DEVELOPMENT OF ELISA FOR DETECTING ANTIBODY AGAINST *BARTONELLA* SPP. SPECIFIC PROTEIN IN CATS AND ITS APPLICATION IN NATURAL INFECTION

Mr. Krissda Boonaramrueng

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Veterinary Medicine
Department of Veterinary Medicine
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2021
Copyright of Chulalongkorn University

การพัฒนาชุดทดสอบอีไลซ่าเพื่อตรวจแอนติบอดีต่อโปรตีนที่มีความจำเพาะของ
เชื้อ*บาร์โทเนลลา* สปีชี่ส์ ในแมว และการประยุกต์ใช้ตรวจวินิจฉัยในการติดเชื้อโดยธรรมชาติ

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

นายกฤษฎา บุญอร่ามเรือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาอายุรศาสตร์สัตวแพทย์ ภาควิชาอายุรศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	DEVELOPMENT OF ELISA FOR DETECTING ANTIBODY AGAINST *BARTONELLA* SPP. SPECIFIC PROTEIN IN CATS AND ITS APPLICATION IN NATURAL INFECTION
By	Mr. Krissda Boonaramrueng
Field of Study	Veterinary Medicine
Thesis Advisor	Associate Professor ROSAMA PUSOONTHORNTHUM, D.V.M., M.Sc., Ph.D., DTBVM.
Thesis Co Advisor	Associate Professor CHANNARONG RODKHUM, D.V.M., Ph.D, DTBVP. Associate Professor Anchanee Kubera, B.Sc., M.Sc., Ph.D Navapon Techakriengkrai, DVM., M.Sc., Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

--- Dean of the FACULTY OF VETERINARY SCIENCE

(Professor SANIPA SURADHAT, D.V.M., Ph.D., DTBVP.)

DISSERTATION COMMITTEE

--- Chairman

(Professor SOMSAK PAKPINYO, D.V.M., Ph.D.,DTBVM.)

--- Thesis Advisor

(Associate Professor ROSAMA PUSOONTHORNTHUM, D.V.M., M.Sc., Ph.D., DTBVM.)

--- Thesis Co-Advisor

(Associate Professor CHANNARONG RODKHUM, D.V.M., Ph.D, DTBVP.)

--- Thesis Co-Advisor

(Associate Professor Anchanee Kubera, B.Sc., M.Sc., Ph.D)

--- Thesis Co-Advisor

(Navapon Techakriengkrai, DVM., M.Sc., Ph.D.)

--- Examiner

(Associate Professor ACHARA TAWATSIN, B.Sc., M.Sc)

--- Examiner

(Prapaporn Jongwattanapisan, D.V.M., Ph.D., DTBVM.)

--- External Examiner

(Professor Sathaporn Jittapalapong, D.V.M., Ph.D.)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

กฤษฎา บุญอร่ามเรือง : การพัฒนาชุดทดสอบอีไลซ่าเพื่อตรวจแอนติบอดีต่อโปรตีนที่มีความจำเพาะของเชื้อ*บาร์โทเนลลา* สปีชี่ส์ ในแมว และการประยุกต์ใช้ตรวจวินิจฉัยในการติดเชื้อโดยธรรมชาติ. (DEVELOPMENT OF ELISA FOR DETECTING ANTIBODY AGAINST *BARTONELLA* SPP. SPECIFIC PROTEIN IN CATS AND ITS APPLICATION IN NATURAL INFECTION) อ.ที่ปรึกษาหลัก : รศ. สพ.ญ. ดร.รสมา ภู่สุนทรธรรม, อ.ที่ปรึกษาร่วม : รศ. น.สพ. ดร.ชาญณรงค์ รอดคำ,รศ. ดร.อัญชณี คูเบอร่า,อ. น.สพ. ดร.นวพล เตชะเกรียงไกร

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินปัจจัยเสี่ยงและความสัมพันธ์ระหว่างการติดเชื้อ *Bartonella* spp. และสถานะของภูมิคุ้มกัน โดยใช้แมวที่ติดเชื้อ retrovirus เป็นแบบจำลอง และเพื่อผลิตและพัฒนารีคอมบิแนนท์โปรตีนแอนติเจนจำเพาะของเชื้อ *B. henselae* (17kDa และ GroEL) สู่ชุดตรวจ ELISA สำหรับการตรวจหาแอนติบอดีต่อการติดเชื้อ *Bartonella* spp. ในแมว โดยเก็บตัวอย่างเลือดแมว 161 ตัวอย่างจากคลินิกและโรงพยาบาลสัตว์ในเขตกรุงเทพฯ และปริมณฑล ในช่วงระหว่างปี 2560-2563 โดยทำการศึกษาสถานะการติดเชื้อ *Bartonella* spp. (โดยวิธี PCR และ IFA serology), การตรวจเลือด, การติดเชื้อ retrovirus (FIV และ FeLV) และระดับ T lymphocyte subsets ($CD4^+$, $CD8^+$ และ อัตราส่วน $CD4^+$ ต่อ $CD8^+$) จากการศึกษาพบความชุกของเชื้อ *Bartonella* spp. เท่ากับ 16.1% โดยวิธี PCR และ 94.9% โดยวิธี IFA serology และยังพบว่าแมวที่มีอายุมากกว่า 1 ปีมีความเสี่ยงที่จะพบผลทางซีรั่มวิทยาเป็นบวกมากกว่าแมวที่อายุน้อยกว่า 1 ปี (OR 4.296; 95%CI: 1.010 - 18.275) นอกจากนี้ยังพบระดับ $CD8^+$ สูงขึ้นอย่างมีนัยสำคัญในแมวที่ให้ผลซีรั่มวิทยาเป็นบวก ($p = 0.026$) และยังพบการลดลงอย่างมีนัยสำคัญของอัตราส่วนของ $CD4^+$ ต่อ $CD8^+$ ในแมวที่ติดเชื้อ *Bartonella* spp. ร่วมกับเชื้อ retrovirus ($p = 0.041$) นอกจากการศึกษาความชุกและปัจจัยเสี่ยงในแมวแล้วในการศึกษานี้ยังได้พัฒนาชุดทดสอบสำหรับตรวจหาแอนติบอดีต่อเชื้อ *Bartonella* spp. ในแมว โดยใช้โปรตีนจำเพาะชนิด 17-kDa และ GroEL ของเชื้อ *B. henselae* โดยทำการโคลนยีน *17-kDa* และ *groEL*ในเวกเตอร์ pET28b และ pH6HTC และชักนำให้เกิดการผลิตรีคอมบิแนนท์โปรตีนโดยใช้ IPTG จากนั้นรีคอมบิแนนท์โปรตีนจะถูกทำให้บริสุทธิ์โดยแอฟฟินิตี้โครมาโตกราฟีโดยใช้เมทริกซ์ชนิด HisTrap และยืนยันความสามารถการเกิดปฏิกิริยาทางภูมิคุ้มกันโดยวิธี immunoblot assay หลังจากนั้นใช้รีคอมบิแนนท์โปรตีนดังกล่าวที่ความเข้มข้นเท่ากับ 1.25 ไมโครกรัม/มล., ซีรั่มของแมว และคอนจูเกตแอนติบอดี (Goat anti-cat IgG) เจือจางที่ 1:200 และ 1: 12,000 มาพัฒนาชุดทดสอบโดยใช้หลักการอินไดเรกอีไลซ่า และทดสอบในตัวอย่างซีรั่มของแมวที่ได้จากภาคสนาม (ตัวอย่างซีรั่มวิทยาเป็นบวกจำนวน 12 ตัวอย่าง และตัวอย่างซีรั่มวิทยาเป็นลบจำนวน 7 ตัวอย่าง) โดยชุดทดสอบอีไลซ่าที่พัฒนาในการศึกษานี้สามารถให้ความไวได้ที่ 75% และ 83.33% และความจำเพาะ 57.14% และ 71.43% ในการตรวจหาแอนติบอดีชนิด IgG ต่อรีคอมบิแนนท์โปรตีนชนิด 17-kDa และ GroEL ตามลำดับ นอกจากนี้เมื่อนำผลทดสอบที่เป็นบวกต่อโปรตีนอย่างน้อยหนึ่งตัวยังพบว่าให้ความไวและความจำเพาะเป็นที่น่าพอใจ (91.67% และ 42.86%) โดยสรุปการศึกษานี้พบว่าแมวที่มีอายุมากกว่า 1 ปีมีความเสี่ยงสูงที่จะพบผลทางซีรั่มวิทยาเป็นบวกต่อเชื้อ *Bartonella* spp. และยังพบว่าแมวที่มีภูมิคุ้มกันบกพร่องหรือติดเชื้อ retrovirus อาจทำให้ การติดเชื้อ*Bartonella* spp. ในแมวแย่ลงได้ นอกจากนี้การศึกษานี้ยังสามารถแสดงความสามารถของรีคอมบิแนนท์โปรตีนชนิด 17-kDa และ GroEL ของเชื้อ *B. henselae* ในการเป็นตัวเลือกที่น่าสนใจสำหรับการพัฒนาการทดสอบทางซีรั่มวิทยาต่อเชื้อ *Bartonella* spp. ในแมวได้อีกด้วย

สาขาวิชา	อายุรศาสตร์สัตวแพทย์	ลายมือชื่อนิสิต ....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

5875515431 : MAJOR VETERINARY MEDICINE
KEYWORD: Bartonella spp., feline retrovirus, indirect ELISA, risk factors, recombinant protein, cats

Krissda Boonaramrueng : DEVELOPMENT OF ELISA FOR DETECTING ANTIBODY AGAINST *BARTONELLA* SPP. SPECIFIC PROTEIN IN CATS AND ITS APPLICATION IN NATURAL INFECTION. Advisor: Assoc. Prof. ROSAMA PUSOONTHORNTHUM, D.V.M., M.Sc., Ph.D., DTBVM. Co-advisor: Assoc. Prof. CHANNARONG RODKHUM, D.V.M., Ph.D, DTBVP.,Assoc. Prof. Anchanee Kubera, B.Sc., M.Sc., Ph.D, Navapon Techakriengkrai, DVM., M.Sc., Ph.D.

This study aims to evaluate the risk factor and an association between *Bartonella* spp. infection and immune status by using retroviral-infected cats as an immunocompromised model and to develop the recombinant *B. henselae* specific antigen protein (17kDa and GroEL)-based ELISA test for antibody detection against *Bartonella* spp. infection in cats. From 2017 to 2020, 161 client-owned clinical healthy cats at veterinary clinics and hospitals in the Bangkok metropolitan area were recruited and tested for *Bartonella* spp. infection statuses (PCR and IFA serology), blood profiles, feline retroviral statuses (FIV and FeLV), and T lymphocyte subsets ($CD4^+$, $CD8^+$, and $CD4^+$ to $CD8^+$ ratio). In this investigation, the prevalence of *Bartonella* spp. polymerase chain reaction at the veterinary clinics and hospitals in the Bangkok metropolitan area was 16.1% and the seroprevalence was 94.9%. Cats older than one year were more at risk of being seropositive than cats younger than one-year-old (OR 4.296; 95%CI: 1.010 - 18.275). The $CD8^+$ percentage was significantly higher in seropositive cats (p = 0.026). There was a significant reduction of $CD4^+$ to $CD8^+$ ratio between cats and concurrent *Bartonella* spp. and retrovirus-infected cats (p = 0.041). Regarding diagnostic tools, it is necessary to develop a sensitive diagnostic test that is specially developed for veterinary use to screen for bartonellosis in cats. This study used 17kDa and GroEL, *B. henselae* specific proteins (BSP), have been identified as immunodominant antigens and proposed as new diagnostic targets. These two genes, *17kDa* and *groEL* were cloned in pET28b and pH6HTC vectors and expressed under IPTG induction. The recombinant proteins were purified by affinity chromatography using HisTrap matrix. Both purified proteins showed the immunoreactivity to seropositive cat serum by immunoblot assay. The 17-kDa and GroEL recombinant antigen proteins were also deployed to develop the antibody detection tool against *Bartonella* spp. infection in cats by indirect ELISA assay. The optimum concentrations of recombinant antigens, cat serum, and conjugated antibody (Goat anti-cat IgG) dilutions were 1.25 µg/ ml, 1:200, and 1: 12,000 for both newly developed ELISAs. The 17-kDa and GroEL-based ELISA were also tested in selected field cat sera (12 seropositive and 7 seronegative sera) and showed 75 % and 83.33 % sensitivity and 57.14% and 71.43% specificity in the IgG antibody detection. Moreover, the combination of positive results for at least one protein indicates satisfactory sensitivity and specificity (91.67 and 42.86%). In summary, this study showed a higher risk of seropositivity against *Bartonella* spp. in cats older than one-year-old and cats that were immunocompromised or retrovirus-infected may debilitate *Bartonella* spp. infection in cats. Our study also indicates that the recombinant 17-kDa and GroEL proteins are promising candidates for the development of serological detection tests of *Bartonella* spp. infection in cats.

Field of Study:	Veterinary Medicine	Student's Signature ....
Academic Year:	2021	Advisor's Signature ....
		Co-advisor's Signature ....
		Co-advisor's Signature ....

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Associate Professor Dr. Rosama Pusoonthornthum and Associate Professor Anchanee Kubera, my advisors, whose expertise and patient guidance are useful and pushed me to sharpen my knowledge and brought my work to a higher level. I would also like to thank Dr. Navapon Techakriengkrai, my co-advisor for their guidance in laboratory and their collaborative effort during writing the thesis. Furthermore, I would like to thank the rest of my co-advisor, Association professor Channarong Rodkhum for his advice and kindness assistance.

Beside my advisor, I would like to thank my thesis committee, Professor Dr. Somsak Pakpinyo, Professor Dr. Sathaporn Jitapalapong, Associate Professor Achara Tawatsin, and Dr. Prapaporn Jongwattanapisan for their meaningful comments and guidance. My special thanks should be given to my funding source, the 90th Anniversary of Chulalongkorn University, Ratchadapisek Sompote Fund and Feline Health and Infectious Disease Research Unit for Excellence, Chulalongkorn University for supporting the scholarship throughout the course of this dissertation.

I wish to thank various people in Department of Veterinary Medicine for their contribution to my research; Miss Pimontip Jamsawang (P'Tip), Miss Wanthanee Jaijumnong (P' Nee), Mr. Sarawoot Mooljuntee (P' Ped), Mrs. Rungnapa Promsombut (P' lek), people in Department of Veterinary Microbiology for their help in Laboratory techniques; Mrs. Waree NiyomThum (P' Pew), Mrs. Thitima Tripipat (P'Nhueng), and people in CU feline center; Dr. Siwaporn Pengpis, Mrs Arporn Narathip (Auntie Mam), who helped me in handling, and collecting the samples and data.

I would also thank my lab mates in Department of Genetics, Faculty of Science, Kasetsart University; N' Pueng, N' Benz and others for their support, and spending time together. These made me comfortable even we did not know before. Finally, I wish to thank my family, for their support. They supported me even they did not know much what I learned. And important person; my girlfriend, Donut, who completedmy happiness and gave me encouragement to do everything successfully.

Krissda Boonaramrueng

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

TABLE OF CONTENTS

Page

ABSTRACT (THAI) iii

ABSTRACT (ENGLISH) iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF TABLES viii

LIST OF FIGURES ix

Chapter 1 Introduction 10

1.1 Important and rationale 10

1.2 Literature review 13

1.3 Objectives of the Study 46

1.4 Research Hypothesis 46

1.5 Advantages of Study 47

1.6 Keywords 47

Chapter 2 *B. henselae* specific antigen protein production 48

2.1 Materials and Methods 48

2.2 Results 52

2.3 Discussions 58

Chapter 3 Development of ELISA using *B. henselae* specific antigen proteins 61

3.1 Materials and Methods 61

3.2 Results 68

3.3 Discussions 73

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Chapter 4 Feline bartonellosis and the association with feline retroviral infection 76

4.1 Materials and Methods 76

4.2 Results 79

4.3 Discussions 86

Chapter 5 General Discussion and Conclusions 91

REFERENCES 95

Appendix 117

VITA 137

LIST OF TABLES

Page

Table 1.1 Prevention Recommendations in immunocompromised persons. 45

Table 2.1 Primers of *17kDa and groEL* genes 51

Table 3.1 Solution preparation 65

Table 3.2 Area under the ROC curve 71

Table 3.3 Comparison of clinical performance of recombinant protein-based ELISAs 71

Table 4.1 Complete blood count, blood chemistry, *Bartonella* spp. PCR result, and number of cats in each category 80

Table 4.2 Association (odds ratio) between gender, age, flea infestation, and retrovirus status, with IFA results of *Bartonella* spp. antibody status 82

Table 4.3 Association (odds ratio) between gender, age, flea infestation, retrovirus status, and antibody status with *Bartonella* spp. PCR results. 83

Table 4.4 Median of $CD4^+$ and $CD8^+$T lymphocyte percentage, and $CD4^+$ to $CD8^+$ ratio of each category. 84

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF FIGURES

Page

Figure 2.1 Verification of *17-kDa* transformants by colony PCR. 53

Figure 2.2 Verification of *groEL* transformants by colony PCR. 53

Figure 2.3 Identification of recombinant protein expressed colonies. 54

Figure 2.4 Identification of recombinant protein in lysate fractions. 55

Figure 2.5 Optimization of recombinant protein purification. 56

Figure 2.6 Dot blot analysis of recombinant proteins. 57

Figure 3.1 Diagram of Indirect ELISA. 62

Figure 3.2 Diagram of workflow of indirect ELISA. 62

Figure 3.3 Scatter plot and 2X2 table of r17-kDa-based ELISA results 69

Figure 3.4 Scatter plot and 2X2 table of rGroEL-based ELISA results 70

Figure 3.5 ROC curve analysis and clinical performance of r17-kDa-based ELISA 72

Figure 3.6 ROC curve analysis and clinical performance of rGroEL-based ELISA 72

Figure 4.1 Column scatter plot of $CD4^+$ to $CD8^+$ ratios in 4 groups of cats with different retrovirus and *Bartonella* spp. statuses. 85

Chulalongkorn University

Chapter 1

Introduction

1.1 Important and rationale

Bartonellosis is a zoonotic disease caused by *Bartonella* spp. infection, which has been reported for more than 20 species (Guptill, 2010a). *Bartonella* spp. is a gram-negative bacilli, fastidious and intra-erythrocytic bacteria (Baldani et al., 2014). *Bartonella* spp. is distributed worldwide with several reports of infection in both human and other mammal species, especially in cats (Guptill, 2010a; Sykes and Chomel, 2014). More than 10 *Bartonella* species have been reported with human infection potential (Guptill, 2010a; Baldani et al., 2014). *Bartonella* spp. can cause a wide variety of diseases in humans, especially in immunocompromised patients (Koehler, 2008). In human patients with severe immunosuppression due to human immunodeficiency virus (HIV) infection, organ transplantation, or chemotherapy, *Bartonella* spp. infection has been reported as the cause of vascular proliferative lesion called bacillary angiomatosis (BA) and usually developed at the late stage of infection (Koehler, 2008). In Thailand, *Bartonella* spp. in HIV-infected patients have been reported since 2008 (Paitoonpong et al., 2008).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

In contrast to humans, feline bartonellosis is usually a subclinical bacteremic stage, though it could easily spread infection worldwide. The prevalence of Bartonellosis in cats is varying from zero up to 90% in some areas. In Thailand, almost 30% of cats were reported to be bacteremic (Maruyama et al., 2001), either by

polymerase chain reaction (PCR) or bacterial culture in which around 15% resided in the Bangkok area (Maruyama et al., 2001; Inoue et al., 2009; Assarasakorn et al., 2012). Similar to humans, feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) infection, commonly known as immunosuppressive diseases in cats (Hosie et al., 2009; Lutz et al., 2009), have also been considered as a risk factor for clinical feline bartonellosis. Several investigators have studied the relationships of bartonellosis in cats and these infections (Glaus et al., 1997; Buchmann et al., 2010). However, they could not find any FIV/FeLV influences on *Bartonella* spp. infection in cats (Glaus et al., 1997; Buchmann et al., 2010).

Diagnosis of *Bartonella* spp. in both cats and humans are rather challenging as there is no single test, which can prove clinical bartonellosis. A combination of consistent clinical abnormalities and the presence of *Bartonella* spp. in the blood is required for the clinical confirmation (Agan and Dolan, 2002). However, *Bartonella* spp. are fastidious, they require a special environment and prolong incubation period for culture and specific equipment and primer for DNA detection. Though, the combination of clinical suspicion and the positive serological test is the main criteria of clinical human bartonellosis, although being seropositive may not be interpreted as an active infection (Agan and Dolan, 2002; Guptill, 2010a). On the other hand, it is difficult to interpret serological test in cats due to the high seroprevalence in cat population, notably in Thailand with warm and humid environment (Guptill, 2010a; Sykes and Chomel, 2014).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Many serologic methods have been developed for cat scratch disease (CSD) diagnosis such as Immunofluorescence assays (IFA), enzyme-linked immunosorbent assays (ELISA), and western blot. According to IFA-based test, the sensitivity was vary between 14%- 100% and specificity was varied between 34% and 100% (Sander et al., 2001). Variation of the results have been described by difference of antigen preparation. IFA using non-cocultivated *Bartonella henselae* cells was found lower antibody titer than IFA using co-cultivated *Bartonella henselae* with Vero cells (Tsuneoka et al., 1998). Moreover, different culture conditions might affect the antigens' expression (Sander et al., 2001). Serological investigations by ELISA method were reported as high specificity (90%-99%) and variable sensitivity (10%-95%). Using *Bartonella henselae* whole-cell antigen for western blot analysis, multiple protein bands were recognized as immunoreactivity but variation of immunoreactivity profiles in each sample were noticed (Sander et al., 2001). Nevertheless, using specific proteins antigen of *Bartonella henselae*, recombinant 17-kDa *B. henselae* antigen protein, the agreement between western blot and IFA method was 92% with IFA-positive human serum samples and 88% for IFA-negative human serum samples (Anderson et al., 1995). Furthermore, the sensitivity and specificity of ELISA based test using recombinant 17-kDa *B. henselae* antigen protein were 71.1% and 93.0%, respectively (Loa et al., 2006). Besides using single 17-kDa of *B. henselae* antigen protein, a combination with recombinant Chaperonin GroEL (GroEL) *B. henselae* antigen protein was conducted. A sensitivity of 82.8% and a specificity of 83.9% of combination of

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

recombinant 17-kDa and GroEL *B. henselae* antigen protein ELISA test were observed in human serum samples (Ferrara et al., 2014).

According to the variation of diagnosis test for *Bartonella* spp. infection in both human and cats, this study aim to develop the recombinant protein expression protocol of 17-kDa and GroEL *B. henselae* antigen protein and apply to ELISA-based test for *B. henselae* infection in cats. This study also proposes to investigate an association between *B. henselae* infection and feline immunodeficiency virus, (FIV) infection in cats, together with their immune and clinical status.

1.2 Literature review

1.2.1 History

Bartonella bacilliformis is the first recognized species in this genus. *B. bacilliformis* was reported by Alberto Barton, Peruvian microbiologist, in 1909 (Barton, 1909). This species was reported as endemic infection is south America and caused 2 forms of clinical symptoms, Oroya fever and Carrion's disease. A phylogenetic analysis of the citrate synthase enzyme (*gltA*) sequence shows a deep ancestral lineage for *B. bacilliformis* (Saenz et al., 2007; Minnick and Anderson, 2015). Furthermore, there were some historical reports that showed the mimicked sign of this infection, called verruga peruana, since pre-Inca period (Schultz, 1968). In addition, one of landmarks of *B. baciliformis* discovery is the experiment by Daniel A. Carrión, Peru medical student. He did a proof of association between Oroya fever and Carrion's disease. Thus, this disease was named after him (Schultz, 1968).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

However, the most ancient evidence of *Bartonella* spp. infection in human was discovered in 4,000-year-old dental pulp of primitive human remains (Drancourt et al., 2005; Fournier et al., 2015). *Bartonella quintana* was recognized in that study. It was believed that body louse-transmitted was a major route of infection in that historical period (Fournier et al., 2015). Later, *Bartonella quintana* was report in as a causative of Trench fever which had been reported since world war I. Furthermore, this fever still found and reported after the World war I.

In contrast, the most earlier report of cat scratch disease, which cause by *Bartonella henselae*, is addressed by Robert Debré (Debré, 1950). However, the causative agent still not recognized in that period. It took several years until 1991-1992. Gram-negative bacillus was addressed. However, earlier agent was falsely informed as *Afipia felis*. Then, there were evidence that promoted *Rochalimaea henselae* as a causative agent. In 1993, the genera *Bartonella, Grahamella* and *Rochalimaea* were proposed to combine as *Bartonella* genus (Brenner et al., 1993). *Bartonella* genus which had single species for decade, have been gradually found more variety of species.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.2.2 Bacteriology

Bartonella spp. is bacteria member of Phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family *Bartonellaceae*, and the genus *Bartonella. Bartonella* spp. infects a variety of mammalian host species. Different *Bartonella* spp. have adapted to specific hosts and can occasionally cause disease in

incidental hosts (Sykes and Chomel, 2014). Nowadays, there are reports more 45 species and 5 subspecies (Okaro et al., 2017; Gutiérrez et al., 2020). Moreover, *Bartonella* is still expanding their animal reservoirs and arthropod vectors ranges. A variety of animal species has been identified as reservoir hosts, including rodents, bats, terrestrial animals, and marine animals, such as beluga whales and sea turtles (Okaro et al., 2017).

Bartonella species are gram-negative bacilli or coccobacilli, fastidious and intraerythrocytic bacteria (Inoue et al., 2009; Baldani et al., 2014). The Gram and Gimenez stain are acceptable used but these are not stained well with safranin and not stained with acid fast (Okaro et al., 2017). *Bartonella* colonies are tiny which growing between 0.2-1 mm in diameter. After several passages, morphology and character of these bacteria may changeable. Colonies may show smooth surface and more fast-growing than early passages. Conventional biochemical tests for bacterial identification are not helpful for these bacteria (Minnick and Anderson, 2015). Some *Bartonella* species have been reported that may have flagella and pili even though most of them are not motile(Minnick and Anderson, 2015). These organisms are cultivable but require heme from the enriched-media or their erythrocyte's host.

Bartonella species carry a single, circular chromosome and varies in size. The average of chromosome is 1.9±0.5 Mbp. Rodent-related species have been reported to have larger genome than human-specific species (*B. bacilliformis*). The shorter size is reported that relate with distinctive genetic features such as intracellular niche and

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

specialized host species (Moran, 2002; Minnick and Anderson, 2015). Most of *Bartonella* species have a VirB/VirD4 type IV secretion system (T4SS), and a Trw T4SS (Dehio, 2005; Okaro et al., 2017). Surface appendages of some *Bartonella* species are known as comprised of trimeric autotransporter adhesins (TAAs). However, there are reports that some species may have plasmids and episomal DNA elements in them. While the plasmids' functions are still not well described, episomal DNA elements are proposed their relationship with the evolution of these organisms. *Bartonella* bacteriophage-like particles (BLP) are result from episomal DNA elements and proposed as horizontal gene transfer (Okaro et al., 2017).

