental period.

In Cat C, though no bacteremia was observed, slight increases of the IgG antibody titer (1:64) were observed on days 0, 41, and 63.

Cytokine mRNA Expressions in PBMC Derived from the Cats

In Cat A, the level of IL-4 mRNA expression was suppressed on day 12, when the first peak of bacteremia was observed (Fig. 2). Following the first bacteremia, the expression of the cat's IL-4 mRNA remained at a high level (days 30 to 101). In contrast, the IFN-γ mRNA expression level remained lower level.

In Cat B, after the first (day 12) and second (day 75) peaks of bacteremia, slight increases in the IL-4 mRNA expression were observed on days 43 and 89, respectively, although both the levels of the IL-4 mRNA expression and the number of bacteria were much lower than in Cat A. As in Cat A, few mRNA expressions of IFN-γ were detected in Cat B.

In Cat C, no bacteremia was observed, and the levels of both IL-4 and IFN-γ mRNA remained at lower levels during the examination.

Figure 3 shows the kinetics of the TNF-α mRNA expressions in PBMC from the cats during the experimental period. Down-regulations of the TNF-α mRNA expressions were observed in Cats B and C on day 12,

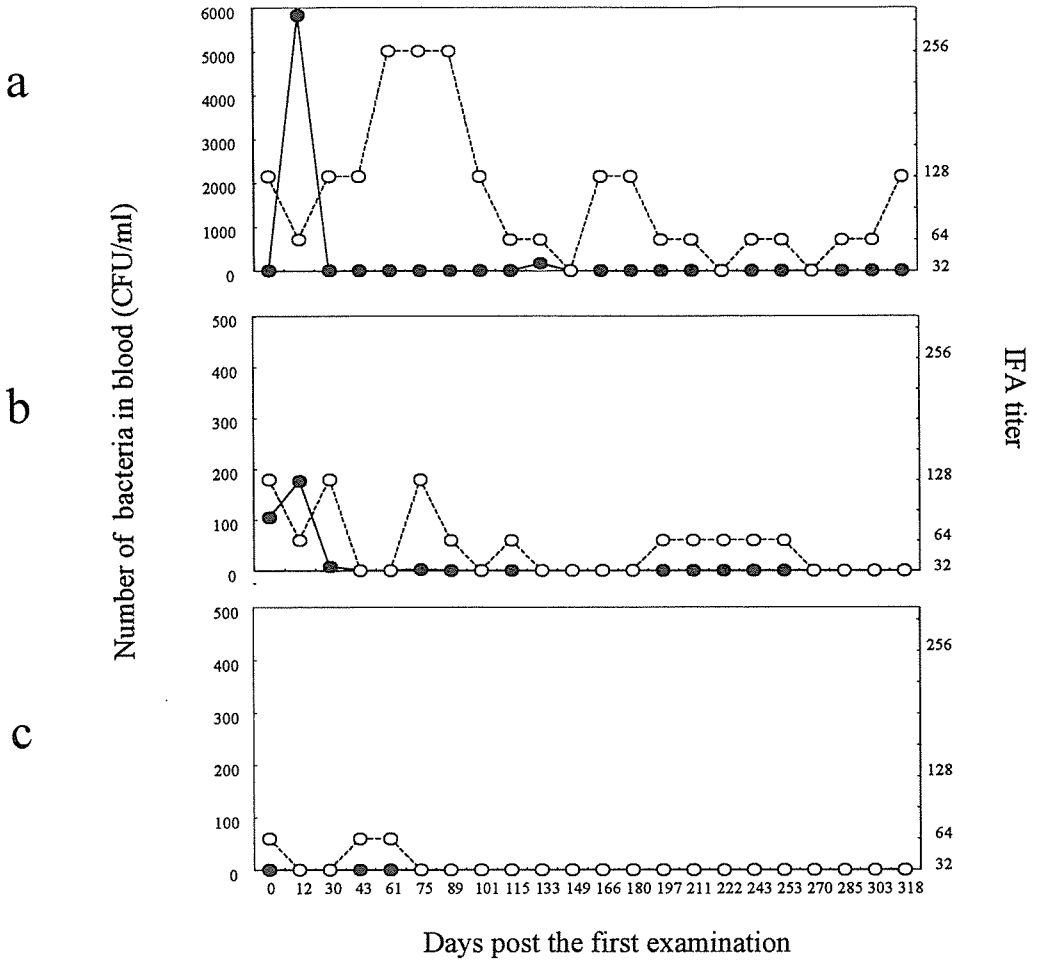

Fig. 1. Time course of bacteremia and IgG response against *B. henselae* in naturally infected cats; Cats A (a), B (b) and C (c). The number of viable cell counts in peripheral blood and the titer of specific antibodies to *B. henselae* (Houston-1) were monitored for 318 days. The number of bacteria was expressed as colony-forming units (CFU) per ml of blood (closed circle). *B. henselae*-specific IgG titers were determined by IFA (open circle).

when Cat B showed a peak of bacteremia. Following the down-regulation, the levels of TNF-α mRNA expression recovered gradually by day 101. Cat A, in which the highest levels of bacteremia had been detected among the three cats, showed comparatively lower levels of TNF-α mRNA expression throughout almost the entire examination.

Discussion

In the present experiment, two of three naturally infected cats (Cats A and B) showed relapsing bacteremia and persistent infections. A similar phenomena has been reported in previous reports on naturally infected cats (1) as well as on cats experimentally infected with the peripheral blood from naturally infected cats (16, 17). In addition, specific-pathogen free (SPF) cats inoculated with multiple strains containing genetically distinct organisms also showed repeated bacteremia infections (10, 30). Comparing these data, cats infected with the monoclonal organisms were demonstrated to show only a short duration of bacteremia without relapse (1, 9, 24). These results clearly indicate that the existence of multiple, genetically distinct organisms may be involved in the establishment of chronic and relapsing bacteremia in *B. henselae* infected cats. In support of this hypothesis, we have shown that the organisms with different restriction enzyme fragment length polymorphism (RFLP) patterns of genomic DNA emerged during the different bacteremic episodes in the same cat (12).

