DISSERTATION

DEVELOPMENT AND APPLICATION OF NEW DIAGNOSTIC ASSAYS FOR

THE DETECTION OF BARTONELLA HENSELAE INFECTION IN CATS

Submitted by

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ABSTRACT

DEVELOPMENT AND APPLICATION OF NEW DIAGNOSTIC ASSAYS FOR

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Many species of the bacteria in the genus *Bartonella* are well known as zoonotic pathogens in humans and are increasingly recognized in a wide range of animals. To date, several mammals are known to serve as natural reservoirs of a variety of *Bartonella* spp.. *Bartonella henselae* is one of the most common species and primarily causes cat-scratch disease in humans. The cat plays a role as a predominant reservoir of this organism. *Bartonella henselae* infection in cats is widespread in most countries due to transmission via the cat flea, *Ctenocephalides felis*. Seroprevalence rates as high as 90% and bacteremia rates as high as 80% have been reported in flea infested healthy cats. While most infected cats show no signs of disease, some develop non-specific manifestations such as fever. Thus, laboratory assay results are necessary to aid in the diagnosis of the clinical feline bartonellosis.

Serology, culture, and PCR assays for the amplification of *Bartonella* spp. DNA are the assays most widely available commercially. Results of the currently available assays are commonly reported as positive or negative and because of the high prevalence rates of infection, positive assay results are often not associated with the clinical manifestations of *B. henselae* infection. In some infectious disease, levels of bacteremia are associated with clinical manifestations of disease. While quantitative *B. henselae* bacterial culture has been performed in some research cats, test results take several weeks to return and quantitative culture has not been used to evaluate clinically ill cats in the field. PCR assay and antigen assay results can be

returned quickly. However, levels of *B. henselae* DNA or antigen in the blood of cats have not been evaluated for association with clinical manifestations of disease, particularly fever. Thus, the primary goals of the research described in this dissertation were to develop new quantitative diagnostic assays and to apply them to an at-risk cat population in Thailand in order to determine if the assays would aid in the diagnosis of fever caused by *B. henselae*. The 2 diagnostic assays developed were 1) a quantitative real-time PCR (qPCR) and 2) an antigen-capture ELISA (AC-ELISA). Both assays can be used to determine the quantity of *B. henselae* in feline blood and can have results returned within one day.

The experiments describing the titration of a new qPCR for amplification of the *groES* gene of *B. henselae* in blood of cats are presented in Chapter 3. The qPCR was shown to have high sensitivity and could detect at least 52 CFU/ml of *B. henselae* in blood accurately. The assay was also shown to be highly specific and reproducible.

The experiments to titrate a new *B. henselae* AC-ELISA are described in Chapter 4 of the dissertation. Rabbit polyclonal antibodies against the CSU-1 isolate of *B. henselae* were produced and purified for specific use in this research. After the AC-ELISA was optimized, the assay has shown to be specific for detection of *B. henselae* antigen and detect at least 1.25 ng/μl of *B. henselae* antigen in feline blood. However, when applied to blood collected from experimentally-inoculated cats, the AC-ELISA detected only 50% of cats shown to be positive by the qPCR assay and so it was concluded the assay had lower analytical sensitivity.

The prevalence of several *Bartonella* spp. infections (*B. henselae*, *B. clarridgeiae*, and *B. koehlerae*) that use *C. felis* as the vector generally correlates to the infestation prevalence. Thailand is located in a tropical area where the climate is appropriate for *C. felis*. However, available information regarding *Bartonella* infection of client-owned cats and *C. felis* collected

from those cats is minimal. Thus, Chapter 5 of the dissertation reports the DNA prevalences of *Bartonella* spp. and other blood borne infections, including hemoplasmas and *Rickettsia felis* in blood and fleas of client-owned cats which lived in Bangkok, Thailand. The prevalence rates of *Bartonella* spp. DNA in blood and flea samples were 17% and 32%, respectively. *Bartonella henselae* and *B. clarridgeiae* were amplified most commonly. Hemoplasmas were commonly found in cats and fleas, as well, but *R. felis* was absent. The results of this preliminary study were used to design the subsequent field study to collect samples from cats with and without fever.

Fever is the most common clinical sign in cats experimentally infected with *B. henselae*. To determine the clinical utility of the *B. henselae* qPCR (Chapter 3) and AC-ELISA (Chapter 4), these assays were applied to the blood samples collected from healthy cats and cats with fever of unknown origin in Bangkok, Thailand (Chapter 6). Risk factors that might be associated with fever were also determined. Using the qPCR, cats with fever were significantly more likely than afebrile cats to have *B. henselae* DNA in blood supporting the hypothesis that *B. henselae* is associated with fever in naturally infected cats. Cats with fever had 50X higher median bacterial counts of *B. henselae* than those of afebrile cats but the results were not statistically different between groups because of the wide range. However, these findings suggest that cats with high bacterial loads were more likely to develop fever but a larger sample size is needed. The AC-ELISA was shown to be less sensitive than the qPCR assay with only about 30% of the qPCR positive samples being positive by AC-ELISA. Because of the low overall sensitivity, the AC-ELISA did not provide enough information to determine an association with fever. However, the sample set collected in this chapter and the prototype AC-ELISA results could be used in future experiments designing more sensitive assays.

The research described in this dissertation raises the knowledge in the field of feline bartonellosis. The results provide additional prevalence results for cats in one area of Thailand, provides additional information that *B. henselae* infection in cats can be associated with fever, and suggests that use of qPCR results are superior to non-quantitative test results to make an association between *B. henselae* and fever.

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CHAPTER 1

Literature Review

1.1 Taxonomy

Bartonella spp. are gram-negative bacteria that belong to alphaproteobacteria on the basis of 16S rDNA sequences. More than 20 species and subspecies have been discovered in a wide range of domestic and wild animals and the number of species identified is still expanding. *Bartonella* spp. are highly adaptive and co-evolved organisms in mammalian reservoir hosts. Each species of *Bartonella* is typically identified and designated in at least one reservoir host (Table 1-1).

1.2 Bacteriological Features

Bartonella spp. are small (0.3-0.6 x 0.3-1.0 μm) aerobic fastidious slow-growing pleomorphic bacilli or coccobacilli. Microscopically, *B. clarridgeiae* and *B. bacilliformis* have flagella, which facilitates erythrocyte invasion, while flagella are not apparent in other *Bartonella* spp., such as *B. henselae*. The organism in infected tissues can be stained with Warthin-Starry silver, and the organism in blood can be evaluated by blood culture. Blood is usually frozen to induce red blood cell lysis before being plated onto agar containing fresh blood of sheep, rabbit, or horse. An inoculated plate is incubated for at least one week at 35 - 37 °C in 5% carbon dioxide environment. Primary isolates of some *Bartonella*, such as *B. henselae*, *B. clarridgeiae*, *B. vinsonii*, or *B. elizabethae* have colonies with a white, rough, dry, raised appearance and pit the medium (Boulouis et al., 2005). Biochemical profiles of *Bartonella*,

including catalase, oxidase, urease and nitrate reductase are negative except for the production of

peptidases, which varies slightly among species (Breitschwerdt and Kordick, 2000).

Table 1-1 *Bartonella* species and subspecies, primary reservoir hosts, vectors, and known accidental hosts (Chomel and Kasten, 2010)

Species	Primary reservoir	Vector	Incidental host
B. alsatica	Rabbit	Rabbit flea (potential) (*Spilopsyllus cuniculi*)	Human
B. bacilliformis	Human	Sand fly (*Lutzomia verrucarum*)	None
B. clarridgeiae	Cat	Cat flea (*Ctenocephalides felis*)	Human, dog
B. elizabethae	Rat	Rat flea (*Xenopsylla cheopis*)	Human, dog
B. grahamii	Wild mice	Rodent fleas	Human
B. henselae	Cat	Cat flea (*Ctenocephalides felis*)	Human, dog, horse, marine animals
B. koehlerae	Cat	Cat flea (*Ctenocephalides felis*)	Human, dog
B. melophagi	Sheep	Sheep ked (*Melophagus ovinus*)	Human
B. quintana	Human	Body louse (*Pediculus humanis*)	Cat, dog
B. rochalimae	Canids	Fleas (potential) (*Pulex irritans*, *Pulex simulans*)	Human, dog
B. vinsonii subsp. *arupensis*	White-footed mouse	Unknown	Human
B. vinsonii subsp. *berkhoffii*	Dog, coyote	Unknown	Human, cat
B. washoensis	California ground squirrel	Flea (*Oropsylla montana*)	Human, dog

Identification of *Bartonella* strains and species of the isolate can be performed using

molecular methods by amplification of selected genes such as 16S rRNA, 16S - 23S rRNA

spacer region, or protein-encoding genes e.g. citrate synthase, followed by restriction fragment

length polymorphism (RFLP) or DNA sequencing.

1.3 Clinical Relevance

Following the discovery of *Bartonella* spp., the knowledge of the organism has been expanded in numerous publications. Many *Bartonella* spp. were apparently defined as zoonotic pathogens since the early 1990s. At least eight *Bartonella* species or subspecies, including *B. henselae*, *B. koehlerae*, *B. elizabethae*, *B. clarridgeiae*, *B. vinsonii* subsp. *arupensis*, *B. vinsonii* subsp. *berkhoffii*, *B. grahamii*, and *B. washoensis* have been recognized as zoonotic agents in humans, and are responsible for a wide spectrum of symptoms in multiple organ systems (Chomel et al., 2004). Most reservoir animal hosts typically have subclinical infections or have minimal clinical signs, but infections may cause severe disease in incidental hosts. Host responses to infection range from subclinical or asymptomatic to significant clinical manifestations depending on virulence of the *Bartonella* spp., host immunity and host adaptation. *Bartonella bacilliformis* and *B. quintana* are human-specific. Infection with *B. bacilliformis* causes Carrion's disease that is frequently reported in South America. The acute phase, also known as Oroya fever, is characterized by an acute fatal hemolytic anemia, and the chronic vasoproliferative phase called verruga peruana, in which the patients develop cutaneous nodular vascular eruptions. Infection in animals has never been reported. *Bartonella quintana* is not considered to be zoonotic; but the organism is the etiologic agent of trench fever and bacillary angiomatosis (BA), characterized by a vascular proliferative lesion, that is commonly observed in immunocompromised patients (Koehler and Tappero, 1993). In addition, *B. quintana* has been an etiology of endocarditis and chronic bacteremia in homeless people (Brouqui et al., 1999). Infection with *B. quintana* may have mild illness with low morbidity and limited mortality. Humans are also considered the primary reservoir host for *B. quintana*, which is transmitted by the human body louse. However, *B. quintana* DNA has been identified from

blood of animals such as feral cats (Breitschwerdt et al., 2007b), dogs with endocarditis (Kelly et al., 2006), healthy Golden Retrievers and Golden Retrievers with lymphoma (Duncan et al., 2008), and a captive-bred cynomolgus monkey (*Macaca fascicularis*) (O'Rourke et al., 2005).

Cats are a major reservoir host for *B. henselae* and potentially *B. clarridgeiae*. *Bartonella henselae* is generally isolated or DNA amplified from blood of cats, and most infected cats have no clinical signs. Nevertheless, mild to severe clinical signs have been documented in naturally and experimentally *B. henselae* infected cats. In humans, *B. henselae* infection results in self-limiting illness as cat-scratch disease (CSD) or severe clinical diseases e.g. BA. *Bartonella henselae* is the primary etiologic agent of CSD, which is commonly characterized by fever and localized lymphadenopathy. In typical CSD patients, a papule and then a pustule usually develops within 7-12 days after a cat scratch at the inoculation site. A regional lymphadenopathy develops within a few weeks after infection and can persist a few weeks to several months. Abscessed lymph nodes are reported occasionally. Fever, malaise, and muscle ache are often reported. Less than 10% of CSD patients may develop atypical CSD. A broad spectrum of disease manifestations of atypical CSD are currently recognized, such as fever of unknown origin (FUO), peliosis hepatis, BA, Parinaud's oculoglandular syndrome, encephalitis, neuroretinitis, osteomyelitis, and endocarditis (Florin et al., 2008). *Bartonella henselae* infection was also reported to be a cause of peliosis hepatis in dogs (Kitchell et al., 2000).

Bartonella clarridgeiae antibodies were reported in a suspect case of CSD, in a patient with a chest-wall abscess due to CSD, and in some patients with lymphadenopathy (Kordick et al., 1997a; Margileth and Baehren, 1998; Sander et al., 2000). However, significant cross-reactivity between *B. henselae* and *B. clarridgeiae* was identified (Tsuneoka et al., 2004). Based upon the serological evidence, *B. clarridgeiae* may be an infrequent agent of CSD and other

clinical syndromes in people. While frequently detected in cats and dogs, associations with clinical disease are unclear.

B. vinsonii subsp. *berkhoffii* was originally isolated from a dog with endocarditis (Breitschwerdt et al., 1995), but the role of dogs for serving as a reservoir host for *B. vinsonii* subsp. *berkhoffii* or other *Bartonella* spp., such as *B. henselae*, or *B. clarridgeiae* is poorly defined. However, there are increasing reports that this organism is associated with clinical illnesses in humans. For example, *B. vinsonii* subsp. *berkhoffii* was isolated from a patient with endocarditis (Roux et al., 2000), and patients with arthritis, fatigue, neurologic or neurocognitive problems such as headaches, insomnia, memory loss, or incoordination (Breitschwerdt et al., 2007a; Breitschwerdt et al., 2008; Breitschwerdt et al., 2010). Most of these individuals had animal contact and arthropod exposure as potential risk factors. Recently, *B. henselae* and *B. vinsonii* subsp. *berkhoffii* have been isolated or DNA amplified from several body fluids, including pleural, pericardial, and abdominal effusions from dogs with idiopathic cavitary effusions or constrictive pericarditis (Cherry et al., 2009), synovial fluid from a polyarthritic dog, and subcutaneous seroma fluids from a traumatic dog (Diniz et al., 2009b). Although the extent to which effusions are caused by *B. henselae* and *B. vinsonii* subsp. *berkhoffii* is unconfirmed, the bacteria may be a cause in the development of various effusions (Breitschwerdt et al., 2010). Alternatively, it is possible that dogs are previously infected with *Bartonella* spp., and the bacteria accumulate in fluids due to other illnesses. Further studies are required to define the role of *Bartonella* spp. as a cause of effusive disease in dogs. DNA from several *Bartonella* spp. was also amplified from the saliva of healthy and sick dogs (Duncan et al., 2007; Kim et al., 2009).

Beside *B. vinsonii* subsp. *berkhoffii* and *B. henselae* that have been associated with endocarditis in humans, *B. elizabethae* (Daly et al., 1993), *B. koehlerae* (Avidor et al., 2004), *B.*

vinsonii subsp. *arupensis* (Fenollar et al., 2005), *B. alsatica* (Raoult et al., 2006), which are host adapted *Bartonella* for rat, cat, mouse, and rabbit, respectively, have been documented in endocarditis cases so far. Furthermore, *B. vinsonii* subsp. *berkhoffii*, *B. henselae*, and *B. clarridgeiae* (Chomel et al., 2001; Chomel et al., 2009b) have also been reported from dogs with endocarditis. *Bartonella grahamii* for which a rodent is a reservoir has been associated with patients of neuroretinitis (Kerkhoff et al., 1999). Recently, several novel *Bartonella* spp. have been isolated from sick patients and animals, such as *B. tamiae* (rodent reservoir) from patients with fever, myalgia, fatigue, and headache (Kosoy et al., 2008), or *B. rochalimae* (fox reservoir) was isolated from a woman with bactermia, fever, and splenomegaly (Eremeeva et al., 2007). *Bartonella rochalimae* has also been isolated from gray foxes, healthy dogs, and a dog with endocarditis (Henn et al., 2007; Diniz et al., 2009a; Henn et al., 2009).

1.4 Course of Infection in the Mammalian Reservoir Host

Bartonella spp. are hemotropic and endotheliotropic organisms. The infection causes prolonged bacteremia and periodically induces a relapsing bacteremia in reservoir hosts. The presence of *B. henselae* in erythrocytes of bacteremic cats was first demonstrated (Kordick and Breitschwerdt, 1995), and invasion of *B. henselae* into feline erythrocytes was described a few years later (Mehock et al., 1998). The course of *Bartonella* spp. infection in mammalian reservoir hosts was subsequently described in detail by the rat model of *B. tribocolum* infection (Dehio, 1999; 2001; Schülein et al., 2001).

In the rat model, after animals were infected with *B. tribocolum* by intravenous inoculation, bacteria were rapidly cleared from circulation or maintained below detectable levels for about four days. Bacteria might be unable to infect erythrocytes during early stages of

infection, but they seemed to colonize a primary niche which is not clearly identified. However, it has been suggested that endothelial cells are an important localization of the primary niche (Dehio, 2005). After release of *B. tribocolum* from the primary niche into the bloodstream, the organism is competent for erythrocyte interaction. The organism can adhere to mature erythrocytes, followed by invasion and replication within a membrane-bound compartment of erythrocytes until a critical density is reached. The intraerythrocytic bacteria are sustained for the remaining lifespan of the infected erythrocytes (Schülein et al., 2001). Large numbers of *B. tribocolum* are usually released from the primary niche into the blood circulation on approximately day five post-inoculation. Bacteria can re-infect the primary niche which triggers further bacterial release, and followed by waves of re-infection. These observations suggested periodic sequestration in one or more tissue sites followed by re-entry into the vasculature and a relapsing pattern of bacteremia. The 5-day period of human trench fever caused by *B. quintana* is probably described by the bacterial re-infection. A relapsing pattern of bacteremia was also demonstrated in cats infected with *B. henselae* by blood transfusion with unpredicted period (Kordick and Breitschwerdt, 1997), but this phenomenon has not been confirmed in humans, dogs, and other reservoir animals.

The certain life cycle of *Bartonella* spp. is different from species to species probably due to specific paths of pathogen-host adaptation, but the overall concept of this course of infection is conserved among members of the genus *Bartonella* (Figure 1-1).
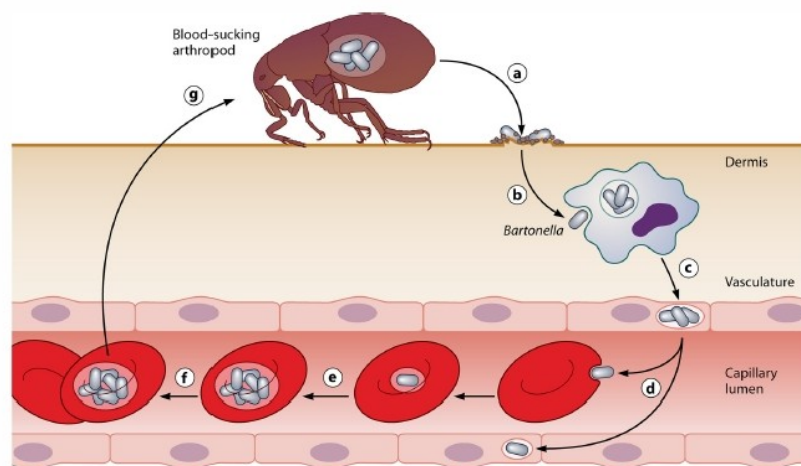
Figure 1-1. Common infection strategy of the bartonellae (Harms and Dehio, 2012). The figure illustrates the general concept of reservoir host infections with *Bartonella*. Following transmission by an arthropod vector (a), the bartonellae entry into migratory cells and are transported to the primary niche (b) colonize into the vascular endothelium (c), where the bacteria persist intracellularly. From the primary niche, the bacteria are seeded into the bloodstream (d), where they invade erythrocytes and reinfect the primary niche. After limited replication inside the red blood cell (e), they persist in the intraerythrocytic niche (f) competent for transmission by a bloodsucking arthropod (g).

Intracellular bacteria seem to have immunological privilege. Therefore, *Bartonella* spp. can persist in the bloodstream for several weeks without causing an abnormality to the experimental rat. Lifespan of infected erythrocytes is similar to that of uninfected cells therefore immunological responses are unlikely to recognize the infected erythrocytes. Antibodies produced by the rat may neutralize bacteria released from the primary niche and protect the infection of additional erythrocytes as well as the re-infection of the primary niche. The bacteremia level of *B. tribocolum* in rats decreases below the detectable level after 10 weeks post-inoculation. A similar duration of bacteremia has been observed in experimental model of *B. vinsonii* subsp. *berkhoffii* infection in dogs (Pappalardo et al., 2001), but longer period of

bacteremia was observed in experiment cats inoculated with *B. henselae* feline strain or *B. clarridgeiae* (Yamamoto et al., 2002a; Yamamoto et al., 2003). To date, mechanisms that maintain bacterial persistence are not clearly understood. The intracellular colonization within erythrocytes and endothelial cells is probably a potential strategy for bacterial persistence, evasion from host immune responses, distribution throughout tissues of host, and transmission by blood-sucking arthropod vectors (Chomel et al., 2009a).

Bartonella henselae* was demonstrated to infect human CD34+ progenitor cells *in vitro* (Mändle et al., 2005). This finding suggests that bacteria may infect bone marrow cells as well as the progenitor cells of erythrocytes. An *in vitro* study indicated that *B. henselae* can infect microglial cells, which are professional macrophages (Muñana et al., 2001). Other reticuloendothelial cells, such as dendritic cells, monocytes, tissue macrophages are capable of carrying bacteria and migrating through many organs. Infection of these monocytes and professional macrophages may allow *Bartonella* to localize to injured tissues as a component of the inflammatory process (Breitschwerdt et al., 2010). Besides red blood cells, the monocytes and professional macrophages are also questioned as being the primary niche of *Bartonella* infection, as well (Chomel et al., 2009a). However, further studies are needed for supporting this hypothesis. It appears that mechanisms of host adaptation to *Bartonella* spp., including endothelial cell localization and erythrocyte and macrophage infection may be related to the diversity of disease manifestations among individual hosts. *Bartonella* spp. may preferentially infect the erythrocytes of a given mammalian host without causing a disease, while endothelial cells may become infected and leaded to severe disease or lesion during incidental infection of non-reservoir hosts. For example, human infected with cat-specific *B. henselae* may cause BA

lesions where bacteria are identified in close association with proliferating endothelial cells (Dehio, 2005). Further studies need to be conducted to confirm this hypothesis.

1.5 Pathogenicity Factors Involved in Adaptation to the Mammalian Host

The pathogenicity factors of *Bartonella* spp. have been studied from the complete genomic sequences of 4 species of *Bartonella*, including *B. henselae*, *B. quintana*, *B. bacilliformis*, and *B. tribocorum*. Comparative genome analysis revealed that only 959 genes are conserved in all the genomes, which is known as small core genome that reflects specific adaptations and preferences of these bacteria to host-integrated metabolism, such as hemin (iron-containing porphyrin). This substance is enriched in the host niches of *Bartonella* e.g. the intracellular space of erythrocytes and the mid-gut lumen of bloodsucking arthropods. An in vitro study documented the requirement of hemin for growth of *B. quintana* due to the presence of multiple genes encoding hemin binding and hemin uptake protein (Alsmark et al., 2004). A similar result was observed in the rat model of *B. tribocorum* infection using a large-scale signature tagged mutagenesis screen (STM), in which several of the hemin-uptake genes were identified as important for intraerythrocytic infection (Saenz and Dehio, 2005). The STM also confirmed that the majority of pathogenicity factors are dependent on the core genome of the four available genome sequences. All other genes that are not strictly conserved in all four sequenced *Bartonella* spp. are excluded from the core genome and constitute the accessory genome, which are organized in genomic islands. Some of the genome islands, such as type IV secretion systems and bacterial adhesins are important for *Bartonella*-host cell interaction and *Bartonella*-host adaptation (Saenz et al., 2007).

1.5.1 Type IV Secretion Systems

Type IV secretion systems (T4SSs) are ancestrally related to bacterial conjugation machines of gram-negative bacteria that mediate the transfer of DNA and protein among diverse species of bacteria and from the bacterial cytoplasm directly into the host cell cytoplasm (Christie et al., 2005). In addition to *Bartonella* spp., T4SSs represent crucial pathogenicity factors for many important human pathogens such as *Helicobacter pylori*, *Legionella pneumophila*, *Bordetella pertussis*, and *Brucella melitensis*. T4SSs are recognized prominently for its capacity of *Bartonella* to adapt to their host. The accessory genome of *Bartonella* encodes at least three distinct T4SSs, which all are absent from the genome of *B. bacilliformis*. Thus, this human pathogen is placed an isolated position in the *Bartonella* phylogeny as the only representative of an ancestral lineage. All other species of *Bartonella* evolve in a separate modern lineage and represent highly host-adapted pathogens of limited virulence potential. *Bartonella* spp. of the modern lineage encode at least one of the closely related T4SSs, VirB/VirD4, Vbh, or Trw that involves diverse important functions in the colonization, invasion, and persistence within either endothelial cells or erythrocytes (Schulein and Dehio, 2002; Seubert et al., 2003; Schröder and Dehio, 2005). The bacterial effector proteins associated with vascular endothelial cell functions for establishing chronic infection are translocated by the VirB/VirD4 and Vbh, which are VirB-like T4SSs (Schmid et al., 2004; Saenz et al., 2007), while the interaction with erythrocytes is related to pilus-associate variant surface proteins expressed by the Trw (Seubert et al., 2003). Trw produces multiple variant pilus subunits critically involved in the invasion of erythrocytes. The adapted T4SSs in modern lineage lead to diminished virulence and pathogenicity in a host, bacteria thus persist for a long-term period.

1.5.2   Trimeric Autotransporter Adhesions

Bacterial adhesins are commonly associated with the pathogenesis of most bacterial infections. Adhesins can bind to extracellular matrix proteins of host cells, and assist the translocation of effectors from a bacterium into the host cell via T4SSs. The function of *Bartonella* trimeric autotransporter adhesions (TAA) is non-pilus-associated on the bacterial surface, and they are orthologous in distinct species, i.e. BadA (340 kDa protein) in *B. henselae* (Riess et al., 2004), and the Vomp family (4 of 100 kDa-proteins) in *B. quintana* (Zhang et al., 2004). The *Bartonella* TAA plays roles in host endothelial cell binding by *B. henselae* (Riess et al., 2004; Kaiser et al., 2008), and in autoaggregation and collagen binding by both *B. quintana* (Zhang et al., 2004) and *B. henselae* (Riess et al., 2004). *Bartonella* TAA is known to represent significant virulence factors, and they are also immunodominant antigens that are rapidly notified by the host immune system (Riess et al., 2004). However, it has been suggested that *Bartonella* spp. escape the immune system of host by an increase in frequency of recombination of repetitive DNA sequences in the stalk domains of BadA and Vomp (Linke et al., 2006). For example, recombination and deletion of Vomp genes result in generation of altered Vomp (antigenic variation; altered the protein expressed), or loss of surface expression of one or more Vomp (phase variation; no longer expressed the protein on its surface). These antigenic and phase variation strategies can be utilized by *Bartonella* spp. to bind to different host cell types. The bacteria can evade immune system and survive in the host. This strategy facilitates the organism to multiply and reach the high density in the bloodstream which is then passed from the mammalian host to the hematophagous arthropod vector in order to be transmitted to a new host.

In addition to the function of host cell binding of BadA and Vomp, they are involved in eliciting a proangiogenic host cell response that leads to generating vascular skin lesions known

as BA. These lesions are specific to immunocompromised patients with *B. henselae* and *B. quintana* infections (Koehler et al., 1992). The mechanism of BA angiogenesis is not completely described. However, BadA and Vomp expression is thought to be required for activation of HIF-1 and secretion of proangiogenic cytokines e.g. vascular endothelial growth factor. It is hypothesized that the localization of *Bartonella* in the tissues where hemin is limited induces formation of new vessels that would provide this necessary substance. Other virulence factors of *Bartonella* TAA are further studied.

1.6 Strategies Utilized by *Bartonella* to Escape the Host Immune Response

In addition to strategy of phase and antigenic variations as described in section 1.5, *Bartonella* spp. are capable of evading the host immune response by their diverse strains and species that can co-infect the host. Different species of *Bartonella* and several strains of *B. henselae* were commonly identified in cats (Gurfield et al., 1997). *B. henselae* strains of human isolates are more relatively limited (Dillon et al., 2002). Based on sequences of 16S rDNA, the major strains that infect cats belonged to genotype II (Marseille), but most strains involved in CSD patients were within the genotype I (Houston I) (Bergmans et al., 1996; Dillon et al., 2002). Recent studies using pulsed-field gel electrophoresis, multilocus sequence typing (MLST), multispacer typing (MST), and multiple locus variable number tandem repeat analysis (MLVA) support a high level of diversity of feline strains (Iredell et al., 2003; Li et al., 2006; Arvand et al., 2007; Monteil et al., 2007; Bouchouicha et al., 2009). Other possible strategies involved in an evasion of host immune responses include outer membrane protein variation (Kyme et al., 2003) and genetic variation, in which many genetic variants were detected in primary isolates of *B. henselae* from different geographical regions (Berghoff et al., 2007).

Host immune responses require humoral and cell-mediated immunity for eliminating *Bartonella* spp. Specific antibodies appears to be important for clearance of bacteria in the initial infection with a *Bartonella* species, but it is suppressed during the relapsing bacteremia (Kabeya et al., 2006). Infection in dogs with *B. vinsonii* subsp. *berkhoffii* also documented immune suppression due to cyclic CD8+ lymphopenia, defects in monocytic phagocytosis, and impaired antigen presentation within lymph nodes (Pappalardo et al., 2000; Pappalardo et al., 2001). It may be possible that infection with *Bartonella* spp., particularly in an incidental host, might induce systemic and local immune suppression that facilitate the persistent infection (Chomel et al., 2009a).

1.7 Zoonotic Implication

Cats and dogs are considered the relevant reservoir hosts for *B. henselae* and *B. vinsonii* subsp. *berkhoffii*, respectively that can infect humans. Because many naturally infected cats have subclinical infections, they play a role as efficient carriers that transmit bacteria to humans. Most of *B. henselae*-infected cases in human are primary associated with history of cat scratches (Zangwill et al., 1993). However, infection can occur in some patients lacking history of scratches. In these cases, transmission from cat to human via a cat bite is questioned. *Bartonella* spp. DNA was recently detected from skin, claw beds, and gingiva of cats from high *Ctenocephalides felis* prevalence area in the United States (Lappin and Hawley, 2009). *Bartonella henselae* DNA was also amplified from saliva and nail samples collected from feral cats in Korea (Kim et al., 2009). These findings did not prove the transmission competency associated with saliva or biting, and the presence of *Bartonella* spp. DNA may be caused by contamination. However, cares should be taken to avoid bites, scratches, and contamination of

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saliva of cats into open wounds, particularly in immunocompromised individuals. *Bartonella* spp. DNA was also detected from oral swabs collected from dogs (Duncan et al., 2007), therefore people should aware of contaminating wounds with saliva of dogs, as well. Infection can occur in some patients that never previously contact with cats. Other arthropod vectors or animal hosts may possibly be involved in transmission, but these have not been well-defined.

Human cases of *Bartonella* infections are mostly people who are veterinarians or are otherwise exposed to animals or arthropods. Due to extensive contact with a variety of animal species, careers involving veterinary medicine appear to have an occupational risk of being exposed to *Bartonella* infection. Regardless of unclarified causation data to date, these individuals should increase precautions to avoid animal bites or scratches, particularly from unknown animals, arthropod bites and feces (fleas, ticks, and lice), and direct contact with blood and other body fluids from sick animals. The higher risk of exposure should be concerned particularly when people are handling feral or rescued animals. A veterinarian reported headaches, fatigue, and intermittent paresthesias after a needle stick during a fine-needle aspiration in a dog with diffuse cutaneous masses. *Bartonella vinsonii* subsp. *berkhoffii* genotype I was amplified from blood of the patient and cultured isolates, and seroconversion to *B. vinsonii* subsp. *berkhoffii* genotypes I and III were found after 30 days post-infection (Oliveira et al., 2010). Therefore, veterinary professionals and staffs should avoid even needle sticks and cuts. Beside of *B. henselae* and *B. vinsonii* subsp. *berkhoffii*, other bacteria in this genus i.e. *B. clarridgeiae* and *B. koehlerae* have been detected in cat fleas (Rolain et al., 2003b), and *B. quintana* DNA has been amplified from ticks (Chang et al., 2001), therefore people should also aware of arthropod vectors that may possibly transmit these organisms.

In 2006, the American Association of Feline Practitioners (AAFP) released a panel report on various issues of *Bartonella* spp. infections (Brunt et al., 2006). The AAFP recommendation for avoiding zoonotic bartonellosis and *Bartonella* spp. infections of cats include:

- Maintain an appropriate flea-control program year-round.

- A healthy cat at the age over 1 year and free from fleas can be adopted, by a family that has an immunocompromised member.

- Immunocompromised individuals should avoid contact with cats of unknown health status.

- Scratches and bites should be avoided.

- Cat claws should be trimmed regularly, but declawing of cats is not required.

- Cat-associated wounds should be washed promptly and thoroughly with soap and water and medical advice sought.

- Although *Bartonella* spp. have not been shown to be transmitted by saliva, cats should not be allowed to lick open human wounds.

1.8 Feline Bartonellosis

B. henselae (previously known as *Rochalimaea henselae*) was first isolated and identified from a cat in the 1990's (Regnery et al., 1992). Currently, four other species, including *B. clarridgeiae*, *B. koehlerae*, *B. quintana*, and *B. bovis* have been known to infect cats (Brunt et al., 2006). Cats serve as reservoir hosts for three of these five species: *B. henselae*, *B. clarridgeiae*, and probably *B. koehlerae*. The DNA of *B. henselae* and *B. clarridgeiae* are commonly isolated or amplified from blood of cats from various geographical regions. *Bartonella koehlerae* was

previously isolated from two naturally infected cats in northern California (Droz et al., 1999), one cat in France (Rolain et al., 2003a), and one cat in Israel (Avidor et al., 2004). Recently, *B. koehlerae* DNA was amplified from blood of two cats in Thailand (Assarasakorn et al., 2012). *Bartonella quintana* was isolated from the dental pulp of a cat (La et al., 2005), and was isolated from 2 barn cats (Breitschwerdt et al., 2007b). *Bartonella bovis* is the host adapted species for ruminants that has been isolated from a few cats (Chomel et al., 2004).

1.8.1 *Bartonella spp. and Vectors*

Transmission for many *Bartonella* spp. is primarily related to arthropod vectors, as listed in Table 1-1. For *B. henselae* transmission among cats, the cat flea (*Ctenocephalides felis*) has been known to be the dominant vector.

Ctenocephalides felis

Ctenocephalides felis is responsible for the transmission of *B. henselae*, *B. clarridgeiae*, and potentially *B. koehlerae* among cats. Since *B. henselae* was first identified from cat fleas (Koehler et al., 1994), they were consequently proved as a main vector of *B. henselae* infection in cats (Chomel et al., 1996). In the study of Chomel et al. (1996), specific-pathogen-free (SPF) kittens were experimentally infected with *B. henselae* after infected fleas infested on them. Whereas SPF kittens housed with bacteremic kittens in the environment without fleas were not infected. A subsequent study (Foil et al., 1998) demonstrated that cats were bacteremic if feces of infected fleas were inoculated intradermally, but bacteremia did not occur by infected fleas which were deposited in retention boxes or cats were fed with infected fleas. The role of fleas as arthropod vector for *B. henselae* was determined in another experiment (Higgins et al., 1996). It

had been shown that *B. henselae* replicated within the flea gut and survived for up to 9 days after infection, while bacteria were shed in the flea feces at the same time. A subsequent study confirmed that *B. henselae* can multiply in the cat fleas (Finkelstein et al., 2002). The bacteria level in the flea feces increased and reached the level of detection after the fleas had fed on the non-infected recipient cat for 6 days. *Bartonella henselae* persisted in the flea feces in the environment for at least 3 days. Therefore, flea feces seem to be the potential source of infection for cats as same as humans, in which flea feces contaminate cat claws and probably inoculated *B. henselae* through skin by scratches. To date, the primary mode of transmission for *B. henselae* in cats suggests that fleas are required for replication of bacteria in the flea gut and excretion in the flea feces which preserve bacteria for a few days. A recent study strongly supported that the monthly use of topical 10% imidacloprid-1% moxidectin reduced flea infestation and prevented flea transmission of *B. henselae* to treated cats over the period of 84 days when compared to untreated cats (Bradbury and Lappin, 2010).

Although the role of fleas as biological vector and flea feces as the source of bacterial transmission has been well documented, other possible routes of *B. henselae* transmission are questioned e.g. flea bite. Further study is needed to determine the possibilities whether *B. henselae* can replicate within the salivary gland of cat fleas and can be spread via saliva during a flea bite or blood suckling period.

Concurrent infection of *Bartonella* spp. in cats and their fleas has been commonly found. DNA of *B. henselae* and *B. clarridgeiae*, and co-infection with these two species has been reported from *C. felis* from different locations, including France, United Kingdom, United States, Canada, Australia, and Thailand (Rolain et al., 2003b; Shaw et al., 2004; Lappin et al., 2006; Kamrani et al., 2008; Barrs et al., 2010; Foongladda et al., 2011; Assarasakorn et al., 2012).

Bartonella clarridgeiae was found to be the predominant species amplified from *C. felis* in French Polynesia (Kernif et al., 2011). *Bartonella koehler*ae was the minor species found in *C. felis* from France and Thailand (Rolain et al., 2003b; Assarasakorn et al., 2012). Increasing reports of *Bartonella* spp. infection in both cats and their fleas support the evolutionary relationship between the reservoir host and the arthropod vector, and indicate the risk of *Bartonella* spp. transmission among cats.

1.8.2   *Experimental Model of Infection in Cats*

The course of *B. henselae* infection in cats had been studied by different models of experiments. Many experiments demonstrated that cats could be infected with *B. henselae* by intravenous (IV) inoculation, intradermal (ID) inoculation, or flea exposure. Duration of bacteremia in experimentally infected cats varied dramatically from weeks to months. Based upon sequential blood culture, some cats had a consistent and predictable pattern of bacteremia, whereas in other cats, bacteremia occurred in a random fashion. In the study of Abbott et al. (1997), kittens inoculated with *B. henselae* by ID or IV route became bacteremic by 1-3 weeks following inoculation, and bacteremia persisted in most cats for 1-8 months. The number of infected cats was higher in the group inoculated by ID route than the group inoculated by IV route. This might suggest the greater efficiency of the ID route or the low inoculum dose of the IV route. One naturally infected cat in this study appeared to be cyclically bacteremic and had the stable antibody titer over the period of 24 months. Antibodies could be detected at the same time as the onset of bacteremia, a few weeks preceding bacteremia, or several weeks after detectable bacteremia. Antibodies subsequently remained a steady titer for several months. However, no clinical or hematologic abnormalities were observed in any of the infected cats. In

another study, cats were inoculated IV with different inoculum dose of *B. henselae*. Clinical abnormalities, bacteremia, antibodies against *B. henselae*, histopathologic lesions were observed over the 32-week period of postinoculation (Guptill et al., 1997). All inoculated cats were bacteremic within 2 weeks. Bacteremia persisted for 16 weeks in most infected cats, but one cat remained bacteremic until 32 week after infection. Some cats developed transient fever within 2 hours and recurrent fever with mild anorexia by 1-3 week after infection. *Bartonella*-specific IgM and IgG were seen in all infected cats. A virulent feline strain (LSU16) of *B. henselae* was documented with apparent clinical signs in cats after infection (O'Reilly et al., 1999). All of the cats inoculated ID either with pure culture, infected blood, or flea feces became bacteremic and had fever by 1-3 weeks postinoculation. Lethargy and anorexia also developed concurrently during febrile periods. Bacteremia peaked at 14 to 28 days post-inoculation in all cats. This study demonstrated inflammatory skin lesions associated with *B. henselae* inoculation and occurrence of lymphadenopathy in most cats. The lesions resolved and disappeared in next 2 weeks. All of infected cats developed high level of antibodies against *B. henselae*. The results from the study of Yamamoto et al. (2002) revealed different responses of cats to the different strains of *B. henselae*. Cats could be infected with feline strain or Houston 1 strain of *B. henselae*, however only feline strain was able to induce clinical signs, such as fever. Although all infected cats developed bacteremia, the duration of bacteremia was much longer in cats inoculated with feline strain than the cats inoculated with Houston 1 strain. Recently, cats exposed to *B. henselae*-infected fleas were found to develop fever and lethargy, whereas *B. henselae*-infected cats by IV inoculation were clinically normal (Bradbury and Lappin, 2010). One of these cats also developed severe clinical signs, including myocarditis, pericardial and pleural effusions, and cholangiohepatits.

