THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Agricultural Biotechnology)

DEGREE

Agricultural Biotechnology | Interdisciplinary Graduate Program

FIELD | **PROGRAM**

TITLE: Molecular Epidemiology of *Bartonella* Species in Stray Cats Resided in Temples in Bangkok Areas

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THESIS

MOLECULAR EPIDEMIOLOGY OF *BARTONELLA* SPECIES IN STRAY CATS RESIDED IN TEMPLES IN BANGKOK AREAS

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Agricultural Biotechnology)
Graduate School, Kasetsart University
2009

Saowaluck Jitchum 2009: Molecular Epidemiology of *Bartonella* Species in Stray Cats Resided in Temples in Bangkok Areas. Master of Science (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Sathaporn Jittapalapong, Ph.D. 120 pages.

The pet ownership is risk for various diseases which their pets can serve as a reservoir of internal and external parasites capable of being transmitted to humans. Cat scratch disease (CSD) is an infectious disease which caused by *Bartonella* species. Cat can serve as a host for these bacteria and cat fleas (*Ctenocephaledes felis*) from infected cat can distribute the pathogen among the cats. The objectives of this study were to investigate the infective rate of *Bartonella* infection in stray cats resided in monasteries of Bangkok metropolitan by the PCR assay and find out association between *Bartonella* infection and risk factors. The PCR result was shown 803 from 1,488 cats infected with *Bartonella* species. A total of 1,488 samples were detected as *B. henselae* 35% (521/1,490), *B. clarridgeiae* 15.26% (227/1,490) and mixed infection 3.7% (55/1,490). The statistical analysis results were shown significant between risk factors and infection. Poor environmental condition was associated with *Bartonella* infection ($p = 0.01$). The other factors comprising age, sex, health condition, ectoparasite and density condition were not related to the infections. The positive samples of *Bartonella* species were found in 432 monasteries from 50 districts. Two species of *Bartonella*, *B. henselae* and *B. clarridgeiae* were found from the overall districts (100%). The result showed that stray cats were crucial reservoirs and can transmit the pathogen to housed cats and human who live in the same environment. The gain basis knowledge is useful for the prevention and control of distribution in both animals and humans from the infection of *Bartonella* species.

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Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

For the accomplishment of my research, I am sincerely indebted to my committee members,

To Assoc. Prof. Dr. Sathaporn Jittapalapong, my advisor, for leading me into this field. Be grateful for his kind, patience, support, suggestion for completely writing of thesis and allowing me to practice and develop the laboratory ability. His benevolence leads me to achievement of the research.

To Assoc. Prof. Dr. Patamaporn Amavisit, my co-advisor, for indispensable suggestion, kindness and great support. My research is not complete if lack of the valuable advice of molecular technique and bacteriology from her.

To Assist. Prof. Dr. Sirichai Wongnakpech, my co-advisor, for kind-heartedness, encouragement and helpful for statistical analysis. The statistical advice from him has fulfilled the achievement of my research.

The study was plentiful facilities provide at the Section of Molecular Laboratory, Department of Parasitology, Faculty of Veterinary, Kasetsart University. I would like to thank the staff at Department of Parasitology, Faculty of Veterinary medicine, Kasetsart University and laboratory colleagues whom gave me for the generous help in everything and kindness suggestion. I do thank to my graduated friends from Agricultural Biotechnology, Faculty of Interdisciplinary Graduate Program in Agricultural Biotechnology, for their friendship, help, cheerfulness and great support.This thesis is supported by the Center for Agricultural Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education.

Finally, I truthfully thank my family who always gave me the chance to do everything, wish well, perpetual support and encouragement.

Saowaluck Jitchum

September 2009

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LIST OF ABBREVIATIONS

× *g*	=	Acceleration gravity
°C	=	Degree(s) Celsius
µg	=	Microgram(s)
µl	=	Microliter(s)
µm	=	Micrometer(s)
B.c.	=	*Bartonella clarridgeiae*
B.h.	=	*Bartonella henselae*
bp	=	Base pair(s)
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
DW	=	Distilled water
EDTA	=	Ethylenediamine tetraacetic acid
et al	=	*et alli*
EtBr	=	Ethidium Bromide
g	=	Gram(s)
L (l)	=	Liter(s)
LB	=	Luria Bertani (broth)
M	=	Mole
ME	=	Microscopic examination
mg	=	Milligram(s)
min	=	Minute(s)

LIST OF ABBREVIATIONS (Continued)

ml	=	Milliliter(s)
mM	=	Micrometer(s)
ng	=	Nanogram(s)
nm	=	Nanometer(s)
PCR	=	Polymerase chain reaction
pH	=	Negative logarithm of hydrogen ion activity
RFLP	=	Restriction fragment length polymorphism
rRNA	=	ribosomal Ribonucleic acid
rpm	=	Round(s) per minute
U/µl	=	Unit(s) per microliter
U/g	=	Unit(s) per gram
UDW	=	Ultrapure distilled water
UV	=	Ultraviolet
V	=	Volts
v/v	=	Volume by volume
w/v	=	Weight by volume
w/w	=	Weight by weight

MOLECULAR EPIDEMIOLOGY OF *BARTONELLA* SPECIES IN STRAY CATS RESIDED IN TEMPLES IN BANGKOK AREAS

INTRODUCTION

The member in genus *Bartonella* (Class *Proteobacteria* and alpha subdivision) is a blood parasite and causes bartonellosis by infecting wild, domestic animals and humans. *Bartonella* species are intraerythrocytic bacteria which found in humans and animals (Gray *et al*., 1990). *Bartonella* spp. is gram-negative, aerobic bacilli, fastidious, hemotropic with more than 25 species and subspecies described (Maurin *et al*., 1997, Boulouis *et al*., 2005, Chomel *et al*., 2006) such as rats (Heller *et al*., 1998, Bermond *et al*., 2000), mice (Welch *et al*., 1999), rabbits (Heller *et al*., 1999), cats (Koehler *et al*., 1994, Kelly *et al*., 1998, Droz *et al*., 1999), dogs (Breitschwerdt *et al*., 1995), voles (Birtles *et al*., 1995) and coyotes (Chang *et al*., 2000). These organisms are emerging zoonotic pathogens that have been isolated from humans and animals in many countries. In humans, *Bartonella* causes various diseases including Carrion's disease, trench fever, cat scratch disease, bacillary angiomatosis, hepatic peliosis, endocarditis, neuroretinitis and chronic bacteremia (Jacomo *et al*., 2002). Arthropods are the important vectors, such as sand fly (*Lutzomyia verrucarum*) and *B. bacilliformis* (Carrion's disease) (Ihler, 1996), body louse (*Pediculus humanus*) and *B. quintana* (trench fever and bacillary angiomatosis) (Raoult and Roux., 1999), rat flea (*Xenopsylla cheopis*) and *B. elizabethae* (Breitschwerdt and Kordick, 2000), cat flea (*Ctenophaledes felis*) (*B. henselae*) (Chomel *et al*., 1996).

Cat scratch disease (CSD) is an emerging zoonosis caused by *Bartonella henselae* (Chomel, 2000). The first described of the disease is in France (Debré *et al*., 1950) and the etiology was not identified until 1992. The serological and microbiological studies have been developed and revealed *B. henselae* (formerly *Rochalimea henselae*) involved in CSD (Regnery *et al*., 1992, Dolan *et al*., 1993). Cats act as a reservoir host. Cat fleas (*Ctenocephaledes felis*) play the role as vector among the cats (Chomel *et al*., 1999, Boulouis *et al*., 2005). Scratching and biting

from infected cat can transmitted the pathogen in human. After the exposure, CSD will form typically manifests as a benign lymphadenitis in immunocompetent person. Typical CSD found in all ages particularly in children. The clinical signs such as the purulent and infected with lymph node, as bacillary angiomatosis, and hepatic peliosis in immunodeficiency patients were found in the co-infection with other bacteria, lead to the wide pathogenesis of disease manifestations such as endocarditis bacteremia, osteolytic lesions, pulmonary nodules, neuroretinitis and neurologic disease (Adal, 1995, Margileth, 1995, Maurin *et al*., 1997, Jacomo *et al*., 2002). Some of these manifestations may be fatal especially in immunodeficient patients (Cockerel *et al*., 1987).

Although, the gold standard for diagnosis of bacteria in genus *Bartonella* is culture technique but this pathogen is slow growth and fastidious. The modern technology, serology and PCR were developed and often replace for routine diagnosis since these methods are faster and more sensitive than the culture technique. Many previous studies reported on epidemiology of this pathogen worldwide. In Asian countries, the prevalences of *Bartonella* infections are 7.2% (50/620) in Japan (Maruyama *et al*., 2000), 64.3% (9/14) in Indonesia (Marston *et al*., 1999), 61.3% (19/31) in Philippines (Chomel *et al*., 1999), 19.1% (25/131) in Taiwan (Chang *et al*., 2006). In Thailand, the prevalence of *B. henselae* in stray and pet cats have been found at 27.9% (76/275) (Maruyama *et al*., 2001) and 5.5% (9/163) and 1.2% (2/163) in humans (Maruyama *et al*., 2000). In Bangkok Metropolitan areas, the population of stray cats is still questionable particularly in monasteries since the number of stray animal seems to be continuously rising due to economic crisis or recession (Jittapalapong *et al*., 2003). The lack of health care in stray cats for example deworming program, vaccination and nutrition, make them play an important role in public health problems such as zoonoses and reservoir of diseases.

This study, we use molecular technique to investigate the infective rate of *Bartonella* infection in stray cats resided in the monasteries in 50 districts of Bangkok and determine factors associated with *Bartonella* infection with Chi-square test. The

result of this study will be beneficial for the prevention and control program of *Bartonella* infections in both animals and humans.

OBJECTIVES

1. To determine the infective rate of *Bartonella* infection in stray cats resided in the monasteries in Bangkok metropolitan areas by PCR and light microscopic examination

2. To identify factors associated with *Bartonella* infection among stray cats

LITERATURE REVIEW

1. The overview of *Bartonella* species

1.1 The history of *Bartonella* species

Bartonella species has been studied since 1909. *Bartonella* species were affecting on human health and *B. quintana* can be found in a 4000 year old human tooth (Drancourt *et al*., 2005). The genus of these bacteria is named after Alberto Leonardo Barton Thompson, who first discovered *B. bacilliformis* which cause of the Oroya fever or Carrión's disease in Peru in 1909 (Schouls *et al*., 1999, Mogollon-Pasapera *et al*., 2008).

As the advanced diagnostic tools and methods, the reclassification of genera *Grahamella* (Birtles *et al*., 1995) and *Rochlimaea* (Brenner *et al*., 1993) were merged into genus *Bartonella* in 1992. The first human bartonellosis (Oroya fever or Carrión disease) has been reported in Peru which has two stages of symptoms. Symptoms of acute or subacute phase are fever, anemia and jaundice, and then a chronic phase recognized as verruga peruana (Peruvain wart) (Mogollon-Pasapera *et al*., 2008). At the present, the member of genus *Bartonella* comprises more than 25 species and subspecies. Many species and subspecies of *Bartonella* are recognized as the causative agents of human diseases, such as *B. bacilliformis*, *B. quintana*, *B. henselae*, *B.elizabethae, B. koehlerae, B. vinsonii* subsp. *berkhoffii, B. vinsonii* subsp. *arupensis, B. grahamii,* and *B. washoensis* (Daly *et al*., 1993, La Scola and Raoult, 1996, Anderson and Neuman, 1997, Kerkhoff *et al*., 1999., Welch *et al*., 1999, Karem *et al*., 2000, Roux *et al*., 2000, Kosoy *et al*., 2003, Avidor *et al*., 2004).

Bartonella has become the emerging zoonoses which affects on medical and veterinary health due to a few factors such as the reduction of host immune system in immunocompromised patients, organ transplant and cancer therapy (Boulouis *et al*., 2005). In addition, the co-infection with many pathogens has been

reported in HIV-patients (Koehler *et al*., 2003) and *Borrelia* species (Eskow *et al.,* 2001, Posiadly *et al*., 2003). Table 1 shows the *Bartonella* species can cause in humans and clinical manifestations of disease.

Table 1 The *Bartonella* species can cause disease in humans and clinical manifestations of disease.

Species	Year of description	Main vectors	Main reservoirs	Main human disease[1]	References
B. bacilliformis	1909	Sand fly	Human	Carrion's disease	Barton
B. quintana	1915	Body louse	Human	Trench fever CSD BA Bacteremia Endocarditis Septicemia	Strong
B. henselae	1992	Cat flea	Cat	CSD BA CA Bacteremia Endocarditis Septicemia	Regnery
B. elizabethae	1993	Oriental rat flea, rodent flea	Rat	Endocarditis Retinitis	Daly

Table 1 (Continued)

Species	Year of description	Main vectors	Main reservoirs	Main human disease[1]	References
B. grahamii	1995		Wild mice	Retinitis	Birtles
B. washoensis	1995	Unknown (flea?)	California ground squirrel	Fever	Regnery
B. vinsonii subspecies berkhoffii	1996	Unknown (tick?)	Coyote, dog	Myocarditis Endocarditis	Kordick
B. clarridgeiae	1996	Cat flea	Cat	CSD	Lawson
B. alsatica	1999	Unknown (flea?)	Rabbit	Endocarditis	Heller
B. vinsonii subspecies qrupensis	1999	Unknown (flea, tick?)	White-footed mouse	Bacteremia	Welch
B. rochalimae	2007	Unknown (flea?)	Unkonown	Bacteremia	Eremeeva

[1] CSD = Cat Scratch Disease, BA = Bacillary angiomatosis, CA = Chronic adenopathy

Source: Mogollon-Pasapera *et al* (2008)

1.2 Bacteriology

1.2.1 Morphology and Biochemical test

The bacteria in member of family *Bartonellaceae* is a gram-negative, short pleomorphic coccobacillary or bacillary rods (0.6μm × 1.0 μm) (Boulouis *et al*., 2005). The bacterium is biochemically inert with oxidase, catalase, urease and nitrate reductase negative except for the production of peptidase so it is difficult to isolate with biochemical test (Breitshwerdt *et al*., 2000). The colonies are small, delicate, aerobic and grow slowly which take from 5 to 15 days and up to 45 days on primary culture to form visible colonies on enriched blood-containing media (Boulouis *et al*., 2005), as dependent on highly blood-containing (Chomel *et al*., 2004). The optimal temperature of growth is 35-37˚C except *B. bacilliformis* prefers 28 ˚C.

In red blood cells, May-Grünwald Giemsa staining can be used to identify these small organisms. In infected tissues with *Bartonella* infection, Warthin-Starry silver impregnation stain exposes small bacilli which likely cluster of organisms.

1.2.2 Phylogenetic of *Bartonella* species

The genus *Bartonella* is bacteria in phylum Proteobacteria, class alpha-proteobacteria, order Rhizobials, familly *Bartonellaceae.* The evolution of bacteria in genus *Bartonella* has homology in α-proteobacterial species including genera *Brucella*, *Agrobacterium* and *Rhizobium* in Figure 1.

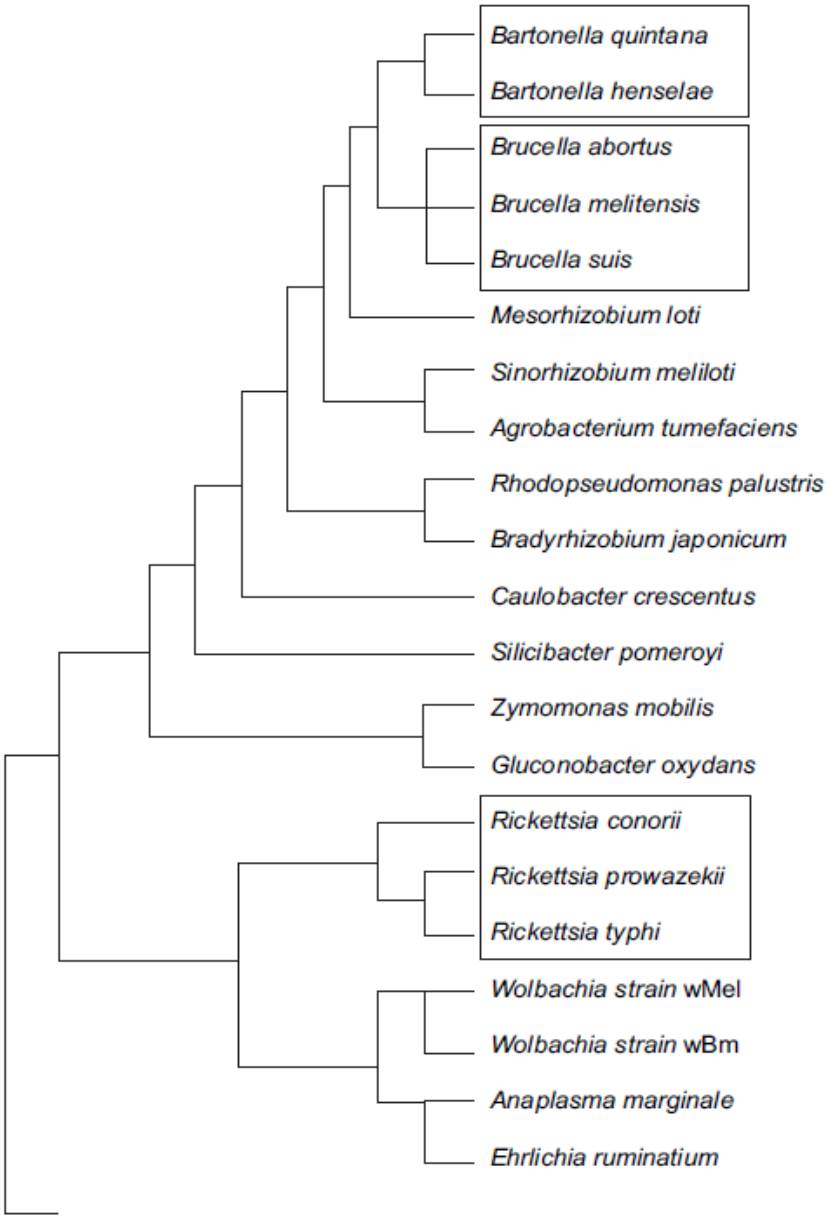


Figure 1 Phylogenetic tree reconstruction inferred using the maximum likelihood method for α-proteobacterial species of which the complete genome sequence is known. Only topology show *Bartonella, Brucella* and *Rickettsia* genera are highlighted.

Source: (Sallstrom *et al*., 2005; Ehrenborg, 2007)

As no distinguishing phenotypic characteristics have been described for *Bartonella* species, the identification and phylogenic classification are based on genetic studies. The different methods have been used to determine the relationship of member in genus *Bartonella*. Many studies have been used 16S rRNA, *ftsZ*, *gltA* and *gro*EL for phylogenetic.There are many molecular genetic methods for differentiation of strain and species of *Bartonella* such as RFLP (restriction fragment length polymorphism) of genes encoding citrate synthase, 16S rRNA, 16S-23S rRNA interspacer region, PCR (polymerase chain reaction). In recently, the amplification of 16S-23S rRNA intergenic spacer region (ITS) (Roux and Raoult, 1995) or protein coding genes such as citrate synthase (*glt*A), the heat shock protein (*gro*EL), the riboflavine (*rib*C), the RNA polymerase beta subunit (*rpo*B), a cell division protein (*fts*Z) and a 17 kDa antigen (La Scola *et al*., 2003) were used for identification of *Bartonella* species and strain (Boulouis *et al*., 2005).

1.3 Bartonellosis and vectors

Bartonellosis is an infection caused by *Bartonella* species which are facultative intracellular bacteria and report worldwide distribution. *Bartonella* spp. has been reported in canid, felid, ruminants and human. *Bartonella* species cause various human diseases including Carrion's disease, trench fever, cat scratch disease, bacillary angiomatosis, hepatic peliosis, endocarditis, chronic bacteremia and neuroretinitis (Jacomo *et al*., 2002). *B. bacilliformis*, the etiologic agent of Carrión disease, is transmitted by the sand fly (*Lutzomyia verrucarum*) in the Andes Mountains in Peru, Columbia, and Ecuador (Ihler, 1996). *B. quintana*, the agent of trench fever and bacillary angiomatosis, is found worldwide and is transmitted by the human body louse (*Pediculus humanus*) (Raoult and Roux, 1999). *B. henselae* is associated with cats, which serve as its reservoir (Regnery *et al*., 1992; Koehler *et al*., 1994); the cat flea (*Ctenocephalides felis*) was demonstrated to be a vector (Chomel *et al*., 1996). Other *Bartonella*-flea associations are apparent, for example, 61% of rat fleas (*Xenopsylla cheopis*) were found infected with *Bartonella* spp., including a known human pathogen, *B. elizabethae* (Breitschwerdt and Kordick, 2000). As for

many vector-borne disease agents, a wide range of mammalian reservoir hosts including, sand flies, fleas and body lice, are involved in the natural cycle of various *Bartonella spp*. (Jacomo and Raoult, 2002). Fleas (phylum Arthropoda, order Siphonaptera and class Insecta) can be found worldwide and are biological vectors of several important zoonoses which transmit the agents between animals and human hosts. Cat fleas (*Ctenocephalides felis*) are importance vector of *Bartonella* spp. including *B. clarridgeiae, B. henselae, B. koehlerae*, *B. quintana* (Bergmans *et al*., 1997).

