Pathogenesis of Bartonella Henselae in the Domestic Cat: Use of a PCR-based Assay for the Detection and Differentiation of B. Henselae Genotype I and Genotype II in Chronically Infected Cats.

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PATHOGENESIS OF \textit{Bartonella henselae} IN THE DOMESTIC CAT: USE OF A PCR-BASED ASSAY FOR THE DETECTION AND DIFFERENTIATION OF \textit{B. henselae} GENOTYPE I AND GENOTYPE II IN CHRONICALLY INFECTED CATS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences through the Department of Veterinary Microbiology and Parasitology

by

Alma F. Roy
B. S., Louisiana State University, 1968
M. S., College of St. Francis, 1990
August 2000
To all of my grandchildren that
they may understand the value of education
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ABSTRACT

*Bartonella henselae* is a zoonotic agent in which the domestic cat serves as the natural reservoir, and humans acquire potentially serious infections associated with this microorganism. The purpose of this research is to contribute to the understanding of the pathogenesis of *B. henselae* in the domestic cat using a molecular approach.

Using sequence differences in a portion of the 16S rRNA gene between *B. henselae* genotype I, and *B. henselae* genotype II, a nested polymerase chain reaction (nPCR) was designed and used to investigate various phases of feline bartonellosis. The nPCR detected 3.2 organisms per milliliter of blood which is below the detection limits of standard bacterial culture. *Bartonella henselae* LSU 16 genotype II, *Bartonella henselae* Baby genotype II, *Bartonella henselae* 87-66 genotype I, and *Bartonella henselae* Houston-1 genotype I were used in this study to infect cats. The PCR assay detected *Bartonella* DNA in 40 blood samples that were culture negative. The bacteremia as determined by PCR lasted for a period of 1 to 9 weeks longer than determined by culture methods in 10 of the 16 cats. An episode of relapsing bacteremia occurred in two cats during the infection. Of the twenty-three cats examined, *Bartonella* DNA was detected in various tissues from 10 of the 23 cats. The spleen of nine of the 10 cats was positive for *Bartonella* DNA. The other tissues in which *Bartonella* DNA was detected included bone marrow, lymph node, kidney, lung, liver, brain, and heart valve. Histopathological lesions associated with nonspecific antigenic stimulation were seen in the cats but organisms could not be visualized in tissue. RNA expression analysis using the RT-PCR assay with primers specific for the 16S rRNA and the citrate synthase gene (*gltA*) of *Bartonella* detected no *Bartonella* RNA.
expression in the tissue of infected cats. *Bartonella* genotypes remained the same throughout the period of the acute bacteremia and in the recurring bacteremia in two cats as determined by the PCR assay. The persistent *Bartonella* DNA detected in tissue was *B. henselae* genotype II. *B. henselae* genotype I was not detected in any of the infected cats.
CHAPTER ONE  
REVIEW OF LITERATURE

Description and History of the Genus  
*Bartonella*

**Taxonomy.** The genus *Bartonella* is named for A. L. Barton who described *Bartonella bacilliformis* in 1909 as an intraerythrocytic bacterium. *Bartonella bacilliformis* was at one time the only species in the genus *Bartonella*. The genus *Bartonella* and *Grahamella* were the only two genera in the family *Bartonellaceae* that were in the Order *Rickettsiales*. With DNA-DNA hybridization techniques, the taxonomy and classification of the family *Bartonellaceae* was changed and the current *Bartonella* species, other than *B. bacilliformis*, changed names and taxonomy several times before being finally placed into the current order of *Bartonellaceae*. The 1984 edition of Bergey's Manual of Systematic Bacteriology divided the order *Rickettsiales* into three families namely, *Rickettsiaceae, Bartonellaceae*, and *Anaplasmataceae* (Weiss 1984). *Bartonella bacilliformis* was the only member of the genus *Bartonella*, and *Grahamella* was the other genus in the family *Bartonellaceae*. The *Rickettsiaceae* family initially included three genera, *Rickettsia, Coxiella*, and *Rochalimaea*. The first and current *Bartonella* species (other than *B. bacilliformis*), *B. quintana*, was first named *Rickettsia quintana* in 1917 as proposed by Schmincke, and is the causative agent of trench fever (Maurin 1996). *Rickettsia quintana* was then transferred to the genus *Rochalimaea* by Krieg in 1961 (Maruin 1996). The next *Rochalimaea* named was *R. vinsonii* isolated from voles (*Microtus pennsylvanicus*) in Canada (Weiss et al. 1978). The third *Rochalimaea* species, *R. henselae* had been visualized in tissue sections of lesions with Warthin-Starry staining but had not been successfully cultured.
or identified (Angritt et al. 1988; Leboit et al. 1988). Using the polymerase chain reaction (PCR) and oligonucleotide primers complementary to 16S ribosomal RNA genes of eubacteria, Relman amplified the 16S ribosomal gene fragment of *Rochalimaea henselae* directly from tissue samples of patients with bacillary angiomatosis (Relman et al. 1990). Slater et al. (1990) and Welch et al. (1992) then isolated the organism on solid medium supplemented with horse or human blood from the blood of patients infected with the human immunodeficiency virus (HIV), transplant recipients, immunocompetent patients with bacteremia and a patient with peliosis hepatis (Slater et al. 1990; Welch et al. 1992). Based on DNA-DNA hybridization data and comparison of existing 16S rRNA gene sequences, *Rochalimaea* and *Coxiella* were removed from the *Rickettsiaceae* family, and the genus *Rochalimaea* were renamed *Bartonella* (Brenner et al. 1993). The genus *Rochalimaea* was transferred from the family *Rickettsiaceae* to the family *Bartonellaceae* and united with the genus *Bartonella*. The family *Bartonellaceae* was removed from the order *Rickettsiales* (Brenner et al. 1993). The current genus *Bartonella* was created. *Bartonella* species were removed from the order *Rickettsiales*, and the genus *Bartonella* and *Grahamella* were combined. Closely related genera based on the sequence homology of the 16S rRNA gene are the *Brucella* and the *Agrobacterium* (Regnery et al. 1992; Anderson and Neuman 1997). The genus *Bartonella* is in the family *Bartonellaceae* and belongs to the alpha-2-subdivision of the class *Proteobacteria*. There are presently 14 recognized species of *Bartonella*. Included in the group is *B. henselae*, *B. bacilliformis*, *B. quintana*, *B. elizabethae* (all human pathogens), *B. vinsonii*, *(B. vinsonii* subspecies
berkhoffi), B. grahamii, B. taylorii, B. doshiae, B. talpae, B. peromysci, B. clarridgeae, B. koehlerae, B. alsatica, B. tribocorum.

**Description of the genus.** *Bartonella* species are small (0.6 by 1.0 um) gram negative rods that are often slightly curved. *Bartonella* species in general are biochemically inert (Bergey’s Manual, Ninth edition). They are oxidase, catalase, and urease negative, aerobic, and highly fastidious. They do not produce acid from carbohydrates. *B. bacilliformis* possesses polar flagella whereas *B. henselae* and *B. quintana* display twitching motility due to the presence of pili (Welch and Slater, 1999). The genome sizes have been estimated to be $1.5 \times 10^6$ bp for *B. quintana* and $2 \times 10^6$ bp for *B. vinsonii* (Meyers et al. 1980) and the guanine plus cytosine content of the genomes for *Bartonella* species is estimated at 39 to 40% (Weiss et al.1978; Tyeryar et al.1973; Welch et al.1992). Plasmids have not been described (Kreuger et al. 1995). A bacteriophage particle has been identified in both *B. bacilliformis* (Umemori et al. 1992) and *B. henselae* (Anderson et al.1994). Gene sequences of *B. henselae* published are those encoding the citrate synthase, 16S rRNA (Daly et al. 1993; Regnery et al. 1993), 5S rRNA, 23S rRNA genes, intergenic regions between rRNA genes (Minnick 1994), a 60-kDa antigen in *B. henselae* (Anderson et al.1993) and a unique 17-kDa gene (Anderson et al. 1995).

**Bartonella species and human disease.** The disease syndrome attributed to *B. bacilliformis* is bartonellosis or Carrion’s disease. Carrion’s disease is geographically confined to the South American Andes region. Its regional occurrence is the result of the limited distribution of the sand fly (*Lutzomyia verrucarum*), the vector. Carrion’s disease is a biphasic disease consisting of an acute hemolytic anemia (Oroya fever) and
a chronic form (verruga peruana) that presents with vascular proliferative skin lesions. Evidence from the artifacts of pre-Columbian culture suggests that the verruga peruana form of Carrion's disease was present in Ecuador at least 1,000 years before the arrival of Europeans (Anderson and Neuman 1997).

*Bartonella quintana*, the causative agent of trench fever was first identified among battlefield troops in World War I (Review by Anderson and Neuman 1997). It was estimated that over 1 million troops were affected. The disease was characterized by a 5-day relapsing fever, with severe and persistent pain in the shins, and although rarely fatal, it resulted in prolonged disability. The disease also affected troops in World War II. Trench fever was a debilitating febrile illness characterized by fever, rash, bone pain and splenomegaly. The disease is often associated with poor sanitation and personal hygiene, and is communicable by means of the body louse (*Pediculus humanus*). Infection is thought to be transmitted from lice to humans via the arthropod excreta, which enters the body through broken skin (Maurin and Raoult 1996). Since World War II infections associated with *B. quintana* were rare until the description of infections in human immunodeficiency virus (HIV)-infected patients. The reason for the reemergence of *B. quintana* infections after many years of apparent absence is unclear (Relman 1998).

In addition to causing diseases with which *Bartonella* has been historically connected, members of the genus *Bartonella* have been isolated from a range of emerging diseases with a wide array of clinical syndromes. *B. henselae* is associated with diseases with granulomatous features such as cat scratch disease, and vascular proliferative disease, such as bacillary angiomatosis, peliosis, and epidemics of
bacteremic illness. *B. quintana* is associated with bacteremic illness, endocarditis, and bacillary angiomatosis and peliosis, but not cat scratch disease. *B. quintana* infection is epidemiologically associated with homelessness, low income, and exposure to lice (Koehler et al. 1997). *B. quintana* infections are seen in HIV-infected patients, chronic alcoholics, and homeless individuals (Drancourt et al. 1993; Koehler et al. 1992; Spach et al. 1993).

*B. henselae* is associated with bacteremic illness, endocarditis, bacillary angiomatosis, peliosis, and cat scratch disease. *B. henselae* infections are epidemiologically linked to cat and flea exposure (Koehler et al. 1997). The most common *Bartonella* infection in humans is cat scratch disease (CSD). In the United States the number of CSD cases is estimated to be approximately 25,000 annually (Welch and Slater 1999). Typical CSD occurs in about 89% of the cases and is usually a self-limiting disease with fever and lymphadenopathy, usually preceded by an erythematous papule at the inoculation site (Welch and Slater 1999). Most cases of CSD are resolved within 2-6 months (Margilith et al. 1992). About one-third of the patients have a fever, and about one-sixth develop lymph node suppuration (Welch and Slater 1999). Inflammatory reactions include granulomata and stellate necrosis seen on histopathological examination of the nodes (Welch and Slater 1999). Atypical CSD occurs in about 11% of the cases. Complications resulting from atypical CSD include encephalitis, retinitis, hepatic and splenic abscesses, pneumonia osteomyelitis, lytic bone lesions, conjunctivitis, and central nervous system involvement (Anderson 1997).

Other *Bartonella* species involved in human disease include *B. elizabethae*, which was isolated from a single patient with endocarditis (Daly et al. 1993), and *B.
clarridgeiae, which has been isolated from a patient with cat scratch disease (Kordick et al. 1997a). *Bartonella vinsonii* subsp. *berkhoffii* was reported in a case of endocarditis in a human that was blood culture-negative and afebrile (Roux et al. 2000). In that case, Bartonella DNA was detected by PCR from valvular material removed during aortic valve replacement. The bacterium was never cultured.

**Bartonella species not related to human disease.** *Bartonella vinsonii*, *B. grahamii*, *B. taylorii*, *B. doshiae*, *B. talpae*, *B. koehlerae*, and *B. peromysci* have all been identified in a wide range of mammalian species, including felines and rodents (Birtles et al. 1995; Heller et al. 1997; Chomel et al. 1995; Droz et al. 1999) but are all presently considered nonpathogenic to humans. *B. vinsonii* has been isolated from vole and canines and designated *B. vinsonii* subsp. *vinsonii* and *B. vinsonii* subsp. *berkhoffii*, respectively (Kordick and Breitschwerdt 1998). *B. vinsonii* subsp. *vinsonii* was first isolated in 1946 from the spleen of a vole and was not described again until 1995 when *B. vinsonii* subsp. *berkhoffii* was isolated from a dog with vascular endocarditis (Breitschwerdt and Kordick 1995). In North Carolina and Virginia 3.6% of 1,920 dogs were seropositive for *B. vinsonii* subsp. *berkhoffii* (Pappalardo et al. 1997). A study by Chang et al. (1998) demonstrated the prevalence of antibodies (7% to 51%) to *B. vinsonii* subsp. *berkhoffii* in coyotes (*Canis latrans*) from parts of northern California. The seroprevlaence of *Bartonella* in coyotes in California was from the coastal counties and thought to be vector transmitted from wildlife to domestic dogs (Chang et al. 1998).

**Bartonella henselae**

**Background and history.** *Bartonella henselae*, a member of the family *Bartonellaceae* has emerged as an important microorganism in the last 5 years.
*Bartonella henselae* is a zoonotic agent causing a wide array of human disease syndromes with the domestic cat as a major reservoir for the organism. In humans, *B. henselae* is associated with cat scratch disease, bacillary angiomatosis, and bacillary peliosis. The search for the infectious agent responsible for these new emerging diseases and the connection to the domestic cat evolved slowly and involved clinical medicine, traditional microbiology, and genetic approaches. The various manifestations of CSD have been recognized for over 100 years but “la maladies des griffes du chat” was not identified as a syndrome until 1950 (Debre et al, 1950). The search for the etiologic agent of CSD has been long with the first described clinical cases in 1931 by Debre and Semelaigne and published in 1950 (Shinall 1990). Organisms that have been reported as the potential etiologic agent of the disease included a herpes-like virus (Kalter et al.1969), a hemagglutinating virus (Turner et al. 1959), *Chlamydia* (Emmons et al. 1976), and an acid-fast bacillus (Boyd 1961). Despite the unknown etiology, a clinical diagnosis of CSD was based on the presence of three criteria: 1) history of animal contact with the presence of a scratch or an inoculation lesion of the skin, eye or mucous membrane; 2) a positive CSD skin test (CSD antigen); 3) regional lymphadenopathy with negative laboratory results for other causes of lymphadenopathy; and 4) characteristic histopathologic changes consistent with CSD in a biopsied lymph node (Margileth 1988).

In 1988, the Armed Forces Institute of Pathology named *A. felis* as the agent of CSD (Regnery and Tappero 1995). Wear et al. (1983), reported finding bacteria in lymph nodes of cat scratch disease patients meeting the criteria by using the Warthin-Starry silver stain. Then in 1988, English et al. (1988) reported the isolation, of a
bacterial agent from a lymph node of a CSD patient's. The isolate, *Afipia felis*, was named after the Armed Forces Institute of Pathology (*Afipia*), the source of the original isolate, and *felis* referring to the presumed vertebrate vector of CSD (Wear et al. 1983; English et al. 1988; Regnery and Tappero 1995). Upon further investigation, other laboratories could not recover isolates of *A. felis* from CSD patients, and a serological link between *A. felis* and cats could not be demonstrated (Bergmans et al. 1995; Regnery and Tappero 1995).

The next part of the story and the true etiologic agent of CSD emerged when patients infected with human immunodeficiency virus (HIV) developed cutaneous and subcutaneous vascular lesions containing bacillary organisms seen on Warthin-Starry silver stain described as indistinguishable from bacillary organisms seen in CSD patients (Stoler et al. 1983, Koehler et al. 1993; Tappero et al. 1993). Zangwill et al. (1993), reported that patients with bacillary angiomatosis had a household kitten under the age of 1 year and reported traumatic contact with a cat. Relman (1990) identified DNA from bacillary angiomatosis skin lesions that was not necessarily identical to but was related to *Rochalimaea quintana*. Independently but about the same time in Houston, Texas, *Rochalimaea*-like organisms were isolated from the blood of HIV-infected patients with relapsing fever of unknown origin. In Oklahoma, Welch et al. (1992) isolated a similar organism and coined the new species designation *R. henselae* in recognition of the contribution of Diane Hensel, who initially isolated several of the organisms in Oklahoma (Regnery et al. 1992a). Regnery et al. (1992b) then developed an indirect fluorescence antibody (IFA) using the Houston-1 isolate of *R. henselae* and found high antibody titers to *R. henselae* in patients identified with
HIV-infected bacillary angiomatosis and later in CSD patients (Tappero et al. 1992; Regnery et al. 1992). This was the first laboratory evidence that *R. henselae* was associated with CSD. *R. henselae* was isolated directly from the lymph nodes of two CSD patients and identified in 1993 by Dolan et al. Using archival sources 93% of the skin-test positive CSD patients tested positive by IFA to *Rochalimaea* antigen (Szelc et al. 1992). Also, pasteurized exudate collected from suppurative CSD lymph nodes used in the skin test as antigen was shown by PCR to contain *Rochalimaea* nucleic acid sequences and *R. henselae* sequences in particular (Perkins et al. 1992; Anderson et al. 1993).

A role for the cat in the life cycle of *R. henselae* was proposed following the discovery of *R. henselae* as the etiologic agent of CSD. Involvement of the cat in the life cycle of *B. henselae* was demonstrated by Bartonella-specific IFA antibodies in 39 of 48 cats living in households reporting human CSD in a study by Zangwill, 1993. After detecting anti-*Bartonella henselae* antibodies in a cat by IFA, Regnery et al. (1992a) isolated *B. henselae* from the blood of a single cat not linked to human illness. *R. henselae* was isolated from the blood of cats whose owners were diagnosed with bacillary angiomatosis established the cat as a reservoir for *R. henselae* infection (Koehler et al. 1994). Cat ownership was then considered a risk factor for diagnosis of CSD and bacillary angiomatosis. In the last decade the reservoir, the possible vectors, the diseases, the epidemiology, the genetic characteristics, and many other aspects of *B. henselae* have been studied. Despite all these years, many aspects of feline bartonellosis are still unclear.
B. henselae, the causative agent of cat scratch disease (CSD), has been linked by epidemiologic and serologic studies to cats (Zangwill, et al. 1993; Childs et al. 1994; Chomel et al. 1995; Demers et al. 1995; Koehler et al. 1994). Contact with cats, especially kittens, has been shown to be a significant risk factor (Zangwill et al. 1993). In the vast majority of CSD cases, patients have been bitten or scratched by a cat. In a small percentage of CSD patients, no history of animal contact was reported (Caithers et al. 1969).

The role of the flea (Ctenocephalides felis) as a vector for transmission has been explored. B. henselae has been amplified by PCR from the flea gut after 24 hours and nine days after feeding on cat blood seeded with B. henselae (Higgins et al. 1996). Using an IFA assay Higgins et al. (1996) were able to observe B. henselae in dissected guts of fleas after 3 hours and at 9 days after infectious feeding. Qualitatively the number of fluorescing bacteria were more plentiful in flea guts at 9 days. Experimental transmission of B. henselae between cats by way of a cat flea vector has been demonstrated (Chomel et al. 1996). The route of the exposure from cat to cat was not clear until an experimental infection of domestic cats with B. henselae by inoculation of flea feces alone (Foil et al. 1998). Direct transmission of B. henselae from cat fleas to humans has not been demonstrated. Reports of tick bites before infection with B. henselae have been reported (Lucey et al. 1992).

Cat scratch disease accounts for approximately 24,000 cases annually in the United States, with an estimated 2,000 hospital admissions (Jackson et al. 1993). The estimated health-care cost of CSD in 1992 in the U.S. is $12 million per year (Jackson et al. 1993). The peak incidence of cases of CSD is in persons between ages 2 and 14.
The incidence is reported to be higher in males (60%) and in whites (Klein et al. 1994) and is seasonal with most cases recognized in the second half of the year (Carithers 1985). Individual reports from numerous countries suggest a worldwide distribution of CSD (Flexman et al. 1995; Ueno et al. 1995; Waldvogel et al. 1995).

**Bartonella henselae and Cats**

**Prevalence.** Prevalence studies performed in both cats and human patients to determine seroprevalence of *B. henselae* use the indirect fluorescent-antibody test (IFA), the enzyme-linked immunoabsorbent assay (ELISA), and the western blot analysis. Several prevalence studies done in cats have shown the sensitivity (86.2% ELISA versus 88% IFA) and specificity (95.9% ELISA versus 94% IFA) when measuring IgG between the IFA and ELISA to be similar (Guptill et al. 1997; Regnery et al. 1996). Other studies, done by Bergmans et al. (1997) on human patients have pointed out the pitfalls and fallacies of serological tests and showed preparation of *Bartonella*-specific antigen influenced the outcome of the test, and measurement of IgM (71%) was shown to have a greater sensitivity than that of IgG (40.9%) serology. In other studies *B. quintana* and *B. clarridgeiae* have been shown to serologically cross-react with *B. henselae* antigens thus influencing seroprevalence of *B. henselae* in cats (Childs et al. 1995; Kordick et al. 1997a; Freeland et al. 1999). Nevertheless, the prevalence of *B. henselae* in cats as determined by serologic evidence using *Bartonella*-specific antigens and by the presence of bacteremia in cats has been reported and varies according to the type of cat population (pet or stray), age of the animal, gender, region of the country as influenced by weather patterns and flea infestation of cats. Chomel et
al. (1995) found impounded and former stray cats were 2.86 times more likely to be bacteremic than pet cats and young cats (<1 year old) were 1.64 times more likely than adult cats to be bacteremic, and bacteremic cats were more likely than nonbacteremic cats to be infested with fleas. In a study by Zangwill et al. (1993) in Connecticut, 46% of the pet cats not associated with human disease and 81% of cats living in households reporting human CSD were seropositive. Childs et al. (1995) found 44.4% (n=9) of feral cats, 11.8% of stray cats (n=195), and 12.5% of cats (n=24) in veterinary hospitals were seropositive in Baltimore, Maryland and that cats are more likely to acquire and transmit *B. henselae* in the first year of their life. Childs et al. (1995) found that 28% of the cats in a group of 1,370 cats from an animal shelter in Maryland had antibodies to *B. henselae*. Jamerson et al. (1995) found a higher seroprevalence in southeastern United States, Hawaii, coastal California, the Pacific Northwest and the south central plains 54.6%, 47.4%, 40.0%, 34.3%, and 36.7%, respectively. Alaska, the Rocky Mountain-Great Plains region, and the Midwest had low average prevalence of 5.0%, 3.7%, and 6.7%, respectively. The overall seroprevalence throughout these regions was 27.9% (n=628) (Jameson et al. 1995). The seroprevalence of *B. henselae* in cats in this study appeared to be influenced by climate. Foley et al. (1998) surveyed a group of 11 catteries from diverse geographical locations in North America and found a seroprevalence of 35.8%.

*Bartonella henselae* has a worldwide distribution. The seroprevalence varies widely among different countries and geographic regions within countries. Seroprevalence of *B. henselae* ranges from 3.7% to 65.4% in the United States, and from 6 to 22% in Japan (Yoshida et al. 1996; Breitschwerdt and Kordick 1995; Childs, et al.1994;
Chomel et al. 1995, Jamerson et al. 1995). In Australia the seroprevalence is 33%, in Egypt 12%, and in Portugal 7% (Ueno et al. 1995; von Alleerberger et al. 1995). In a study in the Netherlands by Bergman et al. (1997), using the enzyme-linked immunoassay 50% (n=113) of sheltered cats were seropositive, 56% (n=50) of pet cats were seropositive, and 22% of sheltered cats were bacteremic; none of the specific-pathogen free cats were seropositive. In an Indonesian cat population Marston et al. (1999) found 54% (n=70) were positive by IFA and 43% (n=14) of feral cats were culture positive for *B. henselae*. The prevalence of *B. henselae* in Germany determined by blood culture from household cats was 13% (n=100) (Sander et al. 1997). In this study young, female cats (24 months of age or younger) were more likely to be found positive for *B. henselae*.