In addition, host immune responses may also contribute to establishing relapsing and chronic *B. henselae* infection in cats. The mechanisms by which *B. hense-*

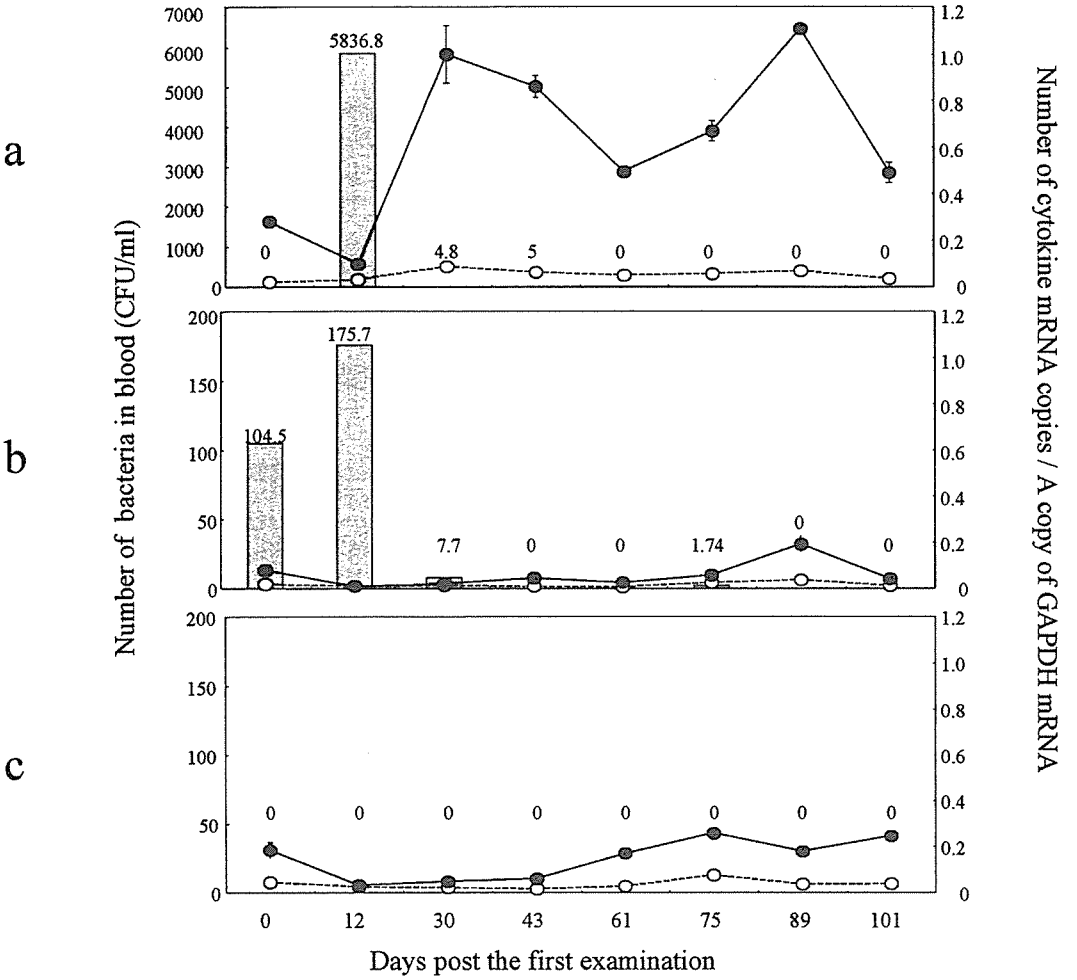

Fig. 2. Time course of bacteremia and levels of cytokine mRNA expressions in naturally infected cats; Cats A (a), B (b) and C (c). PBMC from each cat was stimulated with Con. A for 4 hr and the total RNA was extracted. The quantitative PCR was performed by using a LightCycler. To correct for differences in RNA quality and quantity among the samples, data were normalized using the ratio of the target cDNA concentration to that of GAPDH. The number of bacteria was expressed as colony-forming units (CFU) per ml of blood (closed bar; the numbers were labeled on top of the bars). The amounts of the normalized IFN-γ (open circle) and IL-4 (closed circle) cDNA were expressed as a ratio of the target cDNA values against those of GAPDH.

lae escape the antibacterial defenses of the host, however, remain unknown. In the present study, the levels of bacteremia in the blood decreased after increases of the IgG titer to *B. henselae*. Previous reports demonstrated that the humoral immune responses, including *Bartonella*-specific IgG, significantly contribute to bactericidal activities (9, 26). Antibodies may play some role in the elimination of organisms from infected cats. However, the specific antibody was not sufficient to completely eliminate the organisms from cats, because relapsing bacteremia was observed despite the specific antibody production.

CMI is believed to play an important role in the elimination of intracellular pathogens, including *B. henselae*. However, there have been no reports concerning CMI in cats infected with *B. henselae* because of the lack of analysis systems for feline cytokine expressions. In this study, we chose the IFN-γ and IL-4 mRNA expressions as indicators of the Th1 and Th2 immune responses, respectively, and monitored the activations in naturally *B. henselae* infected cats. We showed that the IL-4 expression was selectively promoted in the infected cats, indicating activation of the Th2 immune response and resulting in the emergence of relapsing bacteremia. Comparing this, the expressions of IFN-γ mRNA remained at low levels regardless of the bacteremia in these cats. In the case of mice, Th1 immune responses were selectively induced in *Bartonella*-stimulated cells,

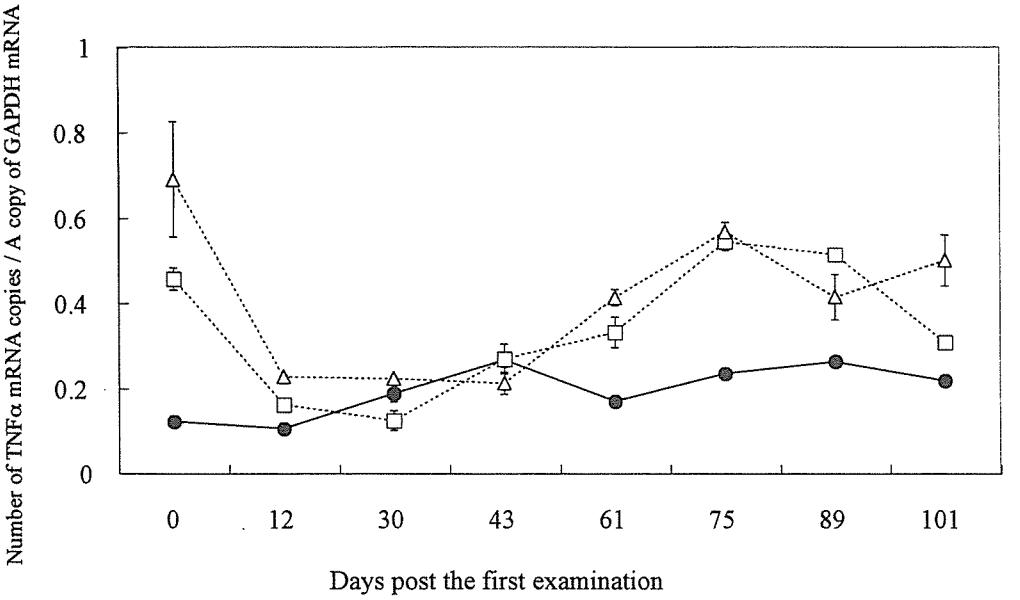

Fig. 3. Time course of the levels of TNF-α mRNA expressions in naturally infected cats; Cats A (closed circle), B (open square) and C (open triangle). PBMC from each cat was stimulated with Con. A for 4 hr and the total RNA was extracted. The quantitative PCR was performed by using a LightCycler. To correct for differences in RNA quality and quantity among the samples, data were normalized using the ratio of the target cDNA value to that of GAPDH. The amounts of normalized TNF-α cDNA were expressed as a ratio of the target cDNA values against those of GAPDH.

and IFN-γ-activated macrophages effectively eliminated the organisms from the infected cells (2, 20). Thus, CMI, including an activation of macrophages, is believed to play a critical role in the complete elimination of the organisms from infected animals. These results suggest that the selective induction of Th2 immune responses may contribute to establishing the persistent infection of *B. henselae* in naturally infected cats.