Cat Scratch Disease

Many of domestic and wild animals are reservoirs for *Bartonella* species in nature such as dogs, rats, mice, rabbits, cattle, mountain lions and Florida panthers (Birtles *et al*., 1995; Brenner *et al*., 1993;Heller *et al*., 1998; 1999; Bermond *et al*., 2000; Droz *et al*., 1999; Kelly *et al*., 1998; Koehler *et al*., 1994; Lawson and Collins, 1996; Breitschwerdt *et al*., 1995; Chang *et al*., 2000; McNee *et al*., 1916; Gray *et al*., 1990). Cats are the reservoir of many species of *Bartonella* such as *B. clarridgeiae* (Kordick *et al*., 1997), *B. koehlerae* (Droz *et al*., 1999; Yamamoto *et al*.,2002) and *B. bovis* (Bermond *et al*., 2002), formerly *B. weissi* (Regnery *et al*., 2000). In cats, there have been isolated at least three species of *Bartonella* species, *Bartonella henselae* (formerly *Rochalimaea henselae*), *B. koehlerae* and *B. clarridgeiae* which may cause diseases in humans (Jacomo *et al*., 2002, Chomel *et al*.,1995).

B. henselae and *B. clarridgeiae* are the main etiology of cat scratch disease and bacillary angiomatosis. These organisms are emerging health concern in many countries such as the United States. The Center for Disease Control and Prevention (CDC) estimated an overall prevalence of 2.5 cases per year in every 100,000 people. The CSD is a common cause of subacute or chronic lymphadenopathy with 80% of all cases (Carithers *et al*., 1985 and Margileth *et al*., 1992). The risk groups for CSD are people who had a close contact with cats. Most CSD was found in the age of patients less than 21 years. CSD that occurs in immunocompetent patients can characterized by self-limiting lymphadenopathy which usually resolves in 2 to 4 months. However in some patients the disease can be persisting for up to 2 years. In addition, *Bartonella henselae* has also been associated with several of disease syndromes in immunocompromised individuals, alcoholics, or organ transplant recipients, particularly AIDS patients, including bacillary angiomatosis and endocarditis (Adal *et al*., 1994; Maurin *et al*., 1997, Schwartzman, 1992; Slater *et al*., 1992).

Cats are the major reservoir of B. henselae since approximately 40% of domestic cats harbor active infections and 80% are tested seropositive from previous exposure (Chomel *et al*., 1995). Actually, cats are close to humans and the owners normally keep their cats in the house particularly in their bedrooms. The habit of cat is usually outdoors, therefore cats can be infected by ectoparasites and endoparasites. Moreover, animals have more risk of infection by their parasites than housed animals. However, the housed cats have a chance to be via infected by stray cats via infected tick, fleas and lice or other parasites which play an important role in spread of infection among cat population.

The transmission of *B. henselae* among cats depends on an arthropod vector such as *Ctenocephalides felis* (Chomel *et al*., 1996, Higgins *et al*., 1996). After that, the bacteria in blood stream grow to the high level (10^4 to 10^6 CFU/ml) and persist long-term infections within the red blood cells of host (Mehock *et al*., 1998). *B. hensela* can be found in other tissues such as liver, brain, kidneys, heart, and lymph nodes (Kordick *et al*., 1999). Infected cats showed such as fever and lesions on internal organs (Greene *et al*., 1996). *B. henselae* is capable of hiding from the host

immune system by stay inside host erythrocytes and sometimes inside macrophages. *Bartonella* can infect red blood cell and cause prolonged intraerythrocytic bacteremia in mammalian reservoir host (Dehio, 2004). The bacterium is intracellular bacteria that may invade and persist in red blood cell and endothelial cell of host for increasing its amount. Blood-sucking arthropods transmitted the pathogen to the others (Greub and Raoult, 2002, Ehrenborg, 2007). Schulein *et al*., (2001) demonstrated model of infection with *Bartonella* in rats with *B. tribocorum* (Figure 2). The study showed the bacterial persistence strategy adapted to a non-hemolytic intracellular colonization of erythrocytes that preserves the pathogen for efficient transmission by blood-sucking arthropods.

The infection of *Bartonella henselae* was shown in Figure 3. The transmissions among cats are by cat fleas and transmitted to humans by cat scratch, cat bite, contaminated claws and tooth and flea bites. The most frequently pathogenesis in normal host is lymphadenitis but in immunocompromised patient, is initiated bacillary angiomatosis, hepatitis peliosis and endocarditis.

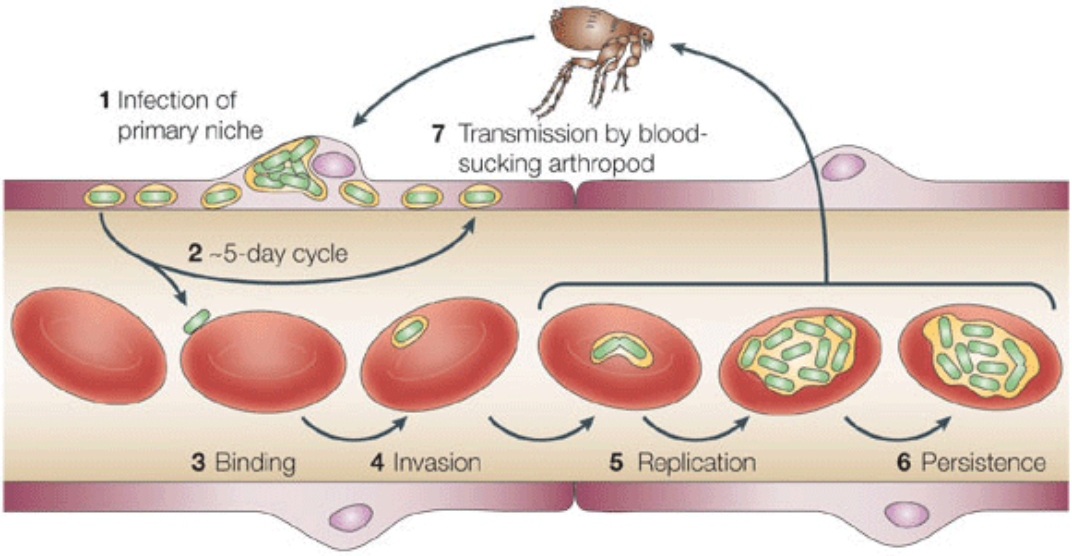


Figure 2 (1) The primary niche of bacterial colonization is still poorly defined but is considered to include the vascular endothelium as a major constituent. (2) At five-day intervals, bacteria are released from the primary niche into the bloodstream, from where they can reinfect the primary niche to start another infection cycle, or (3) where they bind to erythrocytes, (4) invade, (5) replicate in an intracellular membrane-bound compartment, and (6) finally persist in a non-replicative intra-erythrocytic state for several weeks. This strategy is considered to be a specific adaptation to transmission by blood-sucking arthropods.

Source: Dehio (2005)

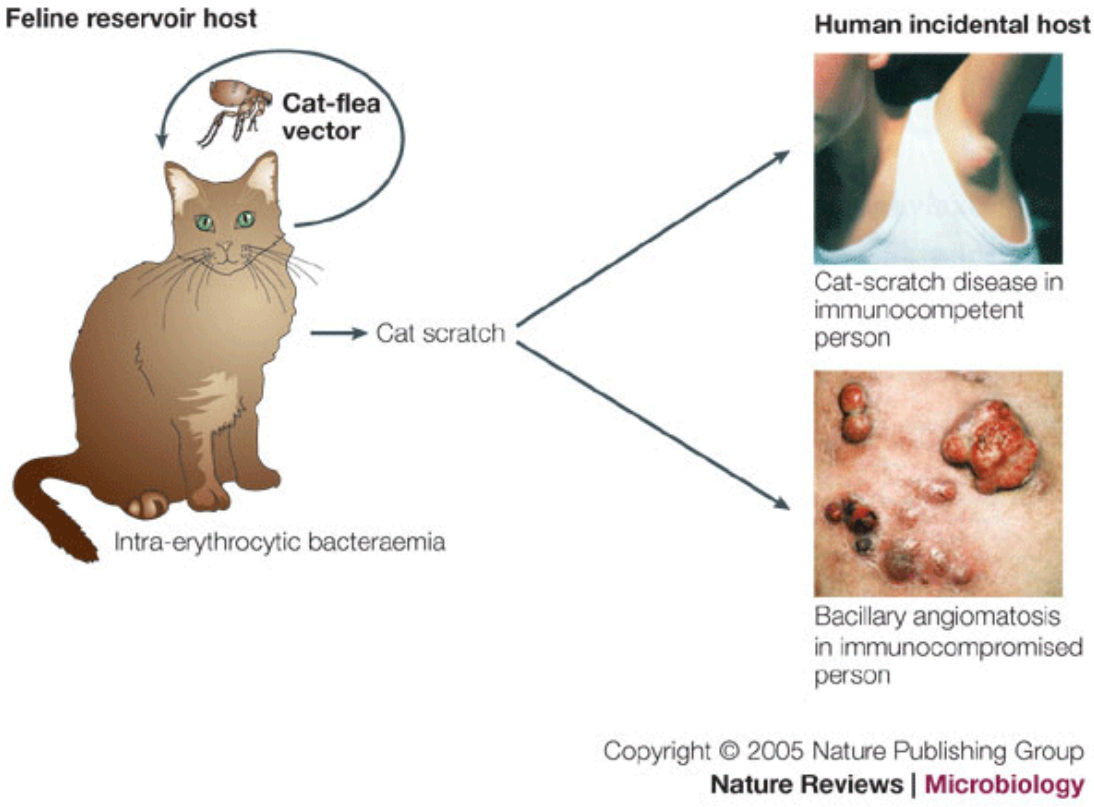


Figure 3 *Bartonella henselae* causes a sub-clinical intra-erythrocytic bacteremia in its feline reservoir host. Through the bite of an infected cat flea or through direct contact trauma (cat scratch), the pathogen is transmitted from cat to cat or from cat to human, respectively. Depending on the immune status of the human host, *B. henselae* can lead to different clinical manifestations, including cat-scratch disease (a febrile lymphadenopathy) in immunocompetent individuals, or bacillary angiomatosis and peliosis in immunocompromised patients.

Source: Dehio (2005)

1.4 Treatment

The treatments for *Bartonella* infections in humans were different among their species. The clinical signs of *Bartonella* infection show different stages such as acute, relapsing or chronic phase. The host red blood cells can be infected by both extracellular and intracellular depend on *Bartnella* species. Drugs recommendation for treatment of *Bartonella* infection was showed in Table 1.

In humans, treatment is different for immunocompetent patients having classical symptoms of cat scratch disease with angiomatous proliferative diseases (Koehler and Tappero, 1993). For immunocompetent patients, numerous antimicrobial agents have been advocated for the treatment of typical CSD. However, most instances, antibiotics do not appear to improve the *Bartonella* infection (Rolain *et al*., 2004). In *Bartonella* endocarditis, effective antibiotic therapy should include an aminoglycoside for a minimum of two weeks (Raoult *et al*., 2003). In immunocompromised patients with bacillary angiomatosis or bacillary peliosis, the effectiveness of treatments was varied (Koehler and Tappero, 1993). Overall, tetracyclines, erythromycin, rifampin, azithromycin, doxycycline or a combination of these antibiotics are effective and should be administered in these patients for at least six weeks and be followed up for 4 to 6 months in those who have relapses (Margileth, 2000, Rolain *et al*., 2004). In cats, antimicrobial agents are not commonly used or recommended for treatment or prevention of *B. henselae*, since antibiotic treatments tested to date may reduce the level of bacteremia but do not clear the cats from their infection (Kordick *et al*., 1997; Regnery, 1996). Additionally, the minimal effectiveness of these antimicrobial agents could be explained by the fact that *Bartonella* species are intracellular organisms. In dogs, no study has been performed to determine the efficacy of antibiotics for treatment of *Bartonella* infection. However, it is likely that antibiotics such as doxycycline (10 mg/kg/day) or tetracycline could reduce the level of bacteremia during chronic infections, but should be administered for prolonged periods of time (4–6 weeks). Fluoroquinolones alone or in combination with amoxicillin have also elicited a positive therapeutic response in dogs (Breitschwerdt *et al*., 2004), as repeated *B. vinsonii* subsp. *berkhoffii* antibody

titers became negative after treatment. However, antibiotic therapy may not be very effective when the lesions of endocarditis are already well established.

In cats, a few experimental studies have been conducted to determine therapy for bacteremia in cats, from which it has been suggested than it is possible to treat these animals with amoxicillin. Results of the study were variable with bacteremia apparently in all cases so it was not possible to completely suppress or eliminated bacteremia, and it was necessary to have repeated treatment sessions. However, there are the suggestions if treatment is attempted, it should be prolonged and combined with eradication of fleas on all animals in the household and the premises in an attempt to avoid re-infection (Brunt *et al*., 2006).

1.5 Prevention

There are the recommendations from the American Association of Feline Practitioners (AAFP) Panel were adapted from Guidelines for Preventing Opportunistic Infections Among HIV-Infected Persons (Kaplan *et al*., 2002) and the AAFP Panel Report on Zoonoses (Brown *et al*., 2002).

1. Flea control should be initiated and maintained year-round.

2. If a family member is immunocompromised and a new cat is to be acquired, adopt a healthy cat >1 year of age and free from fleas.

3. Discuss the advantages and disadvantages of testing healthy cats for *Bartonella* spp. infections.

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

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

6. Scratches and bites should be avoided (including rough play with cats).

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

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

2. Epidemiology of *Bartonella* species

An epidemiology of *Bartonella* species in domestic, wild animals and humans have been reported from many parts of the world (Chomel, 2000). The prevalence of *Bartonella* species in each country is different. The difference of geographic areas was affected to endemic distribution. The geographic distribution of the pathogen may be depend on temporary or permanent climatic alterations such as global warming (Huarcaya *et al*., 2004). For *B. henselae*, the prevalence of bacteria has reported high prevalence in warm and humid region where flea infestation is high (Chemoweth *et al*., 2004).

In the United States, *B. henselae* caused of CSD and bacillary angiomatosis is an emerging health concern with an estimated 22,000 new cases per years and 2,000 of these require hospitalization (Jackson *et al*., 1993). In Netherlands, an estimated of CSD cases was 2,000 cases per year (12.5 cases/100,000 persons) (Bergmans *et al*., 1997). In Thailand, the previous study has reported the prevalence of *Bartonella* in stray cats at 27.6% (Maruyama *et al*., 2001). In humans, the seroprevalence has been found around 5.5% (9/163) for *B. henselae* – IgG and 1.2% (2/163) for *B. henselae* – IgM (Maruyama *et al*., 2000).

Table 2 - 7 show data of investigations in different continents. Data of surveys concerning cat populations in Europe are summarized in tables 2, 3 and 4. Table 5 for Asia and Oceania, table 6 for Americas and table 7 for Africa and the Middle East were also shown (Boulouis *et al*., 2005).

Table 2 Surveys of *Bartonella* spp. infections (bacteremia or antibodies) in domestics in France, Germany and the Netherlands.

Country (location)	**Survey**	**Cat population**	***Bartonella* spp.**	**Percentage of prevalence (positive/total)**	**Reference**
France(Paris)	B	Pets	*B.spp.*	16.5 (72/436)	Gurfield *et al*., 2001
			B.h.	H: 15.3 (11/72)	
				M: 50 (36/72)	
				H+M: 2.8 (2/72)	
			B.c.	20.8 (15/72)	
			B.h.+B.c.	2 (8/72)	
	S	Pets	*B.h.+B.c*	41.1 (179/436)	
France (Lyon)	B	Pets	*B. spp.*	8.1 (8/99)	Rolain *et al*., 2004
			B.h.	H: 75 (6/8)	
				M: 0 (0/8)	
			B.c.	25 (2/8)	
France (Nancy)	B	Strays	*B.spp.*	53 (50/94)	Heller *et al*., 1997
			B.h.	H: 34 (17/50)	
				M: 36 (18/50)	
			B.c.	30 (15/50)	
France (Marseille?)	B	Strays	*B.spp.*	62.3 (38/61)	La Scola *et al*., 2002
			B.h.	H: 39.5 (15/38)	
				M: 18.4 (7/38)	
			B.c.	42.1 (16/38)	
Germany	S	Pets	*B.h.*	15 (107/713)	Haimerl *et al*., 1999
Germany (Freiburg)	B	Pets	*B.h.*	13 (13/100)	Sander *et al*., 1997
Germany (Berlin)	B	Pets/Strays	*B.h.*	10.4 (20/193)	Arvard *et al*., 2001
				Pets: 1 (1/97)	
				Strays: 18.7 (19/96)	
				H: 5 (1/20)	
				M: 90 (18/20)	
			B.c.	5 (1/20) (a stray cat)	

Table 2 (Continued)

Country (location)	Survey	Cat population	*Bartonella* spp.	Percentage of prevalence (positive/total)	Reference
The Netherlands	B	Shelter	*B.h.*	22 (25/113)	Bergman *et al*., 1997
				H: 24 (6/25)	
			B.h./B.c.	M: 40 (10/25)	
			B.c.	16 (4/25)	
	S	Shelter/Pets	*B.h.*	35–60 (Not given)	
				Pets: 56 (28/50)	
				Shelter: 50 (56/113)	

S: seroprevalence, B: bacteremia prevalence, *B.h.*: *Bartonella henselae*, *B.c.*: *Bartonella clarridgeiae*, *B.spp.*: *Bartonella* species, H: type I (Houston I), M: type II (Marseille).

Table 3 Surveys of *Bartonella* spp. infections (bacteremia or antibodies) in domestics in northern and central Europe.

Country (location)	Survey	Cat population	*Bartonella* spp.	Percentage of prevalence (positive/total)	Reference
Austria	S	Pets	*B.h.*	33.3 (32/96)	Allerberger *et al*., 1995
Czech Republic	B	Pets/Shelter/Stray	*B.h.*	8 (5/61)	Melter *et al*., 2003
				M:100 (5/5)	
				Pets 0 (0/34)	
				Shelters 5 (1/21)	
				Stray: 66.6 (4/6)	

Table 3 (Continued)

Country (location)	Survey	Cat population	*Bartonella* spp.	Percentage of prevalence (positive/total)	Reference
Denmark	B	Pets/Shelter	*B.h.*	22.6 (21/93) H: 5 (1/21) M: 95 (20/21) Pets: 18.2 (8/44) Shelter: 26.5 (13/49)	Chomel *et al*., 2002
	S	Pets/Shelter	*B.h.*	45.6 (42/92)	
Denmark (North Zealand)	B	Strais	*B.h.*	44 (11/25)	Engbeak and Lawson, 2004
Norway	B	Pets	*B.spp.*	0 (0/100)	Bergh *et al*., 2002
	S	Pets	*B.h. (EIA)*	1 (1/100)	
			B.h. (IFA)	0 (0/100)	
Poland	S	Shelters	*B.h.*	86 (31/36)	Posiadly *et al*., 2003
Sweden	S	Pets	*B.h.*	1 (3/292)	Hjelm *et al*., 2002
Sweden (Stockholm and southern Sweden)	B	Pets	*B.h.*	2.2 (2/91)	Engvall *et al*., 2003
Switzerland	S	Pets	*B.h.*	8.3 (60/728)	Glaus *et al*., 1997
United Kingdom (Bristol and Southwest UK)	B	Pets	*B.h.*	9.4 (34/360) H: 6 (2/34) M: 88 (30/34) H+M: 6 (2/34)	Birtles *et al*., 2002
United	S	Pets	*B.h.*	40.6 (28/69)	Barnes *et al*., 2000
Kingdom	S	Feral	*B.h.*	41.8 (33/79)	
United Kingdom	B	Pets	*B.h.*	11.4 (40/351)	Laycock *et al*., 2001

S: seroprevalence, B: bacteremia prevalence, *B.h.*: *Bartonella henselae*, *B.c.*: *Bartonella clarridgeiae*, *B.spp.*: *Bartonella* species, EIA: ELISA, IFA: immunofluorescence, H: type I (Houston I), M: type II (Marseille).

Table 4 Surveys of *Bartonella* spp. infections (bacteremia or antibodies) in domestics in Italy and Portugal.

Country (location)	**Survey**	**Cat population**	***Bartonella* spp.**	**Percentage of prevalence (positive/total)**	**Reference**
Italy (Lonbardia) (3 urban, 3 rural areas)	B	Stray	*B.spp.*	18 (140/769)	Fabbi *et al*., 2004
			B.h.	H: 20.6 (27/131)	
				M: 61.1(80/131)	
				H+M:18(24/131)	
	S	Stray	*B.h.*	38 (207/540)	
Italy (northern)	B	Stray	*B.h*	23 (361/1585)	Fabbi *et al*., 2004
				26 (Not given)	
			B.c.	52 (Not given)	
	S	Stray	*B.h.*	39 (553/1416)	
Italy (Tuscany)	B	Pets/cattleries	*B.spp.*	0 (0/28)	Ebani *et al*., 2002
	S	Pets/cattleries	*B.h.*	23 (98/427)	
Italy (Reggio Emilia)	B	Pets	*B.h.*	9.7 (24/248)	Cabassi *et al*., 2002
Portugal	S	Pets	*B.h.*	6.7 (1/14)	Childs *et al*., 1995

S: seroprevalence, B: bacteremia prevalence, *B.h.*: *Bartonella henselae*, *B.c.*: *Bartonella clarridgeiae*, *B.spp.*: *Bartonella* species, H: type I (Houston I), M: type II (Marseille).

Table 5 Surveys of *Bartonella* spp. infections (bacteremia or antibodies) in domestics in Asia and Oceania.