Based upon data of the experimental infection, humoral and cell-mediated immunity plays a role in elimination, suppression, and control of *Bartonella* spp. infection in cats. As shown in a study, activation of cell mediated immune response which was detected by IFN-γ and TNF-α production, was important in elimination of *B. henselae* from bacteremic cats (Kabeya et al., 2009). Because prolonged or relapsing bacteremia is typically seen in cats, it is believed that the natural immunity might only suppress, but not eliminate infection from these cats. Cross-protection from re-infection with different strains was documented in a challenge study (Yamamoto et al., 2003). Cats primarily infected with *B. henselae* type I were cross-protected against the challenge with *B. henselae* type II, but cross-protection was not observed in cats primarily infected with *B. henselae* type II and subsequently challenged with *B. henselae* type I. The duration of bacteremia in cats primarily inoculated with *B. henselae* type I was apparently shorter than cats inoculated with either *B. henselae* type II or *B. clarridgeiae*. Another observation in this study was the shorter bacteremia duration and the lower level of bacteremia after challenging with different strains from the primary inoculation (Yamamoto et al., 2003). The study of Yamamoto et al. (2002b) demonstrated that the duration of *B. koehlerae* bacteremia was significantly shorter than that of cats inoculated with *B. clarridgeiae*, and a bacteremia relapse was not detected.

1.8.3   Prevalence of Bartonella spp. Infection in Cats

Epidemiological surveys on *Bartonella* spp. infections in cats have been performed and documented in several countries throughout the world i.e. the Americas, Europe, Asia, and Oceania (Boulouis et al., 2005). In general, bacteremia was detected by culture of blood and subsequently confirmed by PCR assays. Seroprevalence was determined by ELISA or IFA. *B.*

henselae and *B. clarridgeiae* are the most common *Bartonella* infections in cats from many areas. *B. koehlerae* is rarely found, and has currently been reported from California, France, Israel, and Thailand. Prevalence of bacteremia and seroprevalence of *B. henselae* varies dramatically according to geographical location and cat populations. For example, prevalence rate of bacteremia may range from 8.1 - 62.3% in pet and stray cats from 2 different cities in France (La Scola et al., 2002a; Rolain et al., 2004b). Seroprevalence may dramatically vary from 1% (Bergh et al., 2002; Hjelm et al., 2002) to 93% (Nutter et al., 2004). Based on several studies, seroprevalence is commonly about double the bacteremia prevalence in the same population (Chomel et al., 1995; Guptill et al., 2004). In the United States, an increase in seroprevalence of *B. henselae* likely paralleled an increase of warmth and precipitation (Jameson et al., 1995). It has been suggested that climate and humidity are important factors for an increased number of potential arthropod vectors (fleas). The prevalence of *Bartonella* spp. DNA amplified directly from blood of cats by PCR assays was commonly higher in feral or stray cats than that of pet cats within the same region (Solano-Gallego et al., 2006; Kamrani et al., 2008; Juvet et al., 2010; Kelly et al., 2010; Staggemeier et al., 2010). *Bartonella clarridgeiae* bacteremia in cats has been reported from many countries, and prevalence rates of *B. clarridgeiae* bacteremia are usually much lower than *B. henselae* bacteremia (Boulouis et al., 2005). Co-infection with *B. henselae* and *B. clarridgeiae* in same cats are usually found in many areas (Gurfield et al., 2001; Lappin et al., 2006; Barrs et al., 2010; Juvet et al., 2010; Assarasakorn et al., 2012).

Bartonella henselae feline isolates are generally differentiated into genotype I (Houston) and genotype II (Marseille), based upon variations in the 16S rDNA gene sequence (Bergmans et al., 1996; La Scola et al., 2002b). Prevalence rate in cats for each genotype varies among different regions and cat populations (Gurfield et al., 1997; Heller et al., 1997; Maruyama et al.,

2000; Fabbi et al., 2004; Guptill et al., 2004). It has been suggested that *B. henselae* feline strains have higher level of diversity than those that are isolated from humans, and genetic variations between isolates may also relate to virulence in people (Dillon et al., 2002; Iredell et al., 2003).

The multiple-locus variable number tandem repeat analysis (MLVA) has been recently developed for *B. henselae* typing (Monteil et al., 2007). This technique has been demonstrated to have the highest discriminatory power compared with other typing techniques proposed (Iredell et al., 2003; Li et al., 2006; Arvand et al., 2007). The recent study has shown that genotype I and II isolates shared no common MLVA profile (Bouchouicha et al., 2009). MLVA profiles were differentiated into group A and B. Group A exclusively consisted of genotype II feline isolates. Group B was composed of all human isolates which was further divided in 2 subgroups, *Ba* (exclusively constituted by genotype I isolates, including the strain Houston I) and *B*b (belonged to genotype II). It was also suggested that genotype I isolates could be phylogenetically derived from genotype II isolates located in group B but not in group A. The most recent study showed that MLVA profiles can be used as specific geographical markers for feline *B. henselae* isolates.

1.8.4   *Clinical Implication of B. henselae Infection*

Bartonella spp. have been known as stealth pathogens in reservoir-adapted animals. Most of cats with natural infection are usually subclinically infected and have limited pathology associated with infection. However, a wide variety of disease manifestations associated with *B. henselae* infection may appear in cats. None of clinical signs were documented in either experimentally or naturally infected cats with *B. koehlerae* (Yamamoto et al., 2002b). Based on experimental studies, clinical abnormalities, including fever, lymphadenopathy, inflammatory skin lesions, lethargy, anorexia, mild neurologic signs, and reproductive disorders had been

developed in cats inoculated with some strains of *B. henselae* (i.e. genotype II or LSU16). However, the pathogenesis of many disease manifestations remains unclear. Fever is the most common clinical sign. Self-limiting transient fever can develop within 2 hours after inoculation, but usually during 1-3 weeks following infection of cats with *B. henselae* via needle inoculation (Guptill et al., 1997; Kordick et al., 1999; O'Reilly et al., 1999; Yamamoto et al., 2003). Fever usually persists less than a week. Development of fever can be delayed to 3 months after cats were exposed to infected cat fleas (Bradbury and Lappin, 2010). Skin lesions (erythema, swelling, and pustule) at the site of inoculation and localized or generalized lymphadenopathy were also found (O'Reilly et al., 1999). Some cats developed transient anemia and transient mild neurologic dysfunction, such as nystagmus, tremors, focal motor seizures, and behavior changes. Reproductive failure in female cats, including stillbirths, lack of pregnancy, or pregnancy only after repeated breedings was also reported following experimental infection with *B. henselae* (Guptill et al., 1998). In a recent experiment, cats inoculated with *B. henselae* IV remained non-clinically ill, but exposure to *C. felis* resulted in myocarditis in one cat (Bradbury and Lappin, 2010). This finding suggested that the natural arthropod vector might be important for pathogenicity of the organism and disease pathogenesis. In addition to the development of clinical abnormalities, cats inoculated with *B. henselae* and *B. clarridgeiae* by blood transfusion also developed histopathologic findings in various tissues, including peripheral lymph node hyperplasia, splenic follicular hyperplasia, lymphocytic cholangitis/pericholangitis, lymphocytic hepatitis, lymphoplasmacytic myocarditis, and interstitial lymphocytic nephritis (Kordick et al., 1999), splenic microabscesses, hepatic abscess, hepatic necrosis, focal pyogranulomatous nephritis, and interstitial myocarditis (Guptill et al., 1997). These findings are likely to specify the cell-mediated immunologic response following *B. henselae* infection.

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Cats that are naturally infected with *B. henselae* have been found to develop clinical abnormalities. For example, *B. henselae* has been implicated as a potential cause of anterior uveitis in cats (Lappin et al., 2000), which was confirmed by the evidence of intraocular antibody production against *B. henselae* and a response to antibiotic treatment. Fever commonly develops in experimentally infected cats and the presence of *Bartonella* spp. DNA in blood of client-owned cats has shown to be associated with fever (Lappin et al., 2009). Occasionally, *B. henselae* infection associated with endocarditis was described in bacteremic cats (Malik et al., 1999) or a negative-culture seropositive cats (Chomel et al., 2003). Based on serological studies in low-prevalence areas, seropositivity in cats from Switzerland was associated with urological diseases and stomatitis (Glaus et al., 1997). Seropositive cats in Japan were more likely to have lymphadenitis and gingivitis, especially those co-infected with the feline immunodeficiency virus (Ueno et al., 1996). A recent study from a veterinary hospital found that culture results of *Bartonella* spp., but not serologic test results, was strongly associated with gingivostomatitis in naturally infected cats (Sykes et al., 2010). However, establishment of a causation of disease manifestations in cats from endemic areas is more difficult and usually inconclusive. Due to high number of seropositive cats, many studies supported that antibody tests should not be used alone to determine the cause of clinical illnesses associated with *Bartonella* spp. infection in cats. For example, detection of antibodies against *Bartonella* spp. infection by ELISA or western blot was not able to predict fever (Lappin et al., 2009), uveitis (Fontenelle et al., 2008), gingivitis, or stomatitis in cats (Quimby et al., 2008; Dowers et al., 2010). In addition, PCR results were also not associated with stomatitis and uveitis (Quimby et al., 2008; Dowers et al., 2010). Owing to neurologic disease induced by *Bartonella* spp. infection, the seroprevalence against *B. henselae* was high in healthy cats and the cats with or without neurologic disease, and there was no

difference among groups (Pearce et al., 2006). The findings of *B. henselae* DNA and *Bartonella* spp. C values, representing intrathecal antibody production, in cerebrospinal fluid of client-owned cats with neurologic disease in a subsequent study (Leibovitz et al., 2008) suggest that *B. henselae* infection might involve with neurologic disease in cats. Furthermore, stress is questioned to induce the self-limiting fever in *B. henselae* bacteremic cats following minor surgical procedures (Breitschwerdt et al., 2010). Hyperglobulinemia, particularly polyclonal gammopathy was recently found to be significantly associated with seropositivity to *Bartonella* species in privately owned cats (Whittemore et al., 2012). An increase in globulin concentration by 1 mg/dl was associated with a 4.37-fold increase in the risk of seropositivity. Chronic infection and coinfections with more than one *Bartonella* sp. are speculated to cause hyperglobulinemia.

Clinical signs are rarely reported from cats naturally-infected with *B. clarridgeiae*. However, *B. clarridgeiae* was recently isolated from a cat with lymphadenopathy and fever from Italy (Capitta et al., 2010). *Bartonella vinsonii* subsp. *berkhoffii* was isolated from the osteomyelitis lesion in a carpal joint of a cat after 18 months of amputation of original osteomyelitis lesion in a digit in the rear leg (Varanat et al., 2009). This finding suggests that cats, like dogs and human patients, might be more likely to develop clinical disease when infected with a non-reservoir-adapted *Bartonella* spp.

1.8.5   *Diagnosis*

Detection of *Bartonella* spp. infection in cats is commonly performed by many methods available in laboratories. *Bartonella* spp. infection can be identified by isolation method using

culture of blood or tissues, or amplification of *Bartonella* DNA in blood, tissue, and body fluids by PCR assays, or detecting *Bartonella*-specific antibodies in serum.

Culture

Blood culture is the definitive diagnosis that ultimately proves the active infection in cats. Because cats usually have a high level of intraerythrocytic bacteremia, isolation of *Bartonella* spp., e.g. *B. henselae*, *B. clarridgeiae*, from blood of cats is much easier than isolation of those organisms from other animals that are accidental hosts or do not serve as a reservoir-adapted host. Primary culture from blood sample may take from 5 days and up to a few weeks to obtain visible colonies and to ensure the growth of the organism. Because of the slow growth of these bacteria, standard biochemical testing for identification may not be applicable. Clinical use of culture for diagnosis is limited because of the fastidious nature of the organisms, the requirement of specialized laboratory equipment, and less sensitivity compared to molecular methods. Negative blood culture results are found in many seropositive cats. In this case, it is possible that the infection is absolutely eliminated; the bacteremia is intermittent; the number of organisms is below the limit of detection; or the organisms die during the storage of sample. Positive blood culture results do not prove the cat is clinically ill from the infection. Currently, an insect-based liquid culture medium, also known as *Bartonella-Alphaproteobacteria* growth medium (BAPGM) has been formulated and used for culture of blood of cats, dogs, and humans (Maggi et al., 2005). The medium is efficient and supports the growth of at least seven *Bartonella* spp., and it increases the sensitivity of the culture technique.

Polymerase Chain Reaction

Amplification of *Bartonella* spp. DNA from blood, body fluids, or tissues of cats has been currently used to prove infection due to high sensitivity and specificity. Several PCR assays designed to amplify different protein-encoding genes, such as citrate synthase (*gltA*) (Norman et al., 1995), riboflavin synthase (*ribC*) (Johnson et al., 2003), as well as the 16S - 23S rRNA intergenic spacer region (ITS) (Jensen et al., 2000; Maggi and Breitschwerdt, 2005), are commonly been used for blood of cats. The species of *Bartonella* amplified from assays targeting protein-encoding genes can be identified by restriction fragment length polymorphism (RFLP) or DNA sequencing. The PCR assays targeting the ITS can differentiate the species of *Bartonella* by different product size that was amplified in a single run of assay. PCR assays have been more successful to detect infection when compared with the culture technique. They also provide more rapid results than those from culture. However, positive PCR results confirm presence of *Bartonella* spp. DNA, but do not prove living organisms or clinically ill caused by infection. Intermittent bacteremia, previous use of antibiotics, the number of organisms under the limit of detection, or inhibitory or interfering substances in biologic specimens can induce false-negative results. False-positive results may occur in fluid or tissue being tested from blood contamination. Use of PCR assays is also limited because of the requirement of specialized laboratory and laboratory personnel. PCR assays are generally expensive to perform, and they are not standardized among laboratories.

A combinational approach consisting of liquid pre-enrichment culture of *Bartonella* spp. in *Bartonella*/alpha-*Proteobacteria* growth medium (BAPGM), sub-inoculation of the liquid culture onto agar plates, followed by DNA amplification using PCR assays was shown to increases the sensitivity of PCR-based detection due to an increase in bacterial number. This

approach assists in improvement in the isolation of *Bartonella* spp. when compared to traditional inoculation of blood agar plates. This method also facilitates the detection and subsequent isolation of single and co-infections with *B. henselae* and *B. vinsonii* subsp. *berkhoffii* in the blood of naturally infected dogs. Therefore, the use of a combinational approach of pre-enrichment culture and PCR may assist in the diagnosis bartonellosis in other animals, as well (Duncan et al., 2007).

Serological Testing

Immunofluorescent antibody assay (IFA), enzyme linked immunosorbent assay (ELISA), or Western blot immunoassay have been used to detect antibodies against *Bartonella* spp. in serum of cats. IFA and ELISA are commonly used in many epidemiological studies. Western blot immunoassay has been used to determine the *Bartonella*-specific immunodominant antigens recognized by the humoral immune response of cats (Freeland et al., 1999; Chenoweth et al., 2004). Antibody tests are inexpensive and can be performed quickly, but they are of limited diagnostic value in cats. Many infected cats are likely to be seropositive against *B. henselae*, but serological testing has low positive predictive value (< 50%) for identification of bacteremia (Chomel et al., 1995; Fabbi et al., 2004; Guptill et al., 2004). Therefore, a positive antibody test result only suggests exposure to *Bartonella* spp., but it does not prove current infection. Due to high negative predictive value (> 85%) (Chomel et al., 1995; Gurfield et al., 2001; Fabbi et al., 2004; Guptill et al., 2004), animals without antibodies against *B. henselae* are more likely to be non-bacteremic. Bacteremia in seronegative cats has been reported in some cases, thus a negative test result does not exclude current infection. Because antibodies against *B. henselae* antigens may cross-react with antigens of *B. clarridgeiae* or other species, positive test results cannot

discriminate *Bartonella* spp. that infects a cat. Because of these limitations for serologic testing, bacterial isolation and PCR assay are necessary to identify the species of *Bartonella*.

Use of *Bartonella* spp. antibody tests (IgG, IgM) was attempted to determine illness, including uveitis, stomatitis, neurological disease, and fever, that might be potentially associated with *Bartonella* spp. infection (Pearce et al., 2006; Fontenelle et al., 2008; Lappin et al., 2009; Dowers et al., 2010). However, seropositivity and antibody titer did not correlate with any of clinical diseases. Therefore, proving feline bartonellosis by detection of antibody is not recommended.

The Current Consensus for Clinical Diagnosis

The consensus opinion for diagnosis of clinical bartonellosis in cats created by the AAFP (Brunt et al., 2006) recommends that there is no single test result from currently available assays that can prove clinical bartonellosis in cats. The following findings should be combined in order to diagnose a cat, including presence of a syndrome reported to be associated with *Bartonella* spp. infection; exclusion of other causes of the clinical syndrome; detection of a positive *Bartonella* spp. test (culture, PCR assay, or serology); and response to administration of a drug with presumed anti-*Bartonella* activity. Due to limitation of each diagnostic test, results should be interpreted with caution. In addition, because there is high prevalence of bacteremia in cat populations in endemic areas, the panel in the AAFP does not recommend testing healthy cats whether they are exposed or actively infected.

1.8.6   Treatment and Prevention

Because the diagnosis of feline bartonellosis has not been standardized, optimal antimicrobial protocols for use in clinically ill cats have not been ultimately defined. The information of current treatment in cats was commonly obtained from human studies. In vitro, many strains of feline isolates were susceptible to antibiotics, but antibiotic susceptibilities correlated poorly with clinical efficacy in human patients with *Bartonella* infection (Maurin et al., 1995; Kordick et al., 1997b; Rolain et al., 2004a). Discrepancies between in vitro and clinical data may due to the pathogenesis of the individual syndrome. As found in typical CSD in immunocompetent people, which usually have a low number of organisms in involving lymph nodes. There may be different niches that *Bartonella* reside in the host e.g., sequestration in erythrocytes (Rolain et al., 2004a), or other intracellular locations. It is possible that the clinical manifestations of disease may relate to the immune response of the host for clearance of the organism. Failure of antibiotic treatment may be caused by replication rates of *Bartonella* spp. (Schulein et al., 2001, Seubert et al., 2002). It had been shown in studies of *B. tribocorum* infection of rat that the organism maintained in a non-replicating state after a short period of intraerythrocytic replication (Schülein et al., 2001). Many antimicrobial drugs can penetrate cells very well, but they may not be effective to stationary state of bacteria.

Several antibiotics including doxycycline, enrofloxacin, amoxicillin, amoxicillin-clavulanate, tetracycline HCl, erythromycin, and rifampin were used to treat bacteremia in experimentally infected cats (Greene et al., 1996; Guptill et al., 1997; Kordick and Breitschwerdt, 1998). Results of the studies revealed that none of the drug could completely clear bacteremia in all infected cats. Bacteremia was apparently eliminated in some cats. Clinical efficacy was also variable among each drug. In addition, relapsing bacteremia was found in some

cats with initially negative for blood culture results. Successful treatment with doxycycline or azithromycin in individual cats with uveitis was documented (Lappin and Black, 1999; Ketring et al., 2004). Both antibiotics have broad antibacterial spectrums and also modulate immune responses in human studies, therefore positive therapeutic responses may be related to anti-inflammatory effects in cats (Culic et al., 2002; Webster and Del Rosso, 2007).

Based upon the available data so far, the AAFP panel (2006) indicates that the most appropriate antibiotic treatment regimens for feline *Bartonella* infections have not been documented. The panel recommends no treatment of healthy *B. henselae* seropositive cats. The reasons of not treating healthy cats are based upon the high prevalence of bacteremia in cat populations in flea endemic regions, the doubtful pathogenic role of the organism, and the risk of antibiotic resistance of bacteria. However, a bacteremic (by positive blood culture or PCR assay) cat with unexplained illness related to *B. henselae* infection or indefinite diagnosis would warrant consideration of antimicrobial treatment. Doxycycline administered at 10 mg/kg, PO, every 12-24 hours, or amoxicillin-clavulanate at 22 mg/kg, PO, every 12 hours for 7 days (Green et al., 1996; Brunt et al., 2006) may be considered as the first choice for *Bartonella*-positive cats. Treatment should be continued if there is a positive response. Optimal duration of therapy for any drug has not been determined; however, prolonged treatment for a minimum of 2 weeks and at least 1 week past resolution of clinical illness may be applied. Fluoroquinolone such as enrofloxacin may be considered because treatment of enrofloxacin for 14 days was shown to be effective for the clearance of *Bartonella* spp. DNA in blood of all positive cats for at least 3 months (Bradbury and Lappin, 2010). Azithromycin was previously used to treat CSD patients but the recent *in vitro* study (Biswas et al., 2010) showed that azithromycin was the least active antibiotic for nine feline *B. henselae* isolates and one human isolate when compared to

pradofloxacin and enrofloxacin. For five *B. henselae* isolates tested in this study, Azithromycin was active only until the second passage in our study, while pradofloxacin and enrofloxacin were active until at least the fifth passage. Azithromycin is currently considered contraindicated.

Because cats with clinical bartonellosis are likely to be bacteremic, care should be taken to avoid being bitten or scratched while administering drugs. The optimal follow-up time for repeat testing has not been established. Treatment of clinically ill cats should be combined with eradication of fleas on all animals in the household and the premises in order to avoid re-infection. The AAFP panel recommendations (Brunt et al., 2006) for decreasing the likelihood of pet cats becoming infected with *Bartonella* spp. include: maintain an appropriate flea-control program year-round; be cautious about adding stray cats or cats from shelters to the household without controlling fleas; and keep cats indoors to minimize exposure to fleas and other possible vectors. Application of effective flea control products is highly recommended. For example, monthly use of topical 10% imidacloprid - 1% moxidectin has demonstrated to reduce flea infestation and prevent flea transmission of *B. henselae* to cats (Bradbury and Lappin, 2010).

Prevention of *Bartonella* infection by vaccination is not currently available. However, naturally infected cats have known to infect with various species and strains of *Bartonella* spp., and the immune responses may vary for each individual organism. Therefore, development of multivalent vaccine is suggested for immunizing cats (Yamamoto et al., 2003).

Recommendations for testing and treating healthy cats were stated in the Guidelines for Preventing and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents (Kaplan et al., 2009). The Centers for Disease Control and Prevention (CDC) do not recommend to test healthy cats because there is not enough evidence indicating the benefit to cats or their owners from routine culture or serologic testing of the pet for *Bartonella* infection. Also, because

elimination of *Bartonella* spp. infection is difficult and because of the evidence of re-infection in some cats, there is no proven benefit to treat healthy cats even in cases with any *Bartonella* positive test result. In addition, the development of antimicrobial resistance should be concerned if the failure in clearance of the infection happens after the administration of antibiotics.

References

Alsmark, C.M., Frank, A.C., Karlberg, E.O., Legault, B.A., Ardell, D.H., Canbäck, B., Eriksson, A.S., Näslund, A.K., Handley, S.A., Huvet, M., 2004. The louse-borne human pathogen *Bartonella quintana* is a genomic derivative of the zoonotic agent *Bartonella henselae*. Proc. Nat. Acad. Sci. USA. 101, 9716-9721.

Arvand, M., Feil, E.J., Giladi, M., Boulouis, H.J., Viezens, J., 2007. Multi-locus sequence typing of *Bartonella henselae* isolates from three continents reveals hypervirulent and feline-associated clones. PLoS One 2, e1346.

Assarasakorn, S., Veir, J.K., Hawley, J.R., Brewer, M.M., Morris, A.K., Hill, A.E., Lappin, M.R., 2012. Prevalence of *Bartonella* species, hemoplasmas, and *Rickettsia felis* DNA in blood and fleas of cats in Bangkok, Thailand. Res. Vet. Sci. 93, 1213-1216.

Avidor, B., Graidy, M., Efrat, G., Leibowitz, C., Shapira, G., Schattner, A., Zimhony, O., Giladi, M., 2004. *Bartonella koehlerae*, a new cat-associated agent of culture-negative human endocarditis. J. Clin. Microbiol. 42, 3462-3468.

Barrs, V.R., Beatty, J.A., Wilson, B.J., Evans, N., Gowan, R., Baral, R.M., Lingard, A.E., Perkovic, G., Hawley, J.R., Lappin, M.R., 2010. Prevalence of *Bartonella* species, *Rickettsia felis*, haemoplasmas and the *Ehrlichia* group in the blood of cats and fleas in eastern Australia. Aust. Vet. J. 88, 160-165.

Bergh, K., Bevanger, L., Hanssen, I., Løseth, K., 2002. Low prevalence of *Bartonella henselae* infections in Norwegian domestic and feral cats. APMIS 110, 309-314.

Berghoff, J., Viezens, J., Guptill, L., Fabbi, M., Arvand, M., 2007. *Bartonella henselae* exists as a mosaic of different genetic variants in the infected host. Microbiology 153, 2045-2051.

Bergmans, A.M., Schellekens, J.F., Van Embden, J.D., Schouls, L.M., 1996. Predominance of two *Bartonella henselae* variants among cat-scratch disease patients in the Netherlands. J. Clin. Microbiol. 34, 254-260.

Billeter, S.A., Levy, M.G., Chomel, B.B., Breitschwerdt, E.B., 2008. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. Med. Vet. Entomol. 22, 1-15.

Biswas, S., Maggi, R.G., Papich, M.G., Keil, D., Breitschwerdt, E.B., 2010. Comparative activity of pradofloxacin, enrofloxacin, and azithromycin against *Bartonella henselae* isolates collected from cats and a human. J. Clin. Microbiol. 48, 617-618.

Bouchouicha, R., Durand, B., Monteil, M., Chomel, B., Berrich, M., Arvand, M., Birtles, R.J., Breitschwerdt, E.B., Koehler, J.E., Maggi, R., 2009. Molecular epidemiology of feline and human *Bartonella henselae* isolates. Emerg. Infect. Dis. 15, 813-816.

Boulouis, H.J., Chang, C., Henn, J.B., Kasten, R.W., Chomel, B.B., 2005. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. Vet. Res. 36, 383-410.

Bradbury, C.A., Lappin, M.R., 2010. Evaluation of topical application of 10% imidacloprid-1% moxidectin to prevent *Bartonella henselae* transmission from cat fleas. J. Am. Vet. Med. Assoc. 236, 869-873.

Breitschwerdt, E.B., Kordick, D.L., 2000. *Bartonella* infection in animals: carriership, reservoir potential, pathogenicity, and zoonotic potential for human infection. Clin. Microbiol. Rev. 13, 428-438.

Breitschwerdt, E.B., Kordick, D.L., Malarkey, D.E., Keene, B., Hadfield, T.L., Wilson, K., 1995. Endocarditis in a dog due to infection with a novel *Bartonella* subspecies. J. Clin. Microbiol. 33, 154-160.

Breitschwerdt, E.B., Maggi, R.G., Chomel, B.B., Lappin, M.R., 2010. Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. J. Vet. Emerg. Crit. Care 20, 8-30.

Breitschwerdt, E.B., Maggi, R.G., Duncan, A.W., Nicholson, W.L., Hegarty, B.C., Woods, C.W., 2007a. *Bartonella* species in blood of immunocompetent persons with animal and arthropod contact. Emerg. Infect. Dis. 13, 938-941.

Breitschwerdt, E.B., Maggi, R.G., Nicholson, W.L., Cherry, N.A., Woods, C.W., 2008. *Bartonella* sp. bacteremia in patients with neurological and neurocognitive dysfunction. J. Clin. Microbiol. 46, 2856-2861.

Breitschwerdt, E.B., Maggi, R.G., Sigmon, B., Nicholson, W.L., 2007b. Isolation of *Bartonella quintana* from a woman and a cat following putative bite transmission. J. Clin. Microbiol. 45, 270-272.

Brouqui, P., Lascola, B., Roux, V., Raoult, D., 1999. Chronic *Bartonella quintana* bacteremia in homeless patients. N. Engl. J. Med. 340, 184-189.

Brunt, J., Guptill, L., Kordick, D.L., Kudrak, S., Lappin, M.R., 2006. American Association of Feline Practitioners 2006 Panel report on diagnosis, treatment, and prevention of *Bartonella* spp. infections. J. Feline Med. Surg. 8, 213-226.

Capitta, P., Zobba, R., Masala, G., Cocco, R., Tola, S., Parpaglia, M.L.P., 2010. Isolation and characterization of *Bartonella* Strains in cats in Italy. Transbound. Emerg. Dis. 57, 201-204.

Chang, C.C., Chomel, B.B., Kasten, R.W., Romano, V., Tietze, N., 2001. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. J. Clin. Microbiol. 39, 1221-1226.

Chenoweth, M.R., Greene, C.E., Krause, D.C., Gherardini, F.C., 2004. Predominant outer membrane antigens of *Bartonella henselae*. Infect. Immun. 72, 3097-3105.

Cherry, N.A., Diniz, P., Maggi, R.G., Hummel, J.B., Hardie, E.M., Behrend, E.N., Rozanski, E., DeFrancesco, T.C., Cadenas, M.B., Breitschwerdt, E.B., 2009. Isolation or molecular detection of *Bartonella henselae* and *Bartonella vinsonii* subsp. *berkhoffii* from dogs with idiopathic cavitary effusions. J. Vet. Intern. Med. 23, 186-189.

Chomel, B.B., Abbott, R.C., Kasten, R.W., Floyd-Hawkins, K.A., Kass, P.H., Glaser, C.A., Pedersen, N.C., Koehler, J.E., 1995. *Bartonella henselae* prevalence in domestic cats in California: risk factors and association between bacteremia and antibody titers. J. Clin. Microbiol. 33, 2445-2450.

Chomel, B.B., Boulouis, H.J., Breitschwerdt, E.B., 2004. Cat scratch disease and other zoonotic *Bartonella* infections. J. Am. Vet. Med. Assoc. 224, 1270-1279.

Chomel, B.B., Boulouis, H.J., Breitschwerdt, E.B., Kasten, R.W., Vayssier-Taussat, M., Birtles, R.J., Koehler, J.E., Dehio, C., 2009a. Ecological fitness and strategies of adaptation of *Bartonella* species to their hosts and vectors. Vet. Res. 40, 29.

Chomel, B.B., Kasten, R.W., 2010. Bartonellosis, an increasingly recognized zoonosis. J. Appl. Microbiol. 109, 743-750.

Chomel, B.B., Kasten, R.W., Floyd-Hawkins, K., Chi, B., Yamamoto, K., Roberts-Wilson, J., Gurfield, A.N., Abbott, R.C., Pedersen, N.C., Koehler, J.E., 1996. Experimental transmission of *Bartonella henselae* by the cat flea. J. Clin. Microbiol. 34, 1952-1956.

Chomel, B.B., Kasten, R.W., Williams, C., Wey, A.C., Henn, J.B., Maggi, R., Carrasco, S., Mazet, J., Boulouis, H.J., Maillard, R., 2009b. *Annals of the New York Academy of Sciences*. Ann. N. Y. Acad. Sci. 1166, 120-126.

Chomel, B.B., Mac Donald, K.A., Kasten, R.W., Chang, C.C., Wey, A.C., Foley, J.E., Thomas, W.P., Kittleson, M.D., 2001. Aortic valve endocarditis in a dog due to *Bartonella clarridgeiae*. J. Clin. Microbiol. 39, 3548-3554.

Chomel, B.B., Wey, A.C., Kasten, R.W., Stacy, B.A., Labelle, P., 2003. Fatal case of endocarditis associated with *Bartonella henselae* type I infection in a domestic cat. J. Clin. Microbiol. 41, 5337-5339.

Christie, P.J., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S., Cascales, E., 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu. Rev. Microbiol. 59, 451-485.

Cotté, V., Bonnet, S., Le Rhun, D., Le Naour, E., Chauvin, A., Boulouis, H.J., Lecuelle, B., Lilin, T., Vayssier-Taussat, M., 2008. Transmission of *Bartonella henselae* by *Ixodes ricinus*. Emerg. Infect. Dis. 14, 1074-1080.

Culic, O., Erakovic, V., Cepelak, I., Barisic, K., Brajsa, K., Ferencic, Z., Galovic, R., Glojnaric, I., Manojlovic, Z., Munic, V., 2002. Azithromycin modulates neutrophil function and circulating inflammatory mediators in healthy human subjects. Eur. J. Pharmacol. 450, 277-289.

Daly, J.S., Worthington, M.G., Brenner, D.J., Moss, C.W., Hollis, D.G., Weyant, R.S., Steigerwalt, A.G., Weaver, R.E., Daneshvar, M.I., O'Connor, S.P., 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J. Clin. Microbiol. 31, 872-881.

Dehio, C., 1999. Interactions of *Bartonella henselae* with vascular endothelial cells. Curr. Opin. Microbiol. 2, 78-82.

Dehio, C., 2001. *Bartonella* interactions with endothelial cells and erythrocytes. Trends Microbiol. 9, 279-285.

Dehio, C., 2005. *Bartonella*-host-cell interactions and vascular tumour formation. Nat. Rev. Microbiol. 3, 621-631.

Dillon, B., Valenzuela, J., Don, R., Blanckenberg, D., Wigney, D.I., Malik, R., Morris, A.J., Robson, J.M., Iredell, J., 2002. Limited diversity among human isolates of *Bartonella henselae*. J. Clin. Microbiol. 40, 4691-4699.

Diniz, P., Billeter, S.A., Otranto, D., De Caprariis, D., Petanides, T., Mylonakis, M.E., Koutinas, A.F., Breitschwerdt, E.B., 2009a. Molecular documentation of *Bartonella* infection in dogs in Greece and Italy. J. Clin. Microbiol. 47, 1565-1567.

Diniz, P.P.V.P., Wood, M., Maggi, R.G., Sontakke, S., Stepnik, M., Breitschwerdt, E.B., 2009b. Co-isolation of *Bartonella henselae* and *Bartonella vinsonii* subsp. *berkhoffii* from blood, joint and subcutaneous seroma fluids from two naturally infected dogs. Vet. Microbiol. 138, 368-372.

Dowers, K.L., Hawley, J.R., Brewer, M.M., Morris, A.K., Radecki, S.V., Lappin, M.R., 2010. Association of *Bartonella* species, feline calicivirus, and feline herpesvirus 1 infection with gingivostomatitis in cats. J. Feline Med. Surg. 12, 314-321.

Droz, S., Chi, B., Horn, E., Steigerwalt, A.G., Whitney, A.M., Brenner, D.J., 1999. *Bartonella koehlerae* sp. nov., isolated from cats. J. Clin. Microbiol. 37, 1117-1122.

Dubey, J.P., Bhatia, C.R., Lappin, M.R., Ferreira, L.R., Thorn, A., Kwok, O.C.H., 2009a. Seroprevalence of *Toxoplasma gondii* and *Bartonella* spp. antibodies in cats from Pennsylvania. J. Parasitol. 95, 578-580.

Dubey, J.P., Lappin, M.R., Kwok, O.C.H., Mofya, S., Chikweto, A., Baffa, A., Doherty, D., Shakeri, J., Macpherson, C.N.L., Sharma, R.N., 2009b. Seroprevalence of *Toxoplasma gondii* and concurrent *Bartonella* spp., feline immunodeficiency virus, and feline leukemia virus infections in cats from Grenada, West Indies. J. Parasitol. 95, 1129-1133.

Duncan, A.W., Maggi, R.G., Breitschwerdt, E.B., 2007. A combined approach for the enhanced detection and isolation of *Bartonella* species in dog blood samples: pre-enrichment liquid culture followed by PCR and subculture onto agar plates. J. Microbiol. Methods 69, 273-281.

Duncan, A.W., Maggi, R.G., Breitschwerdt, E.B., 2007. *Bartonella* DNA in dog saliva. Emerg. Infect. Dis. 13, 1948-1950.

Duncan, A.W., Marr, H.S., Birkenheuer, A.J., Maggi, R.G., Williams, L.E., Correa, M.T., Breitschwerdt, E.B., 2008. *Bartonella* DNA in the blood and lymph nodes of Golden Retrievers with lymphoma and in healthy controls. J. Vet. Intern. Med. 22, 89-95.

Eremeeva, M.E., Gerns, H.L., Lydy, S.L., Goo, J.S., Ryan, E.T., Mathew, S.S., Ferraro, M.J., Holden, J.M., Nicholson, W.L., Dasch, G.A., 2007. Bacteremia, fever, and splenomegaly caused by a newly recognized *Bartonella* species. N. Engl. J. Med. 356, 2381-2387.

Fabbi, M., De Giuli, L., Tranquillo, M., Bragoni, R., Casiraghi, M., Genchi, C., 2004. Prevalence of *Bartonella henselae* in Italian stray cats: evaluation of serology to assess the risk of transmission of *Bartonella* to humans. J. Clin. Microbiol. 42, 264-268.

Fenollar, F., Sire, S., Raoult, D., 2005. *Bartonella vinsonii* subsp. *arupensis* as an agent of blood culture-negative endocarditis in a human. J. Clin. Microbiol. 43, 945-947.

Finkelstein, J.L., Brown, T.P., O'reilly, K.L., Wedincamp, J., Foil, L.D., 2002. Studies on the growth of *Bartonella henselae* in the cat flea (Siphonaptera: Pulicidae). J. Med. Entomol. 39, 915-919.

Florin, T.A., Zaoutis, T.E., Zaoutis, L.B., 2008. Beyond cat scratch disease: widening spectrum of *Bartonella henselae* infection. Pediatrics 121, e1413-e1425.

Foil, L., Andress, E., Freeland, R.L., Roy, A.F., Rutledge, R., Triche, P.C., OREILLY, K.L., 1998. Experimental infection of domestic cats with *Bartonella henselae* by inoculation of *Ctenocephalides fells* (Siphonaptera: Pulicidae) feces. J. Med. Entomol. 35, 625-628.

Fontenelle, J.P., Powell, C.C., Hill, A.E., Radecki, S.V., Lappin, M.R., 2008. Prevalence of serum antibodies against *Bartonella* species in the serum of cats with or without uveitis. J. Feline Med. Surg. 10, 41-46.

Foongladda, S., Inthawong, D., Kositanont, U., Gaywee, J., 2011. *Rickettsia*, *Ehrlichia*, *Anaplasma*, and *Bartonella* in ticks and fleas from dogs and cats in Bangkok. Vector Borne Zoonotic Dis. 11, 1335-1341.

Freeland, R.L., Scholl, D.T., Rohde, K.R., Shelton, L.J., O'Reilly, K.L., 1999. Identification of *Bartonella*-specific immunodominant antigens recognized by the feline humoral immune system. Clin. Diagn. Lab. Immunol. 6, 558-566.

Glaus, T., Hofmann-Lehmann, R., Greene, C., Glaus, B., Wolfensberger, C., Lutz, H., 1997. Seroprevalence of *Bartonella henselae* infection and correlation with disease status in cats in Switzerland. J. Clin. Microbiol. 35, 2883-2885.

Greene, C.E., McDermott, M., Jameson, P.H., Atkins, C.L., Marks, A.M., 1996. *Bartonella henselae* infection in cats: evaluation during primary infection, treatment, and rechallenge infection. J. Clin. Microbiol. 34, 1682-1685.