1.2.3 Epidemiology

Many *Bartonella* species are well-adapted to wild range of animal, including cats, dogs, rabbits, deer, elk, foxes, coyotes, ruminants, bats, rodents, and marine animals, such as beluga whales and sea turtles (Guptill, 2010a; Baldani et al., 2014; Okaro et al., 2017). In comparison to other vector-borne diseases, *Bartonella* species can transmitted to several hosts as reservoir and accidental host. Prolonged bacteremia in clinically normal reservoir hosts is considered as key of increasing chances of blood-sucking arthropods for uptake the organism to transmit to other hosts (Baldani et al., 2014). For examples, Cats considered as a primary reservoir for many species including, *B. henselae, B. claridigeae,* and *B. koehlerae.* Coyotes and dogs are considered mammalian reservoirs for *B. vinsonii* subsp. *Berkhoffii. B. bacilliformis and B. quintana,* are known as only two species infect in human as

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

reservoir host. However, co-infection of more than one species is showed in cats, dogs, cattle, and rodents. Recently, diverse, and novel species of *Bartonella* have been reported in bats as reservoir.

Nowadays, *Bartonella* species are considered as emerging pathogens. *B. bacilliformis, B. quintana, and B. henselae* are major pathogens in human bartonellosis (Okaro et al., 2017). There was a study in Veterinarians who were determined high risk to *Bartonella* spp. exposure. There were the two third of veterinarian who were seropositive to at least one *Bartonella* spp. and the half of them were seropositive to more than one species (Oteo et al., 2017). Among patients presenting with fever of unknown origin, 18% of patients had evidence of infection detected by culture, IFA, or PCR (Koehler et al., 2003). Furthermore, 3% of patients had evidence of both HIV and *Bartonella* spp. infection (Koehler et al., 2003). In South Africa, there were 10% of outpatient who were bacteremia by PCR technique from HIV clinic (Frean et al., 2002). These relationship between HIV and *Bartonella* spp. infection was also shown. There were 4/5 HIV patients had concordant genome pattern to their companion cats (Chang et al., 2002).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Bartonella bacilliformis have the sandfly (*Lutzomyia verrucarum*) as vectors and endemic in Andes Mountains in Peru (Guptill, 2010a; Okaro et al., 2017). *B. bacilliformis* infections in South America have numerous outbreak reports in the last 20 years and occur in lower altitudes and in historically non-endemic areas (Minnick and Anderson, 2015). Related sand flies were reported to associated with these

outbreaks. There was study showed that the incident of *B. bacilliformis* infection was 12.7 per 100 person-years and found the highest rate in children under 5 years (Chamberlin et al., 2002). The insecticide application is the best control for *B. bacilliformis* infections (Chamberlin et al., 2002).

In contrast to *B. bacilliformis*, *B. quintana* are worldwide distribution and have human body lice (*Pediculus humanusi*) as vectors. Poor sanitation environments, such as war zone are key of transmission (Guptill, 2010a; Okaro et al., 2017). *B. quintana* were found 20% in Seattle, USA, 14% in France, and 34.5% in Japan in homeless people with body lice (Jackson et al., 1996; Brouqui et al., 1999; Sasaki et al., 2021). *B. quintana* were also reported 10% in people who abused intravenous drugs in Baltimore, USA (Comer et al., 1996). In addition, the prevalence were highest (86.5%) in people who worked with lice in a laboratory (Brouqui et al., 1999). Moreover, *B. quintana* are associated with endocarditis in people. Among 48 cases of *Bartonella*-associated endocarditis were *B. quintana* for 38 cases and *B. henselae* for 10 cases. In patients with *B. quintana* endocarditis, 57.8% were homeless, 60.5% were alcoholic, and 36.1% had contact with body lice. Fever (89.5%) and aortic valve-involvement (65.8%) were common characters of patients (Fournier et al., 2001). Furthermore, *B. quintana* were also reported as the cause of endocarditis in dogs (Kelly et al., 2006). In addition, *B. quintana* were also related with baciliary angiomatosis and lymphadenopathy in HIV-infected people (Foucault et al., 2006).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

In case of *B. henselae,* it is worldwide distribution and linked to cats as primary reservoirs. The major vectors are cat fleas (*Ctenocephalides felis*). Higher prevalence of *B. henselae* have been reported in humid and warm climate areas which promote fleas' growth (Guptill, 2010a; Okaro et al., 2017). Prevalence of *Bartonella* species in cats is varying from zero up to 90% in some area. In Thailand, almost 30% of cats were reported to be bacteremic (Maruyama et al., 2001), either by polymerase chain reaction (PCR) or bacterial culture in which are 10.4%-27.6% (Maruyama et al., 2001; Inoue et al., 2009; Assarasakorn et al., 2012). In human, there are 5.5% of Immunoglobulin G (IgG) seropositive and 1.2% of IgM seropositive in Thai blood donor group (Maruyama et al., 2000a). The incidence of cat scratch disease was reported as 3.7 cases/ 100,000 persons in USA and 2,000 cases/y or 12.5 cases/ 100,000 persons in Netherland (Chomel et al., 2004). Furthermore, two genotype of *B. henseale* were described. There were suggestions that genotype I was more frequently observed in humans whereas most of the cats were infected with genotype II. However, one study showed that European and American isolates of both human and feline origins were genotype II and isolates from Asian and Australian were genotype I (Bouchouicha et al., 2009).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Besides the three species, there are more than 20 rodent-borne *Bartonella* spp. were also reported. The prevalence in rodents was vary between zero to 90% in some area or countries (Gutiérrez et al., 2015a). There are more than 98 rodent species were reported as reservoirs of many *Bartonella* species and variants. At least 6 rodent-borne species were reported the association with human illnesses, including *B. grahamii*,

B. elizabethae, B. tribocorum, B. vinsonii subsp. *arupensis, B. washoensis,* and *B. tamiae* (Buffet et al., 2013). The diversity symptoms were reported as endocarditis (Daly et al., 1993; Fenollar et al., 2005), myocarditis (Kosoy et al., 2003), fever and neurologic disorders (Welch et al., 1999), intraocular neuroretinitis (Kerkhoff et al., 1999), meningitis (Probert et al., 2009), splenomegaly (Eremeeva et al., 2007), and lymphadenopathy (Buffet et al., 2013; Oksi et al., 2013). For *B. elizabethae*, this species was noticed in homeless and IV drug users. One third of drug users were seropositive to *B. elizabethae* in Baltimore, 39% in Stockholm, and 46% in New York. However, only 12.5% in homeless people in Los Angelis were seropositive (Chomel et al., 2004).

1.2.4 Transmission and pathogenesis

Overall, the infection cycle of *Bartonella* spp. begins with inoculation by bloodsucking arthropods, such as fleas in cats. Following, the Bartonellae establish the primary niche. The Bartonellae are released into the circulation from the primary niche and invade erythrocytes in a series of processes spanning from adhesion to invasion and intracellular persistence, allowing for continuous vector transmission (Eicher and Dehio, 2012; Harms and Dehio, 2012).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

One of the cornerstones of *Bartonella* spp. infection is the persistence of *Bartonella* spp. in a primary niche before intraerythrocyte-stage infection (Eicher and Dehio, 2012; Harms and Dehio, 2012). Despite the fact that there is no proof of how this stage works, endothelial cells and migratory cells are thought to be crucial cells in this step. Lymphocytes or mononuclear phagocytes might be the migratory cells of

Bartonella transport, demonstrating how *B. henselae* can endure the macrophage destruction for few days. In murine model, *B. henselae* infection demonstrated that transportation might take place through the lymphatic system and lymphocytes or mononuclear phagocytes. Other investigations have suggested that hematopoietic progenitor cells or erythroblast cells, which are nucleated progenitors of erythrocytes, are parts of the primary niche. (Eicher and Dehio, 2012; Harms and Dehio, 2012).

The recurrence of *Bartonella* infections in reservoir hosts, cats, and following treatment or clearance by the host immune system suggests that the primary niche is preserved. However, it is unclear in general features of *Bartonella* infections that periodic seeding of bacteria from the primary niche into the circulation over several-day intervals. Several In vitro investigations have shown that *B. henselae* may infect a variety of cell types, including endothelial cells, endothelial progenitor cells, epithelial cells, hematopoietic progenitor cells, and monocytes/macrophages, including microglial cells (Eicher and Dehio, 2012; Harms and Dehio, 2012). These characters might be linked to a number of illnesses and symptoms. The interaction of *Bartonella* spp. with nucleated host cells (especially endothelial cells) has been extensively investigated as a vital function in primary niche colonization and may potentially be involved in secondary colonization.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

After colonizing, the bacteria are injected into the circulation from the primary niche, where they invade erythrocytes by adhesion, deformation, invasion, and eventually intraerythrocytic persistence. Adhesion was discovered to be an important

element in host specificity. (Seubert et al., 2001; Harms and Dehio, 2012). It is clear that partners may act as receptors in the adhesion process. Actin, α and β subunits of spectrin, Band 3 protein, glycophorin A, and monomeric and dimeric glycophorin B have all been linked to deformation and invasion phases. The Band 3 protein binds the erythrocyte membrane to the underlying spectrin-actin cytoskeletal network. Pathogenic parasites frequently use disconnection of this network to degrade surface integrity. At the areas where *B. henselae* attached, erythrocytes acquired progressive indentations and invaginations. The invasion site has not been resolved and continues to supply entrance points for *Bartonella*. Deformin, a bacterial factor, has also been shown to be released into the erythrocyte membrane, causing membrane invaginations (Harms and Dehio, 2012). Its mechanism, however, remains unclear. Intraerythrocytic persistence of *Bartonella* spp. did not appear to reduce erythrocyte life span. In cats, the proportion of infected erythrocytes ranges from 1% to 5%. Bacteremia progresses differently depending on the *Bartonella* and host species, immune status, and infection stage.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.2.4.1 Immune Evasion and Immunomodulation

Bartonella spp. uses an "anti-immunology" strategy to escape a host immune response and colonize the intraerythrocytic niche. *Bartonella* can avoid detection as a bacterial pathogen by hiding and modifying pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) or flagella. Current evidence strongly shows that *Bartonella* stimulates IL-10 production as part of its

immunological regulation. This cytokine responds by inhibiting the functioning of multiple immune cells, interfering with both innate and adaptive immunity (Dehio, 2004; Eicher and Dehio, 2012; Harms and Dehio, 2012).

1.2.4.2 Virulence factors

1.2.4.2.1 Trimeric autotransporter adhesins (TAAs)

Trimeric autotransporter adhesins (TAAs), well-known gram-negative bacteria virulence factors, are significant in pathogens like *Yersinia* (YadA), *Haemophilus* (Hia), and *Neisseria* (NadA). TAAs bind to host proteins on cell surfaces, in the extracellular matrix (ECM), or as circulating factors. (Harms and Dehio, 2012). BadA, a trimeric autotransporter adhesin of *Bartonella henselae*, forms 240 nm long hair-like filament trimers on the bacterial surface. BadA plays an important role in bacterial autoagglutination, adhesion to host cells inhibition of phagocytosis, and proangiogenic transcriptional program in target cells. (Dehio, 2004; Eicher and Dehio, 2012; Harms and Dehio, 2012).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.2.4.2.2 VirB-like Type IV secretion systems

The VirB/D4 T4SS and Bartonella effector proteins

The VirB/D4 T4SS of *Bartonella* is one of the most notable virulence factors. The VirB/D4 T4SS obviously plays an important role during infections with a wide range of *Bartonella* species by translocating Beps (*Bartonella* effector proteins) into host cell. Although several subgroups of Beps have been described, comparisons across subsets are unclear. The VirB/D4 T4SS and Beps modify the cellular activities and

manage an intracellular niche. (Schulein and Dehio, 2002; Dehio, 2005; Harms and Dehio, 2012).

The Trw T4SS

Trw T4SS is associated with erythrocyte infection rather than primary niche, and also control specificity adhesion process to the host' s erythrocyte of *Bartonella* spp. (Dehio, 2001; Harms and Dehio, 2012).

Outer membrane proteins

According to current research, outer membrane proteins, particularly OMP43, might perform as adhesions. One or more *Bartonella* outer membrane proteins contribute to host cell adhesion and contact-dependent proinflammatory activation in endothelial cells. (Dehio, 2004; Harms and Dehio, 2012).

GroEL

GroEL are a conserved family of chaperonins whose purpose is to regulate protein folding and relevance during infection is not understand. GroEL was discovered to be immunogenic and localize to the outer membrane of *B. henselae*. In addition, *Bartonella* GroEL serves as a mitogenic and antiapoptotic factors (Harms and Dehio, 2012).

Autotransporters and filamentous hemagglutinins

Classical autotransporters are similar to TAAs and are important virulence factors of various bacterial pathogens. However, autotransporters during *Bartonella*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

pathogenesis remain mystery. Filamentous hemagglutinins are secreted and very similar to that for classical autotransporters (Eicher and Dehio, 2012).

Hemin binding proteins (Hbps)

The hemin binding proteins (Hbps) of *Bartonella* are a collection of porin-like outer membrane proteins that bind hemin on the bacterial surface. The lack of antigenicity of Hbp may be a factor of *Bartonella* reservoir host adaptation. (Harms and Dehio, 2012).

1.2.5 Human bartonellosis

People infected with *Bartonella* spp. infection are suffering from various clinical illnesses. However, Immunocompetent individuals may have more localized and self-limited illness. The common symptoms in humans are cat scratch disease (CSD), bacillary angiomatosis, peliosis, relapsing fever with bacteremia, endocarditis, optic neuritis, pulmonary, hepatic, and splenic granulomas; osteomyelitis and many others (Massei et al., 2000; Massei et al., 2004; Florin et al., 2008; Guptill, 2010a). However, infections in immunocompromised individuals may debilitate and can be fatal.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.2.5.1 Disease in immunocompetent patients

Carrion's disease, cat-scratch disease, chronic lymphadenopathy, and trench fever are commonly known diseases of *Bartonella* spp. infection. Other symptoms that have been recorded include culture-negative endocarditis, bacilliary angiomatosis, bacilliary peliosis, vasculitis, and uveitis (Cheslock and Embers, 2019). Recently, many idiopathic disorders, such as hallucinations, weight loss, muscular exhaustion, partial

paralysis, pediatric acute-onset neuropsychiatric syndrome (PANS), and other neurological symptoms, have been identified (Cheslock and Embers, 2019). The likelihood of this pathogen's involvement in breast tumorigenesis is concerning and need more investigation (Cheslock and Embers, 2019).

Carrion's disease can be classified into two clinical stages, including acute and chronic stages (Maguiña et al., 2009). The acute form of the disease (Oroya fever) is characterized by fever, hemolytic anemia, headache, pallor, myalgia, and arthralgia. This form is often seen in non-native populations in the endemic area. Oroya fever lasts between 1 and 4 weeks and can range from mild to deadly. In untreated patients, mortality rates could range from 44% to 88%. The prevalence of secondary infection is linked to the majority of death. Salmonellosis, various bacteria, mycobacteria, protozoa (*Toxoplasma* and amoebae), fungus (*Histoplasma*, *Pneumocystis*), and viruses such as herpes and hepatitis B are the opportunistic pathogens. Infection during pregnancy can cause transplacental infection, which can result in fetal mortality, abortion, and maternal death (Maguiña et al., 2009; Scorpio and Dumler, 2015). The second phase, known as verruga peruana, occurs between 2 weeks and several years following the acute phase and is recognized by cutaneous nodular angioproliferative lesions. Chronic bacteremia usually occurs in people from endemic area. These lesions can last for several months, but the prognosis for complete healing is promising at this point. When the eruptions arise, they are typically accompanied with minor clinical symptoms such as fever, malaise, osteoarticular pain, and headache. Lesions could

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

vary in size and number and may show as red or purple papules. Nodules often bleed easily, also known as "blood warts". Three classical types of lesions have been recognized: miliary form (<3mm), mular form (>5mm), and diffuse form. Verruga peruana eruptive lesions usually resolve on their own, however these might take a lengthy period. Recurrent lesions are uncommon (Maguiña et al., 2009).

Trench fever is generally not deadly. The incubation period ranges from 5 to 20 days. This symptom is distinguished by three to five febrile episodes and prolonged 4 to 5 days in each. Severe headaches, shin pain, lethargy, anorexia, stomach discomfort, restlessness, and insomnia are common clinical signs. One of the most causes of culture-negative endocarditis in patients is *B. quintana* endocarditis. Homeless people have been reported to develop *Bartonella* endocarditis. *B quintana, B henselae*, or both are the most common causes of *Bartonella* endocarditis (Maguiña et al., 2009; Scorpio and Dumler, 2015)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Cat scratch disease, CSD, usually presents as mild and self-limiting lymphadenitis. However, they may deliberate to severe and/or systemic form, including Parinaud's oculoglandular syndrome, encephalopathy, convulsions, osteomyelitis, retinitis, arthritis, hepatitis, splenitis, erythema nodosum, pulmonary nodules, and pleurisy (Maguiña et al., 2009). Besides *B. henselae, B. clarridgeiae* and *B. grahamii* might be the causing agents of CSD (Scorpio and Dumler, 2015). A classical CSD is characterized by a papular or pustular cutaneous lesion at the puncture site. Incubation period is range from 3 to 10 days after the cat scratch or bite.

Regional lymphadenomegaly usually develops during next 2 weeks. The lymphadenopathy healed after a median of 7 weeks. The suppurative lymphadenopathy occurs in 10% to 15% of individuals. Moreover, some patients report pain, weight loss, nausea, and vomiting, as well as splenomegaly and low-grade pyrexia. Persistent lymphadenopathy (6-24 months) may develop in 20% of individuals (Maguiña et al., 2009). Atypical CSD develop in about 5% - 10% of patients. Parinaud's oculoglandular syndrome is the most frequent. Nevertheless, retinitis, meningitis, encephalitis, osteolytic lesions, and thrombocytopenic purpura can also occur. Encephalopathy is the significant consequences of CSD. It generally starts 2 to 6 weeks after the onset of lymphadenopathy but frequently cure with a full recovery and few or no sequelae (Scorpio and Dumler, 2015).

1.2.5.2 Disease in immunocompromise patients

Infection with *Bartonella* spp. can cause unusual infestations in individuals with significant immunosuppression such as HIV infection, organ donation, or chemotherapy (Koehler, 2008). Bacillary angiomatosis (BA) is a kind of vascular proliferative lesion caused by *Bartonella* spp. infection in late stage of HIV infection. These vascular forms can arise from various organs, including skin, bone, brain parenchyma, lymph nodes, bone marrow, gastrointestinal and respiratory system. Skin lesions are the most often affected site of BA lesions. Cutaneous BA is a small dark red or purple papule. Lesions are also brittle and bleed easily (Maguiña et al., 2009; Scorpio and Dumler, 2015). In HIV-infected individuals, lytic bone lesion should be considered osseous BA as a

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

primary differential diagnosis. Other forms of BA in HIV-infected patients are splenic and hepatic bacillary peliosis, gastrointestinal and respiratory tract bacillary angiomatosis, and lymph node bacillary angiomatosis. BA developed in an HIV-infected patient may associated with seizures and facial nerve deficit (Koehler, 2008). Bacteremia with or without endocarditis has also been described in HIV-infected people (Koehler, 2008).

1.2.6 Feline bartonellosis

Feline bartonellosis is often diagnosed as asymptomatic bacteremia, however infection can spread globally. Five *Bartonella* species, including *B. henselae, B. clarridgeiae, B. koehlerae, B. quintana,* and *B. bovis*, have been shown to infect cats naturally. Nevertheless, *B. koehlerae, B. quintana*, and *B. bovis* are infrequently recorded (Guptill, 2010b). In experimental cats, self-limiting febrile illness, mild to moderate transitory anemia, and temporary neurologic impairment were reported (Breitschwerdt, 2008). Most recent investigations have found that *B. henselae* infection is related with fever, lymphadenopathy, stomatitis, and gingivitis (Guptill, 2010a; Guptill, 2010b). Since *Bartonella* bacteremia is typically persistent, numerous chronic disorders in cats have been hypothesized as associated bartonellosis. However, no link has been established between naturally infected cats and anemia, gingivostomatitis, neurologic disorders, or uveitis (Guptill, 2010b). Naturally, infective endocarditis is uncommon in cats, although a few instances have shown a relationship with *Bartonella* infection. DNA of *B. henselae* was found in the aortic valves of cats with endocarditis,

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

as well as endothelial cells in the myocardium (Guptill, 2010b; Sykes and Chomel, 2014).

1.2.7 Bartonellosis in Dogs

Dogs have been reported the infection with *B. henselae, B. vinsonii subsp.berkhoffii, B. koehlerae, B. clarridgeiae, B. elizabethae, B. washoensis, B. quintana, B. bovis, B. volans*-like, and *B. rochalimae* (Brenner et al., 1993; Pérez et al., 2011; Diniz et al., 2013; Álvarez-Fernández et al., 2018; Müller et al., 2018; Cheslock and Embers, 2019). *Bartonella* spp. is most likely transmitted between dogs by vectors such as fleas and ticks. Nonetheless, there is little evidence to indicate a direct transmission from dogs to people (Cheslock and Embers, 2019). *Bartonella* spp. is a major cause of blood-culture–negative endocarditis in dogs (Guptill, 2010a; Sykes and Chomel, 2014). Dogs are most usually infected with *Bartonella vinsonii* subsp. *berkhoffii* or *B. henselae* (Sykes and Chomel, 2014). Additionally, cardiac arrhythmias, granulomatous rhinitis, peliosis hepatis, meningoradiculoneuritis with dermatitis or panniculitis, and granulomatous lymphadenitis have also been related with *B. vinsonii* subsp. *berkhoffii* and *B. henselae* (Guptill, 2010a). Even though most of these infections are assumed to be accidental infection, dogs are probably a reservoir of a few *Bartonella* species, *B. henselae* and *B. vinsonii* subsp. *berkhoffii* (Cheslock and Embers, 2019).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.2.8 Bartonellosis in other animals

Bartonella henselae, B. vinsonii subsp. *berkhoffii*, and novel *Bartonella* also been documented in horses. They may be accidental infection and transmitted between horses by biting flies, ticks, and lice (Cheslock and Embers, 2019). Additionally, *Bartonella bovis* appears to be widespread in dairy and beef cattle, clinical problems in cattle have not been demonstrate yet. Sheep have also been reported the infection of *B. melophagi* and could be cause of human clinical problems (Cheslock and Embers, 2019). Moreover, *B. alsatica* are found in rabbits. *B. alsatica* has been associated with blood-culture-negative endocarditis and lymphadenitis in closed contact people. Both sheep and rabbits are considered as reservoir for human (Cheslock and Embers, 2019). In addition, non-human primates have been considred as reservoir for human, i.e., *B. quintana* infection. Furthermore, there were reported the *Bartonella* spp. infection in aquatic animal; sea otter, whales, and porpoise (Guptill, 2010a). Otherwise, a number of rodent species have been discovered to be infected with various *Bartonella* spp. and variants throughout the world. Human clinical symptoms, like endocarditis, have been attributed to several rodent-associated *Bartonella* spp. and variants. In recent knowledge, more than 20 species are thought to be rodent-associated *Bartonella* spp. (Gutiérrez et al., 2015a). Interestingly, while bats play important role in several emerging diseases, bats and bat flies are also thought to be infected with *Bartonella* spp. and reservoir for human infection(Gutiérrez

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

et al., 2015a; Bai et al., 2018). A relationship between bat and human infection were showed in a study from Bai et al. (2018).

1.2.9 Diagnosis

Diagnosis of *B. henselae* infection in both cats and human are challenging because there is no single test result can prove clinical bartonellosis. The combination of consistent clinical abnormalities and detection of *B.henselae*. presence are used (Agan and Dolan, 2002). Although serology test may not interpret as active infection but these methods are the main stays of diagnosis in human medicine (Agan and Dolan, 2002). On the other hand, it is difficult to interpret serology test in cat's infection due to high prevalence of seropositive in population of cat in warm and humid environment area, particularly in Thailand (Jameson et al., 1995; Sykes and Chomel, 2014).

There are many methods for diagnose *B. henselae* infection in cats. First, culture of blood or tissues in special media and environments for *B. henselae* may take over 6-8 weeks of incubation (Brunt et al., 2006; Guptill, 2010a). Amplification of *Bartonella* DNA by PCR assay of tissue and body fluids is main tool for detection this organism in both human and veterinary medicine because there are not requirement for special techniques like cultural methods but experience and specific primer are required (Brunt et al., 2006; Sykes and Chomel, 2014). Finally, antibodies in blood, aqueous humor, or CSF can be used to screen individual cats for *Bartonella* infection;

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

these tests are commercially accessible in the United States and certain other countries (Brunt et al., 2006).

1.2.9.1 Clinical laboratory findings and pathologic findings

Clinical manifestations are brief and vary, ranging from mild to absent, and are difficult to identify (Guptill, 2010b). Because bartonellosis is a zoonotic disease, veterinarians may be asked to test healthy cats that belong to immunocompromised owners or *Bartonella*-infected patients. Complete blood counts, serum biochemical testing, and urine analysis revealed no abnormalities in the experimentally infected cats. During the early stages of infection, some cats developed temporary fever and anemia, whereas others developed chronic eosinophilia and neutrophilia, particularly during periods of skin inflammation. Acute and chronic infected cats may have lymphoid hyperplasia, and small foci of lymphocytic, pyogranulomatous, or neutrophilic inflammation in numerous tissues, including lung, liver, spleen, kidney, heart (Guptill, 2010a; Guptill, 2010b; Sykes and Chomel, 2014).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Detecting *B. henselae* in erythrocytes of infected cats is not effective for diagnosis especially with using conventional staining methods. Using fluorescent antibody detection methods were reported to detect Intraerythrocytic *B. henselae* in naturally infected cats. In addition, extracellular *B. henselae* have been documented in peripheral blood and other tissues of infected cats using immunocytochemical and immunohistochemical methods (Guptill, 2010a; Guptill, 2010b). In tissues samples,

Bartonella are stained positively with silver stains such as the Warthin–Starry stain (Breitschwerdt, 2008).

1.2.9.2 Culture method

Culture method is the gold standard to prove current infection in cats (Brunt et al., 2006). However, this method is not main stay to use for clinical diagnosis due to fastidious characters of these bacteria (Brunt et al., 2006). Furthermore, intermittent bacteremia in cats are common (Guptill, 2010a). So, culture is not sensitive method by this reason. On the other hand, positive blood or tissue culture from healthy cat are not related their clinical signs so culture method should be indicated in sick cat who suspected or has high risk of *B. henselae* infection (Brunt et al., 2006; Guptill, 2010a). In addition, blood samples from early course of infection and prior antibiotic therapy are suggested (La Scola and Raoult, 1999).