Country (location)	Survey	Cat population	*Bartonella* spp.	Percentage of prevalence (positive/total)	Reference
Australia (Syney)	B	Pets/Feral	*B.h.*	35 (27/77) Pets: 16 (3/18) Feral: 40 (24/59)	Branley *et al*., 1996
Australia (Melbourne)	B	Pets	*B.h.*	13.2 (45/342)	Ng S.O. and Yates, 1997
New Zealand (Auckland)	B	Pets	*B.h.*	17 (8/48)	O'Halloran *et al*., 1998
Japan	S	Pets	*B.h.*	15.1 (30/199)	Ueno *et al*., 1996
Japan (Kanangawa, Saitama Prefactures)	S	Pets	*B.h.*	9.1 (43/471)	Maruyama *et al*., 1998
Japan	S	Pets	*B.h.*	8.8 (128/1447)	Maruyama *et al*., 2003
Japan	B	Pets/Pound	*B.h.*	9.1 (3/33)	Maruyama *et al*., 1996
Japan	B	Pets	*B.spp.* *B.h.* *B.c.* *B.c.*+*B.h.*	7.2 (50/690) H: 95.5 (43/45) M: 2.2 (1/45) 8 (4/50) 2 (1/50)	Maruyama *et al*., 2000
Indonesia	B	Stray	*B.h.*	43 (6/14)	Marston *et al*., 1999
(Jakarta)	S	Stray	*B.h.*	54 (40/74)	
Philippines (Manilla)	B	Stray	*B.spp.* *B.h.* *B.c.*	61 (19/31) H: 68.4 (13/19) 10.5 (2/19)	Chomel *et al*., 1999
Singapore	S	Stray	*B.h.*	47.5 (38/80)	Nasirudeen and Thong, 1999

Table 5 (Continued)

Country (location)	Survey	Cat population	*Bartonella* spp.	Percentage of prevalence (positive/total)	Reference
Thailand	B	Pets/Stray	*B.spp.*	27.6 (76/275)	Maruyama *et al*., 2001
			B.h.	83 (63/76)	
			B.c.	11.8 (9/76)	
			B.h.+*B.c.*	5.3 (4/76)	
			B.h.	H: 71.6 (48/67)	
				M: 13/67 (19.4)	

S: seroprevalence, B: bacteremia prevalence, *B.h.*: *Bartonella henselae*, *B.c.*: *Bartonella clarridgeiae*, *B.spp.*: *Bartonella* species, H: type I (Houston I), M: type II (Marseille).

Table 6 Surveys of *Bartonella* spp. infections (bacteremia or antibodies) in domestics in the Americas.

Country (location)	Survey	Cat population	*Bartonella* spp.	Percentage of prevalence (positive/total)	Reference
Brazil	S	Pets?	*B.h.*	46 (Not given)	Cited in Velho *et al*., 2003
Chile (Valdivia)	S	Pets	*B.h.*	71 (54/76)	Zaror *et al*., 2002
			B.c.	18.6 (Not given)	
Canada	S	Pets	*B.h.*	17.8 (43/242)	Leighton *et al*., 2001
USA/Canada	S	Pets	*B.h.*	27.9 (175/628)	Jameson *et al*., 1995
USA	S	Pets/Shelter	*B.h.*	28.2 (370/1314)	Childs *et al*., 1995
USA (Baltimore)	S	Stray/Vet.Hosp.	*B.h.*	13 (77/259)	Childs *et al*., 1994
USA (Florida)	S	Feral/Sttray	*B.h.*	33.6 (186/553)	Luria *et al*., 2004

Table 6 (Continued)

Country (location)	**Survey**	**Cat population**	***Bartonella* spp.**	**Percentage of prevalence (positive/total)**	**Reference**
USA (North	S	Feral	*B.h.*	93 (93/100)	Nutter *et al*., 2004
Carolina)	S	Pets	*B.h.*	75 (57/76)	
USA	B	Pets/Stray	*B.h.*	39.5 (81/205)	Chomel *et al*., 1995
(California)					
	S	Pets/Stray	*B.h.*	Pets: 21.4 (24/112) Stray: 61.3 (57/93)	
	S	Pets/Stray	*B.h.*	81 (165/205)	
USA	B	Pets	*B.h.*	24 (65/271) H: 28.6 (14/49) M: 65.3 (32/49) H+M:6.1 (3/49)	Guptill *et al*., 2004
			B.c.	(0)	
	S	Pets	*B.h.*	51 (138/271)	

S: seroprevalence, B: bacteremia prevalence, *B.h.*: *Bartonella henselae*, *B.c.*: *Bartonella clarridgeiae*, *B.spp.*: *Bartonella* species, H: type I (Houston I), M: type II (Marseille).

Table 7 Surveys of *Bartonella* spp. infections (bacteremia or antibodies) in domestics in Africa and the Middle East.

Country (location)	Survey	Cat population	*Bartonella* spp.	Percentage of prevalence (positive/total)	Reference
Egypt	S	Pets	*B.h.*	12 (5/42)	Childs *et al*., 1995
Israel	S	Pets	*B.h.*	39.5 (45/114)	Baneth *et al*., 1996
Israel	B	Stray	*B.h.*	83 (40/48)	Avidor *et al*., 2004
			B.c.	15 (7/48)	
			B.k.	2 (1/48)	
Jordan	S	Pets	*B.h.*	36 (55/153)	Al-Majali, 2004
				True prevalence 32	
South Africa	S	Shelter	*B.h.*	21 (11/52)	Kelly *et al*., 1996
	B	Pets	*B.h.*	3.2 (1/31)	Pretorius *et al*.,
				H: (1/1)	1999
Zimbabwe	S	Pets/Shelter	*B.h.*	24 (28/119)	Kelly *et al*., 1996
	B	Pets	*B.h.*	8 (2/25)	Kelly *et al*., 1998

S: seroprevalence, B: bacteremia prevalence, *B.h.: Bartonella henselae*, *B.c*.: *Bartonella clarridgeiae*, *B.k*.: *Bartonella koehlerae*, *B.spp*.: *Bartonella* species, H: type I (Houston I), M: type II (Marseille).

3. Diagnosis of *Bartonella* infection

In the past, cat scratch disease in human was clinically diagnosed by detection of the enlarged lymph node and small vesicle or granuloma at the inoculation site (Boulouis *et al*., 2005). The criteria for CSD diagnosis such as: epidemiological data involving cat contact, cat scratches or bite, owning a cat, possible contact with fleas or other blood sucking arthropods, the presence of a cutaneous inoculation site, regional lymphadenopathy and a granuloma of lymph node or a positive skin test (Fig. 4) (Ehrenborg, 2007). At present, the advanced of technology and method such as serologic testing and PCR are use to diagnose.

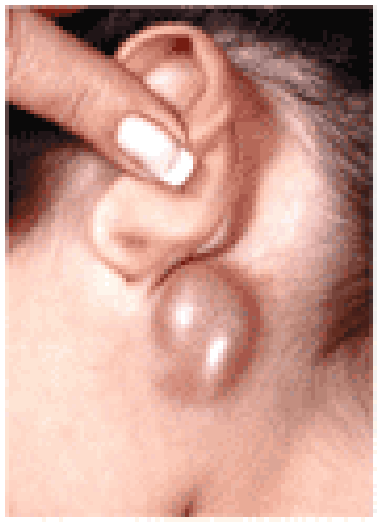


Figure 4 Lymphadenopathy in humans caused by *Bartonella* infections

Currently, there are many methods are available and developed for differentiated and diagnosed bacterial agents such as *Bartonella spp.* from other agents. Each technique has different sensitivity and specificity for each pathogen. Presently, serological techniques are most widely used, but their weak points are due to their cross-reactions with *Chlamydia* or other bacterial species (Drancourt *et al*., 1995, La Scola and Raoult, 1996, Maurin *et al*., 1997) and sometime with variable sensitivities (Maurin and Raoult, 1996) have been reported. Histological examination

is useful for the diagnosis of bacillary angiomatosis and peliosis hepatis, but is not suitable for other clinical manifestations of *Bartonella* infections (Maurin and Raoult, 1996). Finally, biochemical procedures, such as cell wall fatty acid analysis, failed to discriminate *Bartonella* spp. (Daly *et al*., 1993, Welch *et al*., 1992, Drancourt *et al*., 1995). Because of the implication of *Bartonella* in a variety of animal hosts, arthropod vectors, and human diseases, it would be useful to develop species- and strain-specific molecular tools, for dignostic and epidemiologic purposes.

In humans, clinical diagnosis of cat scratch disease is based on detection of an enlarged lymph node and possibly the presence of a small vesicle or granuloma at the inoculation site. However, clinical diagnosis of atypical forms of CSD and other emerging syndromes associated with *B. henselae* infection or other zoonotic *Bartonella* species is not easy and requires laboratory diagnostic means.

Diagnosis technique for bartonellosis

3.1 Microscopic and ultra examination

- 3.1.1 Blood smear
- 3.1.2 Histopathologic examination
- 3.1.3 Electron microscopy

3.2 Serologic technique

- 3.2.1 ELISA (enzyme-linked immunosorbent assay)
- 3.2.2 IFA (Immunofluorescence assays)

3.3 Bacterial isolation or PCR assay

- 3.3.1 Culture
- 3.3.2 Single PCR
- 3.3.3 Nested PCR
- 3.3.4 Real-time PCR

Table 8 Key issues for isolating main emerging bacteria

Group	Medium		Condition for incubation		
	Axenic Specific medium	**Living Sysytem (Embryonated egg cell line)**	**Low temperature (<37 ºC)**	**O2 and CO2 conditions**	**Extended incubation**
Alpha 1 Proteobacteria		*Ehrlichia sp.* *Rickattsia sp.* *Chlamydia sp.*	ELB agent ("*Rickettsia felis*") (28 ºC)		*Ehrlichia sp.*
Alpha 2 Proteobacteria	*Afpia sp.*	*Afipia sp* *Bartonella sp.*	*Bartonella bacilliformis* (28 ºC)		*Bartonella sp.*
Spirochetae	*Borrelia sp.*		*Treponema pallidum*		
Delta-Xi Proteobacteria				*Camphylobacter sp.* (microaerophilic) *Helicobacter sp.* (microaerophilic)	*Helicobacter pylori*
Gamma Proteobacteria	*Legiosella sp.*	*Lagionella sp.*	*Yersinia pestis*		
Mycobacteria	*Mycobacterium sp.*		*Mycobacterium leprae*	*Mycobacterium maimoense* (microaerophilic)	*Mycobacterium sp.*

Table 8 (Continued)

Group	Medium		Condition for incubation		
	Axenic Specific medium	**Living Sysytem (Embryonated egg cell line)**	**Low temperature (<37 ºC)**	**O2 and CO2 conditions**	**Extended incubation**
Mycoplasmas Gram-positive bacteria	*Mycoplasma sp.*	*Tropheryma whipplei*		*Clostridium dofficile* (anaerobic)	*Mycoplasma fermentans* *Tropheryma whipplei*

Source Houpikian and Raoult (2002)

Blood culture

In general, using combined different types of medium, both of solid and liquid media, increases the effectiveness of culture, possibly because of a preference of the bacterium for one type of medium over another or simply from the increased sensitivity obtained by culturing a large volume of specimen (Houpikian and Raoult, 2002). For *Bartonella* species, first isolation of *B. elizabethae, B. quintana*, and *B. henselae* was also achieved on blood agar (Slater *et al*., 1990). But the disadvantage of broad-spectrum media are clear for the fact that some emerging bacteria would not have been isolated without specific media specimen (Houpikian and Raoult, 2002). *Bartonella spp.* are slow, fastidious growth characteristics; therefore the diagnosis of *Bartonella*-associated illnesses is unpredictable.

Bartonella spp. grows on the surface of host erythrocytes but can be grown axenically on blood agar. The blood of infected cat is sometimes to culture difficult because this pathogen is fastidious. The bacteria are suggested to culture on

fresh rabbit blood agar and incubated at least 4 weeks at 35 ºC with 5% CO_2 environment.The molecular technique is also used to identify of *Bartonella* species.

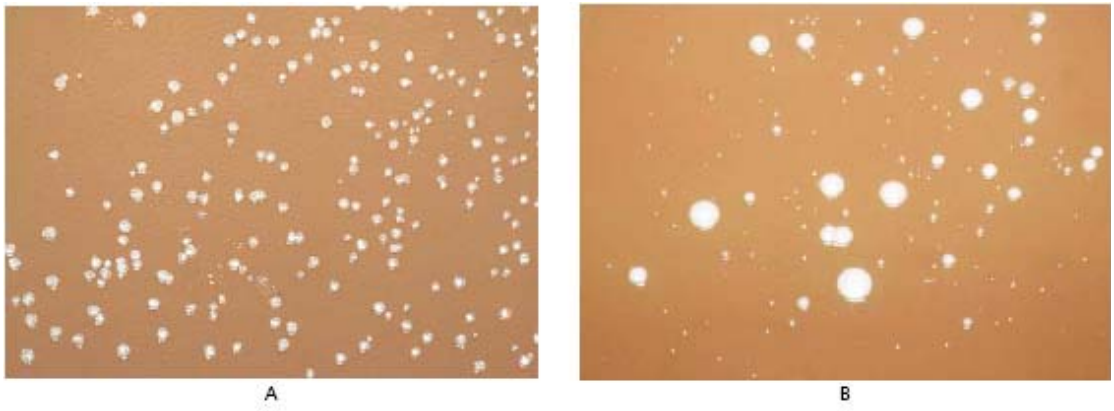


Figure 5 Primary isolation of *B. henselae* and *B. quintana* from biopsy tissue of cutaneous bacillary angiomatosis Lesions. Primary isolation of *B. henselae* from a cutaneous bacillary angiomatosis lesion reveals colonies that are uniform in size, elevated, rough, gray, and deeply embedded in the chocolate agar (Panel A). Primary isolation of *B. quintana* from a cutaneous bacillary angiomatosis lesion on chocolate agar reveals colonies that are flat, round, smooth, shiny, opaque, and of heterogeneous size and that never appear rough or cause pitting of the agar (Panel B).

Source Koehler *et al* (1997)

Cultures of clinical material obtained from patients are usually negative for bacteria due to the fastidious nature of *Bartonella* species, especially when samples have been obtained from patients already treated with antimicrobial agents (La Scola and Raoult, 1999). In negative culture cases, using classical isolation media, the presence of epidemiological factors such as scratches or bites, owning a cat, possible contact with rodents, or fleas, ticks or other blood sucking arthropods may lead to *Bartonella* specific testing (serology, culture or PCR).

In dogs and cats, clinical diagnosis is not usually easy, as the clinical spectrum of *Bartonella* infection is not fully elucidated. *Bartonella* infection should be suspected in dogs with endocarditis, especially if affecting the aortic valve (MacDonald *et al*., 2004). It also should be suspected in dogs with prolonged or intermittent fever, lethargy, unexplained lameness, or unexplained granulomatous disease. Similarly, veterinarians should consider performing a diagnostic test for *Bartonella* infection in sick dogs, when there is clinical or epidemiological suspicion of vector exposure. Thrombocytopenia, anemia, neutrophilic leukocytosis, and eosinophilia are the most commonly detected in dogs seropositive for *B. vinsonii* subsp. *berkhoffii* (Breitschwerdt *et al*., 2004). In all cases, suspicion of *Bartonella* infection is mainly established through serological tests, which provides evidence of *Bartonella* exposure (Jackson *et al*., 1993; Jacomo *et al*., 2002).

Blood smear

In the past, morphologic methods are used to detect new microorganisms and for diagnosis of infections caused by pathogens that are not routinely cultured. The advantage of microscopic examination is rapid and easy which can used in patients who have unknown diseases. However, the disadvantage of this method is its low sensitivity and specificity. The detection in blood smear is useful to differentiate multiple organisms from non-sterile site's culture.

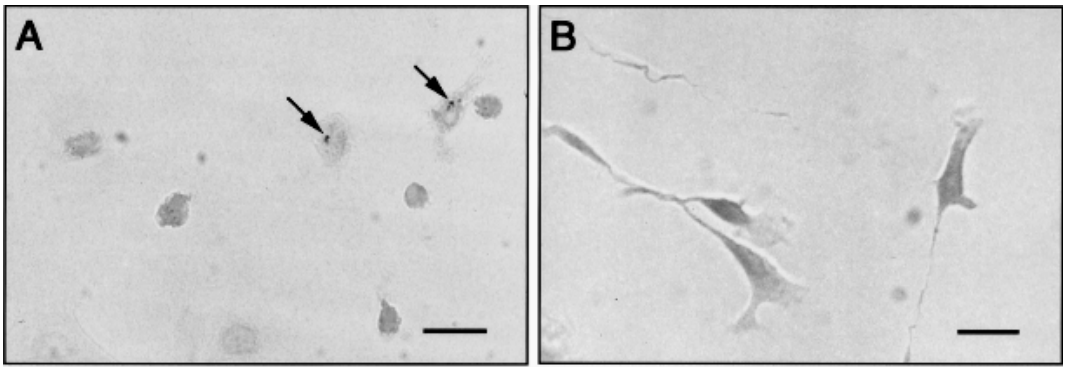


Figure 6 (A) Microglial-cell-enriched cultures, 14 days after inoculation with *B. henselae*, display evidence of infection based on positive Gime´nez staining for bacteria. Bacterial aggregates are identified in the perinuclear region of the cells (arrows). (B) Astrocyte-enriched cultures, 14 days after inoculation with *B. henselae*, reveal no evidence of cellular infection when stained by the Gime´nez method. (Bars = 25 mm)

Source Muňana *et al* (2000)

Histopathologic examination

Normally, all bacteria are not detected in hematoxylin and eosin (H&E) stained tissue sections. However, in bacillary angiomatosis sections which identified as *Bartonella* spp., H&E can demonstrate clumps of finely particulate basophil material.

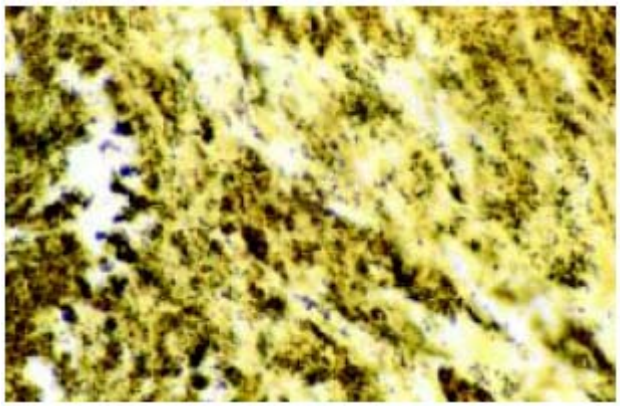


Figure 7 Demonstration of *Bartonella henselae* in cardiac valve of a patient with blood culture-negative endocarditis. The bacilli appear as black granulations (Warthin Starry, original magnification X250).

Source Houpikian and Raoult (2002)

Gram stain has also been proven useful to routinely diagnose *H. pylori* and *H. heilmanii* in the gastric mucosa of patients with gastritis, as well as that of *B. henselae* in cardiac valves (Marshall, 1983, Raoult *et al*., 1996). Silver impregnation is among the most useful methods for detecting bacteria, especially for that stained weakly with a tissue Gram stain. Thus, bacillary angiomatosis lesions were found to contain clusters of bacilli on Warthin-Starry staining 2 years before the etiologic role of *B. henselae* was elucidated. With the same stain, this bacterium was also detected in cardiac valves of patients with endocarditis (Maurin and Raoult, 1996).

Electron microscopy

Among morphologic technique, transmission and scanning electron microscopy (EM) has substantial advantages resulting from its high flexibility and sensitivity (Curry, 2000). Negative staining is a rapid EM method that can be useful in patients with persisting or unexplained disease. Moreover, its specificity and sensitivity can be enhanced by using immunocapture assay. EM can resolve details many hundreds of time smaller than can be seen through light microscopes, and

resolution of major taxonomic features can help to characterize new microorganisms (Curry, 2000). Nevertheless, limitations of EM such as its availability, cost, and need for experienced staff. In addition, EM requires the basic of histology and ultrastructure of the examined tissue examined and organisms and is time-consuming, since each specimen must be examined individually (Curry, 2000).

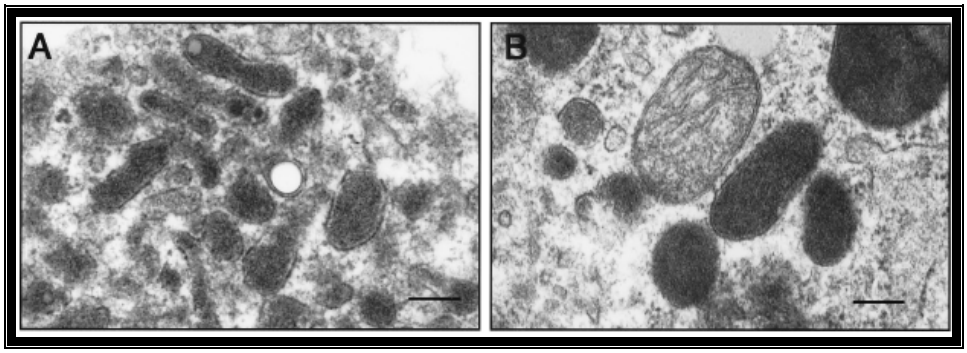


Figure 8 Electron photomicrographs of bacterial organisms within microglial cells 14 days after inoculation. (A) Intracellular aggregate of bacteria with morphologic characteristics similar to those previously reported for *Bartonella* species; (B) a group of bacteria adjacent to a mitochondrion. Bars = 0.09 mm.

Source Muňana *et al* (2000)

Serological test

By showing rising antibody titers or seroconversion, serology can provide indirect evidences for causal relationships between a disease and a newly identified bacterium. Conversely, in the absence of serologic evidence, the role of a cultured organism should be interpreted cautiously, as shown by the example of *Afipia felis,* which was first thought to be the cause of cat-scratch disease, but was finally identified as a water contaminant (La Scola and Raoult, 1999). Serology is also useful to assess the involvement in human diseases of microorganisms that had been initially recovered from the environment, such as novel *Legionella* species, or from

animal hosts, as for the tick-associated bacteria *Borellia burgdorferi* or *Rickettsia slovaca* (Raoult *et al*., 1997). Further, serology is a valuable tool for exploring the bacterial diseases spectrum of a bacterium. Thus, serologic tests contribute to the recognition of *B. henselae* as the main agent of cat-scratch disease (Regnery *et al*., 1992)

Immunofluorescence assays (IFA)

The most widely used serodiagnostic tool for *Bartonella* infections in cats are immunofluorescence assays (IFA). Although, specific and sensitive has a number of drawbacks. This assay lends itself poorly to large numbers of samples and is time-consuming and costly. Furthermore, quantitation of IFA requires that titrations be performed, which increases the cost of the test.