Guptill, L., Slater, L., Wu, C.C., Lin, T.L., Glickman, L.T., Welch, D.F., HogenEsch, H., 1997. Experimental infection of young specific pathogen-free cats with *Bartonella henselae*. J. Infect. Dis. 176, 206-216.

Guptill, L., Slater, L.N., Wu, C.C., Lin, T.L., Glickman, L.T., Welch, D.F., Tobolski, J., HogenEsch, H., 1998. Evidence of reproductive failure and lack of perinatal transmission of *Bartonella henselae* in experimentally infected cats. Vet. Immunol. Immunopathol. 65, 177-189.

Guptill, L., Wu, C.C., HogenEsch, H., Slater, L., Glickman, N., Dunham, A., Syme, H., Glickman, L., 2004. Prevalence, risk factors, and genetic diversity of *Bartonella henselae* infections in pet cats in four regions of the United States. J. Clin. Microbiol. 42, 652-659.

Gurfield, A.N., Boulouis, H.J., Chomel, B.B., Heller, R., Kasten, R.W., Yamamoto, K., Piemont, Y., 1997. Coinfection with *Bartonella clarridgeiae* and *Bartonella henselae* and with different *Bartonella henselae* strains in domestic cats. J. Clin. Microbiol. 35, 2120-2123.

Gurfield, A.N., Boulouis, H.J., Chomel, B.B., Kasten, R.W., Heller, R., Bouillin, C., Gandoin, C., Thibault, D., Chang, C.C., Barrat, F., 2001. Epidemiology of *Bartonella* infection in domestic cats in France. Vet. Microbiol. 80, 185-198.

Harms, A., Dehio, C., 2012. Intruders below the radar: molecular pathogenesis of *Bartonella* spp. Clin. Microbiol. Rev. 25, 42-78.

Heller, R., Artois, M., Xemar, V., De Briel, D., Gehin, H., Jaulhac, B., Monteil, H., Piemont, Y., 1997. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in stray cats. J. Clin. Microbiol. 35, 1327-1331.

Henn, J.B., Gabriel, M.W., Kasten, R.W., Brown, R.N., Koehler, J.E., MacDonald, K.A., Kittleson, M.D., Thomas, W.P., Chomel, B.B., 2009. Infective endocarditis in a dog and the phylogenetic relationship of the associated "*Bartonella rochalimae*" strain with isolates from dogs, gray foxes, and a human. J. Clin. Microbiol. 47, 787-790.

Henn, J.B., Gabriel, M.W., Kasten, R.W., Brown, R.N., Theis, J.H., Foley, J.E., Chomel, B.B., 2007. Gray foxes (*Urocyon cinereoargenteus*) as a potential reservoir of a *Bartonella clarridgeiae*-like bacterium and domestic dogs as part of a sentinel system for surveillance of zoonotic arthropod-borne pathogens in northern California. J. Clin. Microbiol. 45, 2411-2418.

Higgins, J.A., Radulovic, S., Jaworski, D.C., Azad, A.F., 1996. Acquisition of the cat scratch disease agent *Bartonella henselae* by cat fleas (Siphonaptera: Pulicidae). J. Med. Entomol. 33, 490-495.

Hjelm, E., McGill, S., Blomqvist, G., 2002. Prevalence of antibodies to *Bartonella henselae*, *B. elizabethae* and *B. quintana* in Swedish domestic cats. Scand. J. Infect. Dis. 34, 192-196.

Iredell, J., Blanckenberg, D., Arvand, M., Grauling, S., Feil, E.J., Birtles, R.J., 2003. Characterization of the natural population of *Bartonella henselae* by multilocus sequence typing. J. Clin. Microbiol. 41, 5071-5079.

Jameson, P., Greene, C., Regnery, R., Dryden, M., Marks, A., Brown, J., Cooper, J., Glaus, B., Greene, R., 1995. Prevalence of *Bartonella henselae* antibodies in pet cats throughout regions of North America. J. Infect. Dis. 172, 1145-1149.

Jensen, W.A., Fall, M.Z., Rooney, J., Kordick, D.L., Breitschwerdt, E.B., 2000. Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. J. Clin. Microbiol. 38, 1717-1722.

Johnson, G., Ayers, M., McClure, S.C.C., Richardson, S.E., Tellier, R., 2003. Detection and identification of *Bartonella* species pathogenic for humans by PCR amplification targeting the riboflavin synthase gene (*ribC*). J. Clin. Microbiol. 41, 1069-1072.

Juvet, F., Lappin, M.R., Brennan, S., Mooney, C.T., 2010. Prevalence of selected infectious agents in cats in Ireland. J. Feline Med. Surg. 12, 476–482.

Kabeya, H., Sase, M., Yamashita, M., Maruyama, S., 2006. Predominant T helper 2 immune responses against *Bartonella henselae* in naturally infected cats. Microbiol. Immunol. 50, 171-178.

Kabeya, H., Umehara, T., Okanishi, H., Tasaki, I., Kamiya, M., Misawa, A., Mikami, T., Maruyama, S., 2009. Experimental infection of cats with *Bartonella henselae* resulted in rapid clearance associated with T helper 1 immune responses. Microbes Infect. 11, 716-720.

Kaiser, P.O., Riess, T., Wagner, C.L., Linke, D., Lupas, A.N., Schwarz, H., Raddatz, G., Schäfer, A., Kempf, V.A.J., 2008. The head of *Bartonella* adhesin A is crucial for host cell interaction of *Bartonella henselae*. Cell Microbiol. 10, 2223-2234.

Kamrani, A., Parreira, V.R., Greenwood, J., Prescott, J.F., 2008. The prevalence of *Bartonella*, hemoplasma, and *Rickettsia felis* infections in domestic cats and in cat fleas in Ontario. Can. J. Vet. Res. 72, 411-419.

Kaplan, J.E., Benson, C., Holmes, K., Brooks, J.T., Pau, A., Masur, H., 2009. Guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents. MMWR Recomm. Rep. 58 (RR04), 1-198.

Kelly, P., Rolain, J.M., Maggi, R., Sontakke, S., Keene, B., Hunter, S., Lepidi, H., Breitschwerdt, K.T., Breitschwerdt, E.B., Raoult, D., 2006. *Bartonella quintana* endocarditis in dogs. Emerg. Infect. Dis. 12, 1869-1872.

Kelly, P.J., Moura, L., Miller, T., Thurk, J., Perreault, N., Weil, A., Maggio, R., Lucas, H., Breitschwerdt, E., 2010. Feline immunodeficiency virus, feline leukemia virus and *Bartonella* species in stray cats on St Kitts, West Indies. J. Feline Med. Surg. 12, 447-450.

Kerkhoff, F.T., Bergmans, A.M.C., van Der Zee, A., Rothova, A., 1999. Demonstration of *Bartonella grahamii* DNA in ocular fluids of a patient with neuroretinitis. J. Clin. Microbiol. 37, 4034-4038.

Kernif, T., Parola, P., Davoust, B., Plaire, L., Cabre, O., Raoult, D., Rolain, J.M., 2011. *Bartonella clarridgeiae* in Fleas, Tahiti, French Polynesia. Emerg. Infect. Dis. 17, 1773-1775.

Ketring, K.L., Zuckerman, E.E., Hardy Jr, W.D., 2004. *Bartonella*: a new etiological agent of feline ocular disease. J. Am. Anim. Hosp. Assoc. 40, 6-12.

Kim, Y., Seo, K., Lee, J., Choi, E., Lee, H., Hwang, C., Shin, N., Youn, H., young Youn, H., 2009. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in cats and dogs in Korea. J. Vet. Sci. 10, 85-97.

Kitchell, B.E., Fan, T.M., Kordick, D., Breitschwerdt, E.B., Wollenberg, G., Lichtensteiger, C.A., 2000. Peliosis hepatis in a dog infected with *Bartonella henselae*. J. Am. Vet. Med. Assoc. 216, 519-523.

Koehler, J.E., Glaser, C.A., Tappero, J.W., 1994. *Rochalimaea henselae* infection: a new zoonosis with the domestic cat as reservoir. JAMA 271, 531-535.

Koehler, J.E., Quinn, F.D., Berger, T.G., LeBoit, P.E., Tappero, J.W., 1992. Isolation of *Rochalimaea* species from cutaneous and osseous lesions of bacillary angiomatosis. N. Engl. J. Med. 327, 1625-1631.

Koehler, J.E., Tappero, J.W., 1993. Bacillary angiomatosis and bacillary peliosis in patients infected with human immunodeficiency virus. Clin. Infect. Dis. 17, 612-624.

Kordick, D.L., Breitschwerdt, E.B., 1995. Intraerythrocytic presence of *Bartonella henselae*. J. Clin. Microbiol. 33, 1655-1656.

Kordick, D.L., Breitschwerdt, E.B., 1997. Relapsing bacteremia after blood transmission of *Bartonella henselae* to cats. Am. J. Vet. Res. 58, 492-497.

Kordick, D.L., Breitschwerdt, E.B., 1998. Persistent infection of pets within a household with three *Bartonella* species. Emerg. Infect. Dis. 4, 325-328.

Kordick, D.L., Brown, T.T., Shin, K.O., Breitschwerdt, E.B., 1999. Clinical and pathologic evaluation of chronic *Bartonella henselae* or *Bartonella clarridgeiae* infection in cats. J. Clin. Microbiol. 37, 1536-1547.

Kordick, D.L., Hilyard, E.J., Hadfield, T.L., Wilson, K.H., Steigerwalt, A.G., Brenner, D.J., Breitschwerdt, E.B., 1997a. *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). J. Clin. Microbiol. 35, 1813-1818.

Kordick, D.L., Papich, M.G., Breitschwerdt, E.B., 1997b. Efficacy of enrofloxacin or doxycycline for treatment of *Bartonella henselae* or *Bartonella clarridgeiae* infection in cats. Antimicrob. Agents Chemother. 41, 2448-2455.

Kosoy, M., Morway, C., Sheff, K.W., Bai, Y., Colborn, J., Chalcraft, L., Dowell, S.F., Peruski, L.F., Maloney, S.A., Baggett, H., 2008. *Bartonella tamiae* sp. nov., a newly recognized pathogen isolated from three human patients from Thailand. J. Clin. Microbiol. 46, 772-775.

Kyme, P., Dillon, B., Iredell, J., 2003. Phase variation in *Bartonella henselae*. Microbiology 149, 621-629.

La Scola, B., Davoust, B., Boni, M., Raoult, D., 2002a. Lack of correlation between *Bartonella* DNA detection within fleas, serological results, and results of blood culture in a *Bartonella*-
• infected stray cat population. Clin. Microbiol. Infect. 8, 345-351.

La Scola, B., Liang, Z., Zeaiter, Z., Houpikian, P., Grimont, P.A.D., Raoult, D., 2002b. Genotypic characteristics of two serotypes of *Bartonella henselae*. J. Clin. Microbiol. 40, 2002-2008.

La, V.D., Tran-Hung, L., Aboudharam, G., Raoult, D., Drancourt, M., 2005. *Bartonella quintana* in domestic cat. Emerg. Infect. Dis. 11, 1287-1289.

Lappin, M.R., Black, J.C., 1999. *Bartonella* spp infection as a possible cause of uveitis in a cat. J. Am. Vet. Med. Assoc. 214, 1205-1207.

Lappin, M.R., Breitschwerdt, E., Brewer, M., Hawley, J., Hegarty, B., Radecki, S., 2009. Prevalence of *Bartonella* species antibodies and *Bartonella* species DNA in the blood of cats with and without fever. J. Feline Med. Surg. 11, 141-148.

Lappin, M.R., Griffin, B., Brunt, J., Riley, A., Burney, D., Hawley, J., Brewer, M.M., Jensen, W.A., 2006. Prevalence of *Bartonella* species, haemoplasma species, *Ehrlichia* species, *Anaplasma phagocytophilum*, and *Neorickettsia risticii* DNA in the blood of cats and their fleas in the United States. J. Feline Med. Surg. 8, 85-90.

Lappin, M.R., Hawley, J., 2009. Presence of *Bartonella* species and *Rickettsia* species DNA in the blood, oral cavity, skin and claw beds of cats in the United States. Vet. Dermatol. 20, 509-514.

Lappin, M.R., Kordick, D.L., Breitschwerdt, E.B., 2000. *Bartonella* spp antibodies and DNA in aqueous humour of cats. J. Feline Med. Surg. 2, 61-68.

Leibovitz, K., Pearce, L., Brewer, M., Lappin, M.R., 2008. *Bartonella* species antibodies and DNA in cerebral spinal fluid of cats with central nervous system disease. J. Feline Med. Surg. 10, 332-337.

Li, W., Chomel, B.B., Maruyama, S., Guptil, L., Sander, A., Raoult, D., Fournier, P.E., 2006. Multispacer typing to study the genotypic distribution of *Bartonella henselae* populations. J. Clin. Microbiol. 44, 2499-2506.

Linke, D., Riess, T., Autenrieth, I.B., Lupas, A., Kempf, V.A.J., 2006. Trimeric autotransporter adhesins: variable structure, common function. Trends Microbiol. 14, 264-270.

Maggi, R.G., Breitschwerdt, E.B., 2005. Potential limitations of the 16S-23S rRNA intergenic region for molecular detection of *Bartonella* species. J. Clin. Microbiol. 43, 1171-1176.

Maggi, R.G., Duncan, A.W., Breitschwerdt, E.B., 2005. Novel chemically modified liquid medium that will support the growth of seven *Bartonella* species. J. Clin. Microbiol. 43, 2651-2655.

Malik, R., Barrs, V.R., Church, D.B., Zahn, A., Allan, G.S., Martini, P., Wigney, D.I., Love, D.N., 1999. Vegetative endocarditis in six cats. J. Feline Med. Surg. 1, 171-180.

Mändle, T., Einsele, H., Schaller, M., Neumann, D., Vogel, W., Autenrieth, I.B., Kempf, V.A.J., 2005. Infection of human CD34+ progenitor cells with *Bartonella henselae* results in intraerythrocytic presence of *B. henselae*. Blood 106, 1215-1222.

Margileth, A.M., Baehren, D.F., 1998. Chest-wall abscess due to cat-scratch disease (CSD) in an adult with antibodies to *Bartonella clarridgeiae*: case report and review of the thoracopulmonary manifestations of CSD. Clin. Infect. Dis. 27, 353-357.

Maruyama, S., Hiraga, S., Yokoyama, E., Naoi, M., Tsuruoka, Y., Ogura, Y., Tamura, K., Namba, S., Kameyama, Y., Nakamura, S., 1998. Seroprevalence of *Bartonella henselae* and *Toxoplasma gondii* infections among pet cats in Kanagawa and Saitama prefectures. J. Vet. Med. Sci. 60, 997-1000.

Maruyama, S., Kabeya, H., Nakao, R., Tanaka, S., Sakai, T., Xuan, X., Katsube, Y., Mikami, T., 2003. Seroprevalence of *Bartonella henselae*, *Toxoplasma gondii*, FIV and FeLV infections in domestic cats in Japan. Microbiol. Immunol. 47, 147-153.

Maruyama, S., Nakamura, Y., Kabeya, H., Tanaka, S., Sakai, T., Katsube, Y., 2000. Prevalence of *Bartonella henselae*, *Bartonella clarridgeiae* and the 16S rRNA gene types of *Bartonella henselae* among pet cats in Japan. J. Vet. Med. Sci. 62, 273-279.

Maurin, M., Gasquet, S., Ducco, C., Raoult, D., 1995. MICs of 28 antibiotic compounds for 14 *Bartonella* (formerly *Rochalimaea*) isolates. Antimicrob. Agents Chemother. 39, 2387-2391.

Mehock, J.R., Greene, C.E., Gherardini, F.C., Hahn, T.W., Krause, D.C., 1998. *Bartonella henselae* invasion of feline erythrocytes in vitro. Infect. Immun. 66, 3462-3466.

Monteil, M., Durand, B., Bouchouicha, R., Petit, E., Chomel, B., Arvand, M., Boulouis, H.J., Haddad, N., 2007. Development of discriminatory multiple-locus variable number tandem repeat analysis for *Bartonella henselae*. Microbiology 153, 1141-1148.

Muñana, K.R., Vitek, S.M., Hegarty, B.C., Kordick, D.L., Breitschwerdt, E.B., 2001. Infection of fetal feline brain cells in culture with *Bartonella henselae*. Infect. Immun. 69, 564-569.

Norman, A.F., Regnery, R., Jameson, P., Greene, C., Krause, D.C., 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J. Clin. Microbiol. 33, 1797-1803.

Nutter, F.B., Dubey, J., Levine, J.F., Breitschwerdt, E.B., Ford, R.B., Stoskopf, M.K., 2004. Seroprevalences of antibodies against *Bartonella henselae* and *Toxoplasma gondii* and fecal shedding of *Cryptosporidium* spp, *Giardia* spp, and *Toxocara cati* in feral and pet domestic cats. J. Am. Vet. Med. Assoc. 225, 1394-1398.

Oliveira, A.M., Maggi, R.G., Woods, C.W., Breitschwerdt, E.B., 2010. Suspected Needle Stick Transmission of *Bartonella vinsonii* subspecies *berkhoffii* to a Veterinarian. J. Vet. Intern. Med. 24, 1229-1232.

O'Reilly, K.L., Bauer, R.W., Freeland, R.L., Foil, L.D., Hughes, K.J., Rohde, K.R., Roy, A.F., Stout, R.W., Triche, P.C., 1999. Acute clinical disease in cats following infection with a pathogenic strain of *Bartonella henselae* (LSU16). Infect. Immun. 67, 3066-3072.

O'Rourke, L.G., Pitulle, C., Hegarty, B.C., Kraycirik, S., Killary, K.A., Grosenstein, P., Brown, J.W., Breitschwerdt, E.B., 2005. *Bartonella quintana* in cynomolgus monkey (Macaca fascicularis). Emerg. Infect. Dis. 11, 4.

Pappalardo, B.L., Brown, T., Gebhardt, D., Sontakke, S., Breitschwerdt, E.B., 2000. Cyclic CD8+ lymphopenia in dogs experimentally infected with *Bartonella vinsonii* subsp. *berkhoffii*. Vet. Immunol. Immunopathol. 75, 43-57.

Pappalardo, B.L., Brown, T.T., Tompkins, M., Breitschwerdt, E.B., 2001. Immunopathology of *Bartonella vinsonii* (*berkhoffii*) in experimentally infected dogs. Vet. Immunol. Immunopathol. 83, 125-147.

Pearce, L.K., Radecki, S.V., Brewer, M., Lappin, M.R., 2006. Prevalence of *Bartonella henselae* antibodies in serum of cats with and without clinical signs of central nervous system disease. J. Feline Med. Surg. 8, 315-320.

Podsiadly, E., Chmielewski, T., Marczak, R., Sochon, E., Tylewska-Wierzbanowska, S., 2007a. *Bartonella henselae* in the human environment in Poland. Scand. J. Infect. Dis. 39, 956-962.

Podsiadly, E., Chmielewski, T., Sochon, E., Tylewska-Wierzbanowska, S., 2007b. *Bartonella henselae* in *Ixodes ricinus* ticks removed from dogs. Vector Borne Zoonotic Dis. 7, 189-192.

Powell, C.C., Kordick, D.L., Lappin, M.R., 2002. Inoculation with *Bartonella henselae* followed by feline herpesvirus 1 fails to activate ocular toxoplasmosis in chronically infected cats. J. Feline Med. Surg. 4, 107-110.

Quimby, J.M., Elston, T., Hawley, J., Brewer, M., Miller, A., Lappin, M.R., 2008. Evaluation of the association of *Bartonella* species, feline herpesvirus 1, feline calicivirus, feline leukemia virus and feline immunodeficiency virus with chronic feline gingivostomatitis. J. Feline Med. Surg. 10, 66-72.

Raoult, D., Roblot, F., Rolain, J.M., Besnier, J.M., Loulergue, J., Bastides, F., Choutet, P., 2006. First isolation of *Bartonella alsatica* from a valve of a patient with endocarditis. J. Clin. Microbiol. 44, 278-279.

Regnery, R., Martin, M., Olson, J., 1992. Naturally occurring "*Rochalimaea henselae*" infection in domestic cat. Lancet 340, 557.

Riess, T., Andersson, S.G.E., Lupas, A., Schaller, M., Schäfer, A., Kyme, P., Martin, J., Wälzlein, J.H., Ehehalt, U., Lindroos, H., 2004. *Bartonella* adhesin A mediates a proangiogenic host cell response. J. Exp. Med. 200, 1267-1278.

Rolain, J.M., Brouqui, P., Koehler, J.E., Maguina, C., Dolan, M.J., Raoult, D., 2004a. Recommendations for treatment of human infections caused by *Bartonella* species. Antimicrob. Agents Chemother. 48, 1921-1933.

Rolain, J.M., Fournier, P.E., Raoult, D., Bonerandi, J.J., 2003a. First isolation and detection by immunofluorescence assay of *Bartonella koehlerae* in erythrocytes from a French cat. J. Clin. Microbiol. 41, 4001-4002.

Rolain, J.M., Franc, M., Davoust, B., Raoult, D., 2003b. Molecular detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipientis* in cat fleas, France. Emerg. Infect. Dis. 9, 339-342.

Rolain, J.M., Locatelli, C., Chabanne, L., Davoust, B., Raoult, D., 2004b. Prevalence of *Bartonella clarridgeiae* and *Bartonella henselae* in domestic cats from France and detection of the organisms in erythrocytes by immunofluorescence. Clin. Diagn. Lab. Immunol. 11, 423-425.

Roux, V., Eykyn, S.J., Wyllie, S., Raoult, D., 2000. *Bartonella vinsonii* subsp. *berkhoffii* as an agent of afebrile blood culture-negative endocarditis in a human. J. Clin. Microbiol. 38, 1698-1700.

Saenz, H.L., Dehio, C., 2005. Signature-tagged mutagenesis: technical advances in a negative selection method for virulence gene identification. Curr. Opin. Microbiol. 8, 612-619.

Saenz, H.L., Engel, P., Stoeckli, M.C., Lanz, C., Raddatz, G., Vayssier-Taussat, M., Birtles, R., Schuster, S.C., Dehio, C., 2007. Genomic analysis of *Bartonella* identifies type IV secretion systems as host adaptability factors. Nat. Genet. 39, 1469-1476.

Sander, A., Zagrosek, A., Bredt, W., Schiltz, E., Piémont, Y., Lanz, C., Dehio, C., 2000. Characterization of *Bartonella clarridgeiae* flagellin (FlaA) and detection of antiflagellin antibodies in patients with lymphadenopathy. J. Clin. Microbiol. 38, 2943-2948.

Sanogo, Y.O., Zeaiter, Z., Caruso, G., Merola, F., Shpynov, S., Brouqui, P., Raoult, D., 2003. *Bartonella henselae* in *Ixodes ricinus* ticks (Acari: Ixodida) removed from humans, Belluno province, Italy. Emerg. Infect. Dis. 9, 329-332.

Schmid, M.C., Schulein, R., Dehio, M., Denecker, G., Carena, I., Dehio, C., 2004. The VirB type IV secretion system of *Bartonella henselae* mediates invasion, proinflammatory activation and antiapoptotic protection of endothelial cells. Mol. Microbiol. 52, 81-92.

Schröder, G., Dehio, C., 2005. Virulence-associated type IV secretion systems of *Bartonella*. Trends Microbiol. 13, 336-342.

Schülein, R., Seubert, A., Gille, C., Lanz, C., Hansmann, Y., Piémont, Y., Dehio, C., 2001. Invasion and persistent intracellular colonization of erythrocytes: A unique parasitic strategy of the emerging pathogen *Bartonella*. J. Exp. Med. 193, 1077-1086.

Schulein, R., Dehio, C., 2002. The VirB/VirD4 type IV secretion system of *Bartonella* is essential for establishing intraerythrocytic infection. Mol. Microbiol. 46, 1053-1067.

Seubert, A., Hiestand, R., De La Cruz, F., Dehio, C., 2003. A bacterial conjugation machinery recruited for pathogenesis. Mol. Microbiol. 49, 1253-1266.

Shaw, S.E., Kenny, M.J., Tasker, S., Birtles, R.J., 2004. Pathogen carriage by the cat flea *Ctenocephalides felis* (Bouché) in the United Kingdom. Vet. Microbiol. 102, 183-188.

Solano-Gallego, L., Hegarty, B., Espada, Y., Llull, J., Breitschwerdt, E., 2006. Serological and molecular evidence of exposure to arthropod-borne organisms in cats from northeastern Spain. Vet. Microbiol. 118, 274-277.

Staggemeier, R., Venker, C.A., Klein, D.H., Petry, M., Spilki, F.R., Cantarelli, V.V., 2010. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in cats in the south of Brazil: a molecular study. Mem. Inst. Oswaldo Cruz 105, 873-878.

Sykes, J.E., Westropp, J.L., Kasten, R.W., Chomel, B.B., 2010. Association between *Bartonella* species infection and disease in pet cats as determined using serology and culture. J. Feline Med. Surg. 12, 631-636.

Telford, S.R., Wormser, G.P., 2010. *Bartonella* spp. transmission by ticks not established. Emerg. Infect. Dis. 16, 379-384.

Tsuneoka, H., Umeda, A., Tsukahara, M., Sasaki, K., 2004. Evaluation of indirect fluorescence antibody assay for detection of *Bartonella clarridgeiae* and seroprevalence of *B. clarridgeiae* among patients with suspected cat scratch disease. J. Clin. Microbiol. 42, 3346-3349.

Ueno, H., Hohdatsu, T., Muramatsu, Y., Koyama, H., Morita, C., 1996. Does coinfection of *Bartonella henselae* and FIV induce clinical disorders in cats? Microbiol. Immunol. 40, 617-620.

Varanat, M., Travis, A., Lee, W., Maggi, R.G., Bissett, S.A., Linder, K.E., Breitschwerdt, E.B., 2009. Recurrent osteomyelitis in a cat due to infection with *Bartonella vinsonii subsp. berkhoffii* genotype II. J. Vet. Intern. Med. 23, 1273-1277.

Webster, G., Del Rosso, J.Q., 2007. Anti-inflammatory activity of tetracyclines. Dermatol. Clin. 25, 133-135.

Whittemore, J.C., Hawley, J.R., Radecki, S.V., Steinberg, J.D., Lappin, M.R., 2012. *Bartonella* species antibodies and hyperglobulinemia in privately owned cats. J. Vet. Intern. Med. 26, 639-644.

Yamamoto, K., Chomel, B.B., Kasten, R.W., Hew, C.M., Weber, D.K., Lee, W.I., 2002a. Experimental infection of specific pathogen free (SPF) cats with two different strains of *Bartonella henselae* type I: a comparative study. Vet. Res. 33, 669-684.

Yamamoto, K., Chomel, B.B., Kasten, R.W., Hew, C.M., Weber, D.K., Lee, W.I., Droz, S., Koehler, J.E., 2002b. Experimental infection of domestic cats with *Bartonella koehlerae* and comparison of protein and DNA profiles with those of other *Bartonella* species infecting felines. J. Clin. Microbiol. 40, 466-474.

Yamamoto, K., Chomel, B.B., Kasten, R.W., Hew, C.M., Weber, D.K., Lee, W.I., Koehler, J.E., Pedersen, N.C., 2003. Infection and re-infection of domesticcats with various *Bartonella* species or types: *B. henselae* typeI is protective against heterologous challenge with *B. henselae* type II. Vet. Microbiol. 92, 73-86.

Zangwill, K.M., Hamilton, D.H., Perkins, B.A., Regnery, R.L., Plikaytis, B.D., Hadler, J.L., Cartter, M.L., Wenger, J.D., 1993. Cat scratch disease in Connecticut--epidemiology, risk factors, and evaluation of a new diagnostic test. N. Engl. J. Med. 329, 8-13.

Zhang, P., Chomel, B.B., Schau, M.K., Goo, J.S., Droz, S., Kelminson, K.L., George, S.S., Lerche, N.W., Koehler, J.E., 2004. A family of variably expressed outer-membrane proteins (Vomp) mediates adhesion and autoaggregation in *Bartonella quintana*. Proc. Nat. Acad. Sci. USA. 101, 13630-13635.

CHAPTER 2

Research Overview and Specific Aims

2.1 Research Overview

Bartonella infections, particularly *B. henselae* in cats, are commonly distributed in most countries. Many infected cats carry the bacteria without showing clinical signs, but some cats may develop non-specific manifestations of disease. *Bartonella henselae* infection may cause inflammatory effects in multiple systems in the cat's body, including eyes, mouth, and lymph nodes which are the most common types of diseases associated with feline bartonellosis. In rare cases, the disease can cause severe conditions such as hepatitis and endocarditis. Clinical signs in cats are frequently vague or similar to many other types of diseases. Fever is the most common clinical sign particularly in cats experimentally infected with *B. henselae*. Clinical signs alone do not provide enough information for diagnosis. Currently, *Bartonella* spp. infections in cats can be detected by blood culture, molecular techniques, and antibody assays, but positive or negative laboratory test results obtained from these available tests are usually not associated with clinical illness. However, high bacteremia level is thought to be a risk factor for developing clinical signs such as fever. The quantitative real-time PCR (qPCR) is highly sensitive and specific for quantification of microorganism DNA which is correlated to the number of microorganisms in a sample. The antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) can also be a sensitive and specific assay that can rapidly measure antigens of interest with minimal specialized equipment. The quantitative information obtained from both qPCR assay and AC-ELISA is expected to expand the knowledge and information regarding the disease association of *B. henselae* infection in cats.

To date, the quantitative information from qPCR assay and AC-ELISA of *B. henselae* in infected cats are not published. The research goals of this dissertation are therefore to develop two new diagnostic assays, the qPCR and the AC-ELISA for detection of *B. henselae* in blood of cats, and to apply both assays to a cat population in Thailand in order to determine whether or not the assays would aid in the diagnosis of fever caused by *B. henselae*. Chapter 3 (qPCR assay) and Chapter 4 (AC-ELISA) describe the development and titration of the assays. In order to determine the clinical utility of both developed assays, the optimized assays were then applied to a group of client-owned cats which were at risk for *B. henselae* infection in Thailand. Chapter 5 is the preliminary study that determined the prevalence of *Bartonella* infections and other blood borne infections in a client-owned cat population in Bangkok, Thailand. In Chapter 6, a larger sample size of cats in the same area in Thailand that consisted of both febrile and afebrile cats were entered into the study, and the optimized qPCR assay and AC-ELISA were applied to selected blood samples from febrile and afebrile cats. Finally, the future research direction that could be taken to build on the information collected during the completion of this PhD program is described in Chapter 7.

2.2 Specific Aim 1 (Chapter 3: qPCR Assay)

The qPCR is a sensitive, fast, and quantitative assay for amplification of many organisms. The specific aim for the Chapter 3 was to develop a new qPCR assay that is optimized and specific for detection and determination of *B. henselae* bacterial load in blood samples of cats. The analytical performance of the assay, including analytical sensitivity and specificity were also assessed. The qPCR assay is expected to be highly sensitive and specific to detect *B. henselae* DNA in blood of cats.

2.3 Specific Aim 2 (Chapter 4: AC-ELISA)

The AC-ELISA is a quantitative immunoassay that is usually sensitive, specific, and rapid to perform. The level of *B. henselae* antigen in blood of cats has never been assessed by this assay. Therefore, the specific aim of the Chapter 4 was to develop a new AC-ELISA that is able to accurately detect and quantify *B. henselae* antigen in blood of cats. The AC-ELISA is anticipated to be highly sensitive and specific to detect *B. henselae* antigen in blood of cats.

2.4 Specific Aim 3 (Chapter 5: Prevalence in Thailand)

Infections of several *Bartonella* spp. are transmitted among cats via fleas. Risk of flea infestation in cats is commonly high in tropical climate area, including Thailand. However, the information regarding prevalence of *Bartonella* infections in cats and fleas in Thailand is minimal. The specific aim of this chapter is to determine the prevalence of *Bartonella* spp. and other blood borne infections, including hemoplasmas and *R. felis* in client-owned cats and fleas in Bangkok, Thailand. The information of prevalence obtained from this study was used to design a sample size for collecting samples for the subsequent study in the Chapter 6.

2.5 Specific Aim 4 (Chapter 6: qPCR and AC-ELISA with Fever)

Bartonella henselae infection may be a cause of fever of unknown origin in cats. However, diagnosis using currently available techniques may be inconclusive due to high prevalence in the field. The clinical utility for diagnosis of feline bartonellosis using the newly developed qPCR assay and AC-ELISA has not been assessed. The specific aim of the Chapter 6 is therefore to determine the association between results of qPCR assay or AC-ELISA, and fever in client-owned cats from Bangkok, Thailand. Other risk factors that are possibly associated with

fever are also evaluated in this chapter. The hypotheses for the specific aim 4 are that higher bacterial load or antigen level of *B. henselae* will exist in febrile cats compared to afebrile cats and that there is an association between *B. henselae* infection and fever in cats.

CHAPTER 3

Development of Quantitative Real-time Polymerase Chain Reaction for

the Detection of *Bartonella henselae* in Feline Blood

3.1 Summary

Infection of *Bartonella henselae* in cats is commonly detected by molecular methods but a quantitative assay in an attempt to correlate to a clinical manifestation has never been use. A quantitative real-time polymerase chain reaction (qPCR) assay was developed for quantification of *B. henselae* in feline blood. The primers were designed to amplify the 85-bp PCR product of the *groES* gene, which was detected by a specific TaqMan probe. The qPCR reaction was titrated for optimal concentrations of oligonucleotides and reaction conditions. The analytical sensitivity and specificity were determined. The linear dynamic range for accurate quantification was between 5.2 x 10^6 to 52 CFU/ml of blood and the level of detection was 52 CFU/ml. The qPCR assay did not cross-react with other cat-relevant *Bartonella* spp., common bacterial organisms that infect cats, or other bacterial contaminants. The assay was precise and reproducible since the intra-assay and inter-assay coefficients of variance were very low (1.48% and 0.40%, respectively). The recently developed qPCR described here provides a quick and accurate method for quantification of bacteremia level of *B. henselae* in feline blood.

Keywords: qPCR; *Bartonella henselae*; CFU/ml; Cat

3.2 Introduction

In the past few decades, polymerase chain reaction (PCR) assays have been used for detection of *Bartonella* spp. infection in humans and animals. These molecular techniques can detect the bacteria to the species and strain level with good analytical sensitivity and specificity and fast results. Because of high bacterial loads in cats, PCR assays are sufficient for bacterial identification while isolation or culture is not needed. Several conventional polymerase chain reaction (cPCR) assays targeting 16S rRNA (Bergmans et al., 1996) and protein-encoding genes, such as those encoding citrate synthase (*gltA*) (Norman et al., 1995; Birtles and Raoult, 1996), riboflavin synthase (*ribC*) (Bereswill et al., 1999; Johnson et al., 2003), 60 kDa heat shock protein (*groEL*) (Marston et al., 1999; Zeaiter et al., 2002a), cell division protein (ftsZ) (Kelly et al., 1998; Ehrenborg et al., 2000; Zeaiter et al., 2002b), PAP31 and 35-kDa proteins (La Scola et al., 2002b), as well as the 16S - 23S rRNA intergenic spacer region (ITS) (Jensen et al., 2000; Houpikian and Raoult, 2001; Maggi and Breitschwerdt, 2005) have been used for genetic, epidemiologic, and clinical studies. Most concentional PCR (cPCR) assays are sensitive for identification of many species of *Bartonella*, in which species identification can subsequently be performed by sequencing or restriction fragment length polymorphism (RFLP).

Cats infected with *B. henselae* commonly have prolonged bacteremia for months or years (Kordick et al., 1995), therefore amplification of *B. henselae* DNA from blood of cats is usually straight forward and successful. Although the sensitivity of cPCR assay is ideal for the rapid detection of *B. henselae* infections, correlating results of cPCR assay and clinical illness in cats is difficult. This is likely because cats are major reservoirs of *B. henselae* and subclinical infections are common in cats. The current infection of an individual cat may not be associated with clinical signs (Quimby et al., 2008; Dowers et al., 2010). The presence or absence of

Bartonella spp. DNA is therefore not sufficient to conclude that cats are sick because of *Bartonella* spp. infection. The only exception was the presence of *Bartonella* spp. DNA in febrile cats (Lappin et al., 2009). Determination of bacterial counts by routine blood culture is time-consuming and is not sensitive enough to detect fastidious organisms, including *Bartonella* spp. Cats with PCR-positive, but culture-negative test results have often been found. To the best of our knowledge, no existing study addresses the association of clinical illnesses related to *B. henselae* infection and the bacteremia level in cats. Because of wide distribution of *B. henselae* infection, particularly in high flea areas, and the implications in some infected cats, a diagnostic assay that provides faster results and is sensitive enough for detection and quantification of *B. henselae* infection would be useful for diagnosis of feline bartonellosis.

The advanced technology of quantitative real-time PCR (qPCR) enables both detection and quantification of the number of target organisms. The qPCR is at present known to be the most sensitive and precise method for quantification. In addition, the qPCR assay offers fast and reliable quantification of any target sequence in biological sample. The qPCR procedure is performed in the same way following the general principle of PCR, but the key feature is that the amplified product is detected as the reaction progresses in real time. This is beyond the cPCR assay, in which the PCR product is detected at the end of the assay. The amplified product in qPCR assay can be detected using either one of two common methods: 1) fluorescent dyes (SYBR Green) that bind with any double-stranded DNA, and 2) sequence-specific DNA probes (e.g. TaqMan, molecular beacon, minor groove binder); oligonucleotides labeled with a fluorescent reporter that permits detection only after hybridization of the probe with its complementary DNA target. Quantification can be achieved by either an absolute number or relative amount. The qPCR assay is more convenient to perform and quantitative. Because of its

high sensitivity and faster processing, qPCR assay has been chosen as a tool for diagnosis of many pathogenic microorganisms.

Recently, the qPCR assay targeting the citrate synthase gene (*gltA*) has been used for detection of *Bartonella* spp. in feline blood (Mietze et al., 2011). Using this qPCR assay 16.6% of cats were positive for *Bartonella* spp., while only 2.2% were culture positive. The study of Mietze et al. (2011) supported the much higher sensitivity of qPCR assay than that of blood culture but did not quantify the bacterial laod. Additionally, the association between and clinical illness has not been determined.

Because *B. henselae* is the most commonly detected *Bartonella* spp. in cats and appears to be most commonly associated with clinical illness, the current assay was designed to primarily detect *B. henselae*. This chapter describes the development of a qPCR for the detection and quantification of *B. henselae* in blood of cats. The utility of the new qPCR assay for assessing fever in cats is described in Chapter 6. The study hypothesis was that a sensitive and specific qPCR assay could be developed for detection of *B. henselae* in feline blood samples. The objectives of the study were (1) to develop and optimize a new qPCR assay for determination of bacterial load of *B. henselae* in whole blood samples of cats and (2) to assess the analytical sensitivity and specificity of the qPCR assay.

3.3 Material and Methods

3.3.1 Bacteria and Culture Condition

Bartonella henselae strain CSU-1 was the reference strain for optimizing and generating standard curves. Genomic DNA extracted from *B. henselae* isolates and stored at -80 °C was used for assay optimization. The isolates were previously confirmed by the cPCR with the

protocol described formerly (Jensen et al., 2000). For generating standard curves, *B. henselae* was newly grown from whole blood samples of experimentally infected cats. Blood sample (100 μl) was plated onto trypticase soy agar with 10% sheep blood (TSA II; Becton Dickinson Company, Sparks, MD). The plates were then incubated at 37 °C in 5% CO_2 for 7 days. *Bartonella henselae* colonies were collected and suspended in phosphate-buffered saline (PBS) for subsequent uses. The specificity of the qPCR assay was evaluated with DNA extracts of the following bacteria: *B. clarridgeiae*, *B. koehlerae*, *B. vinsonii* subsp. *berkhoffii*, *Mycoplasma haemofelis*, 'Candidatus M. haemominutum', 'Candidatus M. turicensis', *Ehrlichia* spp., and *A. phagocytophilum* which were obtained from the Center for Companion Animal Studies; *Escherichia coli*, *Enterobacter* spp., *Salmonella* spp., *Klebsiella pneumopniae*, *Streptococcus* spp., *Staphylococcus intermedius*, Coagulase-negative *Staphylococcus* spp., *Pseudomonas aeruginosa*, and *Enterococcus* spp. which were kindly provided from the Bacteriology Laboratory at the Colorado State University Veterinary Diagnostic Laboratory.