For pathogens, affecting the expression of cytokines, which is needed for normal protective functioning of the immune response, is one of the major strategies for surviving and multiplying in the host (21). The roles of the organism should be considered in the selective induction of the Th2 immune responses in cats. In the present study, the levels of IL-4 mRNA expression in PBMC increased in proportion to the bacterial loads during the bacteremia, suggesting the possibility that the organism might have some molecules that induce the Th2 immune responses and/or suppress the Th1 immune responses, especially in cats but not in mice.

Members of the genus *Brucella* are facultatively intracellular bacteria that show chronic infections in humans as well as some domestic animals (8, 31). Following invasion of the reticuloendothelial system, the bacteria are known to be able to survive within mononuclear phagocytes. In this case, *Brucella* spp. suppresses the production of TNF-α induced either by their phagocytosis or by exogenously added lipopolysaccharide and favor intracellular multiplication (4). It is possible that down-regulation of the TNF-α expression might also be involved in the selective induction of the Th2 immune responses in *B. henselae*-infected cats, since TNF-α induces the Th1 immune responses by promoting IL-12 production from macrophages (32). Interestingly, the TNF-α expression in Cat A with the highest level of bacterial load was relatively lower than in the other cats, but Cat A had the highest levels of IL-4 mRNA expression. Following this possibility, outer membrane protein 25 secreted from *Brucella suis* showed a high sequence homology at amino acids levels to that of a 31-kDa protein (Pap31) of *B. henselae*, and was demonstrated to be a factor involved in the defect in TNF-α production (7, 11).

In conclusion, the data presented here suggested that PBMC from *B. henselae* naturally infected cats selectively promoted the IL-4 but not the IFN-γ mRNA expression following the increase of the organisms in the blood. To confirm this conclusion, greater numbers of animals should be examined for the analyses of cytokines, including the cytokines examined in this study during the course of *B. henselae* infection in cats.

The selective induction of the Th2 immune responses might contribute to the establishment of persistent infection with *B. henselae* in naturally infected cats. Work is now in progress to examine the mechanisms by which a network of interacting cytokines can be induced, which can result in selective Th2 responses against *B. henselae*.

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Bartonella Spp. in Pets and Effect on Human Health

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Among the many mammals infected with *Bartonella* spp., pets represent a large reservoir for human infection because most *Bartonella* spp. infecting them are zoonotic. Cats are the main reservoir for *Bartonella henselae*, *B. clarridgeiae*, and *B. koehlerae*. Dogs can be infected with *B. vinsonii* subsp. *berkhoffii*, *B. henselae*, *B. clarridgeiae*, *B. washoensis*, *B. elizabethae*, and *B. quintana*. The role of dogs as an important reservoir of *Bartonella* spp. is less clear than for cats, because domestic dogs are more likely to be accidental hosts, at least in nontropical regions. Nevertheless, dogs are excellent sentinels for human infections because a similar disease spectrum develops in dogs. Transmission of *B. henselae* by cat fleas is better understood, although new potential vectors (ticks and biting flies) have been identified. We review current knowledge on the etiologic agents, clinical features, and epidemiologic characteristics of these emerging zoonoses.

Bartonella spp. are fastidious, hemotropic, gram-negative bacteria that are mainly transmitted by vectors. Among the 11 species or subspecies known or suspected to be pathogenic for humans, 6 have been isolated from pet dogs and cats (Table 1). Domestic cats are the principal reservoir for *Bartonella henselae*, the main agent of cat-scratch disease (CSD); *B. clarridgeiae*, which has been suspected in a few cases of CSD; and *B. koehlerae*, recently reported as the cause of human endocarditis (*1,4*). Domestic dogs could be one of the reservoirs for *B. vinsonii* subsp. *berkhoffii* (reported as *B. v. berkhoffii* thereafter) because as it can cause prolonged bacteremia in this species (*5,6*). Dogs can also be infected with *B. henselae*, *B. clarridgeiae*, *B. washoensis*, and *B. elizabethae* (*2*). Recently, 2 cases of endocarditis caused by *B. quintana* were diagnosed (P. Kelly et al., unpub. data). As with human disease, the clinical spectrum of *Bartonella* infection in dogs is expanding (*2*). Fleas play a major role in the transmission of feline *Bartonella* (*7*), but other potential vectors, such as ticks and biting flies have been recently identified to harbor *Bartonella* DNA, including *B. henselae* (*8,9*). This article provides an update on the etiologic agents, new clinical features, and evolving epidemiologic characteristics of these emerging zoonoses. We will not discuss the diagnosis, treatment, and prevention of *Bartonella* infections, as several recent review articles have been written on this subject (*1,2,10*).

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Feline *Bartonella* Species

B. henselae

Since the first isolation of *B. henselae* from a domestic cat in the early 1990s, several studies have been conducted worldwide to determine the importance of cats as a reservoir of this bacterium (reviewed in [2]). Prevalence of infection varies considerably among cat populations (strays or pets) with an increasing gradient from low in cold climates (0% in Norway) to high in warm and humid climates (68% in the Philippines) (*2*). At least 2 genotypes have been identified and designated Houston-1 (type I) and Marseille (previously BATF) (type II) (*1,2*). The respective prevalence of these 2 genotypes varies considerably among cat populations from different areas. *B. henselae* type Marseille is the dominant type in cat populations in the western United States, western Europe (France, Germany, Italy, the Netherlands, United Kingdom), and Australia, whereas type Houston-1 is dominant in Asia (Japan and the Philippines) (reviewed in [2]). However, within a given country, the prevalence may also vary among cat populations. For instance, in France, Marseille type was the most common type in cats from the Nancy and Paris areas, whereas type Houston-1 was the main genotype in cats from Lyon or Marseille (references cited in [2]). However, a few studies in western Europe and Australia have reported that most human cases of CSD were caused by *B. henselae* type Houston-1, despite the fact that type Marseille was found to be the dominant type in the cat population, which suggests that type Houston-1

Table 1. Species and subspecies of *Bartonella* that are confirmed or potential human pathogens

Bartonella sp.	Primary reservoir	Vector	Accidental host	Reference
B. bacilliformis	Human	Sandfly (*Lutzomia verrucarum*)	None	(*1,2*)
B. quintana	Human	Body louse (*Pediculus humanis*)	Cat, dog, monkey	(*1–3*, P. Kelly et al., unpub. data)*
B. elizabethae	Rat (*Rattus norvegicus*)	Oriental rat flea (*Xenopsylla cheopis*)	Human, dog	(*2*)
B. grahamii	Wild mice (*Clethrionomys glareolus, Microtus agrestis, Apodemus flavicollis*)	Rodent fleas	Human	(*1,2*)
B. henselae	Cat (*Felis catus*)	Cat flea (*Ctenocephalides felis*)	Human, dog	(*1,2*)
B. clarridgeiae	Cat	Cat flea	Human?, dog	(*1,2*)
B. koehlerae	Cat	Cat flea	Human	(*2,4*)
B. vinsonii subsp. *berkhoffii*	Coyote (*Canis latrans*), dog (*C. familiaris*)	Unknown (ticks?)	Human	(*5,6*)
B. vinsonii subsp. *arupensis*	White-footed mouse (*Peromyscus leucopus*)	Unknown (fleas?, ticks?)	Human	(*1,2*)
B. washoensis	California ground squirrel (*Spermophilus beecheyii*)	Unknown (fleas?)	Human, dog	(*2*)
B. alsatica	Rabbit	Unknown (flea?)	Human	(D. Raoult, pers. comm.)