Zangwill *et al*., (1993) estimated the sensitivity and specificity of a *B. henselae*-based IFA to be 84% and 96%, respectively. A study comparing two commercially available IFA tests reported that the tests for IgG antibodies to *B. henselae* had higher sensitivities (100% and 85%, respectively) than specificities (70% and 73%, respectively), although this may have been the result of previous exposure to *B. henselae* among the healthy controls designated as the noninfected group (Sander *et al*., 1998). Furthermore, a limiting diagnostic factor in humans is the lack of commercial tests for most rodent-borne zoonotic *Bartonella* species. In cats, serologic testing is also of limited diagnostic value, as many cats (especially stray cats) are likely to be seropositive against *B. henselae* (Chomel *et al*., 1995). Testing is indicated that seronegative cats are more likely not to be bacteremic. Similarly, immunocompromissing persons should require the IFA test to detect antibodies against *B. henselae* before adoption. However, bacteremia in seronegative cats has been reported in a few cases and the antibodies usually cross-react with several *Bartonella* antigens (Chomel *et al*., 2004). Because of these limitations for serologic testing, bacterial isolation or PCR assay are necessary to identify the infecting *Bartonella* spp. In dogs, as for humans, diagnosis of *Bartonella* infection is largely based on the presence of specific antibodies. Testing for various antigens seems to be

appropriate, including *B. vinsonii berkhoffii* and *B. henselae* (Henn *et al*., 2005; Salano-Gallego *et al*., 2004). No formal test evaluation studies have been undertaken to estimate the sensitivities and specificities of the serological tests commonly used for the diagnosis of *Bartonella* infection in cats and dogs. One study investigating the seroprevalence of *B. henselae* and *B. quintana* among pet cats in Jordan did providing the estimation of sensitivity and specificity for the IFA used (Al-Majali, 2004). That author reported that the sensitivities of the *B. henselae* IgG and *B. quintana* IgG IFA tests were 99% and 88%, respectively, while the specificities were 94% and 90%, respectively. These results will have to be validated, however, as it is unclear how infection status of cats was determined and whether they were representative of a population of naturally infected cats.

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) is a useful and powerful method in estimating ng/ml to pg/ml of proteins or materials in the solution, such as serum, urine and culture supernatant. More recently ELISAs have become commercialized available for diagnosis of *Bartonella* infections in humans (Litwin *et al*., 1997) and in cats (Guptill *et al*., 1997). While the ELISA is similar to the IFA in regard of sensitivity (86.2 versus 88%) and specificity (95.9 versus 94%) (Guptill *et al*., 1997; Regnery *et al*., 1996), use of an ELISA has some advantages. For instance, this assay is useful to scream a large numbers of samples and the tests are relatively inexpensive.

Bacterial isolation or PCR assay

Isolation of *Bartonella* spp. from cats or from humans with bacillary angiomatosis is much easier than isolation of those organisms from other animal species or non-immunocompromised individuals. A positive blood culture or culture of other tissue is the most reliable test for definitive diagnosis of active *Bartonella* infection (Guptill, 2003). However, blood cultures may be necessary because of the

relapsing nature of feline *Bartonella* bacteremia (Kordick *et al*., 1999). In humans with cat scratch disease or dogs with *Bartonella* infection, isolation of these bacteria is rarely successful. Isolation of *Bartonella* from blood samples is performed usually by using EDTA tubes. The use of EDTA tubes has the advantage to prevent breakage of the blood tube when subjected to low temperature freezing and avoid the risk of sample contamination during transfer from prior to freezing. Anticoagulated blood is plated onto fresh rabbit blood agar and incubated for at least four weeks at 35 °C with 5% CO_2. Identification of the isolate is performed using molecular technique, such as PCR or partial sequencing of selected genes. Compared with the culture, extraction of DNA from tissue samples and PCR have been more successful as a method of diagnosis of *Bartonella* infections in humans and dogs (Jacomo *et al*., 2002; Koehler and Tappero, 1993; MacDonald *et al*., 2004; Regnery *et al*., 1992). Frozen tissue samples or fresh biopsy specimens can be also tested. PCR of paraffin-embedded tissues is possible with some cumbersome.

PCR amplification and sequence analysis of various genes are now widely used to differentiate *Bartonella* species. The 16S/23S rRNA intergenic spacer region (Houpikian and Raoult, 2001), the heat shock protein (*gro*EL) gene (Marston *et al*., 1999), the citrate synthase gene (*glt*A) (Birtles and Raoult, 1996), the riboflavin synthase achain gene (*rib*C) (Bereswill *et al*., 1999), the cell division protein (*fts*Z) (Ehrenborg *et al*., 2000), and the *pap*31 (Zeaiter *et al*., 2002) gene sequences were used for detecting, identifying, and classifying the phylogenetic properties and subtyping of *Bartonella* isolates.

Single PCR

The single-step PCR assay provides a simple and rapid means of identifying pathogenic *Bartonella* species in humans and companion animals (Jensen *et al*., 2000). This method can be used to directly screen samples from humans or animals, e.g., blood or tissue (Jensen *et al*., 2000). Disadvantage of this method are limitation of ability because it can not differentiate subspecies within different *Bartonella* species (Jensen *et al*., 2000)

Nested PCR

Nested PCR, two pairs of PCR primers were used for a single locus. The first pair amplified the locus as seen in any PCR experiment and the second pair of primers (nested primers) bind to the first PCR product and produce a second PCR product that will be shorter than the first one. The logic behind this strategy is that if the wrong locus were amplified by mistake, the probability is very low that it would also be amplified a second time by a second pair of primers. A nested-PCR offers increased sensitivity over a primary PCR and should be evaluated with currently used methods for the routine detection and speciation of *B. henselae* and *B. clarridgeiae* (Rampersad *et al*., 2005).

Real-time PCR

The real-time PCR assay is a good candidate for a clinical diagnosis. Actually, it is an automated technique that presents many advantages, such as high sensitivity and specificity, less possibilities of contamination, and it allows the quantification of genome copy numbers. This method was described for patients with suspected *Bartonella* endocarditis, and the method was easily and directly applied to the serum sample (Zeaiter *et al*., 2003). Real-time PCR assay is an attractive alternative to block cycler PCR assays. It could be a useful laboratory support that may potentially be standardized as a one-step method for the identification and discrimination of *Bartonella spp.* in clinical samples from patients with clinical evidence of CSD.

The other detection methods such as PCR amplification of the 16S-23S rRNA intergenic region with species-specific primers; restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S genes; RFLP analysis of the PCR-amplified citrate-synthase gene (Jensen *et al*., 2000). These methods are tedious because they require multiple PCR amplification reactions and/or additional sample-processing steps beyond the primary PCR amplification (Kosoy *et al*., 1999).

Other *Bartonella* detection methods that do not rely on multi-step PCR amplifications have been developed such as enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), repetitive extragenic palindromic PCR (REP-PCR), and arbitrarily primed PCR (AP-PCR) (Jensen *et al*., 2000). Although these assays have been successfully implemented in the detection of *Bartonella spp*., they are very sensitive to experimental variation and make reproducibility and standardization difficult. A new typing method known as infrequent-restriction site PCR (IRS-PCR) has been proposed that may become a universal tool for molecular analysis of pathogen species (Yoo *et al*., 1999). The main strategy of IRS-PCR is the selective amplification of the DNA sequence located between both frequently and infrequently occurring restriction sites by using adaptors and primers based on these two enzymes (Sambrook *et al*., 2001). The discriminatory power of IRS-PCR has been shown to be equal to that of pulsed-field gel electrophoresis (PFGE), the method currently used to distinguish *Bartonella spp.* (Yoo *et al*., 1999) recently applied IRS-PCR to clinical isolates of *Actinobacter baumannii* and *Serratia marcescens* and found that IRS-PCR and PFGE are equally discriminatory; however, IRS-PCR is less tedious and less laborious (Yoo *et al*., 1999).

The advantages of broad-range PCR, however, are offset by the problem of microbial DNA contamination. Even after rigorous technical precautions are taken to minimize contamination of PCR reaction, false-positive reactions can occur. Another noticeable limitation of broad-range PCR is the examination of sites that are not normally sterile, such as feces or sputum; use of family- restricted primers, in situ hybridization with specific nucleic probes, or expression library screening with immune sera may help to overcome such limitations (Fredricks and Relman, 1996). Another potential problem is interpretation of the microheterogeneity found in microbial sequences derived directly from host tissues, especially when these sequences become the sole basis for defining the existence of an organism.

MATERIAL AND METHODS

Study areas

The study areas were assigned in 50 districts of Bangkok metropolitan areas between March to May 2004 (Fig. 9). The sample size of stray cats was randomly selected by the simple randomization assay. Of the 439 monasteries located in 50 district of Bangkok metropolitan, 140 monasteries were randomly chosen for blood collection. Three monasteries were randomly chosen from each district. Blood samples were collected 10 samples from each monastery. However, some districts have only 1 or 2 monasteries, therefore 30 and 15 cats samples were collected in each monastery, respectively (Table 9). A total of 1,488 samples were performed and proceed for diagnosis in this study

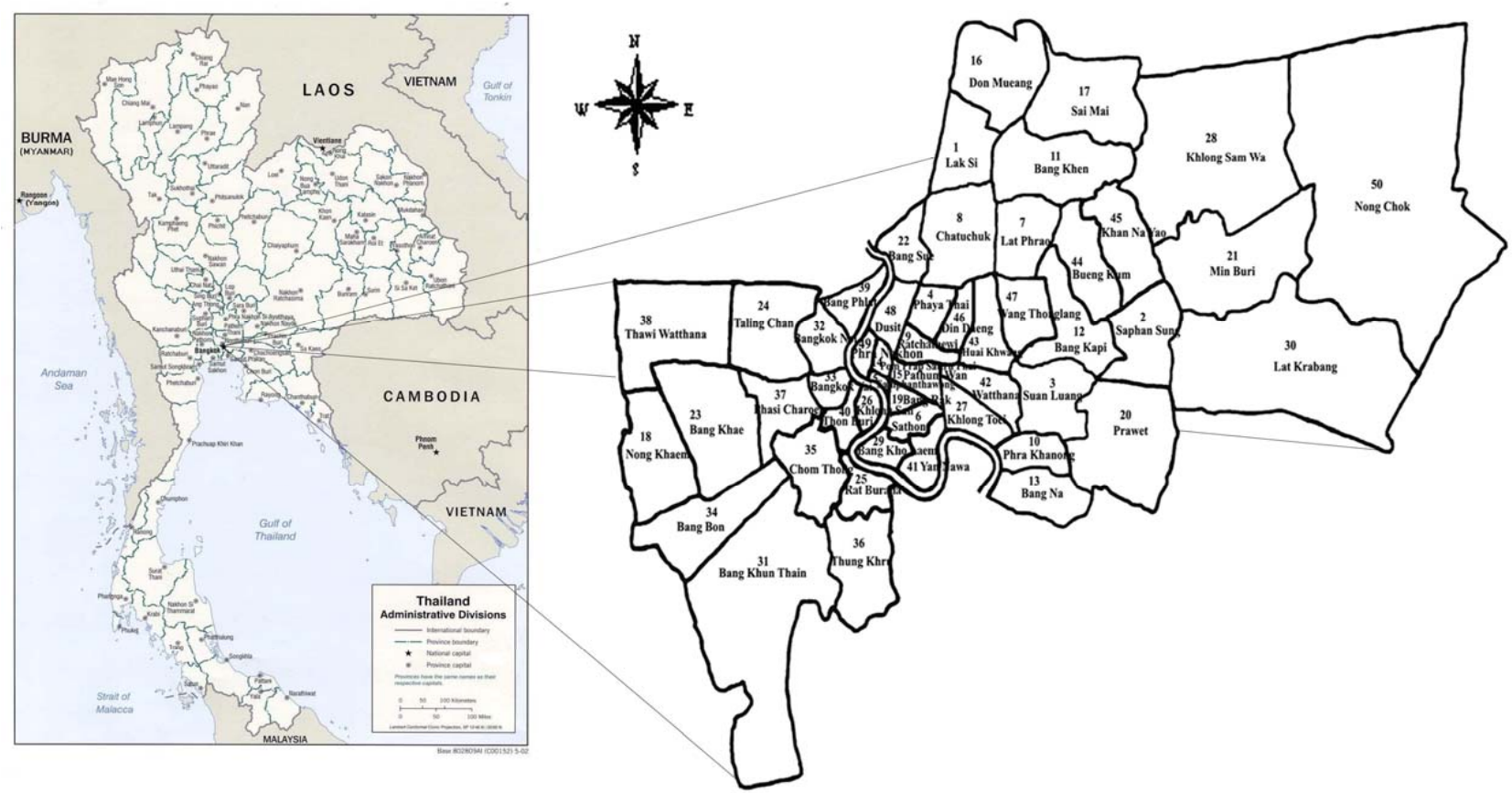


Figure 9 Bangkok metropolitan areas, the study areas of blood samples collection

Samples collection

A total of 1,488 cat blood was collected from stray cats in Bangkok metropolitan. In each, 3-5 ml. of blood were collected from jugular vein (Fig. 10) preserved in sodium citrate vacuum tubes and stored at – 20 ˚C until used for DNA extraction and PCR analysis. Blood smear of blood samples preserved with EDTA were examined for *Bartonella* infection with microscopic. The stray cats were thoroughly examined and record for age, gender, animal condition and environmental details and their ectoparasites. A questionnaire was designed to record these data. Factors associated with *Bartonella* infection are classified as following:

I. Sex status

1 Male

2 Female

II. Age status

1 Young: the ages of cat were three months to two years (≤2)

2 Adult: the ages of cat were between more than two and four years (>2-4)

3 Older: the ages of cat were more than four years (>4).

III. External parasite status

1 Unexposed: no external parasite was found on the cat.

2 Exposed: external parasite was found on the cat such as flea, tick and lice

IV. Health condition

1 Healthy: good body score, no dehydration, no clinical signs, normal mucous membrane

2 Fair: less body score (presenting of crest of Ilium), no dehydration, no clinical signs

3 Poor: weak, dehydration, purulent of ocular or nasal discharge, some clinical signs found (coughing, diarrhea, vomit)

V. Environmental condition

1 Fair: the place was clean, may be slightly filth, but no leftover of the animal feeding and habitation

2 Poor: the place was dirty, poor administration of animal habitation

VI. Density condition

1 Less: the total of stray cats that living in a monastery not more than 20 cat per place

2 Most: the total of stray cats living in a monastery more than 20 cats per place.

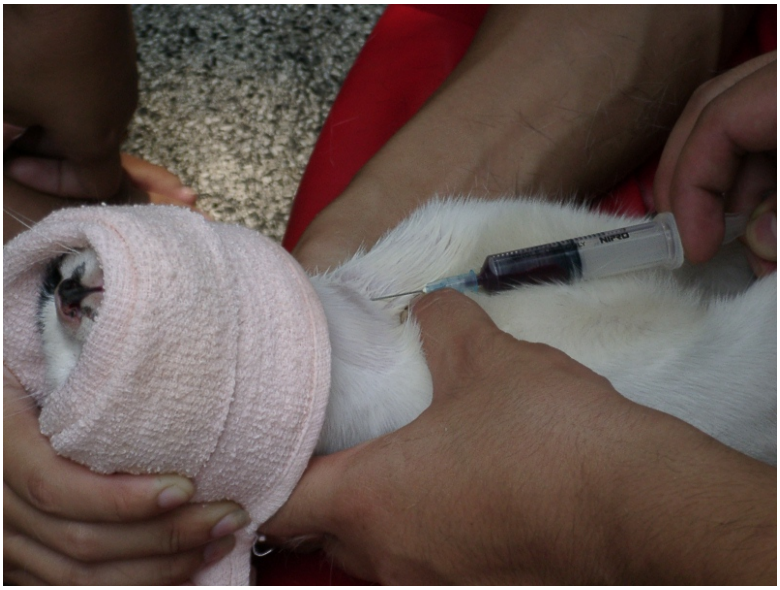


Figure 10 Technique of blood collection from the jugular vein of stray cats.

Diagnosis technique

1. Microscopic examination

A thin blood smear was prepared in the field by fixing with methanol for 2 minutes, staining with Modified Giemsa solution for 5 minutes, washing for 10 minutes in distilled water and examined under light microscope at 1000x power in laboratory room.

2. Molecular detection

2.1 DNA extraction

DNA for PCR amplification was prepared from 100 µl of blood sample by using the phenol – chloroform technique (Sambrook and Russell, 2001). The extracted DNA was stored at – 20 ˚C for using as the PCR template.

2.2 PCR detection (Polymerase Chain Reaction)

The nested-PCR following Rampersad *et al* (2005) for detection of *Bartonella* species in cat blood. Primary PCR primers were P-bhenfa (5'-TCTTCGTTTCTCTTTCTTCA) and P-benr1 (5'-CAAGCGCGCGCTCTAACC). The secondary primers were N-bhenf1a (5'-GATGATCCCAAGCCTTCTGGC) and N-bhenr (5'-AACCAACTGAGCTACAAGCC). All of PCR mixture were performed in 20 µl contained with 0.2 mM each dNTP, 0.5 pmoles/µl each P-bhenfa and Pbenr1, 3 mM $MgCl_2$ reaction buffer, 0.4 pmoles/µl primer, 0.5 units *Taq* polymerase (Invitrogen) and DNA template 2 µl in primary reaction. The nested reaction comprised 0.2 mM each dNTP, 0.5 pmoles/µl each N-bhenf1a and N-bhenr, 1.5 mM MgCl2 reaction buffer, 0.5 units *Taq* polymerase and DNA template 2 µl. The PCR amplifications were performed in a MyCycle™ Thermal Cycler (BioRad Laboratories, USA). Optimized PCR cycle conditions using 94 ˚C 15 s, 48.2 ˚C 30 s and 72˚C 30 s for 35 cycles of the primary-PCR and 94 ˚C 15 s, 56 ˚C 30 s and 72 ˚C 30 s for 35 cycles for the nested-PCR.

2.3 Gel Electrophoresis

After the nested PCR process, the PCR amplification products were analyzed on agarose gel. PCR products were identified by 2% agarose gels by using 1X Tris – borate – EDTA (TBE). The electrophoresis condition was 100 volts for 45 minutes in electrophoresis chamber (MT-108 Pacific Science, Thailand) with 1xTBE buffer. After that, the gel was stained with ethidium bromide buffer for 5 minutes and

then, destained for removed the excess ethidium bromide with distilled water for 15 minutes. The PCR amplification product was visualized under ultra-violet transluminator. The positive with *Bartonella* samples demonstrated a 152 bp fragment for *B. henselae* and a 134 bp fragment for *B. clarridgeiae*.

2.4 Sequencing of PCR amplification products

The positive DNA fragment was extracted and purified from agarose gel by QIAquick® Gel Extraction Kit (QIAGEN, Germany) following manufacturer's instructions for eliminated of excess primers, nucleotides, polymerase and salts. The purified DNA products were sequenced for submitted at Ward Medic, Thailand. The sequences were compared in GenBank® database by used BLASTN software.

Statistical analysis

Our studies used chi – square and Number Cruncher Statistical System (NCSS) ver. 2000 (Kayville, UT) to assess difference. The individual data of stray cats (age, sex, health condition, environmental condition and density condition) were analyzed by statistical analysis. The significant determined by the probability (*p*-value) that an individual cats were infected. If *p* is less than 0.05, it indicates the significant differences.

Table 9 The number of monasteries and cats

Districts	Total number of monasteries [a]	Selected monasteries (% representative)	Cats per monasteries (Average)
Bangkok Noi	32	3(9.4%)	10
Taling Chan	30	3(10%)	10
Phasi Charoen	27	3(11.1%)	10
Thon Buri	25	3(12%)	10
Bang Phlat	23	3(13%)	10
Phra Nakhon	22	3(13.6%)	10
Chom Thong	17	3(17.6%)	10
Dusit	17	3(17.6%)	10
Nong Chok	16	4(25%)	8
Bang Khun Thain	15	4(26.7%)	8
Bangkok Yai	13	3(23%)	10
Lat Krabang	13	3(23%)	9
Samphanthawong	13	3(23%)	8
Pom Prap Sattru Phai	11	4(36.4%)	10
Bang Kho Laem	10	3(30%)	10
Khlong Sam Wa	10	3(30%)	10
Bang Sue	9	3(33.3%)	10
Khlong San	8	3(37.5%)	10
Sai Mai	8	3(37.5%)	10

Table 9 (Continued)

Districts	Total number of monasteries [a]	Selected monasteries (% representative)	Cats per monasteries (Average)
Yan Nawa	8	3(37.5%)	10
Don Mueang	7	3(42.9%)	10
Rat Burana	7	3(42.9%)	10
Pathum Wan	6	3(50%)	10
Prawet	6	3(50%)	10
Bang Khae	6	3(50%)	10
Min Buri	6	3(50%)	10
Nong Khaem	6	3(50%)	10
Bueng Kum	5	3(60%)	10
Bang Kapi	4	3(75%)	10
Bang Khen	4	3(75%)	10
Bang Na	4	3(75%)	10
Bang Rak	4	3(75%)	10
Khlong Toei	4	3(75%)	10
Lat Phrao	4	3(75%)	10
Phra Khanong	4	3(75%)	10
Ratchathewi	4	3(75%)	10
Sathon	4	4(100%)	8
Thung Khru	4	3(75%)	10

Table 9 (Continued)

Districts	Total number of monasteries [a]	Selected monasteries (% representative)	Cats per monasteries (Average)
Huai Khwang	3	3(100%)	10
Khan Na Yao	3	3(100%)	10
Bang Bon	2	2(100%)	15
Chatuchuk	2	2(100%)	15
Din Daeng	2	2(100%)	15
Suan Luang	2	1(50%)	30
Thawi Watthana	2	2(100%)	15
Wang Thonglang	2	2(100%)	11
Watthana	2	2(100%)	15
Lak Si	1	1(100%)	30
Phaya Thai	1	1(100%)	30
Saphan Sung	1	1(100%)	30
Total	439	140 (31.9%)	11.9

[a] total number of monasteries in each district was referred by the Office of National Buddhism.

