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THE DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT FOR THE DETECTION OF *BARTONELLA HENSELAE* INFECTION IN CATS

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CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I
INTRODUCTION

Importance and Rationale

Bartonella were recognized as emerging zoonotic agents in humans and can be isolated from several mammalian reservoirs (Boulouis, et al., 2005). This organism was found by Barton in 1909. After confirmed Barton's discovery in 1913, this bacteria was named to Bartonia in honor of Barton (Cueto, 1996). Then bacteria name was changed to *Bartonella* and used for the only member of the group identified after 1993. *Rochalimaea* (named for Rocha-Lima), a similar group, were recently combined with *Bartonella*. Although these organisms were originally thought to be rickettsiae but *Bartonella* can be cultured on media *in vitro* unlike other rickettsiae (Koehler, et al., 1992; Koehler, et al., 1994).

At least 24 species belong to the genus *Bartonella*. Seventeen *Bartonella* spp. are important causes of human diseases (Anderson and Neuman, 1997; Rolain, et al., 2003; Avidor, et al., 2004; Chomel, et al., 2006; Balakrishnan, et al., 2008). *B. bacilliformis* causes Oroya Fever and Verruga Peruana. *Bartonella henselae* causes cat scratch disease (CSD) and peliosis of the liver (often called bacillary peliosis). *Bartonella quintana* causes trench fever. Both *B. henselae* and *B. quintana* may cause bacillary angiomatosis, infections in homeless populations (Brouqui, et al., 1999), and cause infections in HIV patients (Koehler, 1995; Pons, et al., 2008). Other *Bartonella* spp. that may cause human diseases include *B. vinsonii, B.clarridgeiae, B. tamiae, B. rochalimae* and *B. elizabethae* (Kordick, et al., 1997; Breitschwerdt and Kordick, 2000; Kosoy, et al., 2008)

Thailand is an endemic area of ticks and fleas. These ectoparasites which can transmit *Bartonella* spp. from dogs and cats to its owners (Chomel, et al., 1996; Breitschwerdt and Kordick, 2000). Reports on cats bartonellosis in Thailand were caused by 2 species; *B. henselae* and *B. clarridgeiae* (Boonmar, et al., 1997; Maruyama, et al., 2000; Suksawat, 2008; Jitchum, et al., 2009; Rodkhum, et al., 2010). Cat is one of the most important host species transmitted *Bartonella* spp. to humans. *B. henselae* is the major *Bartonella s*pecies causes bartonellosis in cat and can transmitted cat scratch disease (CSD) to human patients (Breitschwerdt and

Kordick, 2000). The first report of cat scratch disease in Thai patients was presented in 2008 (Paitoonpong, et al., 2008). This problem causes much concern to human health as the zoonotic problem from some of *Bartonella* spp.

Nowadays the major diagnostic method for bartonellosis in human patients is mainly serological test due to difficulties and time consuming of the culture method. However, the serological test for cat bartonellosis is not practical in clinics and not applicable for most veterinarians who need a quick result in their clinical settings. A fast and good screening test for veterinary clinics is needed. The purpose of this study is to develop an in-house ELISA detection method for feline *B. henselae* infection in Thailand.

Hypothesis

Bartonella specific antigenic proteins have high possibility to be used in the development of *Bartonella* spp ELISA antibody detection kit.

Objectives of the study

1. To study the prevalence of *B. henselae* infection in well-cared cats in Thailand.
2. To select *B. henselae* specific antigenic proteins and use for developing a *B. henselae* ELISA antibody detection kit.
3. To validate the developed ELISA antibody detection kit.

Keywords (Thai) : ชุดทดสอบแอนติบอดี บาโทเนลลา เฮนเซลเล แมว อีไลซา
Keywords (English) : antibody detection kit, *Bartonella henselae*, cat, ELISA

Advantages of the study

1. Be able to determine the standard diagnostic method for *Bartonella* spp. identification in Thailand.

2. Be able to use and validate the in-house ELISA antibody detection tool for determining the *B. henselae* infection in cats in Thailand

CHAPTER II
LITERATURE REVIEW

Overview of *Bartonella* spp.

Bacteria of the genus *Bartonella* or formerly known as *Rochalimaea* alpha division of class *Proteobacteria* (α- proteobacteria) represent in the same class of *Rickettsiae, Ehrlichia*, *Brucella* and *Afipia. Bartonella* spp. has been studied since 1909. *Bartonella* spp. affecting human health for example; *B. quintana* can be found in a 4000 years old human tooth (Drancourt, et al., 2005). The genus of these bacteria is named after the discovered *B. bacilliformis* which cause of the Oroya fever or Carrión's disease in Peru by Alberto Leonardo Barton Thompson in 1909 (Baldani, et al., 2014). Seventeen species of *Bartonella* are zoonotic potential including; *B. baciiliformis, B. rochalimaea, B. quintana, B. elizabethae, B. clarridgeiae, B. claridgeiae*-like, *B. grahammi, B. kochleae, B. vinsonii* subsp. *berkhoffii, B. vinsonii arupensis, B. washoensis, B. henselae, B. alsatica, B. doshiae, B. tylorii, B. bovis* and *B. tamiae* but only 7 species were isolated from dogs and cats. These are *B. henselae, B. clarridgeiae, B. koehlerae, B. vinsonii* subsp. *berkhoffii, B. washoensis, B. elizabethae, B. quintana* which also are zoonotic potential (Table 1) (Kordick, et al., 1997; Breitschwerdt and Kordick, 2000; Breitschwerdt, et al., 2007)

Bacterial characteristics

Bartonella spp. are very small as 0.6-1 μm, fastidious, Gram negative, aerobic coccobacilli or bacillary rods with more than 24 described species (Houpikian and Raoult, 2001; Boulouis, et al., 2005; Suksawat, 2008; Tang, et al., 2014). The bacterium is biochemically inert with oxidase, catalase, urease and nitrate reductase negative except for the production of peptidase. It is difficult to identify by standard biochemical test (Breitschwerdt and Kordick, 2000). The colonies were grown slowly with optimal temperature at 35-37˚C and 5% CO_2 condition and took 5 to 15 days and up to 45 days in on primary culture to form visible colonies on enriched blood-containing media (Boulouis, et al., 2005), as dependent on highly blood-containing agar (La Scola and Raoult, 1999).

Table 1: *Bartonella* spp. discovered and their various main-human diseases and clinical abnormalities.

Species	Main vectors	Main reservoirs	Main human Diseases and clinical abnormalities
B. bacilliformis	sand fly	human	Carrion's disease
B. quintana	body louse	human	Trench fever CSD, BA bacteremia endocarditis septicemia
B. henselae	cat flea	cat	CSD, BA, CA bacteremia endocarditis septicemia
B. elizabethae	Oriental flea	rat	endocarditis Retinitis
B. grahamii		wild mice	Retinitis
B. washoensis	unknown	ground squirrel	Fever
B. vinsoii subspecies *berkhoffii*	unknown	coyote, dog	myocarditis endocarditis
B. clarridgeiae	cat flea	cat	CSD
B. alsatica	unknown	rabbit	endocarditis
B. vinsonii subspecies *arupensis*	unknown	mouse	bacteremia
B. rochalimae	unknown	unknown	bacteremia
B. tamiae	unknown	unknown	endocarditis

BA= Bacillary Angiomatosis, CA= Chronic Adenopathy, CSD= Cat Scratch Disease

Modified from: Kempf, 2008 and Kosoy, et al., 2008

Cat Scratch Disease (CSD)

Cat scratch disease is a human disease caused by *B. henselae* which were represented worldwide especially in Asian countries. *B. henselae* was presented in Japan (Murakami, et al., 2002), Korea (Chung, et al., 2005; Kim, et al., 2009), Indonesia (Marston, et al., 1999), the Philippines (Chomel, et al., 1999) and Taiwan (Lee, et al., 1998; Chang, et al., 2006). In Thailand, the first report of *Bartonella* spp. was done by a surveillance of sero-prevalence in cats (Boonmar, et al., 1997). In 2000, prevalence of *B. henselae* in Thailand was confirmed (Maruyama, et al., 2000). In 2008, the first human case was published and it was the first document of *B. henselae* isolation in Thailand (Paitoonpong, et al., 2008). Three cases of endocarditis in human cause of by *B. tamiae* were reported in Khon Kaen province (Kosoy, et al., 2008). Those findings imply that Thailand may be an endemic area of *Bartonella* spp.

Cats are the main reservoirs of many species of *Bartonella* spp. including *B. clarridgeiae* (Kordick, et al., 1997), *B. koehlerae* (Droz, et al., 1999; Yamamoto, et al., 2003) and *B. bovis* (Chomel, et al., 2006), formerly *B. weissi* (Regnery, et al., 2000). In cats, there have been at least six potential zoonotic *Bartonella* spp., including *B. henselae*, *B. koehlerae* (Qurollo, et al., 2014), *B. clarridgeia* (Chomel, et al., 1999; Jacomo, et al., 2002), *B.vinsonii* subsp. *berkhoffii* (Bvb) (Varanat, et al., 2009), *B. quintana* and *B. bovis.* However *B. henselae* and *B. clarridgeiae* are the major cause of cat scratch disease in human patients. Cats and cat fleas were the major transmission of CSD to humans.