*Also reported by O'Rourke LG, Pitulle C, Hegarty BC, Kraycirik S, Killary KA, Grosenstein P, et al. *Bartonella quintana* in cynomolgus monkey (*Macaca fascicularis*). Emerg Infect Dis. 2005;11:1931–4.

strains could be more virulent to humans (2). Cats are usually bacteremic for weeks to months, but some cats have been reported to be bacteremic for >1 year. Young cats (≤1 year) are more likely than older cats to be bacteremic (*11*), and stray cats are more likely to be bacteremic than pet cats (*1,2*).

The clinical description of CSD was first reported in France by Debré et al. in 1950, but the etiologic agent was identified only in 1992 (*1,2,6*). The annual number of cases in the United States has been estimated to be between 22,000 and 24,000, with ≈2,000 cases that require hospitalization, and thousands of cases may occur yearly in Europe. In various studies, the seroprevalence of antibodies to *B. henselae* in healthy persons has ranged from 3.6% to 6% (Table 2) and could be higher in some specific population groups, such as veterinarians, children, or elite orienteers (orienteering is a sport in which participants compete to find points in the landscape using a map and compass). Table 2 gives comparative *B. henselae* seroprevalence data for cat and healthy human populations from selected countries, which suggests that seroprevalence is low in both cats and humans at northern latitudes and increases in warmer climates (*11–24*). Such data are informative and cannot exclude possible serologic cross-reactivity with some other *Bartonella* spp.

Despite the fact that *B. henselae* infection can cause meningitis and encephalitis, only 1 case of a fatal infection has been reported (*5*). CSD is more frequently observed in persons <20 years of age and in persons who own a young cat (<1 year of age, especially if this cat is infested with fleas) or in persons who have been scratched or bitten by a cat (*1,2,6*). In immunocompetent persons, CSD is mainly characterized by a benign regional lymphadenopathy. Usually after a cat scratch, a papule and then a pustule develop within 7 to 12 days at the injection site, followed by a regional lymphadenopathy (usually involving a single lymph node) 1–3 weeks later that can persist for few weeks to several months. Low-grade fever, malaise, and aching are often reported; in some instances, headache, anorexia, and splenomegaly can occur. Abscessed lymph nodes are reported occasionally. In 5% to 9% of CSD patients, atypical manifestations may develop, including Parinaud oculoglandular syndrome, encephalitis, endocarditis, hemolytic anemia, hepatosplenomegaly, glomerulonephritis, pneumonia, relapsing bacteremia, and osteomyelitis.

On the basis of serologic testing or polymerase chain reaction (PCR), several recent publications have associated *B. henselae* with uveitis, focal retinal phlebitis, neuroretinitis, retinal and optical nerve neovascularization, and retinal artery and vein occlusions. Neurologic forms are rare, and patients usually completely recover within 1 year without sequelae. Hepatosplenomegaly and osteolytic bone lesions have been described in persons seropositive for *B. henselae*. Pseudotumoral lesions involving the mammary glands, the liver, or the spleen and, recently, glomerulonephritis and cases of monoclonal and biclonal gammopathy have also been associated with *B. henselae* antibodies. Cases of prolonged fever without adenopathy, chronic fatigue, hemolytic anemia, thrombocytopenic purpura, Henoch-Schönlein purpura syndrome, pleuritis, pneumonia, and even paronychia have been reported in patients who were seropositive for *B. henselae* (*1,2*). Usually, these clinical manifestations disappear in a few weeks to a few months. Bacteremia is rarely detected in immunocompetent persons. Several cases of endocarditis

Table 2. *Bartonella henselae* seroprevalence in various cat and human populations from selected countries*

Country	Cat seroprevalence (%)			Human seroprevalence (%)		
	Stray	Pet	Reference	Healthy	Other	Reference
Sweden	NA	1	(*19*)	1	NA	(*12*)
Japan	NA	8.8–15.1; northern, 0–2; central 10.9–12.6; southern, 18–24	(*20*)	4.5	11.0–15.0 (veterinarians)	(*13,14*)
United States	81	27.9	(*11,21*)	3.6–6	7.0 (veterinarians)	(*15*)
Thailand	27.6†	NA	(*22*)	5.5	NA	(*16*)
Italy	39.0	43.5	(*23*)	NA	8.5–61.6 (children)	(*17*)
Jordan	NA	32.0	(*24*)	NA	NA	(*18*)

*NA, not available.
†Prevalence of bacteremic cats; no data available on seroprevalence.

have been associated with *B. henselae* infection, most frequently in persons with preexisting valvular lesions. Besides *B. henselae*, most human cases of *Bartonella* endocarditis are caused by *B. quintana*, but a few cases of endocarditis or myocarditis have been associated with *B. elizabethae* (1 case), *B. vinsonii berkhoffii* (1 case), *B. vinsonii arupensis* (1 case), *B. koehlerae* (1 case), *B. washoensis* (1 case), and *B. alsatica* (1 case) (Table 3).

In immunocompromised patients, *B. henselae* infection can cause prolonged fever, prolonged bacteremia, or both (*1,2,6*). Bacillary angiomatosis or peliosis is usually observed in highly immunocompromised persons (low CD4 count), who often are infected with HIV. Several severe infections have also been reported in organ transplant recipients (*1,2*).

The clinical spectrum of the infection in cats has not been fully investigated, but naturally infected cats primarily seem to be healthy carriers of the bacterium (*1,2,6*). However, cases of uveitis and rare cases of endocarditis have been molecularly associated with infection caused by *B. henselae*. Seropositive cats were more likely to have kidney disease and urinary tract infections, stomatitis, and lymphadenopathy. In experimentally infected cats, fever, lymphadenopathy, mild neurologic signs, and reproductive disorders have been reported.