RESULTS AND DISCUSSION

Results

1. Microscopic examination (ME)

All of 1,488 of thin blood smear were detected for *Bartonella* spp. by microscopic examination. However, 910 samples were interpreted for infection with ME method due to their debris and precipitation of color stained. *Bartonella* pathogen showed ring or dot shape in the red blood cell under microscpe (Fig. 13). The positive result of *Bartonella* infection by ME was 6.8% (62/910).

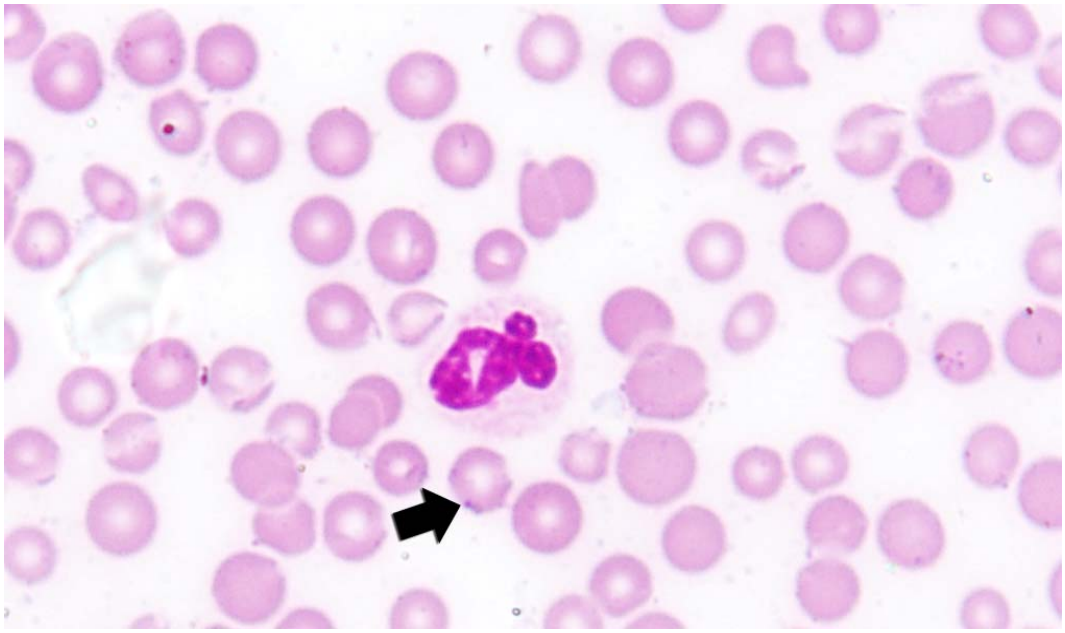


Figure 11 The *Bartonella* infections in feline blood smear (arrow head)

2. Molecular detection

The nested PCR assay was modified to detect and *B. henselae* and *B. clarridgeiae* in blood samples of stray cats. The PCR amplification product of *B. clarridgeiae* was 149 bp and *B. henselae* was 130 bp (Fig. 12). The nucleotide sequences of *Bartonella* spp. were submitted for sequence analysis by using BLASTN comparison algorithm in GenBank®. The results were 99% identical to 16S rRNA gene of *B. clarridgeiae* (DQ 683194.1) (Fig. 13) and 100% identical to 16S rRNA gene of *B. henselae* (DQ529247.1) (Fig. 14).

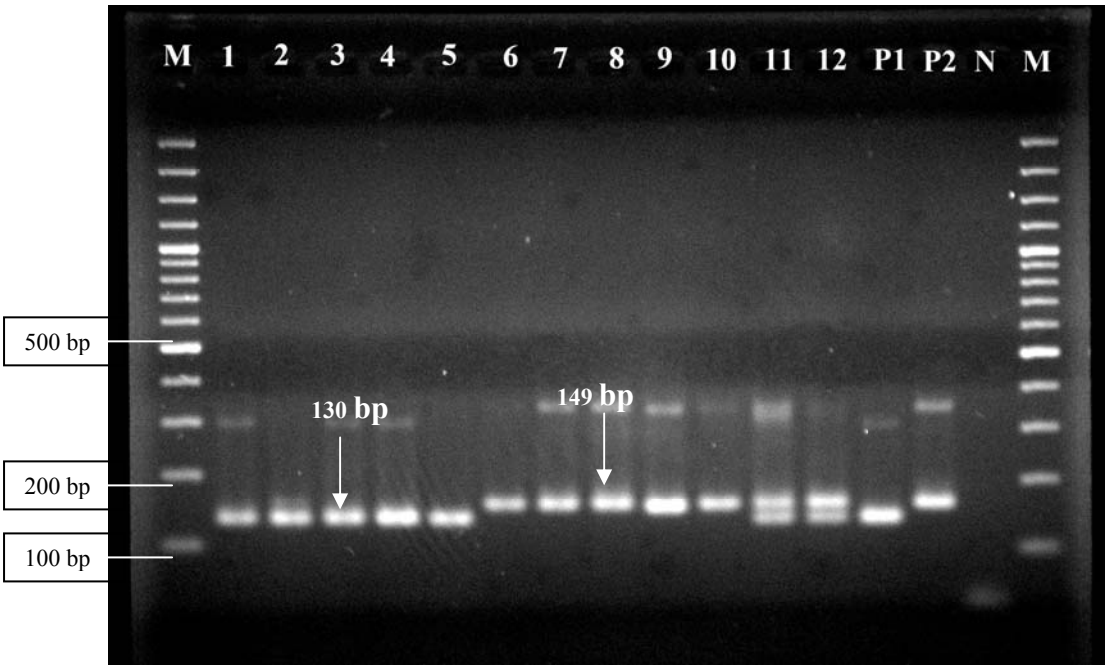
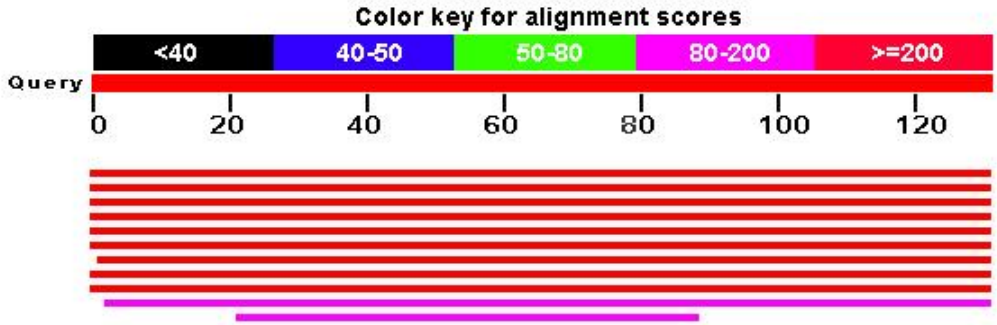


Figure 12 The PCR product of *Bartonella* infection in 2% agarose gel; lane M, GeneRuler 100 bp DNA ladder plus; Lane 1-5, positive of *B. clarridgeiae* (F83, F110, F296, F569 and F1,277); lane 6-10, positive of *B. henselae* (F2, F416, F895, F1, 347 and F1,486); lane11-12, positive of mixed infection of *Bartonella* spp. (F48 and F725); lane P1, positive control of *B. clarridgeiae* (130 bp); lane P2, positive control of *B. henselae* (149 bp); Lane N, negative control.



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ683194.1	Bartonella clarridgeiae 16S-23S ribosomal RNA intergenic spacer, p	235	235	100%	3e-59	99%
EU589237.1	Bartonella clarridgeiae strain M9HN-SHQ 16S ribosomal RNA gene, i	230	230	100%	2e-57	98%
AF312497.1	Bartonella clarridgeiae isolate Houston-2 16S ribosomal RNA gene,	230	230	100%	2e-57	98%
AF312502.1	Bartonella clarridgeiae isolate C 78 16S ribosomal RNA gene, partia	230	230	100%	2e-57	98%
AF312501.1	Bartonella clarridgeiae isolate C 49 16S ribosomal RNA gene, partia	230	230	100%	2e-57	98%
AF167989.1	Bartonella clarridgeiae 16S ribosomal RNA gene, partial sequence;	230	230	100%	2e-57	98%
AF312498.1	Bartonella clarridgeiae isolate C 23 16S ribosomal RNA gene, partia	228	228	99%	6e-57	98%
AF312499.1	Bartonella clarridgeiae isolate C 44 16S ribosomal RNA gene, partia	226	226	100%	2e-56	97%
AF312500.1	Bartonella clarridgeiae isolate C 48 16S ribosomal RNA gene, partia	220	220	100%	9e-55	96%
AF415211.1	Uncultured Bartonella sp. clone BFI7688 16S-23S ribosomal RNA int	137	137	98%	1e-29	86%
DQ003029.1	Bartonella clarridgeiae 16S-23S ribosomal RNA intergenic spacer, p	115	115	51%	5e-23	97%

Figure 13 A BLAST search across multiple DNA databases by using BLASTN software showed that the 130 bp of 16S rRNA gene of *B. clarridgeiae* was homologous to gene of *B. clarridgeiae* at 99% identity (DQ683194.1)**.**

```
CLUSTAL 2.0.11 multiple sequence alignment

BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      ACAAGGTAGCCGTAGGGGAACCTGTGGCTGGATCACCTCCTTTCTAAGGATGATCAAGAA 60


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      TGGGCCTAGGCCTTTTTTGATCTGATTAGACATTGACGGTTTAAAGTCTTATTTAAACCG 120


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      TTGACATATTTTAAACATTCTATGAACCGTGGGTTTTGAATGGAAACTCTGTCCCCTTTA 180


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      GTGATACAGAGCATAACTGTTTTTTATCCATGGTTCATTTGTTTAAAAATTTATAAAAAG 240


BC_Samples      ------------------------------GATGATCCCAAGCCTTCTGGCGATCTGTT 29
DQ683194.1      ----------------------TTTCCAGATGATGATCCCAAGCCTTCTGGCGATCTGTT 38
EU589237.1      ACTAGCCGCCTTCATTTCTCTTTCTTCAGATGATGATCCTAAGCCTTCTGGCGATCTGTT 300
                                       ******** ********************


BC_Samples      TG-ACAAGCCTCTGAGAGGGATGAAGATATTGTTTTCTTTGATCAGATTATGCCGGTAAA 88
DQ683194.1      TGCACAAGCCTCTGAGAGGGATGAAGATATTGTTTTCTTTGATCAGATTATGCCGGTAAA 98
EU589237.1      TGCACAAGCCTCTGAGAGGGATGAAGATATTGTTTTCTTTGATCAGATTATGCCGGTAAA 360
                ** ************************************************************


BC_Samples      GGTTTTCTGGTTTACCCTATAGGGCTTGTAGCTCAGTTGGTT------------------ 130
DQ683194.1      GGTTTTCTGGTTTACCCTATAGGGCTTGTAGCTCAGTTGGTTAGAGCGCGCGCTTGATAA 158
EU589237.1      GGTTTTCTGGTTTACCCTATAGGGCTTGTAGCTCAGTTGGTTAGAGCGCGCGCTTGATAA 420
                ****************************************
```

Figure 14 Multiple sequence alignment of 16S rRNA gene of *B. clarridgeiae* isolated in Thailand (BC Samples) that shown 99% homology with *B. clarridgeiae* isolated in USA (DQ683194.1) and 98% homology with the strain in China (EU589237.1). The alignment was generated by CLUSTAL W (2.0.11) software. A dash (-) indicates a missing residue, and a star (*) indicates a residue conserved in all aligned sequences.

Figure 14 (Continued)

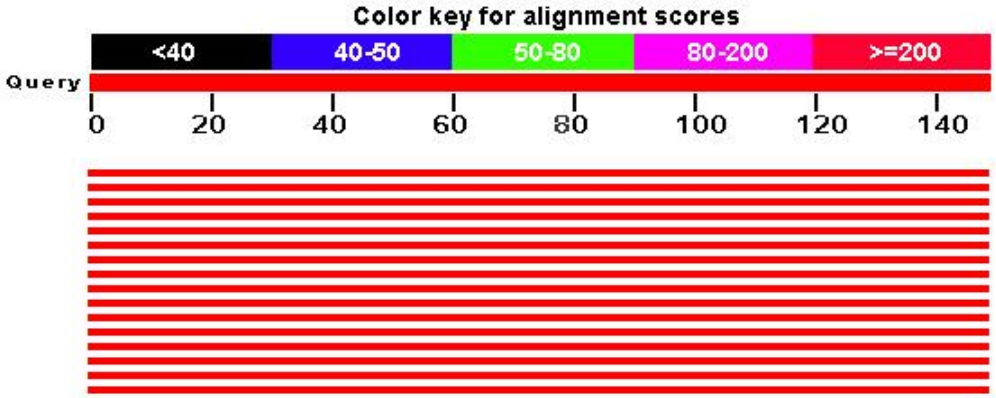
```
BC_Samples      ------------------------------------------------------------
DQ683194.1      GCGTGAGGTCGGAGGTTCAAGTCCTCCCAGGCCCACCAGTTACACGATGCTAAAAGTTGC 218
EU589237.1      GCGTGAGGTCGGAGGTTCAAGTCCTCCCAGGCCCACCAGTTACACGATGCTAAAAGTTGC 480


BC_Samples      ------------------------------------------------------------
DQ683194.1      TATATTGGGAGAGTTGATAATCCCTTACAGGAAATTATTGCCCTTAATAAAACTTTATTT 278
EU589237.1      TATATTGGGAGAGTTGATAATCCCTTACAGGAAATTATTGCCCTTAATAAAACTTTATTT 540


BC_Samples      ------------------------------------------------------------
DQ683194.1      TCTAAAAGCATTCAGAGCTGACATAGAATAGAGCTGACATAGAATTGAGAATCTGACATA 338
EU589237.1      TCTAAAAGCATTCAGAGCTGACATAGAATAGAGCTGACATAGAATTGAGAATCTGACATA 600


BC_Samples      ------------------------------------------------------------
DQ683194.1      GGAATTATTGAAATTGTTTTGGAATTATTGAAATTGTTTTCTATCATTTTAAAAGGCTAA 398
EU589237.1      GGAATTATTGAAATTGTTTTGGAATTATTGAAATTGTTTTCTATCATTTTAAAAGGCTAA 660


BC_Samples      ------------------------------------------------------------
DQ683194.1      AATATTCTGTCTCTATTTTTAAAATAGCATCAGGTGTTTTGTAAGAGTGTGAAGTTTTTA 458
EU589237.1      AATATTCTGTCTCTATTTTTAAAATAGCATCAGGTGTTTTGTAAGAGTGTGAAGTTTTTA 720


BC_Samples      ------------------------------------------------------------
DQ683194.1      AGTGTGAGGTTTTTTATATTTTAGTGTGAGGTTTTTATAAGGGTATGACGTGAGAGCGTT 518
EU589237.1      AGTGTGAGGTTTTTTATATTTTAGTGTGAGGTTTTTATAAGGGTATGACGTGAGAGCGTT 780


BC_Samples      ------------------------------------------------------------
DQ683194.1      TTGACCTGTTTTAGGGGCCGTAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGGGGGT 578
EU589237.1      TTGACCTGTTTTAGGGGCCGTAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGGGGGT 840


BC_Samples      ------------------------------------------------------------
DQ683194.1      CGTCGGTTCGATCCCGTCCGGCTCCACCATAATTTGGTTCATCATTATTGTTAGAAGAAT 638
EU589237.1      CGTCGGTTCGATCCCGTCCGGCTCCACCATAATTTGGTTCATCATTATTGTTAGAAGAAT 900


BC_Samples      ------------------------------------------------------------
DQ683194.1      AGTTATTGCAAGAGATTGAGAGATCTCTTTGCTTGTTCTATTGAAATTGTGAAGAAGAAG 698
EU589237.1      AGTTATTGCAAGAGATTGAGAGATCTCTTTGCTTGTTCTATTGAAATTGTGAAGAAGAAG 960
```

Figure 14 (Continued)

```
BC_Samples      ------------------------------------------------------------
DQ683194.1      ATATATTTCAGACATGTTA---------------------------------------- 717
EU589237.1      GTATATT-CAGACGTTTTTTGCTTGAACTCATTCTTATGAAAGAGATTTTTCTTATGAAA 1019


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      GAGATTTTTAAGAATGGATAGCTTAAAAAGAAGAATGGATGGCTTAAAAAGGTGGCTTAA 1079


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      AAAGATGGCTGTTTTTAAATGAAAATAGTTATTTTTACGCTCTTTTGACGATTGTTACAA 1139


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      CATTATACGATTAAAACATTATACGATAATGATAATAACGATAATAAAAAGAGCTTTCAT 1199


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      TAATAAAAAGAGCTTTCATTAATAATAAAGAGCTTTCATTAATAATAAAGAGCTTTCATT 1259


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      AATAATAAAGAGCTTTCATTGAACTTTCATTGAAGAAGCATTTTGAGCAAAACAGATGTG 1319


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      TCGCAAGGAAGAGCTCAAATTCCTTGCTTATGATTGGCAACTTAACCGTGCCATTGAATA 1379


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      TATCTCGAGAAGTTGGTCTTTTCTGCTGATATTTTTGTTTTAAGTGCCTATTGATGCTAG 1439


BC_Samples      ------------------------------------------------------------
DQ683194.1      ------------------------------------------------------------
EU589237.1      ATTATTTTTAAAAATAATTTTGTATTGATGATTTTGCACGGAATAATTGACGAATGAATA 1499
```

Figure 14 (Continued)

```
BC_Samples      ----------------
DQ683194.1      ----------------
EU589237.1      TGGCAATGAGAATGAT 1515
```



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ832091.1	Bartonella henselae isolate CE5 16S-23S ribosomal RNA intergenic	276	276	100%	2e-71	100%
FJ605499.1	Uncultured Bartonella sp. clone pieterburen 16S-23S ribosomal RNA	276	276	100%	2e-71	100%
DQ529247.1	Bartonella henselae 16S-23S ribosomal RNA intergenic spacer, part	276	276	100%	2e-71	100%
AF312496.1	Bartonella henselae isolate URBHLIE 9 16S ribosomal RNA gene, pa	276	276	100%	2e-71	100%
AF312495.1	Bartonella henselae isolate URBHLLY 8 16S ribosomal RNA gene, pa	276	276	100%	2e-71	100%
AJ457178.1	Bartonella henselae tRNA-Ile gene, IGS and tRNA-Ala gene, isolate	276	276	100%	2e-71	100%
AJ457177.1	Bartonella henselae tRNA-Ile gene, IGS and tRNA-Ala gene, isolate	276	276	100%	2e-71	100%
AJ439688.1	Bartonella henselae partial 16S rRNA gene, 16S-23S IGS, partial 23	276	276	100%	2e-71	100%
AJ439687.1	Bartonella henselae partial 16S rRNA gene, 16S-23S IGS, partial 23	276	276	100%	2e-71	100%

Figure 15 A BLAST search across multiple DNA databases by using BLASTN software showed that the 149 bp of 16S-23S rRNA gene of *B. henselae* was homologous to gene of *B. henselae* at 100% identity (DQ529247.1).

```
CLUSTAL 2.0.11 multiple sequence alignment

BH_Samples      ----GATGATCCCAAGCCTTCTGGCGATCTAGACAAAACAAGTCCACCGTGGGCTTTGAA 56
DQ529247.1      AGATGATGATCCCAAGCCTTCTGGCGATCTAGACAAAACAAGTCCACCGTGGGCTTTGAA 60
FJ832091.1      AGATGATGATCCCAAGCCTTCTGGCGATCTAGACAAAACAAGTCCACCGTGGGCTTTGAA 60
                    ************************************************** 

BH_Samples      AAACGCTTTCCTTGATAAAATTTAAGCGTTTTATAAGAGGATGCCGGGGAAGGTTTTCCG 116
DQ529247.1      AAACGCTTTCCTTGATAAAATTTAAGCGTTTTATAAGAGGATGCCGGGGAAGGTTTTCCG 120
FJ832091.1      AAACGCTTTCCTTGATAAAATTTAAGCGTTTTATAAGAGGATGCCGGGGAAGGTTTTCCG 120
                ************************************************************ 

BH_Samples      GTTTATCCCGGAGGGCTTGTAGCTCAGTTGGTT-------------------------- 149
DQ529247.1      GTTTATCCCGGAGGGCTTGTAGCTCAGTTGGTTAGAGCGCGCGCTTGATAAGCGTGAGGT 180
FJ832091.1      GTTTATCCCGGAGGGCTTGTAGCTCAGTTGGTTAGAGCGCGCGC--------------- 164
                ******************************** 

BH_Samples      ------------------------------------------------------------ 
DQ529247.1      CGGAGGTTCAAGTCCTCCCAGGCCCACCAGTTTATCCATTACTTTCATAAGTGCTTTTAA 240
FJ832091.1      ------------------------------------------------------------ 


BH_Samples      ------------------------------------------------------------ 
DQ529247.1      AAAATAAGTACTTCTAAAAAGATTGCTTCTAAAAAGATTGCTTCTAAAAAGATTGCTTCT 300
FJ832091.1      ------------------------------------------------------------ 


BH_Samples      ------------------------------------------------------------ 
DQ529247.1      AAAAAGCTTATCAAAATTGGCAGGCTTATTGCTTTTGTGTGAGTAATCCAAAGTTAAAGC 360
FJ832091.1      ------------------------------------------------------------ 


BH_Samples      ------------------------------------------------------------ 
DQ529247.1      AAATTAATGGCAAAAAAACAGTTCAAATGCTAAATACTAAGGAGTCAAAATTCCTTGCAA 420
FJ832091.1      ------------------------------------------------------------ 
```

Figure 16 Multiple sequence alignment of 16S rRNA gene of *B. henselae* isolated in Thailand (BH Samples) that shown 100% homology with *B. henselae* isolated in Israel (FJ832091.1) and strain USA (DQ529247.1). The alignment was generated by CLUSTAL W (2.0.11) software. A dash (-) indicates a missing residue, and a star (*) indicates a residue conserved in all aligned sequences.