In veterinary medicine, Isolator TM blood collection tubes (Wampole Laboratories, Cranbury, NJ) are used for *B. henselae* isolation. Blood collected in EDTA and frozen before to culture, on the other hand, was more sensitive than lysis centrifugation tubes for *B. henselae* culture. (Agan and Dolan, 2002; Guptill, 2010a). In *B. henselae* culture, a specific media such as fresh chocolate agar, blood agar, or brain–heart infusion agar supplemented with blood conditions (5% CO_2, temperatures of 35–37 ^{o}C) and a long incubation time of 4-6 weeks are required (Agan and Dolan, 2002; Guptill, 2010a; Baldani et al., 2014). Primary isolates of B. henselae are normally taken 12 to 14 days on blood agar, and longer incubation periods up to 45 days are

occasionally required (Agan and Dolan, 2002). Subsequent sub-cultures often develop faster (3-5 days) than the primary cultures, but colony characters may be different. (Agan and Dolan, 2002). The colonies of *B. henselae* are often rough and firmly embedded in the agar. However, with repeated sub-culture, colony shape frequently smoothes out and bacteria are simpler to disaggregate in suspension. (Agan and Dolan, 2002). Co-cultivated cell culture methods have been shown to be faster and more sensitive than blood agar-based procedures (La Scola and Raoult, 1999).

According to recent findings, the invention of a new pre-enrichment medium for *Bartonella* culture, *Bartonella-Alphaproteobacteria* growth medium (BAPGM), may make be more sensitive diagnostic tool than the past growth medium. (Maggi et al., 2005). In contrast, overwhelming growth of other bacteria may obscure *Bartonell*a

growth even a small amount of contamination with less fastidious bacteria, or false positive culture occurred if residual flea excrement at the site of venipuncture contaminated. As a result, the significance of appropriate sterile technique should be highlighted. (Guptill, 2010b).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.2.9.3 Molecular methods

The polymerase chain reaction (PCR) is a sensitive technique for detecting DNA fragments. PCR for identifying *B. henselae* in blood and tissue samples has been developed for faster detection (Sykes and Chomel, 2014). Because the bacteremia occurs periodically, PCR testing may be no more sensitive than blood culture for detecting active *Bartonella* infection (Sykes and Chomel, 2014; Năsoiu et al., 2015).

Furthermore, the presence of *B. henselae* DNA does not always imply the presence of viable *Bartonella* organisms (Guptill, 2010a). To minimize sample contamination, blood samples for PCR testing should be obtained in a sterile manner, and DNA degradation should be avoided during sample processing.

A number of PCR assays have been developed, including PCR amplification of the 16S-23S rRNA intergenic region using genus- and species-specific primers (Minnick and Barbian, 1997), restriction fragment length polymorphism (RFLP) analysis of PCR-amplified *16S rRNA* genes, *citrate synthase* gene, species-specific amplification of *ftsZ* gene sequences, repetitive-element PCR, and sequence analysis. However, when conventional PCR is utilized in asymptomatic cats, the assays' sensitivity is normally limited. As a result, the utilization of more sensitive methods, such as nested and real-time PCR, may enhance diagnostic sensitivity (Gutierrez et al., 2017). Although the hazards of contamination with this approach are acknowledged, and laboratory precautions are required, nested and real-time PCR testing may boost sensitivity for detection of *Bartonella* DNA in cat blood (Guptill, 2010a). *Bartonella-Alphaproteobacteria* growth medium (BAPGM), a unique pre-enrichment media for culture, is also beneficial in molecular detection. Prior to PCR, pre-enrichment culture is used to increase the sensitivity of PCR procedures (Duncan et al., 2007; Weeden et al., 2017).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.2.9.4 Serology diagnosis methods

Anti-*Bartonella* antibody serological approaches have been described and utilized more than any other diagnostic procedures (Năsoiu et al., 2015). The serologic test has limit to use for identifying the infection. Serum IgG antibodies remain for long periods of time in experimental infection in cats (Guptill, 2010a). Seropositive finding in cats only confirm the exposure. Therefore, serological test findings are not utilized to assess the *Bartonella* spp. infection status of individual cats (Sigirci et al., 2017). The lacking of a relationship between seropositivity and the bacteremia is a cause of experiencing of discrepancy results between PCR analysis or culture and serologic test results (Fabbi et al., 2004).

Lappin et al., 2009 also noted that *Bartonella* species serology test findings were unreliable in predicting bacteremia status because some cats with *Bartonella* species DNA in blood were seronegative and some cats with *Bartonella* species IgG in serum were negative for *Bartonella* species DNA in blood (Lappin et al., 2009). In comparison to bacterial isolation, which may take 4 to 6 weeks, or PCR procedures, serological tests are simple to perform and can be completed in 1-2 days (Năsoiu et al., 2015; Sigirci et al., 2017). The positive predictive value (PPV) of IFA or ELISA (IgG) serologic tests for bacteremia in cats is only 39–46%; false positive tests appear to be frequent, while the negative predictive value (NPV) of serologic tests for bacteremia or the presence of DNA in cat blood is high, at 87–97%. (Guptill, 2010a).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The IFA Test is recommended for young cats and other cats prior to adoption by owners who may be immunocompromised (Năsoiu et al., 2015). Because of the limited positive predictive value compared to the good negative predictive value, the Advisory Board on Cat Diseases found that IFA is more beneficial for exclusion than confirmation of infection (Pennisi et al., 2013; Sigirci et al., 2017).

The use of Western blot assays for serodiagnosis of feline bartonellosis has been advised; however, in a recent study of naturally infected cats, the PPV of a Western blot test for the presence of *Bartonella* DNA in cat blood was shown to be just 18.8% (Lappin et al., 2009; Guptill, 2010a).

1.2.10 Treatment

Bartonellosis has been effectively treated with a range of antibiotics. *In vitro*, most antibiotics are usually effective against *Bartonellae* (Minnick and Anderson, 2015). However, the administration of antimicrobials does not appear to benefit or lessen the duration of the illness in the majority of the cases. Unless the infection is systemic, CSD and trench fever are self-limiting infections that do not require treatment (Minnick and Anderson, 2015). Even with antibiotic treatment, relapses of bartonellosis are common.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Antimicrobial sensitivity varies within the genus, and the antimicrobial susceptibilities of *Bartonella* spp. *in vitro* and *in vivo* do not consistent for a lot of antimicrobials (Baldani et al., 2014). Antimicrobial susceptibilities have been evaluated in the presence of eukaryotic cells as well as in the absence of cells. The difference

in growth medium and intracellular environment had an effect on the level of antibacterial susceptibility (Gadila and Embers, 2021). In general, *Bartonella* spp. are very susceptible to antibacterial drugs *in vitro.* Only fosfomycin, colistin, and vancomycin demonstrate the resistance in agar dilution tests (Maurin et al., 1995). Ketolides, such as telithromycin, macrolides, doxycycline, clarithromycin, and rifampin, are the most effective susceptibility, followed by amoxicillin, cefotaxime, azithromycin, clarithromycin, gentamicin, tetracycline, minocycline, and ceftriaxone (Baldani et al., 2014). The antimicrobials were helpful in promoting clinical outcomes associated with infection, including azithromycin, rifampin, ciprofloxacin, and trimethoprim-sulfamethoxazole, whereas penicillin, cephalosporins, tetracyclines, and erythromycin had little or no clinical effects (Chomel et al., 2004).

In vivo, *B. bacilliformis* is resistant to neosalvarsan and other arsenical compounds. Additionally, Penicillin, streptomycin, chloramphenicol, and oxytetracycline are all potent to *B. bacilliformis* (Baldani et al., 2014). Regarding testing in Vero cell cultures, *B. henselae, B. quintana,* and *B. elizabethae* are thought to have the sensitivity to the macrolides; azithromycin, clarithromycin, dirithromycin, erythromycin, and roxithromycin (Ives et al., 1997; Baldani et al., 2014).

Specific antibiotic resistance mutations in *B. henselae* (azithromycin, gentamicin, enrofloxacin, and ciprofloxacin), *B. quintana*, and *B. bacilliformis* (erythromycin, rifampicin, and ciprofloxacin) have recently been identified (Baldani et

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

al., 2014). Rolain et al. (2004) described the minimal inhibitory concentration (MIC) of various *Bartonella* spp.

To determine the type and duration of antimicrobial therapy, the immunological status of the patient should be taken into consideration (Minnick and Anderson, 2015). Then, antimicrobial therapy for immunocompetent individuals differs from that for immunocompromised ones (Chomel et al., 2004). Antimicrobial therapy for healthy individuals takes around 3 weeks; however, therapy for immunocompromised patients may take many weeks to months, or even a lifetime, to clear the infection (Minnick and Anderson, 2015).

Classical CSD is a self-limiting condition that recovers in 2 to 6 months and fails to respond with the antibiotics. The current evidence does not support the use of antimicrobials to treat CSD. Antimicrobials had no clear effect on either the cure rate or the remission time. In contrast, the effort in reducing the chance of developing systemic symptoms remains controversy. In immunocompetent individuals with mild to severe infections, appropriate monitoring and analgesia are recommended. In significant lymphadenopathy cases, azithromycin (10 mg/kg doses on day 1, and 5 mg/kg between days 2 – 5) may be substituted. Other antimicrobial recommendations include rifampicin (20 mg/kg/day divided into 2 doses for 2 – 3 weeks), ciprofloxacin (20 – 30 mg/kg/day twice a day for 2 – 3 weeks) or trimethoprim/ sulfamethoxazole (10 mg trimethoprim/kg per day in 2-3 times/day for 7 – 10 days) (Blagova and Yanev, 2021).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

In case of bacteremia, recommended treatment should be 3 mg/kg bodyweight gentamicin once a day for 2 weeks, followed by 200 mg doxycycline daily for 4 weeks. All patients treated with chloramphenicol are cured in an outbreak of Oroya fever in the Peruvian Andes. The optimum regimen for the treatment of *B. bacilliformis* bacteremia has been recommended as an initial dose of 50 mg/kg/day chloramphenicol for the first 3 days and a subsequent dose of 25 mg/kg/day till the remission of 14 days of treatment. Nevertheless, patients with *Bartonella* endocarditis are required valvular surgery and have high mortality rate (Angelakis and Raoult, 2014). When *Bartonella* endocarditis is suspected, the American Heart Association recommends ceftriaxone and gentamicin with or without doxycycline, and doxycycline and gentamicin when *Bartonella* endocarditis is confirmed. Endocarditis should be treated with 3 mg/kg/day gentamicin for 2 weeks, followed by 200 mg of doxycycline daily for 6 weeks (Angelakis and Raoult, 2014).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Furthermore, erythromycin's anti-angiogenic effect on microvascular endothelial cells is crucial and considered as the first-line therapy for individuals with bacillary angiomatosis or angioproliferative lesions (Angelakis and Raoult, 2014). Relapses, on the other hand, have been found often in immunocompromised individuals who obtained an inadequate duration of treatment. This patient was recommended to be treated with a 4-week treatment of doxycycline followed by an extended 8 weeks of doxycycline post recurrence. Treatment failure or recurrence has been documented while using nafcillin, dicloxacillin, cefalexin, amoxicillin,

aminoglycosides, and trimethoprim/ sulfamethoxazole, particularly in immunocompromised individuals who were given antibiotics for less than three months. Rifampicin has been advocated as the medicine of choice for the treatment of verruga peruana. Treatment with 10 mg/kg/day rifampicin orally for 10–14 days resulted in an 80% resolution rate, while those treated with 15 mg/kg/day streptomycin for 10 days resulted in a 56% resolution rate. The recommended dose of erythromycin for angioproliferative lesions is 500 mg four times a day for 3 months. In severe infections, 100 mg of erythromycin or doxycycline twice a day is recommended in conjunction with 300 mg of rifampicin twice daily. Recurrence issues following suggested treatment should be substituted with 500 mg erythromycin four times daily or 100 mg doxycycline twice daily for 4–6 months**,** or as long as they are immunocompromised (Angelakis and Raoult, 2014).

Regarding feline infection, it is difficult to eliminate bacteremia in cats by antimicrobials because of the recurrent nature of feline *Bartonella* infections. There is still no proven regimen for clearing *Bartonella* infections in cats. (Guptill, 2010b). Enrofloxacin treatment for 28 days seemed to remove *B. henselae* or *B. clarridgeiae* infection in cats. In contrast, enrofloxacin causes retinal degeneration and blindness in cats. However, recent studies exhibit a high *in vitro* susceptibility of pradofloxacin to *B. henselae*. Unfortunately, a subsequent investigation discovered naturally occurring fluoroquinolone resistance in *Bartonella* isolates, leading to the recommendation that fluoroquinolones not be utilized to treat any *Bartonella*-related human clinical illness.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

(Guptill, 2010b). Doxycycline demonstrated benefits in treating infections caused by *B. henselae* and *B. clarridgeiae*. Higher doses could be more effective in treating feline *Bartonella* infections. Antimicrobials, i.e., erythromycin, amoxicillin, amoxicillin/ clavulanic acid, tetracycline hydrochloride, help lower bacteremia in cats. However, the studies in cats were not observed for a long period of time. (Guptill, 2010b). Furthermore, azithromycin has been widely used to treat feline bartonellosis, despite reports of macrolide-resistant of *Bartonella* spp. Whereas the efficacy of many antibiotics is unclear, there is a danger that frequent treatment of asymptomatic feline *Bartonella* infections will develop in bacterial resistance. Hence, treatment is only recommended in cats with clinical signs. Doxycycline may be the best antimicrobial to be using. The combination of amoxicillin and clavulanic acid may also be beneficial. More controlled studies, however, are required to evaluate antimicrobial strategies for *Bartonella* infections in cats. (Guptill, 2010b).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.2.11 Prevention

The best way to avoid *Bartonella* infections is to avoid encounter with infected animals and arthropod vectors (Guptill, 2010a). In South America, the only known risk factor for *B. bacilliformis* infection is exposure to phlebotomine sand flies (*Lutzomyia* spp.) bites (Minnick and Anderson, 2015). Related sand flies, on the other hand, may act as vectors for the agent (Minnick and Anderson, 2015). Insecticide treatment is the most effective control strategy for sand flies (Minnick and Anderson, 2015). Regarding *B. henselae*, the high incidence (89%) of persistent bacteremia with *B. henselae* has

been documented in self-own cats of CSD patients, while 9%-41% has been reported in control cats from the United States and Japan (Minnick and Anderson, 2015). Cat exposure is the major risk factor for getting *B. henselae* infection (Guptill, 2010a; Guptill, 2010b; Minnick and Anderson, 2015). Reduced exposure to cats during compromised states, antimicrobials for affected individuals, and flea control are all crucial part of the disease's prevention *(Koehler, 2008; Mofenson et al., 2009; Pennisi et al., 2013)*. According to *B. quintana*, they are transferred to humans by body lice (*Pediculus humanus*). Other blood-sucking insects, such as a louse, mite, or flea, are thought to be vectors. Overcrowding and inadequate hygienic conditions promote contact with the vector. Furthermore, being homeless, non-caucasian background, and having alcoholism are risk factors for this infection. Delousing, hygiene measures, and antimicrobial therapy should be recommended to manage this problem (Minnick and Anderson, 2015). For our knowledge, no vaccine is available (Koehler, 2008; Guptill, 2010a; Minnick and Anderson, 2015; Scorpio and Dumler, 2015).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Several *Bartonella* species or subspecies are thought to be zoonotic or potentially zoonotic. Flea and tick control, avoiding encounters that resulting in scratches or bites, thoroughly cleansing bite or scratch wounds, obtaining medical assistance when necessary, and purchasing new pets with known a good health and ectoparasite free are all common precautions for avoiding transmission of *Bartonella* spp. from pets to humans. The Centers for Disease Control and Prevention, the National Institutes of Health, and the Infectious Diseases Society of America Guidelines

for Prevention and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents, as well as HIV-Exposed and HIV-Infected Children, recommend a number of practical measures to reduce the risk of infection, particularly in HIV-infected people (Table 1.1). The Guidelines suggest that there is no evidence that scheduled culture or serologic testing of cats for *Bartonella* infection benefits cats or their owners (Koehler, 2008; Mofenson et al., 2009; Pennisi et al., 2013).

Table 1.1 Prevention Recommendations in immunocompromised persons.

Modified from Koehler (2008); Mofenson et al. (2009); Pennisi et al. (2013)

Recommendations for prevention of infection in immunocompromised persons
• Reducing exposure to vectors of the disease, i.e., the body louse, cats and cat fleas
• Do not allow a cat to lick wounds or cuts.
• Avoid rough play with cats
• Wash any cat-associated wounds promptly
• wash hands after petting and handling pets
• If a new cat is acquired
• Adopt a cat older than1year of age that is in good health and flea-free
• Maintain flea control and minimize flea infestation of pets
• Cats should be kept indoors to avoid exposure to fleas and other possible vectors, and also to prevent other zoonotic risks

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.3 Objectives of the Study

1. To produce the recombinant *B. henselae* specific antigen protein 17-kDa and GroEL.
2. To develop the ELISA diagnostic test using *B. henselae* specific antigen protein 17-kDa and GroEL.
3. To find risk factors and determine an association between *Bartonella* spp. infection and immune status by using retroviral-infected cats as a immunocompromised model.

1.4 Research Hypothesis

1. The recombinant *B. henselae* specific protein: 17-kDa and GroEL can be produced.
2. The recombinant *B. henselae* specific protein: 17-kDa and GroEL can be used to develop a *Bartonella* spp. ELISA test.
3. The performance of this newly developed recombinant *B. henselae* specific protein: 17-kDa and GroEL in the ELISA test is equivalent to or higher than the commercially available IFA test.
4. Immune status is an important factor for developing the concurrent infection of *Bartonella* spp. in clinical healthy retroviral infected cats.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.5 Advantages of Study

1. This study can provide the test kit for the diagnosis of *Bartonella* spp. infection in both retroviral-infected and normal cats.
2. Developed ELISA test kit for detecting antibody against novel BSP will improve the accuracy of diagnostic test of *Bartonella* spp. infection in cats and facilitate as the clinical screening test for cats
3. This study will help to understand the role of the immune status to prognose and increase awareness of clinical sign in *Bartonella* spp. infection in cats.

1.6 Keywords : *Bartonella* spp., cats, feline retrovirus, indirect ELISA, recombinant protein, risk factors

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Chapter 2

B. henselae specific antigen protein production

The purpose of this chapter is to demonstrate the experimental design used to create the recombinant *B. henselae* specific antigen protein; 17-kDa and GroEL. These proteins have been proposed to have strong immunoreactivities and to be used as antigens in human serum-based antibody test kits.

2.1 Materials and Methods

2.1.1 Bacteria and preparation of *B. henselae* genomic DNA

The Bangkok field strain of *B. henselae,* which were collected and stored previously at -80 ^{o}C were used. The culture method was as described previously (Maruyama et al., 2000b). In brief, the stock cultures were thawed at room temperature and spun down to discard the supernatant. The sediments were mixed with 125 µl of isolation medium-199 (Gibco®, USA) and spread onto a 5% sheep blood BHI agar plate. The culture plates were kept in 37 ^{o}C and 5% CO_2 for 2 weeks. The colonies were collected by scraping and processed for DNA extraction following the manufacturing instructions (NucleoSpin® Tissue, Macherey-Nagel GmbH & Co., Germany). The cultures were re-confirmed as *Bartonella henselae* by polymerase chain reaction (PCR) and DNA sequencing. (Jensen et al., 2000; Rampersad et al., 2005).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.1.2 Cat serum samples and IFA testing

Positive and negative cat sera used in this study were defined by using both the commercially available IFA test kit (MegaFLUO® *BARTONELLA* (Megacor Diagnostik GmbH, Austria) and PCR method as previous described. (Jensen et al., 2000; Maruyama et al., 2000b). Sera tested positive on both the IFA test and PCR was considered positive control whereas sera tested negative on both tests was considered negative control. This study was approved by Chulalongkorn University Animal Care and Use Committee (protocol No. 1931953).

2.1.3 Cloning, expression, and purification of 17-kDa and GroEL proteins

The primer used for the cloning of *17-kDa* and *groEL* genes were designed based on the GenBank accession number U23447.3 and U78514.1, respectively (Table 2.1). The *17-kDa* gene was cloned into pET28b (Appendix 1) and the *groEL* genes was cloned into pH6HTC vector (Appendix 2). The recombinant plasmids were transformed into *E. coli* BL21 (DE3) strain by heat shock method at 42 °C for 45 seconds. The transformants were verified by colony PCR and DNA sequencing. The positive colonies were grown in LB broth with appropriate antibiotics according to the expression vectors. The culture was incubated at 37 °C with shaking until the OD_{600} reached 0.4 – 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture with the final concentration 0.05 mM. The cell lysate was analyzed by SDS-PAGE and stained with Instant*Blue*™ (Expedeon, United Kingdom). The expected protein sizes were 17 kDa of 17-kDa protein and 93 kDa of GroEL (60 kDa) with Halotag.

The expected protein bands were excised from gels and analyzed by liquid chromatography-mass spectrometry (LC-MS) analysis at Proteomics Service, Faculty of Medical Technology, Mahidol University, Thailand. The purification of the recombinant proteins was performed by affinity chromatography using Ni Sepharose 6 Fast Flow (GE Healthcare, USA). Imidazole was used to elute the recombinant proteins. For the recombinant 17-kDa protein, it was expressed as the inclusion body. This protein was dissolved in 6M urea prior the purification and refolded in 1X PBS afterward.

2.1.4 Immunoblot

The dot blot was used to confirm the immunoreactivity of the recombinant proteins. Purified proteins were blotted onto nitrocellulose membranes. The membranes were blocked with blocking buffer (3% bovine serum albumin, BSA in 0.1 % TBS-Tween20) and washed 3 times with washing buffer (0.1% TBS-Tween20). The membranes were incubated with 1:500 diluted cat serum as primary antibody and 1:3000 diluted goat anti-cat IgG conjugated with horse radish peroxidase (HRP) as secondary antibody. The clarity ^{TM} western ECL substrate (Bio-Rad, USA) was used to develop the membrane per manufacturer description. The membranes were incubated for 5 minutes before imaging by Fusion Fx7 gel doc (Vilber Lourmat, France).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 2.1 Primers of *17kDa and groEL* genes

Primer name	Vectors	Restriction Enzyme	Sequences
17kda_pET28b_F	pET28b	*Nde*I	5’-TGCCGCGCGGCAGCCATATGATGAAAAAATATAGC-3’
17kda_pET28b_R		*Xho*I	5’-TGGTGGTGGTGCTCGAGAAGTCGGACATCAGATTT-3’
GroEL_pH6_F	pH6HTC	*Sgf*I	5’-TTACTAAGGAGCGATCGCTATGGCTGCTAAAGAAG-3’
GroEL_pH6_R		*Xho*I	5’-CCTCAGTGGTTGGCTCGAGGAAGTCCATGCCGCC-3’

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2.2 Results

Both *17-kDa* and *groEL* genes were successfully cloned and expressed as the recombinant proteins under IPTG induction. Positive colony were confirmed by colony PCR following with DNA sequencing in which the expected band of 476 bp of *17-kDa* and 1644 bp of *groEL* genes were detected (Figure 2.1 and Figure 2.2). Each confirmed clone was induced with the optimum concentration of induction of 0.05 mM IPTG and analyzed by SDS-PAGE. The expected bands of 17-kDa (17 kDa) and GroEL (93 kDa) were detected and confirmed by mass LC-MS analysis at Proteomics Service, Faculty of Medical Technology, Mahidol University, Thailand (Figure 2.3 and Appendix 3). The recombinant 17-kDa protein was found in a water insoluble pellet (Figure 2.4A) whereas the recombinant GroEL protein was in the supernatant (Figure 2.4B). Optimization of purification was done by gradually increasing of imidazole concentration (20-500 mM). The recombiant17-kDa protein was purified and eluted at 250 mM imidazole in 6M urea (Figure 2.5A). The recombinant GroEL was markedly eluted in 100 mM imidazole in 1X PBS fraction (Figure 2.5B). Both proteins were remarkably reactive against IFA-positive cat serum (Figure 2.6).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

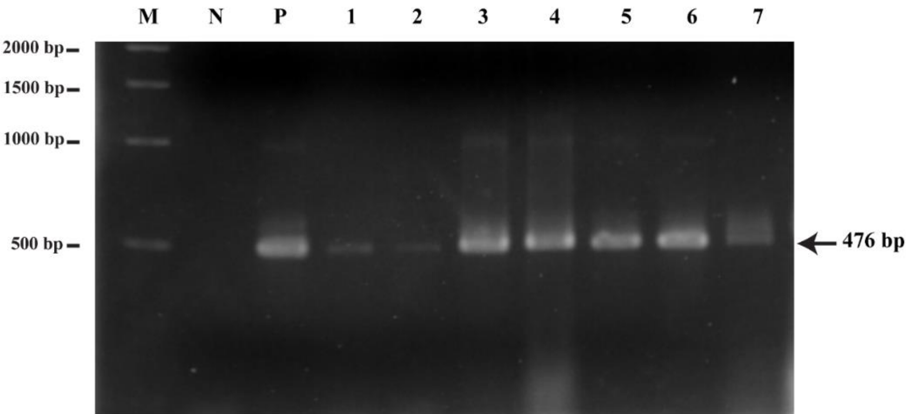


Figure 2.1 Verification of *17-kDa* transformants by colony PCR. M lane is DNA ladder, Lane N is negative control, Lane P is positive control which showed expected 476 bp product size of 17-kDa genes, Lane 3-6 are isolated colonies which showed positivity.

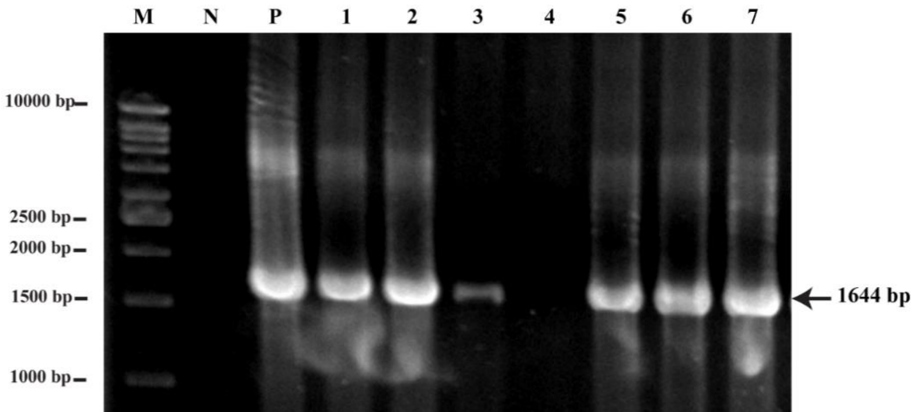


Figure 2.2 Verification of *groEL* transformants by colony PCR. M lane is DNA ladder, Lane N is negative control, Lane P is positive control which showed expected 1644 bp products size of groEL genes, Lane 1, 2, 5, 6, and 7 are isolated colonies which showed positivity.