**Strain variability.** *B. henselae* is a zoonotic agent and has adapted to survive and replicate in the human host (incidental host) and the feline host (natural host) (Relman 1998). Both the diversity of strains within cat reservoirs and that cause various human clinical syndromes has not been compared extensively. *B. henselae* genotype differences have been described using various genotypic methods. *B. henselae* causes a diverse spectrum of diseases in humans that range from self-limiting to severe. Because of the diverse pathology and different host adaptation, strain variability would be expected with *B. henselae*. The difference in the clinical presentation of *B. henselae* in humans and cats suggest that different strains may exist. It is expected that there will be greater strain diversity in the natural host (cat) than within the incidental human host (Relman 1998). The majority of available clinically relevant *B. henselae* strains have

The first isolate of *B. henselae* was named *B. henselae* Houston-1 and was isolated from an HIV-infected patient in Houston and identified as the prototype strain (Regnery et al. 1992). The next human strain was isolated from the blood of febrile patients with AIDS in Oklahoma and designated *B. henselae* ATCC 49793 (Slater et al. 1990). These two strains were identified by genotypic methods including ribosomal RNA gene analysis and were similar. Arvand et al. (1997) isolated the first *B. henselae* from Europe. This group isolated *B. henselae* from the bacillary angiomatosis lesions of an HIV-infected patient in Germany and named it *B. henselae* Berlin-1. Using pulsed-field gel electrophoresis of endonuclease-restricted chromosomal DNA, the Berlin-1 isolate was compared to the *B. henselae* strains isolated from patients in the United States (Arvand et al. 1997). The two strains were indiscernible suggesting that these strains might represent one clone. The Berlin-1 strain was similar to the ATCC 49793 (Oklahoma strain) but did have one differing band, indicating a close relationship between the strains. A feline blood isolate named Berlin-2 was also compared in the Germany study and displayed a digestion profile with at least eight different bands which would be a distinct clone (Arvand et al. 1997). Using the 16S rRNA gene sequence analysis, Bergmans et al. (1994) found a difference of 3 bp located at positions 172 to 175 of the 16S rRNA gene of *B. henselae* from CSD patients. The *B. henselae* isolates were then divided into 2 groups, genotype I and genotype II according to 16S rRNA gene sequence. *B. henselae* genotype I has a TAG sequence and *B. henselae* genotype II has a ATT sequence between the 172 and 175 region of the 16S
rRNA gene (Bergman et al. 1994). Genotype I was identical to the sequence describe by Regnery et al. (1992) as Houston-I and genotype II was identical to the so called “BA-TF sequence found by Relman et al. (1990) in patients with bacillary angiomatosis. Using two type-specific PCRs that could discriminate between B. henselae variants, Bergman et al. (1996) investigated the B. henselae strains in samples from cat-scratch disease patients in The Netherlands. Bergmans et al. (1996) found 32 of 41 Bartonella DNA-positive samples from Dutch patients with CSD contained B henselae genotype I, seven samples contained B. henselae genotype II and 2 samples were found to be negative in both type-specific PCRs. Similarly, Sander et al. (1999) found 23 of 39 of the PCR-positive patients in Switzerland were infected with B. henselae genotype I, and 9 of 39 were infected with B. henselae genotype II. Heller et al. (1999) found genotype I in 59% of Dutch patients and genotype II in 23% of these patients with cat scratch disease.

Further work was done to investigate the genotypes isolated from cats. Several studies suggest that B. henselae genotype II is more common among cats than B. henselae genotype I (Bergmans et al. 1997; Heller et al. 1997). Heller et al. (1997) characterized B. henselae isolates from cats in France and found 34% of the isolates were B. henselae genotype I and 36% of the isolates were B. henselae genotype II. Bergmans et al. (1997) found typed strains of B. henselae isolates from cats in the Netherlands using Bartonella-specific PCR and hybridization assay and by 16S-23S spacer PCR-restriction fragment length polymorphism (RFLP). Three B. henselae types were found according to the RFLP patterns and were labeled A, B, and G (Bergmans et al. 1997). RFLP pattern A corresponds to 16S rRNA genotype I, while RFLP pattern B
corresponds to genotype II. RFLP pattern G did not correspond to either 16S rRNA genotype I or genotype II. RFLP pattern B (genotype II) was found more common within the cats that typed as A or G. The various strains of B. henselae have been shown to influence the immunological response of the host. Drancourt et al. (1996), have documented CSD patients who were seronegative on a standard IFA diagnostic test for *B. henselae* Houston-1 antigen but were seropositive for a Marseille strain of *B. henselae* (Drancourt et al. 1996). Other studies have shown that up to 60% of CSD patients may test negative in conventional *B. henselae* seroassays (Dupon et al.1996; Yoshida et al.1996). Yamamoto et al. (1998) has shown the lack of cross protection between *B. henselae* genotype I and genotype II. Freeland et al. (1999) demonstrated at least three antigenic differences between genotype I strain (Houston-1) and a genotype II strain LSU 16.

**Pathogenesis.** The pathogenesis of *B. henselae* in cats is not clearly understood. The pathogenesis of an infectious disease is influenced by many factors involving the host and the infectious agent. The immunological response of the host, the age, feline genetic differences, additional infections, the dose, the route of entry into the host, virulence factors of the microorganism, and the strain of the microorganism, are some of the factors that influence the pathogenesis of an infectious agent. Clinical signs in experimentally infected animals range from no overt clinical signs to fever, mild anorexia, lymphadenopathy, central nervous signs including abnormal behavior and vacant staring, signs of focal motor seizures, nystagmus, and rigidity (Kordick et al.1999; O’Reilly et al. 1999a). Numerous experimental *B. henselae* infections have been induced in cats and a wide range of clinical responses seen (Green et al. 1996;
Regnery et al. (1996) established a bacteremia and a Bartonella-specific IgG antibody response in 31 cats inoculated with B. henselae Houston-1 strain and did not observe clinical disease in any of the animals. In a study by O'Reilly et al. (1999a), acute clinical disease with bacteremia, antibody response, and adverse clinical signs were established in cats using a pathogenic strain of B. henselae called LSU 16. Bacteremia can be established in most cats within 1 to 2 weeks after inoculation (Regnery et al. 1996; Guptill et al. 1997; O'Reilly et al. 1999a). The magnitude and duration of the bacteremia is varied. The magnitude of bacteremia in experimental infection is reported to be significant and sometimes exceeds 10^6 CFU/ml of blood (O'Reilly et al. 1999a). Bacteremia can persist for weeks to months, and in some cats, a relapsing bacteremia occurs in both experimental and naturally occurring Bartonella infections (Regnery et al. 1996; Guptill et al. 1997; Kordick et al. 1999a). Intervals of culture-negative cats can be random and vary in duration. Kordick et al. (1999) reported intervals of 1 to 4 months of culture negative results before recurrent bacteremia could be detected.

Pathogenesis of B. henselae at the cellular and molecular levels is poorly characterized. Bartonella are most closely related to Brucella based on the phylogenetic relationships of the 16S rRNA gene sequence analysis. Bartonella, like Brucella are considered intracellular pathogens and can cause chronic bacteremia probably because of the ability to survive in macrophages and because of the relative low virulence of these organisms. Several studies have shown attachment and entry of Bartonella into several types of cells. Batterman et al. (1995) has shown attachment to and entry of B. henselae into human epithelial cells. Bundle-forming pili have been
demonstrated by electron microscopy in *B. henselae* and are thought to be an important virulence factor. A strong correlation between degree of attachment to epithelial cells and degree of piliation has been shown (Batterman et al. 1995). Batterman et al. (1995) demonstrated a relationship between piliation and phase variation. The two phases of the colonies noted were dry colonies embedded in the agar and mucoid colonies. *B. henselae* 87-66 (ATCC 49793) produced dry colonies which deeply embedded in the agar whereas *B. henselae* ATCC 49793 colonies were mucoid and did not pit the agar. The heterogeneity in colony types suggests a phase variation and is seen in multiple passages on laboratory media. Batterman et al. (1995) demonstrated that the rough adherent colony type *B. henselae* 87-66 expressed pili and that most of the smooth colony type of *B. henselae* ATCC 49793 had few pili (Batterman et al. 1995). Zbinden et al. (1995) demonstrated intracellular location within the Vero cells mainly around the nuclei. Presently however, mechanisms of entry have not been discovered. Clarridge (1996) demonstrated a cohesive factor in *B. henselae* associated with organisms existing in a tightly packed array. Clarridge suggested that the cohesive growth factor of *B. henselae* might help in resistance to antibiotic penetration and contribute to the recurrence of infection (Clarridge 1996).

Species of *Bartonella* have been shown to have a positive tropism for endothelial cells and red blood cells. Entry of *B. henselae* into the human endothelial cells has been demonstrated by Dehio et al. (1997). In vitro studies have shown *B. henselae* to colonize the vascular tissue with vascular uptake of large bacterial aggregates by engulfment (Dehio et al. 1997). The organism first accumulates and aggregates on the endothelial surface and then enters and forms a structure called an invasome, which
contains large and completely internalized bacterial aggregates (Dehio et al. 1997).

Histological analysis demonstrates the presence of bacterial clumps in close association within proliferating endothelial cells (Dehio et al. 1997). In other human studies it has been shown that *B. henselae* attaches and enters pericytes, macrophages and neutrophils (Anderson et al. 1997; Maurin and Raoult 1996).

Evaluation of bacteremic cats during natural and experimental infections suggests the presence of intracellular, epicellular, and extracellular bacteria in the blood, with erythrocytes invasion being limited to a small percentage of the bacterial population (Mehock et al. 1998). Intracellular location within the cat has been limited to the feline erythrocytes (Kordick and Breitschwerdt 1995). In these studies erythrocytes of persistently bacteremic cats, were studied, and identified 2.9% of the erythrocytes in cat A were infected whereas 6.2% of the erythrocytes in cat B were infected with *B. henselae*. Epicellular or extracellular organisms were not found in the blood of either cat A or B in this study. Kordick and Breitschwerdt (1995) speculated that engulfment of the bacteria by the erythrocyte membrane was a bacterium-induced or that forced endocytosis was involved in the engulfment. In vitro studies have suggested an intracellular location of *B. henselae* in the feline erythrocytes (Mehock et al. 1998). Mehock et al. (1998) demonstrated invasion rates of the feline erythrocytes to be from 2 to 20% of the inoculum. Using laser scanning confocal microscopy to discriminate between intracellular and epicellular *B. henselae* cells, less than 1% of the erythrocytes were determined to be infected. The role that invasion of feline red blood cells plays in the pathogenesis of *B. henselae* has been speculated to have at least four potential benefits: 1) immune evasion, 2) hemin acquisition, 3) survival in the cat flea, and
4) spread to other anatomical sites such as the liver or spleen (Mehock et al. 1998).

Identification of antigenic proteins and subunits of specific virulence factors of *B. henselae* has been limited. Burgess (1998) found that a 43 kDa outer membrane protein was a major adhesin for human umbilical vein endothelial cells. A 17-kDa outer membrane protein from *B. henselae* was shown reactive with human sera from CSD patients (Anderson et al. 1995; Sweger et al. 2000). Freeland et al. (1999) showed the 11.3, 13.3, and 16.9/18.0 kDa antigens, to be immunodominant in 83% (n=482) of seropositive cats naturally infected with *B. henselae*.

In vivo studies have demonstrated low endotoxin potency and diminished induction of neutrophilic oxidative metabolism, degranulation, and chemotaxis by *B. henselae* when compared to *E. coli* and *S. typhi* (Fumarola et al. 1994).

**Mode of transmission, vector.** Several *Bartonella* species have been shown to require an ectoparasite for transmission. *B. bacilliformis* is transmitted by the *Phlebotomus* sand fly, (Schultz et al. 1968), and *B. quintana*, the etiological for trench fever is transmitted by the human body louse, *Pediculus humanus* (Strong et al. 1918). The association of *B. henselae* with the cat flea, *Ctenocephalides felis*, has been shown in several studies (Foil et al. 1998; Higgins et al. 1996; Chomel et al. 1996). In an epidemiological study by Zangwill et al. (1993), the risk factors associated with the development of cat scratch disease were established. Ownership of a kitten was the most significant risk factor noted. The finding of one kitten with fleas helped establish the role of the flea as a biologic or mechanical vector in the transmission of *B. henselae* in cats. Studies have detected *B. henselae* DNA by PCR from fleas of infected cats (Koehler et al. 1994; Bergmans et al. 1997). Higgins et al. (1996) demonstrated that *B.*
henselae was viable after 9 days in flea feces of fleas feed on infected cats. Tompkins (1997) suggested that infection with B. henselae could occur by means of flea feces inoculated into a cutaneous site. Chomel et al. (1996) demonstrated that the cat flea transmits B. henselae from infected cats to noninfected cats by removing fleas from one group to the other. In the study by Foil et al. (1998), experimental infection of cats with B. henselae was established by inoculation of flea feces that had been collected from infected cats.

Clinical manifestations. B. henselae infections in humans result in a wide array of diseases. Because clinical infection in cats can be asymptomatic, natural infections may have gone unnoticed for many years. Early reports suggested that cases of regional and generalized feline lymphadenopathies of unknown cause were associated with Bartonella infections in cats (Kirkpatrick et al. 1989). Argyrophilic bacteria were seen in the lymph nodes of cats with persistent idiopathic lymphadenopathy (Kirkpatrick et al. 1989). Epidemiological studies indicate that a large number of cats have been exposed to B. henselae or are infected with the organism (Childs et al. 1995; Chomel et al. 1995; Jameson et al. 1995; Koehler et al. 1994). Naturally infected cats appear to tolerate chronic infections without obvious clinical abnormalities. Cats can remain asymptomatic and bacteremic for many months (Koehler et al. 1994; Kordick et al. 1995). Several experimental infections have attempted to establish the clinical signs in cats. Clinical presentations in experimentally infected cats have varied from no clinical signs (Regnery et al. 1996; Abbott et al. 1997) to mild clinical signs with fever and histopathologic lesions (Guptill et al. 1997) to acute clinical disease with fever, anorexia, and neurological signs (Kordick et al. 1997b; O'Reilly et al. 1999a). Lappin and Black

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(1999) have reported a case of anterior uveitis in a cat in which antibodies to *Bartonella* species could be detected in serum and aqueous humor. Bacteremia in cats is a clinical presentation that is consistent with infection with *B. henselae*. Bacteremia can be of significant magnitude with counts ranging from 999 to 33,300 CFU/ml (Greene et al. 1996). Bacteremia can last for weeks to years and then relapse (Kordick et al. 1999). In studies by Greene et al. (1996), recrudescent bacteremia in infected cats with lower magnitude (6 to 3,300 CFU/ml) was seen. In the acute phase of the infection in cats, bacteremia was associated with febrile signs, whereas in recurrent episodes of bacteremia, fever not a usual clinical sign (Kordick et al. 1999).

**Persistent infections.** Persistent infections are known to occur in association with many bacteria, viruses, and protozoa. Examples of microorganisms involved in persistent infections include *Salmonella typhi* which can persist in the gall bladder and urinary tract and is intermittently shed in urine and feces; *Mycobacterium tuberculosis*; Herpes simplex; Varicella-Zoster; *Plasmodium vivax*; and *Toxoplasma gondii*. In general, one way of explaining persistent infections is to regard them as a failure of the host’s antimicrobial forces to eliminate the invading microorganism from tissue. Persistent infections cannot by definition be acutely lethal and in fact, they tend to cause only mild tissue damage or disease to the host. In certain acute infections, the patient appears to recover, but there is a later relapse. Persistent infections are not usually significant causes of acute illness but can be of particular importance because 1) They enable the infectious agent to persist in the community. 2) They can be activated in immunosuppressed patients. 3) Some are associated with immunopathological disease i.e. feline leukemia virus, hepatitis B virus, malaria. 4) Some are associated with
neoplasms. 5) Some are immunosuppressive and permit disease caused by other
normally harmless persistent microorganisms. The persistent organism can then
continue to cause pathological changes or to be shed from the body. Bartonellosis in
humans causes a persistent bacteremic illness in patients infected with *B. henselae* and
*B. quintana* (Lucey et al. 1992; Welch, and Slater 1999). Fever with bacteremia is a
common clinical presentation of *Bartonella* infection. Relapsing fever with bacteremia
can occur in both immunocompromised and immunocompetent patients (Lucey et al.
1992). Prolonged and recurring episodes of bacteremia are associated with *Bartonella*
infections in cats. Kordick and Breitschwerdt (1997) demonstrated relapsing
bacteremia in experimentally infected cats in a study where the cats were observed for
213 days. Eleven of these cats were inoculated via blood transfusion and developed
relapsing bacteremia. The reasons for relapsing bacteremia are unclear. Possible
reasons for relapsing bacteremia in cats may result from repetitive evasion of the
immune system through antigenic variation by the organism via alteration of surface
antigens, escape from the phagocytic cell, location in an immunologically privileged
site, or sequestration in the host with intermittent release into the general circulation.
The bacteremia in these cats persisted in the presence of variable antibody titers,
suggesting that *B. henselae* antibodies are not effective in clearing infection. Kordick et
al. (1999), followed a group of cats chronically infected with *B. henselae* for 454 days.
In this study, cats were intermittently bacteremic without obvious clinical signs except
for one cat with symptoms related to the central nervous system that resolved
spontaneously and did not recur (Kordick et al. 1999). Intervals of 1 to 4 months
occurred in which cats were culture negative. Recurrent episodes of bacteremia were not accompanied with fever and lasted for 454 days in two cats. (Kordick et al. 1999)

**Immunological response.** The spectrum of disease seen in humans associated with *Bartonella* species is wide. The ability of *Bartonella* to elicit such different pathologic responses is associated with the immune status of the host. *B. henselae* infection may produce either the granulomatous lymphadenitis that is characteristic of cat scratch disease in immunocompetent patients or vascular proliferation in patients with defects in cellular immunity. The most severe manifestations of *B. henselae* infections occur in individuals with a depressed cellular immune response. *B. henselae* in humans is an intracellular pathogen and multiples within phagocytic cells and is considered a likely candidate for a Th-1 IFN-γ type of response (Anderson and Neuman 1997). The persistence of a Th-1 response to an intracellular pathogen is likely to result in an inflammatory tissue response as seen in infections due to *Bartonella*.

Results from studies of *B. henselae* infections in the murine model (BALB/c) demonstrated granulomatous inflammation in the liver with lesions consisting of lymphocytes, monocytes, and epithelioid cells (Regnath et al. 1998). Mononuclear cells were predominately CD4+ lymphocytes or CD11b+ monocytes. CD8+ lymphocytes were found in low numbers. Karem et al. (1999), found *B. henselae*-specific IgA in fecal samples of infected BALB/c mice 28 days after infection. In addition, a positive delayed-type hypersensitivity and IFN response in mice following in vitro stimulation of splenocytes indicated *B. henselae*-specific cellular responses.

Humoral immunity against *B. henselae* was studied in vitro by Rodriguez-Barradas et al. (1995). In this study, *Bartonella* was killed by complement-mediated cytolysis,
and the presence of specific antibodies did not increase the serum bactericidal activity or complement activation. Nonimmune sera killed more than 99.5% of the inoculum within 2 hours, suggesting that specific antibodies did not contribute to the bactericidal activity of serum. Complement activation proceeded by both classical and alternative pathways. Activation of the alternative pathway is explained by the lipopolysaccharides of gram negative bacteria that activates C3. The classical pathway assays suggested in this study that \textit{B. henselae} activated this pathway independent of the presence of specific antibodies. In this study phagocytosis and production of oxygen radicals, evaluated by flow cytometry, were significantly enhanced in the presence of bacteria previously opsonized with immune sera.

The indirect fluorescent-antibody assay (IFA) is widely accepted as a diagnostic assay for \textit{B. henselae} infection. The limitation of the IFA is the inability to differentiate species-specific serologic reactivity from cross-reactivity with other antigens of the same genus i.e. \textit{B. quintana}. Western blot has been used to dissect the humoral immune response to \textit{B. henselae} antigen in patients infected with \textit{B. henselae}. McGill et al. (1998) used serum specimens from 69 humans diagnosed with \textit{Bartonella} infection (CSD) to investigate subclass level of IgG and other immunoglobulin (Ig) isotypes using western blot assay. The ranges of \textit{B. henselae}-reactive proteins recognized were 17, 48, 69, 97, and 116 k-Da and multiple bands at 200 Da. Reactivity to proteins did not occur in all specimens. In this study, a strong IgG response was detected to total antigen with the response limited to subclass IgG\textsubscript{1}. A lack of IgG\textsubscript{3} and high levels of IgG\textsubscript{1} activity against \textit{B. henselae} in CSD infection suggest increased opsonization activity during CSD rather than complement fixation. Ig isotype differences were
investigated in this same group of infected patients, and IgA was detected in 15 of the 69 patients whereas IgM and IgE antibodies were essentially absent in these patients.

Anderson et al. (1995) identified a strong antibody response in patients with CSD to a 17 kDa antigen he expressed as a fusion protein. This type of antigen would have value as a diagnostic reagent for rapid serologic diagnosis of *Bartonella* infections.

A cellular-mediated delayed-type hypersensitivity has been shown to occur in 95 to 98% of humans with CSD when the Hanger-Rose skin test is used for traditional CSD diagnostics (Szelc-Kelly et al. 1995). Other implications of cellular immune induction was seen in immunocompromised humans when they develop severe disease involving internal organs such as liver, spleen and central nervous system.

The immunological response of cats to *B. henselae* is unclear. The different contributions of antibody and cellular immunity are difficult to determine. The only cell in the cat that *Bartonella* has been associated with is the erythrocyte. An intracellular location of *Bartonella* other than in the erythrocyte has not been demonstrated in the cat. Other difficulties involved in the present interpretation of the immunological response seen in cats are the differences in the route of inoculation and the source of the inoculum. Intravenous, intradermal, intramuscular, and oral routes of inoculation have been performed in experimental infections of *B. henselae* in cats (Abbott et al. 1997; Guptill et al. 1997; Kordick and Breitschwerdt 1997). Sources of inoculum used in these studies include infected blood and urine and pure cultures of *B. henselae* (Abbott et al. 1997; Guptill et al. 1997; Kordick and Breitschwerdt 1997; Regnery et al. 1996). Other difficulties in the interpretation of the immunological response seen in cats is the various strains of *B. henselae* used to experimentally infect
cats, and the number of passages of the culture before the inoculum was prepared. \textit{B. henselae Houston-1}, the prototype strain and a genotype I originally isolated from a human patient, has been used in many studies but genotype I strains are less commonly isolated from cats than are the \textit{B. henselae} genotype II strains (Bergmans et al.1997; Heller et al. 1997). The seroprevalence of \textit{B. henselae} in cats varies throughout regions of the United States and in Europe. Seroprevalence ranges from 3.7\% to 65.4\% in the United States (Jamerson et al. 1994; Childs et al. 1994; Chomel, 1996). An understanding of the role of humoral antibodies and the cellular responses in cats is incomplete. High levels of circulating antibodies to \textit{B. henselae} can be detected in cats with bacteremia. Bacteremic cats have been shown to have higher \textit{B. henselae} antibody titers than nonbacteremic cats (Chomel et al. 1995). Using an enzyme-linked immunosorbent assay (ELISA) for detection of \textit{B. henselae} specific antibodies, Freeland et al. (1999) demonstrated \textit{B. henselae} specific IgM and IgG responses in experimentally infected cats with a pattern of a peak IgM level followed by a rise in IgG antibodies. Western blot analysis has been performed in infected cats to identify the immunodominant antigens. Freeland et al. (1999) identified 24 \textit{Bartonella}-specific antigens recognized by cats during infection, and antigens of the 11.3-13.3 kDa and 16.9/18.0 kDa range were common and useful for an antibody screening test. Using the pathogenic strain \textit{B. henselae LSU 16}, O’Reilly et al. (1999) demonstrated by ELISA measurable levels of IgM at 3 week post exposure and high levels of IgG by 4 weeks postinfection. Of interest in this study was the IgM response that began to wane by week 6 but remained elevated in the infected cats throughout the study, suggesting continued antigenic stimulation. In the study by Greene et al. (1996), cats infected with
B. henselae Houston-1 displayed serological response (IgG) that peaked between 3 to 12 weeks after inoculation. The range of the antibodies demonstrated with IFA (B. henselae Houston-1 antigen) was 128 to 512 with one titer at 4096 in an adolescent cat. After 11-14 weeks, the antibody titer in five of the cats was at or below 64. On rechallenge, the antibody titers increased more rapidly and at a higher level than those after the primary exposure.

The role of antibody responses to B. henselae infection in cats has been demonstrated in several studies. Guptill et al. (1999) compared the immune response of neonatal Specific Pathogen Free (SPF) cats experimentally infected to adult cats (SPF) with a normal mature immune system. Neonates (3-5 days of age) were inoculated with B. henselae intradermally or orally and were bacteremic through 12-16 weeks similar to what occurred in adult cats. Cats inoculated intradermally produced IgG Bartonella-specific antibodies but orally inoculated neonatal cats did not. Cats with or without IgG became abacteremic at the same time suggesting that antibodies are not necessary to clear bacteremia. Parr et al. (1999) demonstrated the role of antibody in limiting clinical disease but not in the prevention of bacteremia and infection with B. henselae. Parr et al. (1999) demonstrated the effects of passive antibody by decreasing clinical disease and delayed onset of bacteremia in experimentally infected cats. The effects of maternal antibodies were demonstrated in kittens from infected queens with maternal antibody. Kittens infected at 6 weeks of age became bacteremic but had decreased clinical disease, whereas kittens from noninfected queens show signs of clinical disease.
Guptill et al. (1998), demonstrated maternal anti-\textit{B. henselae} antibodies in fetuses and kittens of infected mothers at 2 weeks post-partum but were not detected at 10 weeks post-partum.

Homologous protection has been shown in several studies with experimentally infected cats. Regnery et al. (1996) demonstrated homologous protection with \textit{B. henselae} by lack of heterologous protection by \textit{B. quintana}. In this study, cats infected with \textit{B. henselae} failed to develop bacteremia on challenge exposure with \textit{B. henselae} but did support \textit{B. henselae} infection if previously infected with \textit{B. quintana}. Greene et al. 1996), also demonstrated homologous protection with \textit{B. henselae} after secondary challenge. Cats in this study also had higher and more rapid increase in serum antibody titers than after primary inoculation. Yamamoto et al. (1998), demonstrated lack of heterologous protection by various species and types of \textit{Bartonella} in SPF cats. In this study, there was lack of cross protection between \textit{B. henselae} and \textit{B. clarridgeiae} and between \textit{B. henselae} genotype I and II and the wildlife isolate, ‘Humboldt’.