Figure 16 (Continued)

```
BH_Samples      ------------------------------------------------------------
DQ529247.1      AGTGATTTTTACAGCGTCCATTTGGTTGATATAAATTCCAAATGCTCATAGACGTCAATG 480
FJ832091.1      ------------------------------------------------------------

BH_Samples      ------------------------------------------------------------
DQ529247.1      CCTATATGAAACTATCGGTTCAATCATATCGCTTTGAGTTATATAGATTTTGTAATCCCT 540
FJ832091.1      ------------------------------------------------------------


BH_Samples      ------------------------------------------------------------
DQ529247.1      CTTTTGATCGTTTTAAACGCTTTATCCTGATTTAGGGGCCGTAGCTCAGCTGGGAGAGCA 600
FJ832091.1      ------------------------------------------------------------


BH_Samples      ------------------------------------------------------------
DQ529247.1      CCTGCTTTGCAAGCAGGGGGTCGTCGGTTCGATCCCGTCCGGCTCCACCATAAGGTCATC 660
FJ832091.1      ------------------------------------------------------------


BH_Samples      ------------------
DQ529247.1      ATCATTGTTGTAAGAACA 678
FJ832091.1      ------------------
```

Detection by PCR assay

The infective rate of *Bartonella* infection in stray cats resided in monasteries of 50 districts of Bangkok metropolitan areas was 53.96% (803/1,488) (Table 10). The infective rate of *B. henselae* (35%, 521/1,488) was higher than of *B. clarridgeiae* (15.26%, 227/1,488). Mixed infection between *B. henselae* and *B. clarridgeiae* was also found at 3.7% (55/1,488) (Table 10). At least one sample of each district was shown positives of *Bartonella* infection; therefore all districts (50/50) of Bangkok metropolitan areas was infected with these pathogens (Fig. 17 & 18). The distribution of *B. clarridgeiae*, *B. henselae* and mixed infection in Bangkok were demonstrated (Fig. 19 & 20, 21 & 22, and 23 & 24).

The highest number of infection of *Bartonella* spp. was Phasi Charoen district (90%, 27/30). Suan Luang and Phasi Charoen district had the highest number of *B. clarridgeiae* (36.67%, 11/30 and 11/30, respectively). Among districts, Ratchathewi had the highest infection of *B. henselae* (60%, 18/30). The least infection of *Bartonella* spp. was found at Khlong Sam Wa (3.33%). There were no *B. clarridgeiae* infection in 4 districts including Bang Kapi, Nong Khaem, Bangkok Noi and Wang Thonglang. Only one district, Khlong Sam Wa was not infected by *B. henselae*. The infective rate of mixed infection was found at 68% of all districts (34/50).

The risk factors of *Bartonella* infection comprising sex, age, external parasite infection, health condition, environmental condition and density condition were analyzed by statistical program. The association of risk factors and *Bartonella* infaction in monasteries in Bangkok metropolitan areas was showed in Table 11. As the result, *p*-value from the data was significant when *p*-value less than 0.05. Environmental condition was the only factor associated with *Bartonella* infection ($p < 0.05$). Poor environmental condition was related to the infection ($p = 0.014$, $\chi = 8.5358$, $df = 2$). The factors of sex, age, external parasite infection, health condition and density condition were not associated with *Bartonella* infection as shown in Table

12. The results of *Bartonella* infections which distributed among monasteries and districts of Bangkok areas were summaried in Table 13.

Table 10 The PCR result of *Bartonella* infection of stray cats in monasteries of the districts in Bangkok metropolitan areas.

No. of district	Districts	No. of cats	No. of *Bartonella* infection [a] (%)	No.of *B.c.* infection [a] (%)	No. of *B.h.* infecteion [a] (%)	No. of mixed infection [a] (%)
1	Lak Si	30	21(70)	8(26.67)	8(26.67)	5(16.67)
2	Saphan Sung	30	18(60)	2(6.67)	12(40)	4(13.33)
3	Suan Luang	30	25(83.33)	11(36.67)	13(43.33)	1(3.33)
4	Phaya Thai	30	16(53.33)	5(16.67)	9(30)	2(6.67)
5	Samphanthawong	28	11(39.28)	3(10.71)	7(25)	1(3.57)
6	Sathon	30	18(60)	3(10)	14(46.67)	1(3.33)
7	Lat Phrao	30	23(76.67)	7(23.33)	14(46.67)	2(6.67)
8	Chatuchuk	30	9(30)	4(13.33)	4(13.33)	1(3.33)
9	Ratchathewi	30	23(76.67)	3(10)	18(60)	2(6.67)
10	Phra Khanong	30	16(53.33)	8(26.67)	7(23.33)	1(3.33)
11	Bang Khen	30	17(56.67)	1(3.33)	16(53.33)	0
12	Bang Kapi	30	15(50)	0	14(46.67)	1(3.33)
13	Bang Na	30	19(63.33)	2(6.67)	17(56.67)	0
14	Pom Prap Sattru	30	15(50)	4(13.33)	10(33.33)	1(3.33)
15	Pathum Wan	30	20(66.67)	4(13.33)	15(50)	1(3.33)
16	Don Mueang	30	17(56.67)	5(16.67)	11(36.66)	1(3.33)
17	Sai Mai	30	22(73.33)	7(23.33)	14(46.67)	1(3.33)
18	Nong Khaem	30	6(20)	0	6(20)	0
19	Bang Rak	30	15(50)	6(20)	8(26.67)	1(3.33)
20	Prawet	30	18(60)	4(13.33)	11(36.67)	3(10)
21	Min Buri	30	12(40)	3(10)	6(20)	3(10)
22	Bang Sue	30	18(60)	9(30)	8(26.67)	1(3.33)
23	Bang Khae	30	26(86.67)	9(30)	16(53.33)	1(3.33)
24	Taling Chan	30	19(63.33)	7(23.33)	10(33.33)	2(6.66)
25	Rat Burana	30	23(76.67)	8(26.67)	15(50)	0
26	Khlong San	30	19(63.33)	9(30)	10(33.33)	0
27	Khlong Toei	30	14(46.67)	3(10)	10(33.33)	1(3.33)
28	Khlong Sam Wa	30	1(3.33)	1(3.33)	0	0
29	Bang Kho Laem	30	10(33.33)	1(3.33)	9(30)	0
30	Lat Krabang	30	15(50)	4(13.33)	9(30)	2(6.67)
31	Bang Khun Thain	30	10(33.33)	3(10)	4(13.33)	3(10)
32	Bangkok Noi	30	18(60)	0	17(56.67)	1(3.33)
33	Bangkok Yai	30	16(53.33)	1(3.33)	15(50)	0
34	Bang Bon	30	7(23.33)	1(3.33)	5(16.67)	1(3.33)
35	Chom Thong	30	10(33.33)	3(10)	7(23.33)	0
36	Thung Khru	30	10(33.33)	2(6.67)	6(20)	2(6.67)
37	Phasi Charoen	30	27(90)	11(36.67)	13(43.33)	3(10)
38	Thawi Watthana	30	24(80)	7(23.33)	17(56.67)	0
39	Bang Phlat	30	16(53.33)	5(16.67)	10(33.33)	1(3.33)
40	Thon Buri	30	17(56.67)	6(20)	11(36.67)	0

Table 10 (Continued)

No. of district	Districts	No. of cats	No. of *Bartonella* infection [a] (%)	No.of *B.c.* infection [a] (%)	No. of *B.h.* infecteion [a] (%)	No. of mixed infection [a] (%)
41	Yan Nawa	30	14(46.67)	7(23.33)	7(23.33)	0
42	Watthana	30	20(66.67)	6(20)	13(43.33)	1(3.33)
43	Huai Khwang	30	23(76.67)	6(20)	13(43.33)	1(3.33)
44	Bueng Kum	30	19(63.33)	5(16.67)	13(43.33)	1(3.33)
45	Khan Na Yao	30	16(53.33)	1(3.33)	14(46.67)	1(3.33)
46	Din Daeng	30	9(30)	2(6.67)	7(23.33)	0
47	Wang Thonglang	22	2(9.09)	0	2(9.09)	0
48	Dusit	29	11(37.93)	5(17.24)	6(20.68)	0
49	Phra Nakhon	29	16(55.17)	7(24.14)	8(27.59)	1(3.44)
50	Nong Chok	30	19(63.33)	8(26.67)	11(36.67)	0
Total	50	1,488	803(53.96)	227(15.26)	521(35.01)	55(3.70)

[a] *B.c.* = *B. clarridgeiae* infection, *B.h.* = *B. henselae* infection, mixed = co – infection of *B. clarridgeiae* and *B. henselae*

Table 11 Infective rates and risk factors associated with *Bartonella* infection in Bangkok metropolitan areas

Parameter	No. of cats	No. of infected cats	No.of *B.c.* infection (%)*	No.of *B.h.* infection (%)	No.of mixed infection (%)
Sex					
Male	562	315	99(21.38)	199(54.82)	17(3.12)
Female	926	488	128(16.04)	322(53.31)	38(4.28)
Age (years)					
≤ 2	538	302	77(16.70)	203(60.60)	22(4.26)
> 2 to 4	721	379	109(17.81)	245(51.47)	25(3.59)
>4	229	122	41(21.80)	73(46.79)	8(3.62)
External parasite					
Unexposed	988	519	138(16.23)	346(53.89)	35(3.67)
Exposed	500	284	89(21.65)	175(53.85)	20(4.17)
Health condition					
Healthy	1,394	754	214(18.13)	490(54.20)	50(3.72)
Fair	37	19	4(12.12)	12(48)	3(8.82)
Poor	57	30	9(18.75)	19(50)	2(3.63)
Environment condition					
Fair	852	451	110(14.82)	309(56.91)	32(3.90)
Poor	636	352	117(22.54)	212(50)	23(3.75)
Density condition					
Less	261	193	43(19.72)	76(41.08)	9(3.57)
Most	1,227	1,159	184(17.64)	445(56.90)	46(3.89)
District	50	50	46	49	34

* % was number of infected cases per number of non - infection animals

Table 12 The statistical analysis of risk factors associated with *Bartonella* infection in Bangkok metropolitan areas

Parameter	No. of cats	No. of infected cats	Statistic value		
			Chi-square	Degree of freedom	*P* value
Sex			4.1374	2	0.1263
Male	562	315			
Female	926	488			
Age (years)			4.2440	4	0.3739
≤ 2	538	302			
> 2 to 4	721	379			
>4	229	122			
External parasite			4.5107	2	0.1048
Unexposed	988	519			
Exposed	500	284			
Health condition			0.6521	4	0.9570
Healthy	1,394	754			
Fair	37	19			
Poor	57	30			
Environment			8.5358	2	0.0140
Good	852	451			
Poor	636	352			
Density condition			5.1037	2	0.077
Less	261	193			
Most	1,227	1,159			
District	50	50			

Table 13 The infective rates of *Bartonella* infection detected by PCR technique classified by monasteries and districts

District	Monastery	Total	PCR positive (%)		
			B.c. [a]	***B.h.*** [a]	**Mixed** [a]
Lak Si	Lak Si	30	8(26.67)	8(26.67)	5(16.67)
Saphan Sung	Lad Buakao	30	2(6.67)	12(40)	4(13.33)
Suan Luang	Mahabud	30	11(36.67)	13(43.33)	1(3.33)
Phaya Thai	Paiton	30	5(16.67)	9(30)	2(6.67)
Samphanthawon	Trimit	10	2(20)	5(50)	0
	Chakrawat	5	1(20)	0	1(20)
	Samphanthawong	13	0	2(13.33)	1(6.67)
Sathon	Suthiwararam	5	0	1(20)	0
	Lum Charoen Sattha	10	1(10)	5(50)	0
	Barom Sadhol (Don)	5	1(20)	3(60)	0
	Yan Nawa	10	1(10)	5(50)	1(10)
Lat Phrao	Ladplakhao	10	4(40)	1(10)	1(10)
	Sirikamalawad	10	1(10)	6(60)	0
	Sakhon Sun Pracha San	10	2(20)	7(70)	1(10)
Chatuchuk	Sameiennaree	15	3(20)	1(6.67)	1(6.67)
	Tewasoontorn	15	1(6.67)	3(20)	0
Ratchathewi	Prayayung	10	0	5(50)	2(20)
	Thasanaroon Suntrikaram	10	2(20)	8(80)	0
	Dishongsaram	10	1(10)	5(50)	0

Table 13 (Continued)

District	Monastery	Total	PCR positive (%)		
			B.c. [a]	***B.h.*** [a]	**Mixed** [a]
Phra Khanong	Tham Mongkhon Thao Bunrot	10	4(40)	4(40)	0
	Bunrot Thammaram	10	3(30)	2(20)	0
	Ratsatthatham	10	1(10)	1(10)	1(10)
Bang Khen	Bang Bua	10	0	7(70)	0
	Siri Phong Thamma Nimit	10	0	7(70)	0
	Phra Sri Mahathat	10	1(10)	2(20)	0
Bang Kapi	Thep Lila	10	0	7(70)	0
	Phra Kraisi	10	0	4(40)	0
	Si Bunrueang	10	0	3(30)	1(10)
Bang Na	Si-Iam	10	0	5(50)	0
	Phong Phloi Wittayaram	10	1(10)	5(50)	0
	Bang Na Nok	10	1(10)	7(70)	0
Pom Prap Sattru Phai	Disanukaram	10	1(10)	4(40)	0
	Sitaram	10	2(20)	2(20)	1(10)
	KhanikaPhon	5	1(20)	2(40)	0
	Sa Ket	5	0	2(40)	0
Pathum Wan	Patumwanaram	10	2(20)	6(60)	0
	Chai Mongkhon	10	2(20)	4(40)	0
	Dung Khae	10	0	5(50)	1(10)

Table 13 (Continued)

District	Monastery	Total	PCR positive (%)		
			B.c. [a]	***B.h.*** [a]	**Mixed** [a]
Don Mueang	Thep Nimit	10	1(10)	1(10)	0
	Prommarangsi	10	1(10)	5(50)	1(10)
	Khlong Ban Mai	10	3(30)	5(50)	0
Sai Mai	Yu Di Bamrung Tham	10	1(10)	5(50)	1(10)
	Ko Suwannaram	10	4(40)	4(40)	0
	Rat Niyom Tham	10	2(20)	5(50)	0
Nong Khaem	Si Nuan Thammawimon	8	0	0	0
	Wong Lapharam	12	0	4(33.33)	0
	Phai Liang	10	0	2(20)	0
Bang Rak	Mahaphruettharam	10	1(10)	3(30)	0
	Muang Khae	10	3(30)	3(30)	0
	Hua Lumphong	10	2(20)	2(20)	1(10)
Prawet	Kaeopitak Charoentham	10	3(30)	2(20)	0
	Tung Lanna	10	0	6(60)	2(20)
	Tung Saepla	10	1(10)	3(30)	1(10)
Min Buri	Bang Pheng Tai	10	0	6(60)	3(30)
	Lumnok Khwaek	10	0	0	0
	Thong Samrit	10	3(30)	0	0
Bang Sue	Liapratbamrung	10	2(20)	5(50)	0

Table 13 (Continued)

District	Monastery	Total	PCR positive (%)		
			B.c. [a]	***B.h.*** [a]	**Mixed** [a]
	Thongsuttharam	10	2(20)	2(20)	1(10)
	Soi Thong	10	5(50)	1(10)	0
Bang Khae	Muang	10	4(40)	4(40)	0
	Ratbumrung	10	3(30)	6(60)	0
	Promsuwansamukki	10	2(20)	6(60)	1(10)
Taling Chan	Pho	10	3(30)	4(40)	1(10)
	Makok	6	0	1(16.67)	1(16.67)
	Noi Nai	14	4(28.57)	5(35.71)	0
Rat Burana	Bangprakok	10	4(40)	4(40)	0
	Prasertsutthawas	10	2(20)	5(50)	0
	Rat Burana	10	4(40)	4(40)	0
Khlong San	Thongplang	10	2(20)	4(40)	0
	Thongthummachat	10	1(10)	4(40)	0
	Phichayayatikaram	10	6(60)	2(20)	0
Khlong Toei	Khlong Toei Nok	10	1(10)	2(20)	0
	Khlong Toei Nai	10	0	7(70)	0
	Saphanphrakhanong	10	2(20)	1(10)	1(10)
Khlong Sam Wa	Phraya Suren	10	1(10)	0	0
	Chinditwihan	10	0	0	0

Table 13 (Continued)

District	Monastery	Total	PCR positive (%)		
			B.c. [a]	***B.h.*** [a]	**Mixed** [a]
	Lam Kradan	10	0	0	0
Bang Kho Laem	Chan Nok	8	0	0	0
	Ratchasingkhon	12	0	3(25)	0
	Phai Ngoen Chotanaram	10	1(10)	6(60)	0
Lat Krabang	Sutthaphot	10	2(20)	1(10)	2(20)
	Uthai Thammaram	10	0	2(20)	0
	Bueng Bua	10	2(20)	6(60)	0
Bang Khun Thain	Suthamwadi	7	2(28.57)	0	1(14.28)
	Prommarangsi	11	0	0	1(9.09)
	Bua Pan	7	1(14.28)	0	1(14.28)
	Kampang	5	0	4(80)	0
Bangkok Noi	Bangkhunnon	10	0	6(60)	1(10)
	Mai Yaipan	10	0	6(60)	0
	Plengwipassana	10	0	5(50)	0
Bangkok Yai	Deedoud	10	0	3(30)	0
	Tha Phra	10	0	7(70)	0
	Khruea Wan Worawihan	10	1(10)	5(50)	0
Bang Bon	Ninsukharam	15	1(6.67)	5(33.33)	1(6.67)
	Bang Bon	15	0	0	0

Table 13 (Continued)

District	Monastery	Total	PCR positive (%)		
			B.c. [a]	***B.h.*** [a]	**Mixed** [a]
Chom Thong	Sai	10	0	1(10)	0
	Pho Kao	10	2(20)	2(20)	0
	Bang Khun Thain Nai	10	1(10)	4(40)	0
Thung Khru	Phutthabucha	10	0	1(10)	0
	Luang Phor Ophasi	10	1(10)	1(10)	0
	Thung Khru	10	1(10)	4(40)	2(20)
Phasi Charoen	Pradu Bangchak	10	4(40)	4(40)	2(20)
	Chaichimplee	10	3(30)	4(40)	1(10)
	Tanot	10	4(40)	5(50)	0
Thawi Watthana	Komut Phuttha Rangsi	15	4(26.67)	9(60)	0
	Puranawas	15	3(20)	8(53.33)	0
Bang Phlat	Bowon Mongkhon Ratchaworawihan	10	0	3(30)	0
	Chaturamit Pradittharam	10	3(30)	5(50)	0
	Phanu Rangsi	10	2(20)	2(20)	1(10)
Thon Buri	Buppharam Worawihan	10	3(30)	1(10)	0
	Bang Saikai	10	2(20)	6(60)	0
	Waramarttayapansararam	10	1(10)	4(40)	0
Yan Nawa	Dokmai	10	3(30)	1(10)	0
	Dan	10	2(20)	3(30)	0

Table 13 (Continued)

District	Monastery	Total	PCR positive (%)		
			B.c. [a]	***B.h.*** [a]	**Mixed** [a]
	Pariwat	10	2(20)	3(30)	0
Watthana	Thatthong	15	3(20)	8(53.33)	0
	Pasee	15	3(30)	5(33.33)	1(6.67)
Huai Khwang	Phraram Kao Kanchanaphisek	5	1(20)	2(40)	0
	Maichonglom	15	3(20)	9(60)	0
	Uthaitharam	10	2(20)	5(50)	1(10)
Bueng Kum	Nuanchan	10	3(30)	3(30)	0
	Bangtaey	10	2(20)	6(60)	0
	Suwan Prasit	10	0	4(40)	1(10)
Khan Na Yao	Ratsatthatham	15	1(6.67)	9(60)	0
	Khlong Khru	4	0	2(50)	0
	Bunsimunikon	11	0	3(27.27)	1(9.09)
Din Daeng	Kunnatiruttharam	15	1(6.67)	6(40)	0
	Phrom Wongsaram	15	1(6.67)	1(6.67)	0
Wang Thonglang	Bueng Thonglang	15	0	2(13.33)	0
	Samakkhitham	7	0	0	0
Dusit	Prasatbunyawat	10	1(10)	2(20)	0
	Sawatwarisimaram	9	0	1(10)	0
	Thewaratchakunchon	10	4(44.44)	3(33.33)	0

Table 13 (Continued)

District	Monastery	Total	PCR positive (%)		
			B.c [a].	***B.h.*** [a]	**Mixed** [a]
Phra Nakhon	Makutkasattriyaram	10	3(30)	3(30)	1(10)
	Parinayok	10	1(10)	4(40)	0
	Mahannapharam	9	3(30)	1(10)	0
Nong Chok	Mai Charoen Rat	6	1(16.67)	1(16.67)	0
	Saen Kasem	4	1(25)	3(75)	0
	Si Chomphu	10	3(30)	4(40)	0
	Krathumrai	10	3(30)	3(30)	0
Total		1,488	227(15.25)	521(35.01)	55(3.7)