B. clarridgeiae

B. clarridgeiae was first isolated in the United States from the pet cat of an HIV-positive patient (*25*). This *Bartonella* sp. has been less frequently isolated from domestic cats than *B. henselae* because it appears to be more difficult to isolate and is unevenly distributed in cat populations worldwide. A *B. clarridgeiae* prevalence of 17% to 36% among all *Bartonella* isolates was reported in studies conducted in France, the Netherlands, the Philippines, and Thailand (*2,22*). However, *B. clarridgeiae* represented ≤10% of all isolates from domestic cats in the southeastern United States, Japan, or Taiwan (*2*) and has never been isolated in studies conducted in Europe, Australia, and North America (*2*). No specific pathologic features have been associated with natural infection in cats. However, in experimentally coinfected cats (*B. henselae* type II and *B. clarridgeiae*), clinical signs were minimal, and gross necropsy results were unremarkable, but histopathologic examination showed peripheral lymph node hyperplasia, splenic follicular hyperplasia, lymphocytic cholangitis/pericholangitis, lymphocytic hepatitis, lymphoplasmacytic myocarditis, and interstitial lymphocytic nephritis (*26*). In humans, *B. clarridgeiae* has never been isolated or detected by molecular methods. However, *B. clarridgeiae* could be a minor causative agent of CSD, as the presence of *B. clarridgeiae* antibodies were reported in a suspect case of CSD and in a patient with a chest-wall abscess (reviewed in [2]). Furthermore, anti-flagella (FlaA)–specific antibodies against *B. clarridgeiae* were detected by immunoblotting in 28 (3.9%) of 724 patients with lymphadenopathy but in none of 100 healthy controls. However, substantial cross-reactivity between *B. henselae* and *B. clarridgeiae* detected by indirect fluorescence antibody assay was noted in human sera in a recent study from Japan (*2*).

B. koehlerae

B. koehlerae is a *Bartonella* sp. that has rarely been isolated from domestic cats worldwide, as it is a very fastidious bacterium (*2,4*). Until recently, it had been isolated only from 2 cats in California and 1 cat in France (*2,4,27*). The first human case of *B. koehlerae* endocarditis was reported from Israel in 2004 (*2*). Furthermore, these authors were able to isolate *B. koehlerae* from a bacteremic stray cat from that country.

B. quintana and *B. bovis*

A few suspect cases of CSD and cases of bacillary angiomatosis or endocarditis have been associated with *B. quintana,* for which the only risk factor identified was a contact with cats or cat fleas (*3*). Furthermore, the identification of *B. quintana* DNA in cat fleas (*28*) and recently in the dental pulp of a cat (*3*) has raised the question as to whether cats might be a possible source of human infection. However, *B. quintana* has not yet been isolated from naturally infected cats anywhere in the world where epidemiologic studies have been conducted to detect *Bartonella*-bacteremic cats. Similarly, 2 cats infected with

Table 3. Clinical aspects of *Bartonella* infections in humans and dogs

Bartonella sp.	Symptoms	
	Humans	Dogs
B. clarridgeiae	Cat-scratch disease	Endocarditis, lymphocytic hepatitis
B. elizabethae	Endocarditis, neuroretinitis	Lethargy, anemia, weight loss
B. henselae	Cat-scratch disease, endocarditis, bacillary angiomatosis, peliosis hepatis, granulomatous hepatitis, pseudotumoral lesions, arthritis, arthralgia, osteomyelitis, nodules, erythema, cutaneous petechiae, uveitis, neuroretinitis, purpura (Henoch-Schönlein), glomerulonephritis, perionyxis, periodontitis	Granulomatous hepatitis, peliosis hepatis, epistaxis
B. grahamii	Neuroretinitis, bilateral retinal artery branch occlusions	Not diagnosed in dogs
B. koehlerae	Endocarditis	Not diagnosed in dogs
B. vinsonii subsp. *arupensis*	Bacteremia, fever, arthralgia, neurologic disorders, endocarditis	Not diagnosed in dogs
B. vinsonii subsp. *berkhoffii*	Endocarditis	Endocarditis, myocarditis, arrhythmia, uveitis, choroiditis, limping, splenomegaly, polyarthritis, epistaxis
B. washoensis	Fever, myocarditis	Endocarditis
B. quintana	Fever, bacteremia, endocarditis, bacillary angiomatosis	Endocarditis

B. quintana did not become bacteremic but seroconverted (*29*). Subsequently, both cats became bacteremic when challenged with *B. henselae*.

A few cases of *B. bovis* (formerly *B. weissii*) infections have been reported in cats from Illinois and Utah in the United States (*1*). The epidemiologic role of cats for this organism is still unknown.

Dogs as Sentinels for Human Infections?

Dogs can be infected with *B. v. berkhoffii*, *B. henselae*, *B. clarridgeiae*, *B. washoensis*, *B. elizabethae*, and *B. quintana* (*2*, P. Kelly et al., unpub. data). However, the role of dogs as a major reservoir of *Bartonella* spp. is not clear. Current evidence suggests that domestic dogs are more likely to be accidental hosts of various *Bartonella* spp., at least in nontropical regions. Nevertheless, domestic dogs could be one of the reservoirs for *B. v. berkhoffii*, as it causes prolonged bacteremia in this species (*5,6*). The epidemiologic situation is quite distinct between tropical areas where several studies have shown a high prevalence of *B. v. berkhoffii* antibodies, especially in stray dogs, and more northern latitudes, where very low antibody prevalence has been detected in domestic dogs, especially among pets. In sub-Saharan Africa, seroprevalence of 26% in dogs in Senegal and up to 65% in native dogs from Sudan has been reported (*1*). In North Africa, we found that 38% of 147 dogs from Morocco were seropositive for *B. v. berkhoffii* (*30*). In 113 dogs from the Reunion Island, in the Indian Ocean, a seroprevalence of 18% was reported in stray dogs, whereas only 3% of dogs examined at veterinary clinics were seropositive, and no dog was bacteremic (*31*). In Thailand, 38% of sick dogs who exhibited fever, anemia, or thrombocytopenia were seropositive for *B. v. berkhoffii* (*1*). On the contrary, studies in the United States and Europe reported a seroprevalence of <5% in domestic dogs; selected dog populations were at higher risk, including rural dogs and government working dogs (*2*). However, concerns about false-positive results in animals should be raised, as specificity and sensitivity of the tests for dogs have not been fully evaluated. In California, *B. v. berkhoffii* has rarely been isolated from domestic dogs or detected by PCR, whereas coyotes (*Canis latrans*) appear to be a reservoir of this pathogen, as 35% of the coyotes tested in California were seropositive, and 28% of the coyotes tested within a highly disease-endemic region of California were bacteremic (*2*).

In domestic dogs, *B. v. berkhoffii* is a cause of endocarditis (*6*) and, as in humans, the clinical spectrum of the infection attributed to this organism is expanding. *B. v. berkhoffii* is now associated with cardiac arrhythmias, endocarditis and myocarditis, granulomatous lymphadenitis, granulomatous rhinitis, and epistaxis (*6,32*). In both humans and dogs, *Bartonella*-associated cases of endocarditis usually involve the aortic valve and are characterized by massive vegetative lesions (*33*). Based on serologic evidence, infection with *B. v. berkhoffii* may also cause immune-mediated hemolytic anemia, neutrophilic or granulomatous meningoencephalitis, neutrophilic polyarthritis, cutaneous vasculitis, and uveitis in dogs (*2*).