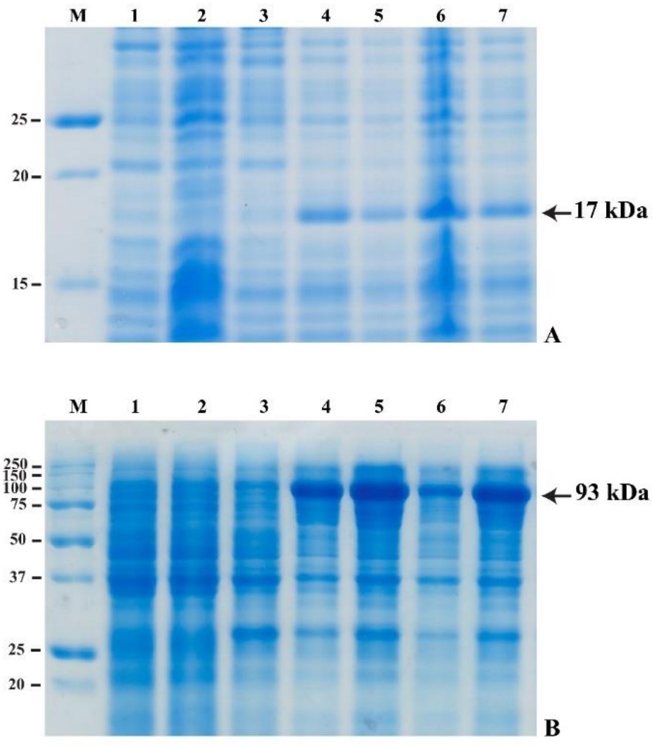


Figure 2.3 Identification of recombinant protein expressed colonies.(A) SDS-PAGE analysis of 17-kDa protein expression of isolated colonies. M lane is protein marker (kDA), Lane1: empty pET28b vector without IPTG, Lane 2: empty pET28b vector with IPTG, Lane 3: clone No. 1 without IPTG, Lane 4: clone No. 1 with IPTG, Lane 5-7: clone No. 2, 3 and 4 with IPTG, Arrowhead shows the expected size of 17-kDa protein. (B) SDS-PAGE analysis of GroEL protein expression of isolated colonies. M lane is protein marker (kDA), Lane 1: empty pH6HTC vector without IPTG, Lane 2: empty pH6HTC vector with IPTG, Lane 3: clone No. 1 without IPTG, Lane 4: clone No. 1 with IPTG, Lane 5-7: clone No. 2,3 and 4 with IPTG. Arrowhead shows the expected size (93 kDa) of GroEL protein (60 kDa with Halotag addition)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

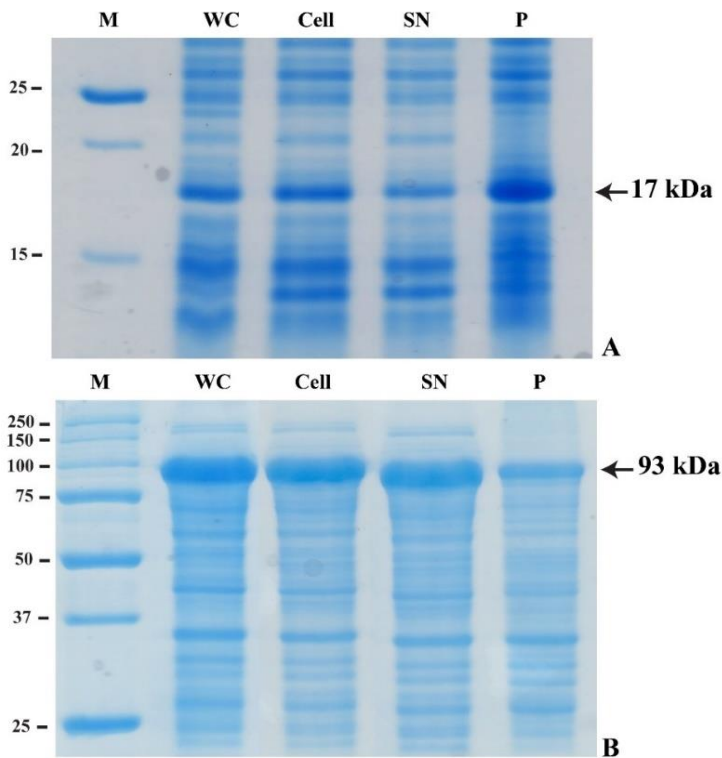


Figure 2.4 Identification of recombinant protein in lysate fractions.(A) SDS-PAGE analysis of 17-kDa protein fraction of bacterial lysate. M lane is protein marker (kDa), WC lane is a whole culture fraction, lys lane is a whole lysate fraction, S/N lane is supernatant fraction and P lane is pellet fraction. The arrow points to the 17 kilodalton site. (B) SDS-PAGE analysis of GroEL protein fraction of bacterial lysate. M lane is protein marker (kDa), WC lane is a whole culture fraction, lys lane is a whole lysate fraction, S/N lane is supernatant fraction and P lane is pellet fraction. The arrow points to the 93 kilodalton site that represent to GroEL protein (60kDa) fused with Halotag (33kDa).

CHULALONGKORN UNIVERSITY

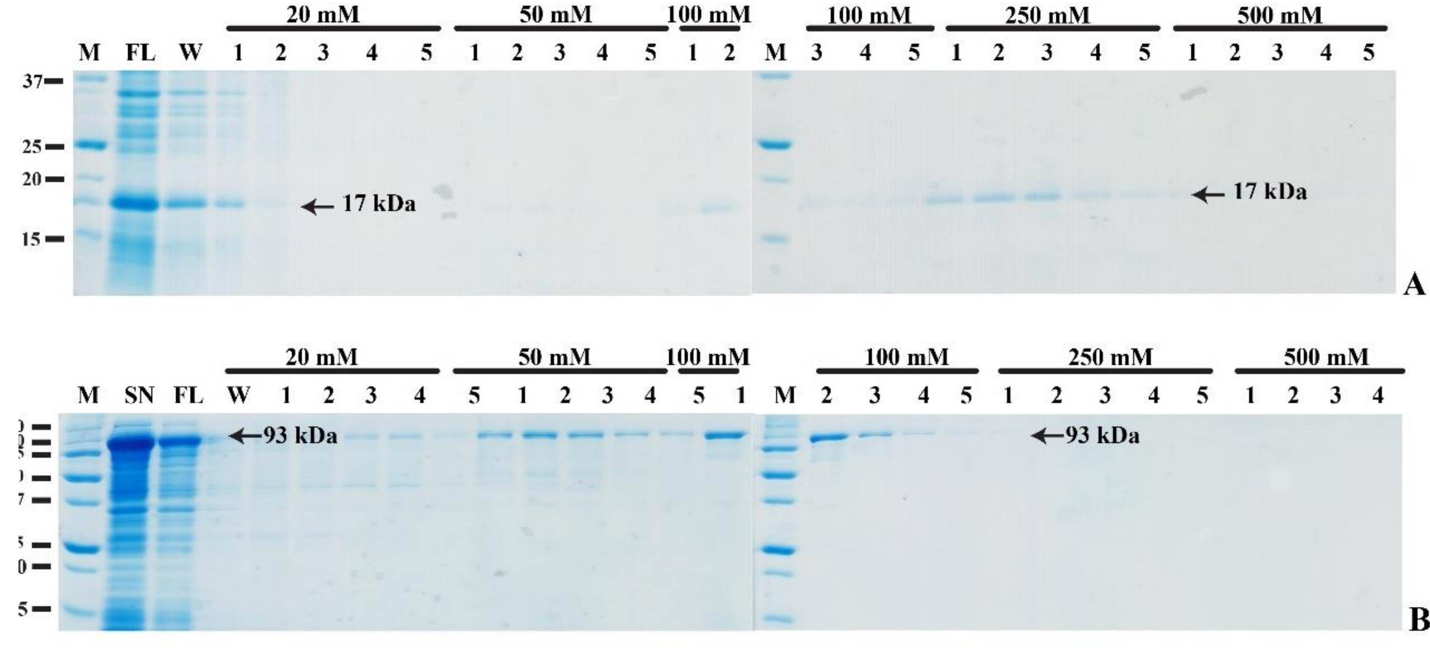


Figure 2.5 Optimization of recombinant protein purification. SDS-PAGE analysis of elution fractions of purified 17-kDa protein (A) and GroEL protein (B) by imidazole. M is protein marker (kDA), FL is Flow-through, W lane is washing, S/N is supernatant. The arrow points to the 17 kilodalton and 93 kilodalton site that represent to 17-kDa protein and GroEL protein (60kDa) fused with Halotag (33kDa).

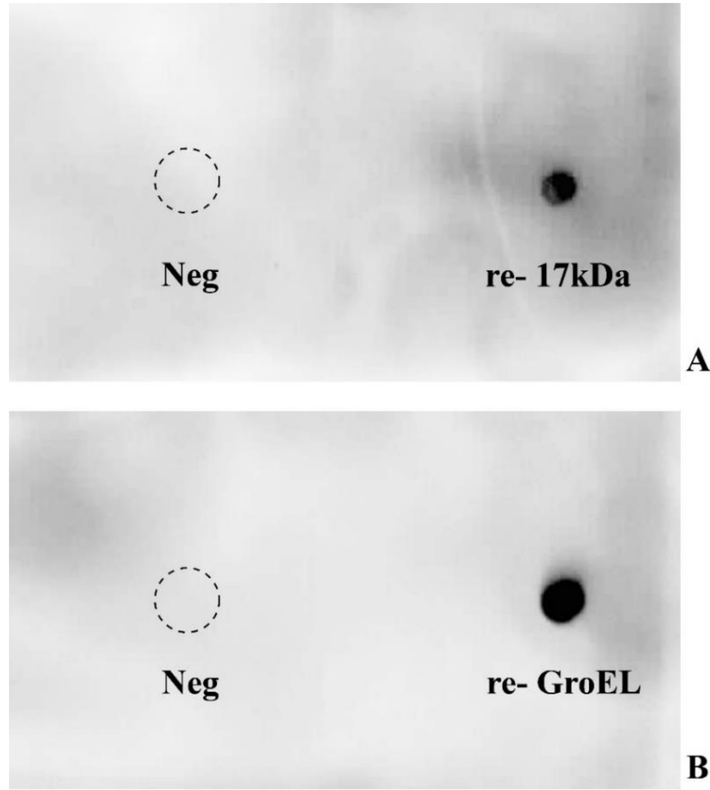


Figure 2.6 Dot blot analysis of recombinant proteins. Recombinant 17-kDa and GroEL proteins showed reactivity against IFA-positive feline serum (A) and (B) respectively.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.3 Discussions

Several proteins are recognized as immunogenic antigens for *B. henselae* (Anderson et al., 1995; Loa et al., 2006; Eberhardt et al., 2009; Saisongkorh et al., 2010). The Two proteins, 17-kDa and GroEL, investigated in this study were also recognized as BSP. The *17-kDa* gene is located in *VirB* operon of type IV secretion systems and related to the exportation of proteins or nucleic acids, and virulence factors in many bacteria (Padmalayam et al., 2000). Type IV secretion system in *Bartonella* spp. is reported to play a pivotal role in intraerythrocytic evasion, a significant phase of infection (Padmalayam et al., 2000; Wagner and Dehio, 2019). On the other hand, GroEL proteins belongs to the heat shock protein family and previously reported as the antigens in a variety of bacterial species including *Bartonella* species (Eberhardt et al., 2009; Saisongkorh et al., 2010; Ferrara et al., 2014). Heat shock proteins are molecular chaperones actively responsive to stress condition such as hypoxia, phagocytosis, or transformation. Nowadays, GroEL of *Bartonella* spp. was reported as suspected virulence factors in several studies. It was found in the outer membrane and might associated with antiapoptotic and mitogenic factors (Dehio, 2005; Harms and Dehio, 2012). However, the molecular mechanism of GroEL was not known (Harms and Dehio, 2012).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The expressions of recombinant 17-kDa and GroEL proteins of *B. henselae* were successfully demonstrated in this study. The *17-kDa* gene was cloned into pET28b plasmid vectors and transformed to competent *E. coli* BL21 (DE3) strains. However,

groEL was successfully cloned to pH6HTC vector. In previous studies, 17-kDA also showed success in PinPoint Xa-2, pTriEx-4 and pMAL-c2X vectors (Anderson et al., 1995; Loa et al., 2006; Ferrara et al., 2014). In contrast, *groEL* was reported success of cloned into pTrcHisB vector (Ferrara et al., 2014). Both pET28b and pH6HTC vectors used in this study contains the T7lac promoter which promote target gene products and have polyhistidine (His) tagged which are benefit for purification. Moreover, pH6HTC vectors contain Halotag® which use for labeling the target expression proteins. So, these selected vectors were selected to clone both genes in the first hand. However, *17-kDa* gene was success cloned in to pET28b vector while *groEL* gene was success cloned into pH6HTC vector.

Marked express level of recombinant 17-kDa was found in pellet of lysate as insoluble protein. They need the dissolved process by 6M urea prior purification. The hydrophobic property of 17-kDa protein was noted. The first 18-20 hydrophobic amino acids of 17-kDa and two lysine residues are common structure in bacterial outer membrane proteins (Anderson et al., 1995; Sweger et al., 2000). However, that process did not disturb the immunoreactive property of this protein as shown by dot blot assay. The same phenomenon is also occurred in previous study using human serum (Loa et al., 2006). Likewise, previous study showed that the immunoreactive against feline serum even their protein are folded in pellet (Satranarakun et al., 2017). On the other hand, recombinant GroEL protein expressed in pH6HTC vector was found in supernatant fraction as soluble protein and immunoreactivity was noted. Previous

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

study also found the similarity phenomena of GroEL expression (Ferrara et al., 2014). GroEL protein is located in both inner and outer membranes of the cell. GroEL protein of several *Bartonella* species were report as mitogenic effect to host cells and also found in supernatant fractions of bacterial culture (Haake et al., 1997; Minnick et al., 2003). However, this study made first reported the expression and detection of immunoreactivity of GroEL against cat sera.

In human serology, using of recombinant 17-kDa has been reported the favorable results for antibody detection in ELISA-based assay (Loa et al., 2006) and immunoblot assay (Anderson et al., 1995). Furthermore, one study in human showed preferential outcomes of antibody detection against *B. henselae* infection by using coordinate of 17-kDa and GroEL (Ferrara et al., 2014). However, the study in veterinary field may still not recognized. Thus, the utilizing of these two recombinant proteins in this study, 17-kDa and GroEL, can be the candidate antigenic proteins for either detection or development of *Bartonella* spp. vaccine for cat in the future.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Chapter 3

Development of ELISA using *B. henselae* specific antigen proteins

According to the limitations of current serological tests, the aim of this chapter is to develop the ELISA diagnostic test using *B. henselae* specific antigen proteins; 17-kDa and GroEL.

3.1 Materials and Methods

3.1.1 Indirect ELISA

Both purified antigen proteins, recombinant 17-kDa and GroEL proteins, were diluted with coating buffer (Bicarbonate/carbonate buffer (100 mM), pH 9.6) and loaded to 96-well plated (Corning®, USA). Coated plates were incubated overnight at 4 °C. Washing process was done by washing buffer (0.05%TWEEN in PBS) and repeated 5 times in each plate. After washing, blocking buffer (1%Bovine serum albumin, BSA in PBS) were added and incubated at 37 °C for 60 minutes. Then, washing process was done. Diluted serum in 1% BSA was added and then incubated at 37 °C for 60 minutes. Then, washing process was repeated. Diluted conjugated antibody (Goat Anti-Cat IgG Fc (HRP), Abcam, UK) was added and incubated at 37 °C for 60 minutes. Then, washing process was repeatedly done. The colorimetric method was developed by adding hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine (TMB Peroxidase EIA Substrate Kit, Bio-Rad, USA) and incubated for 5 minutes. Then, the reaction was stopped by 1N sulfuric acid (1N H_2SO_4). The results were read by spectrophotometer, the optical

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

density at 450 nanometers. The diagram of indirect ELISA description and workflow was shown (Figure 3.1 and Figure 3.2).

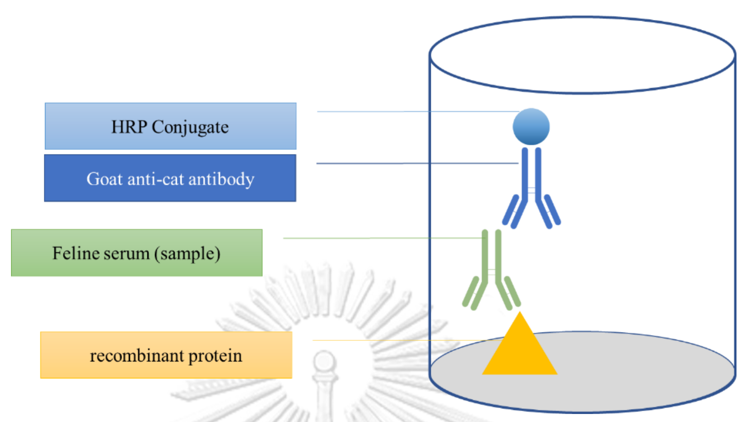


Figure 3.1 Diagram of Indirect ELISA.

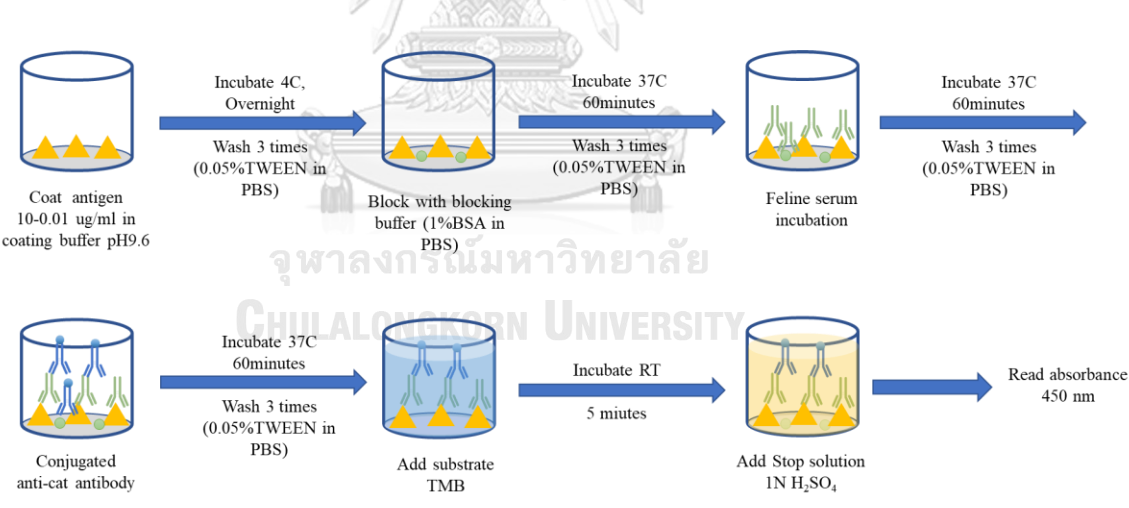


Figure 3.2 Diagram of workflow of indirect ELISA.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.1.2 Reagent formulations (Table 3.1)

3.1.2.1 Coating buffer/ Carbonate buffer

The optimal antigen concentration prepared by diluting the antigen in 0.05 M Carbonate-Bicarbonate, pH 9.6. In brief preparation, 6.0 grams of Sodium Bicarbonate ($NaHCO_3$) and 3.03 grams of Sodium Carbonate (Na_2CO_3) were added in 800 ml of distilled water. Then, adjust the pH by NaOH solution and adjust volume by distilled water until volume is 1 liter.

3.1.2.2 Blocking buffer

The blocking buffer were used for blocking the remaining protein-binding sites in the coated wells and diluting the serum, conjugated antibody. The 1% of bovine serum albumin (BSA) in phosphate buffer saline (PBS) was used in this study.

3.1.2.3 Phosphate buffered saline

PBS is an isotonic buffer and used for many kinds of solutions in biochemistry experiments. To prepare 10x PBS, added 80 grams of sodium chloride (NaCl), 2 grams of potassium chloride (KCl), 14.4 grams of disodium hydrogen phosphate (Na_2HPO_4), and 2.4 grams of potassium dihydrogen phosphate (KH_2PO_4) to 800 ml of distilled water. Then, the solution was adjusted the pH to 7.4 and the volume until volume was 1 liter.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.1.2.3 Washing buffer

The washing buffer was used to remove unbound and excessive components that are capable of interfering with the assay. Preparation of washing buffer was a mixing of PBS and 5% of Tween-20.

3.1.2.4 Serum and conjugated diluent

The diluting of sera and conjugated antibody were used for reducing the non-specific background and exaggerate reaction. The serum and conjugated diluting were prepared by dilute each serum and conjugated in blocking buffer.

3.1.2.5 Substrates/ Chromogen

In this study, conjugated antibody was labeled with horseradish peroxidase (HRP). To detect the HRP, TMB (3,3',5,5'-Tetramethylbenzidine) was used as a substrate. The combination of HRP and hydrogen peroxide causes the oxidation of TMB, resulting in the formation of a blue color.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.1.2.6 Stop solution

For endpoint assays, the immunoreactions were stopped after 5 minutes of incubation before the measurement. The one normality of sulphuric acid (H_2SO_4) was used in this study. To prepare, added 6.9 ml of concentrated sulfuric acid into 250 ml of distilled water. After the stop reaction, the reaction turned to yellow color and read the absorbance at 450nm.

Table 3.1 Solution preparation

Solutions	Component for 1 liter
Coating buffer/ Carbonate buffer	3.03 g Na_2CO_3 6.0 g $NaHCO_3$ 1000 ml distilled water Adjust pH 9.6
Blocking buffer	10 g BSA 1000 ml 1X PBS
10X Phosphate buffered saline	80 g NaCl 2g KCl 14.4g Na_2HPO_4 2.4 g KH_2PO_4 800 ml distilled water Adjust pH 7.4 Adjust with distilled water to 1L
Washing buffer	1000 ml 1X PBS 500 µl Tween 20
Stop solution	27.6 ml of concentrated H_2SO_4 800 ml distilled water Adjust with distilled water to 1L

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.1.3 ELISA condition optimization

3.1.3.1 Checkerboard titration

Preliminary experiments were conducted to establish the optimum amount of purified antigen (0.01-10 µg/ml), serum dilution (1:100 – 1:102,400), and conjugated antibody dilution (1:3000 – 1:192,000). Checkerboard titration method was employed for this study. The preliminary results showed 1.25 µg/ml of purified antigen, 1:200 serum dilution, and 1:12,000 conjugated antibody gave the best signal quality.

3.1.3.2 Quantification of antigen

First, the purified proteins concentration were measured by Bradford assay (biorad). The Bradford assay uses standards to quantify the amount of protein. The concentration range of standards should cover the range of suspected amount of protein. This assay compared the color of sample with the curve of standard protein concentration. After estimate the purified proteins, coating buffer were added to dilute the purified protein until the concentration was 10 µg/ml.

3.1.3.3 Antigen titration

Antigen proteins were diluted with coating buffer to 10 µg/ml. The 96-well plates were prepared. One hundred microliters of coating buffer were load to each well in column number 2-12. Two hundred microliters of diluted protein antigen were load to each well in column number 1. Multichannel pipette was used to perform 2-fold serial dilution from column number 1 to number 11. Positive and negative sera were diluted with blocking buffer to 1: 100. Sera were performed 2-fold serial dilution from row A to row G. the positive and negative sera were done in separated plated. Last, conjugated antibody (goat-anti cat) was diluted to commercial recommended dilution (1:300) and load to each well in 96-well plated. Column 12 and row H were left to blank and received only coating buffer.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.1.3.4 Sera and conjugate titration

The optimum concentration of antigen protein was prepared and load 100 µl of solution to each well. In contrast to previous stage, sera dilution was performed 2-

fold dilution in column 1 to 11 direction. The conjugated antibody dilution was prepared and performed 2- fold serial dilution from 1:3000 to 1:192,000 (row A to G).

3.1.4 Evaluation of Validation

3.1.4.1 Criteria of positive and negative sera

Positive and negative sera were defined by IFA assay. Positive sera were positive to IFA assay. Negative sera were negative to IFA assay.

3.1.4.2 Receiver Operating Characteristics (ROC) analysis

Receiver Operating Characteristics (ROC) analysis were deployed to defined positive and negative cut-off of optical density (OD) value of ELISA results. Area under the curve (AUC) parameter was plotted and calculated. Optimization pair between sensitivity and (1-specificity) which has the greatest distance in a Northwest direction, from the diagonal line, Se = (1–Sp), was considered as the cut-off point.

3.1.5 Evaluation of repeatability

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Intra-plate and inter-plate were tested by 3 replicates. Each result was obtained and calculated mean OD, standard deviation ad coefficient of variation (%CV) to determine the repeatability of ELISA test. Coefficient of variation (%CV) of OD value that equal or less than 15% are acceptable.

3.2 Results

3.2.1 ELISA condition optimization

3.2.1.1 Antigen titration

Checkerboard titration was performed to determine optimum concentration of r17-kDA antigen. The optimum concentration was noted at 1.25 µg/ml. Similar to r17-kDA antigen titration, the optimum concentration of rGroEL was noted at 1.25 µg/ml. The results were shown (Appendix 4-Appendix 7).

3.2.1.2 Sera and conjugating titration

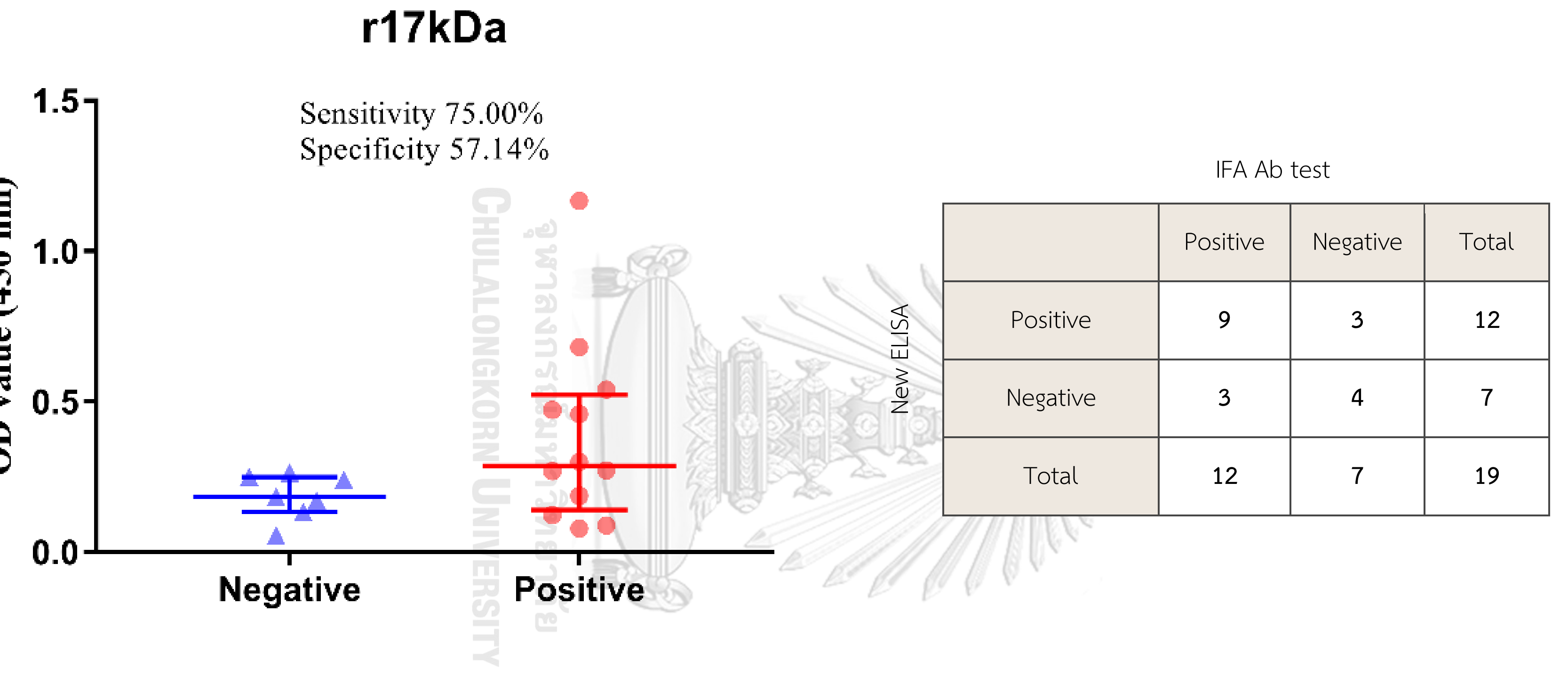
The checkerboard titration method showed the optimum concentration of serum dilution and conjugated antibody were 1:200, and 1:12,000, respectively. The data were shown (Appendix 8-Appendix 11).