[a] *B.c*. = *B. clarridgeiae* infection, *B.h*. = *B. henselae* infection, mixed = co – infection of *B. clarridgeiae* and *B. henselae*

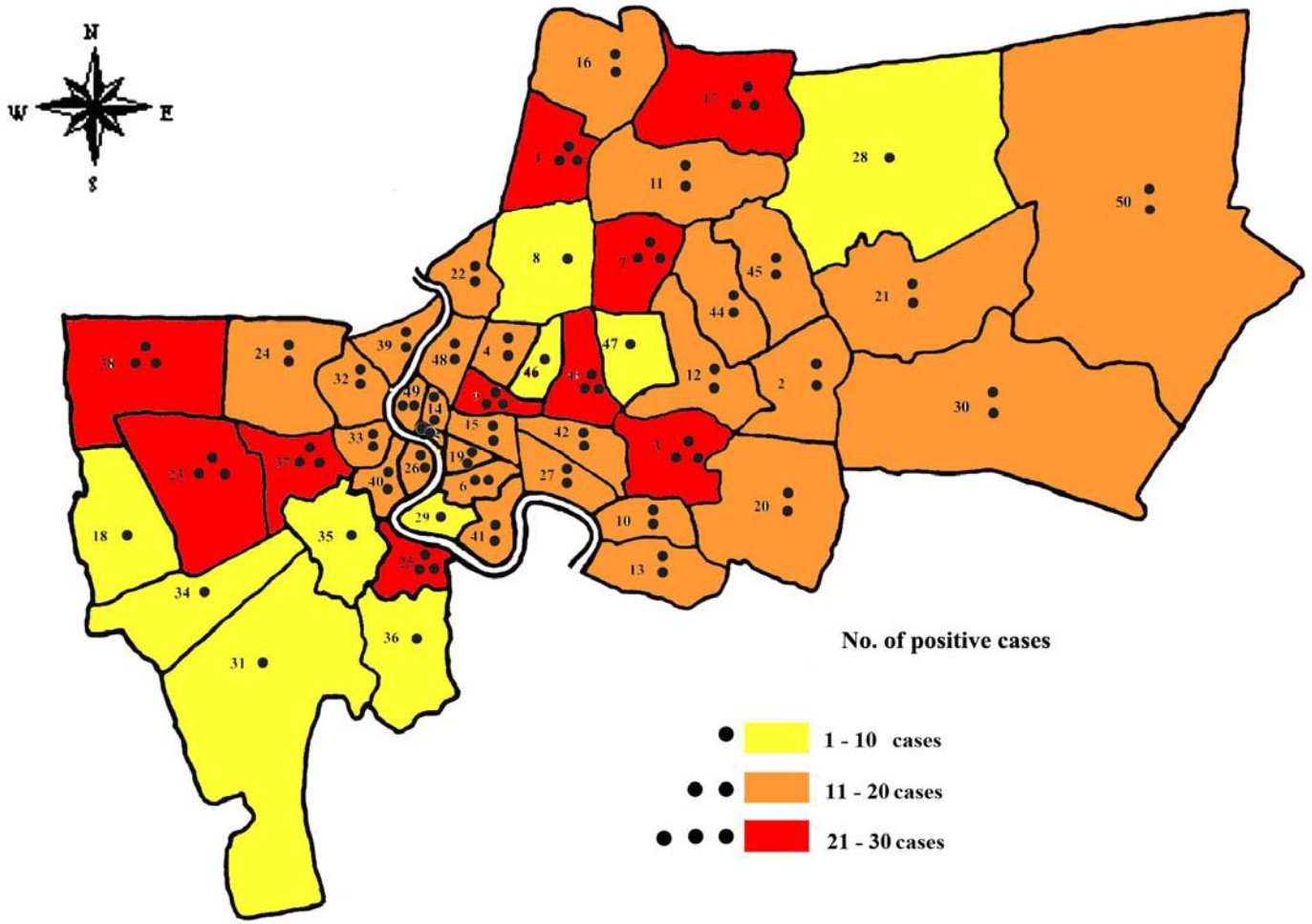


Figure 17 The distribution of *Bartonella* infection in Bangkok metropolitan areas

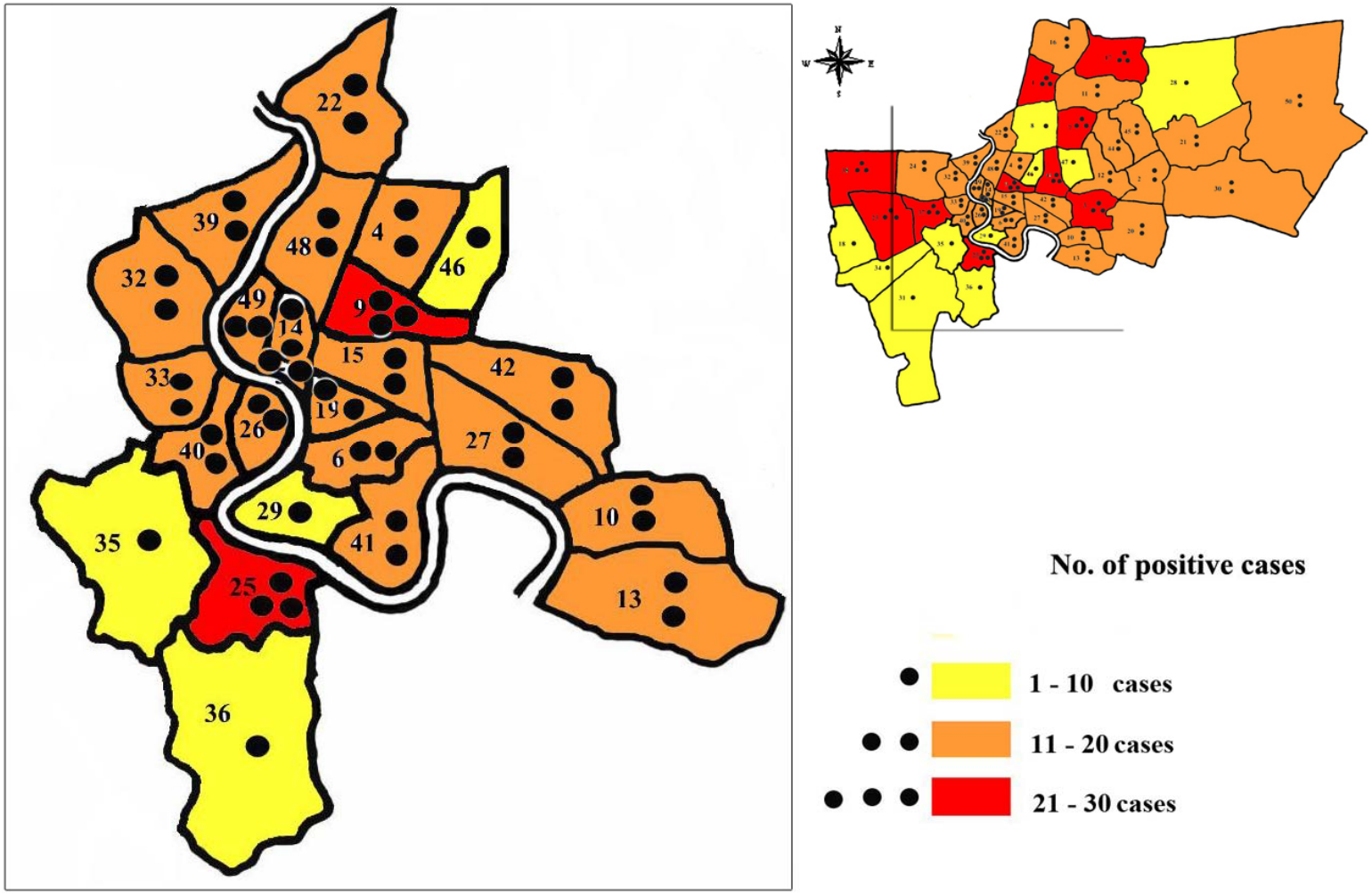


Figure 18 *Bartonella* positive districts of Bangkok located near the river zone

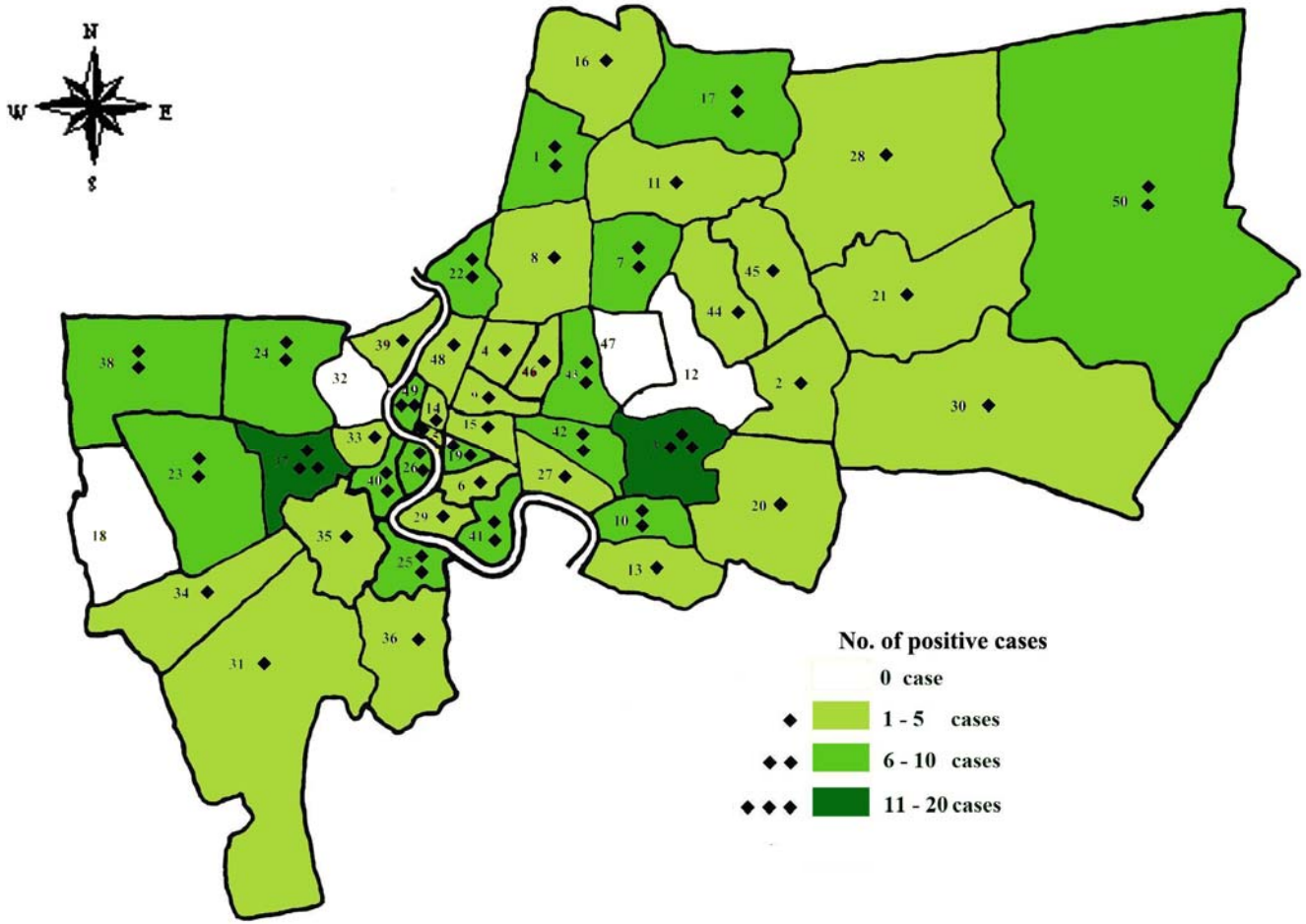


Figure 19 The distribution of *B. clarridgeiae* in Bangkok metropolitan areas

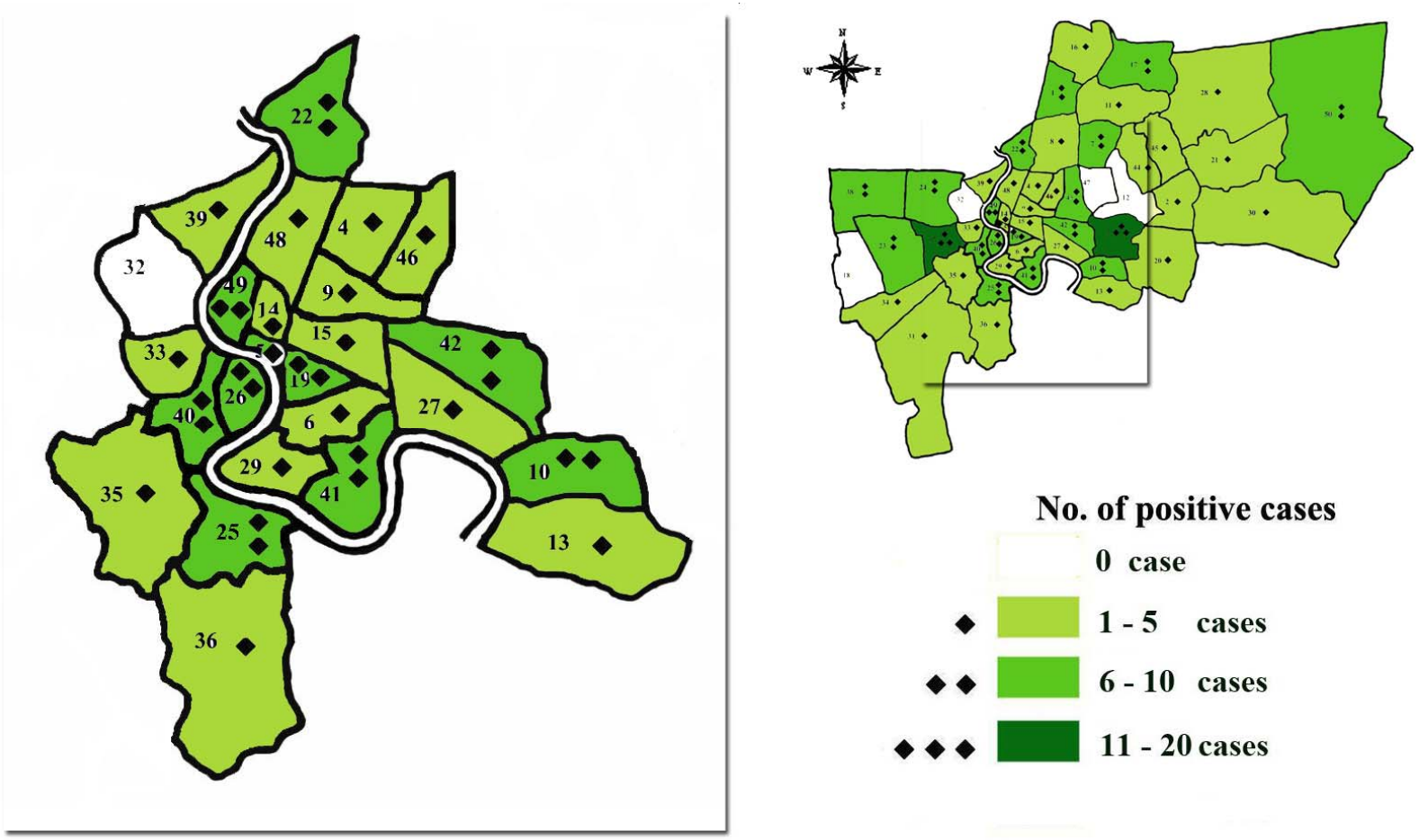


Figure 20 *B. clarridgeiae* positive districts of Bangkok located near the river zone

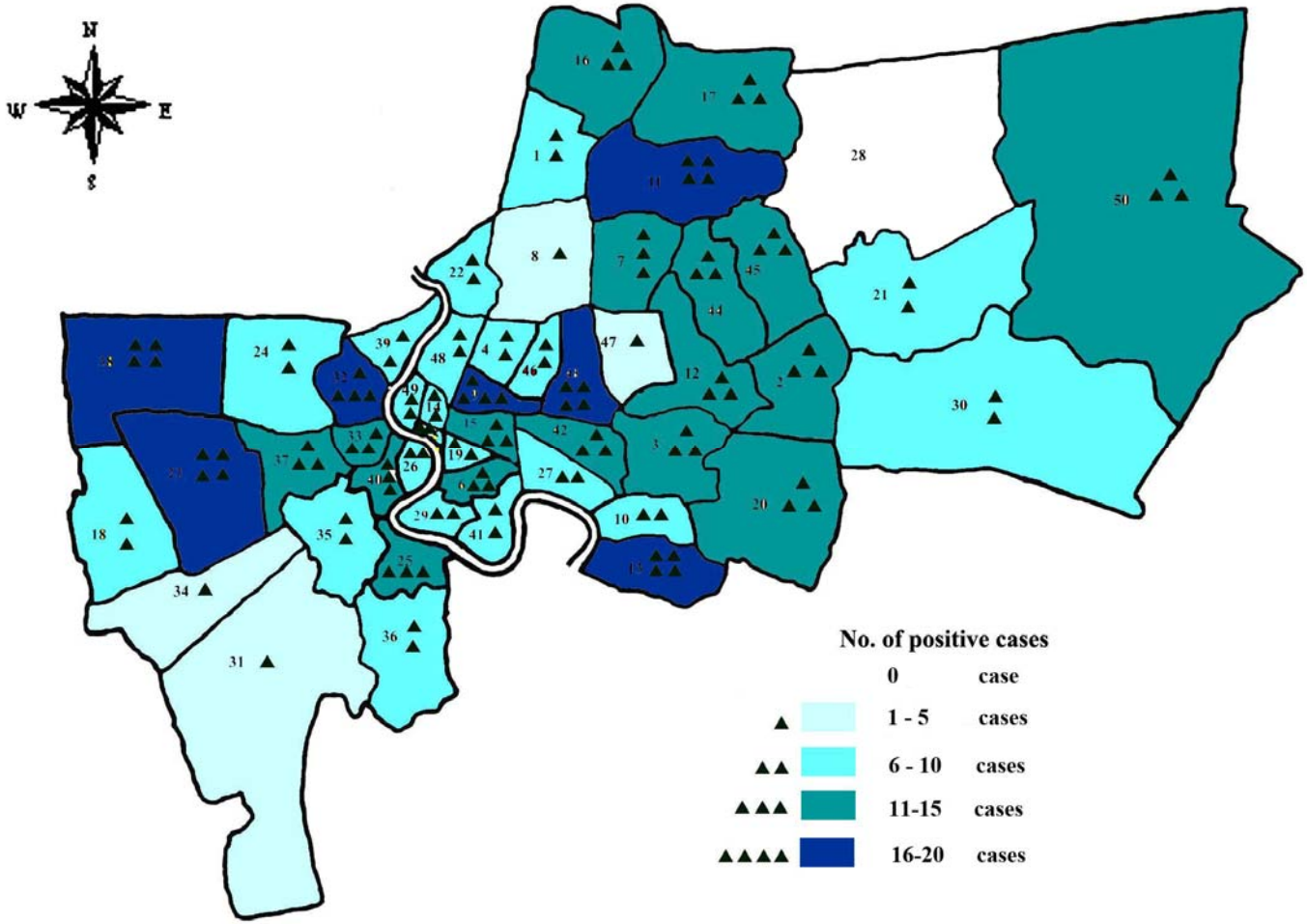


Figure 21 The distribution of *B. henselae* in Bangkok metropolitan areas

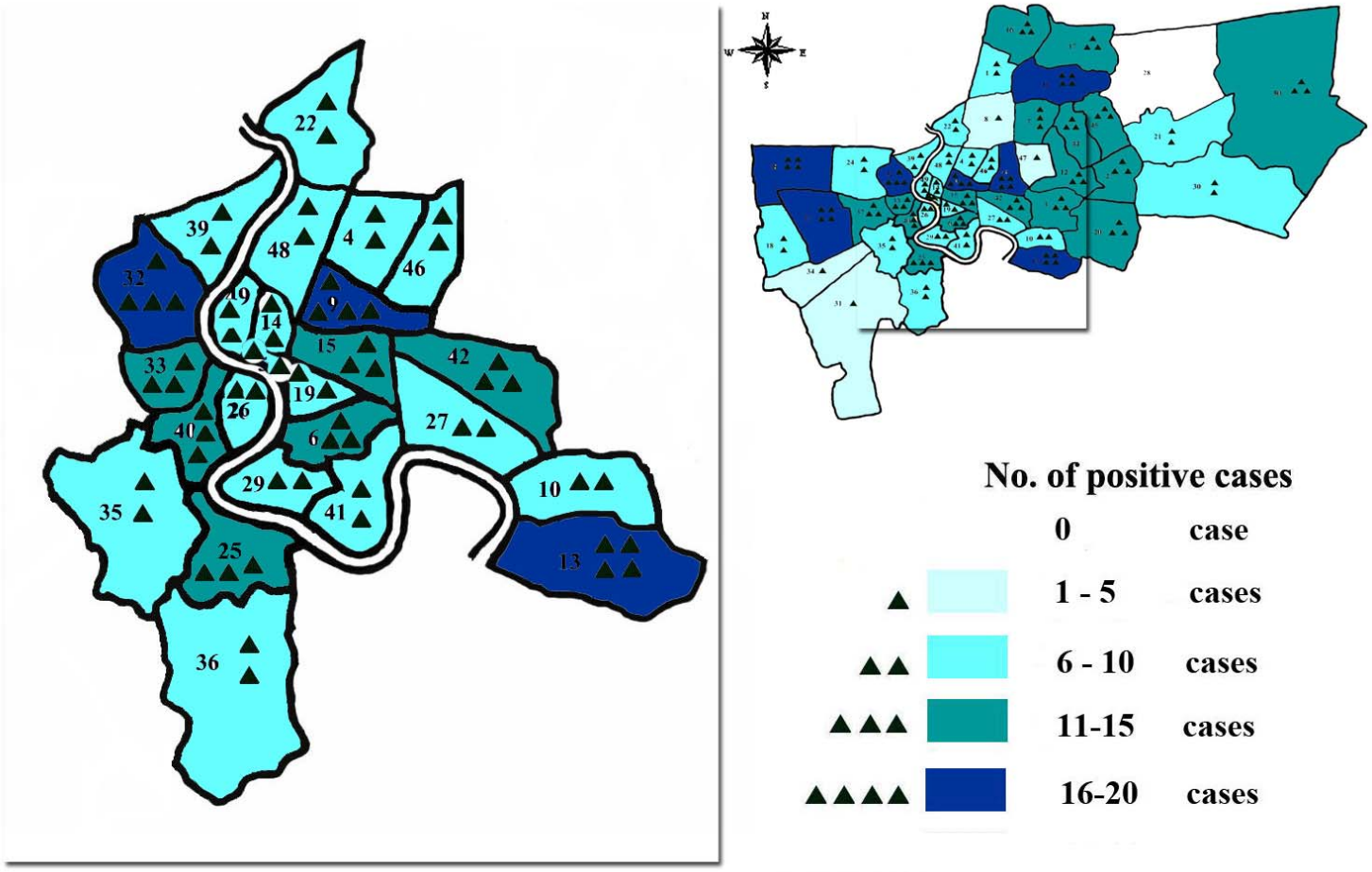


Figure 22 *B. henselae* positive districts of Bangkok located near the river zone