**Therapy and treatment.** \textit{Bartonella} isolates from humans are susceptible to numerous antimicrobial agents including erythromycin, chloramphenicol, rifampin, gentamicin, trimethoprim-sulfamethoxazole, doxycycline, and ciprofloxacin (Maurin and Rauolt 1993; Musso et al. 1995). In vitro resistance to penicillin and ampicillin, tetracycline or vancomycin has been noted (Welch and Slater 1999). There is, however, a poor correlation between in vitro susceptibility and in vivo responsiveness to antibiotics. Minimal inhibitory concentration (MIC) results obtained do not appear to correlate with in vivo antimicrobial efficacy (Maurin et al. 1993). In human infections numerous individual case reports indicate both successful or unsuccessful treatment of
*B. henselae* infection (Margileth et al. 1991; Holley et al. 1991). Response of patients to antibiotics such as trimethoprim-sulfamethoxazole and fluoroquinolones are inconsistent (Welch and Slater, 1999). Human data suggest that higher doses and longer periods of administration of antibiotics than normal are indicated for treatment of *Bartonella* infections. Patients with extensive hepatic involvement and regional lymphadenopathy responded to intravenous gentamicin within 48 hours with no recurrence of symptoms (Bogue et al. 1989). Treatment of healthy patients with typical CSD is supportive because the disease is self-limited. Azithromycin use accelerated the resolution of the lymphadenopathy of typical CSD (Bass et al, 1998; Welch and Slater 1999). In patients with severe multisystemic disease, trimethoprim-sulfamethoxazole, ciprofloxacin or azithromycin is recommended (Margileth et al. 1998). In immunodeficient patients, therapy with erythromycin, clarithromycin, azithromycin, doxycycline, or tetracycline have been found to be effective (Koehler et al. 1997). Treatment in these patients was for 6-8 weeks with retreatment of 4-6 months in infections that relapse. Ciprofloxacin has been used successfully to eradicate *Bartonella* infection in humans (Margileth et al.1992; Holley 1991). The success of ciprofloxacin in treatment of *Bartonella* infections is attributed to the high drug concentration achieved in leukocytes, macrophages, and other tissues and organs frequently colonized by *Bartonella* organisms, including the spleen, liver, lymph nodes, heart, and bone (Kordick et al. 1997b).

Antibiotic therapy for control of *B. henselae* in infected cats has been investigated under controlled experimental condition in several studies. Amoxicillin, enrofloxacin, erythromycin, and tetracycline have been evaluated for efficacy of *B. henselae* infection (Margileth et al. 1991; Holley et al. 1991). Response of patients to antibiotics such as trimethoprim-sulfamethoxazole and fluoroquinolones are inconsistent (Welch and Slater, 1999). Human data suggest that higher doses and longer periods of administration of antibiotics than normal are indicated for treatment of *Bartonella* infections. Patients with extensive hepatic involvement and regional lymphadenopathy responded to intravenous gentamicin within 48 hours with no recurrence of symptoms (Bogue et al. 1989). Treatment of healthy patients with typical CSD is supportive because the disease is self-limited. Azithromycin use accelerated the resolution of the lymphadenopathy of typical CSD (Bass et al, 1998; Welch and Slater 1999). In patients with severe multisystemic disease, trimethoprim-sulfamethoxazole, ciprofloxacin or azithromycin is recommended (Margileth et al. 1998). In immunodeficient patients, therapy with erythromycin, clarithromycin, azithromycin, doxycycline, or tetracycline have been found to be effective (Koehler et al. 1997). Treatment in these patients was for 6-8 weeks with retreatment of 4-6 months in infections that relapse. Ciprofloxacin has been used successfully to eradicate *Bartonella* infection in humans (Margileth et al.1992; Holley 1991). The success of ciprofloxacin in treatment of *Bartonella* infections is attributed to the high drug concentration achieved in leukocytes, macrophages, and other tissues and organs frequently colonized by *Bartonella* organisms, including the spleen, liver, lymph nodes, heart, and bone (Kordick et al. 1997b).

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infections in cats. Studies by Regnery et al. (1996) demonstrated reduction in bacteremia in experimentally infected cats with tetracycline and erythromycin after 34-55 days of treatment. Enrofloxacin-treated cats and amoxicillin-treated cats became abacteremic by day 98 and 127 after infection, respectively (Regnery et al. 1996). Greene et al. (1996), treated cats for 1 week with doxycycline and suppressed the bacteremia but infection was not cleared. These studies are misleading however because of the method of detection of bacteremia and the relapsing nature of *Bartonella* infections. In the studies by Regnery et al. (1996) and Greene et al. (1996) efficacy of treatment was determined by bacteremia as detected by culture, which is not the most sensitive assay for defining bacteremia. Because relapsing bacteremia can occur in cats for long periods, a thorough understanding of the efficacy of antibiotics would involve following infected cats for long periods. Kordick et al. (1997b), treated cats infected with *Bartonella* species using enrofloxacin and doxycycline. In vivo efficacy of both antibiotics was examined in 18 experimentally infected cats and 25 naturally infected cats. Treatment success was achieved in 9 of 14 cats receiving enrofloxacin and 2 of 8 cats receiving doxycycline. Treatment was ineffective in some of the infected cats despite plasma drug concentrations maintained above the MICs of enrofloxacin and doxycycline during each sampling period. Treatment success was based on elimination of bacteremia detected by culture or PCR. Doxycycline was chosen to treat *Bartonella* infected cats because of its solubility in lipids and ability to cross cell membranes, whereas enrofloxacin is known to passively diffuse into bacteria and mammalian cells (Pocidalo 1989). Because cats can be chronically infected with *Bartonella*, treatment success may vary depending on the status of infection i.e. acute versus chronic.
infection. Antimicrobial efficacy may be more successful if given to the acutely infected cats, whereas success in the chronically infected cat may require longer periods of administration and or higher doses.

**Laboratory Diagnosis of Bartonella Species**

**Available test.** Diagnostic tests available for the detection and diagnosis of *B. henselae* in cats include serologic testing, bacterial culture, histopathology with specialized silver staining, and nucleic acid detection using the polymerase chain reaction (PCR). In the acute phase of the disease, cats are usually bacteremic and the organism can be cultured from blood. Clinically, however, cats that are bacteremic often show no clinical signs of disease (Regnery et al.1992).

*B. henselae* can be isolated quite easily from a bacteremic cat using common blood containing media such as chocolate agar and blood agar. Cultures are incubated at 35-37°C in an atmosphere of 5% CO₂. Isolation time varies from 7-35 days. A number of different solid media have been used, including trypticase soy agar with 5% sheep blood, chocolate agar, and heart infusion agar with 5% rabbit blood for the isolation of *B. henselae*. *B. henselae* has characteristic colony morphology on initial isolation. Colony morphology and growth characteristics can be used by those experienced with handling *Bartonella* species to recognize the pathogenic species (Clarridge et al.1995). Colonies are often invaginated, cauliflower-like and imbedded in the agar. It is thought that this autoadherence is due to the presence of genotype IV pili on the surface of the organism (Batterman et al. 1995). Subculturing of the organism results in more rapid colony growth, with visible colonies appearing after 2 to 3 days of incubation and a loss of the autoadherent phenotype (Anderson and Neuman 1997).
**Isolation and Culture.** For many years microorganisms could be visualized in patients with CSD and bacillary angiomatosis but could not be grown in the laboratory. The initial detection of *B. henselae* and its laboratory propagation occurred in 1990 (Slater et al. 1990). Using oligonucleotide primers complementary to the 16S ribosomal RNA genes of eubacteria, a polymerase chain reaction was used to amplify 16S ribosomal gene fragments directly from tissue samples of bacillary angiomatosis (Relman et al. 1990). The PCR fragments were sequenced and identified as an uncharacterized microorganism closely related to *Rochalimaea quintana* (Relman 1990). The first isolation occurred when a curved gram-negative bacillus was isolated from several immunosuppressed patients with persistent fever and bacteremia (Slater et al. 1990). These primary cultures of blood were processed by centrifugation after blood-cell lysis, and colonies were isolated after 15 days on chocolate agar and sheep blood agar (Slater et al. 1990). It is now recognized that *B. henselae* can be cultured on enriched bacteriologic culture medium containing heme and in the presence of 5% CO₂. Success of isolation of *Bartonella* species is variable depending on the time course of the infection and the choice of specimen. In general, isolation of *B. henselae* from blood of patients with CSD who have no evidence of systemic disease was unsuccessful (Anderson and Neuman 1997). On the contrary, patients who are immunocompromised with evidence of systemic disease usually are culture positive for *Bartonella* (Anderson and Neuman 1997). The optimal temperature for growth is 35 to 37°C for *B. henselae*, *B. elizabethae*, and *B. quintana*. *B. henselae* has characteristic colony morphology on initial isolation. Colonies are often imbedded in the agar, and of a cauliflower-like appearance. The organism is often autoadherent and difficult to pick from the surface
of the agar (Batterman et al. 1995). Upon subculturing of the organism, the autoadherent phenotype is lost (Anderson and Neuman 1997). Colony morphology and growth characteristics are unique to Bartonella species and can be easily recognize by anyone trained to do so.

Several types of liquid media have been described that support primary isolation of B. henselae. A defined RPMI 1640 based medium supplemented with pyruvate, hemin, and amino acids was used to isolate B. henselae from blood and tissue specimens of infected patients and a domestic cat (Wong et al. 1995). A blood-free medium with blood-based components has been shown to support the growth of B. henselae (Schwartzman et al. 1993). This medium consists of brucella agar with 6 to 8% Fildes solution (BBL), which is a sterile digest of sheep blood, and 250 μg of hemin, a derivative of heme, which is the iron containing group of hemoglobin. Several commercial systems have also been used to identify Bartonella from blood (i.e. Septi-Chek biphasic system, BACTEC 460, and the BACTEC NR 660 system) (Larson, 1994). BacT/Alert (Organon Teknika, Durham, NC) using tryptic soy broth with 0.03% sodium polyanetholesulfonate (SPS) in CO₂ has been used to isolate Bartonella from the blood of 5 patients who were immunosuppressed (Tierno et al. 1995).

Direct plating of blood collected in EDTA and lysis centrifugation, using Isolator Wampole, (Cranbury, NJ) have been used successfully to isolate B. henselae, B. quintana, and B. claridgeiae. Freezing of blood was shown to increase the sensitivity of detection of B. henselae by increasing the number of CFU/ml recovered (Brenner et al. 1997). It is thought that the freezing lyses erythrocytes and leukocytes and may serve to liberate intracellular organisms or those organisms that may be on the surface.
or cell membrane (Brenner et al. 1997). Blood collected in tubes containing EDTA, frozen at \(-65^\circ\text{C}\) and then plated have been shown to increase the number of CFU/ml compared to blood collected in the Isolator tubes (Brenner et al. 1997). Primary isolation of *Bartonella* from tissue has also been accomplished by grinding tissue and plating on chocolate agar (Dolan et al. 1993).

Cell culture systems have also been used to isolate *Bartonella* from both blood and tissue. Endothelial cell lines have been used to cultivate *B. henselae* and *B. quintana* from skin lesions of patients with bacillary angiomatosis (Koehler et al. 1992). Co-cultivation of *B. henselae* with Vero cells provide substrate to which the *Bartonella* do not autoagglutinate and the cells provide an environment for growth (Dalton et al. 1995). *B. henselae* is grown in Vero cells for preparation of antigen to use in the indirect fluorescent antibody assay (Regnery et al. 1992).

**Identification.** *Bartonella* species can be identified to the genus level by phenotypic characteristics such as growth requirements, gram stain, and colony morphology. Identification of *Bartonella* to species level is somewhat more difficult because in general *Bartonella* species are biochemically inert. Carbohydrate utilization cannot be determined by conventional tests but requires methods that may include genetic analysis. DNA-DNA hybridization and PCR amplification of various genes followed by sequencing or restriction endonuclease analysis of amplicons have been successful in identification of *Bartonella* species.

**Serological assays.** Serological testing is used frequently for the diagnosis of *B. henselae* in cats because these methods are practical and economical methods that can be performed by most laboratories. Serologic tests do have limitations and may not be a
clear diagnostic indicator in some cases. Low antibody levels are detected many times and are not sufficient evidence to determine active or prior infection. Another limitation of some serological tests, is the sensitivity and specificity of the test depending on the antigens used in the assay, the time of specimen collection, the format of the assay, and the indicators used to detect the antibody.

The serological response of the cat to \textit{B. henselae} is not understood partially because \textit{Bartonella} antigens recognized by the cat's immune system have not been fully identified. Lack of understanding of the immunodominant antigens is a controlling factor for the serological assay used for serodiagnosis of \textit{Bartonella} in cats. Serodiagnostic tools such as indirect immunofluorescent assay (IFA); enzyme-linked immunoassay (EIA) and the western blot analysis are all used to determine antibody levels to \textit{Bartonella} in cats and humans. The IFA is probably the most common serologic assay used by laboratories and the sensitivity is reported to be 88% and the specificity is 94% (Regnery et al. 1996). IgG titer of $>1:64$ in a cat is considered a positive titer for \textit{B. henselae}. Studies using the IFA for diagnosis of cat scratch disease however, point out possible weakness of the IFA (Zhinden et al. 1997; Bergmans et al. 1997). In studies by Zhinden et al. (1997); Bergmans et al. (1997) the sensitivity and specificity of the IFA was shown to be affected by the preparation of the \textit{Bartonella} antigen (with or without cocultivation with Vero cells) and the cutoff titer of the test. Bergmans et al. (1997) demonstrated that one-point IgG serology is not diagnostic for \textit{B. henselae} infection in humans and that two-point serology is necessary to detect ongoing \textit{B. henselae} infections. Bergmans et al. (1997) also concluded that IgM serology was superior to IgG serology in diagnosing \textit{B. henselae} in CSD patients.
The serological picture with cats infected with *B. henselae* varies. Cats with *B. henselae* bacteremia were shown to be serologically positive with high titers. Other studies found bacteremic cats with no detectable antibody levels. Results from a study by Bergmans et al. (1997) using the EIA to measure antibody to *Bartonella*, concluded that 20% of the 113 cats enrolled in the study were bacteremic and had anti-*B. henselae* antibodies whereas 30% of the 113 cats had antibodies to *B. henselae* but were not bacteremic, whereas 2% of the 113 were bacteremic but were seronegative. This study concluded that the positive predictive value of the serologic assay for bacteremia is 39%, and the negative predictive value of the serologic assay is 96%.

**Histopathology and Warthin Starry silver stain.** Before culture, serological assay or nucleic acid detection became available, histologic findings with silver stains were used to characterize the causative agent of CSD and bacillary angiomatosis. Early reports in the literature described Warthin—Starry positive bacteria associated with cases of cat scratch disease (Margileth et al. 1984; Kudo et al. 1988). Small pleomorphic bacilli were seen using the Warthin-Starry silver impregnation stain in both the skin at primary inoculation sites and in lymph nodes from patients with CSD (Margileth et al. 1984). Attempts to culture this organism were unsuccessful. Histologic evaluation of lesions in patients with CSD and bacillary angiomatosis revealed similar pleomorphic bacilli on silver stain. The histopathologic findings associated with infections caused by *B. henselae* in humans are described in the literature and are significant. Histopathological findings in lymph nodes depend on the stage of the infection. There may be lymphoid hyperplasia, arteriolar proliferation, and reticulum cell hyperplasia early in the course of the infection (Sander et al. 1999). In
the later stages of the infection, granulomas with central areas of necrosis, multinucleated giant cells, and multiple stellate microabscesses are seen (Bass et al. 1997; Kaschula 1996). The histopathologic changes found in CSD are consistent with granulomatous inflammation with or without central suppuration (Min et al. 1993). The lymph nodes from patients with CSD typically include stellate caseating granulomas, microabscesses, and follicular hyperplasia (Carithers 1985). The follicular hyperplasia may be seen with a distinct germinal center and plasmacytosis in the residual parts of the lymph node (Kudo et al. 1988). Multinucleated giant cells with the zone of histiocytes can be seen (Kudo et al. 1988). Min et al., (1993) identified bacilli of cat scratch disease using a labeled polyclonal rabbit antibody to the outer surface protein of B. henselae. The histopathological findings are typical but not specific for CSD (Sander et al. 1999). Infections caused by other agents, such as lymphogranuloma inguinale caused by Chlamydia trachomatis, atypical mycobacteriosis, yersiniosis, tularemia, brucellosis, certain mycotic and chronic granulomatous diseases of childhood must be considered in the differential diagnosis (Anderson et al. 1994).

Bacillary angiomatosis is a neovascular disorder with the presence of bacilli in the tissue. Cutaneous or subcutaneous lesions are seen in bacillary angiomatosis patients. Characteristic lobular proliferation of blood vessels are seen in the lesions with hematoxylin and eosin stain. Granuloma formation are not seen in patients with bacillary angiomatosis.

Studies of cats seropositive to B. henselae report different findings in regards to histological abnormalities or clinical manifestations. Several studies have shown histological abnormalities in cats experimentally infected with B. henselae (Guptill et
al. 1997; Korkick et al. 1999). Guptill et al. (1997) found histopathologic lesions in multiple organs of experimentally infected cats 8 weeks after infection. In this study, cats were described to have generalized peripheral lymphadenopathy with a marked lymphoid hyperplasia characterized by an expanded paracortex and secondary follicles with germinal centers. Mild to marked lymphoid hyperplasia of the spleen with development of prominent germinal centers was seen in cats infected with a strain of *B. henselae* originally isolated from a cat. Circumscribed aggregates of neutrophils or microabscesses were present throughout the red pulp of the spleen. Two weeks after infection, the liver from this group of cats contained small foci of necrosis in the parenchyma and an increased number of neutrophils in the sinusoids of the liver. Eight weeks after infection small foci of granulomatous inflammation in the liver could be seen. Focal pyogranulomatous nephritis and interstitial myocarditis could be seen in one cat 4 weeks after infection. Few bacteria were seen on Warthin-Starry stains of the spleen of cats examined 2 weeks after infection in this study. In a study done by Kordick et al. (1999), cats were experimental infected with *B. henselae* genotype II and histopathology evaluated after 454 days. In this study peripheral lymph node hyperplasia was seen in all 13 cats, splenic follicular hyperplasia in 9 cats, lymphocytic cholangitis in 9 cats, lymphocytic hepatitis in 6 cats, lymphoplasmacytic myocarditis in 8 cats, and interstitial lymphocytic nephritis in 4 cats. Warthin-Starry silver stains were only suggestive of bacilli in tissue examined in this study.

**Polymerase chain reaction (PCR).** Polymerase chain reaction (PCR) is an in vitro method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. A single nucleic acid target molecule can be amplified until
sufficient material is present to enable detection and identification. Amplification and
detection can be detected in minutes to hours. PCR replaces the conventional process
of biologic amplification i.e. growth in culture, with enzymatic amplification of specific
nucleic acid sequences. The ability to isolate and cultivate microbial pathogens on
artificial medium represents one of the great diagnostic triumphs of the last century.
Cultivation of microbial pathogens from clinical specimens is the mainstay of the
clinical laboratory today. Despite its established value, however, in vitro cultivation has
many drawbacks. The time required for growth of an organism in culture usually
ranges from days to weeks, and definitive tests introduce additional delays. The number
of bacterial species that are isolated on bacterial culture media is limited. It is
estimated that less than 1% of the bacteria present on the earth have been described
using cultivation (Relman et al. 1998).

PCR technology amplifies a selected region of DNA. It involves two
oligonucleotide primers that flank the DNA fragment to be amplified. These primers
hybridize to opposite strands of the target sequence and are oriented so that the DNA
synthesis occurs across the region between the primers. PCR is based on the functions
of DNA polymerase to copy and amplify a strand of DNA. DNA polymerase initiates
elongation of the primer to a longer strand of DNA. The starting material for PCR is
called the target and is a DNA gene segment. The target sample reacts with a master
mix that contains everything needed to synthesize DNA sequences. The PCR assay
consists of three steps per cycle which is repeated 20-40 times. Each cycle doubles the
number of DNA molecules in the previous cycle. Because the copies increase
exponentially, more than 100 million copies can be made in only a few hours. The
three PCR cycles begin with denaturation, in which double stranded material is
denatured into single-stranded material. The second step involves the annealing of a
primer pair. The primer is a small piece of synthetic DNA, an oligonucleotide, known
to complement the DNA sequence of a particular genetic sequence. The primers flank
the target sequence of interest. The primer searches out the separated DNA strands and
anneals to its complementary sequence. The third step of the PCR cycle is extension, in
which the DNA polymerase extends the target sequences between the primers by
addition of bases to the 3' end of the strand.

PCR is a sensitive assay that can detect a single DNA molecule. However, a single
protocol is not appropriate for all target sequences. Each PCR design requires
optimization for best performance of the assay. Factors to consider in the design of the
PCR assay are the reaction components, the denaturation and annealing temperatures,
and the number of cycles performed. Concentrations of reagents in the reaction mixture
such as dNTPs, magnesium chloride, DNA, enzyme, and primers need to be optimized
to maximize efficiency and fidelity of assay. Denaturation of the target template is the
first step in the PCR cycle. Strand separation occurs in this step and the correct
denaturation temperature is critical. Primer annealing temperatures are important and
should be approximately 5°C below the true melting temperature (T_m) of the
amplification primers. The primers used in the assay must be specific only for the DNA
of the target organism. The specificity of the primer increases as the temperature
approaches the correct annealing temperature. Increasing the annealing temperature
enhances discrimination against incorrectly annealed primers and reduces miss
extension of incorrect nucleotides at the 3’ end of the primers.
Since the development in 1984, PCR assays have been used in research and clinical applications. Advantages of nucleic acid techniques include rapid detection of fastidious pathogens, detection of nonculturable agents, and discovery of new organism types and genotypic analysis. A PCR assay was used to discover and understand infections caused by *B. henselae* and to diagnosis cases of bartonellosis. Nucleic acid techniques, including PCR and nucleotide sequencing, were important early in the characterization of *B. henselae* and its association with a variety of disease syndromes. Relman et al. (1990), was the first to use broad-range PCR to detect the 16S rRNA gene sequence of *B. henselae* from tissue of patients with bacillary angiomatosis. *B. henselae* bacilli could be seen on silver stains within the lesions of patients but could not be isolated by culture techniques. Since that time, PCR has been used as a detection and identification method for *B. henselae*. Because of the fastidious nature of many organisms, including *Bartonella* species, and the limitations of cultivation technology, nucleic acid amplification techniques such as PCR are very useful methods to reveal novel microbes associated with disease. The PCR assay has been used to detect *B. henselae* in specimens in multiple human cases involving lymphadenitis, osteomyelitis, retinitis, postpartum coma, encephalopathy, endocarditis, and Parinaud's oculoglandular syndrome in which the cultures were negative (Gottlieb et al.1999; Robson et al.1999; Warren et al.1998; McCormack et al. 1998; Wheeler et al.1997; Drancourt et al.1996; Grando et al. 1999). The discriminatory power in identifying microbes is increased with genotypic methods as compared to cultivation and use of phenotypic methods. Once a microbe is amplified with a PCR assay, the product of that amplification can be confirmed by sequencing to determine the exact nucleic acid sequence of the DNA.
fragment. PCR techniques have been used to determine genotypes of strains involved in cases of CSD and bacillary angiomatosis (Bergmans et al. 1997; Heller et al. 1997; Sander et al. 1999).

Some of the _B. henselae_ genes amplified by PCR are the 16S rRNA gene, the citrate synthase gene (gltA), and the _htrA_ gene encoding a 60 kDa heat shock-like protein. Oligonucleotide primers are designed specifically to be complementary to regions of these genes.

The 16S rRNA gene is often used in nucleic acid detection methods. Multiple copies of a gene encoding a 16S rRNA are found in all eubacterium. The 16S rRNA gene found in these cells is highly conserved with interspersed regions of hypervariable sequences. Broad range eubacterial 16S rRNA primers complementary to the conserved region and flanking the segment of interest can be used in a PCR design to amplify the unknown variable region. The primers anneal to conserved region of microbial DNA, and the polymerase generates new DNA strands, including the variable region that can be sequenced for identity. Analysis of the variable portions permits determination of phylogenetic and evolutionary relationships among organisms, and now forms the basis of a revised system of natural classification (Woese 1987).

Because most bacterial genera and species have a unique 16S rRNA gene sequence, this sequence serves as the molecular fingerprints, and can be used to identify organisms. Pure cultured organisms can be the source of the 16S rRNA targets in PCR, or infected tissue can also be used.

The PCR assay is an extremely sensitive assay and can detect DNA fragments of a particular microorganism that are below the detection limits of culture. The levels of
microbial DNA present in clinical specimens are frequently too low for measurement. PCR can produce sufficient amounts of DNA in order for a microbe to be detected and identified. In theory, the PCR assay can detect as few as one microorganism. The limitations of culture vary with the range of 10 to 100 organisms per ml of fluid. Because each microorganism has a unique complement of DNA, this DNA can help produce an identity of the microbe and allow for differentiation among microorganisms.

The PCR is a specific reaction when a unique set of oligonucleotide primers complementary to regions of the DNA templates are used, and a PCR product of a particular size is generated and seen on gel electrophoresis. In addition, confirmatory methods can be done including sequencing of the amplified product, annealing of a specific oligonucleotide probe to a region of the amplification product, and restriction enzyme cleavage of the amplified product with gel electrophoresis of the digest and restriction fragment length polymorphism analysis. Sensitivity and specificity of the PCR can be increased further by designing a nested PCR. In the nested PCR two reaction are performed using products from the first reactions in the second reaction and increasing the final product. In the nested PCR, products from a primary PCR reaction are used in a second reaction with primers that anneal to regions of the same gene, but at sites internal to the previous priming sites. In a hemi-nested PCR, one primer from the first PCR reaction is used in the second round reaction along with a different primer.