Some other *Bartonella* spp. have infrequently been isolated from domestic dogs. *B. clarridgeiae* and *B. washoensis* were isolated from cases of endocarditis (*1,2*), and *B. henselae* was isolated for the first time from a dog from Gabon (*34*). In the Gabon study, *B. clarridgeiae* was isolated from 5 of 258 dogs tested (1.9%), which suggests a possible reservoir role for this *Bartonella* sp. in Africa (*34*). *B. henselae*, *B. elizabethae*, and *B. clarridgeiae* DNA has also been detected from a few sick dogs with various clinical abnormalities (Table 3) (*1,2,6*). Endocarditis caused by *B. quintana* was recently diagnosed in a dog from the United States and a dog from New Zealand (P. Kelly et al., unpub. data). Two recent studies reported a

B. henselae antibody prevalence of 10% in healthy dogs in the eastern United States (*35*) and a prevalence of 14% of dogs in Zimbabwe (*36*). A much higher prevalence (27%) in sick dogs from the eastern United States was reported (*35*), which contrasts with the low *B. henselae* seroprevalence (<2%) in dogs examined at a university teaching hospital in northern California (*37*). A case-control study conducted on 305 dogs (102 dogs seropositive for *B. henselae*, *B. v. berkhoffii*, or *B. clarridgeiae* and 203 seronegative dogs) suggested an association between the seropositive status and lameness, arthritis-related lameness, splenomegaly, and nasal discharge/epistaxis (*37*).

Unlike the domestic cat, for which clinical manifestations of natural infection is rarely documented, a wide range of clinical and pathologic abnormalities develop in dogs that are very similar to those observed in humans (*32*). Therefore, this species is an excellent sentinel and an important comparative model for human infections. To date, all *Bartonella* spp. identified in sick dogs are also pathogenic or potentially pathogenic in humans.

Beyond the Fleas: New Emerging Vectors

The primary mode of transmission of *B. henselae* to humans is through a cutaneous trauma caused mainly by the scratch of a cat. Transmission is less likely to occur by cat bite; shedding of *B. henselae* in cat saliva has not been clearly documented. The possibility of direct transmission of *B. henselae* to humans by the cat flea is something that has not been proven experimentally and is mainly hypothetical. However, the presence of cat fleas (*Ctenocephalides felis*) is essential for the maintenance of the infection within the cat population (*6*). *B. henselae* has been shown to multiply in the digestive system of the cat flea and survive several days in the flea feces (reviewed in [2]). Experimentally, only cats inoculated with flea feces compared to those on which fleas were deposited in retention boxes or that were fed fleas became bacteremic (*38*). Therefore, the main source of infection appears to be flea feces that are infected by contaminated cat claws.

Beside the cat flea, new possible vectors have been suggested. *Bartonella* DNA, including *B. henselae*, has been detected in *Ixodes ricinus* ticks collected on humans (*9*) and in *I. scapularis* ticks collected in households of persons coinfected with *B. henselae* and *Borrelia burgdorferi* (reviewed in [2]). *B. quintana*, *B. henselae*, and *B. v. berkhoffii* DNA were also detected in questing *I. pacificus* ticks in California, and a few human cases of *B. henselae* infection were temporally related to a tick exposure in the United States (reviewed in [2]). Tick exposure was reported as a risk factor associated with CSD in humans (*39*). Similarly, tick exposure was determined to be a risk factor associated with *B. v. berkhoffii* seropositivity in dogs (*40*). Additional indirect support for ticks as vectors of *B. v. berkhoffii* in dogs relates to serologic or PCR evidence of concurrent infections with various tickborne organisms (*6,33*). The specific role of ticks in *Bartonella* transmission requires additional study, but several recent publications have reported a high prevalence of *Bartonella* spp. infection in ticks from various parts of the world. Finally, *B. henselae* type Marseille DNA was recently detected in a stable fly (*8*).

Conclusion

The number of zoonotic *Bartonella* species identified in the last 15 years has increased considerably. Pets have been identified as a notable reservoir of *Bartonella* species (i.e., cats and *B. henselae* or dogs and *B. v.* subsp. *berkhoffii* in the tropics) and may play an important role as source for human infection. Furthermore, domestic dogs may represent excellent sentinels for *Bartonella* infection because of the wide diversity of the *Bartonella* spp. identified in canines, all of which are human pathogens. A better understanding of the modes of transmission and vectors involved in dog bartonellosis is an urgent priority to implement appropriate parasite control measures for pets.

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Original article

Cat-scratch disease in veterinary-associated populations and in its cat reservoir in Taiwan

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Abstract – In Taiwan, the first human case of cat-scratch disease (CSD) was diagnosed by a serologic test in 1998. Since then, no studies have been conducted to understand the epidemiology of the infection in Taiwan. Therefore, this study is the first epidemiologic survey of CSD in cats and humans in this country. Using veterinary-associated individuals as the study population, it was identified that 1.7% of them were seropositive for *B. henselae*, and residence was the only factor associated with seropositivity. *Bartonella* species were successfully isolated from 25 (19.1%) of the 131 cats tested. Only *B. henselae* and *B. clarridgeiae* were obtained from bacteremic cats. Furthermore, 9.2% of 131 cats were dually-infected with genotypes I and II of *B. henselae*. It is the highest prevalence of co-infection that has ever been reported worldwide. In cats, the seroprevalence was 23.7% by indirect immunofluorescence antibody assay with *B. henselae* Houston-1 (type I) as the antigen. When 12 bacteremic but seronegative cats were re-tested by IFA slides coated with *B. henselae* U-4 antigen (type II), 9 cats were identified to be seropositive. Our study further suggested that using only direct PCR of 16S-23S rRNA intergenic region or the combination of the PCR method and indirect immuno-fluorescence test will be useful to diagnose *Bartonella*-free cats.

***Bartonella* / cat scratch disease / cat / veterinary-associated population / Taiwan**

1. INTRODUCTION

Cat-Scratch Disease (CSD) is a zoonosis as domestic cats are the natural reservoir of this disease [18]. *Bartonella henselae* is the major causative agent of CSD [1, 9, 10, 33]. Though *B. clarridgeiae* has not been isolated from CSD suspected patients up to date, the species is considered to be another possible agent of CSD, based on serological findings [19, 23]. The first CSD case in Taiwan was reported in 1998 [22]. Nevertheless, the official reference laboratory for CSD diagnosis was not established until

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2001 in this country. Until now, no epidemiologic survey was conducted to understand the risks associated with CSD in cats and humans in Taiwan.