3.3.2   DNA Extraction

Genomic DNA was extracted from 200 μl of each bacterial suspension using QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) by following the manufacturer's instructions. A 200-μl aliquot of feline whole blood was used for extraction of total DNA following the protocol described in the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA). The DNA was eluted in 200 μl of elution buffer. All DNA samples were stored at -80 °C until used.

3.3.3   Design of Primers and Probe

The *groES* gene is the target gene of the qPCR assay. The similarity of nucleotide sequences available at GenBank were compared among *B. henselae* and other *Bartonella* spp. The sequences of *B. henselae groES* gene from different strains were analyzed using ClustalW multiple alignment running through Bioedit software version 7.0.9.0 (Hall, 1999). The consensus sequence was subsequently used to design primers and probe by the Primer3 software (Rozen and Skaletsky, 2000) which is available at the Primer-Blast on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Primers were selected corresponding to the following parameters (Hyndman and Mitsuhashi, 2003): PCR product size between 50 and 150 base pairs (bp); melting temperature (T_m) between 58 °C and 60 °C; length between 16 and 21 bp; G+C content between 40% and 60%; and avoiding 4 or more Gs or Cs at the 3' end. Additionally, probe which had the following properties was designed: the length between 20 and 30 bp, T_m between 68 °C and 70 °C, G+C content between 40 and 60%, avoiding 4 or more Gs, and avoiding 5' end G. Complimentary within the primers, between the primers, and between the primers and probe was also evaluated. The oligonucleotides were submitted to the Basic Local Alignment Search Tool (BLAST) at the NCBI website in order to assess the specificity.

3.3.4   Screening of the Primers

Primer sets designed by Primer3 software were initially tested with the SYBR Green chemistry. A standard PCR reaction mixture consisted of: 10 μl of 2X Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA), 2 μl of genomic DNA corresponding to 10 ng of total DNA, 0.6 μl of each primer (final concentration 0.3 μM), and 6.8 μl of PCR water. The PCR

assay was performed using a Mastercycler ep *realplex* (Eppendorf, Hamburg, Germany), which included an initial denaturation for 5 min at 95 °C, followed by 40 cycles of: 10 s at 95 °C for denaturation and 30 s at 60 °C for annealing step and data acquisition step. The reactions were assayed in triplicates. No template controls were included in all plates.

3.3.5   *Optimal Annealing Temperature*

The temperature gradient mode was performed with the Mastercycler ep *realplex*. The PCR products were detected with SYBR Green I to determine the optimal annealing temperature. The reaction mixture was prepared as that described in section *3.3.4*. The PCR conditions were at 95 °C for 5 min, followed by 40 cycles of: 10 s at 95 °C and 30 s at gradient temperatures between 56 °C and 62 °C for annealation. The most optimal temperature was subsequently determined by narrower gradient temperatures between 58 °C and 60 °C. The temperature that yielded high fluorescent intensity (ΔRn) and low quantification cycle (C_q) was chosen as the optimal annealing temperature. The threshold was automatically set at 10 times the standard deviations above the noise of the baseline emission calculated from cycles 3 to 15.

3.3.6   *PCR Product Confirmation*

The PCR products were determined by both melting curve analysis and gel electrophoresis. Analysis of DNA melting curves were acquired on the Mastercycler ep *realplex* by measuring the fluorescence of SYBR Green I during a linear temperature transition from 60 °C to 95 °C at 0.2 °C/s. Fluorescence data were converted into melting peaks by the thermocycler software. The PCR products were loaded on 3% agarose gel and stained with EZ-Vision (AMRESCO, Solon, OH) and Rediload dye (Invitrogen, Grand Island, NY). The gels

were run in 1X Tris-borate-EDTA (TBE) buffer at 120 V for 40 min before it was visualized on a UV transilluminator. The amplicon DNA in gels was purified using DNA Clean&Concentrator-5 (Zymo Research Corporation, Irvine, CA), and submitted for DNA sequencing at Macromolecular Resources, Colorado State University (Fort Collins, CO). The sequences obtained were compared with sequences in GenBank database at NCBI.

3.3.7 *Optimization of Primer and Probe Concentrations*

Reactions were performed using combinations of three different final concentrations, including 0.5, 0.6, and 0.7 µM of forward and reverse primers (Table 3-1). The TaqMan probe was used for detection of amplified products. Each primer combination was tested in triplicates which had a reaction mixture containing 10 µl of 2X TaqMan Fast Advanced Master Mix (Applied Biosystems, Carlsbad, CA), 2 µl of total DNA, 0.8 µl of probe (final concentration 0.2 µM), and PCR water added up to 20 µl. The reactions were performed at 50 °C for 2 min, 95 °C for 1 min, followed by 40 cycles at 95 °C for 10 s and optimal annealing temperature for 30 s, as determined in the section *3.3.5*. The C_q value of a positive reaction was defined as the cycle number at which the fluorescence was first detectable above the threshold. This number correlates inversely to the initial amount of DNA in the template. A negative result was defined where no amplification occurred. For each sample, mean C_q was calculated from triplicate reactions. The primer concentrations were considered to be optimal when the following conditions were met; high fluorescence, low C_q value, low standard deviation between replicates, and lack of primer dimers.

Table 3-1   Primer optimization using the checkerboard titration. Nine combinations of forward and reverse primer concentrations (0.5, 0.6, and 0.7 µM) were assayed in reactions using constant concentration (0.2 µM) of probe and same reaction condition.

#1 F 0.5, R 0.5	#2 F 0.6, R 0.5	#3 F 0.7, R 0.5
#4 F 0.5, R 0.6	#5 F 0.6, R 0.6	#6 F 0.7, R 0.6
#7 F 0.5, R 0.7	#8 F 0.6, R 0.7	#9 F 0.7, R 0.7

F: forward primer concentration (µM), R: reverse primer optimization (µM)

Once the primer concentrations were optimized, the qPCR was further optimized with respect to the probe concentration. Multiple reactions were set up to provide three replicates for each of the reactions using probe concentration of 0.10, 0.15, or 0.20 µM. The assays were performed in the reaction containing optimal primer concentrations and all the other mixtures in the unchanged conditions. The optimal probe concentration was selected from the low concentration that acquired the minimum C_q value and low background.

3.3.8   Generation of Standard Curves

Suspension of *B. henselae* strain CSU-1 isolates which was isolated from positive feline blood and grown on blood agar as described in the section *3.3.1* were serially diluted. After a 10-fold serial dilution of bacterial suspension was performed, an aliquot (100 µl) from each dilution was plated onto duplicated blood agar plates for viable counts. The plates were incubated in the condition as previously performed. The viable number of the colonies was counted on day 7 of incubation, and calculated for CFU/ml. An aliquot (100 µl) from each dilution of bacterial suspension was also spiked in 100 µl of feline whole blood. The spiked blood samples were then extracted for total DNA by QIAamp DNA Blood Mini Kit. The total DNA of all spiked samples

with known amount of *B. henselae* was performed in the optimized qPCR assay and condition in order to generate standard curves.

The thermocycler software was used to construct standard curves in which C_q values were plotted on the *y* axis and known amount (CFU/ml) of *B. henselae* in spiked samples was plotted on the *x* axis. The absolute amount of *B. henselae* level in individual feline blood samples was determined by extrapolating the C_q value of unknown sample with the standard curve.

3.3.9 Determination of Efficiency and Analytical Sensitivity

The qPCR efficiency was calculated from the slope of standard curves, which was calculated according to the following equation: efficiency $= 10^{(-1/\text{slope})}$-1. Other parameters, including the correlation coefficient (R^2) and Y-intercept were also determined by the analysis mode of the thermocycler. The linear dynamic range was determined by the range of *B. henselae* concentrations in spiked samples which had C_q values fitting on the standard curves. The limit of detection (LOD) was defined by the lowest amount of *B. henselae* on the standard curve which was detected by the qPCR assay, and this amount was also referred to the limit of quantification of the assay.

3.3.10 Determination of Analytical Specificity

The specificity of the qPCR assay was evaluated with three other *Bartonella* spp. that are genetically related or capable of infecting cats, or bacteria that are able to cause similar clinical signs or are clinically relevant co-infections, or bacteria that are potential contaminants in feline blood, as listed in the section *3.3.1*. The DNA tested was either genomic DNA extracted from bacterial isolate of the microorganisms or total DNA extracted from feline blood positive with

any microorganism. The DNA was assayed with the qPCR assay in the same plate, and *B. henselae* CSU-1 was used as the positive control.

3.3.11 Determination of Repeatability

Inter-assay and intra-assay variations were determined using the spiked whole blood samples which were used for generation of standard curves. The samples were performed in triplicates within a qPCR assay to determine the intra-assay variation, and the same set of the samples was repeatedly performed in separate runs on 3 consecutive days in order to determine the inter-assay variation.

Coefficient of variance (CV) was calculated from each of replicated samples as followed: standard deviation (SD) of the triplicate C_q / mean of the triplicate C_q x 100. Intra-assay CV was the mean of mean CVs of three separate days. The inter-assay CV was calculated by SD of the overall mean of three separate days / grand mean x 100.

3.4 Results

3.4.1 Selection of Primer and Probe

The nucleotide sequences of the *groES* gene of *B. henselae* was similar to the corresponding gene of *B. quintana* (94% identity), but no significant similarity was found when compared to the sequence of *B. clarridgeiae.* The sequence identity was unable to be compared with *B. koehlerae* because of unavailability of the *B. koehlerae groES* sequence on the GenBank.

Sequences of *groES* gene from four strains of *B. henselae* were available in the GenBank database, including accession number NC_005956, BX897699, AJ749669, and U96734. The ClustalW analysis showed 100% similarity among sequences (Figure 3-1). The consensus

sequence was used to design the primers and probe. Three sets of primers that were designed by Primer3 software were selected for initial screening. A probe was designed to anneal with DNA sequences within a specific region amplified by these sets of primers. The oligonucleotides, including primers and probe had 100% homology with the region of *B. henselae* groES gene when compared with sequences in the GenBank.

All of these sets of primers amplified a single product of their expected size as determined by gel electrophoresis. One of the three sets of primers was chosen for assay optimization. The selected primer set was: forward primer, 5' -CTC TCG ATG ACA ATG GGA- 3' (18 bp) and reverse primer, 5' -ACT TCG GTT CCA GAC CAT- 3' (18 bp). The TaqMan probe was 5' -FAM-TCC AAA CAA GAT ACG GTC CCC TG-BHQ1- 3' (23 bp). The probe was labeled with FAM (6-carboxyfluorescein) as the reporter dye at the 5' end and BHQ-1 (Black Hole Quencher) as the quencher at the 3' end.

3.4.2   PCR Product Confirmation

The amplification of *B. henselae* DNA from isolates showed a single sharp peak of the qPCR product at 75.3 °C (Figure 3-2). However, multiple melting peaks were produced for the PCR products of total DNA from negative blood samples.
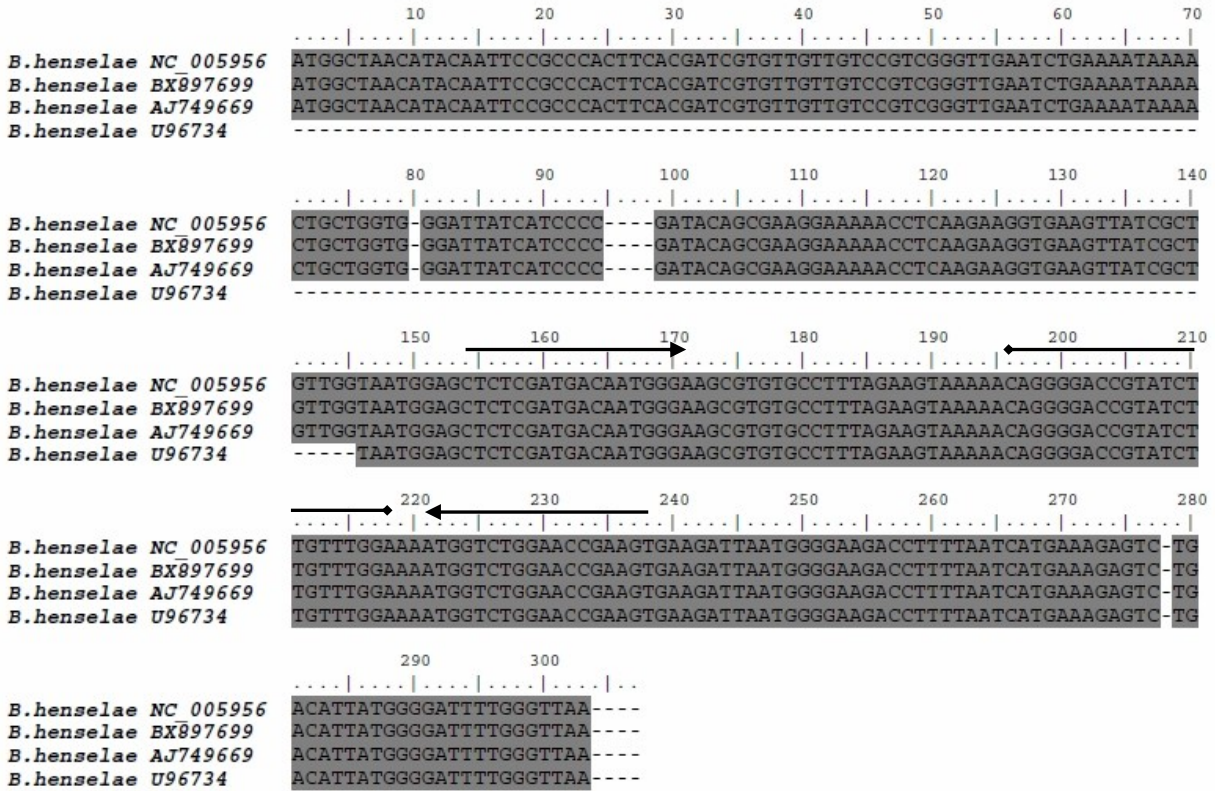
Figure 3-1. Nucleotide sequence alignment of the *groES* gene. Four sequences of *B. henselae* were obtained from the GenBank: NC005956, BX897699, AJ749669, and U96734. The alignment was generated using ClustalW program for maximum homology. →, forward primer; ←, reverse primer; ◆—◆, probe. Dashes indicated gaps.
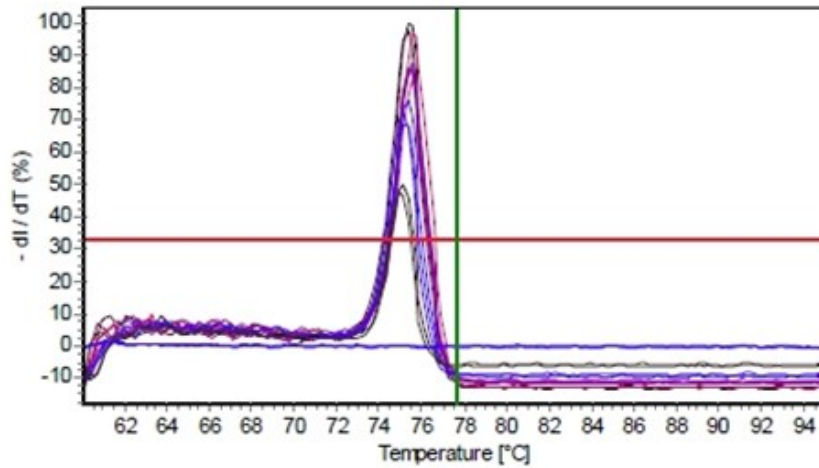
Figure 3-2. Melting curve analysis. A single peak of the PCR product of *B. henselae* isolates was produced at the temperature of 75.3 °C.

The qPCR assay resulted in a single PCR product with the length of approximately 85 bp as determined by gel electrophoresis (Figure 3-3). Non-specific amplicons or primer-dimer formation were not detected. DNA was extracted from gel, submitted for DNA sequencing, and showed 95% homologous similarity with *B. henselae* (GenBank accession number AJ749669, BX897699, and U96734) (Figure 3-4).
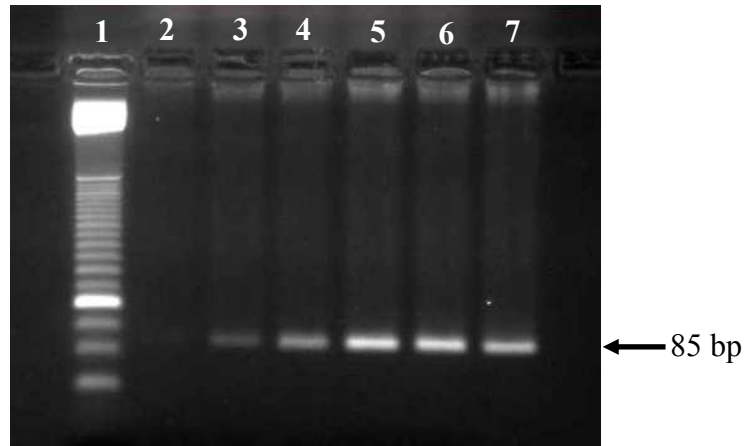
Figure 3-3. Gel electrophoresis. The qPCR products of the *groES* gene amplified from *B. henselae* strain CSU-1 standards was analyzed on gel electrophoresis. The size of the qPCR product is 85 bp in length. Lane 1, 25-bp DNA ladder (Invitrogen, Grand Island, NY); lane 2-7, the qPCR products of *B. henselae* standards ranging from 52 to 5.2 x 10^6 CFU/ml, respectively.
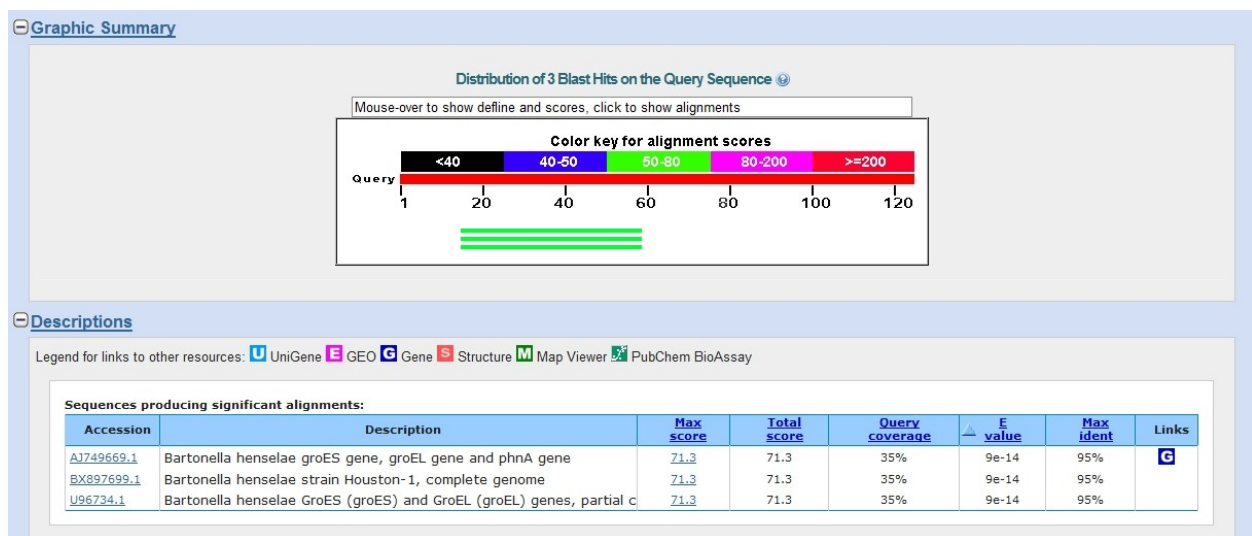
Figure 3-4. DNA sequence BLAST results. The DNA sequence of *B. henselae* strain CSU-1 qPCR product was compared with sequences in the GenBank. BLAST result indicated that the qPCR product was specific to *B. henselae* with 95% similarity with the GenBank accession number AJ749669, BX897699, and U96734.

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3.4.3 Optimal qPCR Assay Condition

High fluorescence signal and low C_q values were observed at the gradient temperature of 59.0 °C and 60.3 °C in the initial analysis (Figure 3-5). The subsequent analysis with the narrow gradient temperatures showed that the annealing temperature at 60 °C produced the PCR product with the highest magnitude of fluorescence emission signal (Figure 3-6A) and the lowest C_q value (Figure 3-6B). After several reactions were performed using combinations of different primer concentrations within the same plate, these combinations resulted in a narrow range of fluorescence intensity (Figure 3-7A) and C_q value (Figure 3-7B). The range of C_q values was between 28.95 and 29.65. From the results, the primer combination of 0.6/0.6 µM was chosen because this combination provided high fluorescence intensity, low C_q, and consistency results.

A

B
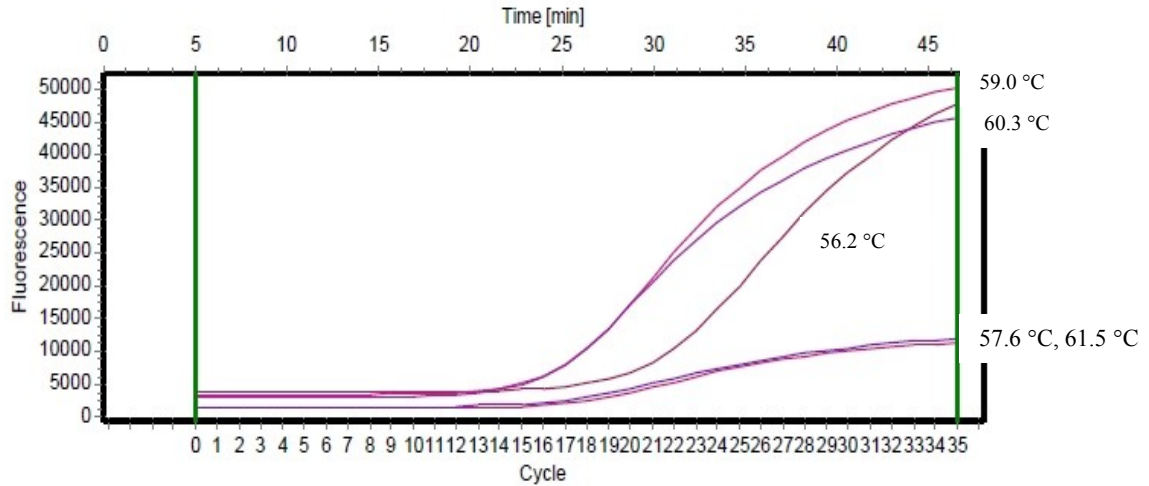
Column	Temperature (°C)	C_q SYBR
1	56.2	16.96
2	57.6	16.83
3	59.0	13.66
4	60.3	13.32
5	61.5	15.93

Figure 3-5.   Gradient temperature analysis (1). The annealing temperature was varied from 56 °C to 62 °C for each well (from 1 - 6) of the plate. (A) fluorescence versus cycle number indicate that the temperature of 56.2 °C, 59.0 °C, and 60.3 °C generated high fluorescence signal (B) the lowest C_q values of the qPCR products were corresponded to the annealing temperatures of 59.0 °C and 60.3 °C.

A

B
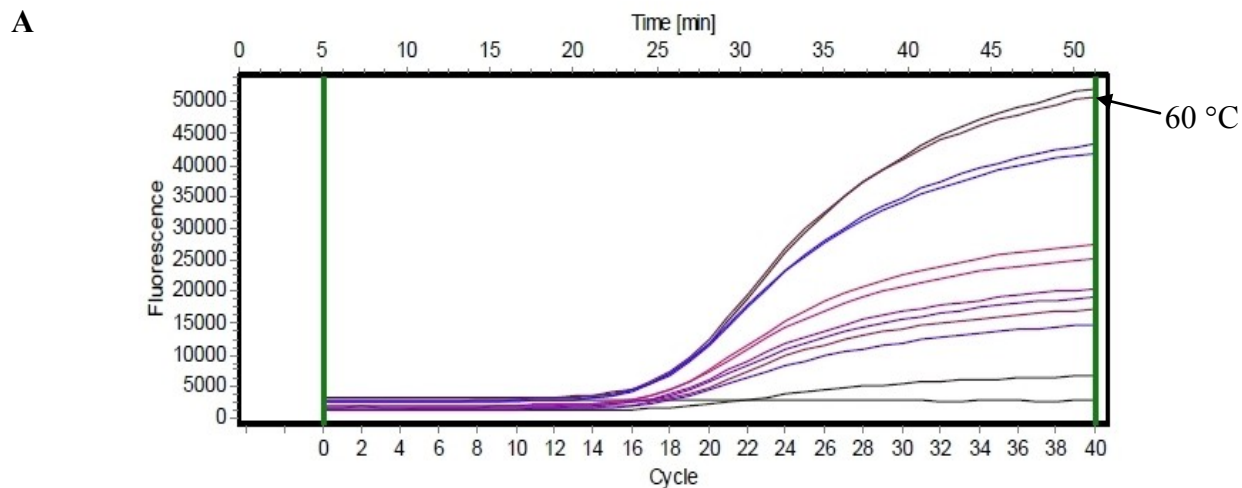
Column	Temperature (°C)	C_q SYBR
1	58.0	NTC
2	58.1	18.33
3	58.2	14.64
4	58.4	14.48
5	58.7	15.85
6	58.9	15.07
7	59.2	15.16
8	59.5	15.34
9	59.7	15.52
10	59.9	16.04
11	60.0	14.30
12	60.0	14.36

Figure 3-6. Gradient temperature analysis (2). The annealing temperature was varied from 58.0 °C to 60.0 °C for each well (from column 1 - 12) of the plate. (A) Amplification plots showing fluorescence versus cycle number indicate that the temperature 60 °C produced the qPCR product with the highest fluorescence signal (B) C_q values corresponding to annealing temperatures indicates that the lowest C_q value of the qPCR product was produced at the temperature 60 °C.

A
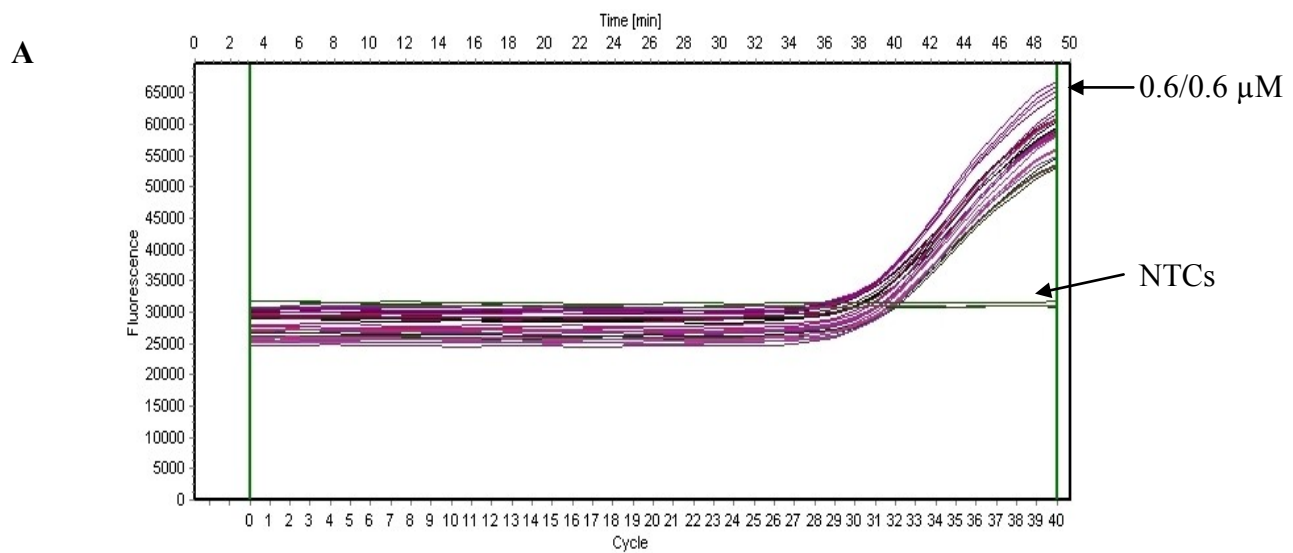
Primer combination (μM)	Mean C_q	S.D. C_q
0.5 / 0.5	29.14	0.20
0.5 / 0.6	29.49	0.18
0.5 / 0.7	29.65	0.11
0.6 / 0.5	28.95	0.18
0.6 / 0.6	**29.19**	**0.07**
0.6 / 0.7	29.20	0.34
0.7 / 0.5	29.10	0.04
0.7 / 0.6	29.13	0.05
0.7 / 0.7	29.26	0.10

B (label at left of table)

Figure 3-7. Optimization of primer concentrations. Primer concentrations were optimized using the forward/reverse primer combination at 0.5, 0.6, 0.7 μM and the constant probe concentration at 0.2 μM (A) Amplification plots showing fluorescence signal versus cycle number of 9 primer concentration combinations indicated that the 0.6/0.6 μM combination generated the qPCR product with highest fluorescence intensity (B) Mean values and standard deviation (S.D.) of Cq values corresponding to each primer combination showed that mean C_q was similar among combinations. However, the 0.6/0.6 μM combination produced the qPCR product with good C_q value and low variation.

Among three different concentrations of probe compared (Figure 3-8), the concentration of 0.10 µM was too low for the assay because the fluorescent intensity was obviously lower and the C_q value was higher than those of 0.15 and 0.20 µM. The concentration of 0.20 µM produced the highest fluorescence, but high fluorescence background was also observed and triplicate results were not consistent. Therefore, the probe concentration of 0.15 µM was considered as the proper concentration due to optimal fluorescence intensity, optimal C_q value, and consistent results.

Probe (µM)	Mean C_q	S.D. C_q
0.10	29.86	0.11
0.15	**29.63**	**0.09**
0.20	29.55	0.17
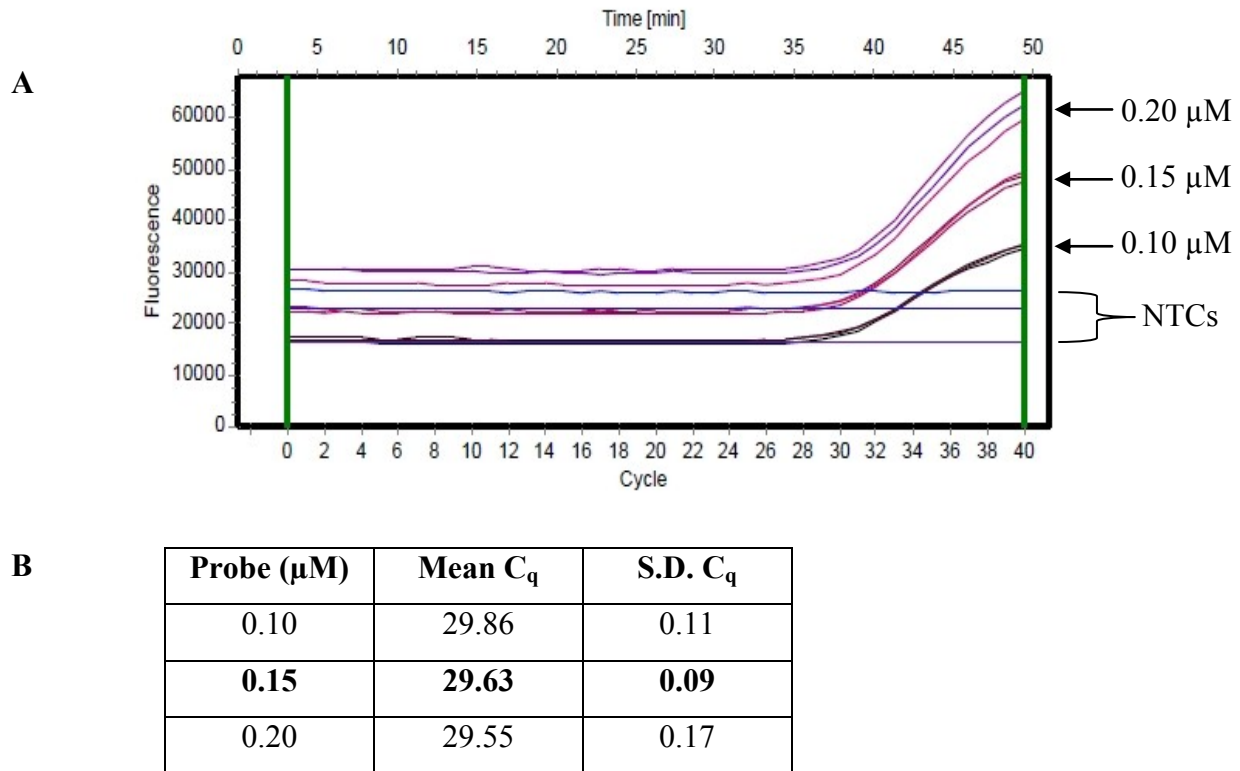
Figure 3-8. Optimization of probe concentration. Probe concentration of 0.10, 0.15, and 0.2 µM was determined in reactions with constant forward and reverse primer concentration of 0.6 µM (A) Amplification plots showing fluorescence signal versus cycle number indicated that probe concentration 0.20 µM generated the qPCR product with higher fluorescence intensity than 0.15 and 0.10 µM (B) Mean values and standard deviation (S.D.) of Cq values corresponding to each probe concentration showed that mean C_q was similar among probe concentrations, but the mean C_q from probe concentration 0.15 µM had the lowest variation.

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The optimized qPCR assay was prepared in a 20-µl mixture of the followings: 10 µl of TaqMan Fast Advanced Master Mix, 1.2 µl of forward and reverse primer (final concentration 0.6 µM), 0.6 µl of probe (final concentration 0.15 µM), 2.0 µl of template DNA, and 5 µl of PCR-graded water. No template controls (NTCs) were included at the same time and under the same conditions to detect the presence of contaminating DNA. Each of samples was assayed in triplicates. The thermocycling protocol included 50 °C for 2 min, 95 °C for 1 min, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C.

3.4.4 CFU Determination

By taking into account the volumes used for DNA extraction and viable cell counts, the number of viable colonies spiked in a volume of blood sample was calculated in CFU/ml of blood. The number of bacteria spiked in blood sample standards ranged from 5.2×10^6 to 52 CFU/ml of blood.

3.4.5 Analytical Performance of qPCR Assay

A log plot of standard samples is showing in Figure 3-9A. The linear dynamic range of the assay determined from a standard curve was composed of 6 logs of *B. henselae* levels ranging from 5.2×10^6 to 52 CFU/ml (Figure 3-9B). Serial dilutions in the tested range demonstrated that the C_q value correlates linearly with the amount of spiked bacteria ($R^2 = 0.994$). The average slope of the standard curve was within -3.954 ± .0.11. The average PCR efficiency was approximately 79% ± 0.3%. The LOD of the qPCR assay was the minimum amount of *B. henselae* that was amplified on the standard curve, which was 52 CFU/ml.

A

B

Slope	-4.020
Y-Intercept	36.65

R^2	0.994
Efficiency	0.77
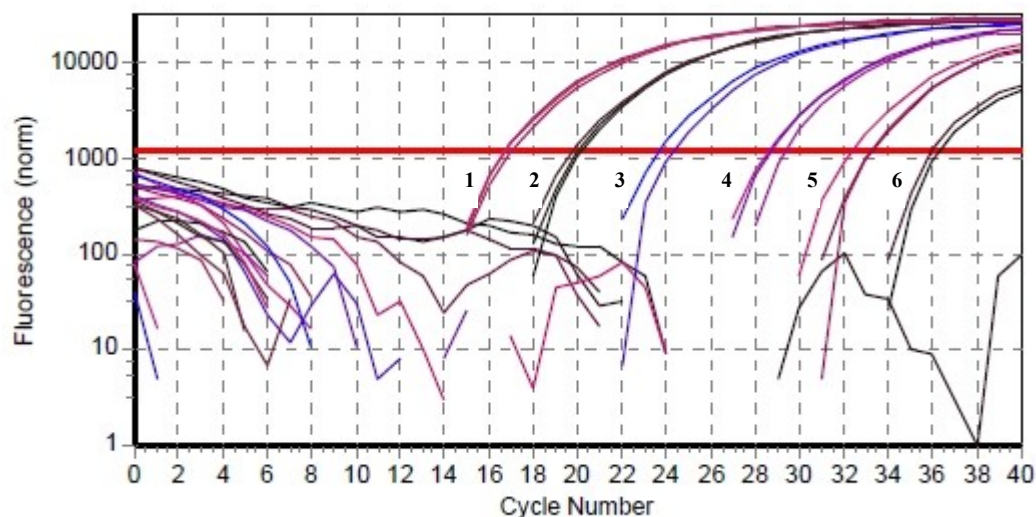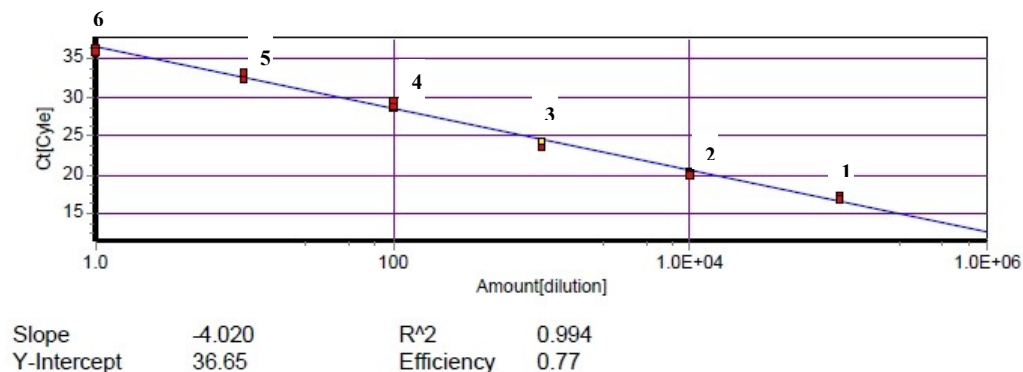
Figure 3-9. Standard curve for the qPCR assay. The qPCR efficiency and quantification of unknown blood samples of cats were determined from standard curves. Known amount of *B. henselae* (CFU/ml) was spiked in feline whole blood, in which the total DNA was subsequently extracted. (A) Log plot of the fluorescence intensity versus cycle number of standards ranging from 5.2×10^6 to 52 CFU/ml: number 1 to 6 (B) Simple linear regression plot of the C_q values (from A) versus log of known amount of *B. henselae* in standards. The qPCR descriptors, including slope, R^2, Y-intercept, and efficiency analyzed by the thermocycler are shown.