PCR assays have a high analytical sensitivity. However, high analytical sensitivity does not necessarily translate into high clinical sensitivity. Clinical performance of an assay such as PCR depends on various factors, including the method of sample collection, preparation to ensure adequate number of target molecules, the presence of
inhibitors of the enzymatic processes of amplification, and the time at which the sample is collected. The volume of sample tested has a direct bearing on clinical performance if the target copy number is low. An analytical sensitivity of a sample with only one target copy will be positive in less than 70% of all reactions performed (Persing 1993). In this hypothetical system, a sample would have to contain an average of 10 targets to ensure that 99% of all specimens contain a single copy for detection. Sensitivity also depends on the number of target molecules in the organism of interest. If multiple copies of the target are present in a single organism, the volume of sample may not be as critical.

A variety of components within clinical specimens may inhibit the PCR by interacting with components of the enzymatic reaction. Inhibitors can interact with the nucleic acid or with the polymerase and inhibit the assay. Heme at 0.8 uM and its metabolic products are known inhibitors of DNA polymerase. Cerebrospinal fluid, urine, and sputum also contain uncharacterized inhibitors of the Taq DNA polymerase. Reagents traditionally used in the purification of nucleic acids such as EDTA, detergents, sodium dodecyl sulfate, chaotropes, and guanidinium HCl are inhibitory to the amplification enzymes.

The fundamental goals of sample preparation include release of nucleic acid, stabilization of nucleic acid against degradation, removal of inhibitors, and concentration of target molecules. Various protocols for extraction and purification of sample DNA are available. A variety of methods to release the nucleic acid from microorganisms include boiling in distilled water or PCR buffer, detergents, sodium hydroxide, freeze-thaw, SDS-proteinase K, perchloric acid, enzymes, sonication and
heat. Additional extraction steps with phenol-chloroform remove amplification inhibitors. Methods of concentration usually include use of alcohol precipitation. Target capture includes matrices such as magnetic particles, glass, Sephadex, and diatomaceous earth, which bind nucleic acids in chaotropic solutions. Following binding of the nucleic acids to the solid-phase matrix, the impurities and amplification inhibitors are removed by centrifugation, washing, and elution of nucleic acids into a compatible buffer. Commercial kits such as InstaGene matrix Genomic DNA Isolation Kits (Bio-Rad Laboratories, Inc., Hercules, CA) and Qiagen Blood and Tissue Kit (Qiagen Inc., Chatsworth, CA) are available for extraction of DNA and are relatively simple and automated.

The advantages of PCR for laboratory diagnosis of *B. henselae* can be summarized in terms of sensitivity, specificity (identification), and turnaround time. A PCR assay for *B. henselae* will detect the microorganism in cases where culture may be negative because of detection limits and because of the fastidious nature of *B. henselae*. Identification of *B. henselae* and the various genotypes can be achieved with a high discriminatory level using confirmatory methods of sequencing or Southern blot using a probe. Identification can be accomplished either from a DNA fragment amplified from blood or tissue or from an isolate from culture.

**Reverse transcription-polymerase chain reaction (RT-PCR).** This procedure involves the reverse transcription of RNA and PCR amplification. The enzyme reverse transcriptase converts RNA to complementary DNA (cDNA) and then the cDNA is amplified in the PCR reaction. The RT-PCR has been widely used in research laboratories as a rapid and sensitive technique for the detection, quantitation, and
cloning of specific mRNA and gene expression. The analysis of RNA expression can determine if cells are in an active growing state, or in a non-growing state, i.e. dormant resting state or nonviable state. RT-PCR is the most sensitive technique to determine the presence or absence of RNA templates or to determine gene expression. RT-PCR is used in clinical diagnostic laboratories for detection and quantitation of infectious microorganisms, cancer cells, and genetic disorders. Two different techniques are commonly used for RT-PCR: the two step RT-PCR or the one step RT-PCR. In the two step RT-PCR, cDNA synthesis is performed with reverse transcriptase derived from avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MuLV) and Tth DNA polymerase from the first step. In the second step, a PCR with DNA polymerase is used. The two step reaction requires that the reaction tube is opened after the cDNA synthesis and reagents are added for the PCR part of the procedure. In the one step RT-PCR the cDNA and PCR reaction are performed without addition of reagents between synthesis of cDNA and PCR.

**Understanding the Pathogenesis of *Bartonella henselae* in the Domestic Cat**

Research problem: *Bartonella henselae* is a serious zoonotic agent and is the etiologic agent for a wide spectrum of disease in humans. The domestic cat is the reservoir for *B. henselae* and a risk factor for contracting infections in which *B. henselae* is the etiologic agent. The pathogenesis of *B. henselae* in cats is incompletely understood. To reduce the risk of contracting *B. henselae* from a domestic cat, a better understanding of the infection in cats is needed. Many aspects of feline bartonellosis are unclear in particular the persistent nature of the infection. *B. henselae* is a fastidious microorganism that has proven to be difficult to detect by standard bacterial culture.
Laboratory methods with high sensitivity and specificity are needed to study the acute and chronic phase of these infections in cats. Understanding the acute and chronic phase of the infection in cats will help reduce the risk of humans contracting *Bartonella* from infected cats.

**Purpose:** Study the pathogenesis of *B. henselae* in the domestic cat using a molecular approach. This study will investigate the bacteremic and chronic phase of *B. henselae* genotype I and genotype II infections in cats using a nested polymerase chain reaction (PCR).

**Hypothesis:** A molecular assay such as polymerase chain reaction (PCR) is a useful assay for detecting fastidious microorganisms involved in infectious diseases. In cats with *B. henselae* infections, *Bartonella* DNA persists longer than can be detected by standard bacterial culture and may explain the chronicity of these infections.

**Research objectives:**
- Design a molecular assay that is sensitive and specific for the species of *Bartonella* that are known to infect cats, i.e. *Bartonella henselae* genotype I and genotype II and *Bartonella clarridgeiae*.
- Determine if PCR assay can be used in biological samples, i.e. blood and tissue from experimentally infected cats.
- Use PCR assay to follow experimentally infected cats in acute phase (bacteremic phase), during recurrent episode of bacteremia and in postmortem tissue.
  Compare PCR assay to bacterial culture.
- Determine if *B. henselae* DNA persists in tissue of experimentally infected cats that are abacteremic.

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• Determine if there is any histopathology associated with *B. henselae* infection in cats.

• Determine the RNA expression of *B. henselae* found in tissue in cats chronically infected using RT-PCR.

• Investigate *B. henselae* genotype differences in experimentally infected cats during bacteremia, relapsing bacteremia and in tissue.
CHAPTER TWO
EVALUATION AND USE OF A NESTED PCR IN CATS EXPERIMENTALLY INFECTED WITH BARTONELLA HENSELAE GENOTYPE I AND BARTONELLA HENSELAE GENOTYPE II

Introduction

The importance of *Bartonella henselae* as an emerging pathogen is recognized more each day by veterinarians, cat owners, and physicians. *Bartonella henselae* causes a wide spectrum of diseases and diverse pathology in humans and the domestic cat acts as a reservoir of this zoonotic disease. Diseases in humans primarily depend on the immune status of the patient. Disease ranges from granulomatous features as in cat scratch disease to vascular proliferative lesions as in bacillary angiomatosis. Cat scratch disease, the most common disease caused by *B. henselae* in immunocompontent patients, is usually a localized self-limiting disease that resolves in time. Sequela to cat scratch disease occurs in 14% of cases and manifests in serious clinical conditions (Regnery and Tappero 1995). Unusual manifestations that have been reported include cases of encephalitis, retinitis, conjunctivitis, hepatic granulomas, osteomyelitis, pulmonary disease, and endocarditis (Regnery and Tappero 1995). Bacillary angiomatosis and bacillary peliosis, caused by *B. henselae* in patients whose immune system is compromised, involve multiple organs and are serious life threatening diseases. *B. henselae* has been shown to establish persistent infection with an intravascular focus displayed as a bacteremia or endocarditis (Relman 1998).

The domestic cat population in the United States numbers approximately 60 million, and 15 to 25 million cats may potentially be infected with *B. henselae* (Kordick et al. 1995). The domestic cat is a common pet in many households and has the potential to
act as a reservoir for human infections. *B. henselae* genotype I, *B. henselae* genotype II, and *B. clarridgeiae* have been isolated from cats and are also associated with human disease (Sanders et al. 1999). Epidemiological studies indicate that a high percentage of cats worldwide have been naturally exposed to or are infected with *B. henselae* (Childs et al. 1994; Jamerson et al. 1995). *B. henselae* has a worldwide distribution. In the United States seroprevalence varies and is as high as 65.4% in some regions (Childs et al. 1994; Jamerson et al. 1995). *B. clarridgeiae* infections in cats are less common with isolation rates reported to be approximately 10% in certain regions (Lawson and Collins 1996; Kordick et al. 1997a).

Clinical disease in cats experimentally infected with *Bartonella* species varies and is thought to be strain related (Relman 1998). In several studies, isolation of *B. henselae* genotype II from cats was more common than isolation of *B. henselae* genotype I (Gurfield et al. 1997; Sander et al. 1998b). Identifying and characterizing the strains or genotypes of *B. henselae* would be useful in studies of the pathogenesis of feline bartonellosis. Cats are reported to be coinfected with *B. henselae* and *B. clarridgeiae* or *B. henselae* genotype I and II (Gurfield et al. 1997). It is not known if there is a difference in virulence or pathogenicity associated with the various species or genotypes of *Bartonella* in cats.

Given the endemicity of *Bartonella* infection in cats, understanding feline bartonellosis is important to those involved in veterinary medicine and human medicine. Because cats can be persistently and asymptptomatically infected with *B. henselae* with intermittent bacteremia, recognizing and understanding feline bartonellosis is important (Regnery et al. 1992c). The bacteremia seen in *B. henselae* infection in cats can be of
significant magnitude in acute or chronic infection, or in chronic infection (Kordick and Breitschwerdt 1997). The likelihood of transmission of *B. henselae* from cats to humans is thought to be highest during times when cats are bacteremic (O’Reilly et al. 1999a).

The pathogenesis of *B. henselae* in the cat is not understood. Assays currently available to identify cats infected with *Bartonella* species include bacterial culture, serological assay, nucleic acid amplification methods, and Warthin-Starry silver stain. Bacterial culture for *Bartonella* species is not ideal and can be limited because of the fastidious nature of organism, the time required for isolation, and the detection limits of culture. Kordick et al. (1999) demonstrated the limitations of culture by amplification of *Bartonella* DNA from the blood of culture negative cats.

The limitations of serological assays include differentiation of exposure from active infection, inconsistent results because of the lack of understanding of the antigenic variability within the *Bartonella* species, and incomplete knowledge of the feline immunological response to this pathogen. Immunological responses in cats to *B. henselae* vary. Chomel et al. (1995) demonstrated that two percent of bacteremic cats in northern California were seronegative. Korkick et al. (1995) observed cats with relapsing bacteremia in conjunction with variable high antibody titers. Other studies have demonstrated a relapsing bacteremia without a peak in IgM level or a rise in IgG level (O’Reilly et al. 1999a).

Argyrophilic stains such as the Warthin-Starry silver stain have been used to detect *Bartonella* in tissue, however, the sensitivity of the test is low (46%) and often fails to
identify organisms in tissues from which the organism had been cultured and from bacteremic cats (Brouqui et al. 1997; Slater et al. 1994).

Polymerase chain reaction assays are important diagnostic and research tools because these assays are highly sensitive and specific. PCR assays are used for the detection and characterization of pathogenic microorganisms associated with infectious diseases. PCR assays are useful to amplify DNA from organisms that are difficult to culture and to detect small amounts of DNA. There are numerous reports in the literature of diagnosis of *Bartonella* infections in humans in which blood and organ cultures were negative, but *B. henselae* DNA was amplified from tissue (Holmberg et al. 1997; Warren et al. 1998; Sander et al. 1998b). The discovery of *Bartonella* as the etiological agent associated with CSD, bacillary angiomatosis was first made with PCR assays, and these assays continue to be a useful laboratory tool for diagnosing these infections (Relman 1993).

A PCR assay for the identification of species and genotypes would assist in characterization of the various strains involved in feline bartonellosis. Our objective was to develop a tool that could be used to detect *Bartonella* in numbers below the limits of culture and to differentiate the various *Bartonella* species and genotypes. In this study, a nested PCR was developed using the 16S rRNA gene of *Bartonella* species that is sensitive and is genus, species, and genotype specific. Experimentally infected cats were cultured and assayed by PCR during the acute and chronic phases of the infection. The application of PCR will assist our understanding of the pathogenesis of *Bartonella* species in the cat populations.
Materials and Methods

Experimental animals. Specific-pathogen-free (SPF) cats (n=16) were bred at Louisiana State University (n=4) or were purchased from Liberty Laboratories (Liberty Corners, NJ) (n=9) or Harlan Sprague-Dawley (Madison, Wis.) (n=3). Cats were culture negative for *B. henselae* and antibody negative for *B. henselae* by western blot analyses. Cats were housed individually or grouped in an environmentally controlled setting.

Bacterial strains. *Bartonella* strains were grown on chocolate agar or blood agar (TSA w/5% rabbit blood agar plates) (Remel, Lenexa, Kansas) and the other microorganisms were grown on blood agar (TSA w/5% sheep blood agar plates) (Remel, Lenexa, Kansas). Bacteria used to evaluate this PCR assay included *Bartonella henselae* 87-66 (ATCC 49793), *Bartonella henselae* Houston-1 (ATCC 49882), *Bartonella quintana* (ATCC VR-358), *Bartonella clarridgeiae* (ATCC 700095), and *Bartonella elizabethae* (ATCC 49927) obtained from the American Type Culture Collection (Rockville, MD). *Bartonella henselae* LSU 16 (O’Reilly et al. 1999a) and *Bartonella henselae* Baby (Parr et al, 1999) were isolated at Louisiana State University from naturally infected cats. *Escherichia coli, Pseudomonas aeruginosa, Bordetella bronchiseptica, Brucella abortus, Pasteurella multocida, Staphylococcus aureus* and *Streptococcus pneumoniae* were obtained from the Louisiana Veterinary Medical Diagnostic Laboratory, School of Veterinary Medicine, Louisiana State University.

Experimental infections. Cats inoculated with *B. henselae* LSU 16, Baby, and Houston-1 strains were given approximately 1x10^7 CFU/ml. Cats inoculated with strain *B. henselae* 86-77 were given approximately 1x10^9 CFU/ml. All cats were injected
intradermally with 0.6 ml of the respective inoculum divided among six sites on the skin of the lateral trunk. Animal inoculations were conducted with a second-passage preparation of organism. In one group of twelve cats, the cats were divided into four groups of three. Six cats were infected with *B. henselae* LSU 16 genotype II, three cats were infected with *B. henselae* Baby genotype II, three cats were infected with *B. henselae* 87-66 genotype I, and three cats were infected with *B. henselae* Houston-1 genotype I (Parr et al. 1999). The second group of 8 cats was in a *Bartonella henselae* passive antibody study and were eventually infected with *Bartonella henselae* LSU 16. Of this group, four cats were given anti-*B. henselae* LSU 16 thirty minutes prior to inoculation with *B. henselae* LSU 16, one cat was given anti-*B. henselae* Houston-1 30 minutes prior to inoculation with *B. henselae* LSU 16 and three cats were inoculated with *B. henselae* LSU 16 (Brown et al. 1998). This group of 8 cats was challenged a second time approximately 12 weeks after the first inoculation. Four cats in this group previously challenged with *B. henselae* LSU 16 were given three injections of 10 mg/kg of methylprednisolone (Upjohn, Kalamazoo, MI) intramuscularly at two week intervals. Experimental infections, passive antibody, and drug administrations were conducted in the laboratory of Dr. K. L. O’Reilly.

**Collection of blood specimens.** Peripheral blood was collected weekly by the staff from Dr. K. L. O’Reilly laboratory beginning one week after inoculation and until the time of necropsy. A total of 362 blood specimens were collected in the study. Before each collection, cats were anesthetized with Telazol (Fort Dodge, Ames, Iowa) at the recommended dosage. Blood was collected aseptically by jugular puncture and placed in Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) containing EDTA for PCR.
assay and in pediatric lysis-centrifugation isolator tubes (Wampole Laboratories, Cranbury, NJ) for culture. Blood collected in EDTA tubes was frozen at -20°C overnight, and DNA extractions were performed the next day followed by PCR assay.

**Blood cultures.** Blood cultures were performed by staff in laboratory of K.L. O'Reilly on blood collected in pediatric Isolator® tubes. A 10-μl aliquot of blood was removed from the Isolator® tube and serially diluted. Dilutions (30 μl) were inoculated onto chocolate agar and incubated at 37°C in 5% CO₂ for 7 days. Colony counts were recorded as the number of CFU per milliliter of blood. Isolates were confirmed to have phenotypic characteristics consistent with *Bartonella henselae*.

**Defining bacteremia.** Bacteremia in experimentally infected cats was determined by isolation of *Bartonella* from a blood specimen (culture-positive) or amplification of *Bartonella* DNA from a blood specimen (PCR-positive).

**Extraction of DNA from whole blood specimens.** DNA was extracted from 200-μl of whole blood collected in a EDTA vacutainer tube using the QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit (Qiagen Inc. Valencia, CA). The Blood and Body Fluid Spin Protocol was followed according to manufacturer directions.

**Extraction of DNA from bacterial strains.** Approximately 10 CFU of each bacterial isolate were taken from the agar with an inoculating loop, placed into 2 ml of PBS, vortexed and centrifuged. The bacterial pellet was suspended in 180 μl of Buffer ATL supplied in the QIAamp DNA Mini Kit and stirred vigorously. Twenty μl of proteinase K (supplied in kit) was added and the mixture was incubated at 56°C for approximately 1 hour. The QIAamp tissue protocol was then followed beginning with the addition of the AL lysis buffer.
**Extraction controls.** A control included all reagents without a DNA sample and were processed exactly as described above to ensure that extraction buffers and reagents were not contaminated with target DNA. Blood from negative cats were periodically extracted and checked for contamination. Blood from positive cats were periodically extracted as positive controls.

*Bartonella genus, species, and type-specific oligonucleotide primers for PCR.*

The target gene chosen for amplification was the 16S rRNA gene of *Bartonella.* By identifying conserved and hypervariable regions within the 16S rRNA gene, primers that are genus specific, that are species specific, and that are type specific were designed. The oligonucleotide primers were designed by Ron Tapp using an alignment of the 16S rRNA gene sequence for *Bartonella* species and other closely related bacteria (Tapp 2000) (Figure 1).

The primer sequence and nucleotide position are shown in Table 1. For amplification of genus specific *Bartonella* DNA, forward primer pair Bhen16FF and reverse primer UB16SDR9 were used, generating an 823 bp fragment. A second reverse primer that is *Bartonella* genus specific Bhen16SBBR 5’-CACATGCAA GTCGAGCACACTCTTTTAGAG-3’ was also designed to be used with the forward Bhen16FF primer to amplify a 567 bp fragment.

The primer sets for each *Bartonella* species and genotype and the sizes of the amplification products are indicated in Table 2. For amplification of species and genotype I-specific *Bartonella* DNA, the forward primer Bhen16SGG and reverse primer Bhen16SBBR were used. For amplification of species and genotype II-specific *Bartonella* DNA, the forward primer Bhen16SHH and reverse primer Bhen16SBBR
Figure 1. A map of the relative positions of the PCR primers and product sizes for genus, species, *B. henselae* and *B. clarridgeiae*, and *B. henselae* genotype I, *B. henselae* genotype II (Tapp 2000) (Used by permission from Ron Tapp)
### Table 1. PCR Primer Sequences *Bartonella*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Position*</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB16SDR9</td>
<td>854-877</td>
<td>GCCCCCGTCAATTCCCTTGTGAGTTT</td>
</tr>
<tr>
<td>Bhen16SFF</td>
<td>52-98</td>
<td>CACATGCAAGTCGAGCACACTCTTTTAG AG</td>
</tr>
<tr>
<td>Bhen16SBBR</td>
<td>648-673</td>
<td>CACTCACCTCTTCCACACTCAAGATA</td>
</tr>
<tr>
<td>Bhen16SGG</td>
<td>159-196</td>
<td>CAAATTTGTGCTAATTACCCTATACGTCC TTAG</td>
</tr>
<tr>
<td>Bhen16SHH</td>
<td>159-197</td>
<td>GAAATTTGTGCTAATTACCCTATACGTCC TATTTG</td>
</tr>
<tr>
<td>Bhen16SCC</td>
<td>159-196</td>
<td>GAAATTTGTGCTAATTACCCTATACGTCC TACT</td>
</tr>
</tbody>
</table>

* Nucleotide positions are based on the reported 16S rRNA-gene sequence for *E.coli*

### Table 2. Primer Set and Product Size

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>PCR Product</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bhen16SFF-UB16SDR9</td>
<td>823 bp</td>
<td><em>Bartonella</em> genus</td>
</tr>
<tr>
<td>Bhen16SFF-Bhen16SBBR</td>
<td>567 bp</td>
<td><em>Bartonella</em> genus</td>
</tr>
<tr>
<td>Bhen16SGG-Bhen16SBBR</td>
<td>477 bp</td>
<td><em>Bartonella henselae</em> Genotype I</td>
</tr>
<tr>
<td>Bhen16SHH-Bhen16SBBR</td>
<td>477 bp</td>
<td><em>Bartonella henselae</em> Genotype II</td>
</tr>
<tr>
<td>Bhen16SCC-Bhen16SBBR</td>
<td>477 bp</td>
<td><em>Bartonella clarridgeiae</em></td>
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</table>
were used. For amplification of *Bartonella clarridgeiae* DNA, the forward primer Bhen16SCC and reverse primer Bhen16SBBR were used. The synthetic oligonucleotides were purchased from GeneLab (Louisiana State University, Baton Rouge, LA).

For the nested reaction, PCR primers were used in a primary reaction and a subsequent second reaction to amplify a region internal to the previous priming site. The *Bartonella* genus specific forward primer Bhen16SFF was used in combination with reverse primer UB16SDR9 to amplify an 823 bp fragment. In a second reaction forward primer Bhen16SFF was used in combination with the inner reverse primer Bhen16SBBR to generate a 567 bp *Bartonella* genus-specific fragment. The species and type-specific *Bartonella* nested PCR was done with combinations of the outer primers Bhen16SFF-UB16SDR9 and in the second reaction inner primers Bhen16SGG, Bhen16SHH or Bhen16SCC and Bhen16SBBR.

**PCR amplification of DNA.** Reaction mixtures were prepared under a hood in a room separate from the extraction process. A separate set of pipettes and aerosol-barrier pipette tips were used for reagent preparation. PCR amplification was performed in a final volume of 50 µl by using a reaction mixture containing deoxynucleoside triphosphates (200µM each), 1.75 mM MgCl₂, 0.2µM of each primer, 2.5 U of *Taq* DNA polymerase, AmpliTaq™ Gold, (Perkin Elmer), 4 µl of DNA template and 32.6 µl of Perkin Elmer buffer. The PCR amplifications were carried out on a Model 9600 Thermal-Cycler Perkin-Elmer/Applied Biosystems (Foster City, CA). Amplification reaction consisted of 10 min. at 95°C; 30 cycles of 94°C for 1 min., 66°C for 1 min., 72°C for 1 min., and a final elongation step at 72°C for 10 min. A second
amplification reaction of 20 cycles with the sample conditions as previously stated was performed using inner primers and 1 μl of DNA from the first PCR reaction. Amplicon carryover was prevented by using aerosol barrier pipette tips and by separation of extraction area, reagent mixture preparation, and amplification and electrophoresis area. As a negative control, PCR reagents without DNA template were included in each amplification assay. Positive controls of extracted *Bartonella* species were also used in each amplification assay.

**Detection of PCR products.** The PCR-amplified products were detected by electrophoresis on a 2% agarose gel in Tris-borate-EDTA buffer and visualized with ethidium bromide and UV light transillumination. Sixteen microliters of the final product was analyzed in each lane of the gel. A Low DNA Mass™ ladder (Life Technologies, Gaithersburg, MD) with six blunt-ended DNA fragments of 2000, 1200, 800, 400, 200, and 100bp was used as the molecular marker.

**Sensitivity of PCR.** To determine the minimum amount of *Bartonella* DNA that could be detected by this PCR assay specific for the 16S rRNA gene of *B. henselae*, genomic *Bartonella* DNA was extracted from a *B. henselae* culture using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) protocol for bacteria. The total DNA extracted from culture was measured by absorbance at 260 nm on a Beckman DU 640 Spectrophotometer (Beckman Instrument, Inc. Hercules, CA) using the Warburg-Christian concentration method. Serial ten fold dilutions of the extracted DNA were made in the elution buffer from the Qiagen kit. PCR amplification was performed on each dilution to determine the limits of detection. To determine the minimum number of organisms that could be detected by PCR assay of the 16S rRNA gene of *B. henselae*,

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serial dilutions of *B. henselae* organisms diluted in PBS and *B. henselae* diluted in sterile cat blood were prepared. Each dilution was extracted using the QIAamp DNA Mini Kit, and PCR amplification performed. A quantitative culture was performed on each dilution. The results of PCR and culture were compared to determine limits of detection of PCR.