3.2.2 Validation

3.2.2.1 ROC analysis

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Total 12 positive and 7 negative sera were used. ROC analyses were plotted and analyzed. The cut-off OD value of this tested was noted at 0.186067 (Figure 3.3). The similar positive and negative sera that used in 17-kDa were also used for determining cut-off of rGroEL ELISA. ROC analyses were plotted and analyzed. The cut-off OD value of this tested was noted at 1.9276 (Figure 3.4).

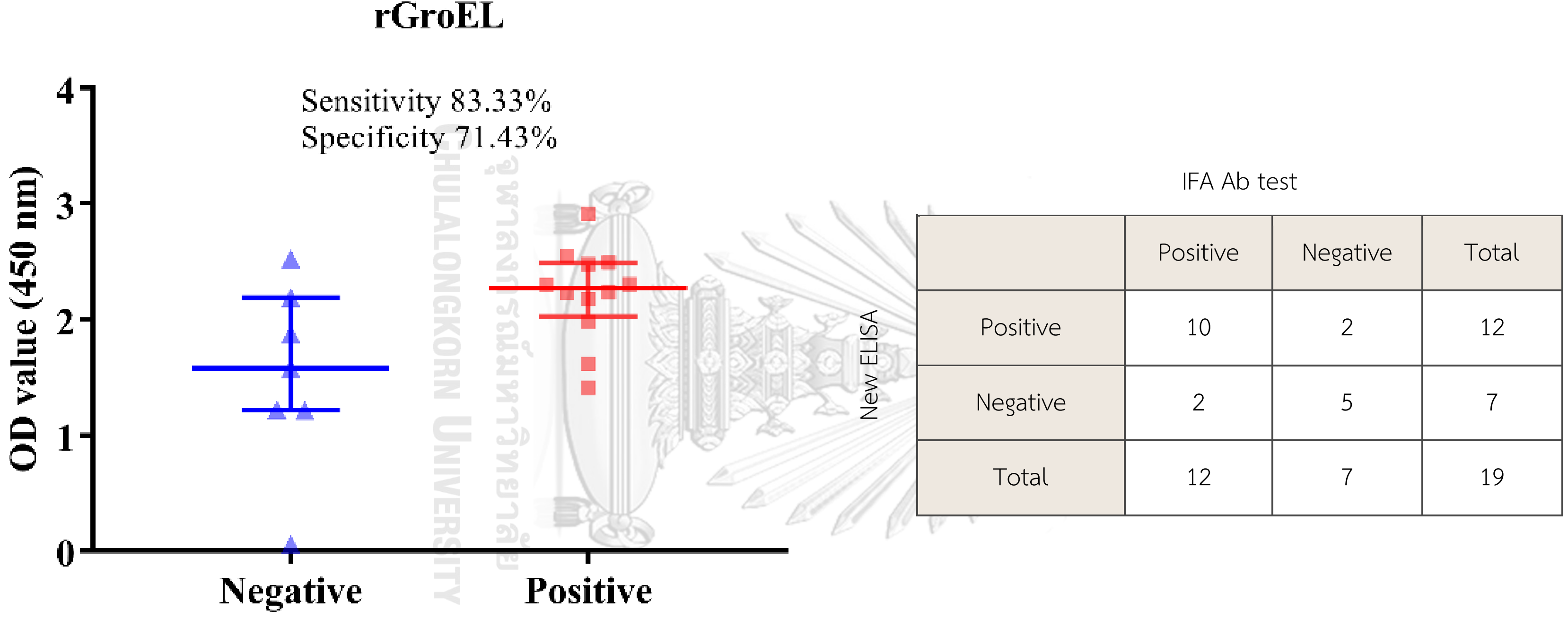


IFA Ab test (columns); New ELISA (rows)

New ELISA	Positive	Negative	Total
Positive	9	3	12
Negative	3	4	7
Total	12	7	19

Figure 3.3 Scatter plot and 2X2 table of r17-kDa-based ELISA results

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY



New ELISA	IFA Ab test: Positive	Negative	Total
Positive	10	2	12
Negative	2	5	7
Total	12	7	19

Figure 3.4 Scatter plot and 2X2 table of rGroEL-based ELISA results

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2.2.3 ROC analysis and Clinical performance of ELISA tests

An ELISA using recombinant 17-kDa and GroEL as the coating antigen were analyzed by Receiver Operating Characteristics (ROC) curves. The recombinant 17-kDa based ELISA demonstrated 75% and 57.14% of sensitivity and specificity with area under the curve (AUC) of 0.75 (Table 3.2, Table 3.3 and Figure 3.5). Similar to recombinant 17-kDa, recombinant GroEL based ELISA showed 83.33% and 71.43% of sensitivity and specificity with AUC of 0.798 (Table 3.2, Table 3.3 and Figure 3.6). Furthermore, both ELISA results were combined and showed an increased in the sensitivity and specificity of 91.67% and 42.86%, respectively.

Table 3.2 Area under the ROC curve

Area under the ROC curve	17-kDa	GroEL
Area	0.75	0.7976
Standard Error	0.1164	0.1187
95% confidence interval	0.5219 to 0.9781	0.565 to 1.03
P value	0.0759	0.0346

Table 3.3 Comparison of clinical performance of recombinant protein-based ELISAs

Statistic	r17kDa-based ELISA	rGroEL-based ELISA	At least 1 positive
Sensitivity	75.00% (42.81- 94.51%)	83.33% (51.59- 97.91%)	91.67% (61.52-99.79%)
Specificity	57.14% (18.41- 90.10%)	71.43% (29.04- 96.33%)	42.86% (9.90-81.59%)
PPV	75.00% (54.56- 88.23%)	83.33% (60.14- 94.31%)	73.33% (58.61-84.23%)
NPV	57.14% (29.24- 81.14%)	71.43% (39.34- 90.60%)	75.00% (27.61-95.63%)

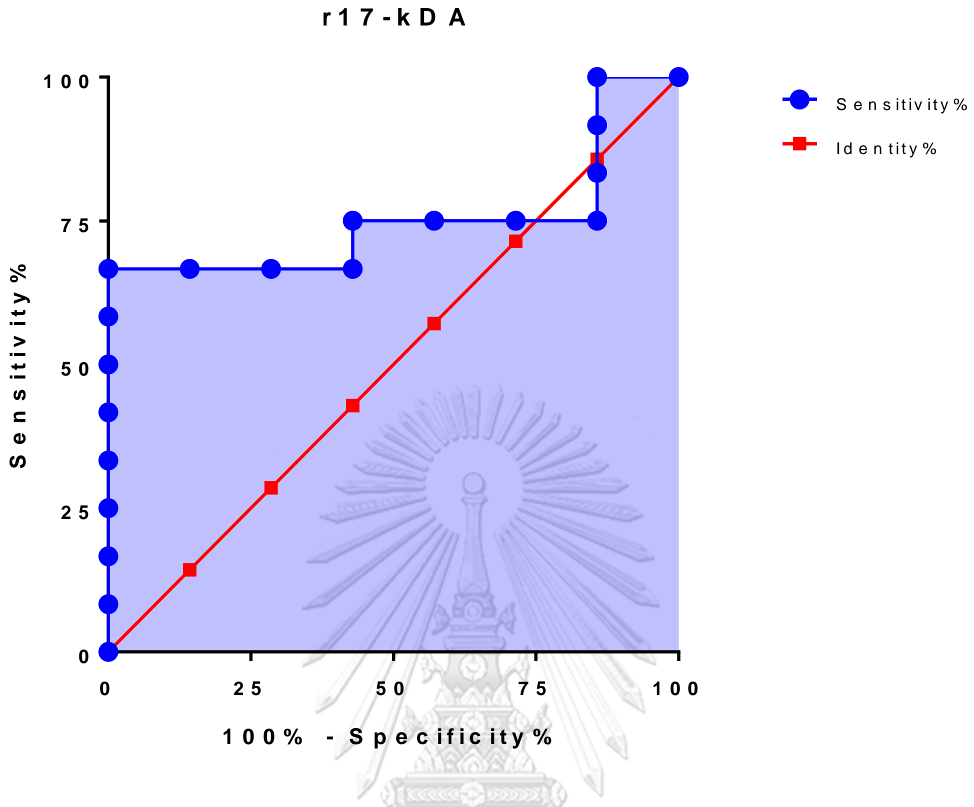


Figure 3.5 ROC curve analysis and clinical performance of r17-kDa-based ELISA

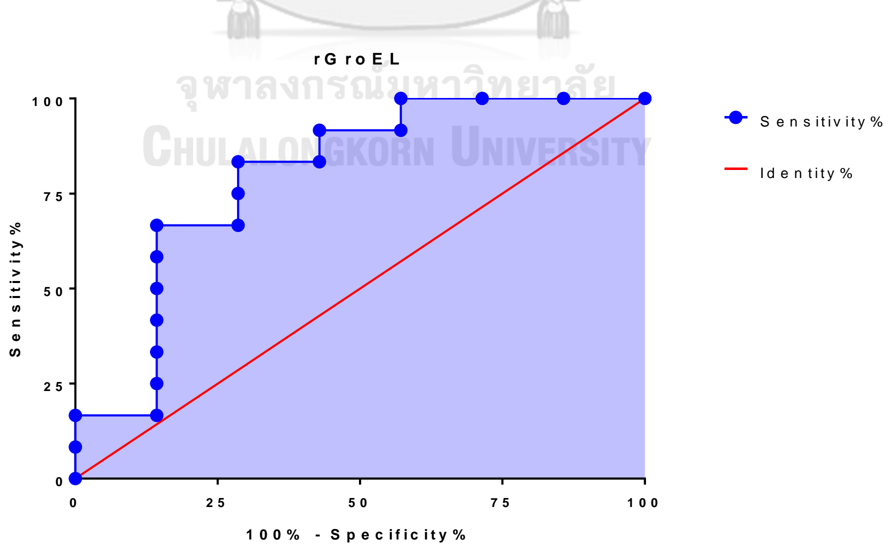


Figure 3.6 ROC curve analysis and clinical performance of rGroEL-based ELISA

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.2.3 Repeatability

A total of 12 positive and 7 negative sera were tested with 3 replicates. Coefficient variations were calculated. The r17-kDa showed %CV of intraplate range from 0.63% to 13.51%, with median value 5.24%. The rGroEL showed %CV of intraplate range from 0.41% to 11.58%, with median value 2.69%. For interplate repeatability, the r17-kDa showed %CV range from 1.03% to 25.54% with median value 5.74% and the rGroEL showed %CV range from 1.75% to 12.49% with median value 2.69% (Appendix 12-Appendix 19).

3.3 Discussions

In human, different serology testings for *Bartonella* spp. demonstrated variation in sensitivity and specificity. According to IFA-based test, the sensitivity of serology test varied between 14%-100% and specificity was varied between 34% - 100% (Sander et al., 2001). Serological investigations for detection of IgG by ELISA method in human were also reported with high specificity (90%-99%) and variable low sensitivity (10%-95%) (Sander et al., 2001; Agan and Dolan, 2002). The different results has been described by the difference in antigens preparation of each test. However, higher sensitivity and specificity were reported in IgM detection ELISA methods(Barka et al., 1993; Agan and Dolan, 2002). Nevertheless, The Advisory Board on Cat Diseases had recommended that IFA is more useful for exclusion rather than for confirmation of the infection because of the low positive predictive value when compared with the good negative predictive value (Pennisi et al., 2013; Sigirci et al., 2017) and the test often

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

can cross-reactivity with other pathogens such as *Coxiella burnetii, Chlamydia* spp.*, Rickettsia rickettsii, Ehrlichia chaffeensis, Treponema pallidum, Francisella tularensis,* and *Mycoplasma pneumonia* (McCool et al., 2008).

Bartonella henselae specific protein (BSP) based serologic tests were studied to reduce cross-reactive bacterial antigen and avoid handling an infectious material (Werner et al., 2008). Several BSP were reported to be immunoreactive (Kabeya et al., 2003; Loa et al., 2006; Werner et al., 2006; McCool et al., 2008; Wagner et al., 2008; Eberhardt et al., 2009; Feng et al., 2009; Saisongkorh et al., 2010; Ferrara et al., 2014; Satranarakun et al., 2017). Using recombinant 17-kDa as the selective protein for *Bartonella* spp. test has reported the favorable results for IgG antibody detection in ELISA-based assay (Loa et al., 2006) and immunoblot assay (Anderson et al., 1995). Furthermore, one study in humans showed preferential outcomes of IgG and IgM antibody detection against *B. henselae* infection by using combination of 17-kDa and GroEL (Ferrara et al., 2014). The specificity and sensitivity of human sera-based IgG detection ELISA were previously reported and range from 76-93% and 65.7-71% for recombinant 17-kDa protein (Loa et al., 2006; Ferrara et al., 2014). Similar to Ferrara et al. (2014), our developed antibody tests demonstrated appropriate results with the sensitivity (75% and 83.33%) and specificity (57.14% and 71.43%) for IgG antibody detection in cat sera by 17-kDa and GroEL-based ELISA. The antibody tests showed satisfactory immunoreactive performances and can recognized *Bartonella* spp. which can be used as screening detection and still need further development. However, due

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

to the small sample size in this study, additional cat serum samples from various disorders should be required to investigate and determine the specificities of this antibody test in the future.

Chapter 4

Feline bartonellosis and the association with feline retroviral infection

This chapter is the study of *Bartonella* spp. infection in cats and the association risks between retroviral infection and *Bartonella* spp. infection in cats. The purpose of this chapter was to find the prevalence and the association factors of *Bartonella* spp. infection in cats and relationship between feline retrovirus and *Bartonella* spp. infection in cats.

4.1 Materials and Methods

4.1.1 Cat samples

One hundred and sixty-one cat blood samples were collected during 2017-2020 at the veterinary clinics and hospitals in Bangkok metropolitan and vicinity area. The specific veterinarians checked general condition of cats and take data including fleas, ectoparasite prevention, and lifestyle of the cats from the owners. Three mls of whole blood was collected and placed into EDTA, heparinized and serum tubes, for CBC, blood chemistry and FeLV/FIV commercial test kit (Witness®, Zoetis). The remaining amount of blood sample were processed for serum collection, DNA extraction and then stored at – 20 °C for further studies. Moreover, all cats were categorized into 4 groups depending on their retroviral and *Bartonella* spp. infection status. Group 1 were clinically healthy cats negative to both *Bartonella* spp. PCR and retrovirus; Group 2 were cats negative to *Bartonella* spp. PCR but positive to retrovirus; Group 3 were cats positive to *Bartonella* spp. PCR but negative to retrovirus; and Group

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4 were cats both positive to *Bartonella* spp. PCR and retrovirus detection. The CD4+ to CD8+ ratios were compared among groups to investigate the influence of immunosuppression by retroviral infections on *Bartonella* spp. This study was approved by Chulalongkorn University Animal Care and Use Committee (protocol No. 1931953).

4.1.2 Detection of *Bartonella* spp. DNAs by PCR

Whole blood in EDTA were processed for DNA extraction by using commercial DNA extract kit (NucleoSpin® Blood, MACHEREY-NAGEL GmbH & Co.). Species-specific primer-PCR (SSP-PCR) of the 16S-23S rDNA was conducted for detection of *Bartonella* species as previous described (Jensen et al., 2000; Rampersad et al., 2005). The 16S-23S rRNA intergenic region sequence were amplified with PCR primers P-bhenfa (5'-TCTTCGTTTCTCTTTCTTCA-3') and P-benr1 (f5'-CAAGCGCGCGCTCTAACC-3') and nested primers N-bhenf1a (5'-GATGATCCCAAGCCTTCTGGC-3') and N-bhenr (5'-AACCAACTGAGCTACAAGCC-3'). DNA sequencing was also analyzed for the *Bartonella* spp. confirmation.

4.1.3 Indirect fluorescent-antibody (IFA) assay

Serology confirmation for *Bartonella spp.* was performed by commercially IFA test kit, MegaFLUO® *BARTONELLA* (Megacor Diagnostik GmbH, Austria) as company recommended. The IFA titer higher than or equal to 1:64 was considered as seropositive samples.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.1.4 Flow cytometry analysis

$CD4^+$, $CD8^+$ and CD4:CD8 ratio were used to evaluate immune status of enrolled cats (Pappalardo et al., 2000; Hosie et al., 2009; Kabeya et al., 2009). One hundred microliters of EDTA blood per sample were added into 5 mls polystyrene tube. To lyse red blood cell, 3 mls of lysing buffer (BD Pharm Lyse™,USA) added and incubated in 37 °C for 30 minutes. Then, centrifuged at 250 relative centrifugal force (rcf), 4 °C for 5 minutes and discarded the supernatant. The remaining pellet washed with 3 mls of wash buffer (PBS +1% w/v bovine serum albumin (sigma Aldrich, UK) + 0.1% sodium azide (sigma Aldrich, UK). Then, centrifuged at 1500 rcf, 4 °C for 5 minutes and discarded the supernatant. The pellet was stained with 5 µl of anti-feline CD4-FITC, anti-feline CD8-PE (Bio-Rad, USA) for 30 minutes at 37 °C. The samples were washed to remove excess staining by 3 mls of wash buffer and centrifuged at 1,500 rcf, 4 °C for 5 minutes and discarded the supernatant. To preserve stained samples before flow cytometry measurement, 100 µl of 2% of paraformaldehyde in 1% PBS added and kept in 4 °C.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.1.5 Statistical analysis

Association risk factors with *Bartonella* spp. infection; age, fleas, sex, retrovirus infection, were analyzed by Chi square and odds ratio (OR) and 95% confidence interval (CI) to determine the strength of association. The OR was considered significant by 95% CI that did not include a value of 1.0. Hematological and biochemistry parameters were also compared with independent t-test. Association risk between non-infection and infection groups. $CD4^+$ cells, $CD8^+$ cells, and $CD4^+$ to $CD8^+$ ratio were

compared among groups by Mann-Whitney and Kruskal-Wallis test and post-hoc analysis. A *p*-values less than 0.05 was considered as statistical significance.

4.2 Results

One hundred and sixty-one clinically healthy cats were included, 73 (45.3%) were male and 88 (54.7%) were female. The median age was 1.5 years old and the range was 2 months to 12 years old. Among 27 cats who were positive for feline retrovirus, 11 cats (6.8%) tested positive for FIV, 18 cats (11.2%) tested positive for FeLV, and 2 cats tested positive for both FeLV and FIV. There were 26 positive cats (16.1%) and 135 negative cats (83.9%) for *Bartonella* spp. by nested PCR. However, the presence of antibodies against *Bartonella* spp. as detected by IFA was high, with 150 (94.9%) cats testing positive and 8 (5.1%) cats being negative.

Mean ± SD of total white blood cell counts was 14,231.21±5,757 cells/ µl. The mean hematocrits percentage was 34.78±7.71%. The mean platelet counts was 138,883±75,150 cells/ µl. Mean serum blood urea nitrogen and creatinine values were 28.18±12.92 and 1.45±0.68 mg/dl, respectively. Mean ± SD of serum alanine aminotransferase and alkaline phosphatase were 75.91±74.50 and 42.67 ±31.71 units/L, respectively. Mean serum total protein, albumin, and globulin were 7.51±0.83, 2.90±0.33, and 4.61±0.90 mg/dl, respectively (Table 4.1).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 4.1 Complete blood count, blood chemistry, *Bartonella* spp. PCR result, and number of cats in each category

Parameters	Mean ± SD	*Bartonella* spp. PCR Negative Mean ±SD	*Bartonella* spp. PCR Positive Mean ±SD	*p*-value
WBC ($x10^3$cells/ µl)	14.23 ± 5.76	14.09±5.90 n = 128	14.91±5.03 n = 26	0.51
HCT (%)	34.78 ± 7.71	35.13±7.96 n = 128	33.03±6.21 n = 26	0.206
PLT ($x10^3$cells/ µl)	138.88±75.15	137.34±76.72 n = 128	146.5±67.80 n = 26	0.573
BUN (mg/dl)	28.18±12.92	28.36±13.89 n = 131	27.22±5.68 n = 25	0.687
Creatinine (mg/dl)	1.45 ±0.68	1.45±0.73 n = 132	1.46±0.27 n = 26	0.970
Total protein (mg/dl)	7.51±0.83	7.51±0.86 n = 126	7.55±0.66 n = 24	0.838
Albumin (mg/dl)	2.90±0.33	2.91±0.34 n = 126	2.90±0.25 n = 24	0.859
Globulin (mg/dl)	4.61±0.90	4.61±0.94 n = 124	4.65±0.67 n = 24	0.832
ALT (units/L)	75.91 ±74.50	78.39±79.06 n = 132	63.35±43.59 n = 26	0.348
ALP (units/L)	42.67 ±31.71	41.51±30.44 n = 125	48.96±38.03 n = 23	0.302

*Significance difference when *p*-value less than 0.05

Abbreviations: WBC = white blood cell count, HCT = hematocrit, PLT = platelet count, BUN = blood urea nitrogen, ALT = serum alanine aminotransferase, ALP = serum alkaline phosphatase, SD: standard deviation

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Blood profile including total white blood cell count, hematocrit, platelet count, blood urea nitrogen, creatinine, total protein, albumin, globulin, and ALT did not show any significant difference between *Bartonella* spp. PCR statuses (Table 4.1). Cats more than 1 year old were significantly associated with having *Bartonella* spp. seropositive (OR 4.296; 95% CI 1.010-18.275) (Table 4.2). However, the cats' ages were not related to PCR detection status (OR 2.480; 95% CI 0.700-8.793) (Table 4.3). Feline retroviral infections (positive to either FeLV or FIV), gender, and flea infestation status were not associated with both *Bartonella* spp. serostatus and PCR results (Table 4.2 and Table 4.3).

Median values of $CD4^+$ and $CD8^+$ T lymphocytes values were 15.175% (range 1.28-48.46%) and 8.350% (range 1.24- 41.88%), respectively. Median of the $CD4^+$ to $CD8^+$ ratio was 1.853 (range 0.323- 5.631). Median of the $CD8^+$ percentage of FIV negative (8.100%, range 1.24- 41.88) was significantly lower than FIV positive cats (14.300% range 6.38- 26.57) (p = 0.026). Median of the $CD4^+$ to $CD8^+$ ratio of FIV negative cats (1.896, range 0.323- 5.065) was significantly higher than for FIV positive cats (1.119, range 0.633-5.631) (p = 0.045). The $CD8^+$ percentage of *Bartonella* spp. seronegative (5.495%, range 2.08- 7.25%) was significantly lower than for seropositive cats (8.740%, range 1.24- 41.88%) (p = 0.024) (Table 4.4).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 4.2 Association (odds ratio) between gender, age, flea infestation, and retrovirus status, with IFA results of *Bartonella* spp. antibody status

Variables		*Bartonella* spp. Ab status		OR	95% CI	
		Negative n (%)	Positive n (%)		Lower	Upper
Gender (n=158)	Male	1 (1.4)	70 (98.6)	0.163	0.020	1.360
	Female	7 (8.0)	80 (92.0)			
Age (n=158)	<1 year	4 (11.1)	32 (88.9)	4.296	1.010	18.275
	≥ 1 year	4 (3.3)	118 (96.7)			
Fleas (n=158)	No	5 (5.4)	87 (94.6)	1.207	0.278	5.237
	Yes	3 (4.5)	63 (95.5)			
Retroviral status (n=158)	Negative	8 (6.1)	123 (93.9)	ND	ND	ND
	Positive	0 (0)	27 (100)			

Abbreviations: OR: odds ratio, 95% CI: 95% confidence interval, SD: standard deviation, Fleas: flea infestation, ND: not determined

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 4.3 Association (odds ratio) between gender, age, flea infestation, retrovirus status, and antibody status with *Bartonella* spp. PCR results.

Variables		*Bartonella* spp. PCR		OR	95% CI	
		Negative n (%)	Positive n (%)		Lower	Upper
Gender (n=161)	Male	61 (83.6)	12 (16.4)	0.962	0.414	2.233
	Female	74 (84.1)	14 (15.9)			
Age (n=161)	<1 year	33 (91.7)	3 (8.3)	2.480	0.700	8.793
	≥ 1 year	102 (81.6)	23 (18.4)			
Fleas (n=161)	No	84 (88.4)	11 (11.6)	2.246	0.958	5.267
	Yes	51 (77.3)	15 (22.7)			
Retroviral status (n=161)	Negative	112 (83.6)	22 (16.4)	0.885	0.279	2.813
	Positive	23 (85.2)	4 (14.8)			
Ab status (n = 158)	Negative	7 (87.5)	1 (12.5)	1.400	0.165	11.885
	Positive	125 (83.3)	25 (16.7)			

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Abbreviations: OR: odds ratio, 95% CI: 95% confidence interval, SD: standard deviation, Fleas: flea infestation, Ab: antibody, ND: not determined

Table 4.4 Median of CD4$^+$ and CD8$^+$T lymphocyte percentage, and CD4$^+$ to CD8$^+$ ratio of each category.