Only *B. henselae* was positive in the qPCR assay (Figure 3-10). None of signals was observed in the reactions of other *Bartonella* spp. and non-*Bartonella* related bacteria. The specificity was confirmed by gel electrophoresis, and there were no PCR product of any organism except *B. henselae* found on gel.
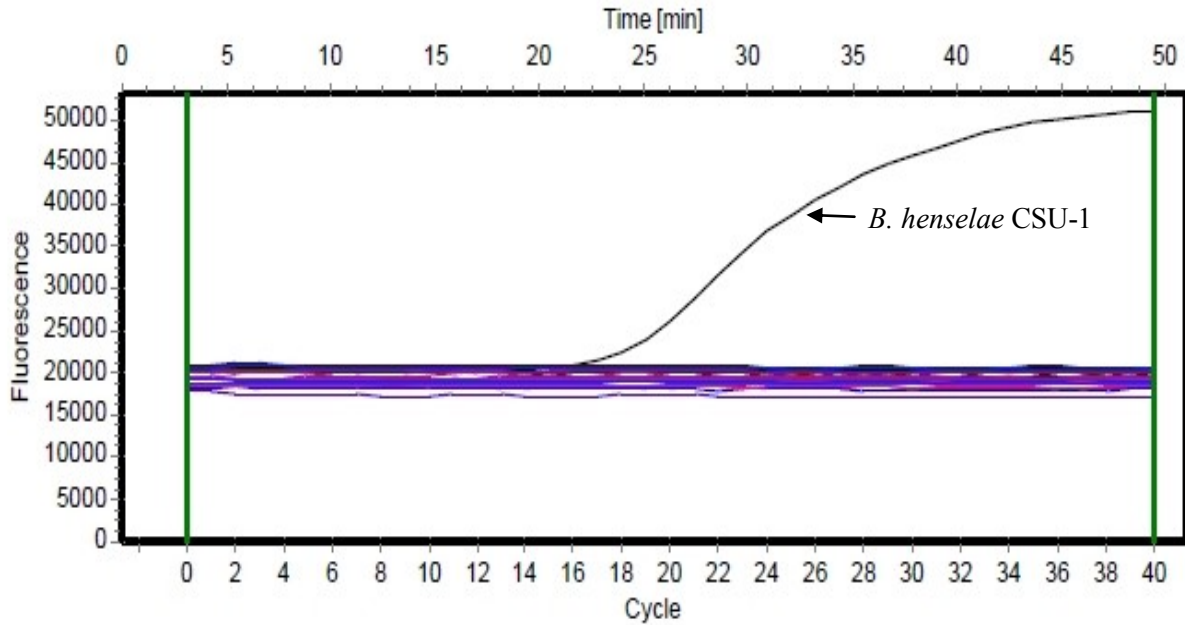
Figure 3-10. Determination of the assay specificity. The amplification plot indicates that *B. henselae* strain CSU-1 was the only organism amplified from the qPCR, while other *Bartonella* spp. or bacteria were not amplified.

After the assay was replicated in three runs on three consecutive days, the intra-assay CV and the inter-assay CV were 1.48% and 0.40%, respectively (Table 3-2).

Table 3-2   Repeatability determined by intra-assay and inter-assay variations. Standard samples ranging from 5.2 x 10^6 to 52 CFU/ml were assayed in triplicates on the same plate for 3 consecutive days.

Standard (CFU/ml)	Day 1			Day 2			Day 3		
	Mean C_q	S.D. C_q	CV	Mean C_q	S.D. C_q	CV	Mean C_q	S.D. C_q	CV
5.2 x 10^6	16.93	0.18	1.09	16.80	0.10	0.57	16.88	0.43	2.53
5.2 x 10^5	20.08	0.21	1.06	20.01	0.04	0.19	19.77	0.70	3.54
5.2 x 10^4	24.06	0.48	2.00	24.31	0.45	1.85	24.23	0.39	1.59
5.2 x 10^3	28.95	0.41	1.42	28.74	0.16	0.55	28.65	0.30	1.04
5.2 x 10^2	33.05	0.53	1.62	32.82	0.85	2.60	32.68	0.46	1.41
5.2 x 10^1	36.20	0.25	0.70	36.10	0.73	2.02	37.80	0.36	0.95
Overall mean C_q	26.54			26.46			26.67		
Mean CV	1.31			1.30			1.84		
Grand mean	26.56								
SD of the grand mean	0.11								
[a] Intra-assay variation: CV	**1.48%**								
[b] Inter-assay variation: CV	**0.40%**								

[a] The mean of mean CVs of three separate days
[b] SD of the overall mean of three separate days/grand mean x 100

3.5 Discussion

B. henselae is the major species of *Bartonella* that infects cats, and *B. henselae* DNA has more frequently been amplified from feline blood than DNA of *B. clarridgeiae* and *B. koehlerae.* In addition, *B. henselae* appears to be the most likely *Bartonella* spp. to be associated with illness in cats. Because of their fastidious and slow growth, routine blood culture is not the optimal method for clinical use. Because molecular techniques are more sensitive and return results faster than routine blood culture, they are frequently used for identification of *Bartonella* spp. infection. Since the *Bartonella*-specific PCR assay was first reported, several different PCR

assays with high specificity and sensitivity have subsequently been developed. Recent studies showed the usefulness of cPCR assays for detection of *Bartonella* spp. DNA from blood of cats in many areas (Lappin et al., 2006; Solano-Gallego et al., 2006; Kamrani et al., 2008; Barrs et al., 2010; Juvet et al., 2010; Kelly et al., 2010; Staggemeier et al., 2010). These assays have been used as one-step analysis or combined with nucleotide sequencing to identify species of *Bartonella*. These assays had enough sensitivity and specificity for field studies, but most of the time they lacked the capability of proving the clinical illness related to the infection (Quimby et al., 2008; Dowers et al., 2010).

More recently, a qPCR assay have also been used to amplify the citrate synthase gene of *Bartonella* spp. in feline blood (Mietze et al., 2011), but the quantification was not mentioned. Therefore, the qPCR assay described herein has the distinct advantage of being able to quantify the bacterial load of *B. henselae* in a single-step procedure.

The qPCR assay in this study was developed to be specific to *B. henselae* due to its high prevalence and perceived clinical relevance. Primers used in previously published studies are commonly able to amplify many *Bartonella* spp. within the same PCR conditions. For example, the primers targeting *gltA* gene could amplify the DNA of four *Bartonella* spp. (Norman et al., 1995), or the primers targeting ITS amplified the DNA of six *Bartonella* spp. (Jensen et al., 2000). However, the new primers and the corresponding probe designed in this study were specific to only *B. henselae*.

The DNA sequences of oligonucleotides in this study were specific to the region within a protein-encoding gene, the *groES* gene. This gene is a single-copy gene in *B. henselae* chromosomal DNA. In general, sequences of protein-encoding gene have interspecies variability, but are conserved at the intraspecies level. This was true with the *groES* gene of *Bartonella* spp.

The *groES* gene was chosen because this gene has higher percentage of similarity among different strains than other genes. All *B. henselae groES* sequences available in GenBank had 100% identity (Figure 3-1), confirming that the sequences of this gene are homologous among known strains of *B. henselae*. The nucleotide sequence of the *groES* of *B. henselae* strain CSU-1, which is the reference strain in this study, is not available at the GenBank. However, this study showed that *B. henselae* strain CSU-1 had 95% homologous similarity with other *B. henselae* strains.

SYBR Green I dye was used as detection system in the initial assay development for screening of primers because it is sensitive, easy to use, and inexpensive. The SYBR Green I is also a good tool for observing primer dimers or unspecific amplicons by melting curves generated after PCR amplification. The SYBR Green I allows for quantification, but its specificity is less than that of TaqMan PCR assays. In this study, using *B. henselae* isolates as template DNA, the melting curve analysis presented fluorescence signal peak around the T_m of the PCR products, and PCR product with the expected size was observed on gel electrophoresis. Primer dimers were not present. However, many non-specific amplicons were identified when total DNA from negative blood samples was used as the template. These non-specific amplicons were detected by strange peaks of fluorescent signals in melting curve analysis and the unpredicted sized of PCR fragments on gel electrophoresis. SYBR Green I can bind to double-stranded DNA independently, thus specific and non-specific products amplified can be both detected (Deprez et al., 2002). Therefore, the use of SYBR Green I was discontinued to avoid non-specific detection, and the probe-based qPCR was used instead. The probe-based PCR assay is usually not as sensitive as the SYBR green assay, but it increases the specificity of the qPCR assay. The primers designed in this study were predicted to amplify a single product of 85 bp in

length. The qPCR assay yielded the PCR product of the predicted size (Figure 3-3), which was subsequently confirmed by nucleotide sequencing (Figure 3-4).

While theoretical annealing temperatures were determined in the process of primer design as described above, the gradient PCR was used to determine the actual optimal annealing temperature and primers specificity. Using the gradient temperature mode of the thermocycler, various temperatures could be set up in the same assay. The annealing temperature of 60 °C (Figure 3-5) was selected in this study because of high fluorescence signal and lowest C_q provided. This temperature ensured higher sensitivity of the assay than the other temperatures tested.

To determine the optimal primer and probe concentrations, the checkerboard assays were performed. The C_q values of the qPCR assay using different primer concentrations showed a narrow variation of 0.7 cycles (Figure 3-6B). The observed C_q differences are due to the variation of primer concentrations, as these were the only variable reaction parameters. The concentration of the forward and reverse primers had a slight effect to the C_q values, since the C_q values did not change dramatically. The qPCR products were also evaluated by gel electrophoresis to exclude any combination that might amplify primer dimers. Each of the primer concentration combinations generated a specific amplicon as determined by gel electrophoresis, and no primer dimer was observed in any combination. As 0.6 μM of each primer generated the amplification curve with the highest fluorescence intensity, low C_q, and consistency results (Figure 3-6A). This combination was therefore chosen for the optimal reaction. After the primer concentrations were optimized, the optimal probe concentration was evaluated. Although the highest fluorescence signal was observed for the probe concentration of 0.2 μM (Figure 3-7A), but the C_q difference between 0.15 and 0.2 μM was only 0.08 (Figure 3-7B). The C_q values of

0.15 µM concentration was more consistent than that of 0.2 µM concentration, and lower fluorescence background was observed. Owing to the cost of a probe-based qPCR assay which is strongly dependent on the cost of the probe, the lower probe concentration was chosen except when the higher probe concentration gave a significantly lower C_q value and/or resulted in a significant improvement of the signal intensity. In this study, the probe concentration of 0.2 µM did not significantly improve the performance of the qPCR assay, so the probe 0.15 µM concentration was chosen for the optimal reaction.

The qPCR assay will be used to determine the correlation of bacterial load and disease state, therefore quantification is necessary. In order to perform the quantification, the absolute quantities of the standards must first be known by some independent method. Standards with known amount of *B. henselae* are considered the critical factor for accurate quantification. In this study, the amount of *B. henselae* in the standards was measured by viable plate counts of bacteria (CFU/ml), in which only living bacteria are counted. This method was chosen because the CFU/ml is more frequently used for bacteria and is more clinically relevant for interpretation than the copy numbers, which are commonly used for viruses. Viable plate counts have advantages over other bacterial quantification methods such as turbidimetric measurement due to its higher sensitivity, easy to perform, and living cells being of interest. However, long incubation periods are needed for these fastidious bacteria. In addition, care must be taken to avoid pipetting errors during dilution over several orders of magnitude and plating, and plates may be contaminated during incubation. Once the quantity of standards was known, unknown samples are able to be compared with the standard curve and extrapolate a value. By performing the absolute quantification, the minimal optimization and validation is required. However, the errors from inaccurate pipetting and instability of the diluted standards must be considered.

In this study, the six orders of magnitude of standards created the linear dynamic range that provided accurate quantifications from 52 to 5.2 x 10^6 CFU/ml of blood. The initial amount of DNA below this limit may be inaccurately quantified. False negative results can be occurred in samples containing very low amount of DNA. The sensitivity of the qPCR assay is 52 CFU/ml. The sensitivity of the qPCR was comparable to the cPCR by Jensen et al. (2000). In that cPCR assay, all blood samples containing 50 to 100 CFU/ml *B. henselae* were detected, and the sensitivity decreased when the number of *B. henselae* was below 50 CFU/ml. The qPCR was shown to be similarly sensitive as the assay of Norman et al. (1995), in which the LOD was 40 CFU/ml. The standard curves in this study were reproducible with both low intra-assay and inter-assay variations.

Generally, the acceptable assay efficiency is between 90% and 110%. Low efficiency may impact the assay sensitivity and the limit of detection. Although the qPCR in this study was optimized, the efficiency of the assay was approximately 79%. However, even though the efficiency was below the generally acceptable value, the analytical sensitivity was comparable with other previous assays. The efficiency of the qPCR may be not significantly problematic for the use of the assay in a field study. The low efficiency of a PCR assay may be caused by primer dimer formation, probe quality, insufficient optimization, PCR inhibitors in samples, inaccurate pipetting, or assay design i.e. poor primer/target binding, too long or too much secondary structure of PCR product. In this study, primer dimer formation and assay design were considered the least likely possibilities for low efficiency. Primer dimers were not detected, and the assay was designed following the general guidelines. If the optimization is not sufficient, nonspecific products or insufficient product may be produced, which directly impact the PCR efficiency. The primers and probe were only the reagents optimized in this study because a

commercially available master mix was used for convenience. It is possible that additional reaction optimization may be needed such as magnesium concentration. Otherwise, the use of different commercially available master mixes should be compared. Since the spiked whole blood samples were used for preparing standards, PCR inhibitors may be left over from the DNA extraction, leading to the low assay efficiency. Poor laboratory technique, particularly pipetting errors can also increase variation of C_q values.

When evaluating the specificity of the qPCR assay, no cross-reactivity with other species of *Bartonella* or other bacterial microorganisms was found. Because co-infection with more than one organism in healthy or sick cats is commonly found, this probe-based qPCR assay ensures that no false positive results will occur. Other *Bartonella* spp. such as *B. quintana* were unavailable at the time of testing, thus the cross-reactivity with the species beyond the list was incomplete.

In conclusion, the qPCR assay in this study has been a successful method providing a new diagnostic tool for detecting and quantifying *B. henselae* in feline blood. This chapter described the development procedures, optimization, analytical sensitivity, and analytical specificity of the qPCR. Optimizing the qPCR assay is very important to ensure its efficiency as well as specificity. The sensitivity of the qPCR has shown to be comparable with cPCR assays. It is believed that the assay described here would be useful in clinical purposes for diagnosis of *B. henselae* in cats. However, some limitations of the qPCR assay, including low efficiency and the quality of standards should be considered for interpretation of results. The question of whether bacteremic level of *B. henselae* correlates with clinical illnesses is addressed in Chapter 6.

3.6 Acknowledgements

References

Barrs, V.R., Beatty, J.A., Wilson, B.J., Evans, N., Gowan, R., Baral, R.M., Lingard, A.E., Perkovic, G., Hawley, J.R., Lappin, M.R., 2010. Prevalence of *Bartonella* species, *Rickettsia felis*, haemoplasmas and the *Ehrlichia* group in the blood of cats and fleas in eastern Australia. Aust. Vet. J. 88, 160-165.

Bereswill, S., Hinkelmann, S., Kist, M., Sander, A., 1999. Molecular analysis of riboflavin synthesis genes in *Bartonella henselae* and use of the *ribC* gene for differentiation of *Bartonella* Species by PCR. J. Clin. Microbiol. 37, 3159-3166.

Bergmans, A.M., Schellekens, J.F., Van Embden, J.D., Schouls, L.M., 1996. Predominance of two *Bartonella henselae* variants among cat-scratch disease patients in the Netherlands. J. Clin. Microbiol. 34, 254-260.

Birtles, R.J., Raoult, D., 1996. Comparison of partial citrate synthase gene (*gltA*) sequences for phylogenetic analysis of *Bartonella* species. Int. J. Syst. Bacteriol. 46, 891-897.

Deprez, R.H.L., Fijnvandraat, A.C., Ruijter, J.M., Moorman, A.F.M., 2002. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. Anal. Biochem. 307, 63-69.

Dowers, K.L., Hawley, J.R., Brewer, M.M., Morris, A.K., Radecki, S.V., Lappin, M.R., 2010. Association of *Bartonella* species, feline calicivirus, and feline herpesvirus 1 infection with gingivostomatitis in cats. J. Feline Med. Surg. 12, 314-321.

Ehrenborg, C., Wesslén, L., Jakobson, Å., Friman, G., Holmberg, M., 2000. Sequence variation in the *ftsZ* gene of *Bartonella henselae* isolates and clinical samples. J. Clin. Microbiol. 38, 682-687.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95-98.

Houpikian, P., Raoult, D., 2001. 16S/23S rRNA intergenic spacer regions for phylogenetic analysis, identification, and subtyping of *Bartonella* species. J. Clin. Microbiol. 39, 2768-2778.

Hyndman, D.L., Mitsuhashi, M., 2003, PCR primer design, In: Bartlett, J.M.S., Stirling, D. (Eds.) PCR protocols (Methods in molecular biology). Human Press, New Jersey, pp. 81-88.

Jensen, W.A., Fall, M.Z., Rooney, J., Kordick, D.L., Breitschwerdt, E.B., 2000. Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. J. Clin. Microbiol. 38, 1717-1722.

Johnson, G., Ayers, M., McClure, S.C.C., Richardson, S.E., Tellier, R., 2003. Detection and identification of *Bartonella* species pathogenic for humans by PCR amplification targeting the riboflavin synthase gene (*ribC*). J. Clin. Microbiol. 41, 1069-1072.

Juvet, F., Lappin, M.R., Brennan, S., Mooney, C.T., 2010. Prevalence of selected infectious agents in cats in Ireland. J. Feline Med. Surg. 12, 476–482.

Kamrani, A., Parreira, V.R., Greenwood, J., Prescott, J.F., 2008. The prevalence of *Bartonella*, hemoplasma, and *Rickettsia felis* infections in domestic cats and in cat fleas in Ontario. Can. J. Vet. Res. 72, 411-419.

Kelly, P.J., Moura, L., Miller, T., Thurk, J., Perreault, N., Weil, A., Maggio, R., Lucas, H., Breitschwerdt, E., 2010. Feline immunodeficiency virus, feline leukemia virus and *Bartonella* species in stray cats on St Kitts, West Indies. J. Feline Med. Surg. 12, 447-450.

Kelly, T.M., Padmalayam, I., Baumstark, B.R., 1998. Use of the cell division protein *FtsZ* as a means of differentiating among *Bartonella* species. Clin. Diagn. Lab. Immunol. 5, 766-772.

Kordick, D.L., Wilson, K.H., Sexton, D.J., Hadfield, T.L., Berkhoff, H.A., Breitschwerdt, E.B., 1995. Prolonged *Bartonella bacteremia* in cats associated with cat-scratch disease patients. J. Clin. Microbiol. 33, 3245-3251.

La Scola, B., Liang, Z., Zeaiter, Z., Houpikian, P., Grimont, P.A.D., Raoult, D., 2002. Genotypic characteristics of two serotypes of *Bartonella henselae*. J. Clin. Microbiol. 40, 2002-2008.

Lappin, M.R., Breitschwerdt, E., Brewer, M., Hawley, J., Hegarty, B., Radecki, S., 2009. Prevalence of *Bartonella* species antibodies and *Bartonella* species DNA in the blood of cats with and without fever. J. Feline Med. Surg. 11, 141-148.

Lappin, M.R., Griffin, B., Brunt, J., Riley, A., Burney, D., Hawley, J., Brewer, M.M., Jensen, W.A., 2006. Prevalence of *Bartonella* species, haemoplasma species, *Ehrlichia* species, *Anaplasma phagocytophilum*, and *Neorickettsia risticii* DNA in the blood of cats and their fleas in the United States. J. Feline Med. Surg. 8, 85-90.

Maggi, R.G., Breitschwerdt, E.B., 2005. Potential limitations of the 16S-23S rRNA intergenic region for molecular detection of *Bartonella* species. J. Clin. Microbiol. 43, 1171-1176.

Marston, E.L., Sumner, J.W., Regnery, R.L., 1999. Evaluation of intraspecies genetic variation within the 60 kDa heat-shock protein gene (*groEL*) of *Bartonella* species. Int. J. Syst. Bacteriol. 49, 1015-1023.

Mietze, A., Morick, D., Köhler, H., Harrus, S., Dehio, C., Nolte, I., Goethe, R., 2011. Combined MLST and AFLP typing of *Bartonella henselae* isolated from cats reveals new sequence types and suggests clonal evolution. Vet. Microbiol. 148, 238-245.

Norman, A.F., Regnery, R., Jameson, P., Greene, C., Krause, D.C., 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J. Clin. Microbiol. 33, 1797-1803.

Quimby, J.M., Elston, T., Hawley, J., Brewer, M., Miller, A., Lappin, M.R., 2008. Evaluation of the association of *Bartonella* species, feline herpesvirus 1, feline calicivirus, feline leukemia

virus and feline immunodeficiency virus with chronic feline gingivostomatitis. J. Feline Med. Surg. 10, 66-72.

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132, 365-386.

Solano-Gallego, L., Hegarty, B., Espada, Y., Llull, J., Breitschwerdt, E., 2006. Serological and molecular evidence of exposure to arthropod-borne organisms in cats from northeastern Spain. Vet. Microbiol. 118, 274-277.

Staggemeier, R., Venker, C.A., Klein, D.H., Petry, M., Spilki, F.R., Cantarelli, V.V., 2010. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in cats in the south of Brazil: a molecular study. Memórias do Instituto Oswaldo Cruz. 105, 873-878.

Zeaiter, Z., Fournier, P.E., Ogata, H., Raoult, D., 2002a. Phylogenetic classification of *Bartonella* species by comparing *groEL* sequences. Int. J. Syst. Evol. Microbiol. 52, 165-171.

Zeaiter, Z., Liang, Z., Raoult, D., 2002b. Genetic classification and differentiation of *Bartonella* species based on comparison of partial *ftsZ* gene sequences. J. Clin. Microbiol. 40, 3641-3647.

CHAPTER 4

Development of Antigen-Capture Enzyme-linked Immunosorbent Assay for the Detection of *Bartonella henselae* in Feline Blood

4.1 Summary

A polyclonal-based antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) was developed for antigen detection of *B. henselae* in blood of cats. Rabbit polyclonal antibodies against *B. henselae* strain CSU-1 were produced. The optimized assay consists of 25 µg/ml of coating IgG and 10 µg/ml of horseradish peroxidase-conjugated IgG per microELISA plate well. Because of non-specific binding between IgG antibodies and non-relevant proteins, preparation of blood samples by hemolysis and subsequent filtration was necessary. The accuracy of the assay was evaluated. The AC-ELISA test results of six experimentally infected cats were compared to results of cPCR assay, qPCR assay, and routine culture. The AC-ELISA appeared to be much less sensitive than the PCR assays because only 50% of the samples positive by both PCR assays were positive by the AC-ELISA. However, the AC-ELISA had high specificity with the false positive rate of <10%. The present format of AC-ELISA is not valid for the clinical use, but the results would be useful for designing more sensitive assays in the future.

Keyword: AC-ELISA; *B. henselae*; Antigen; Cat

4.2 Introduction

Persistent bacteremia in host-adapted reservoirs is a typical characteristic of *Bartonella* spp. infection as well as *B. henselae* infection in cats. *Bartonella henselae* usually colonizes and replicates in a primary niche shortly after animals are infected. Once *B. henselae* is released into blood circulation, bacteria can adhere and invade red blood cells where bacteria replicate and persist for the remaining lifespan of infected cells (Dehio, 2001). *Bartonella henselae* can escape from the immune system of the host because of the intra-cellular location. The antibodies are able to clear extracellular bacteria from bloodstream and prevent bacterial re-invasion into the primary niche or red blood cells (Koesling et al., 2001). Bacteremia in naturally infected cats may last for months or years (Kordick et al., 1995).

Detection of a current *B. henselae* infection in cats is commonly based on culture or amplification of *B. henselae* DNA from blood. Polymerase chain reaction (PCR) assays are reliable methods that have shown to be more sensitive and are more rapid to perform than culture method. However, the laboratory testing using PCR assays can be expensive and requires specialized laboratory facilities and well-trained staffs. Determination of serum antibodies against *Bartonella* spp. by means of immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA) can be performed quickly and inexpensively. However, these serological tests prove only the previous exposure and neither of them is highly correlated with bacteremia. In addition, none of available tests is associated with clinical manifestations related to *B. henselae* infection (Brunt et al., 2006). This chapter introduces a newly developed assay that is simple to perform, is able to reduce the turnaround time, and could potentially be used to accurately prove current *B. henselae* infection in cats.

Several antigen detection assays have been widely used for diagnosis of feline infectious diseases such as feline leukemia virus and *Dirofilaria immitis*. These assays are commercially available, can be performed at the point-of-care, are easy-to-perform, inexpensive, and reliable due to good performances (high sensitivity and high specificity) for diagnosis of particular infections. Detection of antigens can be based on ELISA technique, in which the antigen levels are corresponded to the amount of agent burdens. Because of adequate sensitivity and specificity to produce accurate and reliable results therefore additional tests are generally not required for confirmation. An antigen detection test has never been used for detection of *B. henselae* in cats. The objective of this study is to develop a new antigen capture ELISA for the purpose of detecting *B. henselae* antigens in blood samples of cats. To the best of my knowledge, this is the first polyclonal-based AC-ELISA developed and compared to the currently available assays for the diagnosis of *B. henselae* infection.

4.2.1 General Considerations for the Design and Conduct of Antigen Detection ELISA

Types of ELISA for antigen detection

Typically, there are two different types of ELISA that can be used for antigen detection; antigen-capture ELISA (AC-ELISA) and competitive ELISA (Paulie et al., 2001). The AC-ELISA ELISA (also called sandwich ELISA) is commonly used for detecting specific analyte within complex mixture. Antigens of a targeted analyte are bound between two layers of antibodies, including capture and detection antibodies. The analyte to be measured then must contain at least two antigenic binding sites. The AC-ELISA is therefore confined to the detection of multivalent antigens such as proteins or polysaccharides. The assay also requires that binding of one antibody must not interfere or compete for binding of the other antibody.

The competitive ELISA is an alternative assay for antigen detection. The assay is commonly used if the matched-paired antibodies are not available or the analyte is too small for the AC-ELISA. The competitive ELISA is also used to increase the specificity of an assay when samples contain cross-reacting species. There are several different features for competitive ELISA, and each of these features requires that either antigen or primary antibody must be conjugated to a detection enzyme. The sample is then mixed with the conjugated antigens or conjugated antibodies prior to incubation on the plate. If the plate is coated with unlabeled antigens, the sample antigens compete with coated antigens on the solid phase for binding to the conjugated antibodies. When the plate is coated with unlabeled antibodies, the sample and conjugated antigens compete each other to bind the coating antibodies. Because of the competition between analyte and conjugated reagent, the amount of analyte in the sample is reciprocal to the signal generated.

The AC-ELISA is more frequently used than the competitive ELISA because of higher level of sensitivity and specificity. The sensitivity of the AC-ELISA depends on the affinity of the capture and detection antibodies, while the sensitivity of the competitive ELISA is influenced by the affinity and the amount of antibody used. The AC-ELISA is more specific than the competitive ELISA since it involves at least two antibody recognition sites.

Monoclonal antibody versus polyclonal antibody

The choice of antibody is the primarily important consideration for assay development. Due to the versatility of the AC-ELISA, either monoclonal or polyclonal antibodies can be used in the assay (Leenaars and Hendriksen, 2005). Monoclonal antibodies recognize only single epitope of an antigen therefore they provide high specificity and allow detection of small

differences in an antigen. However, the sensitivity of the assay may be low due to antibody molecules can bind to only one antigenic site. The production of monoclonal antibodies is generally more complicated and time-consuming than polyclonal antibody production.

Conversely, polyclonal antibodies provide higher sensitivity because multiple antibodies can possibly bind to different epitopes on a single antigen molecule. The production of polyclonal antibodies is simple, inexpensive, and requires short period. In order to detect large molecules that have many epitopes, such as bacteria molecules, polyclonal antibodies produced from an animal can be used as a "self-sandwich" ELISA for both capture and detection. However, polyclonal antibodies have a higher risk of cross-reactivity since the epitope is less precisely defined.

Depending on reagents availability, monoclonal antibodies or polyclonal antibodies or combinations of them can be used. Monoclonal antibodies are often used as the capture antibody to generate the highest level of specificity in an assay, and polyclonal antibodies are applied as the detection antibody to enhance the signal to noise ratio. In a different way, coating with polyclonal antibodies can pull down as much of the antigens as possible to increase the sensitivity. Then monoclonal antibodies are used to detect the bound antigens to provide higher specificity. However, the choices of antibodies are empirically selected.

4.2.2   Considerations for Polyclonal Antibody Production

Animal selection

Selection of the animal species for polyclonal antibody production commonly depends on the specific purpose of the experiment, the amount of antiserum needed, and the ease of obtaining blood samples. Rodents and rabbits are often used to produce antibodies for many

research objectives. Female animals are preferred over males for handling, and they are less aggressive in social interactions. Young adults should be used because the immune response is immature at an early age and falls with age after the period of young adulthood (Leenaars et al., 1999).

Antigen preparation

This is one of critical steps in antibody production. The quality and quantity of antigen should be considered. The purity and method of preparation of the immunizing antigens is very important. The antigen to be immunized should have no contamination with endotoxins (i.e. lipopolysaccharide, pyrogens), toxic substances (i.e. urea, citric acid), chemical residues that are used to inactivate the organism (i.e. sodium azide, formaldehyde, β-propiolactone), or extreme pH that can be a risk of toxicity to animals. The quantity of antigen usually depends on the inherent properties of the antigen, the purity of the antigen, the adjuvant used, and the route and frequency of the injection.

Choice of adjuvant

Adjuvants are frequently used to boost the immune response. Freund's Completed Adjuvant containing mycobacteria is used in the polyclonal antibody production because high antibody titers can be induced to almost all types of antigens, especially small peptides with poor immunogenicity. Freund's adjuvants also play a role in depot effect for prolonged exposure of the immune cells by slowly releasing of the antigen from the injection sites (Stills, 2005). However, the inflammatory responses at the sites of injection and the distress in animals are sometimes found after immunization. Adjuvants containing mycobacteria or their components such as cell walls should only be used once per animal because re-exposure to mycobacteria may

result in severe hypersensitivity reactions. Therefore, FCA is limited used for only the initial immunization. The FCA preparation with a low concentration of mycobacteria (0.1 mg/ml) and limited injection dose (0.1 ml per site) are also recommended to minimize subsequent inflammation (Leenaars and Hendriksen, 2005). Alternative adjuvants such as TiterMax, Ribi, Aluminum salts, Quil A, Adjuvax are optional for minimizing side effects on animals, but the immune responses vary by each adjuvant and immunizing antigens.

Immunization protocol

There are several essential issues to be considered (Leenaars et al., 1999; Leenaars and Hendriksen, 2005), including (1) use of an adjuvant (2) route of injection (3) volume to be injected and (4) the immunization schedule. Suggested injection routes depend on the antigen mixture with or without adjuvants and the species of animals. The intramuscular and subcutaneous are major routes of injection. Antigen can be absorbed by lymphatics in this region, but the mixture of antigen and adjuvant may result in local inflammation. The intramuscular route should be avoided for small rodents such as mice. Intravenous administration should be the route of choice for small particulate antigens, including viruses, bacteria, or cells that do not induce anaphylaxis because of wide distribution and increased capture of the antigen by lymphoid tissues. Booster injections should not be administered by the same route used for the primary immunization, and booster injection sites should be distant from previous injection sites. The injection volume should be as small as possible to avoid the adverse reactions and discomfort to animals. The volumes to be injected are based on the use of an adjuvant, and the inoculum should be spread among multiple injection sites in larger animals. Primary injections with very low amounts of antigen (in picograms) are not recommended, since this does not

stimulate the immunologic memory sufficiently, and might induce tolerance to the antigen. The immunization schedule has an influence on the result of the immunization. The time interval between each immunization can affect both the induction of B memory cells and the class switch of B cells from IgM to other antibody classes and subclasses. A booster is generally considered after the antibody titer has plateaued or begun to decline. The endpoint of polyclonal antibody production should be judged when the antibody titer has reached an acceptable level. This should usually occur after a maximum of two boosters.

Blood collection

Because a booster injection into an animal that has previously established a memory response will usually induce a high antibody titre, intermittent bleeding of an immunized animal can help maintaining a high serum antibody level and facilitates the collection of adequate amounts of antibodies. Thus, regular interval blood collections should be performed after a sufficient serum antibody titer has been reached. The volume to be removed per bleeding should not exceed 15% of the total blood volume, which in practice, an amount up to 1% of the total body weight can safely be removed. Animals should not be bled more frequently than once every 14 days when maximum blood volumes are collected. A collection method that does not require anesthesia should be preferred when possible. In a species in which blood sampling in conscious animals is not easy for the operator and stressful for the animal, the use of a sedative to facilitate blood sampling is necessary.

4.2.3   Assay Development Procedure

Assay optimization

Optimization is the process of evaluating and adjusting the physical, chemical and biological parameters of an assay to ensure that the performance characteristics of the assay are best for the intended application. The optimizing process is fundamental and critical to achieve a good quality assay, particularly if the assay is for routine diagnostic use in multiple laboratories. The capture and detection antibodies should be titered in order to select the concentration of each antibody that provides the most excellent results for the assay.

Assay validation

Assay validation is usually performed after the assay has been optimized. This is the process that determines the fitness of an assay for an intended purpose and the performance characteristics of the assay (Jacobson, 1996; OIE, 2010). A number of reference samples are used. Selection, collection, preparation and management of reference samples are essential for the validation. The steps of the assay validation include estimates of the analytical performance characteristics, which are primarily consist of analytical specificity, analytical sensitivity, linear operating range, and repeatability. Analytical specificity is important to determine the ability of the assay to distinguish between the target analyte and other components that may be potentially cross-reactive to antibodies. Analytical sensitivity, synonymous with the lower limit of detection of an analyte, is also required to determine the smallest amount of analyte in a sample that can be detected and distinguished from the result of a matrix background. The preliminary estimate of repeatability is necessary to warrant the precision for further assay development. Two types of precision should be considered, intra-assay and inter-assay repeatability. Intra-assay repeatability

is the reproducibility between wells within an assay. This allows the researcher to run multiple replicates of the same sample on one plate and obtain similar results. Inter-assay repeatability is the reproducibility between assays. Inter-assay precision guarantees that the results obtained will be reproducible over a period of time.

The next step of assay validation is to evaluate the diagnostic performance characteristics which primarily are the establishment of diagnostic sensitivity and diagnostic specificity. Diagnostic sensitivity is the proportion of samples from known infected reference animals that test positive in an assay. Diagnostic specificity is the proportion of samples from known uninfected reference animals that test negative. These estimates are the basis for calculation of other parameters from which inference is made regarding test results such as predictive values. Therefore, it is important that estimate of diagnostic sensitivity and diagnostic specificity are as accurate as possible. These parameters are derived from testing a panel of samples from reference animals of known history and infection status relative to the disease or infection in question, and relevant to the region in which the test is to be used.

4.3 Materials and Methods

4.3.1 Buffers and Reagents

Complete and Incomplete Freund's Adjuvant (Sigma-Aldrich, St. Louis, MO) were used in immunization. The antigen coating buffers assessed in the AC-ELISA were phosphate-buffered saline (PBS), pH 9.0 and carbonate buffer, pH 9.4. The solutions tested for blocking the plate consisted of 2 and 4% bovine serum albumin (BSA) (A-7030; Sigma-Aldrich, St. Louis, MO) and 0.5, 3, and 5% non-fat dry milk (NFDM) (BioRad, Hercules, CA) in coating buffer. The washing buffer was PBS (pH 9) containing 0.05% Tween 20 (Fisher Scientific, Pittsburgh,

PA). The substrate was 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard and Perry Laboratories, Gaithersburg, MD). In addition, 0.18 M sulfuric acid was used for stopping the reaction. The reagents used in SDS-PAGE included Laemmli sample buffer, β-mercaptoethanol, 10X Tris/Glycine/SDS buffer, and Bio-Safe Coomassie Stain (Biorad, Hercules, CA).

4.3.2 Production of Rabbit Polyclonal Antibodies

Rabbits

Two, 4-kg, female New Zealand White rabbits were used for immunization. The rabbits were housed in individual cages and maintained at the Painter Center at Colorado State University. The immunization protocol was approved by the Institutional Animal Care and Use Committee (IACUC). The rabbits were acclimatized for 2 weeks before immunization.

Preparation of B. henselae antigens

Bartonella henselae strain CSU-1 was used for immunizing the rabbits. The bacteria were previously isolated from blood samples of experimentally infected cats. Positive colonies of *B. henselae* were collected into sterile PBS, and stored at -80 °C until used. After being thawed, bacterial cells were disrupted by 30% pulsed sonication for 10 min. The tube was placed in ice water during sonication. The sonicate suspension was centrifuged at 10,000 g for 10 min. The supernatant was discarded, and the pellet was resuspended in sterile deionized water. After vortexing, an aliquot of the crude antigen suspension was removed for measurement of total protein concentration. The rest of the crude antigen suspension was then diluted to an anticipated concentration, aliquoted into single-dose tubes, and stored at -80 °C.

Immunization protocol

Each rabbit was immunized with 100 µg of crude antigen for each injection. An equal volume (0.5 ml each) of the antigen suspension and adjuvant was mixed vigorously until the water-in-oil emulsion was formed. The mixture was immediately administered to the rabbits. Freund's Complete Adjuvant was used in the initial immunization to enhance antibody responses. Thereafter, Freund's Incomplete Adjuvant was used in booster injections. Each rabbit was inoculated subcutaneously in multiple sites with a total of 1 ml of antigen/adjuvant mixture at week 0. Two booster immunizations with the same quantity of antigens were injected to the rabbits by the intramuscular route at weeks 2 and 4. Details of dose, route, quantity, and frequency of immunization are displayed in Table 4-1. The rabbits were adopted in the end of the immunization procedure.

Table 4-1   Immunization protocol of rabbit polyclonal antibody production. Each rabbit was inoculated subcutaneously in multiple sites with a total of 1 ml of antigen/FCA mixture at week 0 of immunization. An equal amount of antigens mixed with FIA was injected to the rabbits by the intramuscular route at weeks 2 and 4 of immunization. A small volume of blood (2 ml) was collected periodically for determination of antisera after immunization. Antisera were collected at week 8, 12, 16, 20 of immunization

Week	Immunization/booster	Dose	Route	Blood volume	Antibody testing
0	Initial immunization (antigen/FCA)	0.1 mLs, 8-10 sites	S.C.	2 ml	AGID
2	First booster (antigen/FIA)	0.5 mLs, 2 sites	I.M.	2 ml	AGID
4	Second booster (antigen/FIA)	0.5 mLs, 2 sites	I.M.	2 ml	AGID
6	NA			2 ml	AGID
8	NA			40-60 ml	AGID
12	NA			40-60 ml	AGID
16	NA			40-60 ml	AGID
20	NA			40-60 ml	AGID

FCA; Freund's Completed Adjuvant, FIA; Freund's Incompleted Adjuvant, AGID; agar gel immunodiffusion, NA; not applicable

Blood collection and processing

Two milliliter of whole blood was collected from each rabbit by lateral ear venipuncture every other week after immunization. Serum was separated by centrifugation at 1,500 g for 20 min, and evaluated for antibodies to *B. henselae* by agar gel immunodiffusion (AGID) test. An approximate 40-60 ml of blood (1% of body weight) was collected from each rabbit at week 8, 12, 16, and 20 of immunization. Serum samples were separated by centrifugation as described above and stored at -20 °C for subsequent processes.

Agar gel immunodiffusion test

Polyclonal antibodies against *B. henselae* were monitored every 2 weeks (from week 0 to 12), followed by once a month (week 16 and 20) with an AGID test following the procedure of the previous studies (Lappin et al., 1989; Simard et al., 2000). The *B. henselae* antigens, positive and negative control sera, and test sera were filled into each individual well on a gel plate. The plate was incubated in a humid chamber at room temperature, and was examined at 24 and 48 h of incubation. A test serum was considered positive if there was an appearance of clearly definable white precipitation line where serum and antigens met each other. Absence of any precipitation lines was recorded as a negative test result.