**Specificity of PCR.** DNA extracted from *Bartonella henselae* 87-66 (ATCC 49793), *Bartonella henselae* Houston-1 (ATCC 49882), *Bartonella quintana* (ATCC VR-358), *Bartonella clarridgeiae* (ATCC 700095), *Bartonella elizabethae* (ATCC 49927), *Bartonella henselae*-LSU 16, and *Bartonella henselae*-Baby, *Escherichia coli*, *Pseudomonas*, *Bordetella bronchiseptica*, *Pasteurella multocida*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were used as templates in individual PCRs with primers Bhen16SDR9, Bhen16SBBR, Bhen16SFF, Bhen16SGG, Bhen16SHH, Bhen16SCC. The specificity of this PCR was also performed with *Brucella abortus* (RB51 strain) DNA because of the close phylogenetic relationship to *Bartonella* species.

**Sequence analysis.** Amplicons derived from isolates of *B. henselae* LSU 16, *B. henselae* Baby, *B. henselae* 87-66, *B. henselae* Houston-1, *B. clarridgeiae* and from blood of one infected cat (cat 86) were sequenced by the Louisiana State University GeneLab using an ABI Prism™ 377 DNA Sequencer (Perkin-Elmer) to confirm the amplification of *Bartonella* DNA.

**Results**

**Specificity of the nested PCR.** The primers Bhen16SFF-Bhen16SDR9 amplified the predicted products of the 823 bp DNA in the first amplification from each of the *Bartonella* species (Figure 2). The primers Bhen16SFF-Bhen16SBBR amplified the
Figure 2. Specificity of Bartonella primers Bhen16SFF-Bhen16SDR9: PCR products (2% agarose gel stained with ethidium bromide)
Lane 1: Low DNA Mass™ Ladder (Gibco)
Lane 2: B. henselae LSU 16
Lane 3: B. henselae Baby
Lane 4: B. henselae 87-66
Lane 5: B. henselae Houston-1
Lane 6: B. quintana
Lane 7: B. elizabethae
Lane 8: B. claridgeiae
Lane 9: E. coli
Lane 10: Pseudomonas aeruginosa
Lane 11: Bordetella bronchiseptica
Lane 12: Pasteurella multocida
Lane 13: Staphylococcus aureus
Lane 14: Streptococcus pneumoniae
Lane 15: Brucella abortus
Lane 16: Reagent control
Lane 17: B. henselae positive control
567 bp DNA in the second amplification of PCR with DNA templates from each of the Bartonella species except B. elizabethae (Figure 3). Products were not amplified with the other microorganisms and negative controls as shown in Figures 2 and 3. Primers Bhen16SGG and Bhen16SBBR amplified the predicted product of the 477-bp DNA of B. henselae genotype I, and primers Bhen16SHH and Bhen16SBBR amplified the predicted product of the 477-bp DNA of B. henselae genotype II, and primers Bhen16SCC and Bhen16SBBR amplified the predicted product of the 477-bp DNA of B. claridgeiae (Figure 4).

**Sensitivity of B. henselae nested PCR.** The sensitivity limit of the assay were determined as the minimum amount of genomic DNA from an isolate of B. henselae and minimum number of B. henselae organisms in spiked cat blood that could be detected. The sensitivity limits of B. henselae genomic DNA of the first reaction with primers Bhen16SFF–Bhen16SDR9 was approximately 6.4 pg or 3.2 x 10³ organism (Figure 5). By using the nested PCR the sensitivity limits of the assay were increased to approximately 6.4 fg, which corresponds to 3.2 organisms (Figure 6). To estimate the quantity of genomic DNA in one cell of Bartonella, the molecular mass of Bartonella bacilliformis (1.76x10⁻¹⁵ g or 1.7 fg or 1.6x10⁶ bp and equal to 1.7 fg) was used. Applying this method of estimation, one Bartonella cell (2x10⁶ bp) is equal to approximately 2 fg. This assay was repeated three times.

Using 200 μl of each dilution of B. henselae in sterile cat blood, the sensitivity limits of B. henselae in cat blood was determined to be one ten-fold dilution beyond the minimal detection limit of culture of 25 CFU/ml. This assay was repeated three times.
Figure 3. Specificity of *Bartonella* primers Bhen16SFF-Bhen16SBBR: PCR products (2% agarose gel stained with ethidium bromide)

Lane 1: Low DNA Mass™ Ladder (Gibco)
Lane 2: *B. henselae* LSU 16
Lane 3: *B. henselae* Baby
Lane 4: *B. henselae* 87-66
Lane 5: *B. henselae* Houston-1
Lane 6: *B. quintana*
Lane 7: *B. elizabethae*
Lane 8: *B. claridgeiae*
Lane 9: *E. coli*
Lane 10: *Pseudomonas aeruginosa*
Lane 11: *Bordetella bronchiseptica*
Lane 12: *Pasteurella multocida*
Lane 13: *Staphylococcus aureus*
Lane 14: *Streptococcus pneumoniae*
Lane 15: *Brucella abortus*
Lane 16: Reagent control
Lane 17: *B. henselae* positive control

567 bp-
Figure 4. Specificity of *Bartonella* species and genotype primers. PCR products (2% agarose gel stained with ethidium bromide)
Lane 1: Low DNA Mass™ Ladder (Gibco)
Lane 2-6: Primer Bhen16SGG-Bhen16SBBR genotype I specificity
Lane 2: *B. henselae* 87-66 genotype I
Lane 3: *B. henselae* LSU 16 genotype II
Lane 4: *B. claridgeiae*
Lane 5: Reagent Control
Lane 6: *B. henselae* 87-66 genotype I positive control
Lane 7-11: Primer Bhen16SHH-Bhen16SBBR genotype II specificity
Lane 7: *B. henselae* 87-66 genotype I
Lane 8: *B. henselae* LSU 16 genotype II
Lane 9: *B. claridgeiae*
Lane 10: Reagent Control
Lane 11: *B. henselae*-LSU 16 genotype II positive control
Lane 12-16: Primer Bhen16SCC-Bhen16SBBR *B. claridgeiae* specificity
Lane 12: *B. henselae* 87-66 genotype I
Lane 13: *B. henselae* LSU 16 genotype II
Lane 14: *B. claridgeiae*
Lane 15: Reagent Control
Lane 16: *B. claridgeiae* positive control

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Figure 5. Sensitivity of *Bartonella* primers Bhen16SFF-Bhen16SDR9: PCR products (2% agarose gel stained with ethidium bromide)
Lane 1: Low DNA Mass™ Ladder (Gibco), Lane 2: 640 ng/ml, Lane 3: 64 ng/ml, Lane 4: 6.4 ng/ml, Lane 5: 640 pg/ml, Lane 6: 64 pg/ml, Lane 7: 6.4 pg/ml, Lane 8-13: blank, Lane 14: *B. henselae* positive control

Figure 6. Sensitivity of *Bartonella* primers Bhen16SFF-Bhen16SBBR: PCR products (2% agarose gel stained with ethidium bromide)
Lane 1: Low DNA Mass™ Ladder (Gibco), Lane 2: 640 ng/ml, Lane 3: 64 ng/ml, Lane 4: 6.4 ng/ml, Lane 5: 640 pg/ml, Lane 6: 64 pg/ml, Lane 7: 6.4 pg/ml, Lane 8: 640 fg/ml, Lane 9: 64 fg/ml, Lane 10: 6.4 fg/ml, Lane 11-12: blank, Lane 13: *B. henselae* positive control
The nested PCR enhanced detection of organisms from the blood of cats experimentally infected with *B. henselae*. The limit of detection in cat 87, when using primer set Bhen16FF-UB162DR9 in the first reaction, was 19,000 CFU/ml. In the first reaction using primers Bhen 16FF-UB162DR9, the assay did not detect amplicons in the blood of cat when the culture results were 1,400 CFU/ml, 1,200 CFU/ml, or 300 CFU/ml. In the second reaction using the inner primers Bhen16SFF-Bhen16SBBR, the nested PCR assay detected amplicons of *B. henselae* in cat 87 below the limits of culture (Figure 7). In the second reaction, the nPCR amplified Bartonella DNA when the culture results were 1,400 CFU/ml, 1,200 CFU/ml, 300 CFU/ml, and when the culture was negative for two consecutive weeks.

**Bacteremia in Experimentally Infected Cats.** In this study, cats experimentally infected with *B. henselae* were followed by PCR and culture beginning one week after inoculation and until time of necropsy. Over the course of this study, 362 blood specimens were used to investigate the bacteremic phase of the infection using PCR assay and bacterial culture. Fourteen of the sixteen cats experimentally inoculated with *B. henselae* Houston-1 did not become bacteremic. Bacteremia in the cats ranged from $1.0 \times 10^2$ to $8.0 \times 10^5$ CFU/ml. The duration of the bacteremia ranged from 5 weeks to 22 weeks (Table 3). Bacteremia was determined by isolation of *Bartonella* from blood or amplification of *Bartonella* DNA from blood. In most samples, the PCR was useful in detecting *Bartonella* DNA after the culture became negative. The PCR assays were positive in 11 of the 14 cats after culture became negative. The PCR assay was positive for periods of 1 to 9 weeks after the culture became negative (Table 3 and Table 5). In
Figure 7. Bartonella DNA. Comparison of nPCR assay and bacterial culture from blood samples from Bartonella henselae experimentally infected cat (87). PCR products from nPCR (2% agarose stained with ethidium bromide)
Lane 1: Low DNA Mass™ Ladder (Gibco)
Lane 2-7 PCR products with primers Bhen16SFF-Bhen16SDR9 and CFU/ml
Lane 2: week-1 Blood sample 19,000 CFU/ml
Lane 3: week-2 Blood sample 1,400 CFU/ml
Lane 4: week-3 Blood sample 1,200 CFU/ml
Lane 5: week-4 Blood sample 300 CFU/ml
Lane 6: week-5 Blood sample 0 CFU/ml
Lane 7: week-6 Blood sample 0 CFU/ml
Lane 8-13 PCR products with primers Bhen16SFF-Bhen16SBBR and CFU/ml
Lane 8: week-1 blood sample 19,000 CFU/ml
Lane 9: week-2 blood sample 1,400 CFU/ml
Lane 10: week-3 blood sample 1,200 CFU/ml
Lane 11: week-4 blood sample 300 CFU/ml
Lane 12: week-5 blood sample 0 CFU/ml
Lane 13: week-6 blood sample 0 CFU/ml
Lane 14: Reagent Control
Lane 15: B. henselae positive control
### Table 3. Results of PCR assay and blood culture from bacteremic cats

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<th>Cat 10&lt;sup&gt;b&lt;/sup&gt; PCR CFU/ml</th>
<th>Cat 83&lt;sup&gt;b&lt;/sup&gt; PCR CFU/ml</th>
<th>Cat 87&lt;sup&gt;b&lt;/sup&gt; PCR CFU/ml</th>
<th>Cat G3&lt;sup&gt;b&lt;/sup&gt; PCR CFU/ml</th>
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<sup>a</sup> post-inoculation
<sup>b</sup> inoculated with *B. henselae* LSU 16 Genotype II
<sup>c</sup> inoculated with *Bartonella henselae* Baby Genotype II
<sup>d</sup> inoculated with *Bartonella henselae* 87-66 Genotype I
<sup>e</sup> inoculated with *Bartonella henselae* Houston-1 Genotype I
<sup>f</sup> ND, not determined

(Table 3 continued)
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<sup>a</sup> post-inoculation  
<sup>b</sup> inoculated with B. henselae LSU 16 Genotype II  
<sup>c</sup> inoculated with *Bartonella henselae* Baby Genotype II  
<sup>d</sup> inoculated with *Bartonella henselae* 87-66 Genotype I  
<sup>e</sup> inoculated with *Bartonella henselae* Houston-1 Genotype I  
<sup>f</sup> ND, not determined  

(Table 3 continued)
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<sup>a</sup> post-inoculation  
<sup>b</sup> inoculated with *B. henselae* LSU 16 Genotype II  
<sup>c</sup> inoculated with *Bartonella henselae* Baby Genotype II  
<sup>d</sup> inoculated with *Bartonella henselae* 87-66 Genotype I  
<sup>e</sup> inoculated with *Bartonella henselae* Houston-1 Genotype I  
<sup>f</sup> ND, not determined  

(Table 3 continued)
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</tr>
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<sup>a</sup> post-inoculation

<sup>b</sup> inoculated with *B. henselae* LSU 16 Genotype II

<sup>c</sup> inoculated with *Bartonella henselae* Baby Genotype II

<sup>d</sup> inoculated with *Bartonella henselae* 87-66 Genotype I

<sup>e</sup> inoculated with *Bartonella henselae* Houston-1 Genotype I

<sup>f</sup> ND, not determined
each of the cats (n=14) that were followed during the bacteremic phase of the infection, the duration of bacteremia, as defined by culture and PCR, lasted for a longer time period when the PCR assay was used to detect circulating *Bartonella* as compared to culture (Table 3). In three cats, the PCR and culture were negative the same week. The culture was positive for 1-2 weeks when the PCR was negative in cat L3 and 321. In two of the experimentally infected cats there was a 1-week interval when the bacteremia was below the detection limits of culture but detected by the PCR assay. In cat 10, the culture negative interval was 2 weeks during the acute bacteremia when the PCR assay was positive. In cat L1 and 361, *Bartonella* species were bacteremic 1-3 weeks post-inoculation. Two of the cats during the time when the culture was negative, the PCR assay were negative one week followed by a positive PCR the next week (Table 3).

Results of the PCR assay were comparable to culture results in most of the specimens. The PCR assay was positive in 135 of the 138 (98%) blood specimens that were culture-positive. The PCR assay was negative from three blood specimens that were culture-positive with 100 colony-forming units per milliliter (Table 3 and Table 5). Of the 223 blood specimens that were culture-negative, PCR assay was positive in 40 (18%) of these specimens (Table 4).

Table 4. Summary of PCR assay and blood culture results (n=362)

<table>
<thead>
<tr>
<th>PCR positive (%)</th>
<th>PCR negative (%)</th>
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<tbody>
<tr>
<td>Culture positive</td>
<td>135/138 (98)</td>
</tr>
<tr>
<td>Culture negative</td>
<td>40/223 (18)</td>
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</table>
**Relapsing bacteremia.** Two cats, cat 86 and cat 321, infected with *B. henselae* LSU 16, a pathogenic strain of *Bartonella* in cats, had an episode of relapsing bacteremia (Table 5). Cat 86 experienced a culture-negative interval of 7 weeks between the two episodes of bacteremia. The second bacteremia in cat 86 lasted for 8 weeks before the blood culture became negative. The PCR assay in cat 86 was positive for 2 weeks longer than the culture in the first episode of bacteremia. During the second bacteremia, both culture and PCR were positive from weeks 13 through 20. Cat 321 was PCR and culture positive from weeks 1 through 6. At week 7, the PCR was positive and the culture was negative. At week 8, both assays were positive again for 3 weeks. After an 11-week culture-negative interval, cat 321 became culture and PCR positive again. The second bacteremia in cat 321 lasted for 4 weeks with positive culture results for 4 weeks and positive PCR results for only three of those weeks.

**Sequence analysis.** The sequence data derived from *B. henselae* LSU 16 and *B. henselae* Baby were that of a *B. henselae* genotype II strain with the base pairs ATT located at positions 172 to 175 of the 16S rRNA gene identical to *B. henselae* genotype II as described by Bergmans et al. (1995) (Figure 8). The sequence data from *B. henselae* Houston-1 and *B. henselae* 87-66 located at positions 172 to 175 of the 16S rRNA gene were that of a *B. henselae* genotype I with the base pairs TAG as described by Bergmans et al. (Figure 8). The sequence data derived from the blood of cat 86 was a *B. henselae* genotype II with the base pairs ATT (Figure 8). The sequence data from the isolate of *B. clarridgeiae* was as reported with the base pairs ACT located at positions 172 to 175 of the 16S rRNA gene (Gene bank X97822) (Figure 8).
Table 5. Results of PCR and blood culture from cats with relapsing bacteremia

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<th>Cat 321⁹</th>
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a post-inoculation
b inoculated with B. henselae LSU 16 Genotype II
c ND, not determined
Figure 8. Partial sequence alignment of 519-bp fragment amplified from 16S rRNA gene of Bartonella henselae LSU 16 genotype II, B. henselae Baby, B. henselae 87-66, B. henselae Houston-1, B. clarridgeiae, and PCR product from blood of experimentally infected cat 86. Region of 172-175 (ATT, TAG) identifies B. henselae genotype II and genotype I. Region of 172-176 (ACT) identifies B. clarridgeiae.
Discussion

A PCR assay that is both sensitive and specific is a good tool for studying persistent infections. The nPCR assay was specific for *B. henselae* LSU 16, *B. henselae* Baby, *B. henselae* Houston-1, *B. henselae* 87-66, *B. clarridgeiae*, and *B. quintana*, *B. elizabethae* was not amplified in the second reaction of the nPCR assay because the reverse primer Bhen16SBBR is not specific for *B. elizabethae*. The sensitivity limits of the PCR assay were improved with the nested design and proved useful in detecting low numbers of organisms. The nested PCR assay was able to detect *Bartonella* DNA in circulating blood of cats that were culture negative. The ability of the PCR to detect *Bartonella* DNA in cats that were culture negative is a function of the sensitivity limits of the PCR as compared to culture. In theory, this nested PCR can detect approximately 3.2 *Bartonella* organisms. The detection limits of culture in this study were approximately 25 CFU/ml, and the limits of the PCR assay were one ten-fold dilution below 25 CFU/ml. Although the amplification of *Bartonella* DNA from the blood of *Bartonella* infected cats may represent dead bacteria, the detection of *Bartonella* DNA in some cats as long as 9 weeks after blood cultures were negative makes the detection of dead bacteria unlikely. Rather, one could postulate that amplification of *Bartonella* DNA in the blood of infected cats long after the blood culture is negative represents a small number of viable organisms circulating in the peripheral blood. One also could speculate that *Bartonella* DNA in the blood of these cats is due to the shedding of microorganisms into the peripheral blood from a sequestered location in the cat such as the endothelial cells of the vascular system.
The PCR assay was able to detect *Bartonella* DNA in 40 samples that were culture negative. Again this could be explained by existence of circulating nonviable organisms or because the sensitivity of the PCR assay is greater than that of culture. The question then becomes the infectious nature of bartonellosis in cats in this stage of the infection. Kordick et al. (1997b) was able to transmit *B. henselae* from blood culture negative cat by inoculation of blood into a SPF cat. This observation indicates that a negative blood culture cannot be used to definitively rule out *Bartonella* infection in cats. If cats are infectious after the culture is negative and PCR is positive, the PCR assay would be a more accurate laboratory assay for detecting cats with *Bartonella*.

Three blood specimens were culture positive and PCR negative. For unknown reason, the PCR assay missed three blood specimens that were culture-positive with 100 colony-forming units per milliliter. This may be explained by the cohesion and aggregation factors that have been reported with *Bartonella* species. Clarridge (1996) reported a degree of cohesiveness in *B. henselae* and *B. clarridgeiae*, and this was correlated with cells existing in a tightly packed array. Despite the fact that the blood was vortexed before sampling, the PCR could have been negative because of the coherent nature of the organism. The PCR assay results were negative for one week followed by a positive result the next week in cats, L1, and 361. The results occurred when the bacteremia was waning and was below the minimum detection limits by PCR or because the result are representative of a false positive test.

The PCR assay did not detect *Bartonella* DNA earlier than the blood culture in the cats with a recurrent episode of bacteremia or in the primary infection in cat L3. Possibly, *if blood were taken within the week before the recurrent episode, a positive*
PCR for Bartonella DNA would have been detected. It is not known how the organisms seed the blood in these infections. If the organism slowly leaks into the circulating blood from a source like the endothelial lining, the PCR may be positive before the culture. If the organism seeds the blood suddenly with a number of organisms large enough for the culture to be positive, the PCR assay would not be positive any sooner than culture. However, because of the turnaround time of the PCR assay compared to culture, the detection of a positive Bartonella cat would be noted at an earlier time.

Specificity was addressed in this PCR assay with the use of specific primers in the first and second reaction of the nested PCR. Specific oligonucleotide primers for Bartonella genus were used in the first reaction by amplifying an 823 bp fragment or a 567 bp fragment from the 16S rRNA gene of Bartonella. In the second reaction, specificity is increased further by the use of another group of specific primers that amplifies a subset from the first reaction because they are complementary to a specific fragment of Bartonella. B. clarridgeiae and Bartonella henselae genotype I and genotype II can be differentiated by the specific primer sets in the second reaction. An assay that can determine genetic differences in strains of Bartonella will be useful in studying the pathogenesis of this organism in cats.

Molecular assays such as PCR give a higher discriminatory power in identification of microorganisms than does identification by phenotypic characteristics (Relman 1993). The products amplified in this PCR reaction were confirmed by sequence analysis that is a very specific method of identification and is based on genetic information, not phenotypic tests as in culture methods. In addition, molecular assays
such as PCR that will amplify and allow for detection of microorganisms missed because of the insensitivity of a particular method is a powerful tool in research and clinical microbiology. This PCR assay has proven to be a useful assay that is both sensitive and specific and will be used in our laboratory for further studies for the understanding of the pathogenesis of *B. henselae* in cats.
CHAPTER THREE
PERSISTENT BARTONELLA DNA IN CATS EXPERIMENTALLY INFECTED WITH BARTONELLA HENSELAE GENOTYPE I AND BARTONELLA HENSELAE GENOTYPE II

Introduction

During the past decade, Bartonella species has emerged as an important human pathogen. Five species, Bartonella henselae, B. quintana, B. clarridgeiae, B. bacilliformis, and B. elizabethae have been associated with human disease. The spectrum of diseases produced by Bartonella species is wide and includes lymphadenopathy, relapsing bacteremia, endocarditis, retinitis, osteolysis, encephalopathy, hemolytic anemia, bacillary angiomatosis, and bacillary peliosis (Anderson and Neuman 1997).

Epidemiological studies have shown that ownership of a cat and being scratched or bitten by a cat are risk factors for acquiring cat scratch disease (Zangwill et al. 1973). Following that study, two species, B. henselae and B. clarridgeiae, were isolated from cats (Gurfield et al. 1997). Prevalence studies have shown that a significant number of cats are subclinically infected with Bartonella, and that cats are a reservoir for human infections involving Bartonella species (Childs et al. 1994; Heller et al. 1997; Jamerson et al. 1995). B. henselae causes the widest spectrum of pathology, depending on the immune system of the host, and includes disease with granulomatous features, vascular proliferative features, and a predominantly intravascular focus (Relman et al. 1990). The only known human disease associated with B. clarridgeiae is cat scratch disease.

Our knowledge of the pathogenesis of B. henselae in the cat is incomplete. It is known that the prevalence of B. henselae infections in cats is high, and that feline bartonellosis can be a persistent infection in cats (Kordick et al. 1999). What is not
understood is the persistent phase of feline bartonellosis. Many aspects of the persistent phase such as the mechanisms of survival, host cell association, or tissue sequestration are unclear.

Clinically infected cats can be bacteremic and asymptomatic for long periods. The bacteremia in cats can be of significant magnitude and duration and can often recur (Kordick and Breitschwerdt 1997). In a study by Kordick et al. (1997b), cats in environmentally controlled settings were culture negative for periods up to 4 months between recurrent episodes of bacteremia. Kordick et al. (1999) observed recurrent episodes of bacteremia for up to 454 days in cats infected with B. henselae or B. clarridgeiae. In humans, relapsing bacteremia is also a characteristic of Bartonella infections and many of these infections evolve into long-term asymptomatic persistent infections (Welch and Slater 1999). The persistence of these infections in both cats and humans is suggestive of a carrier state with sequestration of the microorganism within the host.

During the acute bacteremic phase in cats, B. henselae has been cultured from lymph nodes, heart, lung, spleen, liver, kidney, pancreas, thymus, and bone marrow (Guptill et al. 1997). Clinical signs have been described in experimentally infected cats and include fever, lethargy, and mild neurologic signs consistent with CNS involvement in one cat (O’Reilly et al. 1999a; Guptill et al. 1997; Kordick et al. 1999). Within 2 to 6 weeks after infection, enlarged mandibular or popliteal lymph nodes with lymphoid hyperplasia, a splenic microabscess, and a liver abscess in experimentally infected cats have been described (Guptill et al. 1997). Bartonella can be cultured from many tissues of cats that are bacteremic probably because of the presence of organisms circulating
through tissue. Whether *Bartonella* colonizes or sequesters in tissue after the acute bacteremic phase is unknown. Tissues from blood culture negative cats are often culture negative.

In addition to recurrent bacteremia, there is other evidence to support the belief that *Bartonella* is a persistent infection in cats. This evidence includes failure to eliminate infections readily with the use of antibiotics (Kordick et al. 1997b), pathological changes seen in the tissue of cats infected for long periods with *B. henselae* (Korkick et al. 1999), immunological evidence to suggest continued antigenic stimulation (O'Reilly et al. 1999a) and granulomatous response as seen in some *Bartonella* infections (Guptill et al. 1997). Granulomatous inflammation in the liver of cats infected with *B. henselae* was reported in this study but has not been confirmed as a typical cellular response (Guptill et al. 1997). Granulomatous inflammation has been reported in *Bartonella* infections in humans with CSD, in dogs with granulomatous lymphadenitis and granulomatous rhinitis, and in the liver of mice (Pappalardo et al. 2000, Regnath et al. 1998).

The immunological response of the cat also suggests that feline bartonellosis can be a persistent infection. Some cats respond with typical IgM and IgG antibody patterns of response, while other cats infected with *B. henselae* have been shown to have IgM levels which remain elevated above baseline suggesting continued antigenic stimulation (Freeland et al. 1999). Homologous protection without cross protection by various species and types has been shown (Yamamoto et al. 1998b). Human and murine immune responses to *B. henselae* have been shown to include T-cell activity, IFN-γ production, and macrophage killing activity, suggesting an intracellular location with a
Th1, CD4 type cytokine response (Karem et al. 1999). Freeland et al. (1999) identified a number of *Bartonella*-specific antigens to which strong antibody responses are generated in both experimentally and naturally infected cats. *B. henselae* may evade the cat's immune system by methods of promoting uptake by nonimmune cells such as erythrocytes (Mehock et al. 1998). The strain *B. henselae* LSU16 was found to be resistant to killing by neutrophils when compared to the Houston-1 strain of *B. henselae* (O’ Reilly et al. 1999b).