Cat's status		CD4$^+$ (%)	Range	*p*-value	CD8$^+$ (%)	Range	*p*-value	CD4:8 ratio	Range	*p*-value
All	n=106	15.175	1.28-48.46	ND	8.350	1.24-41.88	ND	1.853	0.323-5.631	ND
FIV	Negative (n=99)	15.710	1.28-48.86	0.099	8.100	1.24-41.88	0.026*	1.896	0.323-5.065	0.045*
	Positive (n=7)	12.120	7.38-16.16		14.300	6.38-26.57		1.119	0.633-5.631	
FeLV	Negative (n=92)	15.050	1.28-48.86	0.955	8.350	1.24-32.28	0.346	1.859	0.455-5.631	0.083
	Positive (n=14)	15.415	2.38-29.58		9.720	2.67-41.88		1.644	0.323-3.306	
Bartonella spp. PCR	Negative (n=85)	15.040	1.28-48.86	0.778	8.310	1.24-32.28	0.269	1.903	0.455-5.631	0.338
	Positive (n=21)	15.290	4.07-26.02		8.770	2.46-41.88		1.654	0.323-4.000	
Bartonella spp. antibody	Negative (n=100)	7.510	5.67-24.03	0.229	5.495	2.08-7.25	0.024*	2.705	1-088-2.667	0.251
	Positive (n=6)	15.415	1.28-48.86		8.740	1.24-41.88		1.806	0.323- 5.631	

Significance difference when p-value less than 0.05

Abbreviations: FIV: feline immunodeficiency virus (witness, Zoetis), FeLV: feline leukemia virus (witness, Zoetis), Neg: negative, Pos: positive,

ND: not determined

In order to find the association between immunocompromised cats and *Bartonella* spp. infection using PCR detection, all cats were divided into 4 groups to compare $CD4^+$ to $CD8^+$ ratio. By the Kruskal-Wallis test, a significant difference was detected among groups (p = 0.034). All pairwise post hoc analysis showed the lowest ratio was found in Group 4 (n=3, median 1.017, range 0.323- 1.027) and was significantly lower than Group 1 (n=70, 1.914, range 0.455- 5.065) (p= 0.041) but was not significantly lower than Group 2 (n=15, 1.469, range 0.633-5.631) and 3 (n =18, 1.864, range 1.021-4.001) (p = 0.308, 0.056) (Figure 4.1).

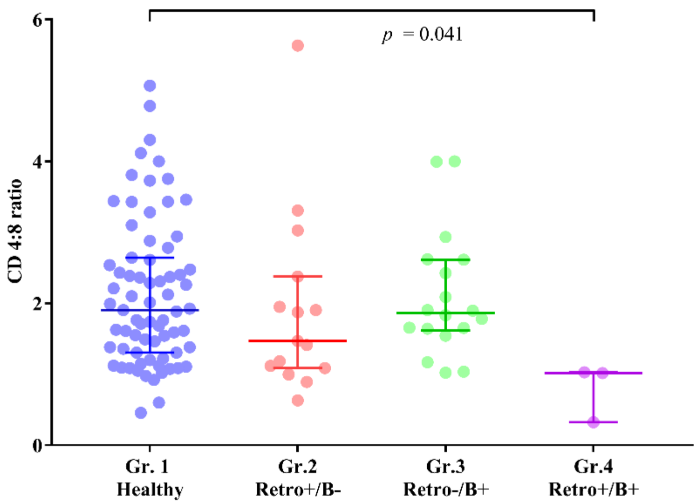


Figure 4.1 Column scatter plot of $CD4^+$ to $CD8^+$ ratios in 4 groups of cats with different retrovirus and *Bartonella* spp. statuses. Gr.1: healthy cats who tested negative for both *Bartonella* spp. PCR and retrovirus; Gr.2: cats who tested negative for *Bartonella* spp. PCR but positive for retrovirus; Gr.3: cats who tested positive for *Bartonella* spp. PCR but negative for retrovirus; and Gr.4: cats who tested positive for both *Bartonella* spp. PCR and retrovirus.

4.3 Discussions

Bartonella spp. is distributed worldwide, with a higher prevalence in humid and warm climate areas (Jameson et al., 1995; Sykes and Chomel, 2014). *Bartonella* spp. infection was high in both domestic and/or stray cats in Asian countries (Inoue et al., 2009). In Thailand, several studies reported *B. henselae* detection in both humans and cats. In 2000, 5.5% of Thai blood donors were seropositive for *B. henselae* (Maruyama et al., 2000a). The prevalence of *B. henselae* in cats, either by nucleic acid detection or bacterial isolation, was reported to be between 10% and 27.6% (Maruyama et al., 2001; Inoue et al., 2009; Assarasakorn et al., 2012). One recent study showed 53.7% bacteremia prevalence in stray cats in the Bangkok metropolitan area (Jitchum et al., 2009). The prevalence of *Bartonella* spp. infection in the clinically healthy cats coming for a health check or neutering between 2017-2020 in the present study was 16.1%. However, the seroprevalence of *Bartonella* spp. infection was as high as 94.9%, which indicates that cats in Thailand had previously been exposed to *Bartonella* spp. infection. High seroprevalences have also been reported for *B. henselae* antibodies, up to 93% in feral cats (Chomel et al., 1995; Nutter et al., 2004; Fontenelle et al., 2008; Assarasakorn et al., 2012). Both bacteremia and high seroprevalence in this study were markedly high compared to those in other countries (Glaus et al., 1997; Fabbi et al., 2004), which may be due to the high temperatures and humidity. Warm temperature and high humidity throughout the year in Thailand encourages the growth and increase infestation rate of arthropod vectors, such as fleas. Although, the presences of

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

antibody do not relate with the presences of bacteremia in cats, seroconversion or four-fold increasing in antibody titer over a 2–3-week period are considered to confirm acute *Bartonella* spp. infection (Chomel et al., 2004; Álvarez-Fernández et al., 2018). However, antibody titers of higher than 1:512 are more likely to be bacteremia (Chomel et al., 1995). The company recommendation of cut-off titer at 1:64 may not support to the clinical healthy cats in Bangkok Metropolitan.

The clinical presentation in naturally infected cats is usually subclinical or only mild clinical signs which may not be observed by the owners (Abbott et al., 1997; Breitschwerdt, 2008; Guptill, 2010a; Sykes and Chomel, 2014). Transient fever generalized or localized peripheral lymphadenomegaly, mild neurologic signs, and reproductive problems were reported in experimental cats (Breitschwerdt, 2008; Guptill, 2010a; Sykes and Chomel, 2014). Other clinical sighs are including uveitis, chronic gingivostomatitis, fever of unknown origin, chronic kidney disease, pancreatitis, chronic rhinosinusitis, and lower urinary tract diseases (Sykes and Chomel, 2014). However, all cats in the present study were all clinically normal healthy cats.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

In previous reports, the risk factors for *Bartonella* spp. infection in cats were flea infestations, outdoor lifestyle, and a multi-cat environment (Gurfield et al., 2001; Guptill et al., 2004; Assarasakorn et al., 2012; Pennisi et al., 2013). Moreover, young cats had a higher risk of *Bartonella* spp. bacteremia or DNA detection (Guptill et al., 2004; Assarasakorn et al., 2012). Interestingly, our results showed that younger cats tend to be less seropositive than the older cats and flea status did not show any significant

association with *Bartonella* spp. infection in this study. These findings are directly in line with previous findings (La Scola et al., 2002; Bai et al., 2015; Gutiérrez et al., 2015b). It is interesting to note that fluctuating bacteremia can cause undetectable levels even when there is persistent bacteremia in cats. Moreover, the findings of bacteremia in cats may not be accompanied by infected fleas (Gutiérrez et al., 2015b). On top of that, the other routes of infection, such as biting and scratching between cats, should also be considered as another important source of *Bartonella* spp. infection.

The clinical manifestations of *Bartonella* spp. infection depend on the host's immune response (Mofenson et al., 2009). Both HIV-infected and other immunocompromised patients were reported to have bacillary angiomatosis and/ or bacillary peliosis more than others (Mofenson et al., 2009) and they were recorded most often in HIV-infected adults with a low number of $CD4^+$ count (fewer than 50 cells/mm^3) (Mofenson et al., 2009). In cats, feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) cause acquired immunodeficiency (Gomez-Lucia et al., 2020). FeLV and FIV infections per se were not directly associated with *Bartonella* spp. infection in this study. The same trend was also observed in other studies (Ueno et al., 1996; Glaus et al., 1997). However, another previous study reported that FeLV was significantly associated with *Bartonella* spp. infection (Buchmann et al., 2010). Therefore, the relationships between feline retroviral infection and *Bartonella* spp. infection remains inconclusive and requires further investigation. One explanation for this inconsistency in retroviral status and *Bartonella* spp. infection may be due to the

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

low number of cats in each stage of FIV infection in the present study. All FIV-positive cats in this study were in the asymptomatic stage with normal $CD4^+$ to $CD8^+$ ratio. In the asymptomatic phase, $CD4^+$ T cell numbers rebound, and the plasma virus load declines to very low levels. Cats in the asymptomatic phase remain healthy for several years. Viral infection gradually suppresses the immune function over years. In the late asymptomatic stage or terminal phase, cats may have clinical signs of opportunistic infections, neoplastic disease, myelosuppression, and neurologic disease (Hartmann, 1998; Harbour et al., 2004; Sykes, 2014) or have *Bartonella* spp. infection later on in life if they are in Aid-related stage or end stage of FIV.

The average percentages of $CD4^+$, and $CD8^+$ T lymphocytes in normal adult cats were reported to be between 31.15-44.5% and 13-27.5%, respectively (Tompkins et al., 1990; Dean et al., 1991; Hoffmann-Fezer et al., 1992; Byrne et al., 2000). The mean $CD4^+$ to $CD8^+$ ratio of T lymphocyte in normal cats is 1.74-3.3 (Tompkins et al., 1990; Dean et al., 1991; Hoffmann-Fezer et al., 1992; Byrne et al., 2000). In FIV-infected cats, the $CD4^+$ T lymphocyte count declines and the $CD8^+$ T lymphocyte count increases, resulting in a gradually decreasing $CD4^+$ to $CD8^+$ ratio (Gomez-Lucia et al., 2020). In FIV-infected cats with cutaneous dermatophytosis, the $CD4^+$ to $CD8^+$ ratio was significantly lower than the ratio in the FIV-infected cats (Reche et al., 2010). In *T. gondii* infection concurrent with FIV infection cats, the $CD4^+$ T lymphocyte count reduced during the first 3 weeks of infection, and no alteration of the $CD8^+$ T lymphocyte count was detected (Lin et al., 1992). The $CD4^+$ to $CD8^+$ ratios in *Bartonella* spp. infected cats

in this study were within normal limits and the grand median of the $CD4^+$ to $CD8^+$ ratio did not differ from what was previously reported ($CD4^+$ to $CD8^+$ ratio = 1.853, range 0.323-5.631).

In this study, the declining trend of $CD4^+$ to $CD8^+$ ratios were observed in both retrovirus-infected cats and *Bartonella* spp. co-infected cats as shown in the earlier studies (Lin et al., 1992; Reche et al., 2010). Furthermore, the lowest $CD4^+$ to $CD8^+$ ratio was also shown in cats with concurrent retroviral and *Bartonella* spp. infected cats. Therefore, the $CD4^+$ to $CD8^+$ ratio should be important parameter when *Bartonella* spp. infection is suspected in retroviral-positive cats.

The present study had some limitations; firstly, the cats in this study may have had undetected concurrent illnesses that altered the population of lymphocytes, and secondly, the retrovirus-infected cats, especially FIV positive cats, had not been classified into the stage of FIV infection due to the low number of FIV-positive cats included. Further investigation of CD expression in different stages of FIV-infected cats should be studied to investigate the effect of the levels of $CD4^+$ and $CD8^+$, and the $CD4^+$ to $CD8^+$ ratio on *Bartonella* spp. infection in the future.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Chapter 5

General Discussion and Conclusions

Bartonella spp. is found all across the world, with a higher incidence in humid and warm climates (Jameson et al., 1995; Guptill, 2010a). People infected with *Bartonella* spp. are suffering from various clinical illnesses. However, immunocompetent individuals may have more localized and self-limited illness (Massei et al., 2000; Massei et al., 2004; Florin et al., 2008; Guptill, 2010a). However, an immunocompromised individuals may debilitate and can be fatal.

Cats considered as a primary reservoir for many species including, *B. henselae, B. claridigeae,* and *B. koehlerae* (Guptill, 2010a). *Bartonella* spp. infection in both cats and people is difficult to detect since there is no one test that can confirm a clinical bartonellosis. Many approaches of detection have been proposed but they are still doubt in their performances. This study showed their clinical important and had developed a new tool as an antibody test that have shown its acceptable sensitivity and specificity. Several investigations in Thailand found *B. henselae* in both people and cats. In this study, the incidence of *Bartonella* spp. infection was 16.1% in clinically healthy cats that came for a health check or neutering between 2017 and 2020. The seroprevalence of *Bartonella* spp. infection, on the other hand, was as high as 94.9% which indicated that cats in Thailand had previously been exposed to *Bartonella* spp. infection. High seroprevalences of *B. henselae* antibodies have also been recorded, with up to 93% in wild cats (Chomel et al., 1995; Nutter et al., 2004;

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Fontenelle et al., 2008; Assarasakorn et al., 2012). Both bacteremia and high seroprevalence were significantly high in this study compared to other countries (Glaus et al., 1997; Fabbi et al., 2004) which might be attributed to Thailand's high temperatures and humidity. Our findings suggested that most cats in Thailand with this high risk can transfer *Bartonella* spp. infection to their owners.

The clinical symptoms of *Bartonella* spp. infection are influenced by the immunological response of the host (Mofenson et al., 2009). In human, both HIV-infected and other immunocompromised individuals were found to have more serious conditions than others (Mofenson et al., 2009), and it was most common in HIV-infected adults with a low $CD4^+$ count (Mofenson et al., 2009). In cats, acquired immunodeficiency is caused by feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) (Gomez-Lucia et al., 2020). In the present study, cats with FeLV and FIV infections per se were not linked to *Bartonella* spp. infection. Other studies have shown the similar pattern (Ueno et al., 1996; Glaus et al., 1997). The stages of FIV infection in cats might also explain the discrepancy in retroviral status and *Bartonella* spp. infection. Cats with terminal phase of FIV may show clinical signs of opportunistic infections (Hartmann, 1998; Harbour et al., 2004; Sykes, 2014). According to previous research, falling $CD4^+$ to $CD8^+$ ratios were also detected in retrovirus-infected cats and *Bartonella* spp. co-infected cats (Lin et al., 1992; Reche et al., 2010). Furthermore, cats in the present study with concurrent retroviral and *Bartonella* spp. infection had the lowest $CD4^+$ to $CD8^+$ ratio. As a result, the $CD4^+$ to

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

$CD8^+$ ratio in retroviral-positive cats may be another predictor of *Bartonella* spp. infection and need to be measured to rule out or rule in *Bartonella* spp. infection in FIV cats.

Therefore, *Bartonella* spp. detection tests are needed to screen the infection in cats. *Bartonella* spp. infection in both cats and people is difficult to detect since there is no one test that can confirm a clinical bartonellosis. Clinical proof requires a combination of compatible clinical tests, the positive serological test is the major criteria of clinical human bartonellosis (Agan and Dolan, 2002; Guptill, 2010a). According to an IFA-based test, the sensitivity ranged from 14% to 100%. Serological examinations using the ELISA technique were shown to have good specificity (90%-99%) and varied sensitivity (10%-95%). Using whole-cell antigen for western blot analysis has also shown different immunoreactivity patterns in each sample. Our developed antibody test using r17-kDa and rGroEL proteins demonstrated a sensitivity of 75% and 83.33% and specificity 57.14% and 71.43%.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Nevertheless, the findings of the present study have to be seen in light of some limitations. First, there were high seroprevalence in a group of enrolled cats. This constraint led this study had a low number of negative sera in validation of ELISA. Second, further investigate in various diseases in cats has not been established. Last, this study recruited only clinically healthy cats which may or may not suffer from unknown concurrent implications. Therefore, additional cat sera of various infectious pathogens, IgM panel antibody, and wide range of infection stage of retroviral- infected

cats should be enrolled to the future study to deduct the range of confounding factors and to increase the specificity of ELISA test.

Finally, this dissertation extends our knowledge of *Bartonella* spp in cats. It revealed the high tendency of zoonotic potential of *Bartonella* spp. in both domestic and stray cats in Bangkok Metropolitan. The $CD4^+$ and $Cd8^+$ ratios were proposed to be another clinical parameter for monitoring the infection in retroviral-infected cats. Though there is difficulty to prove clinical bartonellosis, infection in cats and people, new developed ELISA from this study is original and may raise our new hope for screening *Bartonella* spp. infection in cats and to protect cats' owner from this important zoonosis.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

REFERENCES

Abbott RC, Chomel BB, Kasten RW, Floyd-Hawkins KA, Kikuchi Y, Koehler JE and Pedersen NC 1997. Experimental and natural infection with *Bartonella henselae* in domestic cats. Comp Immunol, Microbiol Infect Dis. 20(1): 41-51.

Agan BK and Dolan MJ 2002. Laboratory diagnosis of *Bartonella* infections. Clin Lab Med. 22(4): 937-962.

Álvarez-Fernández A, Breitschwerdt EB and Solano-Gallego L 2018. *Bartonella* infections in cats and dogs including zoonotic aspects. Parasites & Vectors. 11(1): 624.

Anderson B, Lu E, Jones D and Regnery R 1995. Characterization of a 17-kilodalton antigen of *Bartonella henselae* reactive with sera from patients with cat scratch disease. J Clin Microbiol. 33(9): 2358-2365.

Angelakis E and Raoult D 2014. Pathogenicity and treatment of *Bartonella* infections. Int J Antimicrob Agents. 44(1): 16-25.

Assarasakorn S, Veir JK, Hawley J, Brewer MM, Morris AK, Hill AE and Lappin MR 2012. Prevalence of *Bartonella* species, *hemoplasmas*, and *Rickettsia felis* DNA in blood and fleas of cats in Bangkok, Thailand. Res Vet Sci. 93(3): 1213-1216.

Bai Y, Osinubi MOV, Osikowicz L, McKee C, Vora NM, Rizzo MR, Recuenco S, Davis L, Niezgoda M, Ehimiyein AM, Kia GSN, Oyemakinde A, Adeniyi OS, Gbadegesin YH, Saliman OA, Ogunniyi A, Ogunkoya AB, Kosoy MY and Idanre Bat Festival

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Investigation T 2018. Human exposure to novel *Bartonella* species from contact with fruit bats. Emerg Infect Dis. 24(12): 2317-2323.

Bai Y, Rizzo MF, Alvarez D, Moran D, Peruski LF and Kosoy M 2015. Coexistence of *Bartonella henselae* and *B. clarridgeiae* in populations of cats and their fleas in Guatemala. J Vector Ecol. 40(2): 327-332.

Baldani CD, Santos HA and Massard CL 2014. The Family *Bartonellaceae*. In: The Prokaryotes: Alphaproteobacteria and Betaproteobacteria. Eugene Rosenberg, Edward F. DeLong, Stephen Lory, Erko Stackebrandt, and Fabiano Thompson (eds). Berlin, Heidelberg: Springer Berlin Heidelberg. 81-114.

Barka NE, Hadfield T, Patnaik M, Schwartzman WA and Peter JB 1993. EIA for detection of *Rochalimaea henselae*-reactive IgG, IgM, and IgA antibodies in patients with suspected cat-scratch disease. J Infect Dis. 167(6): 1503-1504.

Barton A 1909. Descripción de elementos endo-globulares hallados en las enfermos de fiebre verrucosa. Cron Med. 26: 7.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Blagova B and Yanev N 2021. Human *Bartonella* infection: a review of literature. J of IMAB. 27(2): 3759-3764.

Bouchouicha R, Durand B, Monteil M, Chomel BB, Berrich M, Arvand M, Birtles RJ, Breitschwerdt EB, Koehler JE, Maggi R, Maruyama S, Kasten R, Petit E, Boulouis H-J and Haddad N 2009. Molecular epidemiology of feline and human *Bartonella henselae* isolates. Emerg Infect Dis. 15(5): 813-816.

Breitschwerdt EB 2008. Feline bartonellosis and cat scratch disease. Vet Immunol Immunopathol. 123(1-2): 167-171.

Brenner DJ, O'Connor SP, Winkler HH and Steigerwalt AG 1993. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. Int J Syst Bacteriol. 43(4): 777-786.

Brouqui P, Lascola B, Roux V and Raoult D 1999. Chronic *Bartonella quintana* bacteremia in homeless patients. N Engl J Med. 340(3): 184-189.

Brunt J, Guptill L, Kordick DL, Kudrak S and Lappin MR 2006. American Association of Feline Practitioners 2006 Panel report on diagnosis, treatment, and prevention of *Bartonella* spp. infections. J Feline Med Surg. 8(4): 213-226.

Buchmann AU, Kershaw O, Kempf VA and Gruber AD 2010. Does a feline leukemia virus infection pave the way for *Bartonella henselae* infection in cats? J Clin Microbiol. 48(9): 3295-3300.

Buffet J-P, Kosoy M and Vayssier-Taussat M 2013. Natural history of *Bartonella*-infecting rodents in light of new knowledge on genomics, diversity and evolution. Future Microbiol. 8(9): 1117-1128.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Byrne KM, Kim HW, Chew BP, Reinhart GA and Hayek MG 2000. A standardized gating technique for the generation of flow cytometry data for normal canine and normal feline blood lymphocytes. Vet Immunol Immunopathol. 73(2): 167-182.

Chamberlin J, Laughlin LW, Romero S, Solórzano N, Gordon S, Andre RG, Pachas P, Friedman H, Ponce C and Watts D 2002. Epidemiology of Endemic *Bartonella bacilliformis*: A Prospective Cohort Study in a Peruvian Mountain Valley Community. J Infect Dis. 186(7): 983-990.

Chang C-C, Chomel BB, Kasten RW, Tappero JW, Sanchez MA and Koehler JE 2002. Molecular epidemiology of *Bartonella henselae* infection in Human Immunodeficiency Virus–Infected patients and their cat contacts, using pulsed-field gel electrophoresis and genotyping. J Infect Dis. 186(12): 1733-1739.

Cheslock MA and Embers ME 2019. Human Bartonellosis: An Underappreciated Public Health Problem? Trop Med Infect Dis. 4(2): 69.

Chomel BB, Abbott RC, Kasten RW, Floyd-Hawkins KA, Kass PH, Glaser CA, Pedersen NC and Koehler JE 1995. *Bartonella henselae* prevalence in domestic cats in California: risk factors and association between bacteremia and antibody titers. J Clin Microbiol. 33(9): 2445-2450.

Chomel BB, Boulouis HJ and Breitschwerdt EB 2004. Cat scratch disease and other zoonotic *Bartonella* infections. J Am Vet Med Assoc. 224(8): 1270-1279.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Comer JA, Flynn C, Regnery RL, Vlahov D and Childs JE 1996. Antibodies to *Bartonella* species in inner-city intravenous drug users in Baltimore, Md. Arch Intern Med. 156(21): 2491-2495.

Daly JS, Worthington MG, Brenner DJ, Moss CW, Hollis DG, Weyant RS, Steigerwalt AG, Weaver RE, Daneshvar MI and O'Connor SP 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J Clin Microbiol. 31(4): 872-881.

Dean GA, Quackenbush SL, Ackley CD, Cooper MD and Hoover EA 1991. Flow cytometric analysis of T-lymphocyte subsets in cats. Vet Immunol Immunopathol. 28(3-4): 327-335.

Debré R 1950. La maladie des griffes de chat. Bull Mem Soc Med Hop Paris. 66: 76-79.

Dehio C 2001. *Bartonella* interactions with endothelial cells and erythrocytes. Trends Microbiol. 9(6): 279-285.

Dehio C 2004. Molecular and cellular basis of *Bartonella* pathogenesis. Annu Rev Microbiol. 58: 365-390.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Dehio C 2005. *Bartonella*–host-cell interactions and vascular tumour formation. Nat Rev Microbiol. 3(8): 621.

Diniz PPVP, Morton BA, Tngrian M, Kachani M, Barrón EA, Gavidia CM, Gilman RH, Angulo NP, Brenner EC, Lerner R and Chomel BB 2013. Infection of domestic dogs in Peru by zoonotic *Bartonella* Species: A cross-sectional prevalence study of 219 asymptomatic Dogs. PLoS Negl Trop Dis. 7(9): e2393.

Drancourt M, Tran-Hung L, Courtin J, Lumley Hd and Raoult D 2005. *Bartonella quintana* in a 4000-Year-Old Human Tooth. J Infect Dis. 191(4): 607-611.

Duncan AW, Maggi RG and Breitschwerdt EB 2007. A combined approach for the enhanced detection and isolation of *Bartonella* species in dog blood samples: pre-enrichment liquid culture followed by PCR and subculture onto agar plates. J Microbiol Methods. 69(2): 273-281.

Eberhardt C, Engelmann S, Kusch H, Albrecht D, Hecker M, Autenrieth IB and Kempf VA 2009. Proteomic analysis of the bacterial pathogen *Bartonella henselae* and identification of immunogenic proteins for serodiagnosis. Proteomics. 9(7): 1967-1981.

Eicher SC and Dehio C 2012. *Bartonella* entry mechanisms into mammalian host cells. Cell Microbiol. 14(8): 1166-1173.

Eremeeva ME, Gerns HL, Lydy SL, Goo JS, Ryan ET, Mathew SS, Ferraro MJ, Holden JM, Nicholson WL, Dasch GA and Koehler JE 2007. Bacteremia, fever, and splenomegaly caused by a newly recognized *Bartonella* Species. N Engl J Med. 356(23): 2381-2387.

Fabbi M, De Giuli L, Tranquillo M, Bragoni R, Casiraghi M and Genchi C 2004. Prevalence of *Bartonella henselae* in Italian stray cats: evaluation of serology to assess the risk of transmission of *Bartonella* to humans. J Clin Microbiol. 42(1): 264-268.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Feng S, Kasten RW, Werner JA, Hodzic E, Barthold SW and Chomel BB 2009. Immunogenicity of *Bartonella henselae* P26 in cats. Vet Immunol Immunopathol. 132(2-4): 251-256.

Fenollar F, Sire S and Raoult D 2005. *Bartonella vinsonii* subsp. *arupensis* as an agent of blood culture-negative endocarditis in a human. J Clin Microbiol. 43(2): 945-947.

Ferrara F, Di Niro R, D'Angelo S, Busetti M, Marzari R, Not T and Sblattero D 2014. Development of an enzyme-linked immunosorbent assay for *Bartonella henselae* infection detection. Lett Appl Microbiol. 59(3): 253-262.

Florin TA, Zaoutis TE and Zaoutis LB 2008. Beyond cat scratch disease: widening spectrum of *Bartonella henselae* infection. Pediatrics. 121(5): e1413-e1425.

Fontenelle JP, Powell CC, Hill AE, Radecki SV and Lappin MR 2008. Prevalence of serum antibodies against *Bartonella* species in the serum of cats with or without uveitis. J Feline Med Surg. 10(1): 41-46.

Foucault C, Brouqui P and Raoult D 2006. *Bartonella quintana* characteristics and clinical management. Emerg Infect Dis. 12(2): 217-223.

Fournier PE, Drancourt M, Aboudharam G and Raoult D 2015. Paleomicrobiology of *Bartonella* infections. Microbes Infect. 17(11-12): 879-883.