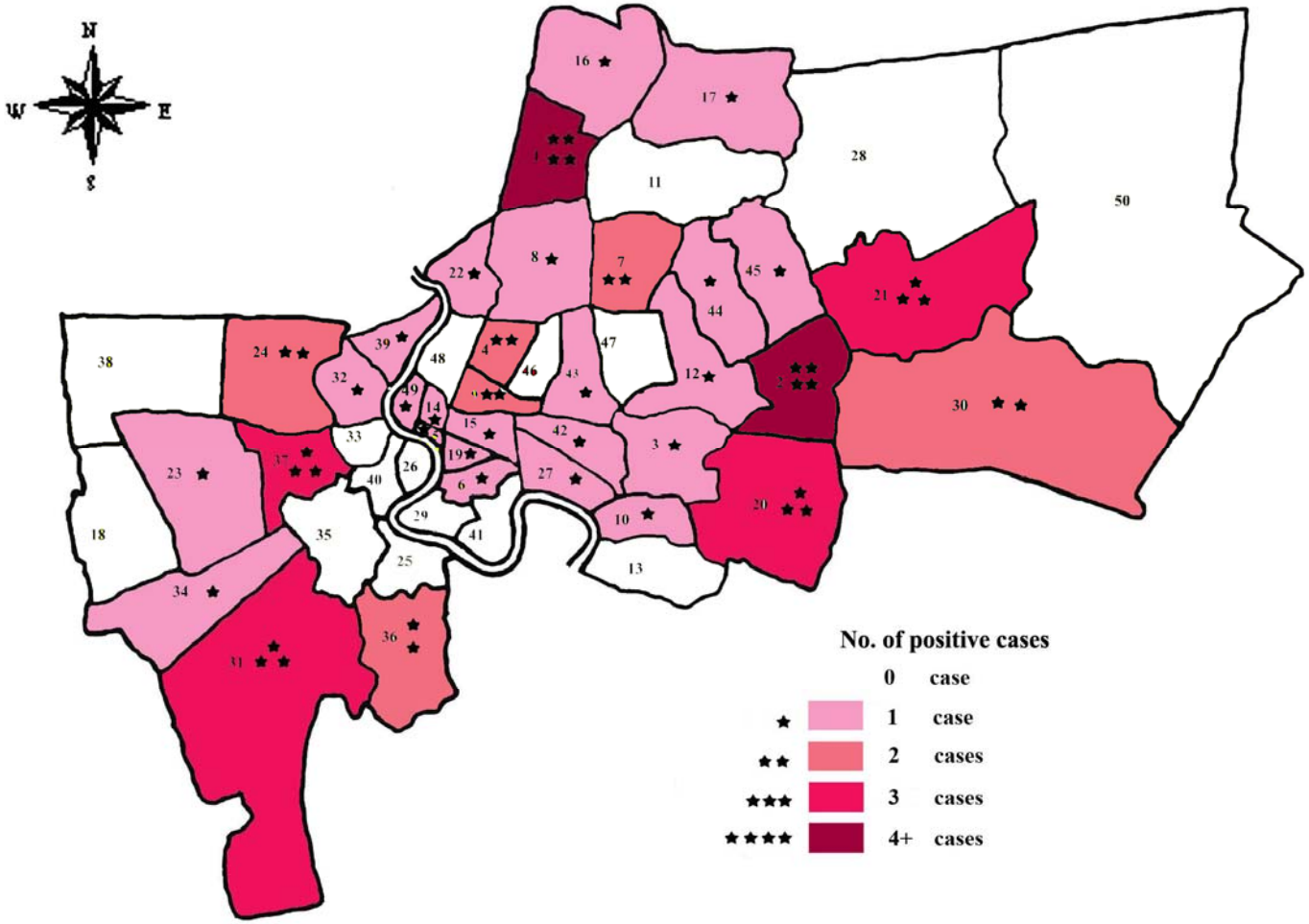


Figure 23 The distribution of mixed *Bartonella* infection in Bangkok metropolitan areas

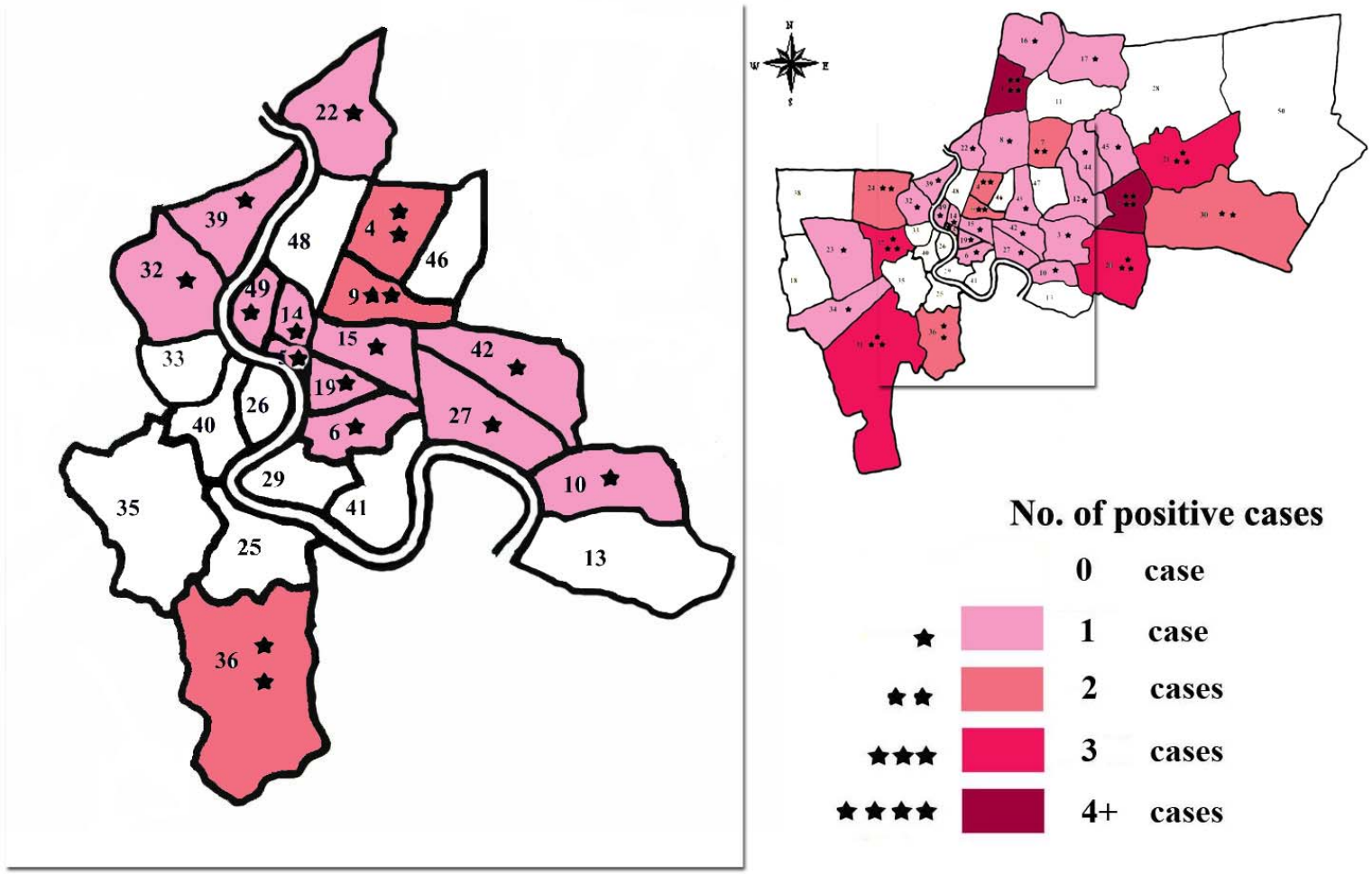


Figure 24 Mixed infection of positive districts of Bangkok located near the river zone

Discussion

Detection of *Bartonella* infection

The gold standard for diagnosis of this disease is blood culture. However, the disadvantage of this method is time consuming with unstable results due to its slow fastidious growth characteristics (Sander, 1998). Currently, the serological and PCR assay were used for detection of *Bartonella* infections since these methods have more specificity and sensitivity than the conventional method.

This is the first study of the pathogen of CSD in the stray cat population in Bangkok metropolitan areas, Thailand. We surveyed the infective rate of *Bartonella* spp. stray cats resided in monasteries in Bangkok areas by nested - PCR. This technique was known as a potentially efficient method to detect and identify *B. henselae* and *B. clarridgeiae* in feline blood (Rampersad *et al*., 2005). We used 16S-23S rRNA gene sequences to study bacterial epidemiology because it has been proposed as a rapid and reliable method for the detection of *Bartonella* species DNA in samples (Maggi and Breitschwerdt, 2005). However, a single of PCR can be used to detect different species within this genus because the variation in ITS sequences among *Bartonella* species (Maggi and Breitschwerdt, 2005). The results of *Bartonella* infection detection were compared between microscopic examination (ME) and PCR assay. The PCR result was shown the infective rate of *Bartonella* at 53.96% compared to 6.8% by ME. Evidently, the sensitivity and specificity of PCR assay were greater than ME. *Bartonella* is a pathogen that has no characteristics mark and it is normally pleomorphic appearance. Moreover, the level of parasitaemia might be an associated factor with sensitivity since this pathogen is inconsistency or the less number in the blood. Therefore, to find this pathogen by ME, the examiner will require more experience.

A BLAST search across multiple DNA databases by using BLASTN software showed that the 130 bp of *B. clarridgeiae* was homologous to *B. clarridgeiae* isolated in USA (DQ683194.1) at 99% sequence identity and 98% homology with the strain in

China (EU589237.1). However, we used primers which designed from conserve sequences but our results were showed 99% homology of *B. clarridgeiae*. Because the variation of Thai isolated *B. clarridgeiae* differ from USA and China strain. Whereas 149 bp of Thai *B. henselae* was homologous to *B. henselae* from Israel (FJ832091.1) and USA (DQ529247.1) at 100% sequence identity.

Epidemiology of *Bartonella* infection in Bangkok metropolitan areas

Our PCR results showed the high infection of *Bartonella* in stray cats population. This is due to cat fleas that served as the most potential vector of this pathogen oftenly found on stray cats in Bangkok areas (Jittapalapong *et al*., 1993) since infected fleas can transmit *Bartonella* spp. among stray cats (Chomel *et al*., 1996). Excluding the vector condition, these stray cats were highly susceptible due to their health since there are no dewormed and vaccination program in these animals. The overall infective rate of *Bartonella* infection in this study was 53.9% (803/1,488) and *B. hensalae* and *B. clarridgeiae* were found at 35% (521/1,488) and 15.3% (227/1,488), respectively. The infective rate in this study was higher than previous report of pet cats infection in Thailand (27.9%) by Maruyama *et al*., (2001). The high infection result was likelydue to the spread among population of stray cat which was the impotance reservoir of this pathogen compared to house cats (Boonmar *et al*., 1997). Previous study (Maruyama *et al*., 2001) was showed co – infection of *B. henselae* and *B. clarridgeiae* at 5.3%. In this study, mixed infections of both species were 3.7% (55/1,488). In addition, there were many investigations of epidemiology of *Bartonella* infection in Asian countries that were varied by countries such as 61% (19/31) of stray cats in Philippines by serological method (Chomel *et al*., 1999), 43% (6/14) of *B. henselae* infection and 21% (3/14) of *B. clarridgeiae* infection of stray cats in Indonesia by IFA method (Marston *et al*., 1999), 47.5% (38/80) of stray cats in Singapore by IFA method (Narisudeen and Thong, 1999) and 7.2% (50/690) of pet cats in Japan by PCR assay (Maruyama *et al*., 2001). Our studies were shown all districts of Bangkok metropolitan areas were endemic for *Bartonella* infection (100%). Our findings supported the previous studies that *B. henselae* and *B. clarridgeiae* were the most common species detected cats. In addition, *B. henselae*

was the predominant species found in Asian countries such as Japan (Maruyama *et al*., 1999), Indonesia (Marston *et al*., 1999), Philippines (Chomel *et al*., 1999) and Singapore (Narisudeen and Thong, 1999). In this study, we found that the number of *B. henselae* infection was higher than *B. clarridegeiae*. The high infection of *B. henselae* in Bangkok metropolitan areas might be public health concern because this pathogen is capable of transmission to humans.

Risk factors associated with *Bartonella* infection

B. henselae were found alive in flea feces for at least 9 days and increased the risk of transmission of *Bartonella* via contact with infected flea feces. (Higgins *et al*., 1996, Foil *et al*., 1998, Finkelstein *et al*., 2002, Brunt *et al*., 2006). Accordingly, the exposure with flea or flea feces was the most importance for transmission of *Bartonella* spp. (Brunt *et al*., 2006). The unsanitary environment of house or monastery lead to increase population of fleas and spreading out of infected fleas. In this study, the poor environmental condition was the only risk factors associated with *Bartonella* infection ($p = 0.01$). Since housed and stray cats share the same environment and their population increase annually. These cats are becoming the importance source of *Bartonella* spp. Our results were indicated that stray cats might be the potential reservoirs of bartonellosis.

Previous studies showed the association between gender of cat and the infection. Zangwill *et al*., (1993) also reported that male cats related to the infection; however, Sander *et al*., (1997) showed that the female cats were associated with the infection. In this study, sex of cats were not associated with the infection ($p = 0.126$) but we found male cats infected with *Bartonella* more than female cats. It might be male cats usually wander and having more opportunuties to be scratched or bitten by other cats while protecting their territories in the limited Bangkok areas (Inoue *et al*., 2009). The other studies were shown young cats associated with the infection (Koehler *et al*., 1994, Sander *et al*., 1997). Although, our studies were indicated that no association between *Bartonella* infection and age group of stray cats but we found young cats (less than 2 years) infected with the pathogen higher than the other groups.

Because young cats might be infect with fleas from their mother during lactation. However, the other risk factors such as ectoparasites, health and density condition were not significantly related with *Bartonella* infection. We found cats with ectoparasite infestation had the higher infected with pathogen than non-infestation cats. Stray cats lived in high density condition were infected with *Bartonella* more than low density condition. Warm and humid environment were associated with *Bartonella* infection (Jameson *et al*., 1995). In addition, other risk factors such as outdoor activity of cats might be related to the infection (Brunt *et al*., 2006) and correspond to our studies since stray cats wander or roam between monasteries and nearby houses. These cats might transmit this pathogen or infected – flea to pet or other stray cats.

CONCLUSION

Bartonella spp. are worldwide emerging zoonoses which transmitted by blood sucking arthropods. *Bartonella henselae*, the causative pathogen of cat scratch disease that transmitted to human by cat scratch, bite or contact with fleas feces. Fleas are the importance vector among cats. Our studies were showed the infective rate of *Bartonella* spp. of stray cats which resided in monasteries in Bangkok metropolitan areas. We found the infective rate of *Bartonella* spp. at 53.96% (803/1,488). A total of 1,488 samples were detected as *B. henselae* 35% (521/1,490), *B. clarridgeiae* 15.26% (227/1,490) and mixed infection 3.7% (55/1,490). The statistical analysis results were shown significant between risk factors and infection. Poor environmental condition was associated with *Bartonella* infection ($p = 0.01$). The other factors comprising age, sex, health condition, ectoparasite and density condition were not related to the infections. The positive samples of *Bartonella* species were found in 432 monasteries from 50 districts. Two species of *Bartonella*, *B. henselae* and *B. clarridgeiae* were found from the overall districts (100%). The result showed that stray cats were crucial reservoirs and can transmit the pathogen to housed cats and human who live in the same environment. *Bartonella* infections among stray cats were cerently existed in stray cats population of Bangkok metropolitan areas. Bangkok are endemic areas of CSD. Control and prevention program will established by control of stray cats population, isolate reservoirs from the population and treatment of the infected cats to prevent the risk of transmission to humans.

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APPENDICES

Appendix A

The standard method

1. DNA extraction protocol; Phenol – Chloroform Extraction of DNA and Ethanol precipitation (Sambrook and Russel, 2001)
2. PCR purification protocol; QIAquick gel extraction kit (QIAGEN)

1. DNA extraction protocol; Phenol – Chloroform Extraction of DNA and Ethanol precipitation (Sambrook and Russell, 2001)

1. DNA was extracted from blood sample 100 µl mixed with denature solution 500 µl by shaken to 5 – 10 minutes.
2. Add chloroform 150 µl and DNA phenol (pH 7.9) 150 µl (chloroform : phenol = 1:1), shaken for 10 minutes.
3. Centrifuge the mixture at 13,000 rpm for 5 minutes to separate the phases.
4. Collected the supernatant for 550 – 600 µl to the clean microtube (1.5 ml), carefully avoiding protein at the aqueous phenol interface at the last collecting.
5. Repeated the same protocol to clean the supernatant (step 2 – 4). In the second time, collected 400 µl of the supernatant and transfer to new microtube (1.5 ml).
6. Precipitated DNA by adding 1,000 µl (1 ml) of absolute ethanol (99.99%), invert gentally upside down and keep in -80°c for 30 minutes or -20°c for overnight.
7. Centrifuge at 13,000 rpm for 10 minutes. Remove the supernatant carefully.
8. To wash the DNA pallet with 75% ethanol. Centrifuge at 13,000 rpm for 5 minutes. Decant the supernatant, and dry the pallet by air.
9. Adding TE pH 8.0 30 µl in tube with dried DNA and dissolve
10. Store DNA at 4°C. For long term storage, place samples at -20°C.

2. PCR purification protocol; QIAquick gel extraction kit (QIAGEN)

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. Recommended: Add 0.5 ml of Buffer QG to QIAQuick column and centrifuge for 1 min.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm).
12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.
14. If the purified DNA is to be analyzed on a gel, add 1 volume of loading dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix B

Reagents and buffers for Phenol – Chloroform extraction and Ethanol precipitation

1. Denature solution (D-solution)

D-solution composed of 4M guanidine thiocyanate, 25mM sodium citrate pH 7, 10% N-lauroylsarcosine and sterilized millique water. The D-solution was transferred at dark bottle and stored at room temperature.

2. 10x Tris buffer (TE)

The stock 10x TE was prepared by dissolved 15.76 g of Tris-HCL and 3.72 g of EDTA in 500 ml of distilled water and adjusted pH to 8.0. The final volume was adjusted to 1,000 ml by distilled water. The 10x TBE was stored at 25°C. The 1x working solution was freshly prepared by diluting the stock 10x TE buffer with distilled water.

Appendix C

Reagents and buffers for agarose gel electrophoresis

Reagents and buffers for agarose gel electrophoresis

1. Gel loading buffer (loading dye)

The loading dye buffer composed of 0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol and 35 ml of ultrapure distilled water. The loading dye solution was kept at 4°C.

2. Tris Borate EDTA buffer (10x TBE)

The stock 10x TBE was prepared by dissolved 108 g of Tris-base in 500 ml of distilled water. After the ingredient was completely dissolved, 55 g of Boric acid and 9.3 g of EDTA, were added into the solution. The final volume was adjusted to 1,000 ml by distilled water. The 10x TBE was stored at 25°C. The 1x working solution was freshly prepared by diluting the stock 10x TBE buffer with distilled water.

3. Working (1x TBE)

Fifty milliliter of 10X TAE was added to 950 ml of distilled water. This solution can be reused three times.

4. Ethidium Bromide (10 mg/ml)

One hundred milligram of Ethidium Bromide was dissolved to 100 ml of 1xTBE. The solution was transferred to dark bottle or aluminum foil wrap box and stored at room temperature.

Appendix D

The sample pictures of stray cat habitation

Apppendix Figure D1 A population of stray cat resided in Wat Wong Lapharam of Nong Khaem district.

Apppendix Figure D2 The stray cat were feeded by a kind nun or monk.

Apppendix Figure D3 The stray cats lived in monk house at Wat Muang of Bang Khae district.

Apppendix Figure D4 The stray cats allowed outdoor and traveled to nearby house where share the same environment.

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

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