In human cases, *B. henselae* DNA has been detected by PCR in atypical *B. henselae* infections involving organs such as heart (endocarditis), brain (encephalitis), lymph nodes, eye (peripapillary angioma), liver, and spleen (Koehler et al. 1997; Gray et al. 1998; McGrath, 1998; Wheeler et al. 1997; Baorto et al. 1998; Stuart and Norwick 1998). *B. henselae* DNA was detected by PCR in the liver of a dog with peliosis hepatis (Kitchell et al. 2000). Regnath et al. (1998) showed that *B. henselae* infected mice cleared the cultivatable organism within the first 6 days of infection from liver and spleen but *Bartonella* DNA could be detected in the liver for at least 3 months.

Whether *Bartonella* has an intracellular niche other than the feline erythrocytes is unknown. An intracellular location of *Bartonella* in cats would be consistent with the persistent nature of feline bartonellosis. In the study by Kordick et al. (1997b), cats chronically bacteremic with *Bartonella* were treated for an extended duration of 25 weeks with enrofloxacin, which is partially converted to ciprofloxacin in the cat, with elimination of infection in 9 of 14 infected cats.

*Bartonella* infections in cats have been reported to cause pathologic changes in major organs in cats (Kordick et al. 1999). Kordick et al (1999) described cats...
experimentally infected with *Bartonella*, that had microscopic inflammatory foci in tissue which represent nonspecific histological changes consistent with systemic dissemination of blood-borne infection. Follicular hyperplasia of lymph nodes and spleen were seen in experimentally infected cats and thought to represent a tissue response to chronic antigenic stimulation, which is consistent with chronic *Bartonella* infection (Kordick et al. 1999). In another study done by Guptill et al. (1997), *Bartonella*-specific antibody-secreting cells were detected in spleen, bone marrow and peripheral lymph nodes in abacteremic experimentally infected cats thirty-two weeks post inoculation. The greatest number of antibody-secreting cells was detected in the spleen in that study.

Often *Bartonella* DNA can be amplified from various types of clinical material in the absence of a positive culture. Whether the *Bartonella* DNA represents microorganisms that are viable or nonviable is not fully understood. The amplification of Bartonella DNA may represent viable organisms lying in a dormant state, viable organisms in numbers below the detection limits of culture or nonviable (dead) organism in tissue. One way to determine if a bacterial cell is viable is to measure the synthesis of RNA. The RT-PCR, which first produces cDNA from RNA and then amplifies the cDNA, is one method for analysis of RNA expression.

The purpose of this study was to determine if persistent *Bartonella* DNA exists in the tissue of abacteremic cats chronically infected with *Bartonella*. A nPCR assay that amplifies a 567-bp fragment of the 16S rRNA gene of *B. henselae* was used to detect *Bartonella* DNA in tissue. RNA expression in the tissue of cats was examined using two different RT-PCR assays. One assay was designed to amplify a 354-bp fragment of
the citrate synthase gene of *B. henselae*, and the other RT-PCR was designed to amplify a 567-bp fragment of the 16S rRNA gene of *B. henselae*. Cats defined as abacteremic by culture and PCR assay were necropsied and tissues taken for isolation and amplification of *Bartonella*. Results were complied from SPF cats experimentally infected with *B. henselae*. The findings of *Bartonella* DNA detected in tissue after the bacteremic phase of the infection are discussed in this study.

**Materials and Methods**

**Experimental animals.** Tissue used in this study were taken from twenty-three cat followed in the study. Sixteen of the cats were the same as referred to in Chapter 2. Four additional cats used in this study were bred at Louisiana State University as specific-pathogen-free cats (SPF) and three additional cat used in this study were purchased from Harlan Sprague-Dawley (Madison, Wis.) as specific-pathogen-free (SPF) cats.

**Bacterial strains.** As described in Chapter 2

**Infection in cats.** Methods the same as in Chapter 2

**Collection of tissue specimens.** Cats were euthanized by cardiac puncture with overdose of Beuthanisa (Schering Plough, Canada). Standard necropsies were performed on all cats. Tissue was collected using a separate set of sterile instruments for each tissue. Representative fresh tissue samples were taken for bacterial culture, PCR analysis, and RNA extraction and formalin fixed tissues were taken for histopathology. For culture and PCR analysis tissue was placed in a sterile whirpak bag and placed on ice immediately. Tissue for RNA extraction was immediately frozen in
liquid nitrogen. Tissue for histopathology was fixed in 10% neutral buffered formalin for paraffin embedding.

**Cultures of tissues.** Postmortem tissue specimens were collected aseptically on each cat at necropsy using a separate set of sterile instruments for each tissue collected. Eight tissues including liver, spleen, bone marrow, heart valve, lung, brain, lymph node, and kidney and blood were taken from each cat. Each tissue was ground in a tissue grinder and a representative sample was cultured on enriched chocolate agar (Remel, Lenexa, Kansas) and brucella broth with 6 to 8% Fildes solution and 250 μg of hemin per ml (Schwartzman et al 1993). Broth cultures were observed weekly for growth. Broths were gram stained and subcultured on chocolate agar before discarding. All cultures were incubated at 37°C in 5% CO₂ for 3 weeks.

**Extraction of DNA from tissue.** Extraction of DNA from tissue was performed using the Tissue Protocol within the QIAamp DNA Mini Kit (Qiagen Inc. Valencia, CA). A representative section of each tissue was taken from each cat at necropsy. Each tissue section was ground in a tissue grinder and 25 mg (Mettler PM 460) of tissue was used in the Tissue Protocol according to manufacturer directions. The extracted DNA was used as a template in the PCR assays. Extraction controls included tissue from *Bartonella* culture positive and negative cats and a reagent control without a DNA sample.

**PCR amplification of DNA from tissue.** Amplification of DNA was identical to the method described in Chapter 2. The PCR assay used in this study was not a quantitative PCR, therefore, the *Bartonella* DNA detected in the tissue of the cats could not be quantitated. The liver of cat 1 and spleen of cat 87 were chosen at random and the
Bartonella DNA extracted. Extracted DNA was diluted 10-fold in elution buffer and amplified as described in the PCR assay.

Detection of PCR products. Methods the same as in Chapter 2

RNA extraction. Tissue that was positive for DNA fragments of the 16S rRNA gene of Bartonella was used for RNA extraction. RNA extractions were done using the RNAeasy Kit, (Qiagen Inc. Valencia, CA). The procedure recommended by the manufacturer was followed with the exception of treatment with RNase-Free DNAase (Qiagen, Inc. Valencia, CA) for 1 hr at 37°C and a follow-up RNA extraction with the RNAeasy Kit.

RT-PCR of the 16S rRNA gene of B. henselae. The RT-PCR reaction mix was made using the Titan™ One Tube RT-PCR System (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer direction. This method uses the reverse transcriptase avian myeloblastosis virus (AVM) for the first strand synthesis and the Expand™ High Fidelity enzyme blend, which consists of Taq DNA polymerase and Pwo DNA polymerase for the PCR. Primers used in this assay were designed to amplify a 567-bp fragment of the 16S rRNA gene of Bartonella. A nested reaction was done using 4 μl of RNA template in the first reaction with forward primers Bhen16SFF 5’ CACATGCAAGTGCAGACACACTCTTTTAGAG 3’ and reverse primer UB16SDR9 5’ GCCCCCCGTCAATTCTTTGAGTTT 3’. The RT-PCR reaction mixture consisted of two reaction mixtures each of 25 μl. One reaction mixture consisted of 12.5 μl of sterile RNase and DNase free water, 4 μl of deoxynucleoside triphosphates (200 uM each), 2.5 μl dithiothreitol solution (DTT) (100 mM), and 4 μl of template. The second reaction mixture consisted of 10 μl of RT-PCR buffer (Titan), 14
μl of sterile RNase and DNase free water, and 1 μl of Titan Enzyme mix (AMV and Expand™ High Fidelity). Twenty-five μl of reaction mix one and 25 μl of reaction mix two were added together for thermocycling. The amplification reaction consisted of reverse transcription step of 50°C for 30 min., 30 cycles at 94°C for 30 s, 66°C for 30 s, 68°C for 1 min and a hold cycle for 68°C for 2 min. The second reaction (nested PCR) mixture consisted of deoxynucleoside triphosphates (200 μM each), 1.75 mM MgCl₂, 0.2 μM of each primer, 2.5 U of Taq DNA polymerase, AmpliTaq™ Gold (Perkin Elmer), 1 μl of DNA from the first reaction in PE buffer in a total volume of 50 μl. In the second reaction the primers were the forward primers Bhen16SFF and reverse inner primer Bhen16SBBR 5’ CACTCACCTCTTCCACACTCAAGATA 3’. Amplification reaction consisted of 10 min. at 95°C; 20 cycles of 94°C for 1 min., 66°C for 1 min., and 72°C for 1 min., and a final elongation step at 72°C for 10 min. The PCR amplification was carried out on a 9600 Thermal-Cycler Perkin-Elmer/Applied Biosystems (Foster City, CA).

RT-PCR of the citrate synthase gene of B. henselae. A second RT-PCR assay was designed to amplify a 354 bp fragment of B. henselae gltA citrate synthase gene (Regnath et al. 1998). In this assay the outer primer sequences CSN-F1 5’ GGTCCCAACTCTTGCGCTATG 3’ and CSN-R1 5’ CAGCCCGA CACTGGGTGCTAATG 3’ were used in the first reaction. The RT-PCR reaction mixture consisted of two reaction mixtures each of 25 μl. One reaction mixture consisted of 12.5 μl of sterile RNase and DNase free water, 4 μl of deoxynucleoside triphosphates (200 μM each), 2.5 μl dithiothreitol solution (DTT) (100mM), and 4 μl of
template. The second reaction mixture consisted of 10 μl of RT-PCR buffer (Titan), 14 μl of sterile RNase and DNase free water, 1 μl of Titan Enzyme mix (AMV and Expand™ High Fidelity). Twenty-five μl of reaction mix one and 25 μl of reaction mix two were added together for thermocycling. The amplification reaction consisted of a reverse transcription step of 50°C for 30 min., 30 cycles at 94°C for 30 s, 66°C for 30 s, 68°C for 1 min., and a hold cycle for 68°C for 2 min. A nPCR was performed with the inner primers CSN-F2, 5’ ATGCCTAAAAATGTTACAAGA 3’ and CSN-R2 5’ CGTGCTAA TGCAAAAAGAAC 3’. The reaction mixture consisted of deoxynucleoside triphosphates (200μM each), 1.75 mM MgCl₂, 0.2μM of each primer, 2.5 U of Taq DNA polymerase, AmpliTaq™ Gold (Perkin Elmer), 1 μl of DNA from the first reaction in PE buffer in a total volume of 50 μl. The amplification of the second reaction consisted of denaturation of 4 min. at 95°C, and 20 cycles of 1 min at 94°C, 10 sec at 30°C, and 1 min. at 72°C and a final extension step of 10 min at 72°C. PCR amplifications were carried out on a 9600 Thermal-Cycler Perkin-Elmer/Applied Biosystems (Foster City, CA).

**Detection of PCR products.** Methods the same as in Chapter 2.

**Histopathology.** Tissues taken at necropsy included brain, liver, spleen, kidney heart valve, lung, bone marrow, and peripheral lymph node. Representative sections of tissues were fixed in 10% neutral buffered formalin for paraffin-embedding, routinely processed, and 5 um thick sections were stained with hematoxylin and eosin and Warthin-Starry silver stains for histological examination. Dr. R. Bauer performed all of the necropsies and examined the histopathology in this study.
**Statistical analysis.** The chi-square test was used to evaluate the relationship between types of tissue and presence of *Bartonella* DNA in various tissues. The analysis was considered significant at a probability of $P \leq 0.05$.

**Results**

**Results of culture of tissue.** Cats were euthanized from 4 weeks to 17 weeks after the bacteremic phase of the infection. At the time of euthanasia, all cats were both blood culture negative and PCR-negative. A total of 184 tissue specimens were analyzed in this study from the 23 cats. Agar and broth cultures from 184 tissues were negative after incubation for 3 weeks.

**PCR results from tissue analysis.** At the time of necropsy, *Bartonella* DNA was not detected from the peripheral blood of any cat. Although *Bartonella* was not cultured from any tissue, *Bartonella* DNA was amplified in 10 of the 23 cats in at least one tissue specimen (Table 6). The distribution of *Bartonella* DNA in cats was from 1 tissue to all 8 tissues in one cat. In two cats, *Bartonella* DNA was detected in the spleen only. *Bartonella* DNA was amplified from the brain of 3 (30%), from the liver of 5 (50%), from the spleen of 9 (90%), from bone marrow of 7 (70%), from the lung of 4 (40%), the lymph node of 6 (60%), from the kidney of 4 (40%) and from the heart value of 5 (50%) (Table 7). There were no significant differences among tissue with regard to finding *Bartonella* DNA in tissue ($\chi^2 = 4.49$, $P > 0.05$). Two of the four cats receiving passive antibody to *Bartonella* LSU 16 were found to have *Bartonella* DNA in at least five tissue sites. The one cat that received passive antibody to *Bartonella henselae* Houston-1 had *Bartonella* DNA in 7 tissue sites. Three of the four cats injected with methylprednisolone were found to have *Bartonella* DNA in 5 to 8 tissue sites. The cat
Table 6. Results of findings of persistent *Bartonella* DNA found in various tissues of cats experimentally infected with *Bartonella henselae*

<table>
<thead>
<tr>
<th>Cat #</th>
<th>Strain</th>
<th>AB&lt;sup&gt;bc&lt;/sup&gt; MP&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Necropsy&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Bartonella DNA in Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na +</td>
<td>19</td>
<td>+ (1, 2, 3, 4, 5, 7, 8)</td>
</tr>
<tr>
<td>10</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt; na</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>81</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+&lt;sup&gt;c&lt;/sup&gt; na</td>
<td>19</td>
<td>+ (1, 2, 3, 4, 5, 6, 7)</td>
</tr>
<tr>
<td>83</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt; na</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>84</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na +</td>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td>85</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>19</td>
<td>+ (1, 2, 3, 4, 6, 7, 8)</td>
</tr>
<tr>
<td>86</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt; +</td>
<td>4</td>
<td>+ (1, 3, 4, 5, 7,)</td>
</tr>
<tr>
<td>87</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt; +</td>
<td>8</td>
<td>+ (1, 2, 3, 4, 5, 6, 7, 8)</td>
</tr>
<tr>
<td>182</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>&gt;19</td>
<td>-</td>
</tr>
<tr>
<td>184</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>&gt;19</td>
<td>-</td>
</tr>
<tr>
<td>223</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>&gt;19</td>
<td>-</td>
</tr>
<tr>
<td>G3</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>12</td>
<td>+ (2, 7)</td>
</tr>
<tr>
<td>321</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>4</td>
<td>+ (2, 3)</td>
</tr>
<tr>
<td>N3</td>
<td>LSU 16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>18</td>
<td>+ (2)</td>
</tr>
<tr>
<td>L1</td>
<td>Baby&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>11</td>
<td>+ (2)</td>
</tr>
<tr>
<td>361</td>
<td>Baby&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>13</td>
<td>+ (2, 3, 8)</td>
</tr>
<tr>
<td>K3</td>
<td>Baby&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>L2</td>
<td>87-66&lt;sup&gt;**&lt;/sup&gt;</td>
<td>na na</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>N2</td>
<td>87-66&lt;sup&gt;**&lt;/sup&gt;</td>
<td>na na</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>K4</td>
<td>87-66&lt;sup&gt;**&lt;/sup&gt;</td>
<td>na na</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>L3</td>
<td>Houston-1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>na na</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>F5</td>
<td>Houston-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>na na</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>347</td>
<td>Houston-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>na na</td>
<td>29</td>
<td>-</td>
</tr>
</tbody>
</table>

*1x10<sup>7</sup>

**1x10<sup>9</sup>

<sup>a</sup> not infected

<sup>AB</sup><sup>bc</sup> anti-LSU 16 or anti-Houston-1, passively administered

<sup>MP</sup><sup>d</sup> given three methylprednisolone injections 2 weeks apart

<sup>b</sup> anti-LSU 16

<sup>c</sup> anti-Houston-1

<sup>d</sup> given three methylprednisolone injections 2 weeks apart

<sup>e</sup> weeks post-bacteremia, time of necropsy

na-negative

1-liver

2-spleen

3-bone marrow

4-heart valve

5-lung

6-brain

7-lymph node

8-kidney

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Table 7. Results of culture and PCR assay from 10 of 23 cats with persistent *Bartonella* DNA in tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Culture positive</th>
<th>PCR positive</th>
<th>%positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0/10</td>
<td>3/10</td>
<td>30%</td>
</tr>
<tr>
<td>Liver</td>
<td>0/10</td>
<td>5/10</td>
<td>50%</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/10</td>
<td>9/10</td>
<td>90%</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0/10</td>
<td>7/10</td>
<td>70%</td>
</tr>
<tr>
<td>Lung</td>
<td>0/10</td>
<td>4/10</td>
<td>40%</td>
</tr>
<tr>
<td>Lymph node</td>
<td>0/10</td>
<td>6/10</td>
<td>60%</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/10</td>
<td>4/10</td>
<td>40%</td>
</tr>
<tr>
<td>Heart valve</td>
<td>0/10</td>
<td>5/10</td>
<td>50%</td>
</tr>
<tr>
<td>Peripheral Blood</td>
<td>0/10</td>
<td>0/10</td>
<td>0%</td>
</tr>
</tbody>
</table>

with all 8 tissues positive had received both passive antibody and methylprednisolone.

Cats 86 and 321, which had a recurrent episode of bacteremia had *Bartonella* DNA in 5 and 2 tissues respectively. Six of 14 cats that were infected but did not receive either passive antibody or methylprednisolone were found to have *Bartonella* DNA in at least one tissue. The amplified PCR products of *Bartonella* DNA from the tissue of cat 1 liver and cat 87 spleen are shown (Figure 9). *Bartonella* DNA was amplified in the undiluted extracted sample from the liver of cat 1 but was not detected in one 10-fold dilution of the extracted DNA. *Bartonella* DNA was detected in the undiluted and in one 10-fold dilution of extracted DNA from the spleen of cat 87.

**RT-PCR results from tissue.** Using primer specific for a 567-bp fragment of the 16S rDNA gene of *Bartonella* and primer specific for a 354-bp fragment of the citrate synthase gene of *Bartonella*, we were unable to detect any cDNA from cats with persistent *Bartonella* DNA in tissue. RT-PCR assay using the 16S rRNA primers did not amplify any cDNA products from the tissue of the cats but the positive control *Bartonella henselae* was amplified to verify the procedure (Figure 10). The PCR
Figure 9. Bartonella DNA from tissue of experimentally infected cats. PCR products amplified from the 16S rRNA gene fragment of Bartonella species.
Lane 1: Low DNA Mass™ Ladder (Gibco)
Lane 2: Liver cat 1
Lane 3-5: Liver cat 1 (10-fold dilutions)
Lane 6: Spleen cat 87
Lane 7: Spleen cat 87 (10-fold dilution)
Lane 8-13: Spleen cat 87 (10-fold dilutions)
Lane 14: Reagent Control
Lane 15: B. henselae positive control
Figure 10. RT-PCR for detection of cDNA of *Bartonella henselae*. PCR products amplified from 16S rRNA gene fragment of *Bartonella*. Tissue samples from cats experimentally infected with *B. henselae*
Lane 1: Low DNA Mass™ Ladder (Gibco)
Lane 2: Spleen tissue from cat 87
Lane 3: Liver tissue cat 1
Lane 4: Spleen tissue cat N3
Lane 5: Positive control *B. henselae* from PCR
Lane 6: Reagent Blank
Lane 7: Positive control *B. henselae* from RT-PCR
primers for the citrate synthase (gltA) gene amplified the Bartonella henselae, used as positive control, but did not amplify cDNA in tissues from infected cats (Figure 11). We were unable to detect Bartonella specific RNA from the tissue of cats with persistent Bartonella DNA. The sensitivity of either assay was not determined.

**Sequence analysis.** The PCR product from cat 1 heart valve was sequenced for confirmation of Bartonella. The sequence data from the PCR product was identical to the 16S rRNA fragment of Bartonella henselae genotype II with the identifying region ATT. Cat 1 was infected with B. henselae LSU 16 genotype II. (Appendix I).

**Postmortem evaluation and histopathology.** Gross necropsy results from the experimentally infected cats were unremarkable except for one cat had multifocal lung lesions and one cat had hydrocephalus. Of the 23 infected cats tested by PCR and culture, tissues from 19 cats were processed for histopathologic examination. Microscopic lesions were seen in the lymph nodes, liver, spleen, kidney, and bone marrow. Histopathological diagnoses in the infected cats were as follows: lymphoid hyperplasia of the lymph nodes in 16 of the 19 cats; lymphocytic pericholangitis in 14 of the 19; lymphocytic hepatitis in 2 of the 19 cats; splenic lymphoid hyperplasia in 15 of the 19 cats; focal interstitial nephritis in 3 of the 19 cats; focal lymphocytic interstitial pneumonia in 2 of the 19 cats; focal eosinophilic pneumonia was seen in one cat and focal bronchopneumonia was seen in one other cat; bone marrow hyperplasia was seen in 7 of the 19 cats and pulmonary edema in 2 of the 19 cats. The lesions ranged from scant to severe. Bacteria were not visualized in any of the tissue on routine staining or with Warthin-Starry stain on a few selected tissues of some cats.
Figure 11. RT-PCR for detection of cDNA of *Bartonella henselae* with primers CSN-F1 and CSN-R1 and CSN-F2 and CSN-R2. Tissue samples from cats experimentally infected with *B. henselae*
Lane 1: Low DNA Mass™ Ladder (Gibco)
Lane 2: Positive control *B. henselae*
Lane 3: Positive control *B. henselae*
Lane 4: Spleen tissue from cat 87
Lane 5: Liver tissue from cat 1
Lane 6: Spleen tissue from cat N3
Discussion

*Bartonella* DNA was detected in the tissues of experimentally infected cats although *Bartonella* DNA could not be detected in the peripheral blood at the time of necropsy. *Bartonella* DNA was not detected in the blood of these cats at the time of necropsy tissue analysis. Therefore it is doubtful that *Bartonella* DNA detected in tissues was from circulating blood. In three cats, there was a 22-week interval between bacteremia and positive finding of *Bartonella* DNA in tissue. In cats with relapsing bacteremia, *Bartonella* DNA was found in tissues from both cats.

The quantity of *Bartonella* DNA in the tissues of these cats is unknown. *Bartonella* DNA could be detected in one 10-fold dilution of the extracted DNA from the spleen of cat 87 but could not be detected from one 10-fold dilution of extracted DNA from the liver of cat 1. The sensitivity limits of the nested PCR assay were determined to be 6.4 fg or approximately three organisms. Therefore, the number of *Bartonella* organisms in the tissue in cat 87 and 1 would be approximately 30 and 3 microorganisms respectively in the tissue of these cats. All cultures from the tissues of the cats were negative. The culture results may reflect the minimal detection limits of culture or possibility of a dormant nonculturable state of the organism in tissue. The absence of detectable *Bartonella* DNA in some tissues may indicate that there was no *Bartonella* DNA in the tissue of these cats or the detection limits may have been exceeded. Many aspects of the pathogenesis of feline bartonellosis are unclear, including entry of the microorganism into the cat, interaction with the phagocytic cells, the immunological Many aspects of the pathogenesis of feline bartonellosis are unclear, including entry of the microorganism into the cat, interaction with the phagocytic cells, the immunological
response of the cat, predilection for certain tissue, or sequestration in tissue. The cat flea is known to play a role in the transmission of _B. henselae_ from cat to cat. The spread of the microbe through the body or the events that might occur with the interaction with phagocytic cells of the host is open to speculation. Little is known about the virulence factors associated with _Bartonella_ or mechanisms responsible for bacterial adherence, colonization, or invasion or the mechanisms of persistence.