Polyclonal IgG purification

Total IgG was purified from rabbit serum samples by a commercially available kit (Melon Gel IgG Purification Kit; Thermo Fisher Scientific, Rockford, IL). The procedure was performed following the manufacturer's instructions. The purified IgG solutions of two rabbits were pooled, aliquoted, and stored at -20 °C until used. The pooling of antibody ensured

consistency in reagent quality, and reduced variability between assays over time. The protein concentration of the pooled IgG solution was assessed by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The purity of IgG preparations was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (Laemmli, 1970). The IgG sample was diluted in the ratio 1:1 in running buffer. The diluted sample was mixed with an equal volume of solubilizing solution (Laemmli sample buffer) containing 5% β-mercaptoethanol, and the mixture was subsequently boiled for 5 min. After protein denaturation, samples were loaded onto 12.5% polyacrylamide gel (Criterion Tris-HCl gel, Bio Rad, Hercules, CA), and electrophoresed in 1X Tris/Glycine/SDS buffer at 180 V for 1 h and 20 min. The gel was stained overnight with Coomassie brilliant blue (Bio-Safe Coommassie) on a rocking shaker. The gel was destained in distilled water to remove the excessive stain, and visualized with epi-white light illumination by ChemiDoc MP system (Biorad, Hercules, CA). Molecular weight of IgG was compared with a protein ladder (Precision Plus Protein Dual Color Standards, Bio Rad, Hercules, CA).

Conjugation of IgG with peroxidase

Fractions of pooled IgG were conjugated with horseradish peroxidase (HRP) by a commercially available kit (EZ-Link Plus Activated Peroxidase Kit; Thermo Fisher Scientific, Rockford, IL), following the manufacturer's instructions. The conjugate was preserved in 50% glycerol solution, and stored at -20 °C until assayed.

4.3.3 AC-ELISA Procedure

A microtiter plate was coated overnight at 4 °C with 100 µl of rabbit anti-*B. henselae* IgG diluted in coating buffer. The next day, the coating IgG was discarded, and the plate was washed for 4 times with 200 µl of PBS containing 0.05% Tween 20 (PBS-TW). Unoccupied space in wells of the plate was blocked with a blocking reagent for 1 h at 37 °C, followed by washing. Samples were applied to the plate in duplicates, and the plate was incubated at 37 °C for 30 min. After washing, 100 µl of rabbit HRP-conjugated IgG was added. The plate was incubated again at 37 °C for 30 min before the excessive conjugate was removed by washings. The colorimetric reaction was developed by incubation with 100 µl of TMB substrate for 10 min at room temperature. The reaction was stopped with 100 µl of sulfuric acid solution. The optical density (OD) values were read at 450 nm wavelength by a microplate reader. Blank and substrate controls were included in each plate to determine any signal generated from non-binding of antigens and antibodies. The AC-ELISA was illustrated in Figure 4.1.
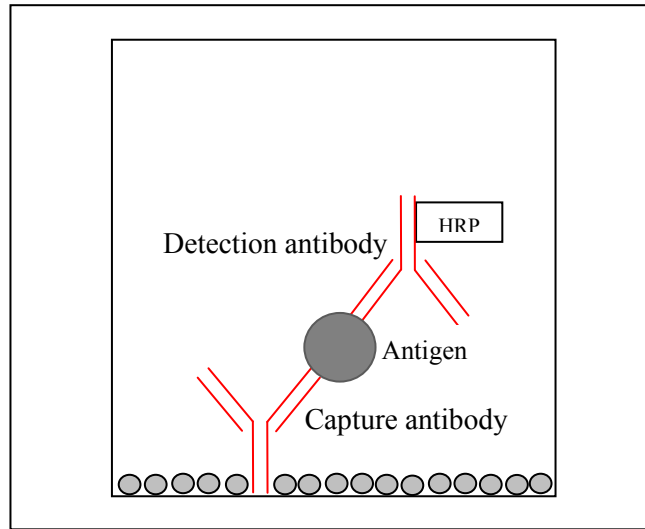
Figure 4-1. AC-ELISA procedure. A microtiter plate is coated with the rabbit anti-*B. henselae* IgG at 4 °C overnight. After 4 times of washing with PBS-TW, the plate is blocked with a blocking reagent for 1 h at 37 °C, followed by washing. Samples are pipetted into the plate in duplicates, and the plate is incubated at 37 °C for 30 min. After washing, the rabbit HRP-conjugated IgG is added. The plate is further incubated at 37 °C for 30 min before it is washed. The TMB substrate is added, and the plated is incubated at room temperature for 10 min. The reaction is stopped with sulfuric acid solution. The OD values are read at 450 nm wavelength by a microplate reader.

4.3.4   Assay Optimization

Titration of capture antibody and conjugated antibody

Different dilutions of rabbit IgG from 1:20 to 1:160 (corresponding to IgG concentrations of 100, 50, 25, and 12.5 µg/ml, respectively) were titrated against 3 different dilutions from 1:20, 1:40 and 1:80 of rabbit conjugated IgG (corresponding to IgG concentrations of 40, 20, and 10 µg/ml, respectively) on the same ELISA plate. The titrated plate layout is shown in Figure 4-2. *B. henselae* antigen 20 ng/µl was applied to each sample well of all combinations. The optimal dilutions of capture IgG and conjugated IgG were determined by the well that gave the highest signal-to-noise ratio (high OD values in sample well, and low background in blank well).

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Figure 4-2. The microtiter plate layout for titration of capture antibody and conjugated antibody. The rabbit IgG was diluted to 1:20 in row A, and 2-fold dilution was made from row B to D. After overnight incubation at 4 °C, the plate was washed and the equal amount of *B. henselae* antigen was added into each sample well. After 30 incubation at 37 °C and washing, the HRP-conjugated rabbit IgG at the dilution 1:20, 1:40, and 1:80 was added into the column 2-3, 4-5, and 6-7, respectively. After incubation and washing, the substrate was added, and then the reaction was stopped. SC, substrate control; B, blank well; dark circle, sample well.

Microtiter plate

Immulon 1B and 2HB (Thermo Scientific, Rochester, NY) were used to compare the optimal plate for the assay.

Coating buffer

Carbonate buffer (pH 9.4) and PBS buffer (pH 9.0) were compared to determine the appropriate coating buffer.

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Blocking reagent

BSA (2 and 4%) and NFDM (0.5, 3, and 5%) in PBS were used to determine the appropriate blocking reagent for the assay.

4.3.5 Sample Preparation

Step 1: Lysis of red blood cells

Red blood cells were lyzed to maximize the release of *B. henselae* into blood sample. Deionized water and RPMI 1640 medium (Gibco-Invitrogen, Grand Island, NY) were compared to determine the proper lysis reagent. A whole blood sample was lysed with 20 volume of a lysis reagent. The tube was vortexed for 10 s and left to stand for 10 min before assayed.

Step 2: Removal of non-relevant proteins from hemolysate samples

Non-specific binding between antibodies and blood components in negative samples was observed. Hemolysate samples were therefore subsequently processed in different ways in order to evaluate the best method that can reduce as many non-relevant proteins as possible and result in high signal-to-noise ratio. Three different methods were determined.

a) *Dilution*: Hemolysate samples were serially diluted 2-fold from 1:10 to 1:80 with PBS-TW before they were assayed.

b) *Centrifugation*: The hemolysate was centrifuged at 10,000 *g* for 10 min. The supernatant was discarded, and the pellet was resuspended with PBS-TW before assayed.

c) *Filtration*: Hemolysate was loaded into a centrifugal filter unit with 5-µm pore size (Ultrafree-MC, Millipore, Billerica, MA). The tube was centrifuged at 10,000 *g* for 10 min to remove cell debris and large molecular particles. The filtrate was gently pipetted out and

discarded while precipitate was left at the bottom of the tube. The precipitate was resuspended with PBS-TW before applied to the plate.

4.3.6 Assay Validation

Calculation of the positive cut-off value

Negative blood samples from 10 known uninfected cats were assayed with the AC-ELISA to calculate a cutoff, which was calculated from average OD value of the negative samples plus 3 times of standard deviations (SD). Samples with absorbance above this value were considered positive.

Determination of analytical sensitivity

A known concentration of *B. henselae* antigen was spiked into a negative blood sample, and serial 2-fold dilutions ranging from 20 to 0.1325 ng/µl was made. Standard curves were generated from these serial dilutions after they were assayed. The concentrations of *B. henselae* antigen (ng/µl) were plotted on the *x* axis and the corresponding absorbance was plotted on the *y* axis. The limit of detection (LOD) was determined by the lowest concentration of *B. henselae* antigen within the linear operating range that was detected by the assay.

Determination of analytical specificity

The specificity was determined using blood samples positive for organisms that maybe potentially cross-reactive with *B. henselae* antigens such as *B. clarridgeiae* and *B. koehlerae*, or those that are causative of bacteremia or as similar clinical illnesses as *B. henselae* infection (i.e. *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum'), or cultivated organisms

that are possibly contaminated in feline blood samples (i.e. *E. coli*, *Enterobacter* spp., *Salmonella* spp., *Klebsiella pneumopniae*, *Streptococcus* spp., *Staphylococcus intermedius*, *Pseudomonas aeroginosa*, and *Enterococcus* spp.). The cultivated organism spiked in a negative blood sample was used when infective blood samples of particular organisms were not available.

4.4 Results

4.4.1 Rabbit Polyclonal Antibodies

There was no evidence of adverse reaction in both rabbits during immunization period. Both rabbits produced polyclonal antibodies against *B. henselae* strain CSU-1 as determined by the AGID test (Figure 4-3). The AGID test was negative in both rabbits on week 0 and 2. Serum of the rabbit A was found positive in AGID test at week 4, 6, 8, 10, and 12 (duration 8 weeks). Weak positive reaction was observed at week 4, and test results were found strong positive in the following weeks. The rabbit B was positive by AGID test at week 6, 8, 10, 12, 16, and 20 (duration 14 weeks). AGID test results were strongly positive from week 8 to 16, followed by weak positive reaction on week 20.

The SDS-PAGE analysis (Figure 4-4) has shown that many non-relevant proteins were eliminated from rabbit antisera after they were purified using the purification kit (Melon Gel). Typically, rabbit IgG molecules were degraded into IgG heavy chain and IgG light chain in the SDS-PAGE analysis. The distinct bands which corresponded to protein with molecular weight of 50 kDa were IgG heavy chains, and the diffused bands corresponding to protein with molecular weight of 25 kDa were IgG light chains. Purified total IgG samples of rabbit A (week 8 and 12) and rabbit B (week 8, 12, or 16) clearly presented the bands of proteins of IgG heavy and light chains (Figure 4-4).
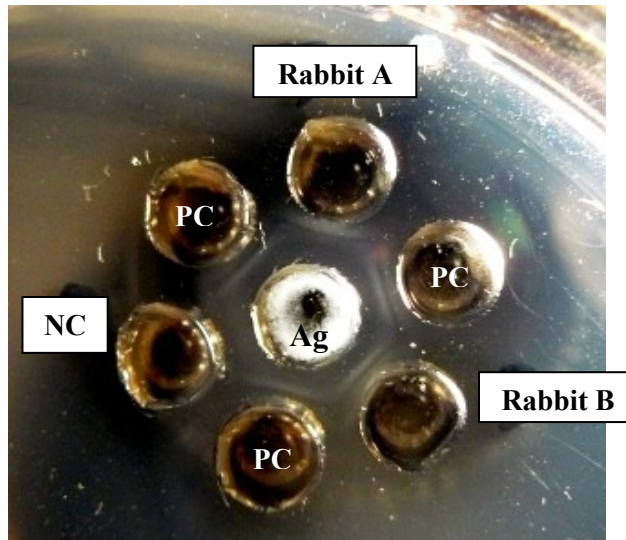
Figure 4-3. AGID test for *B. henselae* antibodies in rabbit sera. A serum sample was considered positive when a white precipitation line appeared between test serum and *B. henselae* antigens. Sera of rabbit A and B were positive for *B. henselae* antibodies, as well as positive controls. Absence of any precipitation lines was recorded as a negative test result, as shown on negative control. Ag, *B. henselae* strain CSU-1 antigens; PC, positive serum control; NC, negative serum control.
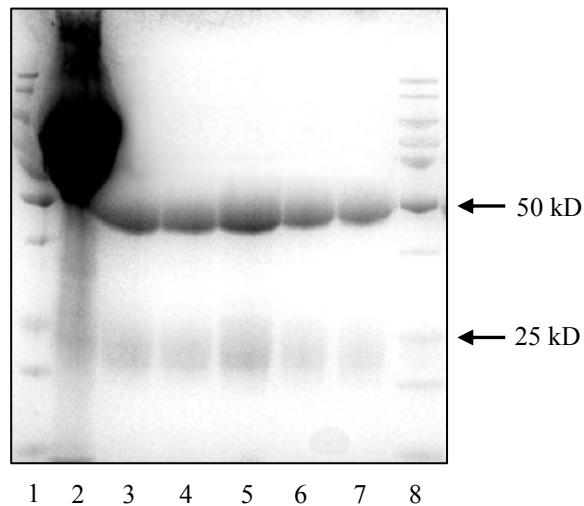
Figure 4-4. Analysis of rabbit purified total IgG by SDS-PAGE. Protein molecular weight marker is shown in lane 1 and 8. The bands corresponding to the molecular weight of 50 kDa, and 25 kDa were IgG heavy chain and light chain, respectively. Lane 2: unpurified serum (control); lane 3-4: IgG fractions of rabbit A from week 8 and 12; lane 5-7: IgG fractions of rabbit B from week 8, 12, 16, respectively.

The pooled IgG was made from the IgG fractions of rabbit A and rabbit B. The concentration of the pooled IgG was 2 mg/ml. A fraction of IgG pool was used for conjugation with HRP, and the final concentration of conjugated IgG was 0.8 mg/ml.

4.4.2 Optimized Assay

A plateau of signal was obtained between the dilution of 1:20 and 1:160 of capture IgG (Figure 4-5). The similar pattern of saturating level was seen in all dilutions of conjugate. The results demonstrated that the capture IgG was saturated on a microtiter plate when the dilution was at least 1:160, and any dilution of capture IgG within this range should generate a similar signal. The 1:20 and 1:40 dilutions of conjugated IgG produced very high OD values at approximately 3.2 and 2.4, respectively. The plateau of OD value generated by the 1:80 dilution of conjugate was about 1.8, which was within an acceptable value. The plate background OD values, developed in wells where there was no antigen, for the 1:20 (mean OD value, 0.6) and 1:40 (mean OD value 0.4) dilutions of conjugated IgG were obviously high, while that of the 1:80 dilution was lower (mean OD value, 0.2). Based on the results the optimized titration for AC-ELISA consisted of capture IgG at 1:80 (25 µg/ml) and conjugated IgG at 1:80 (10 µg/ml).
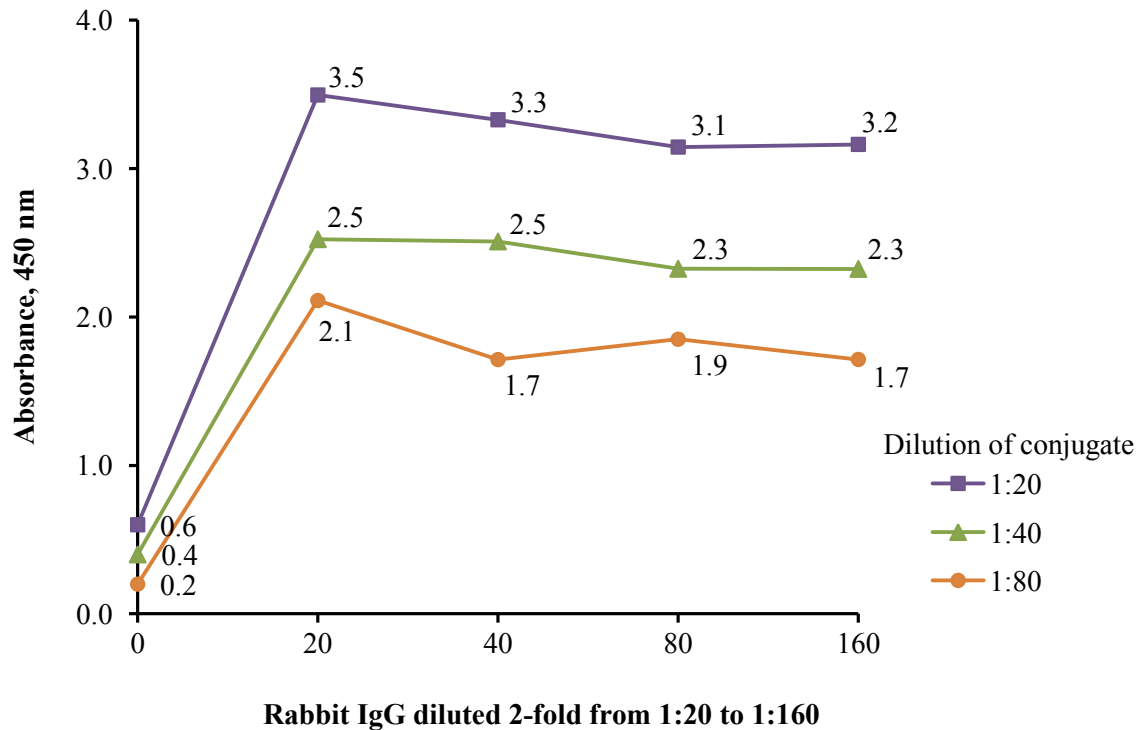
Figure 4-5. Titration of capture and conjugated IgG concentrations. A plate was coated with different dilutions of rabbit IgG at 1:20, 1:40, 1:80, and 1:160. Three different dilutions of rabbit conjugated IgG at 1:20, 1:40, and 1:80 were used as detection antibody.

Comparison of 2 types of microtiter plates revealed that Immulon 2 HB plate provided higher overall absorbance than that of Immulon 1B plate (Figure 4-6). This result confirmed higher binding of capture IgG on Immulon 2HB plate. Using PBS as the coating buffer was likely to provide slightly higher signals on Immulon 2HB than carbonate. In contrast, carbonate gave slightly higher signals than PBS on Immulon 1B plate (Figure 4-6). In addition, PBS generated lower plate background (OD: 0.05 on Immulon 1B; 0.07 on Immulon 2HB) than that of carbonate (OD: 0.07 on Immulon 1B; 0.09 on Immulon 2HB). From the results, PBS was chosen to be used as the coating buffer with Immulon 2HB plate.
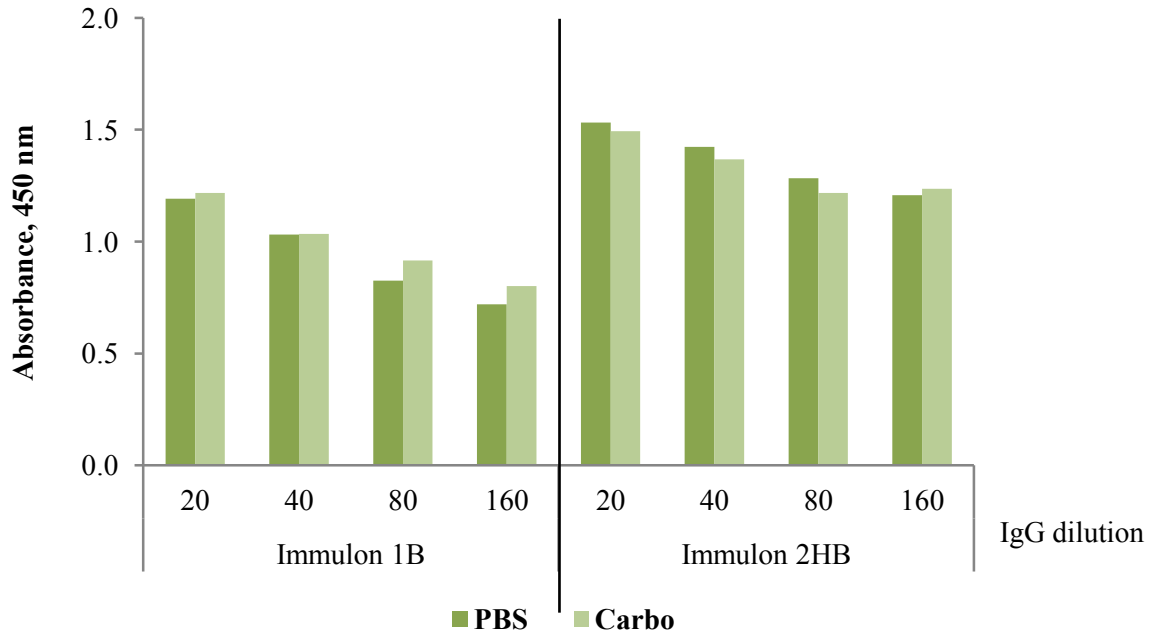
Figure 4-6. Selection of the microtiter plate and coating buffer. The rabbit IgG diluted from 1:20 to 1:160 in either PBS or carbonate buffer was coated on an Immulon 1B plate. The duplicated reaction was performed on an Immulon 2HB. Both plates were subsequently assayed at the same time within the same condition.

Blocking the plate with BSA yielded the plate background OD values ranging from 0.13 - 0.17. However, the plate background OD less than 0.10 was observed when the plate was blocked with NFDM. Among 3 different concentrations of NFDM tested, 0.5% NFDM gave lower OD value about 0.05 - 0.07.

4.4.3 Sample Preparation

Lysis buffer

Lysis of red blood cells with RPMI did not differentiate negative (mean OD, 0.72 ± 0.10) from positive samples (mean OD, 0.65 ± 0.18). The signals were also not different from those generated in controls without lysis (0.57 ± 0.13 for negative samples, and 0.59 ± 0.21 for

positive samples) (Figure 4-7). Using deionized water obviously yielded higher OD value in

positive samples (1.26 $\pm$ 0.25) than that in negative samples (0.96 $\pm$ 0.29). The signals were also

higher than those of RPMI and controls. Deionized water was then selected as lysis buffer.
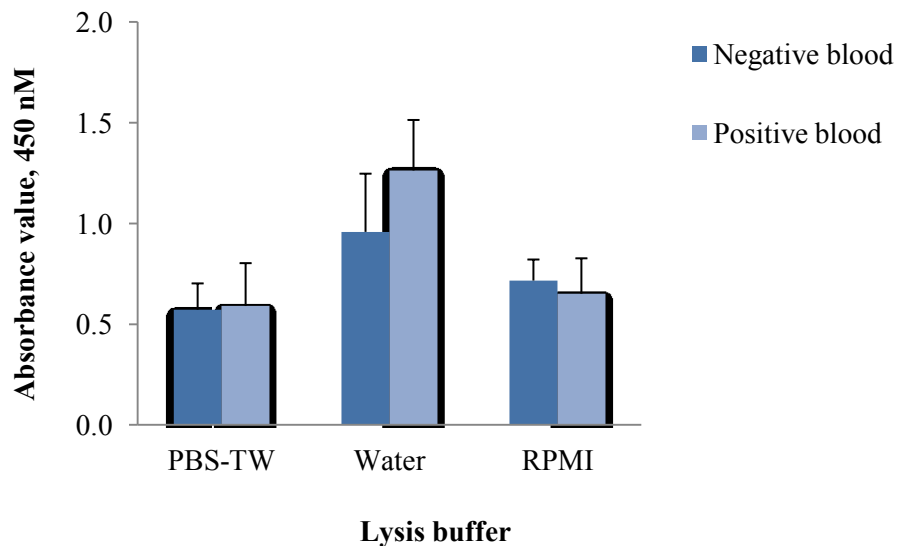
Figure 4-7. Determination of lysis buffer. Positive and negative blood samples were lysed with 20 volume of deionized water and RPMI 1640 medium before they were assayed in AC-ELISA. The results were compared to samples diluted with PBS-TW without hemolysis.

Removal of non-relevant proteins from hemolysate samples

a) Dilution

The absorbance of diluted negative samples were high (OD value, 0.7 - 0.9) for all

dilution levels. The absorbance of positive samples (OD value, 0.9 - 1.2) was not much different

from the negative samples. The results indicated that dilution method did not reduce the effect of

non-specific binding.

b) Centrifugation

The absorbance of negative samples was between 0.3 - 0.5 and that of positive samples was 0.4 - 0.9. Using the centrifugation technique could remove more cross-reactive substances from samples than the dilution method. However, the background signal in negative samples was still unacceptably high.

c) Filtration

The non-specific binding was obviously reduced by the filtration method. The absorbance of negative samples was between 0.2 - 0.4 and that of positive samples was 0.4 - 0.9.

In order to indicate that filtration method was effective for the assay. The samples from 6 experimentally infected cats with known uninfected and infected status were performed. Samples were collected from cats on week 0, 4, 8, 10, and 12 of infection. All samples tested were previously assayed by *Bartonella* specific-cPCR, the qPCR described in Chapter 3, and blood culture.

Results were demonstrated in Table 4-2. The AC-ELISA identified 7 positive samples among the total samples tested (24.1%), culture identified 6 positive samples (20.7%), and both qPCR and cPCR assays identified 12 positive samples (41.4%). Among the 12 samples positive by both PCR assays, 6 were also positive by AC-ELISA. These samples were considered truly positive (sensitivity 50%). The remaining 6 samples negative by AC-ELISA were considered false negative. There were 6 samples positive for both PCR assays and culture. These were also true positives.

For 17 samples found to be negative by both PCR assays, 16 were also negative by AC-ELISA (specificity 94.1%). These samples were considered truly negative. Only 1 sample was

negative by both PCR assays but positive by AC-ELISA. This sample was considered false

positive. None of these 17 samples was positive by culture.

Table 4-2   AC-ELISA results of 6 experimentally infected cats compared with results of qPCR assay, cPCR assay, and blood culture.

Cat	Week	AC-ELISA	qPCR	cPCR	Culture
A	0	-	-	-	-
	4	-	-	-	-
	8	-	-	-	-
	10	-	+ (8)[a]	+	-
	12	+	+ (122)	+	+
B	0	-	-	-	-
	4	-	-	-	-
	8	+	+ (20)	+	-
	10	-	+ (4)	+	-
	12				
C	0	-	-	-	-
	4	-	-	-	-
	8	-	-	-	-
	10	+	+ (40)	+	+
	12	+	+ (35)	+	+
D	0	-	-	-	-
	4	-	-	-	-
	8	+	-	-	-
	10	+	+ (49)	+	-
	12	+	+ (23)	+	+
E	0	-	-	-	-
	4	-	-	-	-
	8	-	+ (10.5)	+	+
	10	-	+ (0.3)	+	+
	12	-	+ (0.4)	+	-
F	0	-	-	-	-
	4	-	-	-	-
	8	-	-	-	-
	10	-	-	-	-

[a] bacterial load (x10^3 CFU/ml)

The results showed that the filtration method could discriminate positive and negative samples better than dilution or centrifugation method. The filtration method was as sensitive as culture method, but less sensitive than PCR assays. The AC-ELISA was less specific than both PCR assays and culture.

4.4.4 *Analytical Performance Characteristics of AC-ELISA*

Cut-off value

A cutoff was assessed from a total of 10 negative blood samples, which OD values ranged from 0.217 - 0.396 (mean $\pm$ SD, 0.292 $\pm$ 0.05). The cutoff was calculated as the average OD value of the selected negative samples plus 3 SDs. This yielded a cutoff value of 0.459.

Analytical sensitivity

A serial dilution of *B. henselae* antigen was used to establish a standard curve (Figure 4-8). Seven doubling dilutions over the range of 0.31 to 20 ng/µl had mean OD values between 0.229 and 1.806. The cutoff was set at 0.459 (from above). Using these criteria, the assay can measure antigens at the range of 1.25 - 20 ng/µl. The result ensures that the amount of *B. henselae* antigen in a sample within this range will be accurately measured. The limit of detection of this assay was 1.25 ng/µl. The linear regression curve was based on OD values between 1.25 and 20 ng/µl; OD = 0.064 (antigen concentration) + 0.619. The standard curve was linear with correlation coefficient (R^2 = 0.9311).
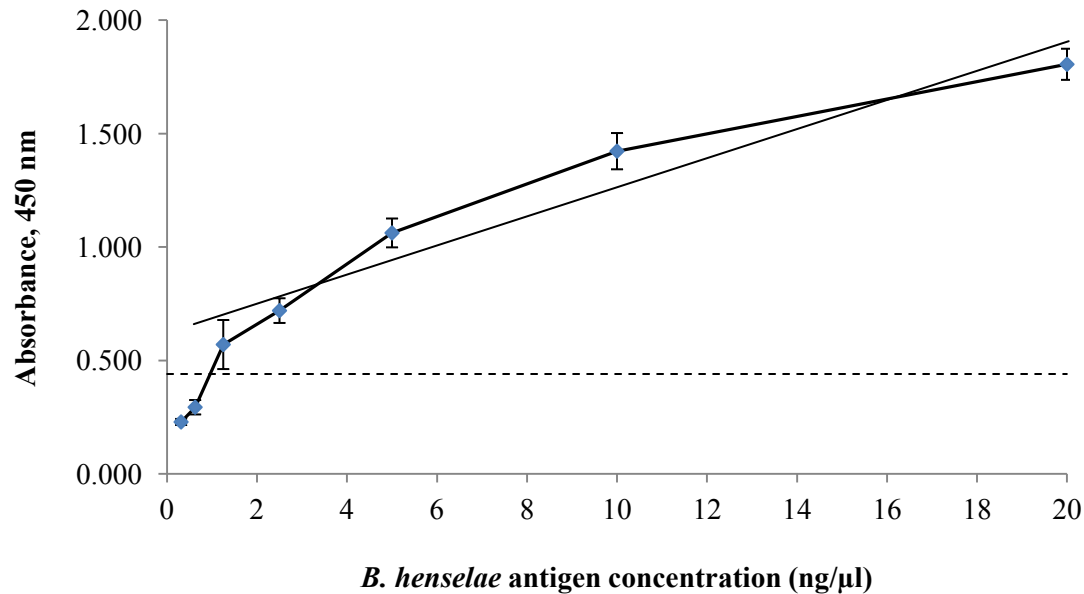
Figure 4-8. Standard curve of for the AC-ELISA. Various concentrations of *B. henselae* antigen over a range between 0.31 and 20 ng/µl were measured from three separate plates run under same conditions. Data points represent the means of OD values, and error bars represent standard deviations. Broken line represents the cutoff (three standard deviations from blank specimen).

Analytical specificity

To determine the analytical specificity, blood samples positive with other *Bartonella* spp. and hemoplasmas, and cultivated organisms of non-related *Bartonella* bacteria were analyzed by the assay. None of these bacteria was positive in the AC-ELISA. These findings indicated that the AC-ELISA would be specific for *B. henselae* and had no cross-reaction with other bacteria.

4.5 Discussion

An ELISA-based assay has been known as a rapid, reliable, easy-to-perform, and inexpensive test. The AC-ELISA described here was developed for the detection of *B. henselae* in blood samples of cats. Because *B. henselae* mostly resides within erythrocytes, evaluation of whole blood is thought to be of higher sensitivity than serum. Polyclonal antibodies are

considered as the choice for this pilot study, due to simplicity, inexpensiveness, and rapidity in antibody production. Additionally, higher sensitivity of the assay may be acquired than using monoclonal antibodies. The rabbits were preferable for antibody production because of the ease of manipulation and the amount of antisera required. This study did not evaluate for predominant antigens of *B. henselae*, therefore the whole-cell antigens were used for immunizing rabbits. Because bacterial cells consist of multivalent antigens, rabbit polyclonal antibodies can be simultaneously used as capture and detection antibodies. Bacterial cells were lysed by sonication before immunizing rabbits for safety and prevention of infection in animals. *B. henselae* strain CSU-1 is the local strain, which was used as the reference strain for antibody production.

Rabbit antisera were screened for antibodies against *B. henselae* by AGID test, but this test cannot evaluate titer of the antibodies. However, the reactivity of antibodies was determined by density of precipitation line in the AGID test. After rabbit antisera were collected, they were purified for total IgG using a commercially available kit, the Melon Gel IgG Purification. The kit provides the affinity method in which IgG can be isolated from serum proteins by negative selection. The gel column contains a proprietary ligand that holds on most non-relevant proteins in serum, while allowing IgG to pass through the column support. Albumin and transferrin are removed by this method. This kit is simple, convenient to perform, and suitable for rabbit serum. Total IgG was highly purified (Figure 4-4). Many proteins were removed from purified IgG solution compared to unpurified serum. Moreover, the reactivity of IgG is stable because elution is not needed.

The AC-ELISA was optimized to ensure high signal, low background, and the highest sensitivity. In this study, the 1:80 dilution (25 µg/ml) of IgG was used for coating the plate, and the 1:80 dilution (10 µg/ml) of conjugated antibody was optimized for detection of antigen-

antibody complex. The selected concentrations in which the capture antibody optimally pairs with the detection antibody would ensure that the assay provides the best signal-to-noise ratio. Several other empirical experiments were performed to optimize the assay. The use of Immulon 2HB plate yielded higher reactivity than Immulon 1B. This is because the hydrophilic surface of Immulon 2HB increases the ability to bind with hydrophilic IgG molecules, while the hydrophobic surface of Immulon 1B primarily binds with hydrophobic molecules. The appropriate coating buffer was determined in this study. The results showed that coating plates with PBS resulted in higher reactivity and lower plate background than carbonate buffer for Immulon 2HB plate. Background caused by non-specific binding of the conjugated antibody to plate can be reduced by using a blocking reagent. Selection of the optimal blocking reagents is empirical for each assay. Protein blockers such as BSA and NFDM are permanent blocker and stabilize surface bound biomolecules. BSA is typically effective on medium and high binding surfaces, as well as many covalent surfaces, but 0.5% NFDM had shown to be stronger blocking reagent than BSA in this study. It is possible that BSA was not optimally functional in the high pH buffer used in the experiments.

Bartonella spp. are intra-erythrocytic organisms. Lysis of red blood cells is capable of releasing organisms and increasing the sensitivity for detection of organisms in samples. Deionized water is hypotonic reagent resulting in hemolysis and leakage of *B. henselae*. In this study, hemolysate could discriminate positive from negative samples, while no difference was found with RPMI-1640 medium. RPMI-1640 medium has been used for the culture of human lymphoid cells for aid in utilizing a bicarbonate buffering system. However, it did not help for lysis of red blood cells in this system.

High background in negative samples was observed from the analysis of hemolysed samples. Negative blood samples which do not have *B. henselae* antigens are supposed to yield low absorbance in the assay. However, OD values of negative blood samples were obviously high (> 0.50). This effect was less likely to result from non-specific binding of conjugated IgG on the plate because the plate background signal was low (< 0.10). There are 2 possibilities that can explain this phenomenon: 1) binding of non-specific IgG and non-relevant antigens in blood samples or 2) cross-reaction between *B. henselae*-specific IgG and non-relevant antigens in blood samples that have as similar epitopes as *B. henselae*. The rabbit IgG used in this study contains *B. henselae*-specific antibodies, and may contain antibodies reactive to impurities in the immunogen, as well as naturally induced antibodies to a variety of non-*B. henselae* antigens from previous exposure. In general, rabbit serum normally contains 5 - 10 mg/ml of total IgG, and only 1 - 2% of the total IgG is specific for the antigen used for immunization to animals. Approximately 50 - 200 μg of specific IgG has been found in 1 ml of serum. Therefore, non-specific antibodies commonly exist in total IgG and might be a primary cause of high non-specific background signal, particularly in samples containing blood or serum. In this study, it is possible that the immunogen may be contaminated with blood components received during bacterial isolate collection from blood agar plate. Contaminants might exist although several washings were applied. Cross-reaction of *B. henselae*-specific IgG and bacteria other than *B. henselae* was determined. The results showed that none of organisms were cross-reactive with the rabbit IgG in the assay. The specificity of rabbit IgG increases the reliability of positive results of the assay.

Non-specific binding of rabbit IgG and non-relevant proteins can affect both analytical specificity and analytical sensitivity of the assay. Analytical specificity would be low because of

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false positives in negative samples that have a lot of non-relevant antigens. In order to increase the analytical specificity, a high cutoff is necessary. Thus, analytical sensitivity will be decreased, and higher proportion of false negative results will be troublesome. The total IgG is usually enough for most assays, but it was not sufficient for this study. In this case, the non-specific antibody binding should be removed by separation of specific antibodies from other non-specific antibodies or using monoclonal antibodies instead. The affinity purification of antibody (Roque et al., 2007) is considered as a method of choice for polyclonal antibody purification in which antiserum contains antibodies with the ultimate in specificity and sensitivity. The affinity purification eliminates all other potentially interfering serum proteins and antibodies that will increase the background and cause cross-reactivity. It leaves behind a population of antibodies that only react with the epitopes chosen for inclusion on the affinity matrix. Therefore, the specific antibody is selected and highly purified while non-specific antibody is discarded. Furthermore, simple sample processing such as hemolysis may only be needed. However, the affinity purification requires a variety of optimization particularly the elution step and a lot of reagents, such as antibody and antigen of interest. Furthermore, antibody reactivity may be lost from elution step in the affinity purification. Modification of the assay by using monoclonal antibody is another option that can reduce the non-specific binding and increase the specificity of the assay. Although polyclonal antibodies have an advantage over monoclonal antibodies for higher sensitivity and versatility; however, in this circumstance, monoclonal antibodies should be the antibody of choice for enhancing the analytical specificity of the assay. Since monoclonal antibodies had distinct advantages over polyclonal antibodies for specific binding to their antigenic determinants.

Due to non-specific binding of the rabbit IgG, subsequent experiments were performed in order to find a method that can remove as many non-relevant molecules as possible while leaving *B. henselae* antigens in samples. Sample processing after lysis of red blood cells, including dilution, centrifugation, or filtration method was performed. The dilution method was not able to decrease the reactivity of non-specific binding. The centrifugation method removed some non-relevant molecules. However, the filtration method of the hemolysate using centrifugal filter unit with a pore size of 5 μm removed more cross-reactive molecules than the centrifugation method, and the signal of negative samples were within an acceptable level. The size of *B. henselae* is 0.3-0.6 x 0.3-1.0 μm (Stramer et al., 2009). Bacterial cells are therefore easily passed through the filter, while molecules bigger than 5 μm were separated out of samples. Molecules in the supernatant which are smaller than bacterial cells are further removed from samples; thus, only molecules that have similar size to *B. henselae* were co-precipitated in the sample to be assayed.

Of 29 known positive and negative samples with PCR assays and blood culture, discordant results of AC-ELISA were found in 20.7% of all samples. Because culture is not practical in many laboratories, and PCR assay has been known as highly sensitive and specific technique. PCR assay was then used for comparison of results of the AC-ELISA. However, viability of bacteria cannot be determined by either PCR assay or AC-ELISA. The samples positive with PCR are thought to be truly positive, as well as samples negative with PCR are thought to be truly negative. The AC-ELISA had a false negative rate of 50% and a false positive rate of 5.9% of when compared to results of PCR assays (Table 4-2). These findings on small number of samples shows that the AC-ELISA has lower diagnostic sensitivity and specificity than the PCR assays. False positivity might be caused by non-specific binding of rabbit IgG and

non-relevant proteins remaining in the processed samples rather than the cross-reactivity with other organisms.

The AC-ELISA was shown to have lower diagnostic sensitivity than the PCR assays. These findings are expected because a high cutoff was selected. Samples that have very low levels of *B. henselae* are detected but the signals are not high enough to be positive. However, other possibilities may result from low analytical sensitivity of the AC-ELISA itself. Low analytical sensitivity may be caused by insufficient optimization of the assay or low avidity of antibodies. In addition, false negativity may be caused by interference of the antigen-antibody interaction by sample matrix, or quality of sample tested because of degradation of antigen during storage. The capture and detection antibodies were already optimized, and the acceptance results were obtained. Therefore, the results highly suggest that low avidity of rabbit IgG for binding with *B. henselae* antigens exist and may be an explanation for the low analytical sensitivity of the assay. If this circumstance is true, substitution with other antibodies will be the solution. The sample matrix probably prevents antibodies for recognizing its designated analyte or the antigen of interest. The antibody is then masked by binding to some matrix component. Antigen degradation might also be possible due to multiple freeze-thaw cycles.