B. henselae transmission among cats is through the exposure of cat flea, *Ctenocephalides felis* [7, 18]. Humans are infected when cat-scratch or bite-wounds are contaminated with feces excreted by infected cat fleas [12, 14]. Given this fact, veterinary professionals seem to be a high-risk population and need to be investigated. Surprisingly, there have not been many epidemiologic studies worldwide on CSD in veterinary-associated populations. The first epidemiologic survey was conducted in veterinarians from the USA [30]. In that study, 6.0% (18/198) of responding veterinarians self-reported a previous diagnosis of CSD, and 3 out of these 18 individual were seropositive for *Bartonella*. Furthermore, year of experience with cats was the only identified variable associated with seropositivity [30]. There were also two serological investigations in veterinary populations in Japan [17, 20]. Kumasaka et al. [20] reported that 15% (35/233) of veterinary professionals were seropositive for *B. henselae*, and young female veterinary assistants and animal beauticians were more likely to be infected. Kikuchi et al. [17] reported that 10.9% (14/129) and 0.8% (1/129) of healthy veterinary students were IgG- and IgM-positive for *B. henselae*, respectively. History of cat-exposure was the main risk factor in this study population [17].

Bartonella infection in cats has been reported from many countries in the world [5]. In Asian countries, the results showed that seroprevalence of *B. henselae* among cat populations in Japan ranged from 9.1 to 15.1% [28, 36], 68% in Philippines [8], 48% in Singapore [29] and 54% in Indonesia [24]. Both *B. henselae* and *B. clarridgeiae* have been isolated from cats in some of these countries, including Indonesia, Thailand, Philippines and Japan [8, 24, 26, 27]. The prevalence of bacteremia ranged from 6.4% to 89% for *B. henselae* [8, 25–27] and 0.7% to 31% for *B. clarridgeiae* [8, 26, 27]. Co-infection with *B. henselae* and *B. clarridgeiae* were reported in cats from the Philippines and Japan [8, 26]. Taiwan is located in the subtropical area, with an average temperature of 22.02 °C and relative humidity of 77.8%, according to the monitoring record of the Central Weather Bureau, Taiwan in 2004. Such an environment is very suitable for growth of fleas, and flea-infested animals, such as stray cats and dogs, are commonly seen all year round. However, there has been no epidemiologic investigation on *Bartonella* infection in cats in Taiwan until now.

In order to elucidate the epidemiologic distribution of CSD in cats and humans in Taiwan, one major objective of this study was to determine the prevalence and risk factors of CSD in veterinary-associated populations. The other objective of the study was to perform a survey in various cat populations to understand which *Bartonella* species is the most prevalent and factors associated with the infection in cats in Taiwan.

2. MATERIALS AND METHODS

2.1. Collection of specimens from humans

A total of 295 human samples were collected from veterinary-associated populations in Taiwan, including 195 whole blood samples and 100 serum samples. The whole blood samples were from 114 volunteers attending a Veterinary Conference in 2002 in Taiwan, and 81 people working at the Veterinary Teaching Hospital of the National Chung Hsing University between September and October, 2002. One hundred serum samples that were originally collected to be tested for leptospiral infection in veterinary professionals, including 29 clinicians, 55 veterinary students and 16 veterinary technicians at National Taiwan University in March, 2002, were also tested for *Bartonella* infection. All subjects were administered a

structured questionnaire to gather demographic, occupational and associated exposure information.

2.2. Collection of specimens from cats

A total of 131 cat samples were collected between March 2001 and May 2003. The cats were from 3 different cat populations in Taiwan, including 30 pet cats, 37 cats from a breeding cat farm in Tainan county and 64 impound cats from a municipal stray animal shelter in Taipei. The breeding cat farm was selected for comparison because its raising environment was under strict ectoparasite control. One to two milliliters of whole blood from each cat were collected in plastic EDTA tubes (Greiner Bio-One VACUETTE® North America, USA) from jugular or saphenous vein. Whole blood samples were centrifuged at 1000× *g* to separate the plasma and blood cells. Sera were prepared from plasma after full speed centrifugation. All samples were frozen at –70 °C before tested. For the pet cats and cats from the breeding farm, descriptive data such as age, sex, neutering history, flea infestation condition of the cats were recorded by investigators through interviewing. In impounded cats, the descriptive data, including estimated age, sex, and flea infestation were recorded by the same investigator at the time of blood sampling.

2.3. Detection of *Bartonella* genomic DNA from human and cat blood

The QIAamp® DNA Blood mini Kit (QIAGEN Inc., Valencia, CA, USA) was used for extraction of DNA from 200 μL of human and cat blood samples. Forty-four cats and 107 human blood specimens were tested by a single step PCR assay aiming at the 16S-23S rRNA intergenic region, as previously described [16]. Forty-four cats were selected from 131 cats by simple random sampling using a table of random number digits, for blind evaluation of sensitivity, specificity, positive predictive value and negative predictive value of the PCR test. A total of 107 human whole blood samples were analyzed because of having enough amount of blood for DNA extraction and PCR analysis. Only whole blood samples were used for DNA extraction. Therefore, serum collected from people for the investigation of leptospirosis were not used for PCR assay. The primer set used in amplification of 16S-23S rRNA intergenic region by a single step PCR assay was BSSPF (5'-CTC TTT CTT CAG ATG ATG ATC C-3') and BSSPR (5'-AAC CAA CTG AGC TAC AAG CCC T-3'). DNA amplification was performed with PCRExpress thermo cycler (HYBAID, Ashford, UK) by the following PCR protocol: 10 min of incubation at 20 °C, followed by 2 min of denaturation at 95 °C and then 45 cycles of 1 min of denaturation at 95 °C, 1 min of annealing at 60 °C, and 30 s of extension at 72 °C. PCR amplification products were identified by ethidium bromide fluorescence after electrophoresis in 3% agarose gels. As described previously by Jensen et al. [15], the amplified fragment was 202 bp for *B. bacilliformis*, 145 bp for *B. clarridgeiae*, 232 bp for *B. elizabethae*, 163 bp for *B. henselae*, 148 bp for *B. quintana*, 251 bp for *B. vinsonii* subsp. *berkhoffii*.

2.4. Isolation of *Bartonella* spp. in cats

After thawing, 100 μL of the blood was inoculated onto two chocolate agar plates (Creative Microbiologicals LTD., Taipei, Taiwan) and incubated at 35 °C, 5% CO_2 for up to four weeks. The agar plates were regularly checked every 3 to 4 days. Identification of *Bartonella*-suspected colonies was based on morphological characteristics and growth time on agar plates. The number of colonies formed on the agar plates was then recorded, and colony-forming units (CFU) per milliliter of blood were calculated to represent the level of bacteremia. When the visible colonies were identified, they were subcultured and confirmed as *Bartonella* at the species level by molecular methods as mentioned above. The original

isolates and their subcultures were frozen at –70 °C for future usage.