_Bartonella_ infections in cats are characterized by an acute phase in which a bacteremia of significant magnitude and duration can be detected by bacterial culture. Chronic infection with recurrent episodes of bacteremia is observed in some cats for long periods. This suggests that there is a subclinical phase of the infection in which some cats harbor the organism and remain persistently infected for years. The cyclic nature of the bacteremia seen in cats infected with _B. henselae_ may represent sequestration of _Bartonella_ organisms in tissues with periodic release into peripheral blood. The nature of the persistence of this microorganism in cats is in question._

_Bartonella_ may use multiple mechanisms or evasive strategies to ensure prolonged survival in the cat. In this study, all cultures from the tissues were negative and no RNA expression was detected although _Bartonella_ DNA could be detected. Does the _Bartonella_ DNA represent dead organisms; is the organism viable in the tissue of cats below the detection limits of culture; is _Bartonella_ in a viable but nonculturable state as reported with other organisms? A viable but nonculturable state occurs in many organisms such as _Vibrio cholerae_ and _Escherichia coli_, _Salmonella enteritidis_, _Shigella sonnei_, and _Legionella pneumophila_, which have lost the capacity to form colonies on culture media yet, remain viable (Dixon et al. 1998).
Detection of *Bartonella* DNA in tissue that is culture negative is not unusual and is seen in other hosts infected with *Bartonella*. In humans infected with *B. henselae*, diagnoses of Bartonella infections are often made by detection of *Bartonella* DNA in culture negative tissue. In cases involving dogs, endocarditis and peliosis hepatis have been diagnosed by amplification of *Bartonella* DNA from culture negative tissue. In a study by Regnath et al. (1998), *B. henselae* infected mice were shown to clear the cultivatable organism within the first 6 days of infection from liver and spleen but *Bartonella* DNA could be detected in liver for at least 3 months. Localization of *Bartonella* in tissue certainly seems logical. Mechanism of localization of *Bartonella* in tissue would depend on cell association. If the microorganisms were free in the plasma, localization in tissue would depend on the ability of the organism to adhere to or grow in vascular endothelial cells. There is evidence that *Bartonella* does adhere to and invade endothelial tissue in humans (Dehio et al. 1997). If *Bartonella* is phagocytized by monocytes this would be a mechanism to carry the organism through tissue as the cells move in and out of the vascular system. The erythrocyte association of *Bartonella* would allow travel through tissue on the erythrocyte. An intraerythrocytic location of a microorganism could be a protective site from phagocytosis by the reticuloendothelial system.

In human studies, *B. henselae* can invade or attach to endothelial cells, macrophages and neutrophils. In the cat, *B. henselae* has only been found within feline erythrocytes (Kordick and Breitschwerdt 1995). An intracellular location that facilitates persistent infections in cats has not been established. However, the persistent nature of *Bartonella* as well as the lack of efficacy of antibiotics to clear infections is suggestive of an
intracellular location as observed with another intracellular organisms, Brucella, which is phylogenetically related to Bartonella (Review by Anderson and Neuman 1997). If Bartonella resides and grows in the macrophage, this mechanism may lead to infection of the organ harboring the macrophages with reseeding of bacteria into the blood by various tissues. In a study by Karem et al. (1999), using the BALB/c mouse as a model, Bartonella could be cultured from tissue of mice infected intraperitoneally 24 hr after infection but not after 6 hours or 7 days. Mice infected with B. henselae by the intraperitoneal route never became bacteremic although Bartonella DNA was detected within 6 hours in the liver and mesenteric lymph node and up to 7 days in spleen, kidney, and liver by PCR. This study suggests uptake of the organism by phagocytes, which then are filtered by organs such as the liver in the mouse model. In cats infected with Bartonella, bacteremia is common and circulating organisms in the blood could localize in organs such as the liver and spleen.

The histopathologic findings in this study are not specific for Bartonella. However, Kordick et al. (1999) reported similar findings in lymph nodes, spleen, liver, and kidney in a group of B. henselae infected cats. In a study by Guptill et al. (1997), histopathologic lesions in multiple organs of infected cats 8 weeks postinfection were reported. Necrotizing granulomas were found in lymph nodes in 1 cat at 8 weeks postinoculation that were similar to what is seen in CSD patient. Guptill et al. (1997) found mild to marked lymphoid hyperplasia of the spleen, marked lymphoid hyperplasia in the lymph nodes, and foci of necrosis throughout the liver, focal pyogranulomatous nephritis, and interstitial myocarditis in a group of Bartonella infected cats. Bacteria were seen infrequently, were extracellular, and not associated
with inflammatory lesions. Similar to the study by Kordick et al. (1999), abscesses or granulomata were not found in tissues from our cats.

*Bartonella*-induced histopathologic alterations of affected lymph nodes from human patients with CSD typically include stellate caseating granulomas, microabscesses, and follicular hyperplasia (Anderson and Neuman 1997). The histopathologic findings in the lymph nodes of patients with CSD depend on the stage of the infection (Sander et al. 1999). Early in the course of CSD infections, lymphoid hyperplasia is seen, while granulomas with necrosis and stellate microabscesses are seen later in these infections (Bass et al. 1997; Kaschula 1996). In human patients with bacillary angiomatosis, a variety of internal organs including the liver, spleen, bone, brain, lungs, and bowel can be affected. The liver or the spleen or both are affected in patients with bacillary peliosis. The affected tissues contain numerous blood-filled partially endothelial cell-lined cystic structures and fibromyxoid stroma with a mixture of inflammatory cells, dilated capillaries, and clumps of bacilli identified by Warthin-Starry staining (Welch and Slater, 1999). Inflammatory reactions to *B. henselae* infection without associated bacillary angiomatosis and or peliosis included granulomatous reactions characterized by nodular collections of histiocytes, lymphocytes and aggregates of neutrophils and karyorrhectic debris suggestive of microscopic abscess formation that resemble lymph node lesions of CSD (Liston et al. 1996). The hydrocephalus found in one cat and the multifocal lung lesions found in another cat are non-related findings and likely not related to the *Bartonella* infection in the experimentally infected cats. The multifocal lung lesions with eosinophils seen among the inflammatory cells is likely to be related to parasite migration or feline asthma.
Regnath et al. (1998) used the mouse model to demonstrate the progression of *Bartonella*-induced histopathologic alterations. Three days postinfection liver lesions were characterized by a few small aggregates of lymphocytes and monocytes that expanded in number and size. In the second week postinfection granulomatous lesions consisting of lymphocytes, monocytes and epithelioid cells were observed. Necrosis in the mouse liver granulomas was not evident as observed in human patients with CSD.

Necrotizing granulomas or abscesses were not found in the cats in this study. The histologic changes are not specific for *Bartonella*. However, the follicular hyperplasia of lymph nodes and spleen as seen in these cats is suggestive of nonspecific tissue response to a chronic antigenic stimulation. The cats in this study were not bacteremic at the time of necropsy although 10 of the cats had persistent *Bartonella* DNA amplified from various tissues, suggesting chronic infection. *Bartonella* DNA was amplified from the spleen of the cats with splenic lymphoid hyperplasia, from the liver of cats with lymphocytic pericholangitis and lymphocytic hepatitis and from the lymph node of the experimentally infected cats with lymphoid hyperplasia of lymph nodes. Three cats had lesions in the kidney, and *Bartonella* DNA was amplified from the kidney of four of the infected cats. Although neurological signs have been observed in cat experimentally infected with *B. henselae* LSU 16 (O’Reilly et al. 1999a), significant lesions were not seen in the brain of cats in this study which were infected with the same strain. Kordick et al. (1999) reported similar histopathological findings in a group of cats experimentally infected with *B. henselae*. *Bartonella* DNA was amplified from the brain of three of the cats. *Bartonella henselae* DNA has been amplified from both dogs and humans with endocarditis. Cats in this study did not have lesions from the
heart valve despite the fact that *Bartonella* DNA was amplified from five cats. Organisms were not seen in any of the tissues analyzed in this study. However, other investigators have reported the difficulty of demonstrating *Bartonella* organisms in tissues. It is difficult to make any correlation between the pathologic changes seen in the tissues of these cats and the *Bartonella* infection. Without locating, *Bartonella* in the lesions of infected cats there is no conclusive evidence that the lesions seen are related to *Bartonella*. However, with the high endemicity of *Bartonella* infections in cats, it is possible that the lesions are related to *Bartonella*. *Bartonella* may evade the immune system so that there are no inflammatory responses seen in the cat despite the fact that the organism is present.

In order to understand the metabolic state or viability of the persistent *Bartonella* DNA found in the tissue of these cats, attempts to demonstrate RNA expression of the citrate synthase gene and the 16S rRNA gene was done. Because there are multiple copies of rRNA (10^4-10^6) per cell, it should be possible to extract RNA and amplify the cDNA of the rRNA of *Bartonella* from the tissue of cats in which persistent *Bartonella* DNA was found. Even if the amount of *Bartonella* DNA found in tissue was low, using rRNA as a target seemed to be a reasonable approach to this problem. Detecting RNA expression of the rRNA of *Bartonella* found in the tissue could have given insight to the metabolic state of the organism. Using primers specific for the citrate synthase gene hoping to amplify cDNA from the reverse transcription of mRNA of the citrate synthase gene was also reasonable. Because the half-life of mRNA is shorter than that of rRNA, mRNA is a better marker for growth and viability. Complementary DNA (cDNA) was not amplified from any of the tissues with either RT-PCR assay. The failure to amplify
cDNA from the rRNA or mRNA of the citrate synthase gene could be because of insufficient numbers of organisms in the tissue and thus insufficient amount of RNA, microorganisms in a dormant state, nonviable organisms, or problems with the activity of RNases. Whether the *Bartonella* DNA amplified from the tissue of the cats in this study represents viable but nonculturable organisms because of a dormant state or because the detection limits of culture remains unknown. All of the cats in this study were abacteremic at the time of necropsy, and some of the cats that were positive for *Bartonella* DNA in tissue had been abacteremic for 19 weeks. In addition, *Bartonella* DNA was amplified from both cats with recurrent episodes of bacteremia suggesting that the organisms do persist in the cat. Further work is needed to characterize the *Bartonella* DNA found in the tissue of cats.
CHAPTER FOUR
COMPARISON OF FINDINGS OF PERSISTENT BARTONELLA DNA IN CATS EXPERIMENTALLY INFECTED WITH BARTONELLA HENSELAE GENOTYPE I AND BARTONELLA HENSELAE GENOTYPE II

Introduction

The domestic cat is the reservoir for *B. henselae* and a risk factor for acquiring cat scratch disease (CSD) (Zangwill et al. 1993). Cats can be chronically infected with relapsing bacteremia for long periods of time (Kordick et al. 1995). Persistent *Bartonella* DNA has been detected in the tissue of cats long after the bacteremic stage of the infection, which suggests a harboring of organism in the tissues of chronically infected cats (Chapter 3).

*Bartonella* are fastidious, slow-growing microorganisms, and distinguishing between the species is difficult. Definitive identification of the species requires molecular techniques. Various molecular methods have been used for identification of *Bartonella* species. *Bartonella henselae* and *B. clarridgeiae* presently are the most common species of *Bartonella* isolated from the blood of cats and the most common species of *Bartonella* isolated from cats is *B. henselae* (Heller et al. 1997). By partial 16S rRNA gene sequence analysis, Bergmans et al. (1996), described genotype I and genotype II of *B. henselae* that differ from each other in three nucleotides located at positions 172 and 175 of the 16S rRNA gene. The sequences of genotype I and genotype II are analogous to the sequences of *B. henselae* Houston-1 described by Regnery et al. (1992) and *B. henselae* “BA-TF” described by Relman et al. (1992), respectively. Two types of *B. henselae*, genotype I and genotype II have been shown to coinfect some cats (Gurfield et al. 1997). In a study by Heller et al. (1997), blood samples from 94 stray cats found that 34% of the isolates from cats were genotype I, 36% were genotype II and 30%
were *B. clarridgeiae*. Bergmans et al. (1996) found that the majority of the lymph nodes from patients with CSD contained *B. henselae* genotype I, while Sander et al. (1999) found *B. henselae* genotype I in 59% of PCR-positive lymph nodes and genotype II in 23% of the PCR-positive lymph nodes of CSD patients. Seven (18%) of the lymph nodes were negative in both genotype-specific PCRs. Sander et al. (1998b) found 16 of 17 isolates of *B. henselae* from blood culture of human patients to be *B. henselae* genotype II. These authors have suggested that there may be a difference in the types of *B. henselae* and host specificity.

In this study, blood and tissues from cats experimentally infected with *B. henselae* genotype I and *B. henselae* genotype II were tested using a PCR assay designed to detect both genotypes of *B. henselae* using minor sequence differences between the genotypes in a region of the 16S rRNA gene of *Bartonella*.

Identifying strains and types of *B. henselae* will be useful in understanding the pathogenesis of these two genotypes.

**Materials and Methods**

**Experimental animals.** Tissue and blood used in this study was from twenty-three cats as reported in Chapter 3.

**Bacterial strains.** Methods the same as in Chapter 3

**Infection in cats.** Methods the same as in Chapter 2 and 3

**Collection of blood and tissue specimens.** Blood was collected with the same methods as in Chapter 2. Tissue was collected with the same methods as in Chapter 3

**Blood and tissue cultures.** Blood was cultured with the same methods as in Chapter 2. Tissue was cultured with the same methods as in Chapter 3.
Extraction of DNA from blood and tissue. Blood was extracted as in Chapter 2. Tissue was extracted with the same methods as in Chapter 3.

PCR amplification of DNA from blood and tissue. A nested PCR was performed using primers previously described (R. Tapp 2000, Master Thesis, Louisiana State University, The Institute for Environmental Studies) to amplify a final product of a 477 bp fragment of the 16S rRNA gene of *Bartonella*. For amplification of genus specific *Bartonella* DNA, forward primer pair Bhen16FF 5’CACATGCAAGTCGAGCACA CTCTTTTAGAG 3’and reverse primer UB16SDR9 5’GCCCCCGTCAATTCCTTTT GAGTTT 3’ were used, generating an 823 bp fragment. A second reverse primer that is *Bartonella* genus specific Bhen16SBBR 5’-CACATGCAAGTCGAGCACA CTCTTTTAGAG-3’ was also designed to be used with the forward Bhen16FF primer to amplify a 567 bp fragment.

For amplification of species and genotype I-specific *Bartonella* DNA, the internal forward primer Bhen16SGG 5’GAAATTTGTGCTAATACC GAATACGTCTTAG 3’and reverse primer Bhen16SBBR were used to amplify a 477 bp fragment of the 16s rRNA gene of *B. henselae*. For amplification of species and genotype II-specific *Bartonella* DNA, the forward primer Bhen1616SHH 5’GAAATTTGTGCTAATAC GGAATACGTCTATTTG 3’and reverse primer Bhen16SBBR were used to amplify a 477 bp fragment. Because genotype I and genotype II fragments were of the same base pair size both primer sets were performed simultaneously in separate reaction for each sample. The synthetic oligonucleotides were purchased from GeneLab (Louisiana State University, Baton Rouge, LA). DNA amplification was carried out in 50-μl reaction volumes. The PCR amplification was carried out on a Model 9600 Thermal-Cycler.
Perkin-Elmer/Applied Biosystems (Foster City, CA). Each reaction mixture consisted of deoxynucleoside triphosphates (200µM each), 1.75 mM MgCl₂, 0.2µM of each primer, 2.5 U of Taq DNA polymerase, AmpliTaq™ Gold (Perkin Elmer), 4 µl of DNA in PE buffer in a total volume of 50 µl. Amplification reaction consisted of 10 min. at 95°C, 30 cycles of 94°C for 1 min., 66°C for 1 min., and 72°C for 1 min., and a final elongation step at 72°C for 10 min. In the first reaction, primers Bhen16SFF and UB16SDR9 or Bhen16SBBR were added. A second amplification reaction of 20 cycles with the sample conditions as previously stated was performed using Bhen16SGG and Bhen16SBBR for genotype I and Bhen16SHH and Bhen16SBBR for genotype II and 1 µg of DNA from the previous PCR reaction. As a negative control, PCR reagents without DNA template were included in each amplification assay. Positive controls of extracted B. henselae genotype I and genotype II were also used in each amplification assay.

**Detection of PCR products.** Methods the same as in Chapter 2 and Chapter 3

**Sequence analysis.** Methods the same as in Chapter 2

**Results**

*Bartonella henselae* genotype I and genotype II DNA detection during bacteremia and in tissue. The *B. henselae* primers specific for genotype I and genotype II were used to determine genotype specificity in the cats during the bacteremic phase of the infection and in the tissue analysis (Figure 12). The bacteremic phase of the infection was followed in 16 of the 23 experimentally infected cats. In nine of the cats, the *B. henselae* genotype specific primers were used to determine if there was a genotype switch during periods of bacteremia. Cats remained infected with
Figure 12. *Bartonella henselae* genotype I and genotype II DNA
Lane 1: Low DNA Mass\textsuperscript{TM} Ladder (Gibco)
Lane 2: Cat L2 infected with *B. henselae* 87-66 genotype I (primers Bhen16SGG-BBR-genotype I specific)
Lane 3: Cat L2 infected with *B. henselae* 87-66 genotype I (primers Bhen16SHH-BBR-genotype II specific)
Lane 4: Reagent Control
Lane 5: *B. henselae* 87-66 genotype I positive control (primers Bhen16SGG-BBR-genotype I specific)
Lane 6: *B. henselae* 87-66 genotype I positive control (primers Bhen16SHH-BBR-type II specific)
Lane 7: Cat 321 infected with *B. henselae* LSU 16 genotype II (primers Bhen16SGG-BBR-genotype I specific)
Lane 8: Cat 321 infected with *B. henselae* LSU 16 genotype II (primers Bhen16SHH-BBR-genotype II specific)
Lane 9: Reagent Control
Lane 10: *B. henselae* LSU 16 genotype II positive control (primers Bhen16SGG-BBR-genotype I specific)
Lane 11: *B. henselae* LSU 16 genotype II positive control (primers Bhen16SHH-BBR-genotype II specific)
the same *B. henselae* genotype as was inoculated (Table 8). Seven cats were infected with *B. henselae* LSU-16 genotype II, and the type specific PCR primers confirmed the *Bartonella* DNA detected to be *B. henselae* genotype II throughout the bacteremia. Three cats infected with *B. henselae* Baby genotype II were also confirmed to be *B. henselae* genotype II DNA throughout the bacteremia. Of the three cats infected with *B. henselae* 87-66 genotype I, all remained infected with *B. henselae* genotype I and the one cat infected with *B. henselae* Houston-1 remained infected with *B. henselae* genotype I (Table 8). None of the cats infected with *B. henselae* 87-66 genotype I or *B. henselae* Houston-1 experienced relapsing bacteremia.

Cat 86 and cat 321 experienced an episode of relapsing bacteremia. Both cats were infected with *B. henselae* genotype II, *B. henselae* genotype II was amplified from both cats during the relapsing bacteremia (Table 9).

Persistent *Bartonella* DNA was found in tissue samples from 10 of 23 of the cats in this study. Of the 10 cats in which *Bartonella* DNA was found, all were *B. henselae* genotype II. Seventeen cats were infected with *B. henselae* genotype II. Of the four cats that became bacteremic with *B. henselae* genotype I, *B. henselae* DNA could not be found in tissues and thus genotype could not be determined (Table 10).

**Sequence analysis.** The sequence analysis of the PCR product from blood specimens of cat 86 taken during the bacteremic phase of the infection confirmed the product was a *B. henselae* genotype II (Table 11). Cat 86 was experimentally infected with *B. henselae* LSU 16 genotype II. The sequence analysis of the PCR product from blood specimen of cat L2 taken during the bacteremic phase of the infection was typical a *B. henselae* genotype I (Table 11). Cat L2 was experimentally infected with
Table 8. Results of *Bartonella henselae* genotype from cats during period of bacteremia

<table>
<thead>
<tr>
<th>Week a</th>
<th>Cat L1 c</th>
<th>Cat 361 c</th>
<th>Cat K3 c</th>
<th>Cat G3 b</th>
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<td>II 3.4x10^5</td>
<td>II 4.0x10^4</td>
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<tr>
<td>2</td>
<td>+ 2.4x10^5</td>
<td>+ 3.5x10^4</td>
<td>+ 3.2x10^4</td>
<td>+ 1.8x10^5</td>
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<td>II 3.0x10^2</td>
<td>II 3.6x10^5</td>
<td>II 1.5x10^5</td>
<td>II 8.0x10^5</td>
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<td>4</td>
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</table>

\(a\) post-inoculation  
\(b\) inoculated with *B. henselae* LSU 16 Genotype II  
\(c\) inoculated with *Bartonella henselae* Baby Genotype II  
\(d\) inoculated with *Bartonella henselae* 87-66 Genotype I  
\(e\) inoculated with *Bartonella henselae* Houston-1 Genotype I  
\(f\) ND, not determined
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<th>Week&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Cat F5&lt;sup&gt;e&lt;/sup&gt; type CFU/ml</th>
<th>Cat 347&lt;sup&gt;e&lt;/sup&gt; type CFU/ml</th>
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<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
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</table>

<sup>a</sup> post-inoculation  
<sup>b</sup> inoculated with B. henselae LSU 16 Genotype II  
<sup>c</sup> inoculated with Bartonella henselae Baby Genotype II  
<sup>d</sup> inoculated with Bartonella henselae 87-66 Genotype I  
<sup>e</sup> inoculated with Bartonella henselae Houston-1 Genotype I  
<sup>f</sup> ND, not determined

(Table 8 continued)
<table>
<thead>
<tr>
<th>Week</th>
<th>Cat N2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cat N3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cat L2&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
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<td>type CFU/ml</td>
<td>type CFU/ml</td>
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<td>I 6.8x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
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<td>+ 4.4x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
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</tr>
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<td>4</td>
<td>+ 2.4x10&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>+ 1.1x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
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<td>II 3.6x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>I 3.5x10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
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<td>II 6.0x10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>I 1.3x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
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<td>I 1.3x10&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>I 8.3x10&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
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<td>+ 1.5x10&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>- 0</td>
<td>I 8.0x10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
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<td>- 0</td>
<td>+ 7.3x10&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
<td>23-29</td>
<td>- 0</td>
<td>- 0</td>
<td>I 1.0x10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> post-inoculation  
<sup>b</sup> inoculated with B. henselae LSU 16 Genotype II  
<sup>c</sup> inoculated with *Bartonella henselae* Baby Genotype II  
<sup>d</sup> inoculated with *Bartonella henselae* 87-66 Genotype I  
<sup>e</sup> inoculated with *Bartonella henselae* Houston-1 Genotype I  
<sup>f</sup> ND, not determined
Table 9. Results of *Bartonella henselae* genotype from cats with relapsing bacteremia

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<tr>
<th>Week</th>
<th>Cat 86&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cat 321&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>2</td>
<td>+</td>
<td>1.7x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>4.3x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>2.2x10&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>6</td>
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<td>1.1x10&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>3.3x10&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>7.0x10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
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<td>II</td>
<td>2.3x10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>1.0x10&lt;sup&gt;2&lt;/sup&gt;</td>
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<sup>a</sup> post-inoculation  
<sup>b</sup> inoculated with *B. henselae* LSU 16 Genotype II  
<sup>c</sup> ND, not determined
Table 10. Results of findings of persistent *Bartonella* DNA in various strains of *Bartonella henselae*

<table>
<thead>
<tr>
<th>Cat</th>
<th>Strain</th>
<th>Type</th>
<th><em>Bartonella</em> DNA in tissue</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>LSU 16*</td>
<td>II</td>
<td><em>(1, 2, 3, 4, 5, 7, 8)</em></td>
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<tr>
<td>10</td>
<td>LSU 16*</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td>81</td>
<td>LSU 16*</td>
<td>II</td>
<td><em>(1, 2, 3, 4, 5, 6, 7)</em></td>
</tr>
<tr>
<td>83</td>
<td>LSU 16*</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td>84</td>
<td>LSU 16*</td>
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<td>85</td>
<td>LSU 16*</td>
<td>II</td>
<td><em>(1, 2, 3, 4, 6, 7, 8)</em></td>
</tr>
<tr>
<td>86</td>
<td>LSU 16*</td>
<td>II</td>
<td><em>(1, 3, 4, 5, 7, )</em></td>
</tr>
<tr>
<td>87</td>
<td>LSU 16*</td>
<td>II</td>
<td><em>(1, 2, 3, 4, 5, 6, 7, 8)</em></td>
</tr>
<tr>
<td>182</td>
<td>LSU 16*</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
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<td>LSU 16*</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td>223</td>
<td>LSU 16*</td>
<td>II</td>
<td>-</td>
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<tr>
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<td>II</td>
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<tr>
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<td><em>(2, 3)</em></td>
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<td><em>(2)</em></td>
</tr>
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<td>Baby*</td>
<td>II</td>
<td><em>(2)</em></td>
</tr>
<tr>
<td>361</td>
<td>Baby*</td>
<td>II</td>
<td><em>(2, 3, 8)</em></td>
</tr>
<tr>
<td>K3</td>
<td>Baby*</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td>L2</td>
<td>87-66**</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>N2</td>
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<td>-</td>
</tr>
<tr>
<td>K4</td>
<td>87-66**</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>L3</td>
<td>Houston-1*</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>F5</td>
<td>Houston-1a</td>
<td>I</td>
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<tr>
<td>347</td>
<td>Houston-1a</td>
<td>I</td>
<td>-</td>
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</table>

*1x10⁷
**1x10⁹

a not infected
1-liver
2-spleen
3-bone marrow
4-heart valve
5-lung
6-brain
7-lymph node
8-kidney

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B. henselae 87-66 genotype I. The Bartonella DNA amplified from the heart valve tissue of cat 1 was typical of a B. henselae genotype II (Table 11). Cat 1 was experimentally infected with B. henselae LSU 16 genotype II.

Table 11. Partial sequence data of Bartonella henselae strains amplified from experimentally infected cats

<table>
<thead>
<tr>
<th>Cat</th>
<th>Specimen</th>
<th>Challenge Strain</th>
<th>Sequence data</th>
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</thead>
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<tr>
<td>Cat 86</td>
<td>blood</td>
<td>B. henselae LSU 16 genotype II</td>
<td>CCTATTGGGA</td>
</tr>
<tr>
<td>Cat L2</td>
<td>blood</td>
<td>B. henselae 87-66 genotype I</td>
<td>CCTTAG- GCA</td>
</tr>
<tr>
<td>Cat 1</td>
<td>heart valve</td>
<td>B. henselae LSU 16 genotype II</td>
<td>CCTATT- GCA</td>
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</tbody>
</table>

Discussion

Use of this PCR assay differentiated between the subtypes of B. henselae in cats experimentally infected with Bartonella henselae. It has been shown that cats can be coinfect ed with B. henselae genotype I and genotype II and or B. clarridgeiae. However, whether certain types are predominant over others during coinfection is unknown. Currently it is not known whether these genotypes are predictive of pathogenicity or virulence.