A limitation of the AC-ELISA is the availability of antibodies. Polyclonal antibodies were obtained from only 2 rabbits, and titer of them was unknown. Thus, only one pair is available for the assay. Because the reactivity of antibodies from both rabbits was observed to be similar in the AGID test, they were pooled and the pooled antibodies were used. This study showed that the pooled antibodies may have low avidity causing low sensitivity of the assay. It is suggested that different sources of antibodies should be obtained in order to find a perfect match

of antibodies. At least 3 rabbits should be immunized, and 3 matches of these antibodies would be compared.

The AC-ELISA described here was optimized and initially validated for detection of *B. henselae* in blood samples of cats. The major advantage of the AC-ELISA is the ability to obtain quick results because it is easy-to-perform and requires only simple laboratory facilities. The weakness of the assay is the non-specific binding of IgG that causes high background in negative blood samples. Therefore, it took a lot of time and creativity until the most acceptable performance characteristics of the assay were achieved. However, the accuracy of the AC-ELISA was not acceptable when compared with the PCR assays. Because of low analytical sensitivity of the AC-ELISA, truly infected cats cannot be detected by this assay, and the AC-ELISA should not be used alone for screening of *B. henselae* infection. It is highly suggested that the AC-ELISA be improved by substitution of the current antibodies with another perfect match pair of antibodies, or development of a monoclonal-based ELISA for ultimate achievement of the assay.

4.6 Acknowledgements

References

Brunt, J., Guptill, L., Kordick, D.L., Kudrak, S., Lappin, M.R., 2006. American Association of Feline Practitioners 2006 Panel report on diagnosis, treatment, and prevention of *Bartonella* spp. infections. J. Feline Med. Surg. 8, 213-226.

Dehio, C., 2001. *Bartonella* interactions with endothelial cells and erythrocytes. Trends Microbiol. 9, 279-285.

Jacobson, R.H., 1996, Principles of validation of diagnostic assays for infectious diseases. In: Manual of standards for diagnostic tests and vaccines, 3rd ed. Office International de Epizooties, Paris, pp. 8-15.

Koesling, J., Aebischer, T., Falch, C., Schülein, R., Dehio, C., 2001. Cutting edge: antibody-mediated cessation of hemotropic infection by the intraerythrocytic mouse pathogen *Bartonella grahamii*. J. Immunol. 167, 11-14.

Kordick, D.L., Wilson, K.H., Sexton, D.J., Hadfield, T.L., Berkhoff, H.A., Breitschwerdt, E.B., 1995. Prolonged *Bartonella* bacteremia in cats associated with cat-scratch disease patients. J. Clin. Microbiol. 33, 3245-3251.

Laemmli, U.K., 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227, 680-685.

Lappin, M.R., Greene, C.E., Prestwood, A.K., Dawe, D.L., Tarleton, R.L., 1989. Enzyme-linked immunosorbent assay for the detection of circulating antigens of *Toxoplasma gondii* in the serum of cats. Am. J. Vet. Res. 50, 1586-1590.

Leenaars, M., Hendriksen, C.F.M., 2005. Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. ILAR Journal 46, 269-279.

Leenaars, P., Hendriksen, C.F.M., De Leeuw, W.A., Carat, F., Delahaut, P., Fischer, R., Halder, M., Hanly, W.C., Hartinger, J., Hau, J., 1999. The production of polyclonal antibodies in laboratory animals. Altern. Lab. Anim. 27, 79-102.

OIE 2010. OIE Terrestrial Manual 2010. In Principles and methods of validation of diagnostic assays for infectious diseases (The World Organisation for Animal Health), pp. 1-18.

Paulie, S., Perlmann, H., Perlmann, P., 2006, Enzyme-linked Immunosorbent Assay, eLS. DOI: 10.1038/npg.els.0004021, 1-4.

Roque, A.C., Silva, C.S., Taipa, M.A., 2007. Affinity-based methodologies and ligands for antibody purification: Advances and perspectives. J. Chromatogr. A. 1160, 44-55.

Simard, C., Richardson, S., Dixon, P., Komal, J., 2000. Agar gel immunodiffusion test for the detection of bovine leukemia virus antibodies: lack of trans-Atlantic standardization. Can. J. Vet. Res. 64, 96-100.

Stills, H.F., 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J. 46, 280-293.

Stramer, S.L., Hollinger, F.B., Katz, L.M., Kleinman, S., Metzel, P.S., Gregory, K.R., Dodd, R.Y., 2009. Emerging infectious disease agents and their potential threat to transfusion safety (Appendix 2). Transfusion. 49, 45S-233S.

CHAPTER 5

Prevalence of *Bartonella* Species, Hemoplasmas, and *Rickettsia felis* DNA in Blood and Fleas of Cats in Bangkok, Thailand

5.1 Summary

Flea infestations are common in Thailand, but little is known about the flea-borne infections. Fifty flea pools and 153 blood samples were collected from client-owned cats between June and August 2009 from veterinary hospitals in Bangkok, Thailand. Total DNA was extracted from all samples, and then assessed by conventional PCR assays. The prevalence rates of *Bartonella* spp. in blood and flea samples were 17% and 32%, respectively, with DNA of *Bartonella henselae* and *Bartonella clarridgeiae* being amplified most commonly. *Bartonella koehlerae* DNA was amplified for the first time in Thailand. Hemoplasma DNA was amplified from 23% and 34% of blood samples and flea pools, respectively, with '*Candidatus* Mycoplasma haemominutum' and *Mycoplasma haemofelis* being detected most frequently. All samples were negative for *Rickettsia felis*. Prevalence rate of *B. henselae* DNA was increased 6.9 times in cats with flea infestation. Cats administered flea control products were 4.2 times less likely to be *Bartonella*-infected.

5.2 Introduction

Ctenocephalides felis (*C. felis*) has been recognized as the vector of several infectious agents that cause disease in cats and humans. The most common pathogens detected in *C. felis* include *Bartonella* spp., hemoplasmas, and *Rickettsia felis*. *Bartonella henselae* and *Bartonella clarridgeiae* are associated with cat-scratch disease and other syndromes in humans and are thought to be the most common species of *Bartonella* in cats (Breitschwerdt et al., 2010). More recently, *Bartonella koehlerae* has been amplified from cats and humans but information concerning its prevalence in cats or fleas is minimal (Droz et al., 1999; Avidor et al., 2004). Most cats with natural *B. henselae* infection are clinically normal. However, clinical findings such as fever, lymphadenopathy, endocarditis, myocarditis, gingivitis, stomatitis, and uveitis have been reported in some cats (Fontenelle et al., 2008; Quimby et al., 2008; Lappin et al., 2009; Perez et al., 2009; Bradbury and Lappin, 2010).

Mycoplasma haemofelis, '*Candidatus* Mycoplasma haemominutum', and '*Candidatus* Mycoplasma turicensis' are three hemoplasma species that can reside on the surface of feline erythrocytes. Some cats with hemoplasma infections develop hemolytic anemia (Sykes et al., 2007), with *M. haemofelis* appearing to be the most pathogenic (Foley et al., 1998). '*Candidatus* M. haemominutum' can be pathogenic in some cats, particularly when co-infections with feline leukemia virus occur (Reynolds and Lappin, 2007). '*Candidatus* M. turicensis' was originally identified in the blood of a Swiss cat with anemia, and has recently been amplified from the blood of client-owned cats in other countries. It has been proven experimentally to induce anemia in some cats (Willi et al., 2005).

R. felis was identified in *C. felis* for the first time in 1990, and it is closely related to the spotted fever group. People infected with *R. felis* may develop signs of fever, headache, myalgia,

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and macular rash. Recently, it was suspected that an Australian family had an illness due to *R. felis* infection after contact with their cats (Williams et al., 2011). However whether clinical illness is associated with natural infection with *R. felis* in cats is unclear (Bayliss et al., 2009).

Since *C. felis* serves as a potential vector of many pathogens, the number of prevalence studies in cats and fleas have been increasing worldwide. The prevalence rates can differ among cat populations and geographical locations. Thailand is a tropical country where *C. felis* infestations are common in cats. Although cats are likely to be exposed to fleas and these flea-borne pathogens, only a few prevalence studies have been reported to date (Maruyama et al., 2001; Parola et al., 2003 and Inoue et al., 2009) and to our knowledge, hemoplasmas have never been reported in Thailand. To provide further information concerning these organisms among cats living in this area, the objective of this study was to determine the prevalence of *Bartonella* spp., hemoplasmas, and *R. felis* in client-owned cats and fleas in Bangkok, Thailand.

5.3 Materials and Methods

5.3.1 *Cats and Fleas*

This study was approved by the Institutional Animal Care and Use Committee at Colorado State University and the owner of each cat gave permission to use the samples. Samples were obtained from client-owned cats that were presented to veterinary hospitals in Bangkok between June and August 2009. Whole blood and serum samples and at least one flea were collected from each cat. Fleas were brushed off each cat and immediately placed into a tube. Questionnaires related to demographic data, body temperature, and health history were completed by the attending veterinarians. All samples were stored at -20 °C and then shipped on cold packs to Colorado State University for assay. The species of fleas were identified by a

veterinarian (SA) using morphology criteria (Menier and Beaucournu, 1998) before deoxyribonucleic acid (DNA) was extracted for analysis in the polymerase chain reaction (PCR) assays.

5.3.2   *DNA Extraction and PCR Assays*

Total DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN). Fleas from each cat were pooled and total DNA extracted as previously described (Shaw et al., 2004). The DNA of blood and pooled-flea samples were assayed in conventional PCR assays for *Bartonella* spp. (Jensen et al., 2000) targeting a fragment of the 16S-23S rRNA intergenic spacer region, hemoplasma spp. (Jensen et al., 2001) targeting a fragment of 16S rRNA gene, and *Rickettsia* spp. (Shaw et al., 2004) that amplified a fragment of *gltA* gene. Samples with a band of 170 base pairs (bp) were differentiated for *M. haemofelis* and 'Candidatus M. turicensis' by DNA sequencing. The 161-bp PCR products for *B. henselae* and *B. koehlerae* were differentiated by assaying the DNA extracts by a *B. koehlerae* specific PCR assay (unpublished data) and confirmed by DNA sequencing (Macromolecular Resources, Colorado State University, Fort Collins, CO). Because *R. felis* IFA slides to use with serology are not commercially available in the USA, PCR alone was used in this study. The amplified products were identified by electrophoresis on agarose gels as described for each assay.

5.3.3   *Bartonella spp. IgG Enzyme-linked Immunosorbent Assay*

Bartonella spp. IgG was measured in sera using an indirect ELISA as previously described (Lappin et al., 2009). Briefly, 100 µl of a 1:64 dilution of each serum sample, a positive serum control, and a negative serum control were pipetted into quadruplicate wells of a

microtitre plate coated with *B. henselae* antigen. Substrate and enzyme control wells were included on each plate. The plates were incubated for 30 min at 37 °C, and subsequently washed three times with 200 µl of PBS solution containing 0.05% Tween-20. One hundred milli liter of a 1:3000 dilution of peroxidase-labeled goat anti-cat IgG (gamma) (Kirkegaard and Perry Laboratories) in PBS-Tween solution was pipetted into appropriate wells. The plate was incubated for 30 min at 37 °C and after another wash step, 100 µl of substrate (TMB; SureBlue Reserve TMB Substrate, Kirkegaard and Perry Laboratories) was pipetted into each well. The enzyme reaction was stopped after 10 min. The optical density of each well was read at 450 nm with an automated micro-ELISA reader. A sample was considered positive for *B. henselae* IgG if the mean OD value was greater than the mean OD value plus 3SD of negative samples at the titer ≥ 64. Titers were estimated by comparison to a standard curve generated from the positive and negative control sample results.

5.3.4   *Statistical Analysis*

Associations between PCR results and risk factors were assessed with Pearson's chi-square or Fisher's exact test. Significance was defined as $P < 0.05$.

5.4 Results

5.4.1   *Client-Owned Cats*

A total of 153 blood and serum samples were collected from cats at various ages. Gender and neuter status were classified into intact female (18.3%), spayed female (29.4%), intact male (27.5%), and castrated male (24.8%) groups. The majority of the cats lived in single cat households (75.2%), and most (54.9%) lived exclusively indoors. Approximately 50% of the cats

had been exposed to fleas but only 32.7% were currently infested. Flea control products had been applied to 44.4% (68 of 153) of the cats; however 20.6% (14 of 68) of these cats had fleas. Conversely, 43.4% (36 of 83) of the cats without flea control had a current flea infestation.

Fever (> 102.5 °F) was detected in 5.9% of the cats when they presented at the hospitals. A number of other health issues were reported in 56.2% of the cats. Problems associated with the urinary system, injuries, the gastrointestinal system, the respiratory system, the integument, and gingivitis were most commonly recorded.

5.4.2 Flea Identification

A total of 226 fleas were collected from 50 cats, and 99% of the individual fleas were *C. felis*. One *Ctenocephalides canis* and one *Pulex irritans* were co-infested on two different cats with *C. felis*.

5.4.3 PCR Results

Of the 153 blood samples, DNA of at least one target organism was amplified from 37.3% with the distribution of results presented in Table 5-1. *Rickettsia* spp. DNA was not amplified from any cat blood sample or flea pool. The overall prevalence of *Bartonella* spp. DNA was 17.0% (26 cats). Of the 50 *C. felis* pools, DNA of at least one target organism was amplified from 56.0%. The overall prevalence of *Bartonella* spp. DNA was 32.0% (16 pools) with the distribution of assay results presented in Table 5-1.

Of the 26 *Bartonella*-positive cats, 12 had *Bartonella*-positive fleas, five had *Bartonella*-negative fleas, and nine had no fleas. Flea control products had previously been applied to seven of these 26 cats. A topical product containing fipronyl (Frontline Plus, Merial) was applied to four cats, selamectin (Revolution, Pfizer Animal Health) to one cat, and a non-specified flea product to two cats. The products were administered consistently in only one of the seven cats.

Table 5-1 *Bartonella* spp. and hemoplasma spp. DNA in feline blood samples and cat fleas.

PCR assays	Number of PCR positive samples (%)	
	Cat blood (n = 153)	Flea pool (n = 50)
All assays negative	96 (62.7)	22 (44)
Any assay positive	57 (37.3)	28 (56)
Any *Bartonella* spp. positive	26 (17.0)	16 (32)
B. henselae alone	12 (7.8)	5 (10)
B. clarridgeiae alone	6 (3.9)	5 (10)
B. koehlerae alone	1 (0.7)	0 (0)
Any hemoplasma positive	35 (22.9)	17 (34)
'*Candidatus* M. haemominutum' alone	22 (14.4)	11 (22)
M. haemofelis alone	4 (2.6)	1 (2)
B. henselae and *B. koehlerae* positive	1 (0.7)	1 (2)
B. henselae and *B. clarridgeiae* positive	1 (0.7)	0 (0)
B. henselae and *M. haemofelis* positive	1 (0.7)	0 (0)
B. henselae and '*Candidatus* M. haemominutum' positive	2 (1.3)	1 (2)
B. clarridgeiae and '*Candidatus* M. haemominutum' positive	1 (0.7)	2 (4)
M. haemofelis and '*Candidatus* M. haemominutum' positive	5 (3.3)	0 (0)
B. henselae, *B. clarridgeiae*, and '*Candidatus* M. haemominutum' positive	0 (0)	1 (2)

Of 12 cats with *Bartonella* spp. DNA amplified from blood and fleas from the same cat, four paired samples were positive for *B. henselae*, three paired samples were positive for *B. clarridgeiae*, and one paired sample was co-infected with *B. henselae* and *B. koehlerae*. Four cats had negative blood, but positive flea extracts, for DNA of *B. henselae* or *B. clarridgeiae*. *B. koehlerae* DNA was amplified from the blood of two cats and extract of one flea pool. By DNA sequencing, the homology percentage of these *B. koehlerae*-positive PCR products ranged from 96% to 99% with the *B. koehlerae* GenBank accession No. AF312490.

Overall, hemoplasma DNA was amplified from 22.9% of the cats; both *M. haemofelis* and '*Candidatus* M. haemominutum' were identified. Haemoplasma spp. DNA was amplified from 34.0% of the flea pools (Table 5-1). Of the 35 hemoplasma-positive cats, seven had hemoplasma-positive fleas. Four of the seven cats had agreement of the hemoplasma species ('*Candidatus* M. haemominutum'). The remaining hemoplasma-positive cats had no fleas or fleas infected with different organisms.

All PCR assays were negative using DNA from *C. canis* and *P. irritans*. However, the cats infested with *C. canis* and *P. irritans* were positive for *B. clarridgeiae* and *M. haemofelis* DNA, respectively.

5.4.4   *Bartonella spp. IgG ELISA*

The overall prevalence of *Bartonella* spp. IgG antibodies in serum was 65.4%. The reciprocal titers ranged from 64 to 1024. When the *Bartonella* spp. IgG and PCR assay results were paired, 44.4% had agreement between ELISA and PCR assay results (20 cats were both IgG and PCR positive and 48 cats were both IgG and PCR negative). Of the 153 cats, 52.3% were IgG positive but PCR negative, and 3.3% were IgG negative but PCR positive.

5.4.5 *Risk Factors Associated with Bartonella spp. and Hemoplasma Infections*

Based on the PCR results, the odds of having *B. henselae* DNA were 4.3 times higher in young cats (< 1 year) than older cats (≥ 1 year) (95% CI = 1.37 - 13.80; *P* = 0.003). The odds of having *B. henselae* DNA were 6.9 times higher in flea-infested cats than cats without flea infestation (95% CI = 2.09 - 26.05; *P* < 0.001). Cats administered flea control products had 4.2 times lower odds of having *B. henselae* DNA (95% CI = 1.20 - 16.46; *P* = 0.010). Lastly, febrile cats had higher odds of having *B. henselae* DNA than cats without fever but the result was not statistically different (OR = 4.3; 95% CI = 0.62 - 22.47; *P* = 0.073). None of risk factors were associated with the PCR results for *B. clarridgeiae* or hemoplasmas.

5.5 Discussion

B. henselae, *B. clarridgeiae*, and *B. koehlerae* are transmitted by *C. felis* and so while prevalence rates for *Bartonella* spp. infection in cats can vary among areas, it often parallels the flea prevalence rates. This is the first study to determine the prevalence of *Bartonella* spp. DNA in both feline blood samples and fleas in Bangkok. In this study, cats with flea infestation were seven times more likely to be infected with *B. henselae* than flea-free cats. The overall prevalence rate (17%) of *Bartonella* spp. DNA in the blood of cats in this study is similar to other studies in which rates varied from 19% to 22% (Jensen et al., 2001; Maruyama et al., 2001; Inoue et al., 2009). The prevalence is lower than that reported by others (Lappin et al., 2006). The prevalence of 32% of *Bartonella* spp. DNA in flea pools is similar to the Australian study of Barrs et al. (2010), lower than the study from the United States (Lappin et al., 2006), but higher than the studies from the United Kingdom and Canada (Shaw et al., 2004; Kamrani et al., 2008).

There was agreement between the *Bartonella* spp. amplified from blood and the corresponding flea pools in 67% of the paired samples. The data support the likelihood of the transmission of *Bartonella* spp. from fleas to cats. However, some *Bartonella* spp. infected cats were infested by *Bartonella*-negative fleas or fleas positive with different *Bartonella* spp. This may due to the fleas feeding on different cats in the same vicinity with different infections (Lappin et al., 2006; Barrs et al., 2010).

Monthly use of an imidacloprid was proven experimentally to prevent the transmission of *B. henselae* among cats (Bradbury and Lappin, 2010). In this study, seven cats owned by 68 clients had *Bartonella* spp. infection although they had received flea control products. The owners may not have complied with optimal treatment recommendations for the products and the cats may have been infected by low level exposure to *Bartonella* spp. infected fleas. It is also possible that some products may not reduce transmission amongst cats, as reported for imidacloprid, or that the cats were infected before the products were administered. Owners should be advised of the potential risks of flea-associated illnesses and instructed in the application of effective flea control products.

This is the first report of *Bartonella* spp. antibodies in cats in Thailand. The seroprevalence rate of 65% is similar to those reported from other areas with high *C. felis* infestation rates (Chomel et al., 1999; Marston et al., 1999; Nasirudeen and Thong, 1999). The ELISA and PCR assay results were in agreement for only 44.4% of the cases and when the PCR assay result was used as the reference test, the positive predictive value and negative predictive value for ELISA were 20% and 90%, respectively. Like previous other studies (Barrs et al., 2010), *Bartonella* spp. antibodies should not be used for the diagnosis of current infection in individual cats.

This study is the first report of *B. koehlerae* infection in cats and *C. felis* in Thailand. Cats are considered to be the reservoir for *B. koehlerae*, but the prevalence rates appear to be low in both cats and fleas (Droz et al., 1999).

B. henselae and *B. clarridgeiae* were commonly amplified in both cats and fleas. Since *B. henselae* is predominant among cats in the area, *B. henselae* infection should be suspected as an underlying cause when cats present with clinical signs associated with bartonellosis. We found that 6% of the cats had fever and 44% were positive for *Bartonella* spp. DNA. A subsequent study will be performed to further assess whether *Bartonella* spp. infections are associated with fever in cats in Thailand.

Just as with *Bartonella* spp., hemoplasma prevalence rates in cats and fleas are increasingly being studied worldwide. Prevalence rates vary among cat populations and geographical areas, ranging from 27% to 40% (Shaw et al., 2004; Tasker et al., 2004; Lappin et al., 2006). In this study, '*Candidatus* M. haemominutum' is the major species found in cats and fleas, while *M. haemofelis* is the subpopulation. Only 11% of hemoplasma-positive cats had the same hemoplasma species in blood and fleas, suggesting that the fleas may be feeding on more than one cat as discussed for the *Bartonella* spp. In addition, current flea infestation did not increase the risk of hemoplasma infection in these cats. *C. felis* may play a minor role on transmission of hemoplasmas in cats, and fleas may merely obtain hemoplasmas via the hematophagous activity. Fighting and biting are proposed to be the natural route of hemoplasma transmission (Sykes, 2010).

The failure to amplify *R. felis* DNA from feline blood has been reported in other studies (Hawley et al., 2007; Barrs et al., 2010). Bacteremia may be transient and intermittent, or *R. felis* could be sequestered in tissues other than blood. *R. felis* antibodies have been detected in serum

of some cats suggesting exposure may be relatively common (Case et al., 2006; Bayliss et al., 2009). However, additional seroprevalence work is needed to further determine the range of *R. felis* exposure in cat populations. Cat fleas are a natural vector for *R. felis*, and the prevalence in *C. felis* has commonly been reported (Parola et al., 2003; Shaw et al., 2004). In this study, *R. felis* DNA was not amplified from any of the fleas. In the only other study of *R. felis* in *C. felis* in Thailand (Parola et al., 2003), the prevalence rate was not reported. Why *R. felis* DNA was not amplified from fleas in this study is unclear. It is possible that *R. felis* infection is very rare or the organism was present but was below the limit of detection of the assay.

In conclusion, cats and fleas in Bangkok are commonly positive for DNA of *Bartonella* spp., hemoplasmas, or a combination of these flea-associated organisms. Because of the potential for illness to be induced by these agents in the cats or humans, flea control for cats is highly suggested in the area.

5.6 Acknowledgements

References

Avidor, B., Graidy, M., Efrat, G., Leibowitz, C., Shapira, G., Schattner, A., Zimhony, O., Giladi, M., 2004. *Bartonella koehlerae*, a new cat-associated agent of culture-negative human endocarditis. J. Clin. Microbiol. 42, 3462-3468.

Barrs, V.R., Beatty, J.A., Wilson, B.J., Evans, N., Gowan, R., Baral, R.M., Lingard, A.E., Perkovic, G., Hawley, J.R., Lappin, M.R., 2010. Prevalence of *Bartonella* species, *Rickettsia felis*, haemoplasmas and the *Ehrlichia* group in the blood of cats and fleas in eastern Australia. Aust. Vet. J. 88, 160-165.

Bayliss, D.B., Morris, A.K., Horta, M.C., Labruna, M.B., Radecki, S.V., Hawley, J.R., Brewer, M.M., Lappin, M.R., 2009. Prevalence of *Rickettsia* species antibodies and *Rickettsia* species DNA in the blood of cats with and without fever. J. Feline Med. Surg. 11, 266-270.

Bradbury, C.A., Lappin, M.R., 2010. Evaluation of topical application of 10% imidacloprid-1% moxidectin to prevent *Bartonella henselae* transmission from cat fleas. J. Am. Vet. Med. Assoc. 236, 869-873.

Breitschwerdt, E.B., Maggi, R.G., Chomel, B.B., Lappin, M.R., 2010. Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. J. Vet. Emerg. Crit. Care. 20, 8-30.

Case, J.B., Chomel, B., Nicholson, W., Foley, J.E., 2006. Serological survey of vector-borne zoonotic pathogens in pet cats and cats from animal shelters and feral colonies. J. Feline Med. Surg. 8, 111-117.

Chomel, B.B., Carlos, E.T., Kasten, R.W., Yamamoto, K., Chang, C.C., Carlos, R.S., Abenes, M.V., Pajares, C.M., 1999. *Bartonella henselae* and *Bartonella clarridgeiae* infection in domestic cats from the Philippines. Am. J. Trop. Med. Hyg. 60, 593-597.

Droz, S., Chi, B., Horn, E., Steigerwalt, A.G., Whitney, A.M., Brenner, D.J., 1999. *Bartonella koehlerae* sp. Nov., isolated from cats. J. Clin. Microbiol. 37, 1117-1122.

Foley, J.E., Harrus, S., Poland, A., Chomel, B., Pedersen, N.C., 1998. Molecular, clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in domestic cats. Am. J. Vet. Res. 59, 1581-1588.

Fontenelle, J.P., Powell, C.C., Hill, A.E., Radecki, S.V., Lappin, M.R., 2008. Prevalence of serum antibodies against *Bartonella* species in the serum of cats with or without uveitis. J. Feline Med. Surg. 10, 41-46.

Hawley, J.R., Shaw, S.E., Lappin, M.R., 2007. Prevalence of *Rickettsia felis* DNA in the blood of cats and their fleas in the United States. J. Feline Med. Surg. 9, 258-262.

Inoue, K., Maruyama, S., Kabeya, H., Kawanami, K., Yanai, K., Jitchum, S., Jittaparapong, S., 2009. Prevalence of *Bartonella* infection in cats and dogs in a metropolitan area, Thailand. Epidemiol. Infect. 137, 1568-1573.

Jensen, W.A., Fall, M.Z., Rooney, J., Kordick, D.L., Breitschwerdt, E.B., 2000. Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. J. Clin. Microbiol. 38, 1717-1722.

Jensen, W.A., Lappin, M.R., Kamkar, S., Reagan, W.J., 2001. Use of a polymerase chain reaction assay to detect and differentiate two strains of *Haemobartonella felis* in naturally infected cats. Am. J. Vet. Res. 62, 604-608.

Kamrani, A., Parreira, V.R., Greenwood, J., Prescott, J.F., 2008. The prevalence of *Bartonella*, hemoplasma, and *Rickettsia felis* infections in domestic cats and in cat fleas in Ontario. Can. J. Vet. Res. 72, 411-419.

Lappin, M.R., Breitschwerdt, E., Brewer, M., Hawley, J., Hegarty, B., Radecki, S., 2009. Prevalence of *Bartonella* species antibodies and *Bartonella* species DNA in the blood of cats with and without fever. J. Feline Med. Surg. 11, 141-148.

Lappin, M.R., Griffin, B., Brunt, J., Riley, A., Burney, D., Hawley, J., Brewer, M.M., Jensen, W.A., 2006. Prevalence of *Bartonella* species, haemoplasma species, *Ehrlichia* species, *Anaplasma phagocytophilum*, and *Neorickettsia risticii* DNA in the blood of cats and their fleas in the United States. J. Feline Med. Surg. 8, 85-90.

Marston, E.L., Finkel, B., Regnery, R.L., Winoto, I.L., Graham, R.R., Wignal, S., Simanjuntak, G., Olson, J.G., 1999. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in an urban Indonesian cat population. Clin. Diagn. Lab. Immunol. 6, 41-44.

Maruyama, S., Sakai, T., Morita, Y., Tanaka, S., Kabeya, H., Boonmar, S., Poapolathep, A., Chalarmchaikit, T., Chang, C.C., Kasten, R.W., 2001. Prevalence of *Bartonella* species and 16s rRNA gene types of *Bartonella henselae* from domestic cats in Thailand. Am. J. Trop. Med. Hyg. 65, 783-787.

Menier, K., Beaucournu, J.C., 1998. Taxonomic study of the genus *Ctenocephalides* stiles & collins, 1930 (Insecta: Siphonaptera: Pulicidae) by using aedeagus characters. J. Med. Entomol. 35, 883-890.

Nasirudeen, A., Thong, M., 1999. Prevalence of *Bartonella henselae* immunoglobulin G antibodies in Singaporean cats. Pediatr. Infect. Dis. J. 18, 276-278.

Parola, P., Sanogo, O.Y., Lerdthusnee, K., Zeaiter, Z., Chauvancy, G., Gonzalez, J.P., Miller, R.S., Telford III, S.R., Wongsrichanalai, C., Raoult, D., 2003. Identification of *Rickettsia* spp. and *Bartonella* spp. in fleas from the Thai-Myanmar border. Ann. N. Y. Acad. Sci. 990, 173-181.

Perez, C., Hummel, J.B., Keene, B.W., Maggi, R.G., Diniz, P.P.V.P., Breitschwerdt, E.B., 2009. Successful treatment of *Bartonella henselae* endocarditis in a cat. J. Feline Med. Surg. 12, 483-486.

Quimby, J.M., Elston, T., Hawley, J., Brewer, M., Miller, A., Lappin, M.R., 2008. Evaluation of the association of *Bartonella* species, feline herpesvirus 1, feline calicivirus, feline leukemia

virus and feline immunodeficiency virus with chronic feline gingivostomatitis. J. Feline Med. Surg. 10, 66-72.

Reynolds, C.A., Lappin, M.R., 2007. "*Candidatus* Mycoplasma haemominutum" infections in 21 client-owned cats. J. Am. Anim. Hosp. Assoc. 43, 249-257.

Shaw, S.E., Kenny, M.J., Tasker, S., Birtles, R.J., 2004. Pathogen carriage by the cat flea *Ctenocephalides felis* (Bouché) in the United Kingdom. Vet. Microbiol. 102, 183-188.

Sykes, J.E., 2010. Feline hemotropic mycoplasmas. Vet. Clin. North Am. Small Anim. Pract. 40, 1157-1170.

Sykes, J.E., Drazenovich, N.L., Ball, L.M., Leutenegger, C.M., 2007. Use of conventional and real-time polymerase chain reaction to determine the epidemiology of hemoplasma infections in anemic and nonanemic cats. J. Vet. Internal Med. 21, 685-693.

Tasker, S., Braddock, J.A., Baral, R., Helps, C.R., Day, M.J., Gruffydd-Jones, T.J., Malik, R., 2004. Diagnosis of feline haemoplasma infection in Australian cats using a real-time PCR assay. J. Feline Med. Surg. 6, 345-354.

Willi, B., Boretti, F.S., Cattori, V., Tasker, S., Meli, M.L., Reusch, C., Lutz, H., Hofmann-Lehmann, R., 2005. Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with hemolytic anemia in Switzerland. J. Clin. Microbiol. 43, 2581-2585.

Williams, M., Izzard, L., Graves, S.R., Stenos, J., Kelly, J.J., 2011. First probable Australian cases of human infection with *Rickettsia felis* (cat-flea typhus). Med. J. Aust. 194, 41-43.

CHAPTER 6

Determination of *Bartonella henselae* Bacterial Load and Antigen Level in

Blood Samples of Febrile and Afebrile Client-owned Cats

6.1 Summary

Some cases of fever of unknown origin in cats may be probably caused by *B. henselae* infection, but correlation of fever and infection has not been clearly documented. A quantitative real-time polymerase chain reaction (qPCR) assay and an antigen-capture enzyme linked immunosorbent assay (AC-ELISA) were recently developed for detecting *B. henselae* infection in cats. The objectives of this study were to determine whether bacterial DNA load and antigen levels of *B. henselae* are associated with fever, and to identify risk factors that might be associated with fever in cats presenting to veterinary hospitals in Thailand. Blood and serum samples were collected from client-owned cats, including 22 with fever of unknown cause and 178 without fever. Total DNA was extracted from each sample and evaluated in *Bartonella* and hemoplasma conventional PCR assays and the new *B. henselae* qPCR assay. Each blood sample was assayed in the AC-ELISA, and each serum sample was tested for *Bartonella* spp. IgG, feline leukemia virus antigen, and feline immunodeficiency virus antibodies. Febrile cats had significantly higher prevalence of *B. henselae* DNA than those of afebrile cats. Higher bacterial load in febrile cats than afebrile cats was detected, but the finding was not statistically significant. Because of low sensitivity of the AC-ELISA, the presence of *B. henselae* antigen was not associated with fever. The present study supports that *B. henselae* infection is associated with fever, and *B. henselae*-infected cats with high bacterial DNA levels are most likely to develop fever.

Keyword: *B. henselae*; Fever; Cat; qPCR; Antigen

6.2 Introduction

Fever is a common clinical sign developed in cats during infections, inflammations, and immunologic or idiopathic diseases. A number of infectious agents, including viruses, bacteria, fungi, and protozoa have been found to be causes of fever in cats. Among these organisms, *Bartonella* spp., hemoplasmas, feline leukemia virus (FeLV), and feline immunodeficiency virus (FIV) are the common organisms that infect cats. They are also included in the lists of diagnosis for febrile cats presenting to the veterinary practitioner. *Bartonella henselae* has been known to commonly infect cats as the primary animal reservoir and results in subclinical infection with prolonged intra-erythrocytic bacteremia. However, some infected cats may develop clinical signs; fever appears to be common and is reported in both experimentally and naturally infected cats. Lymphadenopathy, inflammatory skin lesions, lethargy, anorexia, mild neurologic signs, and reproductive disorders also develop in some experimentally infected cats (Guptill et al., 1997; Guptill et al., 1998; Kordick et al., 1999; O'Reilly et al., 1999; Bradbury and Lappin, 2010). Uveitis, stomatitis, gingivitis, lymphadenopathy, hyperglobulinemia, and urological diseases have been documented in naturally-infected cats (Ueno et al., 1996; Glaus et al., 1997; Lappin et al., 2000; Lappin et al., 2009; Whittemore et al., 2012). Hemoplasmas are the causative agents of infectious anemia in cats. Acute infection with *Mycoplasma haemofelis* and 'Candidatus Mycoplasma turicensis' can cause fever, lethargy, anorexia, and anemia (Foley et al., 1998; Willi et al., 2005). Cats infected with 'Candidatus Mycoplasma haemominutum' are subclinical or develop only minimal clinical signs of acute disease (Foley et al., 1998; Foley and Pedersen, 2001). Fever is also a common clinical sign presenting in cats infected with the retroviruses, FeLV and FIV. Cats may not show any clinical signs in the initial state, but sickness is obviously seen in the chronic state, particularly FeLV which may lead to cancer. Both FeLV

and FIV may cause severe immune suppression in cats resulting in increased risk of opportunistic infections (Pedersen et al., 1989; Hoover and Mullins, 1991).

Diagnosis of *Bartonella* spp. in cats mostly relies on amplification of bacterial DNA from blood of animals due to the high sensitivity and specificity of molecular assays. Routine culture of blood is less frequently used clinically because of delayed turnaround time for results and some culture techniques are less sensitive than some of the PCR assays. Antibody testing by means of immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), or western blot can be performed quickly and inexpensively, but results often are not correlated with current infection or disease manifestations. A recent study revealed higher prevalence of *Bartonella* spp. DNA in febrile cats than afebrile cats (Lappin et al., 2009). Fever caused by infection is an inflammatory response of the host's immune system triggered by exogenous pyrogens. This raises a question regarding level of bacteremia that can stimulate fever. In order to determine estimate the level of bacteremia, a quantitative real-time polymerase chain reaction (qPCR) assay and an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) were recently developed for the amplification of *B. henselae* DNA and antigen, respectively. The hypothesis to be tested is that higher levels of *B. henselae* DNA or antigen will be detected in the blood of febrile cats than those of afebrile cats. The objectives of this study were to determine whether bacterial load and antigen level of *B. henselae* are associated with fever, and to identify risk factors for fever in cats presenting to veterinary hospitals in Thailand.

6.3 Materials and Methods

6.3.1 Client-owned Cats and Sample Collection

Whole blood samples were collected from febrile and aferile client-owned cats presenting to veterinary practitioners in the Small Animal Teaching Hospital of Chulalongkorn University and 6 private veterinary hospitals in Bangkok, Thailand between June 2010 and October 2010. Physical examination was performed by attending practitioners on all cats. Fever was defined as body temperature > 102.5 °F. Only febrile cats with unknown cause of fever on physical examination were included in the study. Cats diagnosed with hyperthermia of known causes, and cats previously treated with antibiotics within two weeks were excluded. One ml of EDTA-anticoagulated whole blood sample and 0.5 ml of serum sample from each cat were stored at -20 °C and shipped on ice packs to the Center for Companion Animal Studies at Colorado State University, where total DNA was extracted from the whole blood, and PCR assays were performed. A questionnaire was completed by the owner and veterinarian at the time of sample collection. The questionnaire provided information concerning signalment, housing location, presence or absence of flea infestation, and flea control.

6.3.2 DNA Extraction

The total DNA was extracted from 200 µl of each blood sample using a commercially available kit (QIAamp DNA Blood Mini Kit, QIAGEN, Valencia, CA) that was performed following the manufacturer's instructions. The extracted DNA was eluted in 200 µl of Tris-EDTA buffer.

6.3.3 Conventional PCR Assays

The conventional PCR assays described previously (Jensen et al., 2000; Jensen et al., 2001) were performed to detect *Bartonella* spp. and hemoplasmas in the total DNA samples. The positive results were detected by electrophoresis on a 3% agarose gel, stained and visualized with UV light. The PCR products of *B. henselae* and *B. koehlerae* yielded the same size of the band, as well as *M. haemofelis* and '*Candidatus* M. turicensis'. The species were confirmed by DNA sequencing, which was performed at Macromolecular Resources, Colorado State University, Fort Collins, CO.

6.3.4 Bartonella henselae-specific Quantitative Real-time PCR Assay

All samples positive for *B. henselae* DNA in cPCR assay were subsequently analyzed in the qPCR assay. In addition, cPCR-negative samples from 6 febrile and 6 afebrile cats were also randomly selected for testing with the qPCR assay. All reactions were performed in triplicates in the PCR mixture consisted of 10 µl of commercially available master mix (2X TaqMan Fast Advanced Master Mix, Applied Biosystems, Carlsbad, CA), 0.6 µM of each primer (forward: 5' CTC TCG ATG ACA ATG GGA 3' and reverse: 5' ACT TCG GTT CCA GAC CAT 3'), 0.15 µM of probe (5' FAM-TCC AAA CAA GAT ACG GTC CCC TG-BHQ1 3'), 2 µl of template DNA in a final 20 µl reaction mix. The PCR was carried out with a thermocycler (Mastercycler ep *realplex*, Eppendorf, Hamburg, Germany) under the following conditions: incubation at 50 °C for 2 min, 95 °C for 1 min, and followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s.