Transmission of *B. henselae* between cats

Cats are the major reservoir of *B.henselae*. It is transmitted among cat by cats fleas **(***Ctenocephalides felis*) via intradermal inoculation during fleas fed (Chomel, et al., 1996) but the transmission by fleas saliva not been reported. The publications confirmed that *Bartonella* DNA has been detected in fleas (Rolain, et al., 2003; Stevenson, et al., 2003). Ticks are one of the propose transmission vectors for *B. henselae* among cats and human (Chang, et al., 2001; Sanogo, et al., 2003). The other concerned vectors for *B. henselae* transmission vectors are lice (Durden, et al., 2004), sand files (Jacomo, et al., 2002) and biting flies (Chung, et al., 2004).

Moreover, there are not shown the transmission during mating between bacteremic female cats and uninfected male. In additional there was no transmission during their sharing food or litter boxes (Abbott, et al., 1997; Guptill, et al., 1997)

Pathogenesis of bartonellosis in cats

After the bacterial inoculation to cats, the bacterium was invaded and persist in red blood cells and endothelial cells of hosts for increasing their amount and distributed to tissues such as liver, brain, kidneys, heart, and lymph nodes (Kordick, et al., 1999). During the infection period *B. henselae* can stay inside red blood cell and macrophage that hide from the detection of host immune system and cause prolonged bacteremia in hosts (Dehio, 2004). The other blood-sucking arthropods can transmit these bacteria to the others (Schülein, et al., 2001; Greub and Raoult, 2002; Ehrenborg, 2007).

Clinical sign of feline bartonellosis

Healthy cat can be a carriers of *B. henselae*, as limited pathology has been associated with natural infection. It has been difficult to observe specify clinical signs of naturally infected cats with *B. henselae* (Chomel, et al., 2006). Based on serologic results, naturally infected cats are more likely to have lymphadenitis and gingivitis. Uveitis may also observed in naturally bartonellosis infection (Lappin and Black, 1999). However the severity of clinical signs can occur especially in those co-infected with the feline immunodeficiency virus or infected with varies strains of *Bartonella* spp. (Mikolajczyk and O'Reilly, 2000). In experimental conditions, cats infected with *B. henselae* mainly type II have developed various clinical signs than the infected with type I. The most commonly reported is fever which can persist for more than a week. Local inflammation (erythema, swelling) at the site of inoculation and localized or generalized lymphadenopathy have also been observed (Yamamoto, et al., 2003).

Zoonotic potential of *Bartonella* spp.

Cat scratch disease (CSD) is now the most common zoonosis worldwide. The incidence of CSD are increasing every year especially in children and young adult (Carithers, 1985). The transmissions of *B. henselae* from cats to humans mainly by cat scratch or cat bite (Carithers, 1985) with contaminated *B. henselae* in claws and tooth and possibly by flea bites (Rolain, et al., 2003). The clinical sign in immunocompetent people are lymphadenitis and fever. However Bacillary angiomatosis (BA) and endocarditis are often observed in immunocompromised people (Koehler and Tappero, 1993; Koehler, 1995). Cat fleas are the major cause of transmission and they spread CSD disease especially to cat owners who has close relationship with their cats. The recommendation on cat flea control is suggested.

Epidemiology of *Bartonella* spp.

An epidemiology of *Bartonella* spp. from domestic, wild animals and human have been reported in many countries. The highest prevalence shown up in areas with arthropod vectors (Guptill, 2003) including to the climate and geographic areas were related to endemic distribution of *Bartonella* spp. (Jacomo, et al., 2002; Vorou, et al., 2007). Al-Majali in 2004 reported high prevalence in warm and humid region where flea infestation is higher than in cold area (Al-Majali, 2004).

In Thailand, Maruyama et al(2001) reported the prevalence of *Bartonella* in stray cats at 27.6% (Maruyama, et al., 2001). In humans, the sero-prevalence 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). Many species of *Bartonella* were identified in Southeast Asian countries (Suksawat, 2016) as present in Table 2.

Table 2: *Bartonella* spp. infections in Southeast Asia.

SEA countries	***Bartonella* spp**	Sources
Thailand	*B. henselae* *B. clarridgeiae* *B. vinsonii* spp. *berkhoffii* *B. elizabethae* *B. tamiae* *B. coopersplainsensis* *B. phoceensis* *B. rattimassiliensis* *B. tribocorum* *B. vinsonii* spp. *arupensis* *B. queenslandensis* *B. grahamii* *B. quintana* *B. taylorii* BK1, KK1 and KK2 (novel genotypes) *B. bovis* *B. rochalimae*	Human, cat Human, cat, cat flea Cat, dog Rat flea Human, chigger mite, tick Rodent Rodent Rat, mice Rodent Rodent Rodent Dog Dog, human Dog Dog Water buffalo Rat flea
Indonesia	*B. henselae* *B. clarridgeiae* *B. phoceensis* *B. elizabethae* *B. rattimassiliensis*	Cat Rodent
The Philippines	*B. henselae* *B. clarridgeiae*	Cat flea
Myanmar	*B. henselae* *B. clarridgeiae*	
Malaysia	*B. henselae , B. clarridgeiae* *B. elizabethae, B. tribocorum* *B. queenslandensis*	Cat flea Rat
Singapore	*B. henselae*	Cat
Lao PDR	*B. clarridgeiae* *B. henselae* *B. tribocorum, B. rattimassiliensis* *B. queenslandensi,* *B. coopersplainsensis*	Cat flea Human Rodent
Cambodia	*B. elizabethae, B. rattimassiliensis,* *B. queenslandensis,* *B.. coopersplainsensis*	Rodent
Vietnam	*B. elizabethae, B. tribocorum* *B. queenslandensis,* *B. rattimassiliensis* *B. coopersplainsensis*	Rat mice

Note: SEA = Southeast Asia countries

Source: Suksawat, 2016 (in press).

Diagnosis of feline bartonellosis and CSD

At present, many methods were developed to differentiate and diagnose *Bartonella* spp. Since clinical presentations associated with bartonellosis in cats are non-specific, the diagnosis of the disease must be confirmed by the several laboratory testing methods. Laboratory testing of bartonellosis includes identification of *Bartonellae* in red blood cells by Giemsa coloration, primary culture technique, molecular genetics methods and serologic testing. The traditional gold standard method for diagnosis feline bartonellosis is bacterial culture method (Hansmann, et al., 2005). Primary culture technique can be sensitive but the technical expertise is required. Because they are fastidious and slow growing, it takes 5-45 days to form visible colonies. As described by Clarridge et al. in 1999, *Bartonella* spp. are cultivated in semisolid nutrient agar containing fresh rabbit blood or sheep blood or horse blood in 5% CO_2 at 35^{o}C. Molecular genetics methods are currently used such as restriction fragment length polymorphism (RFLP) of gene encoding citrate synthase, 16S rRNA or 16S-23S rRNA spacer region, and polymerase chain reaction (Roux, et al., 2000); however, it is costly.

At present, the common clinical diagnostic methods for feline bartonellosis is serological testing. Current serological testing for *Bartonella* spp. antibody includes indirect immunofluorescence assay (IFA) (Pons et al., 2005) and ELISA (Sander et al., 2001). IFA is not practical due to unavailable fluorescent microscope outside research laboratory or veterinary schools. Many studies showed the limitation of IFA which is very low in the sensitivity (Bergmans, et al., 1997; Zbinden, 1998). IFA is not only very low in the sensitivity but also cross reactivity between *Bartonella* spp. (Iralu, et al., 2006) and other bacterias as *Chlamydia* sp. are demonstrated (Maurin, et al., 1997). ELISA can facilitate the diagnosis of this disease as the advantages of the ELISA over IFA are: a) it is more quantitative and not subjective, b) more tests can be performed in a given time, c) it is more sensitive, and d) it does not need an expensive instrument.

According to the limitation of serological test as described, many proteins of *Bartonella* spp. were studied for developed and improved diagnosis tools for bartonellosis in Table 3

Table 3: Overview of studied pathogenic factor proteins of *B. henselae.*

Designation	Characterization	Role in infection process
VirB/D4 T4SS (type 4 secretion system)	virB operon (virB2-10)	Inhibition of apoptosis, NF-kB-dependent proinflammatory host response, invasion process of host cells
Beps	BepA-G	*Bartonella* effector proteins (Beps) translocated via VirB/D4 T4SS, BepA with antiapoptotic properties
OMPs (outer membrane protein)	23-92 kDa OMPs	NF-kB-dependent proinflammatory host response
OMP43	43 kDA OMP	Endothelail cell adherence
Fur	Iron-dependent gene regaulator	Unknown, influence on transcriptional regaulation of pathogenic factors?
Pep31	Hemin-binding protein	Unknown, bacterial replication within host cells?
CycA	Amino-acid transporter	Crucial for intracellular survival
CycA	Amino-acid transporter	Crucial for intracellular survival
VapA5	Virulence-associated protein A5	Crucial for intracellular survival
BadA (*Bartonella* adhesin A)	Trimetric autotransporter adhesin	Endothelial cell adherence, adherence to extracellular matrix, crucial for angiogenic reprograming, immunodominant

Source: Kempf, 2008

Until now, many specific proteins of *Bartonella* spp. from human products were used to develop ELISA test but they showed low sensitivity and specificity (Sander, et al., 2001; Schmiederer, et al., 2001; Litwin, et al., 2004; Loa, et al., 2006) The 17-kDa (VirB)(Anderson, et al., 1995; Sweger, et al., 2000) and outer membrane protein 43 (OMP 43)(Chenoweth, et al., 2004) are two of the few characterized proteins of *B. henselae* that induce antibody responses in hosts. There were many reports on serologic detection of bartonellosis by *B. henselae* specific protein (BSP) as 17-kDa (Loa et al., 2006) and OMP 43 (Burgess and Anderson, 1998; Fuhrmann, et al., 2001; Riess, et al., 2004) but all presented publications are mainly in humans. However the specific proteins as mentioned above should be used for the development of an antibody enzyme-linked immunosorbent assay (ELISA) for the serologic diagnosis of *B. henselae* infections in cats.