Chronic infection with recurrent episodes of bacteremia is observed in some cats for long periods. This suggests that there is a subclinical phase of the infection in which some cats harbor the organism and remain persistently infected for years. The cyclic nature of the bacteremia seen in cats infected with B. henselae may represent sequestration of Bartonella organisms in the cat with periodic release into peripheral blood. The nature of the persistence of this microorganism in cats is in question. Bartonella may use multiple mechanisms or evasive strategies such as intracellular
location, location in sites that are immunologically privileged, or antigenic variation to ensure prolonged survival in the cat.

There are discrepancies between the results of *Bartonella* experimental infections in cats of various investigators. The differences are in the duration and reoccurrence of bacteremia experienced in experimentally infected cats. Different parameters of the infection process such as dose, route of inoculation, response of the host, and strain of the organism could be reasons for some of these discrepancies. In the experimental infection with *B. henselae* Houston-1 by Regnery et al. (1996) and Greene et al. (1996), bacteremia of short duration and without relapse was reported. Kordick et al. (1999) used *B. henselae* genotype II from donor cats to induce prolonged bacteremia with relapse for 454 days in experimentally infected cats. In the study by O'Reilly et al. (1999a), acute clinical disease was established in cats using *B. henselae* LSU 16 genotype II isolated from a naturally infected cat.

Identifying and characterizing the strains or subtypes of *B. henselae* would be useful in the studies of the pathogenesis of feline bartonellosis. *B. henselae* strain typing by genotypic methods will be useful in comparing the diversity of strains within the cat reservoirs with the diversity of strains that cause the various human clinical syndromes. It has been suggested that the majority of human clinical samples associated with cat scratch disease involve *B. henselae* genotype I, while the majority of the isolates from cats are genotype II *B. henselae* (Sander et al. 1998; Bergmans et al. 1996).

In this study, all 10 cats with persistent *Bartonella* DNA detected in tissues were cats infected with *B. henselae* genotype II. *Bartonella* DNA was not found in any of the cats infected with *B. henselae* genotype I despite the fact that the three cats infected
with *B. henselae* 87-66 genotype I were inoculated with a higher dose \(10^9\) versus \(10^7\) of organism than the cats infected with *B. henselae* genotype II. These results suggest that *B. henselae* genotype II may be more pathogenic are more adapted in cats because of the prolong duration of *Bartonella* DNA found in tissues of these cats. Bacteremia was established in all the *B. henselae* genotype II cats. Bacteremia was established in cats infected with *B. henselae* 87-66 genotype I however a higher dose was used. Only one of the three cats inoculated with *B. henselae* Houston-1 became bacteremic. Episodes of recurring bacteremia were seen in the two cats infected with *B. henselae* genotype II. None of the cats infected with *B. henselae* genotype I had episodes of recurring bacteremia during the course of this study. Relative differences in virulence-associated genes and pathogenicity related to strain are still not clear. The finding of persistent *Bartonella* DNA in the tissue of cats infected with *B. henselae* genotype II and the relapsing bacteremia seen in those same cats is suggestive of a virulence-associated strain. The cats with persistent *Bartonella* DNA in tissue were infected with two different isolates of *B. henselae* genotype II and suggest that persistence and chronic infections in cats may be associated with genotype II. Use of genetic tools, to identify disease-associated genes of *B. henselae* will be nice.

Freeland et al. (1999) found differences in absorption antibody profiles with *B. henselae* Houston-1 and *B. henselae* LSU 16 genotype II which may represent differences in immunodominant antigens expressed by these two types of *Bartonella*.

The cats experimentally infected and used in this study were examined by other investigators (Parr et al. 1999) for comparison of clinical disease in the various strains. Parr et al. (1999) described differences in clinical disease, bacteremia, antibody
response, and the number of circulating antibodies to the various strains of *B. henselae*. In general clinical disease was more severe in *B. henselae* genotype II than in *B. henselae* genotype I as defined by fever, lymphadenopathy, lethargy, diarrhea, and anorexia. In addition, cats infected with *B. henselae* LSU 16 displayed neurological signs not observed in the other cats. The IgM response peaked earlier (3 weeks) in the *B. henselae* genotype II strain than in the *B. henselae* genotype I strain (6 weeks). The IgG patterns were also different with the peak IgG level appearing at 9-12 weeks in the genotype II strains and at 7 weeks in the genotype I strains. These results are suggestive that there may be a difference in virulence and pathogenicity among the strains of *B. henselae*. 
CHAPTER FIVE
CONCLUSIONS

Summary of Results

The nested PCR assay developed was successful in amplifying genus, species, and genotypes of Bartonella and was a valuable research tool in this study. The nested PCR design using outer and inner primer sets amplified an 823 bp fragment, a 567 bp fragment, and a 477 bp fragment of the 16S rRNA gene of Bartonella. Sequence analysis of the PCR products were as predicted and were confirmed to be Bartonella henselae. Sensitivity limits of the PCR assay were determined to be 3.2 organisms per milliliter of blood and below the detection limits of culture.

Experimentally infections were established in 23 cats and the PCR assay was able to detect Bartonella in all of the infected cats and in more samples and for longer periods than bacterial culture. The PCR assay was positive in 40 of the blood samples in which the blood culture was negative. The bacteremic phase of the infection was extended by 1 to 9 weeks as defined by the PCR assay.

The chronicity of Bartonella infections in cats was demonstrated by detecting persistent Bartonella DNA in 10 of the 23 cats. Bartonella DNA was detected in 10 cats in multiple tissues. Bartonella DNA were found in the spleen of 9 of the 10 cats. Other tissues in which Bartonella DNA was detected was liver, kidney, brain, lymph nodes, bone marrow, heart valve, and lung. Chronicity of Bartonella infection was demonstrated in two cats with episodes of relapsing bacteremia. Bartonella DNA was found in tissues from both of these cats.

Histopathologic analysis of tissues of infected cat showed reactions that were typical of chronic antigenic stimulation but nonspecific for Bartonella. Lymph nodes from 16
of the 19 infected cats displayed lymphoid hyperplasia and, in 15 of the 19, splenic lymphoid hyperplasia was observed. Specific diagnostic lesions associated with Bartonella infections could not be made from these cats.

Determination of viability of organism detected in these cats with persistent Bartonella DNA was not accomplished by RNA express analysis with the RT-PCR assays. RNA expression of the citrate synthase gene or the 16S rRNA gene was not detected in tissue specimens.

The particular B. henselae genotype used to infect the cats did not change over the course of the acute or chronic bacteremic phase of the infection. The persistent Bartonella DNA detected in tissue in the chronic phase of the infection was determined to be B. henselae genotype II. B. henselae genotype I was not detected in tissue from any of the cats infected with that genotype.

**Future Work**

Many aspects of the pathogenesis of Bartonella henselae are still unclear. Research designed to investigate virulence factors, strain variability, cell association of the organism, and mechanisms of persistence are needed. Understanding the nature of the persistence by determining viability of Bartonella DNA detected in tissue is an interesting area. Is the organism viable but dormant in tissue and the source of relapsing episodes of infection? Moreover, what are the factors that allow the bacteremia to reoccur? Whether B. henselae DNA found in the tissue of cats long after the acute phase of the infection represents sequestration of a viable organism which has lost the capacity to form colonies on culture media or is nonviable and not the source of recurring infection is unknown. The concepts of culturability and viability are
confusing. An organism that is culturable is able to reproduce and an organism that is viable is alive but not necessarily reproducing or culturable in the present state or environment. There are over 30 bacterial species in which a viable but nonculturable response occurs when exposed to adverse environmental factors (Oliver 1999). There is a great deal of study necessary to understand the molecular and physiological basis of dormancy in microorganisms. Understanding the sensing mechanisms used by organisms in various environments inside and outside of the host and the responses generated to modify metabolism is a dominant theme in studying pathogenesis.

Determining if the *Bartonella* DNA found in the cat is a source of clinical bacteremia when sequestered in the host and if there is a tissue predilection for sequestration will help in the understanding of the course of *Bartonella* infection in cats. Therapeutic decisions may evolve around the determination of persistence. Chronic infections may be eliminated by treatment of cats in the acute phase of infection to avoid sequestration.

Using RNA expression as an indicator for growth and metabolism of prokaryotic cells requires adequate amounts of RNA. The amount of *Bartonella* DNA detected in the tissue of cats is probably low and a limiting factor in the recovery of detectable RNA. Another approach to characterizing the *Bartonella* DNA in terms of viability would be to inoculate tissue with detectable *Bartonella* DNA into other animals and observe for signs of infection.

From this research, *Bartonella* DNA was detected by PCR from culture-negative blood of infected cats. The infectious nature of PCR positive and culture negative blood would be interesting to know and could be determined by infecting cats with this blood or using fleas for transmission.
Knowledge of strain variability involved in *Bartonella* infections will allow for comparison of the diversity of strains within cat reservoirs with the diversity of strain that causes various human clinical syndromes. There may be strains that are more pathogenic similar to the strain *B. henselae* LSU-16. Using molecular strain typing methods such as pulsed-field gel electrophoresis of endonuclease-restricted chromosomal DNA or restriction fragment length polymorphism (RFLP) of the intergenic transcribed spacer region may help identify the strains of *Bartonella*. 

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REFERENCES


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Tapp, R. A., 2000. DNA diagnostics for the detection and identification of cat scratch disease causing agents. Thesis Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in Partial Fulfillment of the requirements for the degree of Master of Science in The Institute for Environmental Studies.


**APPENDIX A**

PILOT EXPERIMENT: CULTURE TECHNIQUES FOR ISOLATION OF *BARTONELLA HENSELAE*

<table>
<thead>
<tr>
<th>Cat ID**</th>
<th>Date infected*</th>
<th>Date necropsied</th>
<th>HIA b</th>
<th>BB c</th>
<th>VC d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>46</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>57</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>61</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>62</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63</td>
<td>1/97</td>
<td>6/97</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*B. henselae* positive control + + +

** cats were blood culture negative

* Cats were infected with *B. henselae* Houston-1. Cats were necropsied and tissue taken for culture. Tissue sampled for culture included; lung, liver, spleen kidney, lymph node, bone marrow, tonsil, brain, uterus, ovary, blood, buffy coat, plasma. Blood was collected in Dupont Isolator tube, EDTA vacutainer tube.

b Heart Infusion Agar with 5% Rabbit blood. Cultures were incubated at 37% C in 5% CO₂ for 3 weeks

c Brucella broth with Fildes 250 µg/ml and Hemin 6-8%. Cultures were incubated at 37% in 5% CO₂ for 3 weeks and subcultured on HIA agar

d Vero Cells-tissue was placed in tissue grinder for processing. Subcultures were made from vero cell culture on to HIA agar.
## APPENDIX B
### SUMMARY OF RESULTS OF EXTRACTION TECHNIQUES

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extraction technique</th>
<th>FFDR9&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FFBBR&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bartonella henselae</em> 87-66 genotype I</td>
<td>Qiagen*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bartonella henselae</em> Houston-1 type</td>
<td>Qiagen*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bartonella henselae</em> LSU 16</td>
<td>Qiagen*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bartonella henselae</em> Baby</td>
<td>Qiagen*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bartonella clarridgeiae</em></td>
<td>Qiagen*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ICM-3 (20 cfu/ml)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CTAB**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ICM-3 (20 cfu/ml)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Qiagen*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cat 351-2 (90 cfu/ml)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CTAB**</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cat 351-2 (90 cfu/ml)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Qiagen*</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>*Qiagen Kit for Tissue and Blood Extraction (Qiagen Inc., Chatsworth, CA)</sup>

<sup>**Preparation of Genomic DNA from Bacteria Phenol chloroform CTAB extraction with ethanol precipitation (Current Protocols in Molecular Biology, 1994)</sup>

<sup>a</sup> primers used in the first reaction of the nPCR

<sup>b</sup> primers used in the second reaction of the nPCR

<sup>c</sup> 200ul of blood used as sample
APPENDIX C
COMPARISON OF BLOOD SPECIMENS COLLECTED FROM CATS EXPERIMENTALLY INFECTED WITH BARTONELLA HENSELAE

<table>
<thead>
<tr>
<th>Collection tube</th>
<th>Culture&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dupont Isolator™</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vacutainer Heparin</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Vacutainer EDTA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vacutainer EDTA frozen</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

<sup>a</sup>blood was cultured on Heart Infusion Agar with 5% rabbit blood at 37°C in 5% CO<sub>2</sub> for 3 weeks

<sup>b</sup>polymerase chain reaction done using Bartonella genus and species specific primers in 50 µl volume using reaction mixture containing deoxynucleoside triphosphates (200 µM each), 1.75 mM MgCl<sub>2</sub>, 0.2 µM of each primer, 2.5 U of Taq DNA polymerase, AmpliTaq™ Gold, 4 µl of DNA template and 32.6 µl of Perkin Elmer buffer. The PCR amplifications were carried out on a 9600 Thermal-Cycler Perkin-Elmer/Applied Biosystems (Foster City, CA). Amplification reaction consisted of 10 min. at 95°C; 30 cycles of 94°C for 1 min.; 66°C for 1 min., 72°C for 1 min.; and a final elongation step at 72°C for 10 min.

+ low numbers
++ high numbers
ND not determined
APPENDIX D
RESULTS OF VARIOUS SPECIMENS FOR PCR ASSAY FOR DETECTION OF BARTONELLA HENSELAE

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Number positive by PCR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum*</td>
<td>0/39</td>
</tr>
<tr>
<td>Mouth swab*</td>
<td>0/22</td>
</tr>
<tr>
<td>Whole blood*</td>
<td>12/39</td>
</tr>
</tbody>
</table>

*polymerase chain reaction done using Bartonella genus and species specific primers in 50 µl volume using reaction mixture containing deoxynucleoside triphosphates (200 µM each), 1.75 mM MgCl2, 0.2 µM of each primer, 2.5 U of Taq DNA polymerase, AmpliTaq TM Gold, 4 µl of DNA template and 32.6 µl of Perkin Elmer buffer. The PCR amplifications were carried out on a 9600 Thermal-Cycler Perkin-Elmer/Applied Biosystems (Foster City, CA). Amplification reaction consisted of 10 mins. at 95°C; 30 cycles of 94°C for 1 min.; 66°C for 1 min., 72°C for 1 min.; and a final elongation step at 72°C for 10 min.

* blood drawn in serum vacutainer tube and separated. 12 of the 39 were positive using whole blood.

* sterile cotton swabs were used to swab the mouth of cats. Swabs were placed in sterile phosphate buffered saline for 1 hour and concentrated for PCR assay. All cats in this group were bacteremic as defined by PCR and culture.

* blood was drawn in EDTA vacutainer tube and frozen before PCR assay performed.
## APPENDIX E
### BACTERIAL SPECIES AND REFERENCE STRAINS

<table>
<thead>
<tr>
<th>Genus, Species and Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Gene Bank Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bartonella henselae</em> Houston-1</td>
<td>I</td>
<td>ATCC #49882</td>
<td>AJ223780</td>
</tr>
<tr>
<td><em>Bartonella henselae</em> 87-66</td>
<td>I</td>
<td>ATCC #49793</td>
<td>AJ223780</td>
</tr>
<tr>
<td><em>Bartonella henselae</em> LSU 16</td>
<td>II</td>
<td>LAVMDL*</td>
<td>NA**</td>
</tr>
<tr>
<td><em>Bartonella henselae</em> Baby</td>
<td>II</td>
<td>LAVMDL*</td>
<td>NA**</td>
</tr>
<tr>
<td><em>Bartonella clarridgeiae</em></td>
<td></td>
<td>ATCC #700095</td>
<td>X97822</td>
</tr>
<tr>
<td><em>Bartonella quintana</em></td>
<td></td>
<td>ATCC #VR-358</td>
<td>M73228</td>
</tr>
<tr>
<td><em>Bartonella elizabethae</em></td>
<td></td>
<td>ATCC #49927</td>
<td>L01260</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
<td>ATCC #25330</td>
<td>X06684</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td>ATCC #11303</td>
<td>E05133</td>
</tr>
<tr>
<td><em>Bordetella bronchiseptica</em></td>
<td></td>
<td>LAVMDL*</td>
<td>NA**</td>
</tr>
<tr>
<td><em>Pasteurella multocida</em></td>
<td></td>
<td>LAVMDL*</td>
<td>NA**</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td>LAVMDL*</td>
<td>NA**</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td></td>
<td>LAVMDL*</td>
<td>NA**</td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td></td>
<td>LAVMDL*</td>
<td>NA**</td>
</tr>
</tbody>
</table>

*Louisiana Veterinary Medical Diagnostic Laboratory*

**Not applicable**

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APPENDIX F
INDIRECT FLUORESCENT ANTIBODY ASSAY (IFA) FOR BARTONELLA HENSELAE

Antigen preparation:
1. *Bartonella henselae* LSU 16 grown in Vero Cells for 5 days or until > 80% infected. Antigen for IFA testing was prepared by pelleting and resuspending the microorganisms in 0.5% bovine serum albumin in phosphate-buffered saline (PBS).

Slide preparation
1. 12 well Teflon coated slides were washed with glass washing detergent and rinsed and soaked in 95% ethyl alcohol prior to use.
2. 5 µl of antigens are spotted on slide with capillary tube
3. Antigen is allowed to completely air-dry and are fixed to slides by immersion in clean acetone for 15 minutes, then air-dried.
4. Slides are wrapped and stored in freezer.

IFA Procedure
1. Sul of patient serum is added to 155 µl of Vero cells and allowed to incubate for 30 minutes at 37°C.
2. Twofold dilutions of serum ranging from 1:32 to 1:1024 were made in skim milk.
3. 25 µl of each dilution is added to antigen spot well with positive and negative controls.
4. Incubate slides for 30 minutes at 37°C in a covered dish.
5. Rinse slides in (PBS) for five minutes. Repeat once and rinse in distilled water. Allow slides to air dry.
6. Cover each antigen spot well with 25 µl of FITC-conjugated goat anti-cat conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD).
7. Incubate slides for 30 minutes at 37°C.
8. Rinse slides in PBS for five minutes. Repeat once and rinse in distilled water.
9. Slides are examined at 40x with a fluorescence microscope.

Interpretation of Results: Slides are examined for fluorescing bacilli. A positive titer is taken as 1:64 or greater.
# APPENDIX G
## PERSISTENT BARTONELLA DNA FOUND IN VARIOUS TISSUES BY PCR

<table>
<thead>
<tr>
<th>Cat #</th>
<th>Strain</th>
<th>ABbc</th>
<th>MPd</th>
<th>Necropsy</th>
<th>Bartonella DNA in Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LSU 16*</td>
<td>na</td>
<td>+</td>
<td>19</td>
<td>+ (1, 2, 3, 4, 5, 7, 8)</td>
</tr>
<tr>
<td>10</td>
<td>LSU 16*</td>
<td>+b</td>
<td>na</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>81</td>
<td>LSU 16*</td>
<td>+c</td>
<td>na</td>
<td>19</td>
<td>+ (1, 2, 3, 4, 5, 6, 7)</td>
</tr>
<tr>
<td>83</td>
<td>LSU 16*</td>
<td>+b</td>
<td>na</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>84</td>
<td>LSU 16*</td>
<td>na</td>
<td>+</td>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td>85</td>
<td>LSU 16*</td>
<td>na</td>
<td>na</td>
<td>19</td>
<td>+ (1, 2, 3, 4, 6, 7, 8)</td>
</tr>
<tr>
<td>86</td>
<td>LSU 16*</td>
<td>+b</td>
<td>+</td>
<td>4</td>
<td>+ (1, 3, 4, 5, 7,)</td>
</tr>
<tr>
<td>87</td>
<td>LSU 16*</td>
<td>+b</td>
<td>+</td>
<td>8</td>
<td>+ (1, 2, 3, 4, 5, 6, 7, 8)</td>
</tr>
<tr>
<td>182</td>
<td>LSU 16*</td>
<td>na</td>
<td>na</td>
<td>&gt;19</td>
<td>-</td>
</tr>
<tr>
<td>184</td>
<td>LSU 16*</td>
<td>na</td>
<td>na</td>
<td>&gt;19</td>
<td>-</td>
</tr>
<tr>
<td>223</td>
<td>LSU 16*</td>
<td>na</td>
<td>na</td>
<td>&gt;19</td>
<td>-</td>
</tr>
<tr>
<td>G3</td>
<td>LSU 16*</td>
<td>na</td>
<td>na</td>
<td>12</td>
<td>+ (2, 7)</td>
</tr>
<tr>
<td>321</td>
<td>LSU 16*</td>
<td>na</td>
<td>na</td>
<td>4</td>
<td>+ (2, 3)</td>
</tr>
<tr>
<td>N3</td>
<td>LSU 16*</td>
<td>na</td>
<td>na</td>
<td>18</td>
<td>+ (2)</td>
</tr>
<tr>
<td>L1</td>
<td>Baby*</td>
<td>na</td>
<td>na</td>
<td>11</td>
<td>+ (2)</td>
</tr>
<tr>
<td>361</td>
<td>Baby*</td>
<td>na</td>
<td>na</td>
<td>13</td>
<td>+ (2, 3, 8)</td>
</tr>
<tr>
<td>K3</td>
<td>Baby*</td>
<td>na</td>
<td>na</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>L2</td>
<td>87-66**</td>
<td>na</td>
<td>na</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>N2</td>
<td>87-66**</td>
<td>na</td>
<td>na</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>K4</td>
<td>87-66**</td>
<td>na</td>
<td>na</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>L3</td>
<td>Houston-1*</td>
<td>na</td>
<td>na</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>F5</td>
<td>Houston-1a</td>
<td>na</td>
<td>na</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>347</td>
<td>Houston-1a</td>
<td>na</td>
<td>na</td>
<td>29</td>
<td>-</td>
</tr>
</tbody>
</table>

*1x10⁷
**1x10⁹

*a* not infected

ABbc anti-LSU 16 or anti-Houston-1

MPd given three methylprednisolone injections 2 weeks

banti-*B. henselae* LSU 16

canti-*B. henselae* Houston-1

e weeks post-bacteremia, at time of necropsy

na-not applicable

1-liver
2-spleen
3-bone marrow
4-heart valve
5-lung
6-brain
7-lymph node
8-kidney
APPENDIX H
LETTER OF PERMISSION

Alma Roy
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803

Dear Mrs. Roy,

You have my permission to use Figure 2.1: Primer Design, page 14, that is published in my thesis titled: DNA DIAGNOSTICS FOR THE DETECTION AND IDENTIFICATION OF CAT SCRATCH DISEASE CAUSING AGENTS.

Sincerely,

[Signature]

Ronald Aaron Tapp Jr.
APPENDIX I

SEQUENCE ANALYSIS OF PCR PRODUCT FROM HEART VALVE OF CAT ONE. CAT ONE WAS EXPERIMENTALLY INFECTED WITH B. HENSELAE LSU 16 GENOTYPE II.

1 TACCCATCTCTACGGGAATAAACACAGAGAAA 45
46 TTTGTGCTAATTTACCCTATACCTCCTATTGGAGAAAGATTTATC 91
92 GGAGATGGATGAGCCCGCGTTGGATTAGCTAGTTGCTGAGGAAGTAAC 136
137 GGCTCACCAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCA 181
182 GCCACACTGGGACTGAGACACGGCCCAGACTCTGCTACGGGAGGCA 225
316 GTG AAGATAATGACGGTAACGGGAGAAGGCCGGCTAACCT 359
405 GATTTACTGGCGTAAGGCAGCATGTGATGCGGATATTATAATTCAGA 449
450 GGTGAAATCCCAAGGCTCAACCCTTGAACCTGCCTTGTACTTGGG 494
495 TATCTTGAGTGGAAGAAGGGAG 519
VITA

Alma Faye Mix was born to Alfred and Hilda Mix on February 14, 1946, in Baton Rouge, Louisiana. After graduating from Istrouma High School in 1964, she was admitted to Louisiana State University in Baton Rouge, Louisiana. She graduated with a bachelor of science degree in 1968 from the College of Chemistry and Physics. She became a member of the American Society of Clinical Pathologists and a licensed Medical Technologist. Alma worked at various hospitals, Our Lady of the Lake Regional Hospital, East Ascension Hospital, and Summit Hospital over the years as a medical technologist. In 1974, she was employed by the School of Veterinary Medicine in the Department of Anatomy and then later in the Department of Veterinary Microbiology and Parasitology. She pursued a master of science degree in health care administration in 1988 from the College of St. Francis and graduated in 1990. Alma became an instructor at the School of Veterinary Medicine and was employed by the Louisiana Veterinary Medical Diagnostic Laboratory and the Department of Microbiology and Parasitology. In 1996, Alma was accepted into the graduate program in the Department of Veterinary Microbiology and Parasitology at Louisiana State University and completed the requirements for the degree of Doctor of Philosophy in the summer of 2000.

Alma was married to John Didier Roy in 1968, and they have two beautiful daughters, Amy Jeannine Roy Abadie and Jennifer Lin Roy. She also has two beautiful grandchildren Madeline Elliot Abadie born August 21, 1995 and Olivia Roy Abadie born February 3, 1997.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Alma F. Roy

Major Field: Veterinary Medical Sciences

Title of Dissertation: Pathogenesis of Bartonella henselae in the Domestic Cat: Use of a PCR-based Assay for the Detection and Differentiation of B. henselae Genotype I and Genotype II in Chronically Infected Cats

Approved:

[Signatures]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

June 16, 2000