6.3.5 *Estimation of Bacterial Load by qPCR assay*

A standard curve was performed from a blood sample set spiked with known initial amount of *B. henselae*, ranging from 5.2 x 10^6 to 52 CFU/ml. The absolute amount of *B. henselae* in unknown samples was extrapolated from quantification cycle (C$_q$) values with the standard curve by a simple linear regression model.

6.3.6 *Rabbit Polyclonal Antibody Production*

Bacterial cells of *B. henselae* strain CSU-1 were disrupted by 30% pulsed sonication for 10 min. After centrifugation of the sonicate suspension at 10,000 *g* for 10 min, the supernatant was discarded. The pellet was suspended in sterile deionized water. The crude antigen suspension was then diluted, aliquoted into single doses, and stored at -80 °C until used.

The immunization protocol was approved by the Institutional Animal Care and Use Committee (IACUC). Two female, 4-kg, New Zealand White rabbits were used for immunization. The rabbits were first inoculated subcutaneously in multiple sites with 100 μg of crude antigen mixed with Freund's Complete Adjuvant. They were boosted twice in 2-week intervals by intramuscular injection of the same quantity of antigen emulsified in Freund's Incomplete Adjuvant. Antibodies against *B. henselae* were evaluated every 2 weeks by agar gel immunodiffusion test, as previously described (Lappin et al., 1989; Simard et al., 2000). Approximately, 40 - 60 ml of blood was collected by lateral ear venipuncture for serum separation at week 8, 12, and 16 of immunization. Total IgG was purified from antisera using a commercially available kit (Melon Gel IgG Purification Kit; Thermo Fisher Scientific, Rockford, IL). The procedure was performed following the manufacturer's instructions. The purity of IgG preparations was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE), as previously described (Laemmli, 1970). Purified IgG solutions of two rabbits were pooled, and fractions of the pooled IgG were conjugated with horseradish peroxidase (HRP) by a commercially available kit (EZ-Link Plus Activated Peroxidase Kit; Thermo Fisher Scientific, Rockford, IL), following the manufacturer's instructions. The conjugate was preserved in 50% glycerol solution. Pooled IgG and conjugate were stored at -20 °C until assayed.

6.3.7   *Blood Sample Preparation for AC-ELISA*

Whole blood samples were lysed with 20 volume of deionized water. Suspension was vortexed for 10 s and left to stand for 10 min. Hemolysate was loaded into a centrifugal filter unit with 5-µm pore size (Ultrafree-MC, Millipore, Billerica, MA). The tube was centrifuged at 10,000 g for 10 min. The filtrate was gently pipetted out and discarded. The precipitate was resuspended with PBS-TW before applied to the assay.

6.3.8   *AC-ELISA Procedure*

The same sample set that was tested in the qPCR assay was also tested with the AC-ELISA. A microtiter plate was coated at 4 °C overnight with rabbit anti-*B. henselae* IgG (25 µg/ml) diluted with PBS (pH 9.0). The plate was washed for 4 times with PBS containing 0.05% Tween 20 (PBS-TW) after incubation, and then blocked at 37 °C for 1 h with 0.5% NFDM. Following 4-time washing, samples in quadruplicates were applied to the plate. The plate was incubated at 37 °C for 30 min. After washing, the rabbit HRP-conjugated IgG (10 µg/ml) with PBS-TW was added into wells, and the plate was incubated at 37 °C for 30 min. Washing was applied, and TMB substrate was added into wells. The plate was incubated at room temperature

for 10 min before the reaction was stopped with sulfuric acid solution (0.18 M). The optical density (OD) value was read at 450 nm wavelength by a microplate reader. Blank and substrate controls were included in each plate and each run of the assay.

6.3.9 Calculation of the Positive Cut-off for the AC-ELISA

A sample was determined to positive when OD value was higher than a cutoff which was calculated by average OD value of known negative samples plus 3 times of standard deviations (SD).

6.3.10 Bartonella spp. IgG ELISA

Serum samples were analyzed for *Bartonella* spp. IgG using the protocol described previously (Lappin et al., 2009).

6.3.11 Detection of Retroviral Infection

Serum was used to identify feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) infections using a commercially available test kit (Snap Feline Triple Test, IDEXX Laboratories, Westbrook, ME).

6.3.12 Data Analysis

Continuous variables, including age and bacterial load were expressed as median (range), while body temperature were expressed as mean $\pm$ SD. Comparison of continuous variables between cats with and without fever was analyzed by two sided *t*-test or Wilcoxon Rank Sum Test. Associations between categorical variables and fever were determined by Pearson's Chi-

square test and Fisher's exact test, and *P*-value ≤ 0.05 was considered as significant. Logistic regression analyses were used to evaluate factors that might be associated with fever. The dependent variable was the clinical sign of fever (y/n), and the independent variables included age (> 1 year/≤ 1 year), sex (male/female), housing location (indoor/indoor and outdoor), flea infestation (y/n), and flea control (y/n), *B. henselae* DNA (positive/negative), *B. henselae* antigen (positive/negative), *Bartonella* spp. IgG (positive/negative), "*Candidatus* M. *haemominutum*" DNA (positive/negative), FIV antibody (positive/negative), and FeLV antigen (positive/negative). Variables with a critical α of 0.25 in univariable models were entered as predictor variables in the multivariable model. The final model was identified using backwards selection with a critical α of 0.05. Confounding and interactions were also evaluated. Odds ratios (OR) and 95% confidence intervals (CI) were calculated from logistic regression models. Statistical significance was set as *P* ≤ 0.05. Analyses were accomplished using statistical software (SAS 9.1, SAS Institute, Cary, NC).

6.4 Results

6.4.1 Client-owned cats

Two hundred cats were included in the study, of which 22 were febrile and 178 were afebrile. The median age of febrile cats (1.2 years, range 0.4 - 10) was not significantly different from afebrile cats (2 years, range 0.2 - 17; *P* = 0.20). The majority (169/200) of cats presented to the Small Animal Teaching Hospital of Chulalongkorn, and the other cats were recruited from private veterinary hospitals. The mean body temperature in febrile cats (103.6 ± 0.9 °F) was significantly higher than afebrile cats (100.8 ± 1.01 °F; *P* < 0.001). The age, sex, housing location, flea infestation, and flea control of client-owned cats with and without fever are

described in Table 6-1. There was no significant difference in the proportions of these variables between both groups of cats (Table 6-2).

Table 6-1 Characteristics of client-owned cats. Twenty-two febrile and 178 afebrile client-owned cats presented to one of 7 veterinary hospitals in Bangkok, Thailand in 2010. Data presented as number (%).

Characteristic	Fever (n=22) Number (%)	No fever (n=178) Number (%)
Age (year)		
≥ 1	15 (68.2)	125 (70.2)
< 1	7 (31.8)	53 (29.8)
Sex		
Male	6 (27.3)	81 (45.5)
Female	16 (72.7)	97 (54.5)
Housing		
Indoor and outdoor	11 (50.0)	87 (48.9)
Indoor only	10 (45.5)	89 (50.0)
Unknown	1 (4.5)	2 (1.1)
Flea infestation		
Yes	6 (27.3)	58 (32.6)
No	15 (68.2)	116 (65.2)
Unknown	1 (4.5)	4 (2.2)
Flea control		
Yes	8 (36.4)	61 (34.3)
No	12 (54.5)	111 (62.4)
Unknown	2 (9.1)	6 (3.3)

Table 6-2 Univariable logistic regression analysis of variables that are associated with fever. Characteristics of cats and laboratory findings of 200 client-owned cats that were presenting to one of 7 veterinary hospitals in Bangkok, Thailand in 2010 were analyzed for risk factors of fever.

Variable	Category	OR [95% CI]	*P*-value
Age	≥ 1 year	0.89 [0.34, 2.31]	0.81
	<1 year	Ref.	
Sex	Male	0.45 [0.17, 1.20]	0.11
	Female	Ref.	
Housing location	Indoor and outdoor	1.13 [0.46, 2.78]	0.80
	Indoor	Ref.	
Flea infestation	Yes	0.80 [0.30, 2.17]	0.66
	No	Ref.	
Flea control	Yes	1.21 [0.47, 3.13]	0.69
	No	Ref.	
B. henselae DNA	Positive	2.80 [0.99, 7.96]	0.05
	Negative	Ref.	
B. henselae antigen	Positive	0.40 [0.04, 4.12]	0.44
	Negative	Ref.	
Bartonella spp. IgG	Positive	0.59 [0.16, 1.79]	0.32
	Negative	Ref.	
'Candidatus M. haemominutum' DNA	Positive	0.69 [0.19, 2.48]	0.58
	Negative	Ref.	
FIV antibody	Positive	1.01 [0.22, 4.73]	0.99
	Negative	Ref.	
FeLV antigen	Positive	2.79 [0.91, 8.50]	0.07
	Negative	Ref.	

6.4.2 cPCR Results

Overall prevalence rates of *Bartonella* spp. DNA and hemoplasma DNA were 20% (40/200) and 22% (44/200), respectively. Only DNA of *B. henselae* and '*Candidatus* M. haemominutum' was detected in febrile cats. Five febrile cats were positive for *B. henselae* only, 2 were positive for '*Candidatus* M. haemominutum' only, and 1 was positive for both *B. henselae* and '*Candidatus* M. haemominutum' (Table 6-3). Prevalence rates of *B. henselae* DNA in febrile cats were 27.3%. Of 63 afebrile cats positive for any organism, the majority were positive for '*Candidatus* M. haemominutum' only (20 cats), followed by *B. henselae* only (14 cats). Some afebrile cats were positive for only *M. haemofelis* or *B. clarridgeiae*. *B. koehlerae* or '*Candidatus* M. turicensis' was detected from each of 2 afebrile cats. The DNA of more than one species was also detected in afebrile cats. The prevalence rate of *B. henselae* DNA in afebrile cats was 11.8%. Febrile cats had significantly higher prevalence of *Bartonella* spp. DNA than afebrile cats ($P = 0.05$), but prevalence rate of '*Candidatus* M. haemominutum' DNA was not significantly different between groups ($P = 0.58$) (Table 6-2). The odds of having *Bartonella* spp. DNA in blood was 2.5 times higher for cats infested with fleas compared with those without flea infestation ($P = 0.04$). Age was not significantly associated with the prevalence of *B. henselae* DNA (OR 1.07, 95% CI 0.42 - 2.72; $P = 0.89$); neither was sex (OR 0.88, 95% CI 0.36 - 2.20; $P = 0.77$), housing location (OR 1.66, 95% CI 0.70 - 3.92; $P = 0.25$), or flea control (OR 0.67, 95% CI 0.29 - 1.59; $P = 0.37$).

Table 6-3 Prevalence of *Bartonella* spp. and hemoplasma DNA determined by cPCR assays in blood samples of 22 febrile and 178 afebrile client-owned cats.

Assay	Fever (n=22) Number (%)	Non-fever (n=178) Number (%)
Negative	14 (63.6)	115 (64.6)
B. henselae	5 (22.7)	14 (7.8)
B. clarridgeiae	0 (0)	7 (3.9)
B. koehlerae	0 (0)	1 (0.6)
'*Candidatus* M. haemominutum'	2 (9.1)	20 (11.2)
M. haemofelis	0 (0)	6 (3.4)
'*Candidatus* M. turicensis'	0 (0)	1 (0.6)
Co-infections		
'*Candidatus* M. haemominutum' and *M. haemofelis*	0 (0)	2 (1.1)
B. henselae and '*Candidatus* M. haemominutum'	1 (4.6)	3 (1.7)
B. henselae and *M. haemofelis*	0 (0)	1 (0.6)
B. henselae, '*Candidatus* M. haemominutum', and *M. haemofelis*	0 (0)	2 (1.1)
B. clarridgeiae and '*Candidatus* M. haemominutum'	0 (0)	5 (2.8)
B. clarridgeiae, *B. henselae*, and '*Candidatus* M. haemominutum'	0 (0)	1 (0.6)

6.4.3 qPCR Results

The quantitative performance of the qPCR assay was established by the standard curve which is shown in Figure 6-1. The plot of the log of bacterial load (CFU/ml) versus the C_q showed a linear correlation ($R^2 = 0.997$). The efficiency of the qPCR was 79%, and the limit of detection (LOD) was 52 CFU/ml.
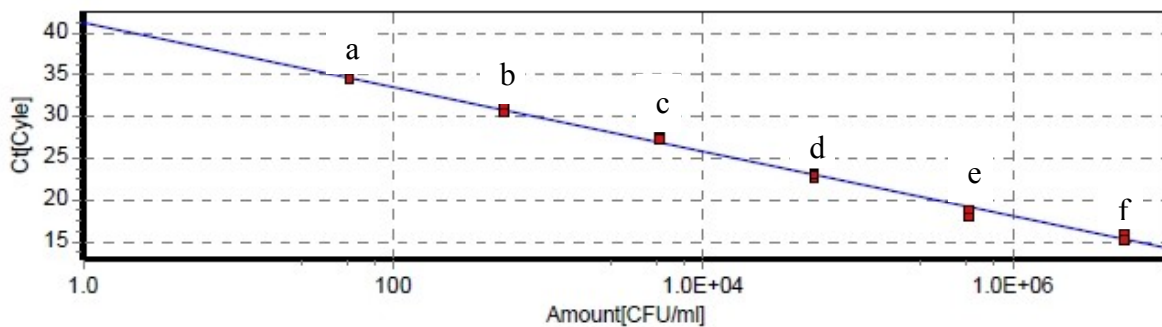

Figure 6-1. Standard curve for the qPCR assay. Six orders of magnitude of *B. henselae*, ranging from 52 (a) to 5.2 x 10^6 (f) CFU/ml, were spiked in feline whole blood samples.

All of the samples that tested positive for *Bartonella* cPCR, including 6 febrile and 21 afebrile cats were also positive by the qPCR assay. Of 6 febrile cats that were positive with qPCR assay, only 1 had bacterial load below the LOD. The median bacterial load of 6 febrile cats was 2470 CFU/ml (range 21 - 7467 CFU/ml). Whereas 11 of 21 afebrile cats positive with qPCR assay had bacterial load which was below the detection limit. The median bacterial load of 21 afebrile cats was 49 CFU/ml (range 19 - 3494 CFU/ml). The median bacterial load of febrile cats was 50 times higher than that of afebrile cats (Figure 6-2), although the difference was not statistically significant ($P = 0.15$). The cPCR-negative samples were all negative with the qPCR assay. In addition, the body temperature was not correlated with the bacterial load ($P = 0.08$).

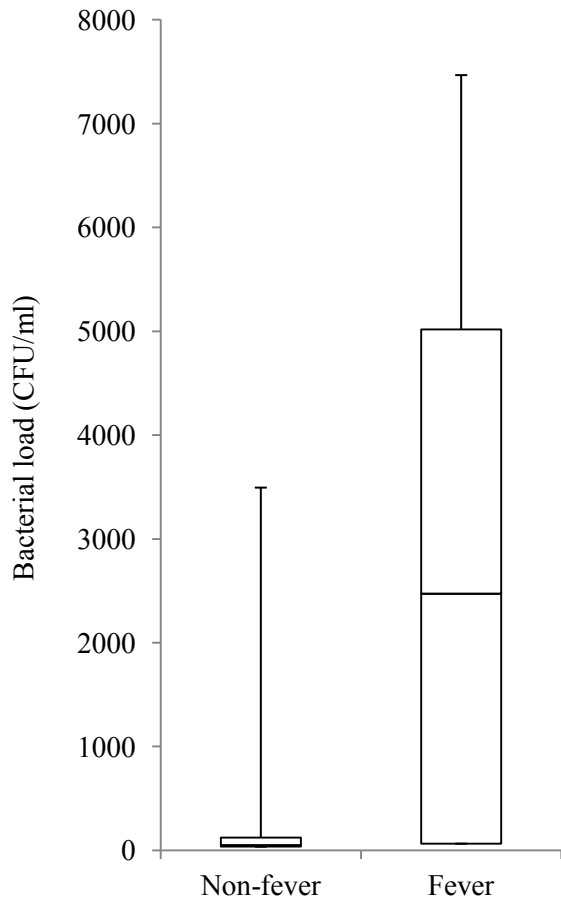**Figure 6-2.** Comparison of *B. henselae* bacterial load between febrile and afebrile cats. Box plots of *B. henselae* bacterial load (CFU/ml) in blood samples of 21 afebrile and 6 febrile client-owned cats presenting to one of 7 veterinary hospitals in Bangkok, Thailand in 2010. The box represents the interquartile range (25-75%), the horizontal line represents the median, and the T-bars represent the range of the data.

6.4.4   AC-ELISA Results

The AC-ELISA was shown to be specific to *B. henselae* since other *Bartonella* spp. and unrelated *Bartonella* bacteria tested for specificity were all negative in the assay. Blood samples of 27 febrile and afebrile cats that were tested positive by both PCR assays were also tested in the AC-ELISA. Overall, 29.6% of cats were positive by the AC-ELISA. The results showed that the AC-ELISA was less sensitive than both PCR assays. Of 6 febrile cats positive with PCR assays, only one (16.7%) was positive with AC-ELISA. Seven of 21 (33.3%) afebrile cats that were positive by PCR assays were also positive with AC-ELISA. The cPCR-negative samples tested were all negative with the AC-ELISA. The odds of developing fever were not significantly different between cats positive and negative with *B. henselae* antigen (Table 6-2).

6.4.5   Bartonella spp. IgG

Sixty-four cats (32%) were positive for *Bartonella* spp. IgG, in which 13 were *B. henselae* DNA positive, 3 were *B. clarridgeiae* DNA positive, 1 were *B. henselae* and *B. clarridgeiae* DNA positive, and 47 were negative for *Bartonella* spp. DNA. The cat that was positive for *B. koehlerae* DNA was negative for *Bartonella* spp. IgG.

6.4.6   FeLV and FIV Results

Overall prevalence rates of FeLV antigen and FIV antibody was 11% (22 cats) and 9% (18 cats), respectively. Of 22 febrile cats, 5 (22.7%) were positive for FeLV antigen, and 2 (9.1%) were positive for FIV antibody. Among 178 afebrile cats, 17 (9.6%) were positive for FeLV antigen, and 16 (9.0 %) were positive for FIV antibody. Neither FeLV ($P = 0.07$) nor FIV ($P = 0.99$) was associated with fever (Table 6-2). Three cats (1.5%) were positive for both FeLV

and FIV, and these cats were not febrile. Of 40 cats that were *Bartonella* spp. DNA positive, 5 (12.5%) were concurrently positive for FeLV, and 2 (5%) were also positive for FIV. The prevalence of FeLV antigen was not associated with the presence or absence of *Bartonella* spp. DNA (OR 1.20, 95% CI 0.32 - 3.7; *P* = 0.78). The FIV antibody was not associated with the prevalence of *Bartonella* spp. DNA (OR 0.47, 95% CI 0.05 - 2.16; *P* = 0.54).

6.4.7 Risk Factors for Fever

Of all variables described in Table 6-2, the variables of sex, FeLV, and *B. henselae* DNA passed screening (*P* < 0.25), and they were included in multivariable model building. However, the final model included only *B. henselae* DNA because this was the main risk factor of interest and was the only significant factor. The odds of developing fever was 2.8 times greater for cats that were positive for *B. henselae* DNA compared with those that were negative (95% CI 0.99 - 7.96; *P* = 0.05).

6.5 Discussion

Cats are prone to develop feline bartonellosis during infection with *B. henselae*, and fever is the most clinical sign documented. In this study, the prevalence of *B. henselae* DNA was almost 3 times higher in febrile than afebrile cats (*P* ≤ 0.05). The results of this study are consistent with those of the previous study which was performed with samples of cats in the United States (Lappin et al., 2009). Fever has been reported in *B. henselae*-infected cats (Guptill et al., 1997; Guptill et al., 1998; Kordick et al., 1999; O'Reilly et al., 1999; Mikolajczyk and O'Reilly, 2000; Bradbury and Lappin, 2010), and has also been documented in *B. henselae*-infected people (Chomel et al., 2006). Although *B. henselae* infection cannot be proved to be the

cause of fever, this study suggests that presence of *B. henselae* DNA in blood is related to fever in cats. The overall prevalence of *Bartonella* spp. DNA was similar to that of the recently published study of cats within the same area (Assarasakorn et al., 2012). *Bartonella clarridgeiae* and *B. koehlerae* were rarely amplified from blood of cats, and those samples were from afebrile cats. Both species of *Bartonella* may be minimal pathogenic organisms for cats. Cats of this area are prone to be infected with many feline *Bartonella* spp.; however, the qPCR assay was designed to be specific to only *B. henselae* due to the highest prevalence rate and clinical relevance in cats.

This study proposes the qPCR assay which is capable of estimating *B. henselae* bacterial load in feline blood. The assay is very specific to *B. henselae*, and the analytical sensitivity is comparable to the currently used cPCR assay (Jensen et al., 2000). The advantage of the new assay is the quantification of bacteria that would be detected earlier than routine culture. Levels of *Bartonella henselae* DNA between cats with and without fever are compared for the first time in this study. The qPCR results demonstrated 100% concordance with the cPCR results. These findings show that the qPCR assay has equivalent diagnostic sensitivity to the cPCR assay. There were 44% (12/27) of the samples tested that had bacterial load lower than the limit of detection of the assay. The least amount of bacterial load detected in these samples was 19 CFU/ml. All of these samples were considered truly positive due to high specificity of the assay, but the detectable bacterial load beyond the detection limit may have risk for inconsistence and imprecision. However, all qPCR-positive results were included for comparing between groups of cats. That is because at least 2 of 3 replicates of each sample were found to be positive. This study suggests that very low bacterial load should be interpreted with caution because the actual quantity was difficult to acquire between replicates with unacceptably large variation. Samples

with very low bacterial load may originally have very low amount of *B. henselae*, but it is possible that DNA might be lost over the period of storage due to the degradation of DNA.

Despite febrile cats had approximately 50 times higher *B. henselae* bacterial load than afebrile cats, there was no significantly different in bacterial load between groups. Lack of statistical significance should be caused by inadequate number of studied cats. Repeating an analysis with a larger sample size is strongly recommended in order to increase the statistical power. The results of the present study suggest that fever is more likely to develop in cats that have high level of bacterial load detected by the qPCR assay. However, cats are natural reservoir for *B. henselae*, afebrile cats can also have estimated bacterial loads. The pathogenesis of fever is generally triggered by endogenous pyrogens (cytokines) that are released from phagocytic cells after they are activated by exogenous pyrogens such as bacterial infection. The higher number of *B. henselae* may be able to induce higher responses of phagocytic cells, and these cats are prone to develop fever. If the bacterial load was proved to be associated with fever, the qPCR assay would be used for diagnosis of fever related to *B. henselae* infection in cats.

The AC-ELISA is an immunoassay that was developed for detection of *B. henselae* antigen in order to evaluate whether antigenemia level is associated with fever induced by *B. henselae* infection. The performance of this AC-ELISA is not adequate for detection of *B. henselae* antigen in feline blood. Of all PCR positive-samples tested, about 30% were found positive with AC-ELISA. The results document that the PCR assays tested have higher sensitivity or better ability to identify cats with *B. henselae* infection than the AC-ELISA. Negative results in PCR assays suggest the absence of infection, but negative results in AC-ELISA may be falsely negative. The results suggest that low analytical sensitivity of the assay may result in low diagnostic sensitivity and low negative predictive value, as well.

The limited analytical sensitivity of the AC-ELISA may be caused by the avidity of the rabbit antibodies used. The antibodies produced from the 2 rabbits showed similar reactivity in the screening test (AGID test). The antibodies of both rabbits were then pooled and used for coating the plate and detecting the antigen of bacteria. In spite of adequate optimization, the pooled antibody itself may not have enough avidity for antigen binding. Antigen, particularly with low amount of antigen will be separated from antibody on the solid phase, and no signal will be generated. The result of this study suggested that selection of a perfect match pair of antibody should be performed using different sources of antibodies. False negatives may result from the degradation of antigen during transport and storage of samples. Results would be more accurate if fresh samples are tested.

Because cross-reaction of polyclonal antibodies and non-relevant antigens in blood sample may occur, the high cutoff value was selected to avoid false positivity from non-specific binding. Concurrently, the high cutoff then decreased the analytical sensitivity that may affect the diagnostic sensitivity of the assay. If the level of analyte to be tested is below the detection limit, the assay will detect that sample as a false-negative test result. Cats infected with *B. henselae* commonly have prolonged, intermittent bacteremia for months or years. It is possible that antigen-negative cats may be experiencing chronic infection with low level bacteremia and antigenemia. Otherwise, cats may be within the window period of *B. henselae* infection, which is the time between first infection and when the test can reliably detect that infection. Because of higher sensitivity of PCR assays, *B. henselae* DNA may be detected earlier than the detection of *B. henselae* antigen. The advantage of the assay is the specificity to *B. henselae* antigens. Low rates of false positive test results should be found. The presence of *B. henselae* antigen in feline blood was shown to be not associated with fever for this sample set. However, because of limited

performance of the AC-ELISA, the assay did not provide enough information to determine an association with fever. In addition, the present AC-ELISA is not suitable for clinical use. Improvement of the sensitivity of the assay is highly suggested, and the performance should be determined by a larger number of the cat population.

In this study, seroprevalence of *Bartonella* spp. IgG was not associated with the prevalence of *B. henselae* DNA. Similar results were documented in a previous study (Lappin et al., 2009). The *Bartonella* spp. ELISA may be used to recognize whether a cat was exposed to *B. henselae*, but it should not be used to test for current infection. In addition, seroprevalence of *Bartonella* spp. IgG was not associated with fever in the cats described here which is the same as the result in the previous study (Lappin et al., 2009). However, there were 29% (47 cats) of PCR-negative cats that were positive by the *Bartonella* ELISA. The combination of PCR and ELISA results resulted in the detection of *Bartonella* in a total of 43.5% (87 cats) in this sample set, while only 20% of the cases could be identified by the PCR alone. The combined tests of PCR and ELISA were suggested to increase the detection of *Bartonella*-infected cats.

The overall prevalence of hemoplasma DNA was similar to the prevalence in cats in the previous study (Assarasakorn et al., 2012). Hemoplasma DNA of either 'Candidatus M. haemominutum' or *M. haemofelis* was commonly found in afebrile cats. 'Candidatus M. turicensis' was first identified in a healthy cat in Thailand in this study. Cats with hemoplasma infections may develop hemolytic anemia, particularly *M. haemofelis* that is more pathogenic (Foley et al., 1998; Sykes et al., 2007), and 'Candidatus M. turicensis' was recently been proven to be cause hemolytic anemia in cats (Willi et al., 2005). 'Candidatus M. haemominutum' was found to be associated with anemia in client-owned cats (Reynolds and Lappin, 2007). Anemic cats having hemoplasma DNA should be considered to be treated with antihemoplasma drug.

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No association was found between the prevalence of *Bartonella* spp. DNA and the prevalence of FIV or FeLV in this study. The absence of association between *Bartonella* spp. IgG and FIV infection is not surprised, and the result is consistent with previous studies (Glaus et al., 1997; Maruyama et al., 1998; Dubey et al., 2009). Rather, a negative association between seroprevalence of *Bartonella* spp. and the prevalence of FeLV antigen was found in this study. Care should be taken in interpreting this result because it was based on a low number of observations (2 cats were positive for *Bartonella* spp. IgG and FeLV).

Logistic regression analysis showed that no risk factors, but *B. henselae* DNA was associated with fever in cats. *Bartonella henselae*-infected cats of any age, sex, housing location, with or without flea infestation, and with or without flea control can develop fever. Infection with hemoplasmas, FeLV, and FIV was evaluated as possible contributing factors to the development of fever in this study. However, none of these infections was associated with fever in these cats. There were 8 febrile cats that were negative in all assays. The predisposing cause of fever in these cats was unknown.

In conclusion, the findings of this study strongly support that *B. henselae* should be considered as an etiologic agent of cats with fever of unknown origin, particularly cats with high bacterial load of *B. henselae*. The qPCR assay is suggested to be used for diagnosis of fever associated with *B. henselae* infection. Because of low sensitivity, the AC-ELISA is not appropriate for clinical use.

6.6 Acknowledgements

References

Assarasakorn, S., Veir, J.K., Hawley, J.R., Brewer, M.M., Morris, A.K., Hill, A.E., Lappin, M.R., 2012b. Prevalence of *Bartonella* species, hemoplasmas, and *Rickettsia felis* DNA in blood and fleas of cats in Bangkok, Thailand. Res. Vet. Sci. 93, 1213-1216.

Bradbury, C.A., Lappin, M.R., 2010. Evaluation of topical application of 10% imidacloprid-1% moxidectin to prevent *Bartonella henselae* transmission from cat fleas. J. Am. Vet. Med. Assoc. 236, 869-873.

Brunt, J., Guptill, L., Kordick, D.L., Kudrak, S., Lappin, M.R., 2006. American Association of Feline Practitioners 2006 Panel report on diagnosis, treatment, and prevention of *Bartonella* spp. infections. J. Feline Med. Surg. 8, 213-226.

Chomel, B.B., Boulouis, H.J., Maruyama, S., Breitschwerdt, E.B., 2006. *Bartonella* spp. in pets and effect on human health. Emerg. Infect. Dis. 12, 389-394.

Dowers, K.L., Hawley, J.R., Brewer, M.M., Morris, A.K., Radecki, S.V., Lappin, M.R., 2010. Association of *Bartonella* species, feline calicivirus, and feline herpesvirus 1 infection with gingivostomatitis in cats. J. Feline Med. Surg. 12, 314-321.

Dubey, J.P., Lappin, M.R., Kwok, O.C.H., Mofya, S., Chikweto, A., Baffa, A., Doherty, D., Shakeri, J., Macpherson, C.N.L., Sharma, R.N., 2009. Seroprevalence of *Toxoplasma gondii* and concurrent *Bartonella* spp., feline immunodeficiency virus, and feline leukemia virus infections in cats from Grenada, West Indies. J. Parasitol. 95, 1129-1133.

Foley, J.E., Harrus, S., Poland, A., Chomel, B., Pedersen, N.C., 1998. Molecular, clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in domestic cats. Am. J. Vet. Res. 59, 1581-1588.

Foley, J.E., Pedersen, N.C., 2001. '*Candidatus* Mycoplasma haemominutum', a low-virulence epierythrocytic parasite of cats. Int. J. Syst. Evol. Microbiol. 51, 815-817.

Glaus, T., Hofmann-Lehmann, R., Greene, C., Glaus, B., Wolfensberger, C., Lutz, H., 1997. Seroprevalence of *Bartonella henselae* infection and correlation with disease status in cats in Switzerland. J. Clin. Microbiol. 35, 2883-2885.

Guptill, L., Slater, L., Wu, C.C., Lin, T.L., Glickman, L.T., Welch, D.F., HogenEsch, H., 1997. Experimental infection of young specific pathogen-free cats with *Bartonella henselae*. J. Infect. Dis. 176, 206-216.

Guptill, L., Slater, L.N., Wu, C.C., Lin, T.L., Glickman, L.T., Welch, D.F., Tobolski, J., HogenEsch, H., 1998. Evidence of reproductive failure and lack of perinatal transmission of *Bartonella henselae* in experimentally infected cats. Vet. Immunol. Immunopathol. 65, 177-189.

Hoover, E.A., Mullins, J.I., 1991. Feline leukemia virus infection and diseases. J. Am. Vet. Med. Assoc. 199, 1287-1297.

Jensen, W.A., Fall, M.Z., Rooney, J., Kordick, D.L., Breitschwerdt, E.B., 2000. Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. J. Clin. Microbiol. 38, 1717-1722.

Jensen, W.A., Lappin, M.R., Kamkar, S., Reagan, W.J., 2001. Use of a polymerase chain reaction assay to detect and differentiate two strains of *Haemobartonella felis* in naturally infected cats. Am. J. Vet. Res. 62, 604-608.

Kordick, D.L., Brown, T.T., Shin, K.O., Breitschwerdt, E.B., 1999. Clinical and pathologic evaluation of chronic *Bartonella henselae* or *Bartonella clarridgeiae* infection in cats. J. Clin. Microbiol. 37, 1536-1547.

Laemmli, U.K., 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227, 680-685.

Lappin, M.R., Breitschwerdt, E., Brewer, M., Hawley, J., Hegarty, B., Radecki, S., 2009. Prevalence of *Bartonella* species antibodies and *Bartonella* species DNA in the blood of cats with and without fever. J. Feline Med. Surg. 11, 141-148.

Lappin, M.R., Greene, C.E., Prestwood, A.K., Dawe, D.L., Tarleton, R.L., 1989. Enzyme-linked immunosorbent assay for the detection of circulating antigens of *Toxoplasma gondii* in the serum of cats. Am. J. Vet. Res. 50, 1586-1590.

Lappin, M.R., Kordick, D.L., Breitschwerdt, E.B., 2000. *Bartonella* spp antibodies and DNA in aqueous humour of cats. J. Feline Med. Surg. 2, 61-68.

Maruyama, S., Hiraga, S., Yokoyama, E., Naoi, M., Tsuruoka, Y., Ogura, Y., Tamura, K., Namba, S., Kameyama, Y., Nakamura, S., 1998. Seroprevalence of *Bartonella henselae* and *Toxoplasma gondii* infections among pet cats in Kanagawa and Saitama prefectures. J. Vet. Med. Sci. 60, 997-1000.

Mietze, A., Morick, D., Köhler, H., Harrus, S., Dehio, C., Nolte, I., Goethe, R., 2011. Combined MLST and AFLP typing of *Bartonella henselae* isolated from cats reveals new sequence types and suggests clonal evolution. Vet. Microbiol. 148, 238-245.

Mikolajczyk, M.G., O'Reilly, K.L., 2000. Clinical disease in kittens inoculated with a pathogenic strain of *Bartonella henselae*. Am. J. Vet. Res. 61, 375-379.

O'Reilly, K.L., Bauer, R.W., Freeland, R.L., Foil, L.D., Hughes, K.J., Rohde, K.R., Roy, A.F., Stout, R.W., Triche, P.C., 1999. Acute clinical disease in cats following infection with a pathogenic strain of *Bartonella henselae* (LSU16). Infect. Immun. 67, 3066-3072.

Pedersen, N.C., Yamamoto, J.K., Ishida, T., Hansen, H., 1989. Feline immunodeficiency virus infection. Vet. Immunol. Immunopathol. 21, 111-129.

Quimby, J.M., Elston, T., Hawley, J., Brewer, M., Miller, A., Lappin, M.R., 2008. Evaluation of the association of *Bartonella* species, feline herpesvirus 1, feline calicivirus, feline leukemia

virus and feline immunodeficiency virus with chronic feline gingivostomatitis. J. Feline Med. Surg. 10, 66-72.

Reynolds, C.A., Lappin, M.R., 2007. "*Candidatus* Mycoplasma haemominutum" infections in 21 client-owned cats. J. Am. Anim. Hosp. Assoc. 43, 249-257.

Simard, C., Richardson, S., Dixon, P., Komal, J., 2000. Agar gel immunodiffusion test for the detection of bovine leukemia virus antibodies: lack of trans-Atlantic standardization. Can. J. Vet. Res. 64, 96-100.

Sykes, J.E., Drazenovich, N.L., Ball, L.M., Leutenegger, C.M., 2007. Use of conventional and real-time polymerase chain reaction to determine the epidemiology of hemoplasma infections in anemic and nonanemic Cats. J. Vet. Intern. Med. 21, 685-693.

Ueno, H., Hohdatsu, T., Muramatsu, Y., Koyama, H., Morita, C., 1996. Does coinfection of *Bartonella henselae* and FIV induce clinical disorders in cats? Microbiol. Immunol. 40, 617-620.

Willi, B., Boretti, F.S., Cattori, V., Tasker, S., Meli, M.L., Reusch, C., Lutz, H., Hofmann-Lehmann, R., 2005. Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with hemolytic anemia in Switzerland. J. Clin. Microbiol. 43, 2581-2585.

Whittemore, J.C., Hawley, J.R., Radecki, S.V., Steinberg, J.D., Lappin, M.R., 2012. *Bartonella* species antibodies and hyperglobulinemia in privately owned cats. J. Vet. Intern. Med. 26, 639-644.

CHAPTER 7

Conclusions and Future Directions

7.1 Conclusions

Bartonella henselae infection is important in cats for both clinical and zoonotic issues. Due to vague clinical signs which may develop in infected cats, diagnostic tests are necessary to help formulate the optimal treatment plan. A major goal of this work was to develop new quantitative assays which have adequate sensitivity and specificity for aid in diagnosis of diseases associated with *B. henselae* infection in cats. This dissertation described how these two new assays, the qPCR and the AC-ELISA were developed and demonstrated the performance of the assays. The qPCR assay performed very well with feline blood. The sensitivity and specificity of the qPCR assay was equivalent to the cPCR assay which is commonly used and considered a reference test. Moreover, the qPCR assay provides advantages over the cPCR assay, including rapidity and quantitative measurement. For the polyclonal-based AC-ELISA, the performances of the assay are not adequate to apply to clinical cases. The sensitivity of the AC-ELISA is inferior to that of the qPCR and the cPCR assays. The results of this study provide additional infectious disease agent prevalence information for cats in Bangkok, Thailand. The application of the qPCR assay to the samples of febrile and afebrile cats in Thailand suggest that using PCR to estimate bacterial load may aid in the diagnosis of fever associated with *B. henselae* infection. The results also provided additional information that *B. henselae* infection in cats can be associated with fever. The AC-ELISA applied to feline blood samples from Thailand did not show the association with fever and this assay should not be used for this purpose at this time. All of the work in this dissertation shows that the qPCR results are superior to non-

quantitative test results to make an association between *B. henselae* and fever. Quantification of *B. henselae* bacterial load in feline blood appears to be helpful for diagnosis of fever in cats and the qPCR should be used to assess for other disease associations. The AC-ELISA in this dissertation was a prototype assay and these results can be used to aid in the development of a more sensitive assay in the future.

7.2 Future Directions

7.2.1 The qPCR assay

While the qPCR assay described here has only moderate efficiency, its sensitivity was adequate for detecting *B. henselae* infection in cats, and is comparable with another currently being used PCR assay. However, if the efficiency of the assay can be improved, the limit of detection could be lower than the present assay. A higher number of samples with very low DNA quantities would likely be detected and additional information would be potentially gained. Several approaches can be performed in order to increase the stringency of assay, such as using different master mixes, adjusting the magnesium concentration, or using PCR enhancer/additives. The qPCR assay showed its ability for being used as a diagnostic tool. Although the statistical power of the study is not enough due to the limited number of samples from the cat population of interest (fever), it is suggested that bacterial load of *B. henselae* is likely to be associated with fever in some cats. A larger sample size is highly required in the next study. A qPCR cutoff value that consistently associated with fever was not determined in this work, but that will be evaluated further when additional samples are collected. The results of the qPCR assay may be evaluated in other clinical problems like uveitis to extend the clinical utility

of the assay. This work suggests that the qPCR assay can be a sensitive and cost-effective tool for diagnosis of feline bartonellosis.

7.2.2 The AC-ELISA

The performance characteristics, particularly the sensitivity of the AC-ELISA in this dissertation, are not adequate to detect *B. henselae* infection in cats. The assay is also not a reliable tool for diagnosis of *B. henselae* associated fever. The most important consideration to improve the assay is the efficiency of antibody. In this dissertation, the rabbit polyclonal antibodies used appeared to contain cross-reactive antibodies that increased the background signal of the assay. This finding significantly reduced the sensitivity and specificity of the assay. Sample processing can only improve the specificity, but the low sensitivity still exists. The use of monoclonal antibodies assay should be the antibody of choice for the AC-ELISA because cross-reaction can ultimately be eliminated. Due to high specificity between specific monoclonal antibodies and antigen, use of monoclonal antibodies is anticipated to decrease background as well as increase the sensitivity of the assay. Monoclonal antibody-based ELISA might be a cost-effective test for *B. henselae* antigen detection. Furthermore, the assay may be developed in the format of a point-of-care test.