2.5. Molecular identification of *Bartonella* species and 16S rRNA genotyping

Three to five colonies suspected to be *Bartonella* spp. were harvested for identification of *Bartonella* species by PCR of the citrate synthase gene (*glt*A gene) with one set of specific primers, namely BhCS.781 (5'-GGG GAC CAG CTC ATG GTG G-3') and BhCS.1137n (5'-AAT CGA AAA AGA ACA GTA AAC A-3'). The PCR products were further processed by restriction fragment length polymorphism (RFLP) analysis with *Taq* I (Biolabs® Inc., USA) and *Hha* I (Takara Biochemicals, Ohotsu, Japan) digestion [31]. Genomic DNA was obtained by boiling bacterial colonies at 100 °C for 10 min. The template DNA was mixed with the reaction solution (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 3 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1% Triton X-100) containing 1 mM of dNTPs, 20 pmol each of sense/antisense primers, 1.25 mg bovine serum albumin (BSA, SIGMA, St. Louis, MO, USA), and 2.5 U of *Taq* polymerase (GeneTeks BioScience Inc., Germany), and adjusted to a final volume of 50 μL. DNA amplification was performed with PCRExpress thermo cycler (HYBAID) with initial denaturation (95 °C, 5 min), followed 35 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 30 s) and extension (72 °C, 2 min), with a single final extension step (72 °C, 5 min). The amplified fragment (379 bp) was subjected to electrophoresis in a 3% agarose (NuSieve® 3:1 agarose, BioWhittaker Molecular Applications, Rockland, ME, USA) gel and stained with 0.6 μg/mL ethidium bromide solution. After confirmation by electrophoresis, the amplicon was digested with *Taq*I and *Hha*I restriction endonucleases. The isolates were identified as *B. henselae* or *B. clarridgeiae* by comparing the standard band patterns of the type strains, *B. henselae* Houston-1 (American Type Culture Collection, ATCC 49882) and *B. clarridgeiae* (ATCC 51734).

Genotyping of *B. henselae* was performed by PCR of the 16S rRNA gene as previously described by Bergmans et al. [2] with minor modifications. The reaction solution of PCR was prepared with two sets of *B. henselae* 16S rRNA gene type-specific primers: 16SF and either BH1 or BH2. DNA amplification was performed with PCRExpress thermo cycler with initial denaturation (95 °C, 3 min), followed 30 cycles of denaturation (95 °C, 20 s), annealing (56 °C, 30 s) and extension (73 °C, 1 min), with a single final extension step (73 °C, 5 min). Amplified products were subjected to electrophoresis in a 3% agarose gel and the gel was stained with 0.6 μg/mL ethidium bromide solution. When the specific band of 185 bp was observed with primers 16SF and BH1, the strain was identified as type I. While the specific band of 185 bp was observed with primers 16SF and BH2, the strain was identified as type II. The strains Houston-1 and U-4 were used as the reference strains of *B. henselae* types I and II, respectively. Strain U-4 was kindly shared by Dr Bruno B. Chomel (University of California, Davis, USA).

2.6. *B. henselae* indirect immunofluorescence antibody test

The antibody titers to *B. henselae* were determined by indirect immunofluorescence antibody test (IFA), using slides respectively made by *B. henselae* Houston-1 (ATCC 49882) and *B. henselae* U4 (University of California, Davis) as antigens [32]. The type strain was cultured on chocolate agar plate at 37 °C in 5% CO_2 for 4 days to grow up a confluent plate of bacteria. The cultured organisms harvested from agar plates were suspended in 0.5 mL phosphate buffer saline (PBS, pH 7.4) and added into 15 mL M199 tissue culture media (SIGMA) with 5% fetal bovine serum (FBS, HyClone® Laboratories Inc., Logan, UT, USA). The bacteriological suspension was inoculated to a 90% confluent Vero cell

(CCRC 60013, Bioresources Collection and Research Center, Taiwan) tissue culture flask (75 cm^2) and incubated at 37 °C with 5% CO_2 for 2 days. After incubation, the tissue culture was washed twice with sterile calcium- and magnesium-free PBS, and then was treated with trypsin for harvesting the infected cells. After using sterile PBS to re-suspended cells, the suspension was centrifuged at 200× *g* for 10 min. Then, the supernatant was discarded and the cells were resuspended in 30 mL growth medium for tissue culture. A volume of 30 μL of suspension containing infected cell was distributed onto each well of 12-hole Teflon printed slides (Electron Microscopy Science, Hatfield, PA, USA), and the slides were incubated at 37 °C with 5% CO_2 overnight. After incubation, the slides were washed twice in PBS, then fixed in acetone and air-dried. The slides were put at –70 °C for storage.

For IFA testing, the frozen sera were thawed at room temperature and treated at 56 °C for 30 min for heat inactivation. The serum samples were serially diluted from 1:32 to 1:512 by twofold dilutions using PBS (with 10% skim milk). Thirty microliters of diluted serum was dropped onto each well of slides previously prepared. The slides were incubated at 37 °C for 40 min and washed with PBS for 10 min. The secondary antibodies used for serological testings in humans and cats were fluorescein-labeled goat anti-human immunoglobulin G and goat anti-cat immunoglobulin G (Kirkegaard® Perry Laboratories Inc., Gaithersburg, MD, USA), respectively. They were diluted at 1:400 in PBS, and the mixture was applied to each well. The slides were incubated at 37 °C for 40 min, washed with PBS for 10 min, and washed again with double distilled water for 10 min prior to reading with a fluorescent microscope (magnification, ×400). The intensity of the bacillus-specific fluorescence was scored subjectively from 1 to 4, and the fluorescence score of ≥ 2 at dilution of 1:64 was considered to be positive. The seronegative cats were tested by IFA using *B. henselae* U4 as the antigen.

2.7. Statistical analysis

The data were analyzed by SAS® version 6.12 and Microsoft Excel. The chi-square test for homogeneity was used to evaluate the association between disease status (bacteremia or seropositivity) and a categorized risk factor, and *P* value was calculated using Yates corrected method or two-tailed Fisher's exact test when expected numbers of observations were less than five.

3. RESULTS

In the veterinary-associated population, 5 (1.7%) of the 295 persons were seropositive for *B. henselae*. All five seropositive individuals had recalled cat or dog exposures during the last 6 months. No major risk factors that we investigated were associated with seropositivity to *B. henselae*. Univariate analysis by Fisher exact test showed that residence was the only factor associated with seropositivity for *B. henselae* ($P < 0.05$) (Tab. I). However, it was observed that only a few samples were from the eastern area of Taiwan. None of the 107 human blood specimens tested were PCR-positive, but 5 of them were seropositive for *Bartonella*. Their antibody titers were all at 1:64, which implied past infection.

Bartonella species were successfully isolated from 25 (19%) of the 131 cats tested (Tab. II). These isolates were confirmed to be *Bartonella* species by PCR/RFLP of the citrate synthase (*gltA*) gene with *Taq*I and *Hha*I digestion. Comparing to the PCR/RFLP patterns of the reference strains, it was identified that one isolate was *B. clarridgeiae*, and 24 isolates were *B. henselae* (Fig. 1). *B. henselae* isolates were further genotyped by PCR of the 16S rRNA gene. Because there were two samples with major fungal contamination that could not be analyzed by genotyping of 16S rRNA gene, the