CHAPTER III
MATERIALS AND METHODS

PART I: General

Animals

Two hundred and fifty healthy cats presented to the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and other private animal hospitals in Bangkok metropolitan with normal physical examination, no history of previous treatment with antibiotic within three months were included. The study was approved by Chulalongkorn University Animal Care and Use Committee number 1031068.

Samples collection

Blood samples were collected from 250 healthy cats from the small animal hospitals in Bangkok metropolitan between November 2010 - November 2011. The amount of 2.5 ml of blood samples were collected by sterile technique from jugular vein. One ml of this placed into 2 EDTA tubes (Eppendorf, USA), 0.5 ml each. First tube was for complete blood count (CBC) and DNA preparation, the second tube was stored at -80°C for bacterial culture (Breitschwerdt and Kordick, 2000). Serum was prepared from the rest amount of blood sample (1.5 ml), and then stored at -80 ^{0}C for further analysis.

Bacterial strain

Bartonella henselae field strain(s) were grown from blood samples taken from healthy cats. They were harvested for further analysis.

E. coli BL 21 strains (Invitrogen, U.S.A.) was prepared for cloning and protein expression as published protocols (Loa, et al., 2006).

PART II : *Bartonella spp.* culture and identification

Bacterial culture (Clarridge, et al., 1995)

All blood samples from the second tube were used for *Bartonella* spp. isolation by bacterial culture which was the gold standard method. Thawed blood samples were centrifuged and the supernatant were drawn off and discarded. Subsequently, the pellet was re-suspended into 125 µl of isolation medium-199 (GIBCO corp.) (Koehler, et al., 1992). The re-suspended pellets was streaked onto Brain-heart infusion agar supplemented with 5% sheep red blood cells and incubated in a humidified chamber at 35^{o}C- 37 ^{0}C with 5% CO_2 condition for 5 to 15 days

The cultured plates were examined for bacterial growth and identified for *Bartonella* spp. base on Gram's stain. All *Bartonella* spp. are represent in gram negative bacilli with cocci-rod shape, the morphology {small colonies with 0.6-1 micron] , and grow slowly (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) and standard biochemical test (Cowan and Steel, 1973). The bacterium was biochemically inert with oxidase, catalase, urease and nitrate reductase negative except for the production of peptidase (Breitschwerdt and Kordick, 2000).

Then, the *Bartonella* culture positive samples were tested with polymerase chain reaction (PCR) (Norman, et al., 1995) DNA sequencing was employed for the *Bartonella* spp. confirmation (Sanger, et al., 1977).

PCR for specific identification of *B. henselae*

DNA preparation for PCR

DNA was extracted from each sample using QIAamp DNA mini kit (QIAGEN, Germany). Cat EDTA blood sample 200 µl or three to five colonies of each bacterial sample was mixed with 100 µl sterile water in a sterile test tube, then 20 µl of proteinaseK and 200 µl of lysis buffer was added and incubate at 56°C for 10 min. Two hundred (200) µl of absolute ethanol is added and transfered into the DNeasy Mini spin column (QIAGEN, Germany). The column was then centrifuged and washed twice with provided washing buffer. Subsequently, 100 µl of provided

elution buffer was added into the column to elute the DNA. Finally, extract DNA samples were kept at -80°C until using for other purposes.

PCR amplification of the oligonucleotide citrate synthase (*gltA*) gene

The PCR was used to detect the DNA of *Bartonella* spp. in both cat blood samples and colonies of bacterial samples as previously described (Norman, et al., 1995).

Table 4: The oligonucleotide primers used in this study.

Target gene	Primers	Oligonucleotide sequences
gltA primer	Forward-BhCS. 781p	5'-GGGGACCAGCTCATGGTGG-3'
	Revrese-BhCS. 1137n	5'-AATGCAAAAAGAACAGTAAACA-3'

All of PCR mixture were performed in 50 µl contained 10x buffer 5 µl, 2.5 mM dNTPs 4 µl, 100 µM each Forward-BhCS 781p and Revrese-BhCS 1137n (Table 4) 0.5 µl of each, 25 mM $MgCl_2$ reaction buffer 0.5 µl , 0.25 units *Taq* polymerase (Invitrogen) 2.5 µl, DNA template 5 µl and nuclease free water 32 µl. The PCR amplifications were performed in a MyCycle™ Thermal Cycler (BioRad Laboratories, USA). PCR cycle conditions were optimized using 95˚C 10 min, and 95˚C 30 s, 57 ˚C 1 min and 72˚C 2 min for 45 cycles then 72˚C for 5 min.

After processing, the PCR products were analyzed on agarose gel. PCR products were identified by 1% agarose in 1X Tris-borate-EDTA (TBE). The electrophoresis condition was 100 volts for 45 minutes in electrophoresis chamber with 1xTBE buffer. Subsequently, the gel was stained with ethidium bromide for 5 minutes and destained by removing the excess ethidium bromide with distilled water for 10 minutes. The PCR amplification product was visualized under an ultra-violet transluminator. The positive *Bartonella* samples demonstrated a 380-bp fragment for *Bartonella* spp.

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 elimination of excess primers, nucleotides, polymerase and salts. The purified DNA products were submitted for sequencing. The resulting sequences were compared using GenBank® database (www.ncbi.nlm.nih.gov) by BLAST software.

PART III: Production of *B. henselae* specific protein (BSP)

Selection of *B. henselae* specific protein (BSP)

The 17-kDa (VirB) characterized proteins of *B. henselae* was selected for development of an antibody enzyme-linked immunosorbent assay (ELISA) for the serologic diagnosis of *B. henselae* infections in cats.

Preparation of BSP

BSP was prepared by overexpression in *E. coli* expression system as following:

1. BSP gene preparation

The entire coding sequence of the published BSP gene was cloned from *B. henselae* genomic DNA by PCR. The set of oligonucleotide primers were synthesized (Table 5).

Table 5: The oligonucleotide primers was designed and used in the present study

Target gene	Primer	Oligonucleotide sequences
17-KDa protein (VirB)	Forward Nde I 17 kDa Bart PS	5'-GGGCATATGAAAAAATATAGCTTAGTCA -3'
	Reverse Xho I 17 kDa Bart PS	5'-GGGCTCGAGAAGTCGGACATCAGATTT -3'

BSP gene was amplified by PCR at conditions: 95 ^{0}C for 3 min. followed by 30 cycles of 95 ^{0}C for 30 s, 55 ^{0}C for 30 s, and 72 ^{0}C for 45 s then 72 ^{0}C for 5 min. The amplified product was digested with Nde I and Xho I and cloned to plasmid pET28a (Novagen, USA). The construct was transformed into competent *Escherichia coli*

(*E. coli*) strain BL 21. Plasmids were purified with a QIAquik mini-prep kit (Qiagen, Germany), and the in-frame DNA sequences were submitted for sequencing at AIT biotech, Singapore. The PCR product was called pET28a- BSP.

2. Expression of the BSP in *E. coli*

2.1 Cloning of 17 kDa gene

The 17kDa prepared from *B. henselae* isolation fields strain was cloned into pET28a expression vector. The feature of this vector contains 6xHistidine tagged at target protein.

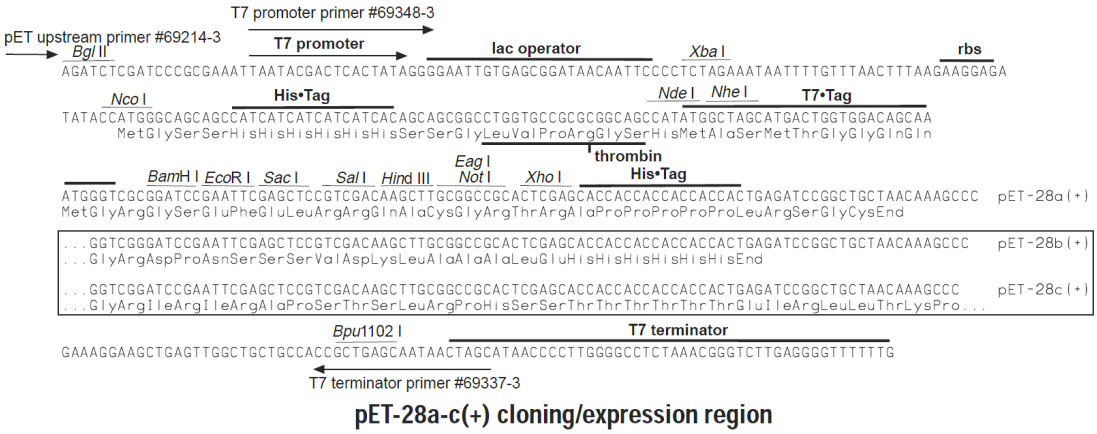

Figure 1: Cloning and expression region of pET28a

Source: www.novagen.com

2.2. Plasmid transformation to *E. coli* using heat shock method

The *E. coli* BL 21 were taken from -80^{o}C freezer. A 100 ng of the recombinant plasmid pET28a was added and mixed with 200 µl of competent cells in a 1.5 ml tube. The tubes were kept on ice for 30 minutes then put into water bath at 42^{o}C for 90 seconds and put tubes back on ice for 5 minutes. The cells were added with 1 ml of LB (with no antibiotic added), incubated for 1 hour at 37^{o}C, and centrifuged for 5 minutes at 3,000 rpm. The media was poured off and the remained of 200 µl was spread on LB plates with 50 µg/ml Kanamycin added and grown overnight. Three colonies of each mutants were picked up and grown in 3 ml LB with 100 µg/ml ampicillin added and incubated at 37^{o}C overnight for plasmid extraction. The plasmid was detected by gel electrophoresis of

0.8% agarose gels. DNA sequencing was performed to confirm 17kDa DNA sequence at AIT Technology, Singapore.

2.3 Optimization of the condition for protein expression

The colonies of *E. coli* BL21 harboring recombinant plasmid pET28a was cultured at 37°C with shaking 180 rpm in LB broth medium containing 50 μg/ml kanamycin. The empty vector transformed into *E. coli* BL21 cells were used as a control. The 1-3% overnight culture of *E. coli* was used as a starter culture and transferred into a new 200 ml LB broth containing 50 μg/ml kanamycin. The cell cultures were grown at 37°C with shaking at 180 rpm until the optical density at 600 nm of the solution of the culture reached 0.4-0.6. The condition of 17 kDa protein expression was verified (Table 6).

Table 6: Condition for 17 kDa protein expression

No.	Condition
1	The IPTG was added to a final concentration of 1 mM to induce the expression at 30°C with shaking at 180 rpm for 4 hours
2	The IPTG was added to a final concentration of 0.1 mM to induce the expression at 30°C with shaking at 180 rpm for 4 hours
3	The IPTG was added to a final concentration of 1 mM to induce the expression at 37°C with shaking at 180 rpm for 4 hours
4	The IPTG was added to a final concentration of 0.1 mM to induce the expression at 37°C with shaking at 180 rpm for 4 hours

Cells were harvested by centrifugation at 6,000 rpm for 10 minutes at 4^{o}C. The whole cell lysate were collected and the supernatant was discarded. The pellet was resuspended in 10 ml of sterile distilled water (SDW). The cell suspension was incubated with 1 mg lysozyme at room temperature for 30 minutes. After incubation, the cells were lysed by sonication using 40% amplitude, pulse on 10 seconds and off 5 seconds for 20 minutes. Sample must be on ice. The sample was centrifuged at 12,000 rpm for 10 minutes at 4^{o}C. After centrifugation, the supernatant was collected and the

pellets was resuspended in 2 ml of SDW as inclusion body fraction. The whole cell lysate, the supernatant and the inclusion body fractions of 17 kDa protein was identified using 12% SDS-PAGE gels.

2.4 Small scale protein expression

The colonies of 17 kDa pET28a were picked and grown in 3 ml LB with 50 µg/ml Kanamycin added and incubated at 37^{o}C overnight. The 1-3% of culture was transferred into a new 10 ml of LB broth containing 50 µg/ml Kanamycin. Cells were allowed to grow at 37^{o}C with shaking at 220 rpm, until OD600= 0.4 (~2-3 hours) then added 0.1 mM IPTG (isopropyl-b-D-thiogalactopyranoside) to grow at 37^{o}C with shaking at 220 rpm for 4 hours. The cells were kept at OD 600= 0.5 and transferred to 1.5 ml tubes, centrifuged at 5,000 rpm for 2 minutes. The supernatant was removed, the pellets were resuspended with 50µl sterile water then 4X sample buffer was added and boiled for 5 minutes. Eight microlitre of sample was subjected in 12% SDS-PAGE gels and visualized by Coomassie brilliant blue stain R-250. Then the large scale protein expression was performed in 100 ml LB broth to increase quantity of protein for protein purification process.

2.5 Extraction of the recombinant BSP from bacteria cell lysate

The bacterial cell pellet was lysed with Bugbuster reagent (Novagen, USA.) and Benzonase (Novagen, USA.) as manufacturer's handbook. The soluble supernatant and insoluble inclusion body pellet were analyzed by SDS-PAGE and Western blotting for the presence of the recombinant BSP.

2.6 Inclusion body solubilization

From protein expression, the inclusion body of 17 kDa protein was solubilized in 8 M urea at room temperature for 2 hours. The supernatant was collected by centrifugation at 12,000 rpm for 10 minutes at room temperature and then transferred into a new tube. The protein solubilized in 8 M urea which was the denatured form of 17 kDa protein was analyzed by 12% SDS-PAGE gels.

2.7 Refolding of purified BSP protein

The denatured of specific BSP recombinant protein in 8 M urea obtained from affinity chromatography was refolded using the concentrator, Amicon® Ultra-4 Centrifugal Filter Units (Merck, Germany) with cut off at 5 kDa. Five hundred milliliters of purified protein was transferred into the concentrator and then centrifuged at 6,000 rpm, 4°C until the supernatant on the top remained at 100 µl. Four hundred microliters of SDW was added and centrifuged at 6,000 rpm at 4°C until the supernatant on the top remained at 100 µl. Four hundred microliters of SDW was added and the total supernatant was collected and analyzed by SDS-PAGE.

2.8 Purification of the recombinant BSP

Either the supernatant or the denatured form of inclusion body of the recombinant BSP was resuspended in binding buffer (5 mmol/L imidazole, 0.5 mol/L NaCl, 20 mmol/L Tris–HCl, and 6 mol/L urea at pH 7.9) and transferred into His-trap-affinity column. The affinity column was prepacked with Protino® Ni-NTA Agarose (Macherey-Nagel, Germany). The column was equilibrated with SDW for 10 column volumes and column buffer (1X PBS solution pH7.4) for 10 column volumes. One ml of protein sample (1.364 mg/ml) was loaded into the column and collected the flow through fraction. After that, the column was washed with column buffer for 5 column volumes. The recombinant BSP was eluted with 1 column volume of elution buffer (1X PBS solution pH7.4 which contains 20, 50, 100 and 200 mM imidazole) and 3 column volumes of elution buffer (1X PBS solution pH7.4 which contains 500 mM imidazole). The flow through, wash and elution fractions were analyzed by 12% SDS-PAGE gels and kept at -20°C.

2.9 Western immunoblotting for detection and confirmation of expressed BSP

The nitrocellulose membrane, two fiber pads, and two Whatman papers (BioRad Laboratories, USA) were placed in a shallow tray filled with Transfer Buffer for a few minutes. The stacking gel was cut off from 12% SDS-PAGE gel with a clean razor blade and soak the gel in Buffer was transferred for a few minutes. The

transfer apparatus gel cassettes was opened with the black panel lying flat on the bottom of the tray filled with Transfer Buffer, the clear panel should be against the side of the tray. The transfer sandwich was prepared on the black panel in the tray filled with Transfer Buffer. one fiber pad, one Whatman papers, SDS gel, nitrocellulose membrane, one Whatman papers, one fiber pad. The air bubbles were removed by rolling a glass tube on the membrane. The sandwich was covered with the clear panel, fasten with the latch, and insert the gel cassette into the electrode module with the black panel facing the black cathode electrode panel. The bio-ice cooling unit was inserted into the buffer chamber, and filled the buffer chamber with Transfer Buffer, transferred for 70 minutes at 4ºC, stirred at a constant current of 100V and then the membrane was washed with distilled water on the shaker for 10 minutes. The membrane was incubated with blocking buffer on a shaker at 37 °C for 30 minutes. The positive cat serum by culture and PCR was used as primary antibody, diluted to blocking buffer (1:5000) and incubated the membrane with the blocking buffer the diluted primary antibody on a shaker for overnight at 4 ºC. The membrane was washed two times with blocking buffer on the shaker for 10 minutes each time. The anti-cat serum was used as secondary antibody (Goat pAb to cat IgG (HRP) (Abcam®, United Kingdom) was diluted to blocking buffer (1:5000) and incubated the membrane with the diluted secondary antibody on a shaker at 37 °C for 1.30 hours. The membrane was washed two times with distilled water on the shaker for 5 minutes each time. The membrane was washed with 0.05% PBS-tween on the shaker for 5 minutes and incubated in AEC Substrate Reagent (4 ml deionized water, 2 drops Acetate Buffer, 1 drop AEC Chromogen, 1 drop hydrogen peroxide) at 37 °C for 15 minutes until clear red insoluble signal was obtained for positive control. The membrane was washed with steriled distilled water.

PART IV Development of enzyme-linked immunosorbent assay (ELISA) for detection of antibody to *B. henselae* in cats

1. Standard serum samples

The positive serum samples were selected by positive of *B. henselae* both culture and PCR methods as same as the criteria for negative serum samples that confirmed for negative for both culture and PCR. The secondary antibody serum was used as control

2. Development of Indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibody to B. henselae in cats (Sangdee, et al., 2012)

The 250 ng of BSP protein was coated on each well of 96-well plate with 100 µl coating buffer (0.35 M sodium bicarbonate, 0.15 M sodium carbonate, pH 9.6) at 4^{o}C overnight. The solution were discarded and washed three times by phosphate buffer saline – tween (PBST; 1x PBS with 0.1% tween 20). The 100 µl of blocking solution (5% skim milk in PBST) were added to each well. The plate was incubated at 37^{o}C for an hour. The blocking solution were discarded and washed three times by PBST. The 100 µl of diluted cat serum (cat serums in PBST at a ratio 1:1000 by volume) were added to each well. The plate was incubated at 37^{o}C for an hour. The solution were discarded and washed three times by PBST. The 100 µl of secondary antibody solution (Goat pAb to cat IgG (HRP) (Abcam®, United Kingdom) in PBST at a ratio 1:5000 by volume) were added to each well. The plate was incubated at 37^{o}C for an hour. The solution were discarded and washed three times by PBST. The 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution (0.01 M sodium acetate, 0.003% hydrogen peroxide and 0.0004 mM TMB in deionized water) were added to each well. The plate was incubated at room temperature for 30 minutes in the dark place. The 50 µl of stop solution (0.1 M sulfuric acid) were added to each well. The optical density at 450 nm of the solution was measured using microplate reader.

3. The validation of the developed in-house *B. henselae* ELISA antibody detection kit

All positive serum samples for *B. henselae* antibody by culture and PCR were used to evaluate the BSP-ELISA. As same as negative serum samples that confirmed by culture and PCR were prepared to evaluation both sensitivity and specificity of BSP–ELISA test kit.

The sensitivity and specificity of the test kit were calculated using the following formula.

	Diagnostic test kit		
Bartonella spp.	Positive	Negative	Total
Positive	True positive (A)	False positive (B)	A+B
Negative	False negative (C)	True negative (D)	C+D
Total	A+C	B+D	A+B+C+D

Sensitivity = (A/(A+B)) x 100

Specificity = (D/(C+D)) x 100

Accuracy = ((A+D)/(A+B+C+D)) x 100

Predictive value positive test = (A/ (A+C)) x 100

Predictive value negative test = (D/(B+D)) x 100

CHAPTER IV
RESULTS

PART I: *Bartonella* spp. culture and identification

Bartonella spp. culture

Blood samples from two hundred and fifty healthy well-care client-owned cats were included. There were 138 female and 112 male cats. All cats were between 5 months to 15 years old with no visible flea infestation. The selected cats had close relationship with their owners and lived mostly in-door. All of them did not show any clinical signs throughout the study. The CBC result was shown in Table 7

Table 7: The Mean± SD of CBC blood profile cat samples

Parameter	Unit	Normal Value*	Cat samples
RBC	$\times 10^6/\mu l$	5-10	7.1 ± 2.8
WBC	$\times 10^3/\mu l$	5.5-19.5	9.8 ± 7.2
Neutrophils	%	45- 66	66.5 ± 2.3
Lymphocytes	%	27-36	25 ± 10
Eosinophils	%	0-4	4 ± 2.5
Basophils	%	0-1	-
Monocytes	%	0-5	0 ± 0.3

*Reference from The Merck Veterinary Manual

The result from *Bartonella* culture were 11 positive samples. All positive bartonellosis cats were Siamese-mixed breed 100% (11/11). Mean age of positive cats was 5±3.7 years. Mean weight of positive cats was 3.5± 0.4 kgs. The positive cats were neutered male 36.3% (4/11), and neutered female 63.7% (7/11). Another signalment of cat samples in the study was presented in Table 8. The positive samples were identified by Gram's staining, morphology as pinpoint colonies (Figure 2), growth characteristic and biochemical test. The bacterium was biochemically inert with oxidase, catalase, urease and nitrate reductase negative (Table 9).

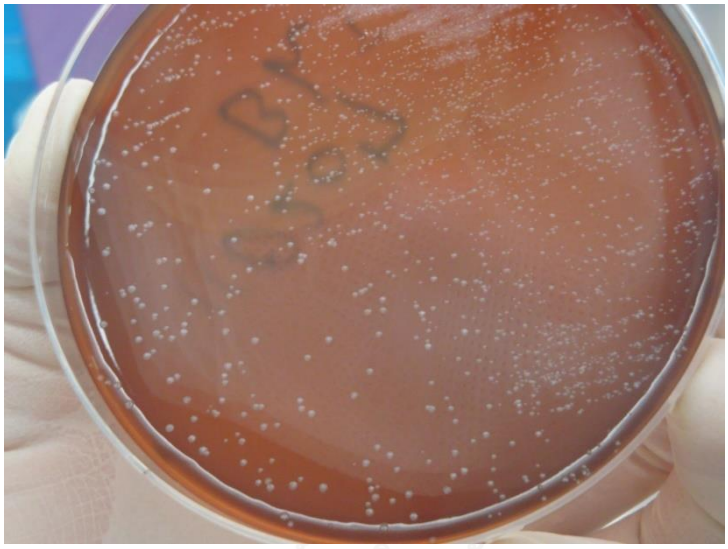

Figure 2: *B. henselae* colonies in blood agar

Table 8: The signalment of cat samples in the study

Criteria		Parameter	Result
Breed	Domestic Short Hair (DSH)	%	100
Sex	Female	%	6.4
	Female sprayed	%	50.6
	Male	%	3.1
	Male castrated	%	39.9
Life style	indoor	%	7
	outdoor	%	2.5
	Mix	%	90.5
Flea control	Spot on	%	97.5
	Oral product	%	0
	collar	%	0
	Not use	%	2.5

Table 9: The biochemical test result of *Bartonella* spp.

Biochemical test	Result
Oxidase	negative
Catalase	negative
Urease	negative
Nitrate reductase	negative

PCR for specific identification of *B. henselae*

All positive samples were identified for *Bartonella* spp. using PCR technique with *gltA* gene (Figure 3) and gene sequencing (Table 10). The resulted indicated the prevalence of *bartonellosis* in well-care client-owned cats in Bangkok metropolitant area between November 2010 - November 2011 was 4.4% (11/250). Among the 11 *Bartonella*-positive cats, 10 (91.9%) of the positive cats were infected with *B. henselae,* only 1 (9.1%) cat was infected with *B. clarridgeiae*.

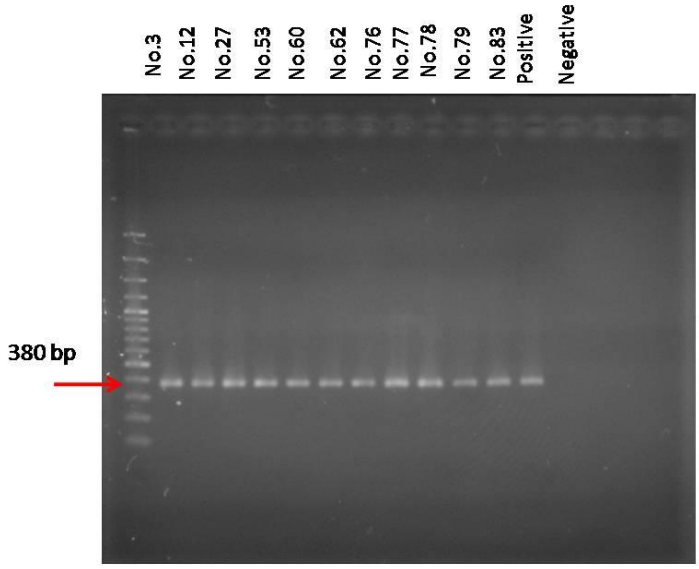

Figure 3: The PCR results of *gltA* gene in 11 well-cares client-owned cats with positive *Bartonella* spp.

Table 10: The results of 11 positive samples DNA sequencing from blood samples

Samples	*Bartonella* spp.	% Similarity
No3	*B.henselae*	99.69%
No12	*B.henselae*	100%
No27	*B. henselae*	100%
No53	*B. henselae*	100%
No60	*B. clarridgeiae*	100%
No62	*B. henselae*	100%
No76	*B. henselae*	99.69%
No77	*B. henselae*	99.69%
No78	*B. henselae*	100%
No79	*B. henselae*	99.69%
No83	*B. henselae*	100%

PART II: Production of *B. henselae* specific protein (BSP)

BSP gene preparation

Cloning of 17 kDa protein gene

The 17 kDa protein gene was cloned into pET28a, it was composed of 18 bp His tag-coding sequence, 27 bp T7 tag-coding sequence, 51 bp sequence of pET28a expression vector, 6 bp restriction size. Therefore, the total length of 17 kDa protein gene was estimation at 20-23 kDa. The DNA sequence of 17 kDa protein gene recombinant was sequenced by AIT Biotech, Singapore. The result present in Figure 4

ATG AAA AAA TAT AGC TTA GTC ACA TTG TTA TCT TTT TGC ATC CCT CAT GCA AAA GCA CAA ACA GCA CCC CTT ACT GAT GAA TAT TAT AAA AAA GCC TTA GAA AAC ACG CAA AAA TTA GAC GTT GCA AAA TCA CAA ACA GCT GAG TCT ATT TAT GAA TCT GCA ACA CAA ACT GCA AAA AAA TTC AGG AAT TAA ACG AGA AGC TTG AAA AGG CTA AAA CAG CTG AAA AAA CCA AAT CTG AAG AAT TGC AGG CTC TTC AAG TAG AGC TCT CTC TTC TCC AAG CAC AGT TGC AAG TGG ATA CTT TAA AAG TTC AGT CCC TTT CTA TGA TTC AAG CAA AAG ATA CAA AAA CAA AAG AAG AAC TGC GTG AAG AGC AAA CGC AGC AAA ATC ATA AAA AAA TTG AAG AAA AAT TAA AAG AAA AAC TTG GGA AAT CTG ATG TCC GAC TTC TCG AG

Figure 4: Amino acid sequence cloning alignment of 17 kDa protein

The cloning sequence of 17 kDa was blast in www.ncbi.com. The result of sequence producing alignment with accession number U23447 shown similarity 99% in *Bartonella henselae* VirB and 99% in *B. henselae* gene for 17 kDa, respectively as show in Figure 5.

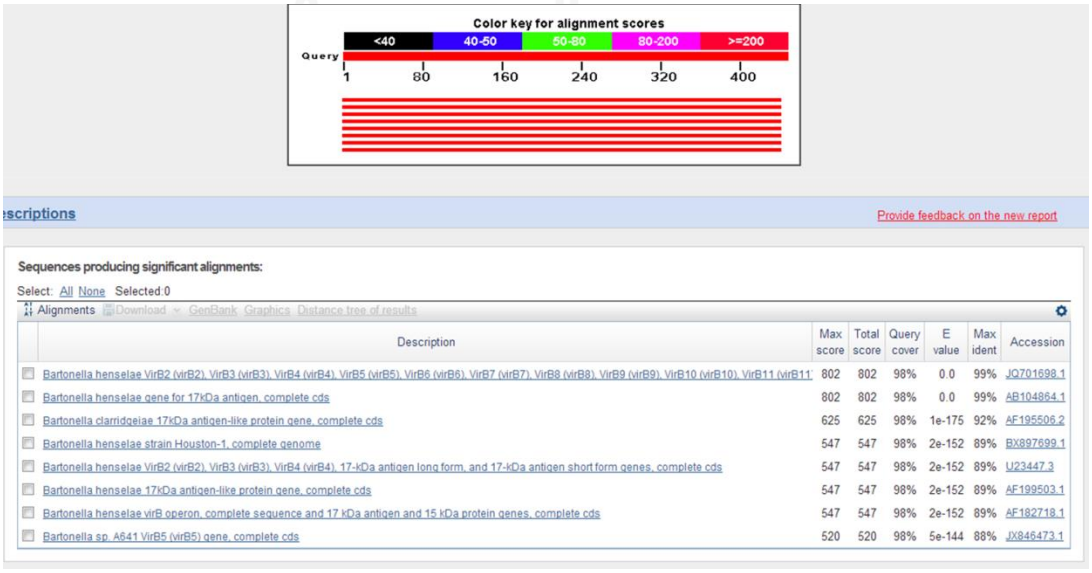

Figure 5: Sequence producing alignment

Small scale protein expression in pET28a

The colonies were verified by PCR and DNA sequencing. The DNA sequences of colony mutants are colonies No. 3, 4. The colonies were picked and grown in 10 ml LB containing 50 µg/ml Kanamycin and 0.1 mM IPTG to induce protein expression of each selected colony. Total proteins were separated on 12% SDS-PAGE and visualized by Coomassie blue-R stain. Figure 6 shows the result of protein expression at 23 kDa in both colonies when compared to non-induced colonies. Large scale protein expression was performed for protein purification.

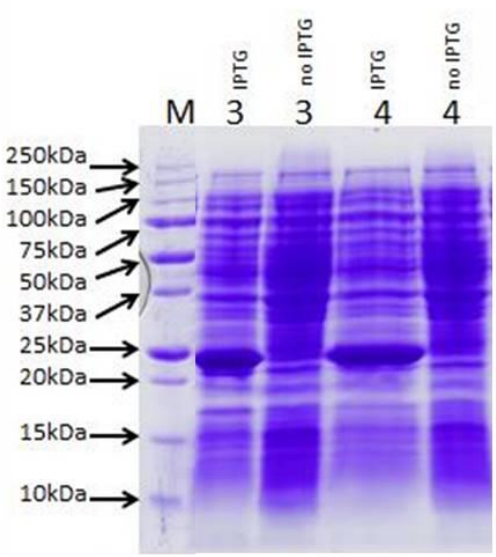

Figure 6: Fraction identification of 17 kDa protein expression with 0.1 mM IPTG in 10 ml LB broth

Lane M was representing to protein molecular weight marker

Large scale protein expression in pET28a

The expression of colony No. 3 was selected and induced by 1 mM IPTG and the cultures were grown at 37^{o}C in LB broth 100 ml. The lysed cell, soluble fractions and inclusion bodies was analyzed on 12% SDS-PAGE (Figure 7). Inclusion bodies were detected by western blotting using anti-cat serum. The SBHPG was expressed as inclusion bodies in *E. coli*.

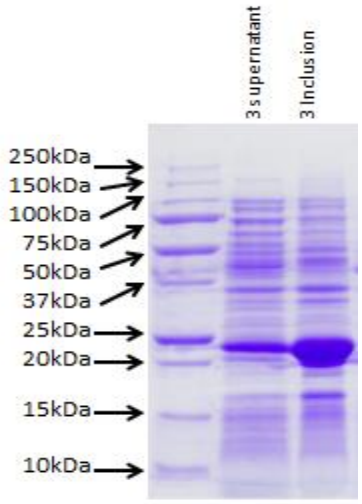

Figure 7: Fraction identification of 17 kDa protein expression with 0.1 mM IPTG in 100 ml LB broth

The extraction and Inclusion body solubilization

The protein extraction and solubilization of inclusion body and supernatant were shown in Figure 8. The product obtained was further performed for protein purification in the next step.

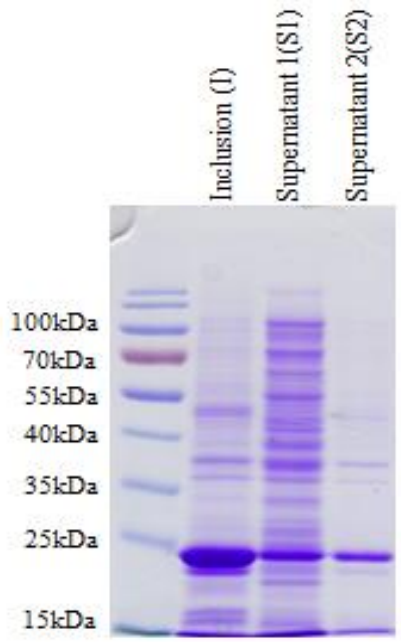

Figure 8: Fraction identification of Inclusion body and supernatant

The first lane: Protein molecular weight marker, Lanes 2, 3 and 4: Inclusion and supernatant after 1st and 2nd re-suspension of bacteria harboring pET28a, respectively.

17 kDa protein purification

After protein expression, the supernatant of 17 kDa protein was purified using His-trap-affinity column. The flow through, wash and elution fractions were analyzed on 12% SDS-PAGE. This result indicated that both inclusion body and supernatant of recombinant BSP protein was successfully purified.

The purify process was performed, the result of purification protein at 23 KDa was represented in Figure 9 and confirmed by Western Immunoblot (Figure 10)

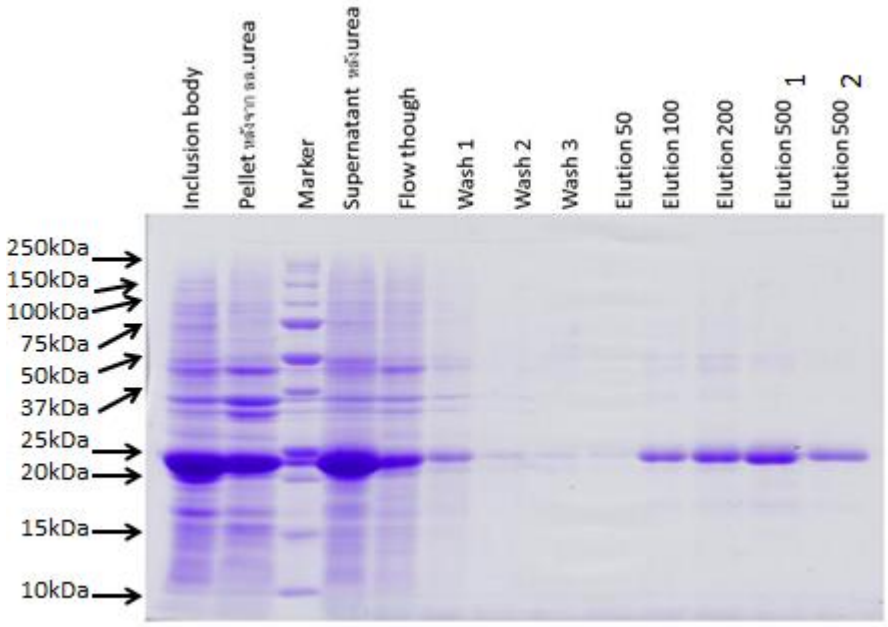

Figure 9: The SDS page analysis of supernatant recombination 17 kDa after solubilized in 8M urea purification

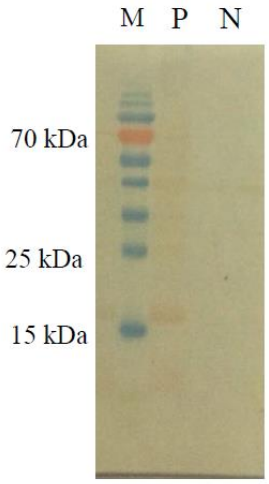

Figure 10: Western immunoblot results of the purified BSP

Lane M: molecular weight marker

Lane P: positive serum Lane N: negative serum

PART III: Development of *B. henselae* ELISA antibody detection method

The 10 positive and 5 negative cat serum samples by culture and PCR confirmation were analyzed by microplate reader (BioRad Laboratories, USA) as presented in Table 10. The results from microplate reader were calculated for sensitivity and specificity as shown in Table 11. However the absorbance of OD result both positive and negative samples were not different.

Table 11: The absorbance result by microplate reader

	No 1	No.2	No.3	No.4	No. 5	No. 6	No.7	No.8	No. 9	No. 10
Positive	0.8083	1.0556	0.4936	1.0646	0.7223	1.1991	0.9184	0.4960	0.8783	1.0221

	No.1	No.2	No.3	No.4	No. 5
Negative	1.6683	1.3883	1.1751	0.6443	1.0420

Control	0.0869

Table 12: The result of sensitivity and specificity

	Diagnostic test kit		
Bartonella spp.	Positive	Negative	Total
Positive	10	0	10
Negative	5	0	5
Total	15	0	15

Sensitivity = (10/(10+0)) x 100 = 100

Specificity = (0/(0+0)) x 100 = 0

Accuracy = ((10+0)/(10+0+5+0)) x 100 = 66.7

Predictive value positive test = (10/ (10+5)) x 100 = 66.7

Predictive value negative test = (0/(0+0)) x 100 = 0

CHAPTER V
DISCUSSIONS

Bartonellosis is an emerging zoonotic disease in many countries. Cat is an important host for *Bartonella* spp. which can be transmitted to cause bartonellosis or cat scratch disease (CSD) in humans. Three species of *Bartonella* has been identified from cats in Thailand including *B. henselae* and *B. clarridgeiae* (Boonmar, et al., 1997; Jitchum, et al., 2009), and *B. kohlerae* (Assarasakorn, et al., 2012). According to zoonotic concerned on bartonellosis, there were many reports of bartonellosis in Thai pateints as *B. henselae, B. tamiae, B. visonii ver berkhoffii* (Suksawat, 2006; Kosoy, et al., 2008; Paitoonpong, et al., 2008)The vectors were most concerned on transmission process of bartonellosis to human due to Thailand was endemic area of tick, flea and other ectoparasites. In the past, there were many reports of prevalence in the major carrier as cats but almost were focus on stray cats because they were most infestrated with fleas and without fleas control programs. The present study reported the prevalence of *Bartonella* spp. in healthy well-care client-owned cats. The prevalence of *Bartonella* infection in cats was different from the previous reported by Jitchum et al in 2009, regarding on different in group of cat, samples size, and target gene used in the PCR. The report shown distribution of *Bartonella* infections among stray cats was higher than the well-care client-owned cats. Therefore, the similarity of report of Jitchum et al., (2009) and this study is the prevalence of *B. henselae* found was higher than *B. clarridgeiae*. The caring of well-care client-owned cats by flea control programs may help to reduce prevalence of *Bartonella* spp. in well-care client-owned cats. The results of the prevalence of *Bartonella* spp. in this study was suggested that Bartonellosis in the well-care client-owned cats and *Bartonella* zoonoses (cat scratch disease) should be also concerned. The awareness on the transmission of *Bartonella* spp. from cats to their owners especially cats which were very close relationship with their owners and owner's family members including children and immune-compromised owners should be more seriously consideration.

Interestingly, all cats in this present study had no flea infestation on visible examination but the prevalence of *Bartonella* spp. has still remained. Most of the cats

(more than 80%) use once a month heartworm and fleas prevention spot on products. This evidence was suggested that the prevalence of cats infected with *Bartonella* spp. may also be from other vectors such as wood louse hunter spider (Mascarelli, et al., 2013) or chigger mite (Kosoy, et al., 2010). The important role of vector for *Bartonella* spp. should be the further objectives for bartonellosis research in the future.

There are many diagnostic methods for detection of bartonellosis in cats including blood culture, PCR, immunofluorescent assays (IFA) and ELISA. For ELISA, many specific proteins of *B. henselae* were used to develop ELISA test including the 17-kDa (VirB), *Bartonella* effector proteins (Beps), outer membrane protein(OMP) (Kempf, 2008), and heat shock protein (GroEL) (Ferrara, et al., 2014). The 17 kDa protein (VirB) is one of the most candidates for a good antigenic proteins highly specific for *B. henselae* infection (Anderson, et al., 1995; Loa, et al., 2006; Eberhardt, et al., 2009; Ferrara, et al., 2014) and used for *Bartonella* antibody detection kit in human (Anderson, et al., 1995; Sweger, et al., 2000). Therefore, the 17 kDa or VirB protein was selected and produced for using in development of indirect ELISA antibody detection for *B. henselae* infection in cats in this present study. This study has been focused on expression of recombinant protein from the VirB gene by using pET28a *E. coli* expression system which differed from expression vectors and primers from other previous study (Loa, et al., 2006; Ferrara, et al., 2014). Thus, our study gives the original method for *Bartonella* VirB protein expression by using the expression vector pET28a. The recombinant VirB protein was expressed 0.1 mM IPTG incubation at 37^{o}C for 4 hours. This VirB was expressed both as inclusion body and supernatant. However, it was produced much more as an inclusion body component. The VirB protein is scarcely soluble due to deposition in inclusion bodies same as experiment of Loa et al. (2006). The result also indicated that the VirB protein found was expressed as inclusion body, because high temperatures during expression and high level expression of recombinant protein in *E. coli* causes aggregated protein formation referred to inclusion body. The inclusion bodies of recombinant protein need to elaborate solubilization, refolding and purification for recover biological activity of protein (Vallejo and Rinas, 2004). Therefore, the inclusion body of VirB protein was solubilized in 8 M urea. The clear supernatant,

obtained from inclusion body solubilization, was successfully purified by affinity chromatography. However, the denatured VirB protein obtained from affinity chromatography may not successfully refolded to remove the denaturant, because the results of our ELISA assay were presented with false positive that may be related to the denature of VirB protein during solubilization processes. In addition, on the false positive the VirB protein on detection of antibody of *B. henselae* could be related to the long period of persistent of antibody levels (Chomel, et al., 2003). A preliminary validation of the antigenic properties of the recombinant protein 17 kDa or VirB in this study was performed by western blot analysis. The results demonstrated positive reactivitiy in all samples.

Loa et al. (2006) also select the 17 kDa (VirB) protein from *B. henselae* (strain ATCC 49822 or Houston strain) for expression in expression vector pTriEx-4. They can be success to express VirB protein by pTriEx-4 and use it for antibody-capture ELISA. Their ELISA test results gave the sensitivity 71.1% and specificity 93% which is different from our results. The ELISA result of VirB from *B. henselae* in our study shown high sensitivity but no specificity when compared to the another ELISA test in human study that showed 81% and 95% on sensitivity and specificity, respectively (Bergmans, et al., 1997). The differences in sensitivity and specificity were also reported low sensitivity and high specificity on ELISA IgG test in human patients at 65% and 91%, respectively(Vermeulen, et al., 2007).

Our VirB specific protein gene was successfully expressed in pET28a expression vector and design specific primers for evaluation ELISA in cat blood samples. The difference between the cat and human serum samples was one of the most parameters which make a different in ELISA results. The other important factor for the differences in results may be due to different methods of antigen preparation. As well as the difference in two different of *B. henselae* Houston and Marseille (Zhoa et al., 2005). His studied contributed a difference on virulence proteins and antigenicity on host response (Zhao, et al., 2005). Antigen of *B. henselae* (ATCC 49882) from patients with cat scratch disease yield 86.9% sensitivity and 96% specificity due to the process of protect *Bartonella* antigen from heat degradation (Not, et al., 1999).

Our VirB protein in the present study is still need more validation. More concern must be on the specificity for the ELISA development. Another important concern is the cross reactivity among *Bartonella* spp. with *Chlamydia* spp. (Maurin, et al., 1997) and different strain of *B. henselae* would be needed to clarify before the development of *B. henselae* validated ELISA test kit for cats. Moreover, *B. henselae* diagnosis is still depending on manually intensive, time consuming assays including culture, micro-immunofluorescence, western blotting or indirect immunofluorescence. Our specific 17 kDa or VirB protein from *B. henselae* field strain in Thai cats is one step further to develop rapid identification of *B. henselae* infection as in ELISA test kit. The potential ELISA will be used for screening non-clinical infected cats and prevent cats owners from get infected by their own cats. Many limitations were found in the development of ELISA in this study including; the need of true negative serum sample from specific pathogen free cats, the cross-reactivity with other bacteria such as *Chlamydia* spp. or different species of *Bartonella* spp. and different strain of *B. henselae* in cat blood samples. The further research should be concerned for all of the limitations.

The evaluation on other specific antigenic protein genes of this bacterial species should be further investigation. The protein expression in difference expression vectors and conditions should also be further performed for discovering the soluble proteins which can be a good candidate for development of ELISA assays for detection of cat antibody to *B. henselae*. The development of high sensitivity and specificity of ELISA can also support for the prevention and control strategies of *B. henselae* infection in cats and cat scratch disease (CSD) in humans.

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APPENDICES

Appendix A: History record of the feline bartonellosis project

เบอร์บัตร (HN) …….	
ผู้ทำการศึกษา	สพ.ญ.ภควัน ศาสตรานรากุล
อาจารย์ที่ปรึกษา	รศ.สพ.ญ.ดร.รสมา ภู่สุนทรธรรม
	รศ.น.สพ.ดร.สถาพร จิตตปาลพงศ์
	ผศ.น.สพ.ดร.ชาญณรงค์ รอดคำ

วันที่…….....เดือน.....พ.ศ.....

ชื่อเจ้าของสัตว์…….....นามสกุล.....

ที่อยู่.....

เบอร์โทรศัพท์.....

ชื่อแมว.....สีขน / รูปแบบ.....ตำหนิ / จุดเด่น.....

พันธุ์ ( ) พันธุ์ไทย ( ) วิเชียรมาศ ( ) โคราช (สีเทา) ( ) ขาวมณี (สีขาว)

( ) ศุภลักษณ์ (สีทองแดง) ( ) อื่น ๆ (โปรดระบุ) .....

( ) พันธุ์ไทยผสม ( ) พันธุ์ Abyssinian ( ) พันธุ์ Russian Blue

( ) พันธุ์ Burmese ( ) พันธุ์ Himalayan ( ) พันธุ์เปอร์เซีย

( ) พันธุ์ ๆ (โปรดระบุ).....

อายุ.....ปี.....เดือน

เพศ ( ) ผู้ ( ) ผู้ตอนแล้ว ( ) เมีย ( ) เมียตอนแล้ว (ตอนเมื่ออายุ.....ปี.....เดือน)

น้ำหนักตัว.....กิโลกรัม ( ) จากการชั่งน้ำหนัก ( ) จากการประมาณน้ำหนัก

ประวัติการเลี้ยงดู

ชนิดของอาหาร ( ) อาหารกระป๋อง ยี่ห้อ( ) อาหารเม็ด ยี่ห้อ ( ) อาหารปรุงเอง

รายละเอียดอื่น ๆ เพิ่มเติม.....

การคุมกำเนิด ( ) ฉีดยาคุม ( ) ทำหมัน ( ) กักบริเวณ ( ) อื่น ๆ (โปรดระบุ).....

ประวัติการทำวัคซีน ( ) โรคพิษสุนัขบ้า ( ) โรคไข้หัดแมว โรคหลอดลมอักเสบและหวัดติดต่อ

( ) โรคลิวคิเมีย ( ) ไม่เคยฉีดวัคซีน

ลักษณะสถานที่เลี้ยงสัตว์ ( ) บ้าน ( ) เทาว์เฮาส์ ( ) ตึกแถว ( ) คอนโด

ลักษณะการเลี้ยงสัตว์ ( ) เลี้ยงปล่อย ( ) ขังกรง ( ) อยู่แต่ในบ้าน

( ) อื่น ๆ .....

การตรวจร่างกายทั่วไป อุณหภูมิร่างกาย .....

( ) มีหมัดบนตัว ( ) มีไรในหู ( ) ต่อมน้ำเหลืองโต

( ) เหงือกอักเสบ ( ) ตาเจ็บ เป็นแผลที่กระจกตา ( ) โลหิตจาง

ประวัติการใช้ยาปฏิชีวนะ

( ) กลุ่มยา..... ( ) ระยะเวลาการใช้.....

( ) ใช้ยาครั้งสุดท้ายเมื่อ.....

การป้องกันหมัด ( ) ใช้ยาหยดหลังทุกเดือน ยี่ห้อ..... ( ) ให้กินยา

( ) ใช้ปลอกคอ ( ) ไม่ได้ทำการป้องกัน

การป้องกันพยาธิภายใน

ถ่ายพยาธิ ( ) เคย (โปรดระบุ)..... ( ) ไม่เคย

ความถี่ในการถ่ายพยาธิ ( ) ปีละครั้ง ( ) 2 ครั้งต่อปี ( ) ทุกสามเดือน ( ) เดือนละครั้ง

Appendix B: Brain-heart infusion agar supplement with 5% blood agar

1) Suspend 80g of powder (Brain-heart infusion agar) in 2L distilled water.
2) Stiller and boil the powder until the contents are dissolved in part.
3) Sterilize and dissolve the contents by using autoclave at 121°C for 15 min.
4) Add sheep bloods into the contents. Adjust 5% blood including-agar plates.
5) Apply the dissolved contents to petri dish aseptically.
6) Keep the agar plates at 4°C until used.

Appendix C: Culture animal bloods

1) Freeze animals blood in the deep freezer (-70~80°C).
2) Thaw the freezing blood in the room temperature, and the 200 μl blood is separated into 1.5 ml tube.
3) Centrifuge these tube at 3600 rpm for 75 min, and discard supernatant from tube.
4) Add medium 199 into tube, and mix.
5) A 100 μl of each was placed on a Brain-heart infusion agar containing 5% sheep blood, and incubate these plates aerobically at 35°C in 5% CO_2 for up to 4 weeks.

If you discover small colonies on the agar plates, pick up one colony, and subsequently subculture on fresh above agar. After pure culturing, all of colonies were harvested by sterile loop and divided into two passages.

The colonies were stocked into 1.5 ml medium 199 containing 10% DMSO (dimethyl sulfoxide) and other way is that remaining of colonies were dissolved into 500μl distilled PBS for DNA extraction.

Appendix D: Components of culture medium for *Bartonella* isolation

Medium 199 (GIBCO corp.)	79 ml
100mM MEM Sodium Pyruvate Solution (GIBCO corp.)	1ml
Fetal bovine serum (GIBCO corp.)	20 ml
total volume	100ml

After mixing the above reagents, sterile the solution by filtration.

Appendix E: Preparation of stock medium for the strains

Medium 199 for *Bartonella* isolation		90 ml
DMSO		10 ml
	total volume	100ml

After stocking a *Bartonella* strain into the stock medium, store this under -70~80°C or liquid nitrogen

Appendix F: DNA extraction

1) For extraction from colonies, use InstaGene Matrix (BIO-RAD corp.).
2) Bacteria resuspended in 500μl PBS are vortexed for 10 sec, and subsequently centrifuge at 13,200rpm for 5 min.
3) After removing supernatant from above tube, add 50μl InstaGene Matrix to the tube and incubate at 56C° for 30 min.
4) Place the tube in a 100C° heat block or water bath for 8min.
5) Vortex the tube for 10 sec. Centrifuge at 13,200rpm for 5min, and take the supernatant from the tube.
6) Adjust the DNA concentration of the supernatant to 20ng/μl.

Appendix G: PCR technique for *gltA* gene

Targeted gene, *gltA* is the most popular gene for molecular characterization of genus *Bartonella*. Product size is expected to be about 380 bp, and the data bases was registered in GeneBank for phylogenetic analysis.

Preparing

Nuclease-free water, positive control DNA (*B. henselae*), negative control (Nuclease-free water), DNA sample

composition	adding volume (µl)
10x Buffer	5
25 mM $MgCl_2$	3
2.5 mM dNTPs	4
Taq polymerase	2.5
100 µM primer	Forward 0.5 Reverse 0.5
Sample DNA	5
Nuclease-free water	32
Final volume	50

1) Stand 0.2 ml tubes on the ice block, and add the above reagents and DNA samples.
2) Set the above tube on the thermal cycler, and run a PCR.
3) After the PCR, confirm the products size by using an electrophoresis. If it is in the right DNA band shows at the portion of above 350bp.