Fournier PE, Lelievre H, Eykyn SJ, Mainardi J-L, Marrie TJ, Bruneel F, Roure C, Nash J, Clave D, James E, Benoit-Lemercier C, Deforges L, Tissot-Dupont H and Raoult D 2001.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Epidemiologic and clinical characteristics of *Bartonella quintana* and *Bartonella henselae* endocarditis: A study of 48 Patients. Medicine. 80(4): 245-251.

Frean J, Arndt S and Spencer D 2002. High rate of *Bartonella henselae* infection in HIV-positive outpatients in Johannesburg, South Africa. Trans R Soc Trop Med Hyg. 96(5): 549-550.

Gadila SKG and Embers ME 2021. Antibiotic susceptibility of *Bartonella* grown in different culture conditions. Pathogens. 10(6): 718.

Glaus T, Hofmann-Lehmann R, Greene C, Glaus B, Wolfensberger C and Lutz H 1997. Seroprevalence of *Bartonella henselae* infection and correlation with disease status in cats in Switzerland. J Clin Microbiol. 35(11): 2883-2885.

Gomez-Lucia E, Collado VM, Miró G, Martín S, Benítez L and Doménech A 2020. Clinical and hematological follow-up of long-term oral therapy with type-I interferon in cats naturally infected with feline leukemia virus or feline immunodeficiency virus. Animals. 10(9): 1464.

Guptill L 2010a. Bartonellosis. Vet Microbiol. 140(3-4): 347-359.

Guptill L 2010b. Feline bartonellosis. Vet Clin North Am Small Anim Pract. 40(6): 1073-1090.

Guptill L, Wu CC, HogenEsch H, Slater LN, Glickman N, Dunham A, Syme H and Glickman L 2004. Prevalence, risk factors, and genetic diversity of *Bartonella henselae*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

infections in pet cats in four regions of the United States. J Clin Microbiol. 42(2): 652-659.

Gurfield AN, Boulouis HJ, Chomel BB, Kasten RW, Heller R, Bouillin C, Gandoin C, Thibault D, Chang CC, Barrat F and Piemont Y 2001. Epidemiology of *Bartonella* infection in domestic cats in France. Vet Microbiol. 80(2): 185-198.

Gutiérrez R, Krasnov B, Morick D, Gottlieb Y, Khokhlova IS and Harrus S 2015a. *Bartonella* infection in rodents and their flea ectoparasites: An overview. Vector Borne and Zoonotic Diseases. 15(1): 27-39.

Gutiérrez R, Nachum-Biala Y, Harrus S and Goodrich-Blair H 2015b. Relationship between the presence of *Bartonella* species and bacterial loads in cats and cat fleas (*Ctenocephalides felis*) under natural conditions. Appl Environ Microbiol. 81(16): 5613-5621.

Gutiérrez R, Shalit T, Markus B, Yuan C, Nachum-Biala Y, Elad D and Harrus S 2020. *Bartonella kosoyi* sp. nov. and *Bartonella krasnovii* sp. nov., two novel species closely related to the zoonotic *Bartonella elizabethae*, isolated from black rats and wild desert rodent-fleas. Int J Syst Evol Microbiol. 70(3): 1656-1665.

Gutierrez R, Vayssier-Taussat M, Buffet JP and Harrus S 2017. Guidelines for the isolation, molecular detection, and characterization of *Bartonella* Species. Vector Borne and Zoonotic Diseases. 17(1): 42-50.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Haake DA, Summers TA, McCoy AM and Schwartzman W 1997. Heat shock response and *groEL* sequence of *Bartonella henselae* and *Bartonella quintana*. Microbiology. 143 (Pt 8): 2807-2815.

Harbour DA, Caney SMA and Sparkes AH 2004. Feline immunodeficiency virus infection. In: Feline medicine and therapeutics. E. Chandler, C. Gaskell, and R. Gaskell (eds). Oxford: Blackwell Publishing. 607-622.

Harms A and Dehio C 2012. Intruders below the radar: molecular pathogenesis of *Bartonella* spp. Clin Microbiol Rev. 25(1): 42-78.

Hartmann K 1998. Feline immunodeficiency virus infection: an overview. Vet J. 155(2): 123-137.

Hoffmann-Fezer G, Thum J, Ackley C, Herbold M, Mysliwietz J, Thefeld S, Hartmann K and Kraft W 1992. Decline in $CD4^+$ cell numbers in cats with naturally acquired feline immunodeficiency virus infection. J Virol. 66(3): 1484-1488.

Hosie MJ, Addie D, Belák S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hartmann K, Lutz H, Marsilio F, Pennisi MG, Radford AD, Thiry E, Truyen U, Horzinek MC and Lloret A 2009. Feline Immunodeficiency: ABCD guidelines on prevention and management. J Feline Med Surg. 11(7): 575-584.

Inoue K, Maruyama S, Kabeya H, Kawanami K, Yanai K, Jitchum S and Jittaparapong S 2009. Prevalence of *Bartonella* infection in cats and dogs in a metropolitan area, Thailand. Epidemiol Infect. 137(11): 1568-1573.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Ives TJ, Manzewitsch P, Regnery RL, Butts JD and Kebede M 1997. In vitro susceptibilities of *Bartonella henselae, B. quintana, B. elizabethae, Rickettsia rickettsii, R. conorii, R. akari*, and *R. prowazekii* to macrolide antibiotics as determined by immunofluorescent-antibody analysis of infected Vero cell monolayers. Antimicrob Agents Chemother. 41(3): 578-582.

Jackson LA, Spach DH, Kippen DA, Sugg NK, Regnery RL, Sayers MH and Stamm WE 1996. Seroprevalence to *Bartonella quintana* among patients at a community clinic in downtown Seattle. J Infect Dis. 173(4): 1023-1026.

Jameson P, Greene C, Regnery R, Dryden M, Marks A, Brown J, Cooper J, Glaus B and Greene R 1995. Prevalence of *Bartonella henselae* antibodies in pet cats throughout regions of North America. J Infect Dis. 172(4): 1145-1149.

Jensen WA, Fall MZ, Rooney J, Kordick DL and Breitschwerdt EB 2000. Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. J Clin Microbiol. 38(5): 1717-1722.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Jitchum S, Wongnarkpet S, Amavisit P, Maruyama S and Jittapalapong S 2009. Detection of *Bartonella* spp. infection of stray cats resided in monasteries of Bangkok Metropolitan areas by polymerase chain reaction technique. Proceedings of the 47th Kasetsart University Annual Conference, Kasetsart, 17-20 March, 2009. Subject: Veterinary Medicine:139-147.

Kabeya H, Maruyama S, Hirano K and Mikami T 2003. Cloning and expression of *Bartonella henselae sucB* gene encoding an immunogenic dihydrolipoamide succinyltransferase homologous protein. Microbiol Immunol. 47(8): 571-576.

Kabeya H, Umehara T, Okanishi H, Tasaki I, Kamiya M, Misawa A, Mikami T and Maruyama S 2009. Experimental infection of cats with *Bartonella henselae* resulted in rapid clearance associated with T helper 1 immune responses. Microb Infect. 11(6): 716-720.

Kelly P, Rolain J-M, Maggi R, Sontakke S, Keene B, Hunter S, Lepidi H, Breitschwerdt KT and Breitschwerdt EB 2006. *Bartonella quintana* endocarditis in dogs. Emerg Infect Dis. 12(12): 1869-1872.

Kerkhoff FT, Bergmans AMC, Zee Avd and Rothova A 1999. Demonstration of *Bartonella grahamii* DNA in ocular fluids of a patient with neuroretinitis. J Clin Microbiol. 37(12): 4034-4038.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Koehler JE 2008. *Bartonella* Infections in HIV-infected Individuals. In: Global HIV/AIDS Medicine. Philadelphia: Elsevier Health Sciences. 425-436.

Koehler JE, Sanchez MA, Tye S, Garrido-Rowland CS, Chen FM, Maurer T, Cooper JL, Olson JG, Reingold AL, Hadley WK, Regnery RR and Tappero JW 2003. Prevalence of *Bartonella* infection among human immunodeficiency virus-infected patients with fever. Clin Infect Dis. 37(4): 559-566.

Kosoy M, Murray M, Robert D. Gilmore, Bai Y and Gage KL 2003. *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. J Clin Microbiol. 41(2): 645-650.

La Scola B, Davoust B, Boni M and Raoult D 2002. Lack of correlation between *Bartonella* DNA detection within fleas, serological results, and results of blood culture in a *Bartonella*-infected stray cat population. Clin Microbiol Infect. 8(6): 345-351.

La Scola B and Raoult D 1999. Culture of *Bartonella quintana* and *Bartonella henselae* from human samples: a 5-Year experience (1993 to 1998). J Clin Microbiol. 37(6): 1899-1905.

Lappin MR, Breitschwerdt E, Brewer M, Hawley J, Hegarty B and Radecki S 2009. Prevalence of *Bartonella* species antibodies and *Bartonella* species DNA in the blood of cats with and without fever. J Feline Med Surg. 11(2): 141-148.

Lin DS, Bowman DD and Jacobson RH 1992. Immunological changes in cats with concurrent *Toxoplasma gondii* and feline immunodeficiency virus infections. J Clin Microbiol. 30(1): 17-24.

Loa CC, Mordechai E, Tilton RC and Adelson ME 2006. Production of recombinant *Bartonella henselae* 17-kDa protein for antibody-capture enzyme-linked immunosorbent assay. Diagn Microbiol Infect Dis. 55(1): 1-7.

Lutz H, Addie D, Belák S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hartmann K, Hosie MJ, Lloret A, Marsilio F, Pennisi MG, Radford AD, Thiry E, Truyen

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

U and Horzinek MC 2009. Feline leukaemia: ABCD guidelines on prevention and management. J Feline Med Surg. 11(7): 565-574.

Maggi RG, Duncan AW and Breitschwerdt EB 2005. Novel chemically modified liquid medium that will support the growth of seven *Bartonella* species. J Clin Microbiol. 43(6): 2651-2655.

Maguiña C, Guerra H and Ventosilla P 2009. Bartonellosis. Clin Dermatol. 27(3): 271-280.

Maruyama S, Boonmar S, Morita Y, Sakai T, Tanaka S, Yamaguchi F, Kabeya H and Katsube Y 2000a. Seroprevalence of *Bartonella henselae* and *Toxoplasma gondii* among healthy individuals in Thailand. J Vet Med Sci. 62(6): 635-637.

Maruyama S, Nakamura Y, Kabeya H, Tanaka S, Sakai T and Katsube Y 2000b. Prevalence of *Bartonella henselae, Bartonella clarridgeiae* and the 16S rRNA gene types of *Bartonella henselae* among pet cats in Japan. J Vet Med Sci. 62(3): 273-279.

Maruyama S, Sakai T, Morita Y, Tanaka S, Kabeya H, Boonmar S, Poapolathep A, Chalarmchaikit T, Chang C-C and Kasten RW 2001. Prevalence of *Bartonella* species and 16s rRNA gene types of *Bartonella henselae* from domestic cats in Thailand. Am J Trop Med Hyg. 65(6): 783-787.

Massei F, Messina F, Gori L, Macchia P and Maggiore G 2004. High Prevalence of Antibodies to *Bartonella henselae* among Italian Children without Evidence of Cat Scratch Disease. Clin Infect Dis. 38(1): 145-148.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Massei F, Messina F, Talini I, Massimetti M, Palla G, Macchia P and Maggiore G 2000. Widening of the clinical spectrum of *Bartonella henselae* infection as recognized through serodiagnostics. Eur J Pediatr. 159(6): 416-419.

Maurin M, Gasquet S, Ducco C and Raoult D 1995. MICs of 28 antibiotic compounds for 14 *Bartonella* (formerly *Rochalimaea*) isolates. Antimicrob Agents Chemother. 39(11): 2387-2391.

McCool TL, Hoey JG, Montileone F, Goldenberg HB, Mordechai E and Adelson ME 2008. Discovery and analysis of *Bartonella henselae* antigens for use in clinical serologic assays. Diagn Microbiol Infect Dis. 60(1): 17-23.

Minnick MF and Anderson BE 2015. *Bartonella*. In: Molecular Medical Microbiology. Second Edition ed. Max Sussman, Dongyou Liu, Ian Poxton, and Joseph Schwartzman (eds). Boston: Academic Press. 1911-1939.

Minnick MF and Barbian KD 1997. Identification of *Bartonella* using PCR; genus- and species-specific primer sets. J Microbiol Methods. 31(1–2): 51-57.

Minnick MF, Smitherman LS and Samuels DS 2003. Mitogenic effect of *Bartonella bacilliformis* on human vascular endothelial cells and involvement of GroEL. Infection and Immunity. 71(12): 6933-6942.

Mofenson LM, Brady MT, Danner SP, Dominguez KL, Hazra R, Handelsman E, Havens P, Nesheim S, Read JS, Serchuck L and Van Dyke R 2009. Guidelines for the prevention and treatment of opportunistic infections among HIV-exposed and

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

HIV-infected children: recommendations from CDC, the National Institutes of Health, the HIV Medicine Association of the Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the American Academy of Pediatrics. MMWR Recomm Rep. 58(Rr-11): 1-166.

Moran NA 2002. Microbial Minimalism: Genome Reduction in Bacterial Pathogens. Cell. 108(5): 583-586.

Müller A, Soto F, Sepúlveda M, Bittencourt P, Benevenute JL, Ikeda P, Machado RZ and André MR 2018. *Bartonella vinsonii* subsp. *berkhoffii* and *B. henselae* in dogs. Epidemiology and Infection. 146(9): 1202-1204.

Năsoiu L, Mitrea I and Ioniță M 2015. Advanced diagnostic methods as tools to investigate the exposure to *Bartonella* infections in cats. Proc Rom Acad (Series B, Suppl. 1):145-150.

Nutter FB, Dubey JP, Levine JF, Breitschwerdt EB, Ford RB and Stoskopf MK 2004. Seroprevalences of antibodies against *Bartonella henselae* and *Toxoplasma gondii* and fecal shedding of *Cryptosporidium* spp, *Giardia* spp, and *Toxocara cati* in feral and pet domestic cats. J Am Vet Med Assoc. 225(9): 1394-1398.

Okaro U, Addisu A, Casanas B and Anderson B 2017. *Bartonella* species, an emerging cause of blood-culture-negative endocarditis. Clin Microbiol Rev. 30(3): 709-746.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Oksi J, Rantala S, Kilpinen S, Silvennoinen R, Vornanen M, Veikkolainen V, Eerola E and Pulliainen AT 2013. Cat scratch disease caused by *Bartonella grahamii* in an immunocompromised patient. J Clin Microbiol. 51(8): 2781-2784.

Oteo JA, Maggi R, Portillo A, Bradley J, García-Álvarez L, San-Martín M, Roura X and Breitschwerdt E 2017. Prevalence of *Bartonella* spp. by culture, PCR and serology, in veterinary personnel from Spain. Parasites & Vectors. 10(1): 553.

Padmalayam I, Karem K, Massung R and Baumstark B 2000. The gene encoding the 17-kDa antigen of *Bartonella henselae* is located within a cluster of genes homologous to the *virB* virulence operon. DNA Cell Biol. 19(6): 377-382.

Paitoonpong L, Chitsomkasem A, Chantrakooptungool S, Kanjanahareutai S, Tribuddharat C and Srifuengfung S 2008. *Bartonella henselae*: first reported isolate in a human in Thailand. Southeast Asian J Trop Med Public Health. 39(1): 123-129.

Pappalardo BL, Brown T, Gebhardt D, Sontakke S and Breitschwerdt EB 2000. Cyclic CD8+ lymphopenia in dogs experimentally infected with *Bartonella vinsonii* subsp. *berkhoffii*. Vet Immunol Immunopathol. 75(1-2): 43-57.

Pennisi MG, Marsilio F, Hartmann K, Lloret A, Addie D, Belak S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hosie MJ, Lutz H, Mostl K, Radford AD, Thiry E, Truyen U and Horzinek MC 2013. *Bartonella* species infection in cats: ABCD guidelines on prevention and management. J Feline Med Surg. 15(7): 563-569.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Pérez C, Maggi RG, Diniz PPVP and Breitschwerdt EB 2011. Molecular and serological diagnosis of *Bartonella* infection in 61 dogs from the United States. J Vet Intern Med. 25(4): 805-810.

Probert W, Louie Janice K, Tucker James R, Longoria R, Hogue R, Moler S, Graves M, Palmer Heather J, Cassady J and Fritz Curtis L 2009. Meningitis due to a "*Bartonella washoensis*"-like human pathogen. J Clin Microbiol. 47(7): 2332-2335.

Rampersad JN, Watkins JD, Samlal MS, Deonanan R, Ramsubeik S and Ammons DR 2005. A nested-PCR with an internal amplification control for the detection and differentiation of *Bartonella henselae* and *B. clarridgeiae*: an examination of cats in Trinidad. BMC Infect Dis. 5: 63.

Reche A, Daniel AG, Strauss TCPL, Taborda CP, Vieira Marques SA, Haipek K, Oliveira LJ, Monteiro JM and Kfoury JR 2010. Cutaneous mycoflora and CD4:CD8 ratio of cats infected with feline immunodeficiency virus. J Feline Med Surg. 12(4): 355-358.

Rolain JM, Brouqui P, Koehler JE, Maguina C, Dolan MJ and Raoult D 2004. Recommendations for treatment of human infections caused by *Bartonella* species. Antimicrob Agents Chemother. 48(6): 1921-1933.

Saenz HL, Engel P, Stoeckli MC, Lanz C, Raddatz G, Vayssier-Taussat M, Birtles R, Schuster SC and Dehio C 2007. Genomic analysis of *Bartonella* identifies type IV secretion systems as host adaptability factors. Nat Genet. 39(12): 1469-1476.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Saisongkorh W, Kowalczewska M, Azza S, Decloquement P, Rolain J-M and Raoult D 2010. Identification of candidate proteins for the diagnosis of *Bartonella henselae* infections using an immunoproteomic approach. FEMS Microbiol Lett. 310(2): 158-167.

Sander A, Berner R and Ruess M 2001. Serodiagnosis of cat scratch disease: response to *Bartonella henselae* in children and a review of diagnostic methods. Eur J Clin Microbiol Infect Dis. 20(6): 392-401.

Sasaki T, Adachi T, Itoh K, Kubota M, Yamagishi T, Hirao M, Isawa H, Oishi K, Shibayama K and Sawabe K 2021. Detection of *Bartonella quintana* infection among the Homeless Population in Tokyo, Japan, from 2013–2015. Jpn J Infect Dis. advpub.

Satranarakun P, Kubera A, Jitapalapong S, Rodkhum C and Pusoonthornthum R 2017. Expression and purification of *Bartonella henselae VirB* protein. J Trop Med Parasitol. 40(1): 8-19.

Schulein R and Dehio C 2002. The VirB/VirD4 type IV secretion system of *Bartonella* is essential for establishing intraerythrocytic infection. Mol Microbiol. 46(4): 1053-1067.

Schultz MG 1968. A History of Bartonellosis (Carrión's Disease). Am J Trop Med Hyg. 17(4): 503-515.

Scorpio DG and Dumler JS 2015. *Bartonella*. In: Manual of Clinical Microbiology. 11th ed. James H. Jorgensen, Karen C. Carroll, Guido Funke, Michael A. Pfaller, Marie Louise

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Landry, Sandra S. Richter, and David W. Warnock (eds). Washington, DC: ASM Press. 873-886.

Seubert A, Schulein R and Dehio C 2001. Bacterial persistence within erythrocytes: A unique pathogenic strategy of *Bartonella* spp. Int J Med Microbiol. 291(6): 555-560.

Sigirci BD, Celik B, Kahraman BB, Gumus B, Metiner K, Adiguzel MC, Ikiz S, Bagcigil AF, Ozgur NY and A. K. S 2017. A comparative study on detection of *Bartonella henselae* infection by culture followed by PCR, nested-PCR and IFA. Kafkas Univ Vet Fak Derg. 23(2): 333-357.

Sweger D, Resto-Ruiz S, Johnson DP, Schmiederer M, Hawke N and Anderson B 2000. Conservation of the 17-kilodalton antigen gene within the genus *Bartonella*. Clin Diagn Lab Immunol. 7(2): 251-257.

Sykes JE 2014. Chapter 21 - Feline Immunodeficiency Virus Infection. In: Canine and Feline Infectious Diseases. Jane E. Sykes (ed). Saint Louis: W.B. Saunders. 209-223.

Sykes JE and Chomel BB 2014. Bartonellosis. In: Canine and Feline Infectious Diseases. Jane E. Sykes (ed). Saint Louis: W.B. Saunders. 498-511.

Tompkins MB, Gebhard DH, Bingham HR, Hamilton MJ, Davis WC and Tompkins WAF 1990. Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat. Vet Immunol Immunopathol. 26(4): 305-317.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Tsuneoka H, Fujii R, Yamamoto K, Fujisawa K, Iino H, Matsuda M and Tsukahara M 1998. Determination of anti-*Bartonella henselae* antibody by indirect fluorescence antibody test--comparison of two types of antigen: non-cocultivated *B. henselae* and cocultivated *B. henselae* with Vero cells. Kansenshogaku Zasshi. 72(8): 801-807.

Ueno H, Hohdatsu T, Muramatsu Y, Koyama H and Morita C 1996. Does coinfection of *Bartonella henselae* and FIV induce clinical disorders in cats? Microbiol Immunol. 40(9): 617-620.

Wagner A and Dehio C 2019. Role of distinct type-IV-secretion systems and secreted effector sets in host adaptation by pathogenic *Bartonella* species. Cell Microbiol. 21(3): e13004.

Wagner CL, Riess T, Linke D, Eberhardt C, Schäfer A, Reutter S, Maggi RG and Kempf VA 2008. Use of *Bartonella* adhesin A (BadA) immunoblotting in the serodiagnosis of *Bartonella henselae* infections. Int J Med Microbiol. 298(7-8): 579-590.

Weeden AL, Cherry NA, Breitschwerdt EB, Cheves AG and Wamsley HL 2017. *Bartonella henselae* in canine cavitary effusions: prevalence, identification, and clinical associations. Vet Clin Pathol. 46: 326-330.

Welch DF, Carroll KC, Hofmeister EK, Persing DH, Robison DA, Steigerwalt AG and Brenner DJ 1999. Isolation of a new subspecies, *Bartonella vinsonii* subsp. *arupensis*, from a cattle rancher: identity with isolates found in conjunction with *Borrelia*

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

burgdorferi and *Babesia microti* among naturally infected mice. J Clin Microbiol. 37(8): 2598-2601.

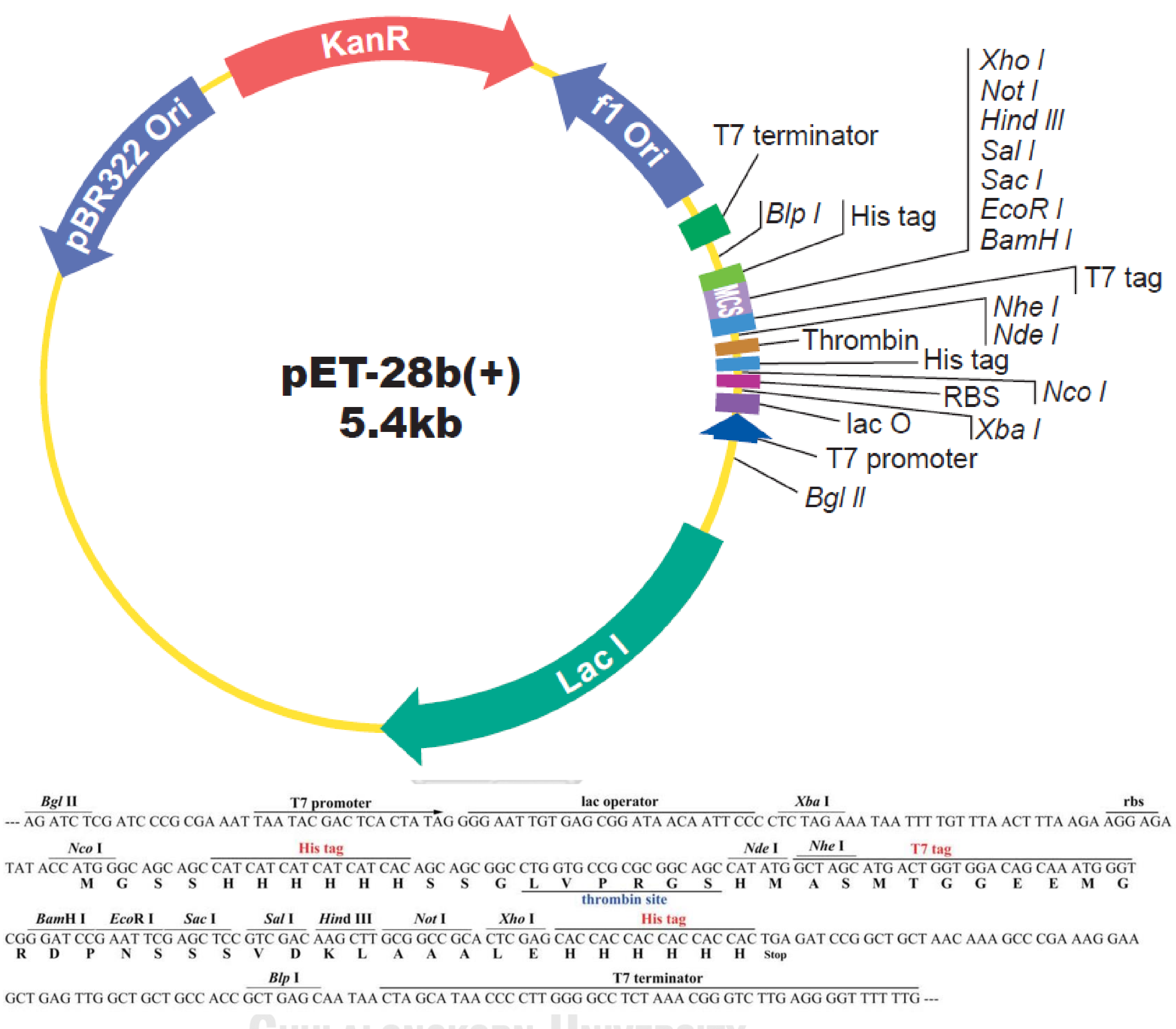
Werner JA, Feng S, Chomel BB, Hodzic E, Kasten RW and Barthold SW 2008. P26-based serodiagnosis for *Bartonella* spp. infection in cats. Comp Med. 58(4): 375-380.

Werner JA, Feng S, Kasten RW, Hodzic E, Chomel BB and Barthold SW 2006. Cloning, characterization, and expression of *Bartonella henselae* p26. Clin Vaccine Immunol. 13(8): 830-836.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

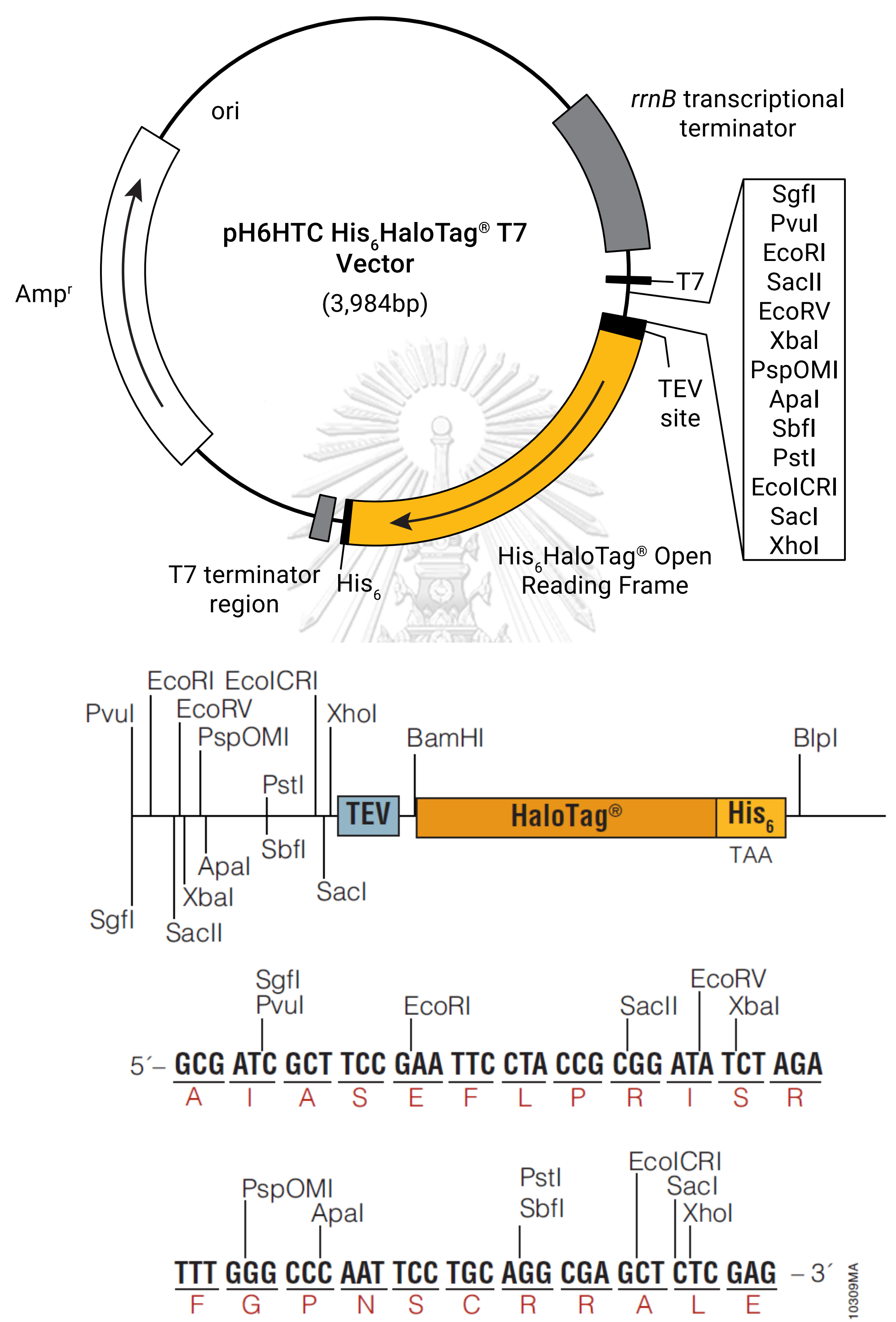
Appendix

Appendix 1 pET28b Vector Map



CHULALONGKORN UNIVERSITY

Appendix 2 pH6HTC His6HaloTag® T7 Vector map



Appendix 3 Mass spectrometry result of recombinant 17-kDa and GroEL proteins

MATRIX SCIENCE MASCOT Search Results

Protein View: AAF00943.1

17 kDa antigen [Bartonella henselae str. Houston-1]

Database:	Bartonella_henselae_22012019
Score:	319
Nominal mass (M_r):	18292
Calculated pI:	9.13

Sequence similarity is available as an NCBI BLAST search of AAF00943.1 against nr.

Search parameters

MS data file:	17K.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)

Protein sequence coverage: 20%

Matched peptides shown in ***bold red***.

1 MAAYISSKER KSMKKYSLVT LLSLFCISHA K**AQTATLTDE** **YYKK**ALENTQ
51 KLDVAKSQTA ESIYESATQT ANKIK**DINNQ** **LANLK**ADTKT KPEQLQALQI
101 ELTLLQAQLQ ADTLK**IQSLA** **MIQAK**DTKTK EELREEQTQK KHEDLQKQLK
151 EKLEKSDVRL

MASCOT Search Results

Protein View: CH60_BARHE

60 kDa chaperonin OS=Bartonella henselae (strain ATCC 49882 / DSM 28221 / Houston 1) OX=283166 GN=groL PE=3 SV=1

Database:	SwissProt
Score:	7321
Nominal mass (M_r):	57589
Calculated pI:	5.10
Taxonomy:	Bartonella henselae str. Houston-1

Sequence similarity is available as an NCBI BLAST search of CH60_BARHE against nr.

Search parameters

MS data file:	groEL.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)

Protein sequence coverage: 40%

Matched peptides shown in ***bold red***.

1 MAAKEVKFGR EARERLLR**GV** **DILANAVK**VT LGPKGRNVVI DKSFGAPRIT
51 K**DGVSVAK**EI ELEDKFENMG AQMLREVASK TNDIAGDGTT TATVLGQAIV
101 QEGVK**AVAAG** **MNPMDLKR**GI DAAVDEVVAN LFKKAKKIQT SAEIAQVGTI
151 SANGAAEIGK **MIADAMEKVG** **NEGVITVEEA** **KTAETELEVV** **EGMQFDRGYL**
201 **SPYFVTNAEK** MVADLDDPYI LIHEKKLSNL QSLLPVLEAV VQSGKPLLII
251 AEDVEGEALA TLVVNKLRGG LK**IAAVKAPG** **FGDRR**KAMLE DIAILTSGQV
301 ISEDVGIK**LE** **NVTLDMLGR**A KK**VNISKENT** **TIIDGAGQKS** **EINAR**VNQIK
351 **VQIEETTSDY** **DREK**LQERLA K**LAGGVAVIR** **VGGATEVEVK** **EK**K**DRVDDAL**
401 **NATR**AAVEEG IVAGGTALL R**AANALTVKG** **SNPDQEAGIN** **IVRRALQAPA**
451 **RQIATNAGEE** **AAIIVGK**VLE NNADTFGYNT ATGEFGDLIA LGIVDPVKVV
501 RSALQNAASI ASLLITTEAM VAEVPKKDTP VPPMPGGGMG GMGGMDF

Appendix 4 OD values of r17-kDA antigen titration of negative serum

17k-Neg serum			1	2	3	4	5	6	7	8	9	10	11	12
Antigen dilution (ug/ml)			10	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0.02	0.01	Blank
serum dilution (1:n)	A	100	0.0821	0.0793	0.0797	0.0704	0.0686	0.0671	0.0591	0.0614	0.0617	0.0576	0.0641	0.0636
	B	200	0.078	0.063	0.076	0.0691	0.0651	0.0575	0.0658	0.0665	0.0563	0.0637	0.0608	0.0632
	C	400	0.0811	0.0694	0.0706	0.067	0.0611	0.0647	0.0576	0.0645	0.0604	0.0637	0.0625	0.0591
	D	800	0.0728	0.0698	0.068	0.0603	0.061	0.0582	0.0645	0.0585	0.0604	0.0701	0.0755	0.0685
	E	1600	0.0888	0.0752	0.0631	0.0612	0.061	0.0647	0.0598	0.061	0.0628	0.0684	0.0658	0.0603
	F	3200	0.075	0.0776	0.0646	0.0687	0.0662	0.0575	0.0709	0.0671	0.0616	0.0716	0.0575	0.0632
	G	6400	0.0899	0.0741	0.0718	0.0702	0.0667	0.0629	0.0585	0.0621	0.0627	0.0603	0.0626	0.0702
	H	Blank	0.0954	0.0692	0.0764	0.0745	0.0617	0.0632	0.0639	0.0734	0.0691	0.0799	0.07	0.0692

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix 5 OD values of r17-kDA antigen titration of positive serum

17k-Pos serum			1	2	3	4	5	6	7	8	9	10	11	12
Antigen dilution (ug/ml)			10	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0.02	0.01	Blank
serum dilution (1:n)	A	100	3.4527	3.4924	3.4708	3.2539	3.0757	2.5839	2.1718	1.9769	1.7524	1.5817	1.5286	1.467
	B	200	3.3748	3.352	3.3326	2.9959	2.6199	2.2369	1.7225	1.5538	1.3764	1.3106	1.1413	1.4139
	C	400	2.9939	3.084	2.9579	2.6359	2.2795	1.7039	1.3184	1.1467	0.9383	0.9061	0.8874	0.9261
	D	800	2.7089	2.7324	2.6276	2.2585	1.715	1.2204	0.9421	0.7888	0.6857	0.5814	0.5757	0.6483
	E	1600	2.244	2.1357	2.011	1.6989	1.227	0.8167	0.6446	0.5805	0.4247	0.4235	0.4042	0.4334
	F	3200	1.6805	1.456	1.3626	1.1523	0.7449	0.522	0.4101	0.3695	0.2714	0.2669	0.237	0.227
	G	6400	1.1267	0.9642	0.9389	0.812	0.4869	0.331	0.2868	0.2544	0.2046	0.2015	0.1764	0.1668
	H	Blank	0.103	0.1041	0.0902	0.0721	0.0729	0.0673	0.0607	0.0706	0.0784	0.0643	0.061	0.0561

Appendix 6 OD values of rGroEL antigen titration of negative serum

groEL-Neg serum			1	2	3	4	5	6	7	8	9	10	11	12
Antigen dilution (ug/ml)			10	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0.02	0.01	Blank
serum dilution (1:n)	A	100	0.0847	0.0851	0.0823	0.136	0.0582	0.0591	0.0598	0.0568	0.0657	0.08	0.0607	0.0615
	B	200	0.085	0.076	0.2189	0.1489	0.1143	0.0567	0.0517	0.0772	0.1265	0.0618	0.0717	0.0686
	C	400	0.065	0.1215	0.0719	0.0624	0.0578	0.0779	0.064	0.06	0.0958	0.0692	0.0836	0.0617
	D	800	0.0682	0.0778	0.0725	0.0611	0.054	0.1235	0.0742	0.0558	0.0979	0.0647	0.0728	0.0886
	E	1600	0.0603	0.0662	0.0811	0.0771	0.0737	0.0758	0.0565	0.0951	0.0639	0.0581	0.0649	0.0694
	F	3200	0.073	0.0675	0.0933	0.0552	0.0571	0.0568	0.0567	0.0559	0.0651	0.0623	0.0675	0.0703
	G	6400	0.0697	0.0698	0.065	0.0649	0.0613	0.0578	0.0758	0.0799	0.0576	0.0667	0.0703	0.0807
	H	Blank	0.1017	0.1136	0.0958	0.0766	0.0754	0.0759	0.1074	0.0585	0.0915	0.0735	0.0908	0.0817

Appendix 7 OD values of rGroEL antigen titration of positive serum

groEL-Pos serum			1	2	3	4	5	6	7	8	9	10	11	12
Antigen dilution (ug/ml)			10	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0.02	0.01	Blank
serum dilution (1:n)	A	100	3.2715	3.1476	3.0438	2.7247	2.4509	2.2662	1.9463	1.864	1.7035	1.6953	1.458	1.3272
	B	200	3.2009	3.2059	3.0118	2.697	2.2952	1.9139	1.7551	1.3594	1.3569	1.4211	1.267	1.3073
	C	400	2.8497	2.961	2.6492	2.1954	1.9554	1.5776	1.2258	0.9663	0.974	0.9895	0.8887	0.9135
	D	800	2.7904	2.6258	2.3628	1.7253	1.4059	0.9826	0.7688	0.6559	0.6177	0.6454	0.5993	0.6304
	E	1600	2.1727	2.0579	1.7096	1.1354	0.9185	0.6525	0.5261	0.4534	0.4147	0.4393	0.3887	0.4221
	F	3200	1.6511	1.4437	1.1292	0.7793	0.572	0.4428	0.3478	0.3141	0.2682	0.2747	0.2847	0.3256
	G	6400	1.0312	0.9709	0.7544	0.4861	0.3777	0.2757	0.2193	0.221	0.1793	0.186	0.1664	0.2113
	H	Blank	0.0882	0.0825	0.1102	0.0699	0.1464	0.0556	0.0623	0.0704	0.0695	0.0711	0.1307	0.1138

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix 8 OD values of antibody titration of r17-kDa negative serum

17k-Neg serum			1	2	3	4	5	6	7	8	9	10	11	12
serum dilution (1:n)			100	200	400	800	1600	3200	6400	12800	25600	51200	102400	Blank
conjugate dilution(1:n)	A	3000	0.0626	0.0669	0.0624	0.0606	0.0732	0.067	0.0673	0.0767	0.0625	0.065	0.0643	0.0698
	B	6000	0.058	0.0586	0.0556	0.056	0.0593	0.0576	0.0544	0.0585	0.0595	0.0583	0.0547	0.0576
	C	12,000	0.0543	0.0557	0.0563	0.0549	0.0591	0.0533	0.0542	0.0538	0.0543	0.0528	0.0534	0.0575
	D	24,000	0.0528	0.0528	0.0539	0.0555	0.0523	0.0526	0.0474	0.0493	0.0549	0.0508	0.0532	0.0514
	E	48,000	0.0626	0.051	0.0511	0.0526	0.053	0.0625	0.0543	0.0468	0.0501	0.053	0.0538	0.0557
	F	96,000	0.0516	0.0507	0.0514	0.0536	0.0521	0.0505	0.0505	0.0517	0.0523	0.0522	0.0509	0.0549
	G	192,000	0.0544	0.0526	0.0543	0.0524	0.0531	0.0528	0.0526	0.0543	0.0537	0.0504	0.0533	0.0513
	H	Blank	0.0536	0.054	0.0532	0.0531	0.0547	0.0522	0.0555	0.0553	0.0567	0.0538	0.0519	0.0581

Appendix 9 OD values of antibody titration of r17-kDa positive serum

17k-Pos serum			1	2	3	4	5	6	7	8	9	10	11	12
serum dilution (1:n)			100	200	400	800	1600	3200	6400	12800	25600	51200	102400	Blank
conjugate dilution(1:n)	A	3000	2.5501	2.1538	1.6704	1.1729	0.7858	0.5359	0.3254	0.2034	0.1555	0.1238	0.0917	0.0649
	B	6000	1.8799	1.5086	1.0996	0.7357	0.486	0.312	0.2006	0.1376	0.097	0.095	0.0723	0.0632
	C	12,000	1.2254	0.9532	0.6723	0.4329	0.2878	0.1912	0.1158	0.1032	0.0846	0.0727	0.062	0.0624
	D	24,000	0.7711	0.5891	0.4508	0.2826	0.1994	0.1341	0.0908	0.088	0.0704	0.0645	0.0544	0.0556
	E	48,000	0.4192	0.3487	0.2556	0.1555	0.1135	0.0859	0.069	0.0688	0.0619	0.0564	0.054	0.0544
	F	96,000	0.2485	0.2092	0.1576	0.1017	0.0844	0.0724	0.0582	0.0577	0.059	0.055	0.0536	0.054
	G	192,000	0.1551	0.1318	0.1082	0.0814	0.064	0.0591	0.056	0.0595	0.0546	0.0558	0.0533	0.0556
	H	Blank	0.0569	0.056	0.0568	0.0576	0.0557	0.0531	0.0564	0.0569	0.0563	0.0535	0.0522	0.0552

Appendix 10 OD values of antibody titration of rGroEL negative serum

groEL-Neg serum			1	2	3	4	5	6	7	8	9	10	11	12
serum dilution (1:n)			100	200	400	800	1600	3200	6400	12800	25600	51200	102400	Blank
conjugate dilution(1:n)	A	3000	0.1499	0.1396	0.1496	0.1422	0.1059	0.1081	0.1177	0.1139	0.1129	0.1086	0.1068	0.1242
	B	6000	0.1013	0.1388	0.1176	0.0825	0.0705	0.066	0.0645	0.0622	0.0685	0.0653	0.0637	0.0762
	C	12,000	0.0759	0.1303	0.1194	0.0631	0.0581	0.0566	0.053	0.0534	0.0602	0.0526	0.0604	0.0624
	D	24,000	0.0652	0.0795	0.0632	0.0553	0.0465	0.0538	0.0509	0.0466	0.0472	0.0464	0.0508	0.0519
	E	48,000	0.0603	0.059	0.0479	0.046	0.0488	0.0472	0.0507	0.05	0.045	0.0531	0.0447	0.0483
	F	96,000	0.053	0.0587	0.0461	0.0454	0.0485	0.0445	0.0478	0.0485	0.0459	0.0502	0.0476	0.0505
	G	192,000	0.0546	0.0551	0.0473	0.0505	0.0453	0.0464	0.0451	0.0472	0.0473	0.0473	0.049	0.0493
	H	Blank	0.0516	0.0508	0.0524	0.052	0.0496	0.0515	0.0484	0.0483	0.0499	0.0504	0.0463	0.0485

Appendix 11 OD values of antibody titration of rGroEL positive serum

groEL-Pos serum			1	2	3	4	5	6	7	8	9	10	11	12
serum dilution (1:n)			100	200	400	800	1600	3200	6400	12800	25600	51200	102400	Blank
conjugate dilution(1:n)	A	3000	3.7973	3.6794	3.5388	3.3614	2.8477	2.0968	1.5388	0.949	0.6334	0.3855	0.2691	0.1054
	B	6000	3.6214	3.4848	3.2561	2.8886	2.2963	1.5514	1.0037	0.6429	0.4128	0.2671	0.1783	0.0698
	C	12,000	3.4222	3.1436	2.8491	2.264	1.6246	1.0662	0.6735	0.4225	0.2678	0.193	0.1381	0.0581
	D	24,000	3.0422	2.6217	2.1553	1.5832	1.0658	0.6128	0.4047	0.2562	0.1633	0.116	0.0926	0.0517
	E	48,000	2.2446	1.8605	1.4658	0.9791	0.6503	0.3827	0.2493	0.1588	0.111	0.0878	0.0703	0.0491
	F	96,000	1.5457	1.2083	0.9397	0.6041	0.4062	0.2313	0.1429	0.0998	0.0714	0.0629	0.0555	0.0497
	G	192,000	0.9809	0.7837	0.5582	0.3694	0.2471	0.1405	0.0992	0.0758	0.0593	0.0585	0.0494	0.0513
	H	Blank	0.0535	0.0522	0.0508	0.0504	0.049	0.0498	0.0503	0.0478	0.0469	0.0507	0.0533	0.0521

Appendix 12 r17-kDa Intra-plate repeatability of positive sera

	1	2	3	4	5	6	7	8	9	10	11	12
	Pos1	Pos2	Pos3	Pos4	Pos5	Pos6	Pos7	Pos8	Pos9	Pos10	Pos11	Pos12
A	0.6124	0.8815	0.289	2.0331	0.5198	0.4008	0.5657	0.8227	0.1929	0.2116	0.8674	0.6469
B	0.5417	0.8272	0.2716	2.018	0.4304	0.3724	0.5389	0.7536	0.1708	0.1924	0.7151	0.5209
C	0.5047	0.8055	0.2858	2.0076	0.4411	0.3626	0.5281	0.7894	0.1612	0.217	0.8273	0.5114
Avg	0.552933	0.838067	0.282133	2.019567	0.463767	0.3786	0.544233	0.788567	0.174967	0.207	0.803267	0.559733
sd	0.054722	0.039148	0.009261	0.012822	0.04882	0.01984	0.019359	0.034558	0.016256	0.012929	0.078943	0.075638
%CV	9.90%	4.67%	3.28%	0.63%	10.53%	5.24%	3.56%	4.38%	9.29%	6.25%	9.83%	13.51%

Appendix 13 r17-kDa Intra-plate repeatability of negative sera

	1	2	3	4	5	6	7
	Neg1	Neg 2	Neg3	Neg4	Neg5	Neg6	Neg7
D	0.0532	0.2678	0.1626	0.3672	0.6651	0.2711	0.6252
E	0.0532	0.2645	0.134	0.3559	0.6175	0.3297	0.6347
F	0.0539	0.2857	0.1598	0.3962	0.6145	0.3168	0.5942
Avg	0.053433	0.272667	0.152133	0.3731	0.632367	0.305867	0.618033
sd	0.000404	0.011407	0.015766	0.020788	0.028388	0.030792	0.02118
%CV	0.76%	4.18%	10.36%	5.57%	4.49%	10.07%	3.43%

จุฬาลงกรณ์มหาวิทยาลัย CHULALONGKORN UNIVERSITY

Appendix 14 r17-kDa Inter-plate repeatability of positive sera

	1	2	3	4	5	6	7	8	9	10	11	12
	Pos1	Pos2	Pos3	Pos4	Pos5	Pos6	Pos7	Pos8	Pos9	Pos10	Pos11	Pos12
A1	0.5622	0.9041	0.4177	1.4027	0.8831	0.6512	1.0339	1.8161	0.6307	0.6044	2.1341	2.1191
A2	0.6465	0.989	0.4685	1.4587	0.8684	0.666	1.085	1.9618	0.7184	0.8341	2.0754	2.1494
A3	0.6156	0.9913	0.4475	1.53	0.9492	0.7274	1.2343	1.9264	0.7642	0.7242	2.2024	2.1107
Avg	0.6081	0.961467	0.444567	1.4638	0.900233	0.681533	1.117733	1.901433	0.704433	0.7209	2.1373	2.1264
sd	0.042648	0.049694	0.025527	0.063803	0.043039	0.040405	0.104133	0.075991	0.067837	0.114886	0.06356	0.020357
%CV	7.01%	5.17%	5.74%	4.36%	4.78%	5.93%	9.32%	4.00%	9.63%	15.94%	2.97%	0.96%

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Appendix 15 r17-kDa Inter-plate repeatability of negative sera

	1	2	3	4	5	6	7
	Neg1	Neg 2	Neg3	Neg4	Neg5	Neg6	Neg7
D1	0.0601	0.6252	0.33	0.7725	0.943	0.6654	1.1749
D2	0.0632	0.619	0.3312	0.8469	0.9529	0.6282	1.1761
D3	0.0633	0.6125	0.3114	0.892	1.0974	0.9109	1.7854
Avg	0.0622	0.6189	0.3242	0.837133	0.997767	0.734833	1.3788
sd	0.001819	0.006351	0.011101	0.060346	0.086427	0.153608	0.352126
%CV	2.92%	1.03%	3.42%	7.21%	8.66%	20.90%	25.54%

Appendix 16 rGroEL Intra-plate repeatability of positive sera

	1	2	3	4	5	6	7	8	9	10	11	12
	Pos1	Pos2	Pos3	Pos4	Pos5	Pos6	Pos7	Pos8	Pos9	Pos10	Pos11	Pos12
A	2.3083	2.4688	1.9432	2.0916	2.2693	1.4035	2.521	2.8508	1.5632	2.2149	2.3931	2.4807
B	2.3147	2.4978	1.9971	2.303	2.3511	1.3627	2.4463	2.9467	1.5811	2.2393	2.4711	1.8636
C	2.2923	2.5044	2.0013	2.2943	2.2778	1.4652	2.6676	2.9474	1.7123	2.2485	2.5711	2.1825
Avg	2.3051	2.490333	1.980533	2.229633	2.2994	1.410467	2.544967	2.914967	1.618867	2.234233	2.478433	2.1756
sd	0.009421	0.015463	0.026454	0.097669	0.036722	0.042134	0.091921	0.045374	0.06647	0.014177	0.072853	0.251977
%CV	0.41%	0.62%	1.34%	4.38%	1.60%	2.99%	3.61%	1.56%	4.11%	0.63%	2.94%	11.58%

Appendix 17 rGroEL Intra-plate repeatability of negative sera

	1	2	3	4	5	6	7
	Neg1	Neg 2	Neg3	Neg4	Neg5	Neg6	Neg7
D	0.0515	2.5898	1.5655	1.2189	1.7218	1.2484	2.1402
E	0.0541	2.5453	1.5521	1.2078	1.8419	1.2854	2.1991
F	0.059	2.4287	1.6195	1.2366	2.0603	1.1069	2.2289
Avg	0.054867	2.521267	1.579033	1.2211	1.874667	1.213567	2.1894
sd	0.003109	0.067929	0.029132	0.01186	0.140121	0.076922	0.036855
%CV	5.67%	2.69%	1.84%	0.97%	7.47%	6.34%	1.68%

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Appendix 18 rGroEL Inter-plate repeatability of positive sera

	1	2	3	4	5	6	7	8	9	10	11	12
	Pos1	Pos2	Pos3	Pos4	Pos5	Pos6	Pos7	Pos8	Pos9	Pos10	Pos11	Pos12
A1	2.30830	2.46880	1.94320	2.09160	2.26930	1.40350	2.52100	2.85080	1.56320	2.21490	2.39310	2.48070
A2	2.53630	2.54400	1.56990	2.20760	2.36300	1.29540	2.63060	2.99710	1.65110	2.12480	2.61630	1.98690
A3	2.32640	2.57370	2.13690	2.28040	2.48920	1.46980	2.73780	3.05070	1.79510	2.44770	2.73280	2.53940
Avg	2.390333	2.528833	1.883333	2.1932	2.373833	1.389567	2.6298	2.9662	1.6698	2.26247	2.580733	2.335667
sd	0.103478	0.044148	0.235316	0.077747	0.0901	0.071877	0.08851	0.084483	0.095592	0.136047	0.140944	0.247777
%CV	4.33%	1.75%	12.49%	3.54%	3.80%	5.17%	3.37%	2.85%	5.72%	6.01%	5.46%	10.61%

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Appendix 19 rGroEL Inter-plate repeatability of negative sera

	1	2	3	4	5	6	7
	Neg1	Neg 2	Neg3	Neg4	Neg5	Neg6	Neg7
D1	0.0515	2.5898	1.5655	1.2189	1.7218	1.2484	2.1402
D2	0.0562	2.5271	1.6066	1.2676	1.8937	1.2463	2.025
D3	0.06	2.3558	1.2947	1.1664	1.6677	0.9638	1.9106
Avg	0.0559	2.4909	1.488933	1.217633	1.761067	1.152833	2.025267
sd	0.003477	0.0989	0.138365	0.041324	0.096351	0.13367	0.093734
%CV	6.22%	3.97%	9.29%	3.39%	5.47%	11.59%	4.63%

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

VITA

NAME	Krissda Boonaramrueng
DATE OF BIRTH	28 September 1984
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University
HOME ADDRESS	24 Thonburi National Housing 3, Rama 2 road, Samaedam, Bangkhuntien, Bangkok, Thailand

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY