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THESIS

MOLECULAR CHARACTERIZATION OF *EHRLICHIA AND* RELATED GENERA IN DOGS AND CATS FROM BANGKOK, THAILAND

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Ehrlichiosis caused by *Ehrlichia* is of veterinary importance worldwide. In Thailand, there has been little information of *Ehrlichia* available on species, drug resistance and pathogenicity at molecular level. Genus-specific primers for *Ehrlichia* and *Anaplasma* were designed and used to amplify the *16S rRNA* gene from naturally infected canine blood samples. Both homology and secondary structure analysis of *16S rRNA* sequences indicated that they were novel *E. canis* and *A. platys* strains. Phylogenetic analysis revealed that the Thai *E. canis* strain was closely related and formed a single cluster with *E. canis* from different countries. *A. platys* found in this study showed close relationship with earlier report of *A. platys* from Thailand. These specific primers were further used to amplify *16S rRNA* genes from stray cat blood samples. However, the PCR products sequencing were failed to identify *Ehrlichia* and *Anaplasma* but surprisingly revealed similarity to other groups of alphaproteobacteria including *Bartonella clarridgeiae*, *Ochrobactrum intermedium* and *Sphingomonas paucimobilis*. Phylogenetic analysis showed that these species closely clustered but different from those of *Ehrlichia* and *Anaplasma*.

In order to understand drug resistance, a gene involved in bicyclomycin resistance, *bcr*, was isolated from *E. canis*-Bangkok by PCR. The hypothetical Bcr protein was analyzed and revealed close relationship with those of drug resistance transporter Bcr/CflA subfamily from bacteria in order Rickettsiales. Topology prediction using hidden Markov model algorithms indicated that the *E. canis*-Bangkok Bcr protein was an 11-transmembrane segment protein with N-terminal out of cell and C-terminal in the cytoplasm. It contained special motifs conserved in drug transporters belonging to major facilitator superfamily (MFS). Phylogenetic analysis showed that the hypothetical Bcr proteins of *E. canis*-Bangkok was closely related to those of *Rickettsia* and were segregated from other 12-TMS proteins. Therefore, they are likely to represent a new member of MFS.

In order to understand pathogenicity, patatin (*pat*) gene from *E. canis*-Bangkok was identified by PCR. Its protein sequence revealed characteristics of the conserved domains of bacterial patatins including those of phathogenic ones. The *pat* gene was then expressed in *E. coli* BL21 (DE3) and the crude enzyme was characterized for phospholipase A activities. The results showed that *E. canis*-Bangkok patatin could hydrolyze phospholipids that shorter than 10 carbon atoms because it could hydrolyze tributylglycerol but not trioleoylglycerol. The enzyme showed high activity at pH 8.0 and at temperature 40 °C which are the condition similar to environment in infected animals. It is purposed that *E. canis*-Bangkok patatin may be involved in hydrolysis of phospholipid membrane of mononuclear cells and enhance pathogenicity by spreading *Ehrlichia* to other cells.

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Danai Pinyoowong

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LIST OF ABBREVIATIONS

µm	=	micrometer
µl	=	microliter
mg	=	milligram
kg	=	kilogram
DNA	=	Deoxyribonucleic acid
PCR	=	Polymerase Chain Reaction
rRNA	=	Ribosomal Ribonucleic Acid
ml	=	milliliter
UV	=	Ultraviolet
bp	=	base pair
ng	=	nanogram
kDa	=	kilo Dalton
kb	=	kilo base-pair

MOLECULAR CHARACTERIZATION OF *EHRLICHIA* AND RELATED GENERA IN DOGS AND CATS FROM BANGKOK, THAILAND

INTRODUCTION

Ehrlichiosis is caused by obligate intracellular Gram-negative bacteria, *Ehrlichia* spp., which are the agents of veterinary and medical disease distributed worldwide. Although ehrlichiosis has been recognized as an infectious disease only in animals for a long time, they are currently known as important emerging zoonosis. Some of which are also regarded as emerging human pathogens. This zoonosis is a tick-borne disease and widely distributed in companion animals.

Recently pet animals, such as dogs and cats, in Thailand, particularly in Bangkok, provided a reservoir for ehrlichiosis. The risk for zoonotic disease is also increased, since they share the same pathogens between pets and humans. Ehrlichiosis in companion animals have been increasingly recognized worldwide. However, there is limit information of ehrlichiosis in dogs and cats in Thailand. To estimate a prevalence, identification and characterization of *Ehrlichia* infection is still questionable for epidemiology. In this study, *Ehrlichia* spp. and related genera such as *Anaplasma* spp. will be determined by molecular technique to verify the species as well as distinguish the possible co-infection. Some genes that are likely to be involved in pathogenicity and drug resistance will be cloned and characterized.

OBJECTIVES

1. To identify *Ehrlichia* spp. and related genera in dogs and cats in Bangkok using molecular techniques on *16S rRNA* genes.

2. To clone and characterize patatin gene and drug resistance transporter Bcr/CflA subfamily gene from Thai *Ehrlichia* strain.

LITERATURE REVIEWS

1. Overview of canine and feline ehrlichiosis

1.1 Classification

Ehrlichiosis is a tick-borne disease caused by obligate intracellular Gram-negative bacteria in genera *Ehrlichia* and *Anaplasma* which parasitize leukocytes and platelet, respectively (Harvey, 1998; Lappin, 1998; Neer, 1998). Previously, *Ehrlichia* spp. lived in family Rickettsiaceae and *Anaplasma* spp. lived in family Anaplasmataceae in order Rickettsiales (Moulder, 1974). At present, these two genera belong to family Anaplasmataceae (Dumler *et al*., 2001) (Figure 1).

Ehrlichia species can be divided into three classes based on the host cells they infected, such as monocytic, granulocytic, and thrombocytic cells (Neer, 1998). On the basis of *16S rRNA* sequence homology and antigenic relationship, three genogroups of *Ehrlichia* have been delineated: (i) *E. canis* genogroup (*E. canis, E. chaffeensis* and *E. ewingii*); (ii) *E. phagocytophila* genogroup (*E. phagocytophila, E. equi*, human granulocytic ehrlichiosis agent (HGE) and *A. platys*); and (iii) *E. senetsu* genogroup (*E. sennetsu, E. resticii* and *E. bovis*) (Breitschwerdt, 2000; Kruss *et al*., 2003; Lappin and Parnell, 2003).

Recently, the genera in families Rickettsiaceae and Anaplasmataceae were reorganized after the study on *16S rRNA* sequences (Dumler *et al*., 2001). The family Anaplasmataceae consists of genus *Ehrlichia, Anaplasma, Neorickettsia* and *Wolbachia* (Dumler *et al*., 2001). Some *Ehrlichia* species including *E*. (*Anaplasma*) *phagocytophila* (composes of *E. equi* and HGE agent), *E*. (*Anaplasma*) *bovis* and *E*. (*Anaplasma*) *platys* were proposed to be included in genus *Anaplasma* (Dumler *et al*., 2001). One was changed into genus *Neorickettsia* which is *E*. (*Neorickettsia*) *sennetsu* (Dumler *et al*., 2001). One species in genus *Cowdria* was reclassified into *Cowdria* (*Ehrlichia*) *ruminantium* (Dumler *et al*., 2001).

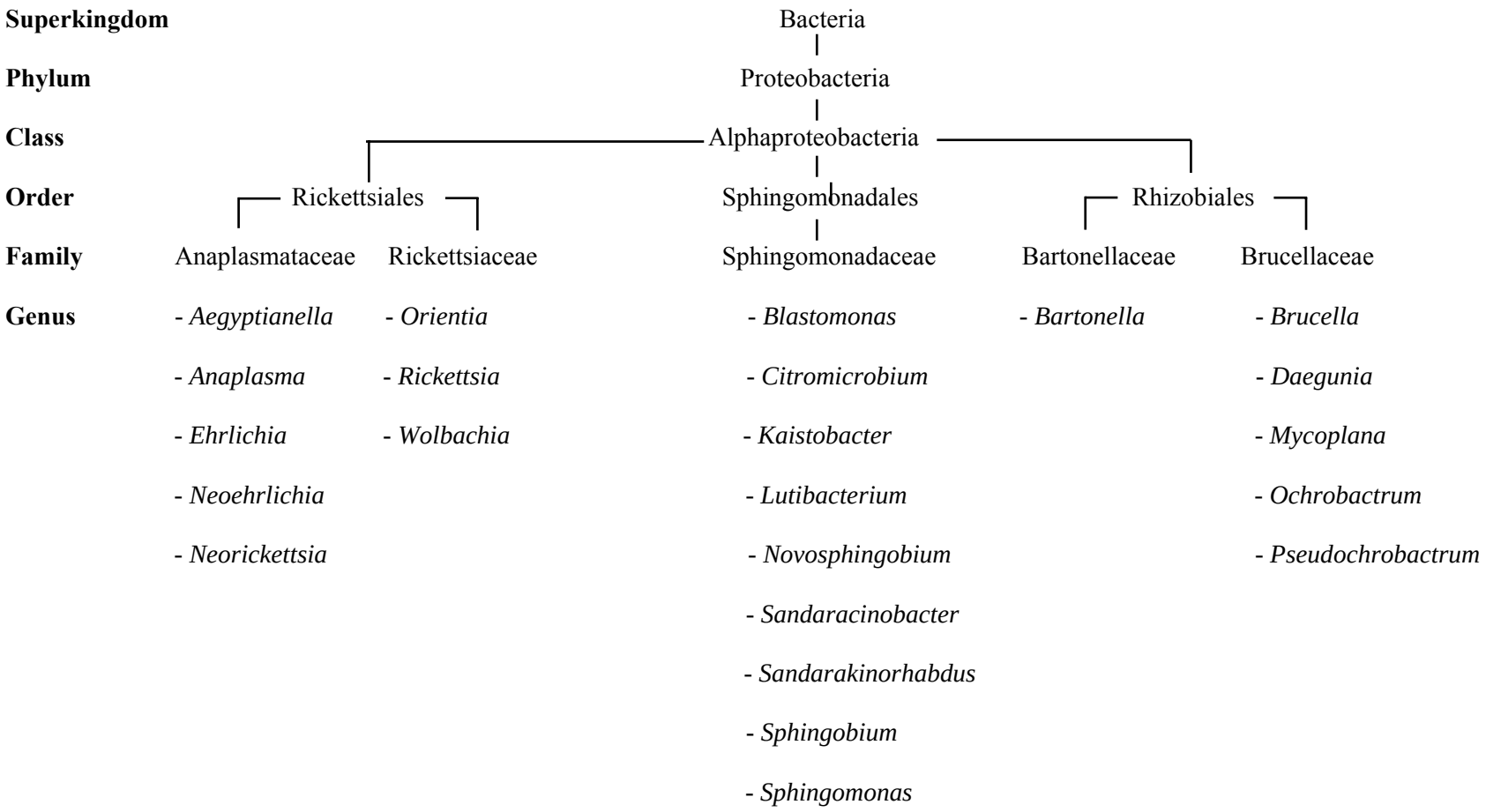


Figure 1 Taxonomic of alpha-proteobacteria

1.2 Life cycle and morphology

Ehrlichia spp. replicate in tick and trematode. They are horizontal transmitted from infected cells of vectors to animals and human blood cells (Rikihisa, 1991). The relationship between organisms, vectors, infected cells, hosts and cross-reactivity of *Ehrlichia* spp. have been explained by Lappin and Parnell (2003).

The distribution of ehrlichiosis is related to the spreading of vectors. *Rhipicephalus sanguineus* (brown dog tick) is a vector of *E. canis* found around the world (Groves *et al*., 1975). *Amblyomma americanum* (lone star tick) is a vector for *E. chaffeensis* and *E. ewingii* found in the United States. These tick species can transmit *Ehrlichia* spp. to wild and domestic animals in Europe, Asia, Africa and South America (Breitschwerdt, 1990).

The life cycle of *Ehrlichia* spp. goes through the three developmental stages of elementary bodies, initial bodies and morulae (Forbes *et al*., 1998). Within a cell small elementary bodies (0.2 to 0.5 μm) develop into larger initial bodies (1.0 to 1.5 μm) and eventually into intracytoplasmic inclusion bodies (morulae: 2 to 5 μm) containing approximately 100 elementary bodies (Kruss *et al*., 2003). Morulae, intracellular inclusions composed of clusters of Rickettsial organisms, are occasionally observed in blood smears during the early stage of infection but rarely in association with chronic infection (Breitschwerdt, 2000). From single organism adheres to the plasma membrane, it invaginates into the host cells, the organism divides by binary fission into progress larger morulae. Organisms may leave the cells when it ruptured or by exocytosis (Greene and Harvey, 1984) (Figure 2). In other intracellular bacteria, Phagosome-Lysosome (P-L) fusion and low acidic pH in phagosome were showed relationship of bacteria to escape from degraded by lysosomal enzyme. In addition, when it can escape from lysosome they can live, multiplication in phagosome and can destroy phagosomal cell membrane by using phospholipase in low acidic pH (Brestad *et al*., 2002).

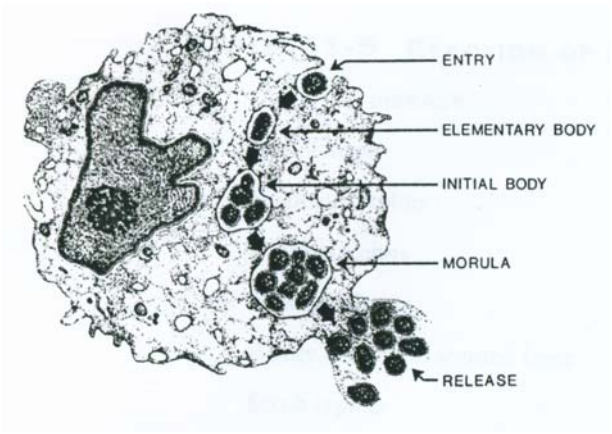


Figure 2 Schematic representation of the growth cycle of ehrlichiae in an infected cell.

Source: Forbes *et al*., 1998.

Most information on pathogenesis was particularly related to *E. canis*. Infection of the vertebrate host occurred when an infected tick ingested a blood meal and salivary secretions contaminated the feeding site. Blood transfusions from infected donors can also transmit the organisms (Wardrop *et al*., 2005) (Figure 2).

Individual animal can be infected with multiple tick-transmitted pathogens or with multiple genotypes of the same pathogenic species. The same tick species can be a vector for several pathogens, however co-infection of individual tick can occur in the same animal (Suksawat *et al.*, 2001a; Shaw *et al.*, 2001).

1.3 Medical importance

Several of the tick-borne infection affecting animals can cause serious disease in human. Ehrlichiosis had been recognized as disease only in animals for a long time, but recently known to be important emerging zoonosis in people (Kruss *et al*., 2003).

Arthropods in general, ticks have evolved as ectoparasites of wild animals. Only a minor of tick species, generally a wide host range, transmit diseases

to domestic animals and humans. The increasing prevalence of tick-transmitted diseases of pets and their owners has been associated with environments and increment in the reservoir of wild host species that now have been associated with human activity (Greig *et al*., 1996; Shaw *et al.*, 2001).

Information of human ehrlichiosis is increasing. At least 5 *Ehrlichia* species including *E. sennetsu* (Misao and Kobayashi, 1995), *E. chaffeensis* (Maeda *et al*., 1987), *Anaplasma phagocytophila* (Chen *et al*., 1994), *E. canis* (Perez *et al*., 1996) and *E. ewingii* (Buller *et al*., 1999) can cause human ehrlichiosis. Previously, infection with *Ehrlichia* species is considered to be host specific (Kruss *et al*., 2003). *E. canis* infects dogs while *E. chaffeensis* infects humans and deers (Kruss *et al*., 2003). It was reported that an isolate microorganism similar to *E. canis* was isolated from a man in Venezuela (Perez *et al.*, 1996). However, human infections with *Neorickettsia sennetsu* had been reported since 1950s cited by Misao and Kobayashi, 1995 but *E. chaffeensis*, *E. ewingii* and *A. phagocytophilum* were found later to infect human (Maeda *et al*., 1987; Chen *et al*., 1994 and Buller *et al*., 1999). The prevalence and full of extent of the diseases in many cases are still unknown.

Human ehrlichiosis can be divided into two groups depending on target cells. When the monocyte is infected, called human monocytic ehrlichiosis (HME) (Anderson *et al.*, 1991) but when granulocyte is infected, called human granulocytic ehrlichiosis (HGE) (Bakken *et al.*, 1994; Chen *et al.*, 1994).

1.4 Veterinary importance

1.4.1 Canine ehrlichiosis

Ehrlichial diseases have emerged as significant problems for human and animals over the past two decades. In 1935, *E. canis* was first discovered in dog in Algeria (Donatien and Lestoquard, 1935). Before the outbreak in military working dogs in Southeast Asia in 1967, canine ehrlichiosis was considered to be a mild disease characterized by fever, vomiting and naso-ocular discharge (Huxsoll, 1990).

The symptom of ehrlichiosis has been divided into three stages including phases acute, subclinical and chronic based on clinical signs and clinicopathologic abnormalities (Neer, 1998). Since then, the disease in dogs have been spread worldwide (Shaw *et al.*, 2001).

Canine ehrlichiosis can be divided into three groups upon *Ehrlichia* species that cause disease and target cells (Greene and Harvey, 1984). Firstly, canine monocytic ehrlichiosis (CME) is caused by *E. canis* and *E. chaffeensis* that infected monocytic cells. Secondly, canine granulocytic ehrlichiosis (CGE) is caused by *A. phagocytophilum* and *E. ewingii* that infected granulocytic cells. Finally, canine cyclic thrombocytopenia (CCT) is caused by *A. platys* that infected thrombocytic cells (Greene and Harvey, 1984).

Canine monocytic ehrlichiosis (CME) is an important fatal tick-borne disease caused by *E. canis* that can spread worldwide (Suksawat *et al*., 2001a; Rodriguez-Vivas *et al*., 2005; Trapp *et al*., 2006). The detection of chronic infection of CME is very difficult because it has the low number of parasites in blood stream (Greene and Harvey, 1984). To identify at the early stage of *Ehrlichia* spp. infection is benefit for dogs to succeed in escaping of canine ehrlichiosis.

Tick-borne ehrlichioses commonly caused by *Ehrlichia* spp. are important problem throughout the world. Household and stray dogs are most at risk for these infections, due to vector-related infections. In Thailand, little information has been reported (Pryor *et al*., 1972; Davison *et al*., 1975; 1978). There has been no report until the beginning of 1972, on the association of serological survey of military personnel and dogs in Thailand and South Vietnam (Pryor *et al*., 1972). Recently three reports described a noticeable increase of ehrlichiosis in dogs in Thailand (Suksawat *et al*., 2001a, 2001b; Parola *et al.,* 2003). Thus, the underlying causes of this confound outbreak remain elusive.

The characteristics in common among infected dogs were infested with tick vectors. The major canine ehrlichial agents, including *E. canis* are all

Rhipicephalus sanguineus tick vectors (Groves *et al*., 1975). In Thailand, *R. sanguineus*, the brown dog tick, is usually retrieved on domestic dogs (Tanskul *et al*., 1983).

Ehrlichiosis is endemic disease in Thailand. These disease destroyed large number of dogs. Diagnosis of ehrlichiosis in Thailand commonly detected by clinical sign including epitaxis, hemorrhagic sign and pale mucous membrane. Evaluation on blood chemistry and detection of morulae on stained blood smears using light microscope are routinely applied in Thailand. To describe infecting *Ehrlichia* spp. may important in clinical and treatment suggestion. Interestingly, we did not culture any *Ehrlichia* isolate from blood samples of dogs because cultivation of *Ehrlichia* spp. is very complex, time-consuming and requiring great amount volume of blood specimen.

1.4.2 Feline ehrlichiosis

The documents of ehrlichiosis in cats are rather rare. It is not known why this disease is under reported. Since cats are less susceptible to clinical disease and they can remove ticks during self-grooming, it may decrease opportunity for disease transmission (Kruss *et al*., 2003). Cats can be infected with one or more species of *Ehrlichia* based on the finding of *ehrlichia*-like organisms within monocellular cells or granulocytic cells with canine ehrlichiosis. The source of infection in cats is unknown. Transmission involving arthropods or mice is postulated (Couto, 2000) or the blood sucking parasite such as mosquito and flea are possible vectors (Lau and Hay, 1993).

Cats have at least two forms of feline ehrlichiosis. Either caused by *E. canis* with inclusion in mononuclear cells (Breitschwerdt, 2000) or caused by *Anaplasma* (*Ehrlichia*) *phagocytophilum* that has inclusion in neutrophils (Dumler *et al*., 2001). The precise route of transmission remains unknown but ticks have been strongly incriminated. Fleas have also been suggested as possible vectors but serologic studies and multiplex PCR assay have not provided well evidence for this

(Lappin and Parnell, 2003). Granulocytic ehrlichiosis was reported in a cat infected with *A. phagocytophilum* in Italy (Tarello, 2005). The symptoms included fever, anorexia, lethargy, dehydration and tachypnea. The inoculation of *A. phagocytophilum* and *E. resticii* into cat showed the development of morulae in neutrophils and mononuclear cells (Misao and Kobayashi, 1995). These feline cases were identified in geographic regions that were endemic for canine, equine or human anaplasmosis (Lappin and Parnell, 2003).

Additional studies are needed to genetically characterize feline *Ehrlichia*, *Anaplasma* and *Neorickettsia* organisms to clarify the future of the pathogenic importance of these organisms in cats.

1.5 Epidemiology

Seroepidemiology study of canine babesiosis and ehrlichiosis in Brazil showed that *R. sanguineous* is the main tick vector transmitted *Ehrlichia* spp. to dogs (Trapp *et al*., 2006). It was also suggested that warmer weather area had highly prevalence of ehrlichiosis. Seroprevalence of infected and associated factors of *E. canis* in dogs in Yucatan, Mexico were sex, aged presented of thrombocytopenia and the distribution of the *R. sanguineus* vector (Rodriguez-vivas *et al*., 2005).

From a public health, *E. canis*, *E. chaffeensis*, *E. equi*, and *E. ewingii* have emerged as pathogens for both dogs and human beings. Recent research indicated that dogs may serve as sentinels for human exposure to *Ehrlichia* species (Greig *et al*., 1996; Pusterla *et al*., 1997). *E. equi* and *E. phagocytophila* can cause HGE in humans (Chen *et al*., 1994) and may cause granulocytic ehrlichiosis in cats, dogs and horses (Johansson *et al*., 1995; Bjoersdorff *et al.*, 1999). However, identification of infection in domestic animals preceded recognition in humans. *Ehrlichia* spp. was amplified from naturally exposed cats in several countries. *Ehrlichia*-like morula was detected in mononuclear cells or neutrophils of naturally exposed cats in Kenya (Buoro *et al*., 1989), United stated (Bouloy *et al*., 1994; Breitschwerdt, 2000), France (Beaufls *et al*., 1995) , Brazil (Almosny and Massard, 1999), Sweden (Bjoersdorff *et al*., 1999),

Italy (Tarello, 2005) Spain (Ortuno *et al*., 2005) and United Kingdom (Shaw *et al*., 2005). Cats experimentally infected with *E. risticii* and *A. phagocytophila* developed morulae in mononuclear cells and polymorphonuclear cells, respectively (Misao and Kobayashi, 1995).

2. Methods for detection of ehrlichiosis

Ehrlichiosis may be suspected on the basis of the clinical history, physical examination, complete blood counts and serum chemistry profiles (Greene and Harvey, 1984; Kruss *et al.*, 2003). Diagnosis of ehrlichiosis can be accomplished by the direct examination of blood smears for the intracellular *Ehrlichia*-like inclusion bodies and polymerase chain reaction (PCR) to detect the etiology in blood and serology methods (Schriefer and Azad, 1994).

Diagnosis of acute canine ehrlichiosis is based on the identification of ehrlichia on blood smears using microscopic examination. The detection of ehrlichia is very difficult in dogs with chronic infection because of low level of parasitemia (Greene and Harvey, 1984). Microscopic detection of *Ehrlichia* spp. on blood film is still the best and most commomly used method for diagnosis of ehrlichiosis. Microscopic detection must be handling by experience pathologists. Microscopic examination can not detect low level of parasitemia and false negative may be found. Clinical presentations associated with ehrlichial diseases in humans and animals are non-specific. The diagnosis of ehrlichiosis must be confirmed by laboratory testing (Neer, 1998). Laboratory diagnosis of ehrlichiosis includes, (i) identification of ehrlichial morula in peripheral blood smears or impression smears prepared from biopsy samples (lung, lymph node or spleen); (ii) detection of *Ehrlichia*-specific antibodies in serum; (iii) cell culture isolation and PCR amplification of ehrlichial DNA from blood or other tissue specimens. (Schaer, 2003).

PCR technique has been accepted and recommended for parasitic diagnostic for routine work and appropriate for detection of carrier hosts, exported or imported animal testing and blood donor screening test (Wardrop *et al*., 2005). In

epidemiological research, molecular evidences from PCR-based technique characterize the genotype of organisms that distinguished between closely related infection agents or to document the patterns of transmission of strains and species within population (Dagnone *et al*., 2003).

Diagnosis of ehrlichiosis by serologic methods such as the indirect fluorescent antibody (IFA) test (Mcbride *et al.*, 2001; Waner *et al.*, 2001) or ELISA have been used to detect *Ehrlichia*-infected macrophages or granulocytes (Kruss *et al.*, 2003). Cross-reacting between *Ehrlichia* species could be occurred (Waner *et al.*, 2001). Coinfections amongst *E. canis, E. chaffeensis, E. ewingii, E. equi, E. platys*, *Rickettsia* species, *Bartonella* species and *Babesia canis* were documented in a kennel of heavily tick infested dog (Kordick *et al.*, 1999). In cats, ehrlichiosis was detected by serological test but cross reaction between species were also reported (Breistchwerdt, 2000).

3. Characterization and phylogenetic analysis of *Ehrlichia* spp.

None of the conventional biological typing methods such as biotyping, phage typing, serotyping and bacteriocin typing could offer an ideal approach for subdividing of microbial species (Towner and Cockayne, 1993). Progress in molecular biology has resulted in the availability of methods which have the potential to study diversity in any microbial species. Molecular methods may be used to approach microbial identification and typing.

PCR from bloods is considerable help in finding specific DNA and in differentiating the various agents (Breitschwerdt *et al.,* 1998; Kordick *et al*., 1999; Kruss *et al*., 2003). Sequence analysis of *16S rRNA* gene is useful for phylogenetic determination (Weisburg *et al*., 1991). This approach has been widely used to identify newly discovered bacteria as well as re-define existing taxonomy (Weisburg *et al*., 1991).

Recently sequence analyses of *16S rRNA* gene, heat shock protein gene and surface protein gene have resulted in a substantial identification and reclassification of the genus *Anaplasma, Ehrlichia, Cowdria, Neorickettsia* and *Wolbachia* (Kruss *et al*., 2003). The *16S rRNA* gene of bacteria is highly conserved, therefore it is a powerful tool for identification new organism (Woese, 1987; Clarridge III, 2004). Obligate intracellular bacteria are difficult to grow and purify for genetic studies. Therefore, PCR amplification and sequencing of *16S rRNA* gene have been used to identify and characterize members of genus *Ehrlichia* (Dumler *et al*., 2001). In North Carolina and Virginia, granulocytic ehrlichiosis, *E. chaffeensis*, in dogs was identified by PCR and *16S rRNA* sequencing (Anderson *et al*., 1991; Dumler and Bakken, 1995). *16S rRNA* sequencing was used to identify differences of *Ehrlichia* agents between dog and human in Venezuela (Unver *et al*., 2001a). Real-time PCR was used to identify different species of *Ehrlichia* and *Anaplasma* in rodents in U.S. military training site/installations in Korea (Chae *et al*., 2003). In Thailand, sequencing of *16S rRNA* gene and some outer membrane proteins were used to identify differences between *Ehrlichia* and *Anaplasma* from dogs (Suksawat *et al*., 2001a; 2001b). At Thai-Myanmar border and Vietnam, using PCR technique and *16S rRNA* sequencing, bacteria in the family Anaplasmataceae were detected from different tick species (Parola *et al*., 2003).

Characterization of *Ehrlichia* spp. and related genera in infected dogs and cats in Thailand has limited documents. In other countries, other animals such as horse (Pusterla *et al.*, 1997), mouse (Muramatsu *et al*., 2005) and human were reported by serology and PCR based method that explained genotyping of *Ehrlichia* spp. using PCR-RFLP technique and DNA sequencing (Carter e*t al.*, 2001). Recently genetic analyses of *16S rRNA* genes (Breitschwerdt, 2000), heat shock protein genes (Eremeeva and Dasch, 2002; Inokuma *et al*., 2004) and surface protein genes (Kim and Rikihisa, 1998; Ohashi *et al*., 2001) have resulted in a reclassification and identification of the genus *Anaplasma, Ehrlichia, Cowdria, Neorickettsia* and *Wolbachia* (Kruss *et al*., 2003). In addition, outer membrane proteins of *Ehrlichia* spp. were different in size and specific sites that presented immunodominant in each species (Unver *et al*., 2001b). The analysis of surface proteins of *E. canis* in dogs

showed 30-kDa (*p30*) of outer membrane protein containing 22 paralogs of multigene family while, *E. chaffeensis* had 28-kDa (*p28*) outer membrane protein (Ohashi *et al*., 2001; Unver *et al*., 2001b). The difference among the outer membrane proteins could be used to identify each species by designing outer membrane protein specie-specific PCR primers (Ohashi *et al*., 2001). Outer membrane proteins found in *E. canis* and *E. chaffeensis* consist of 4 regions called α, β1, β2, γ and δ. In these 4 regions contain hypervariable sequences. Differentiation in δ region mostly showed conserve sequences. Therefore, δ region of *p30-10* gene of *E. canis* was used to identify at species level (Ohashi *et al*., 2001). Outer membrane protein was used to identify *E. canis* in dogs and ticks by PCR assay (Suksawat *et al*., 2001b; Bremer *et al*., 2005). Identification of P28 outer membrane protein by PCR technique was used to identify *E. chaffeensis* and was suggested that the method was very sensitive (Wagner *et al*., 2004).

4. Coinfection of *Ehrlichia* spp. with other vector-borne pathogens

Coinfection of *Ehrlichia* spp. and other Rickettsiae in the same host are relatively common. It was reported that bacteria that shared the same tick vectors could cause coinfection. *Rhipicephalus sanguineus* transmits *E. canis*, *E. platys*, *Babesia canis*, *Rickettsia* spp. and *Bartonella vinsonii* subspecies *berkhoffii* (*Bvb*). *Amblyomma americanum* transmits *E. chaffeensis*, *E. ewingii* and *Rickettsia* spp. *Ixodes scapularis* transmits *E. equi* and *Rickettsia* spp. (Kordick *et al*., 1999). In Thailand, coinfection of *E. canis*, *E. equi* and *E. platys* was previously reported (Suksawat *et al*., 2001a; 2001b). At Thai-Myanmar border and Vietnam, coinfection of *Ehrlichia* spp. and *Anaplasma* spp. from the same species of tick was also reported (Parola *et al*., 2003). Coinfection of *Ehrlichia* spp. and *Anaplasma* spp. was reported in rodents in U. S. military training sites/installation in Korea (Chae *et al*., 2003).

Molecular technique can explain coinfection of vector borne disease. Real-time reverse transcriptase chain reaction was used to detect *Ehrlichia* spp. and *Anaplasma* spp. in dog peripheral blood samples (Sirigireddy and Ganta, 2005). PCR-RFLP was employed to identify and typing of pathogenic microorganisms and

emerging infections (Tarasevich *et al*., 2003). Coinfection of *Ehrlichia spp*. in dogs was identified by PCR-RFLP (Dagnone *et al*., 2003). PCR-RFLP of *p44* gene of HGE from human, equines, canines, small animals and ticks was able to differentiate among the various species that caused of granulocytic ehrlichiosis in the United Stated (Carter *et al*., 2001). To identify *Anaplasma* spp., PCR-RFLP of *16S rRNA* gene was used (Oporto *et al*., 2003). PCR-RFLP could identify differences amongst *Anaplasma* species that caused granulocytic anaplasmosis and infectious thrombocytopenia (Alberti and Sparagano, 2006).

5. *Ehrlichia canis* complete genome

The genome project of *E. canis* strain Jake from the United Stated (Mavromatis *et al*., 2006) had already completed (GenBank accession number: NC007354). Comparative genomics of emerging ehrlichiosis agents, *E. chaffeensis* and *E. canis*, which caused ehrlichiosis in human and dog, respectively has been recently reported (Hotopp *et al*., 2006). Because of complex membrane structures and immune invasion strategies, it could explain how *E. canis* can live in leukocyte cells and is highly sensitivity to tetracycline drug (Mavromatis *et al*., 2006). Moreover, how *E. canis* can escape from phagosomal system is very interesting. There are several genes that involved in survival and resistance to some antibiotics of *E. canis* as follows:

5.1 Genes involved in pathogenicity

Mavromatis *et al*. (2006) determined the complete genome sequences of *E. canis* strain Jake and reported a large set of proteins with pathogen-host interaction that involved in pathogenicity composed of ankyrin, putative type IV secretory pathway virB6 components and FrbL/virB6 plasmid conjugal transfer protein. In addition, it was proposed that enzymes that neutralize reactive oxygen, outer membrane proteins and protein secretion systems were involved in pathogenicity of *Ehrlichia* spp. (Hotopp *et al*., 2006). Patatin was also suggested to involve in

pathogenicity in several bacterial pathogens (Banerji and Flieger, 2004). However, there is no report on patatin investigation in *Ehrlichia* spp.

Patatin was firstly discovered in potato (Andrews *et al*., 1988). It is a plant storage glycoprotein that has the enzymatic activity of lipid acyl hydrolase. This activity can cleave fatty acids from membrane lipids that protected plant from stress and infection (Andrews *et al*., 1988). Phospholipases are ubiquitous and diverse enzymes that mediate various cellular functions, including membrane maintenance, cellular turn over and the generation of the inflammatory response. Phospholipases constitute a diverse subgroup of lipolytic enzymes that share the ability to hydrolyse one or more ester linkages in phospholipids, with phosphodiesterase as well as acyl hydrolase activities (Waite, 1996). Phospholipases are classified into four groups (Group A-D) based on the position at which they cleave the phospholipid. Phospholipase A composed of Phospholipase A1 that cleaves the SN-1 acyl chain and Phospholipase A2 that cleaves the SN-2 acyl chain. Phospholipase B cleaves both SN-1 and SN-2 acyl chains, also known as a lysophospholipase. Phospholipase C cleaves before the phosphate, releasing diacylglycerol and a phosphate-containing head group. Phospholipase C plays a central role in signal transduction, releasing the second messenger inositol triphosphate. Phospholipase D cleaves after the phosphate, releasing phosphatidic acid and an alcohol (Figure 3) (Nelson and Cox, 2000).

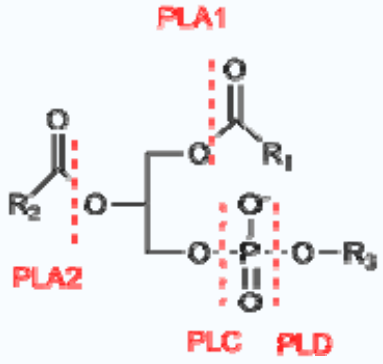


Figure 3 Phospholipase cleavage sites.

Source: Nelson and Cox, 2000.

Enzymes with phospholipase A_2 (PLA_2) activity hydrolyze fatty acids at the sn-2 position. The resulting cleavage products, lysophospholipid and free fatty acid, participate in multiple signaling pathways and are precursors to potent mediators of the host inflammatory response, including the eicosanoids and platelet activating factor (Granata *et al*., 2003). Recently, the number of phospholipases identified in prokaryotic, fungal and protozoan pathogens has been steadily increasing (Sitkiewicz *et al*., 2007). Phospholipases with demonstrated or putative role in virulence have been identified in *Candida* sp. (phospholipase C; PLC) (Kumar *et al*., 2006), *Cryptococcus neoformans* (phospholipase B; PLB) (Cox *et al*., 2001; Ganendren *et al*., 2006), *Plasmodium* sp. (PLA_2) (Bhanot *et al*., 2005) and *Trichomonas vagialis* (Lubick and Burgess, 2004). Therefore, phospholipases are likely to be involved in the membrane disruption process that often occur during host cell invasion (Waite, 1996) such as pH-actvated phospholipase A2 of *Helicobacter pylori* (Berstad *et al*., 2002).

Patatin is the trivial name given to a group of immunologically related glycoproteins with a molecular weight of 40 kDa found practically in all potato (*Solanum tuberosum* L.) cultivars (Andrews *et al*., 1988). Patatin accounts for up to 40% of the total soluble protein present in tubers (Racusen and Foote, 1980; Rosahl *et al*., 1986). Although its physiological function in the tuber is not clearly known, patatin may act to mobilize or degrade lipid during tuber development and sprouting. It has also been suggested to play a role in wound response (Dennis and Galliard, 1974) or in affording a defense by mediating phytoalexin production (Andrews *et al*., 1988) or to be involved in signal transduction by virtue of its phospholipase A_2-like activity (Senda *et al*., 1996). Currently, no direct evidence to support any of these hypotheses is available. Lipolytic enzymes are important biocatalysts due to their ability to utilize a wide range of substrates and hence have a potentially broad spectrum of biotechnological uses (Verger, 1999).

Patatin is present in lipolytic activities in prokaryotes and eukaryotes. Lipolytic enzymes can be classified into eight families based on the conserved sequences, motifs and biological properties included true lipase, GDSL, hormone-

sensitive lipase (HSL) and families III, V-VII (Arpigny and Jaeger, 1999). In addition, the lipolytic enzymes can be classified into three main groups based on substrate specificity included lipase (EC 3.1.1.3), esterase/carboxyl esterase (EC 3.1.1.1) and phospholipase (EC 3.1.1.4) (Verger, 1997). True lipases can be defined as carboxylesterases that catalyze the hydrolysis and synthesis of relatively long chain acylglycerols with acyl chain lengths of > 10 carbon atoms, with trioleoylglycerol as the standard substrate. In contrast, esterase catalyze the hydrolysis of glycerolesters with acyl chain lengths of < 10 carbon atom, with tributylglycerols (tributyrin) as the standard substrate (Verger, 1997), even through lipases as also capable of hydrolyzing esterase substrate (Verger, 1997). Many lipolytic enzymes, including lipase, esterases or carboxylesterase and various types of phospholipase, have been found in a wide range of organisms from bacteria to humans (Arpigny and Jaeger, 1999).

ExoU is the first patatin-like protein (PLP) found in bacteria, *Pseudomonas aeruginosa* (Phillips *et al*., 2003; Sato *et al*., 2005). PLP genes were detected in 55 out of 123 completed bacterial genomes including Gram-negative and Gram-positive bacteria such as *Bacillus* sp., *Brucella* sp., *Rickettsia* sp., *Staphylococcus aureus*, *Yersinia pestis* and many others (Banerji and Flieger, 2004). Some animal and plant pathogens as well as symbiont genomes contain high copy number of PLP genes. The number of PLP genes also varied between different species (Banerji and Flieger, 2004). However, *E. canis* has only one copy of patatin gene in the genome (GenBank accession number: NC007354). The correlation between the number of PLP genes and virulence are not commendable. A high number of PLP genes might be a benefit to the bacterium in interaction with the host, adaptation to various environments and competition with other organisms (Banerji and Flieger, 2004).

In *Toxoplasma gondii*, a protozoa in leukocyte, PLP can protect it from degradation in activated macrophages because the nitric oxide (NO) synthase was reduced and allowed *T. gondii* to survive and replicate in moderately activated macrophage (Mordue *et al*., 2007). The important of NO is defense against microbial

pathogens. NO can be directly bacteriocidal; it can also react with hydrogen peroxide to form peroxynitrite, to enhance killing by the oxidative brust (Waite, 1996).

Phylogenetic analysis of PLPs in *Rickettsia felis* showed that only *R. felis* carried three PLP genes (*pat1*), while other species in the same genus had only single in their genomes. It was suggested that the *Rickettsial* PLPs might play a role in the membranolytic activity of phospholipase A2 (PLA_2) that referred to hemolytic activity which help *Rickettsia* escaped from early phagosomal compartment (Blanc *et al*., 2005). In a component host cell, it has been proposed that the phospholipase A_2 activity in a concert reaction both stimulates the host cell to internalize the rickettsiae and provides a mean for that rickettsia to escape the phagosome and can be released into the cytoplasm (Winkler and Miller, 1980; Winkler, 1986). Furthermore phospholipase A in the destruction of the host cell membrane would be necessary for the exit of rickettsiae from the host cell since *R. prowazekii* has either a phospholipase A or the ability to activate a latent phospholipase A (Winkler and Daugherty, 1989).

The alignments of PLPs from plants and bacteria indicated the conserved amino acid residues that recognized as PLA_2 activity (Blanc *et al*., 2005). Bacterial PLPs were found to possess four conserved domains (Block I-IV). Block I consists of a glycine-rich region with a conserved arginine or lysine residue which probably serves as an oxyanion hole. Block II comprises the hydrolase motif, G-X-S-X-G, with the putative active-site serine. Block III contains a conserved serine, which may be an important structural element as a potential phosphorylation site or due to its capacity to form hydrogen bond. Block IV includes the putative active-site aspartate which is the catalytic dyad (Benerji and Flieger, 2004). Phylogenetic analysis revealed that *R. felis* lived in the same clade of *Rickettsia* and different from *Ehrlichia* species (order Rickettsiaceae) that have single PLP gene (Blanc *et al*., 2005). PLP gene in pathogens or symbionts therefore, can help pathogens lived in host cells.

5.2 Gene involved in drug resistance

Ehrlichioses and anaplasmoses are emerging infectious disease that can cause severe diseases and they are major medical concern. Clinical data on the treatment of these diseases are limited. *Ehrlichia* spp. and *Anaplasma* spp. were considered highly resistant organisms. Only two drugs, doxycycline and rifampin, are effective in treatment (Brouqui and Raoult, 1992).

Oxytetracycline inhibits the synthesis of bacterial protein by impeding the growth of polypeptide chains through prevention of aminoacyl tRNA to 30S ribosomal subunit. As *Anaplasma phagocytophilum* is sensitive to oxytetracycline, its effect on the ability of *A. phagocytophilum* to inhibit Phagosome-Lysosome (P-L) fusion was investigated (Gokce *et al*., 1999). *In vitro* test of antibiotic susceptibility of the organisms are based on microscopic counting of morulae in cells and tissue cultures before and after exposure to serial dilution of antibiotics. These tests are not standardized, not sensitive, time consuming and not well adapted for the screening of new drugs or strains of organisms (Bradley *et al*., 1996).

A gene in *E. canis* strain Jake that involved in drug resistance composed of acriflavin resistance protein, putative ABC-transport system involved in resistance to organic solvent auxillary component, Macrolide-specific ABC-type efflux carrier (MacAB), major facilitator family transporter, ABC transporter (transmembrane region) and bicyclomycin resistance gene. Bicyclomycin resistance (*Bcr*) gene, the drug resistance transporter Bcr/CflA subfamily appeared in the genome (Mavromatis *et al*., 2006). Bicyclomycin is an antibiotic against a broad spectrum of Gram-negative and Gram-positive bacteria. The target of bicyclomycin is *rho* transcription terminator factor. The rho protein is important for termination of many gene products in Gram-negative bacteria. Without rho the cell losses viability (Kohn and Widger, 2005). Antibiotic resistance is a concern for the management of disease in animals. To study and identify antibiotic resistance genes from uncultured or difficult to culture bacteria such as *Ehrlichia* spp. are problematical worked since culture of *Ehrlichia* is very limited (Forbes *et al*., 1998). However, PCR technique can be used to amplify the

target gene (Riesenfeld *et al*., 2004). The identifying antibiotic resistance gene from uncultured or difficult to culture bacteria may contribute to the search for new antibiotics in two ways. Firstly, study of the genetic diversity, enzymology and structure of bacterial antibiotic resistance protein may ultimately lead to the design of compound that inhibits resistance mechanisms thus, extending the useful lifetime of currently available antibiotics. Secondly, gene for antibiotic resistance are often clustered with genes for antibiotic biosynthesis, therefore, study of antibiotic resistance gene may lead to the discovery of biosynthetic pathways encoding potentially novel antibiotics (Anderson *et al*., 2002).

In *E. canis* strain Jake genome showed gene involed in drug resistance a protein with transmembrane helices, Bcr, which associated with drug resistance transporter (Mavromatis *et al*., 2006). Transmembrane (TM) proteins are integral proteins which span the lipid bilayer with portions exposed on both sides of the membrane. Most TM proteins span the membrane by α-helical regions (Cooper and Hauaman, 2007). More than 60 percent of TM proteins in cells are with putative or unknown function. But TM proteins play extremely important life function of the cells. Their functions are closely correlated to their TM topology (Krogh *et al*., 2001; Arai *et al*., 2003). They play a role as pump, channels, receptors and energy transducers and play a role as pharmaceutical targets in the biomedical field (Arai *et al*., 2003). The drug resistance transporter Bcr/CflA proteins are predicted to have 12 membrane-spanning regions and are known as transmembrane protein efflux systems belonging to the class of the major facilitator superfamily (MFS) (Paulsen *et al*., 1996; Putman *et al*., 2000; Paulsen, 2003). Members with known activity include Bcr from *E. coli* (Bentley *et al*., 1993), Blt from *Bacillus subtitlis* (Ahmed *et al*., 1995) and Nor A from *Staphylococcus aureus* (Yoshida *et al*., 1990).

However, membrane proteins are difficult to work with experimentally and very few structures have been demonstrated to date. TM proteins difficulties to express and crystallize because of their amphipathic character (Cooper and Hausman, 2007). Alternative approach to studying these proteins is to use computational method which, has been focused of much research in recent years. Consequently, reliable

techniques for identifying and analyzing TM protein have the potential to accelerate drug discovery efforts. TM topology prediction may be used as a basis for hypothesis to study the function of TM (Wiles *et al*., 2006). TM protein topology prediction approached can be divided into three classes. Firstly, methods that use physiochemical properties, such as hydrophobicity or charge (Hirokawa *et al*., 1998; Jayasinghe *et al*., 2001; Juretic *et al*., 2002). Secondly, model-based methods, such as hidden Markov models (HMMs) (Krogh *et al*., 2001) and neural networks (Rost *et al*., 1996). Finally, methods based on the consensus approach (Arai *et al*., 2004). All three methods are designed to identify potential TM α-helices and to predict the overall in and out topology of the protein in TM.

In Thailand, there are limited information of *Ehrlichia* spp in dogs and cats on the prevalence, molecular characterization, genetic variation, pathogenicity and drug resistance activity. Canine and feline ehrlichiosis could be considered as a serious problem for tick-transmitted infection agents. This study will increase information about the distribution and pathogenicity of ehrlichiosis in dogs and cats in Thailand by using molecular techniques. It will also help to verify coinfection events that routinely occurred during detection. Some genes that likely to be involved in pathogenecity and drug resistance in *Ehrlichia* will be cloned and characterized. The knowledge obtained in this study will be very useful for ehrlichiosis researches of dogs and cats in Thailand.

MATERIALS AND METHODS

1. Blood samples of dogs and cats

Blood samples of dogs and cats using in this study were obtained from Assoc. Prof. Dr. Sathaporn Jittapalapong, Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University. A total of fifty-six dog blood samples were collected from dog diagnosed with clinical ehrlichiosis from Veterinary Diagnostic Laboratory (VDL) center, Bangkok. Sixty-seven cat blood samples were collected from stray cats living in temples around Bangkok.

One milliliter of whole blood was kept in a tube and stored at -20 °C.

2. Microscopic examination

Place a drop of blood from either dog or cat on one side of the slide and spread the drop by using another slide (spreader) to smear the blood. Blood smears should be air-dried, and then dipped into 100% methanol. After one minute, the slides are removed and air-dried. The slide is immersed into 10% Giemsa stain solution for 5-10 minutes, then flushed with tap water and left dry. *Ehrlichia* spp. in cytoplasm of leukocyte was detected on slide by using light microscope at 100 x magnification.

3. Bacterial strains, vectors and culture conditions

Bacterial strains and vectors used in this study are shown in Table 1.

Methods for *E. coli* cultivation were obtained from Sambrook and Russell (2001).

E. coli was grown on LB agar at 37°C overnight or until colonies were clearly visible. Plates could then be stored at 4°C for a month. In liquid media, *E. coli* was grown in LB broth at 200-250 rpm, 37°C and 50 µg/ml amplicillin was used for maintenance of the recombinant strains.

E. coli were maintained for long period by transferring 0.5 ml of overnight growth bacteria to a cryogenic storage tube or microcentrifuge tube with equal volume of sterilized 40% (w/v) glycerol. Then mixed well and stored at -80 °C.

Table 1 Sources of bacterial strains and vectors used in this study

Strain or plasmid	Relevant characteristics	Source/reference
E. coli BL21 (DE3)	F $^-$, *ompT*, *hsdS*$_B$ ($r_B^- m_B^-$), *gal*, *dcm* (DE3)	Novagen
E. coli DH5α	F $^-$ φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk$^-$, mk$^+$), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	Takara
pGEM-T Easy	3'- oligothymine overhanged linear vector, *lacZ*, f1*ori*, Amp^r (3,018 bp)	Promega
pET-15b	T7*lac* N-His•Tag Thrombin Amp^r (5,708 bp)	Novagen
pGEM-T/*bcr*	1.1-kb *Nde*I-*Bam*HI *bcr* gene in pGEM-T Easy	This study
pGEM-T/*pat*	1.0-kb *Nde*I-*Bam*HI *pat* gene in pGEM-T Easy	This study
pET-15b/*bcr*	1.1-kb N-His$_6$*Nde*I-*Bam*HI *bcr* gene in pET-15b	This study
pET-15b/*pat*	1.0-kb N-His$_6$*Nde*I-*Bam*HI *pat* gene in pET-15b	This study

4. DNA and plasmid manipulation

4.1 DNA extraction from whole blood samples

DNA was extracted from 100 µl of whole blood according to the method of Sambrook and Russell (2001). Blood was mixed with 500 µl denature solution (D solution: 4 M guinidinium thiocyanate, 25 mM sodium citrate, pH 7.0 and 0.5% N-lauryl sarcosine), and vortex for 5 to 10 minutes. At this stage cell membrane and protein components were lysed with denaturing solution. Then, 200-300 µl of phenol: chloroform (1:1) was added and mixed by vortex. The sample was centrifuged at 13,000 rpm for 5 minutes and 400-600 µl of the supernatant was transferred to a clean tube. This stage should be repeated for a few times to get rid of proteins as much as possible. After that, DNA was precipitated by adding 2 volumes of absolute ethanol and solution was gently mixed up side down and kept at -80 ºC for 30 minutes. DNA

was precipitated by centrifugation at 13,000 rpm for 15 minutes and washed twice by adding 1 ml of 75% ethanol. DNA pellet was air dried and dissolved in Tris-EDTA (TE) buffer (50 mM Tris, pH 8.0, 100 mM EDTA) and kept at -20ºC until used.

4.2 Isolation of plasmid DNA by alkaline lysis

Plasmid isolation using the alkaline lysis method described by Sambrook and Russell (2001). Plasmid-containing *E. coli* was grown in 3-5 ml of LB broth with 50 µg/ml ampicillin overnight at 250 rpm, 37°C. The cells were harvested by centrifugation at 12,000 rpm and resuspended in ice-cold 100 µl of Solution I (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0). The sample was mixed and incubated on ice for 10-15 min. The bacteria were lysed by adding 200 µl of freshly prepared Solution II [0.2 M NaOH and 1% (w/v) SDS] and mixed by vortex and incubated for 5 min. Then, 150 µl of ice-cold Solution III was added and inverted several times before centrifugation at 12,000 rpm for 5 min. The supernatant was transferred to the new microcentrifuge tube and equal volume of phenol:chloroform:isoamyl alchol (25:24:1) was added. The solution was then mixed by inversion of the tube for 1 min before centrifugation. The top layer of the supernatant was transferred to a fresh tube and two volume of absolute ethanol was added. After final centrifugation for 10 min, the supernatant was removed and the DNA pellet was allowed to air-dry at room temperature. The DNA pellet was suspended in 20-50 µl of TE containing 20 µg/ml RNaseA and kept at 20°C until used.

4.3 Isolation and purification of plasmid DNA by commercial kit

The reaction conditions were set up according to manufacture instructions from GeneJET^{TM} Plasmid Miniprep Kit (Fermentas).

Picked a single colony of *E. coli* from a freshly streaked selective plate to inoculate in 1-5 ml LB medium supplemented with 50 µg/ml ampicillin and incubated for 12-16 h at 200-250 rpm, 37°C. Bacterial culture was then harvested by

centrifugation at 8000 rpm for 2 min at room temperature. The pellet was resuspended in 250 µl of the resuspension solution and was added 250 µl of the lysis solution and mixed thoroughly by inverting the tube 4-6 times until the solution become viscous and clear. After that, 350 µl of the neutralization solution was added and mixed immediately by inverting the tube 4-6 times and then centrifuged for 5 min at room temperature. The supernatant was transferred to the supplied GeneJET^{TM} spin column and centrifuged for 1 min. The flow-through was discarded and the column was placed back. The washing step was repeated twice by adding 500 µl of the wash solution to the GeneJET^{TM} spin column and centrifuged for 30-60 sec and then discarded the flow-through and placed the column back into a fresh microcentrifuge tube. The GeneJET^{TM} spin column was then transferred into a fresh microcentrifuge tube. 50 µl of the elution buffer was added to the center of GeneJET^{TM} spin column membrane and incubated for 2 min and then centrifuged for 2 min at room temperature. The purified plasmid was stored at -20°C until used.

4.4 Purification of PCR products

The reaction conditions were set up according to manufacture instruction from QIAquick PCR purification Kit (QIAGEN).

Five volumes of buffer PB was mixed with 1 volume of the PCR sample and applied to the QIAquick column and centrifuged for 30-60 sec. The flow-through was discarded and the QIAquick column was placed back to the same tube. Then 0.75 ml buffer PE was added into the QIAquick column and centrifuged for 30-60 s. After that, discarded the flow-through and placed the QIAquick column back in the same tube. The column was then centrifuged for an additional 1 min at 13,000 rpm and then placed in a clean microcentrifuge tube. 30 µl of buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O was added into the center of the QIAquick membrane and left for 1 min. DNA was eluted by spining the column for 1 min at 13,000 rpm. DNA was kept at -20 ^{o}C until used.

4.5 Recovery of DNA fragment from agarose gel

The reaction conditions were set up according to manufacture instructions from QIAquick Gel extraction Kit (QIAGEN).

The DNA fragment from the agarose gel was excised by a clean, sharp scalpel. The gel slice was weigh and 3 volumes of buffer QG was added to 1 volume of gel (100 mg ~ 100 µl) and incubated at 50°C for 10 min (or until the gel slice has completely dissolved). After that the solution was applied to the QIAquick column, and centrifuged at 13,000 rpm for 1 min. Discarded the flow-through and placed QIAquick column back in the same collection tube. Added 0.75 ml of buffer PE to QIAquick column and centrifuged at 13,000 rpm for 1 min. Discarded the flow-through and centrifuged the QIAquick column for an additional 1 min at 13,000 rpm and placed QIAquick column in a clean microcentrifuge tube. The DNA was eluted by adding 30 µl of buffer EB (10 mM Tris·Cl, pH 8.5) or deionized water to the center of the QIAquick membrane, let the column stand for 1 min and centrifuged the column for 1 min at 13,000 rpm. DNA was kept at -20 ^{o}C until used.

5. Molecular techniques for gene cloning

5.1 Restriction enzyme digestion

The reactions were set up according to instructions provided by restriction enzyme manufacturers. Typically, digestion conditions consisted of 200 ng-0.5 µg DNA and 10 unit/µl restriction endonuclease, 2xbuffer, which were made up to a total volume of 20 µl with deionized water. The reaction mixture was incubated at 37°C or at appropiate temperature for 1 h up to overnight.

When single restriction enzyme was required to cut the vector, calf intestinal alkaline phosphatase (CIAP) was used to prevent self ligation. After setting

the digestion, 1 µl of CIAP (1 unit/ µl) was added and incubated further at 37°C for 1 h. The reactions with CIAP were terminated by incubation at 70°C for 20 min.

5.2 Ligation of DNA fragment

Digested DNA fragment was annealed with plasmid using T_4 DNA ligase to create recombinant plasmids. The quantity of the ligation mixture was calculated according to equation below. The insert:vector ratio at 2:1 when performing cohesive end ligation and 1:1 when ligated blunt end.

$$\text{ng of insert} = \frac{\text{vector size (kb)}}{\text{insert size (kb)}} \times \text{insert:vector molar ratio} \times \text{ng of vector}$$

Standard ligation reactions consist of x ng of vector and insert 4 µl of 5xligation buffer and 1 unit/µl of T_4 DNA ligase in a final volume of 20 µl. The ligation mix was then incubated at room temperature for 2 h or at 16°C (or 4°C) overnight.

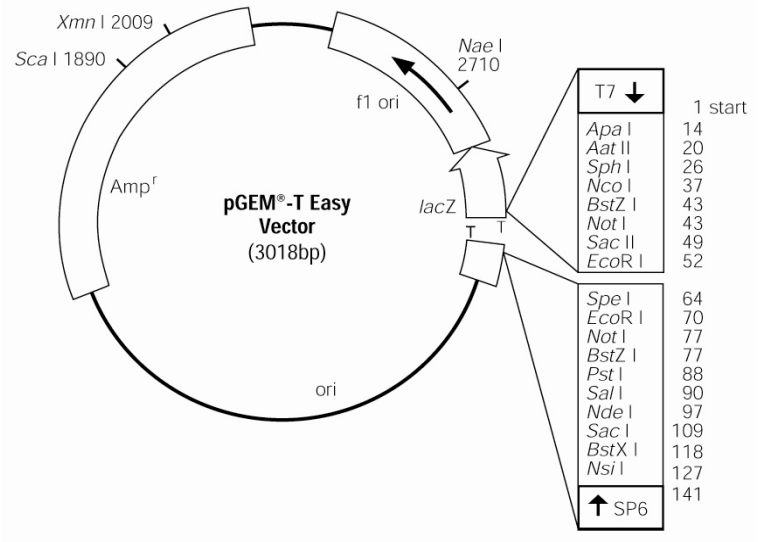
5.3 Agarose gel electrophoresis

The DNA samples were mixed with 6x loading dye at ratio 5:1 and run on 0.8% agarose gel in 1x TAE (50x TAE buffer: 48.4 g Tris-base, 10.9 g Glacial acetic acid, 2.92 g EDTA and Deionized water equal to 1 liter) or 1x TBE (50x TBE buffer: 54.0 g Tris-base, 27.5 g Boric acid, 2.92 g EDTA and Deionized water equal to 1 liter) at 100V for 30-40 min. The agarose gel was stained with 0.5 µg/ml ethidium bromide for 10 min and destained with tap water for further 5 min. The specific size of interested DNA was compared to GeneRuler^{TM} DNA ladder mix (Fermentas, USA) under UV illumination and then photographed.

5.4 PCR cloning in pGEM-T Easy

Ligation reaction of 20 µl was performed containing 50 ng (1 µl) of pGEM-T Easy vector (Figure 4), 250 ng of PCR product, 1x ligation buffer and 3 Weiss units/µl T_4 DNA ligase. The reaction mixture was incubated at 16°C for 16 h or 4°C overnight.

A



B

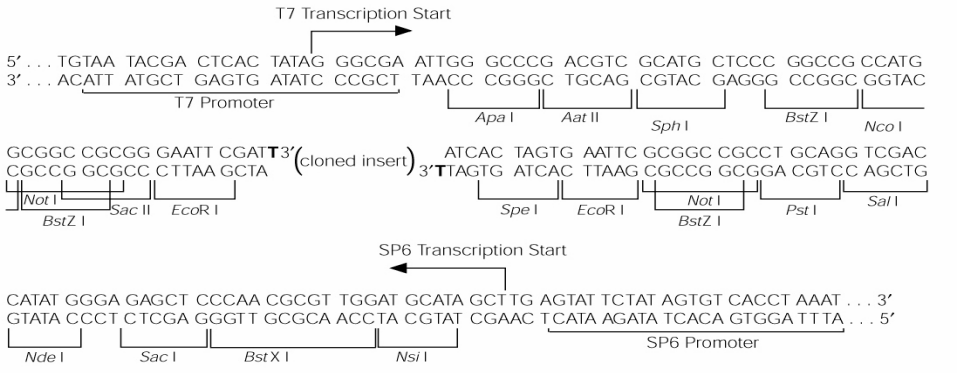


Figure 4 Map of pGEM-T Easy vector. (A) Circle map. (B) Region at promoters and multiple cloning sites.

Source: Promega, 1998.

5.5 Genetic manipulation in *E. coli*

5.5.1 Preparation of completent cell

Competent cells were prepared using the method described by Chung *et al*. (1989). 500 µl of an overnight culture of *E. coli* was added in 50 ml of LB broth and incubated at 250 rpm, 37°C until *E. coli* reached on early exponential phase (OD_{600} at 0.3-0.4). The culture was then transferred to 50 ml centrifuge tube and incubated on ice for 30 min. The cells were harvested by centrifugation at 4,000 rpm, 4 °C for 5 min and resuspended in 5 ml ice cold transformation and storage solution [TSS: LB broth containing 10% PEG (MW 8000), 5% (v/v) DMSO and 50 mM $MgCl_2$, pH 6.5)]. Cells were harvested again and resuspended in 2 ml ice-cold TSS. Cells were aliquot in 100 µl each tube and stored at -80°C or directly used to test the efficiency of transformation.

5.5.2 Introduction of plasmid DNA into *E. coli* by transformation

E. coli transformation procedure by heat-shock method was followed the procedure of Sambrook and Russell (2001).

100-250 ng of recombinant plasmid DNA was added to 100 µl of competent cells. The sample was gently mixed and incubated on ice for 20 min. The plasmid was then heat-shocked at 42°C for 90 sec and immediately transferred the tube on ice for 5 min. 900 µl of LB broth was added and the tube was incubated at 37°C for 1 h before plating on LB agar containing 100 µg/ml amplicillin. In the case of selection by the function of gene *lacZ,* 40 µl of 20 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in dimethyl formmamide (DMF) and 4 µl of 200 mg/ml IPTG (isoprppylthio-β-D-galactoside) were spread on the LB agar plate to select the recombinant clones. The clones were observed after overnight incubation at 37 °C.

6. Primer designs and polymerase chain reaction

6.1 Primer designs

Primers designed and used in this study are shown in table 2

Table 2 Primers for PCR reactions in this study

Gene	Primers	Nucleotide sequences	Ta (°C)
16S rRNA	ATT062F	5'CCTGGCTCAGAACGAACGCT3'	64
	ATT062R	5'GATCCAGCCGCAGGTTCACC3'	64
	ATT066F	5'CCCTGGTAGTCCACGCTG3'	64
	ATT067R	5'CAGCGTGGACTACCAGGG3'	64
pat	ATT068F	5'AGCTCATATGACTAAGTATGTGTTATCAG3' (*Nde*I)	56
	ATT068R	5'AGCTGGATCCTATAAATTCTTAATTGTATCATC3' (*Bam*HI)	56
bcr	ATT069F	5'AGCTCATATGTGTGATATGTCTTCTGATA3' (*Nde*I)	58
	ATT069R	5'AGCTGGATCCATAATAAGCTACCCTAAGTTCC3' (*Bam*HI)	58

6.1.1 Primers designed for *16S rRNA* amplification

Primers for amplification of *16S rRNA* genes of *Ehrlichia* and *Anaplasma* were designed from nucleotide sequences deposited in GenBank database (DQ342324, AF414873, AF414870, AF414869, AB211163, U23503, CR767821, U96436, AB196302 and AF318946). All of the sequences were aligned for the maximum homology by ClustalW version 8.1 (Thompson *et al*., 1994). Conserved regions were selected and specific oligonucleotide primers named ATT062F and ATT062R were derived (Table 2).

6.1.2 Primers design for *patatin* gene

Primers for *patatin* gene amplification were designed from genome sequence database of *E. canis* strain Jake (NC007354), namely ATT068F and ATT068R. *Nde*I and *Bam*HI restriction sites were added on the respective ATT068F and ATT068R for cloning purpose (Table 2).

6.1.3 Primers design for *bcr* gene

Primers for *bcr* gene amplification were designed from genome sequence database of *E. canis* strain Jake (NC007354), namely ATT069F and ATT069R. *Nde*I and *Bam*HI restriction sites were added on the respective ATT069F and ATT069R for cloning purpose (Table 2).

6.2 PCR reactions

DNA isolated from dog and cat blood samples were used as templates to amplify *16S rRNA*, *bcr* and *patatin* genes from *Ehrlichia* spp. by PCR. To find good conditions for PCR amplification gradient PCR were used. 3-6 µl of genomic DNA extracted from dogs and cats blood samples was used as template for each 20 µl PCR reaction mixture (3-6 µl DNA template, 10x PCR buffer, 50 mmM $MgCl_2$, 10 mM dNTPs, 10 pmol forward and reverse primers, 5 U taq DNA polymerase and deionized water equal to 20 µl) in a Peltier thermal cycler (MJ Research, Watertown, MA, USA). The PCR reaction was performed by preceeding at 94^{o}C for 4 min, 30 cycles of 30 s at 94 ^{o}C, 30 s at 55-65 ^{o}C for *16S rRNA* (50-60 ^{o}C for *pat* and 50 – 60 ^{o}C for *bcr*), 1 min at 72^{o}C; and followed by 4 min at 72 ^{o}C.

7. DNA sequencing and phylogenetic analysis

7.1 DNA sequencing

PCR products or recombinant plasmids were purified by QIAquick PCR purification kits (QIAGEN) according to the manufacturer's protocol. DNA sequencing in this study was submitted to Bio-Technology service Unit (BSU), National Science and Technology Development agency (NSTDA), Thailand and MACROGEN Inc., Korea.

7.2 Sequence analysis

The resulting sequences were subjected to Blast program analysis from NCBI homepage to identify the most similarity sequences with the gene sequence from other organisms.

Multiple sequence alignments were performed using the ClustalW version 1.8 (Thompson *et al*., 1994) or CLUSTALW version 1.83 XP (Forbes *et al*., 1998). The differences between nucleotide positions were confirmed by DnaSP version 4.10 (Rozas *et al*., 2003). Protein signal sequence peptide was predicted using SignalP (Bendtsen *et al*., 2004). In the case of Bcr, transmembrane protein topological structures were analyzed using ConPredII (Arai *et al*., 2004), HMMTOP (Tusnady and Simon, 2001), SOSUI (Hirokawa *et al*., 1998), TMHMM (Sonnhammer *et al*., 1998), and TMMOD (Kahsay *et al*., 2005). Motifs were predicted using program MAFFT (Katoh *et al*., 2002).

7.3 Phylogenetic tree analysis

Phylogenetic trees were inferred using neighbor-joining (NJ) analysis by MEGA software version 3.1 (Kumar *et al*., 2004) and software package PHYLIP 3.63. The distance matrix of nucleotide divergences was calculated according to Kimura's two-parameter model. A bootstrap re-sampling technique of 1,000 replications was performed to statistically support the reliabilities of the nodes on the trees.

8. Gene expression and analysis

8.1 Constructions of recombinant plasmids

The PCR products of the full-length patatin and bicyclomycin resistance genes were cloned into pGEM-T Easy and transformed into *E. coli* DH5α. The plasmids were extracted and digested with recombined *Nde*I and *Bam*HI. The inserted

fragment was determined by agarose gel electrophoresis. Then, the recombinant plasmids were sequenced to verify mutation occurred during PCR reaction. The right *pat* and *bcr* gene were then subcloned into pET-15b expression vector and transformed into *E. coli* BL21 (DE3) (Figure 5).

8.2 Induction of gene expression

8.2.1 Lactose concentration

Cells containing the recombinant plasmid were cultured and induced with lactose as described previously (Kim *et al*., 2007). In this experiment, the concentration of lactose was varied to a final concentration of 1.0, 2.0, 3.0 and 4.0 mM respectively**.** The culture was harvested after 4 h and overnight.

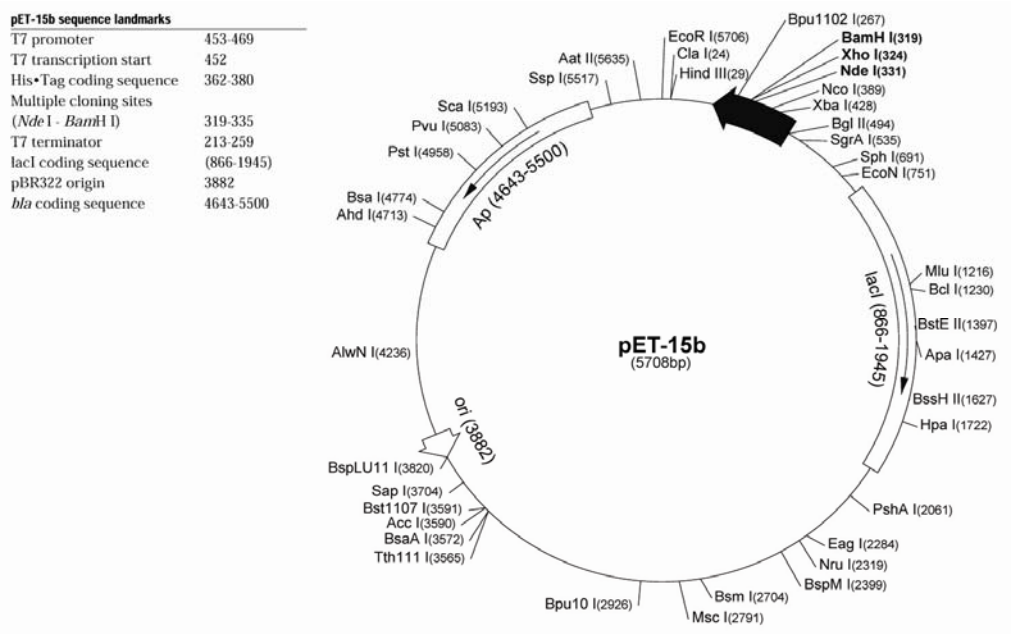
8.2.2 Temperature for expression

The recombinant cells were cultured and induced with lactose as described previously but the inducing temperature was varied. After induced with 1mM lactose, cells were cultured at 25, 30 and 37 °C respectively. The culture was harvested after 4 h and overnight.

8.2.3 Cell culture density

In this experiment, the absorbance of OD_{600} of cell cultures prior to adding lactose was varied. The cultures were induced with 1mM lactose when OD_{600} of culture reached at 0.4, 0.5, 0.6 and 1.0. The culture was harvested after 4 h and overnight.

A



B

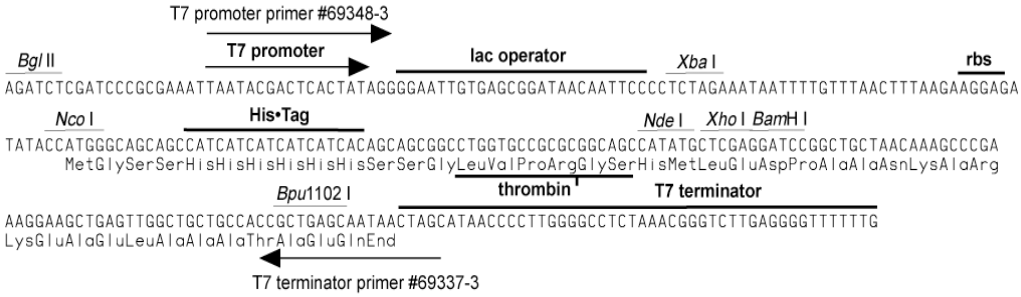


Figure 5 Map of pET-15b vector. (A) Circle map (B) Region at promoter, multiple cloning sites, and 6x histidine tag.

Source: Novagen, 1998.

8.3 SDS-PAGE analysis

SDS-PAGE was performed with 12 % polyacrylamide gel (Leammli, 1970) using Mini-Protein II (Bio-Rad, UK).

8.3.1 Preparation of crude proteins from pellet and supernatant

E. coli BL21 (DE3) harboring the desired plasmid was cultured at 37°C in 50 ml LB media containing 50 µg/ml ampicillin. When the OD_{600} reached about 0.4–0.6, 1 mM lactose was added into the culture. The culture was harvested after further incubation at 4 h and overnight. The cell pellet was resuspended in 1 ml 1X Laemmli buffer (Bio-Rad, UK) and stored at 4°C for further analysis. In the case of supernatant, the cell pellet was suspended in 1 ml of BugBuster® Protein Extraction Reagent (Novagen, Germany), shaken on agitator and centrifuged at 10,000 rpm for 20 min. The supernatant were collected and stored at 4°C for further analysis. The pellet (detection of inclusion bodies) was solubilized by adding 1 ml of BugBuster® buffer.

8.3.2 Sample preparation and protein loading

1X Laemmli buffer was mixed to each sample prior to boil at 100°C for 10 min and loaded on SDS-PAGE. 5 µl of PageRuler™ unstained protein ladder (Fermentas, USA) was used as protein marker. SDS-PAGE was carried out in a Bio-Rad minigel tank in 1X Tris-glycine buffer [(1% (w/v) SDS, 0.25 M tris-base and 1.92 M glycine)] at 30 mA. Protein bands were visualized by staining with protein gel staining solution [(0.3% (w/v) coomassie brilliant blue R-250 in 10% (v/v) acetic acid and 25% (v/v) methanol)] then destained in a protein gel destaining solution [(10% (v/v) acetic acid and 25% (v/v) methanol)].

8.3.3 Preparation of separating gel and stacking gel

The running gel containing 12% (w/v) acrylamide: bis-acrylamide (29:1) (Bio-Rad, UK), 400 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate (APS) and 0.1% (v/v) N, N, N', N'-tetramethyl-ethylene diamine (TEMED). Polymerization occurred under a layer of water-saturated isobutanol. After the separating gel was polymerized, the layer of water was removed. The stacking gel contained 4.2% (w/v) acrylamide: bis-acrylamide (29:1), 120 mM

Tris-HCl (pH 6.8), 0.1% (w/v) APS and 0.1% (v/v) TEMED. The gel was mounted in the vertical electrophoresis apparatus and 1X Tris-glycine buffer was added. The samples were loaded under 1X Tris-glycine buffer.

8.4 Protein purification

Supernatant was prepared as described in 8.3.1 and purified using HiTrap™ Affinity columns (Amersham Biosciences, UK). The sample was mixed with binding buffer (20 mM sodium phosphate, 0.5 M NaCl and 20 mM imidazole, pH 7.4). The syringe or pump tubing of the columns was filled with binding buffer. Subsequently, the stopper of the column was removed and connected to the syringe (with the provided adaptor). After removed the twist-off end and washed the column with 5-10 column volumes of binding buffer, the sample was applied using a syringe fitted to the adaptor or by pumping it onto the column. The column was then washed with at least 5 column volumes of binding buffer or until no material appears in the effluent. The protein was eluted with 5 column volumes of elution buffer (20 mM sodium phosphate, 0.5 M NaCl and 100-400 mM imidazole, pH 7.4). Each fraction was corrected and further analysed by SDS-PAGE.

8.5 Western blot

Proteins on SDS-PAGE were transferred to nitrocellulose membrane in transfer box using 1X transfer buffer [(25mM Tris-HCl, pH 8.3, 192 mM glycine and 20% (v/v) methanol)]. The blot was performed at 100 V, 150 mA for 1 h in cooling system place or at 30 V, 40 mA overnight. The nitrocellulose membrane was incubated in 10 ml blocking buffer [(5% skim milk in 1X TBST (Tris-buffer saline with Tween 20: 50 mm Tris-HCl (pH 8.0), 0.75 M NaCl and 0.25% (v/v) Tween-20)]. Gently agitated using a rocker platform for 1 h at room temperature (at this step the blot can keep in blocking solution at 4°C, overnight). After blocking, washed membrane twice in 20 ml TBST for 5 min with gentle agitation. The membrane was then transffered to a tray containing the AP (alkaline phosphatase)-conjugated antibody (1: 2000) in 10 ml dilution buffer and incubated with gentle agitator for at

least 2 h at room temperature or overnight. Transferred membrane to a tray containing 20 ml 1X TBST and washed three times for 5 min with gentle agitation. Transferred membrane to a tray containing TBS (TBST without Tween 20) and washed for 5 min.

Prepared fresh substrate solution [(500 µl of bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) in 4 ml deionizer water)] immediately before used and mix thoroughly. Added the substrate solution onto the membrane and incubated with agitation at room temperature until the purple color developed (10-30 min). The membrane was then washed in distilled water for 10 min and air-dried membrane on filter paper

9. Enzyme assay for lipolytic activity

Lipolytic activity of patatin-like phospholipase activity was determined by measuring the amount of *p*-nitrophenol product after lipolytic-catalyzed hydrolysis (Lee *et al*., 1993). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute from the *p*-nitrophenyl ester.

9.1 The optimal temperature

The optimal temperature for patatin-like phospholipase activity was determined by gently mixing 90 µl of substrate (1mM butyrate) (dissolved in 50 mM, 0.1% gum arabic and 0.4% Triton X) with 10 µl of recombinant protein. The reaction mixtures were then incubated at different temperatures ranging from 30°C to 60°C for 15 min. The mixtures were transferred to 96-well plate to measure the activity by spectrophotometer. Relative activity was calculated as enzymatic activity at indicated temperature divided by maximal activity at optimal temperature.

9.2 The optimal pH

The buffers used to study optimal pH were 50 mM sodium acetate (pH 3-5), 50 mM MES (pH 5-7), 50 mM HEPES (pH 7-8), and 50 mM Tris-HCl (pH 8-12).

The patatin-like phospholipase activity was measured by gently mixed 90 µl of substrate in different buffer (1mM substrate, 50 mM buffer, 0.1% gum arabic and 0.4% TritonX) with 10 µl of recombinant protein. The mixture was incubated on heating block at 40 °C for 15 min and transfered to 96-well plate to measure the activity by spectrophotometer. The production of *p*-nitrophenol from *p*-nitrophenyl butyrate was monitored at 348 nm.

RESULTS

1. Characterization of *Ehrlichia* sp. from dog blood samples

1.1 Microscopic examination

Microscopic examinations have been used in the detection of Giemsa-stained blood film. In this study, showed positive blood films many infected mononuclear cells with inclusion of *E. canis* in cytoplasm of monocyte (Figure 6). Microscopic examination should be performed in a case of high level of parasitemia. This method has been applied to routine use in other blood parasite.

A **B**

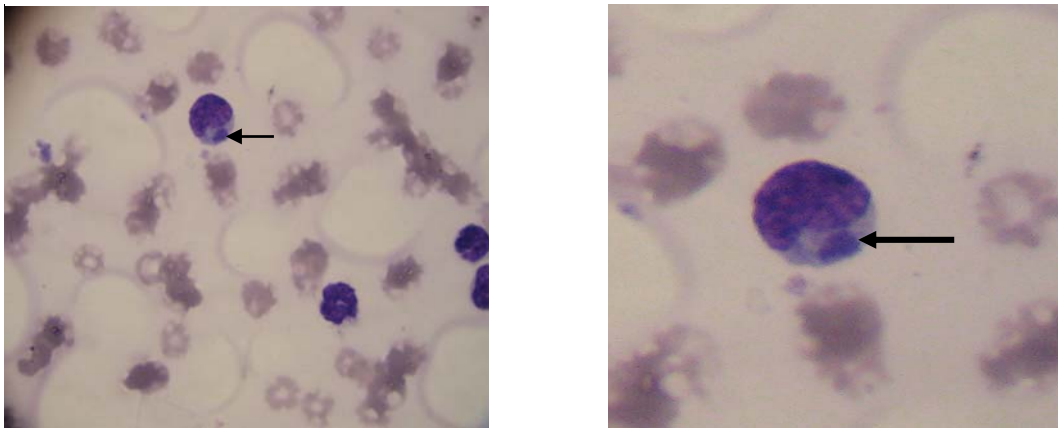


Figure 6 Mononuclear cells with *E. canis* inclusion. A and B are positive blood smear taken from dog affected with ehrlichiosis shows an *E.canis* morulae (arrow) within the cytoplasm of a monocyte (100X).

1.2 Amplification and sequencing of *16S rRNA*

To identify the agents associated with diagnosed canine monocytic ehrlichiosis and canine cyclic thrombocytopenia, it is often based on clinical diagnosis and microscopic examination of peripheral blood. Blood samples from dogs

diagnosed with clinical ehrlichiosis were screened to compare these agents to previously reported strains. Templates were prepared and assayed by PCR with primers ATT062F and ATT062R, which were designed for specific amplification of *Ehrlichia* and *Anaplasma 16S rRNA* gene.

Since the concentration of *Ehrlichia* DNAs in blood samples may vary, 1-12 µl total DNA extracted from 56 dog blood samples were used as templates for PCR reaction. The results showed that all of samples exhibited positive PCR product with different concentration of DNA template. The results showed that DNA template between 3-9 µl could reveal mostly positive PCR products. It is advised to use different number of total DNAs from 1-12 µl when blood samples were extracted to identify *Ehrlichia* spp. by PCR (Table 3).

Approximately 1.5 kb amplicons corresponding to the expected size of targeted 16S *rRNA* gene fragments were obtained (Figure 7). Four randomly selected amplicons from the individual canine blood samples (D1, D9, D27 and D50) were purified and directly sequenced with the same primers. The nearly complete *16S rRNA* gene sequences were obtained. Three of them were identical to consensus *16S rRNA* sequences for *E. canis* and the other was *A. platys* when compared to GenBank database. Two sequences were named as *E. canis*-Bangkok [1,478 bp (51% AT)] and *A. platys*-Bangkok [1,481 bp (52% AT)] and deposited as new *16S rRNA* sequences in GenBank database under accession numbers EF139458 for *E. canis*-Bangkok and EF139459 for *A. platys*-Bangkok (Figure 8A and B).

Table 3 Variation of DNA templates for PCR of *16S rRNA* gene

Sample	DNA template (µl)				
	1	**3**	**6**	**9**	**12**
D1	-	-	+	ND	ND
D2	+	ND	ND	ND	ND
D3	-	-	-	-	+
D4	+	ND	ND	ND	ND
D5	-	-	-	+	ND
D6	-	-	-	+	ND
D7	-	+	ND	ND	ND
D8	-	+	ND	ND	ND
D9	+	ND	ND	ND	ND
D10	-	-	+	ND	ND
D11	-	+	ND	ND	ND
D12	-	+	ND	ND	ND
D13	-	-	-	-	+
D14	-	-	+	ND	ND
D15	-	-	-	+	ND
D16	-	-	-	-	+
D17	-	-	+	ND	ND
D18	-	-	-	+	ND
D19	-	-	-	+	ND
D20	-	-	-	+	ND
D21	-	-	-	+	ND
D22	-	-	+	ND	ND
D23	-	-	-	-	+
D24	-	-	-	-	+
D25	+	ND	ND	ND	ND
D26	-	-	+	ND	ND
D27	-	-	-	-	+
D28	-	-	+	ND	ND
D29	-	-	+	ND	ND
D30	-	+	ND	ND	ND
D31	-	+	ND	ND	ND
D32	-	-	+	ND	ND
D33	-	+	ND	ND	ND
D34	-	-	-	-	+
D35	-	-	-	+	ND
D36	-	-	+	ND	ND
D37	-	-	+	ND	ND
D38	-	-	+	ND	ND
D39	-	-	-	+	ND
D40	-	-	+	ND	ND
D41	+	ND	ND	ND	ND
D42	-	+	ND	ND	ND
D43	-	+	ND	ND	ND
D44	-	+	ND	ND	ND
D45	-	+	ND	ND	ND
D46	-	-	+	ND	ND
D47	-	-	-	+	ND
D48	-	-	-	+	ND
D49	-	-	-	-	+
D50	+	ND	ND	ND	ND
D51	-	+	ND	ND	ND
D52	-	-	+	ND	ND
D53	+	ND	ND	ND	ND
D54	-	-	-	+	ND
D55	-	+	ND	ND	ND
D56	+	ND	ND	ND	ND

\+ = positive PCR product

ND = Not Determined

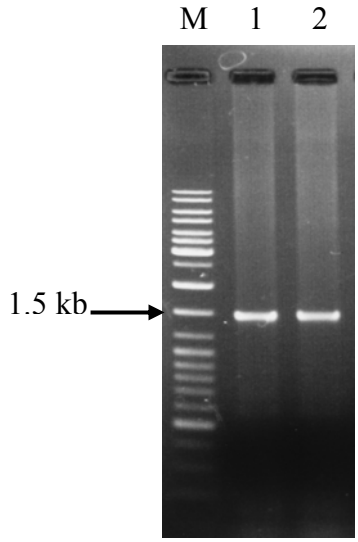


Figure 7 PCR products of 16S *rRNA* gene from *E. canis* and *A. platys*. Lane M, DNA ladder mix (Fermentas, USA); Lane 1 and 2, PCR amplicons of *E. canis*-Bangkok and *A. platys*-Bangkok, respectively.

A

```
   1 tcctggctca gaacgaacgc tggcggcaag cctaacacat gcaagtcgaa cggacaatta
  61 tttatagcct ctggctatag gaaattgtta gtggcagacg ggtgagtaat gcgtaggaat
 121 ctacctagta gtacggaata gccattagaa atggtgggta atactgtata atccccgagg
 181 gggaaagatt tatcgctatt agatgagcct acgttagatt agctagttgg tgaggtaatg
 241 gcttaccaag gctatgatct atagctggtc tgagaggacg atcagccaca ctggaactga
 301 gatacggtcc agactcctac gggaggcagc agtggggaat attggacaat gggcgaaagc
 361 ctgatccagc tatgccgcgt gagtgaagaa ggccttcggg ttgtaaaact ctttcaatag
 421 ggaagataat gacggtacct atagaagaag tcccggcaaa ctctgtgcca gcagccgcgg
 481 taatacggag ggggcaagcg ttgttcggaa ttattgggcg taaagggcac gtaggtggac
 541 tagtaagtta aaagtgaaat accaaagctt aactttggag cggcttttaa tactgctaga
 601 ctagaggtcg aaagaggata gcggaattcc tagtgtagag gtgaaattcg tagatattag
 661 gaggaacacc agtggcgaag gcggctatct ggttcgatac tgacactgag gtgcgaaagc
 721 gtggggagca aacaggatta gataccctgg tagtccacgc tgtaaacgat gagtgctaaa
 781 tgtgaggatt ttatctttgt attgtagcta acgcgttaag cactccgcct ggggactacg
 841 gtcgcaagac taaaactcaa aggaattgac ggggacccgc acaagcggtg gagcatgtgg
 901 tttaattcga tgctacgcga aaaaccttac cacttttga catgaaggtc gtatccctcc
 961 taacaggggg agtcagttcg gctggacctt acacaggtgc tgcatggttg tcgtcagctc
1021 gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa ccctcattct tagttaccaa
1081 caggtaatgc tgggcactct aaggaaactg ccagtgataa actggaggaa ggtggggatg
1141 atgtcaaatc agcacggccc ttatagggtg ggctacacac gtgctacaat ggcaactaca
1201 ataggttgcg agaccgcaag gtttagctaa tccataaaag ttgtctcagt tcggattgtt
1261 ctctgaaact cgagagcatg aagtcggaat cgctagtaat cgtggatcat cacgccacgg
1321 tgaatacgtt ctcgggtctt gtacacactg cccgtcacgc catgggaatt ggcttaactc
1381 gaagctggtg tgctaaccgc aaggaagcag ccatttaagg ttgggttagt gactagggtg
1441 aagtcgtaac aaggtagctg taggtgaacc tgcggctgga t
```

Figure 8 Nucleotide sequences of *16S rRNA* genes. (A) *E. canis*-Bangkok (EF139458); (B) *A. platys*-Bangkok (EF139459).

B

```
   1 tcctggctca gaacgaacgc tggcggcaag cttaacacat gcaagtcgaa cggatttttg
  61 tcgtagcttg ctatgataaa aattagtggc agacgggtga gtaatgcata ggaatctacc
 121 tagtagtatg ggatagccac tagaaatggt gggtaatact gtataatccc tgcgggggaa
 181 agatttatcg ctattagatg agcctatgtt agattagcta gttggtaggg taaaggccta
 241 ccaaggcagt gatctatagc tggtctgaga ggatgatcag ccacactgga actgagatac
 301 ggtccagact cctacgggag gcagcagtgg ggaatattgg acaatgggcg caagcctgat
 361 ccagctatgc cgcgtgagtg aggaaggcct tagggttgta aaactctttc agtggggaag
 421 ataatgacgg tacccacaga agaagtcccg gcaaactccg tgccagcagc cgcggtaata
 481 cggagggggc aagcgttgtt cggaattatt gggcgtaaag ggcatgtagg cggttcggta
 541 agttaaaggt gaaatgccag ggcttaaccc tggagctgct tttaatactg ccagactcga
 601 gtccgggaga ggatagcgga attcctagtg tagaggtgaa attcgtagat attaggagga
 661 acaccagtgg cgaaggcggc tatctggtcc ggtactgacg ctgaggtgcg aaagcgtggg
 721 gagcaaacag gattagatac cctggtagtc cacgctgtaa acgatgagtg ctgaatgtgg
 781 ggacgttttg tctctgtgtt gtagctaacg cgttaagcac tccgcctggg gactacggtc
 841 gcaagactaa aactcaaagg aattgacggg gacccgcaca agcggtggag catgtggttt
 901 aattcgatgc aacgcgaaga accttaccac ttcttgacat ggagattaga tccttcttaa
 961 cggaagggcg cagttcggct ggatctcgca caggtgctgc atggctgtcg tcagctcgtg
1021 tcgtgagatg ttgggttaag tcccgcaacg agcgtaaccc tcatccttag ttgccagcgg
1081 gttaagccgg gcactttaag gagactgcca gtggtaaact ggaggaaggt ggggatgatg
1141 tcaagtcagc acggccctta tggggtgggc tacacacgtg ctacaatggt gactacaata
1201 ggttgcaatg tcgcaaggct gagctaatcc gtaaaagtca tctcagttcg gattgtcctc
1261 tgcaactcga gggcatgaag tcggaatcgc tagtaatcgt ggatcagcat gccacggtga
1321 atacgttctc gggtcttgta cacactgccc gtcacgccat gggaattggc ttaactcgaa
1381 gctggtgcgc caaccgcaag gaggcagcca tttaaggttg ggtcagtgac tagggtgaag
1441 tcgtaacaag gtagctgtag gtgaacctgc ggctggat
```

Figure 8 (Continued)

1.3 Molecular characterization of *E. canis* from Thai dogs

E. canis-Bangkok *16S rRNA* sequence was compared to ten other *E. canis* strains reported from China, Japan, Peru, South Africa, Spain, Venezuela and USA, to confirm the identity of this Thai strain. All sequences were adjusted to the same length of 1,247 base pairs prior to alignment. *E. canis*-Bangkok *16S rRNA* was 100% identical to *E. canis*-VDE and *E. canis*-VHE strains from Venezuelan canine and human hosts, respectively (Unver *et al*., 2001a). The *E. canis* strain sequences showed very close identity ranging from 99.76% to 99.92%. The most polymorphisms were observed between *E. canis*-Bangkok and Lima strains. Four different *16S rRNA* sequence patterns were found among the 11 *E. canis* strains, with polymorphisms at 13 positions that included eight substitutions, one insertion and four deletions (Table 4). Substitutions consisted of six transitions and two transversions. Compared to *E. canis*-Bangkok, (1) Germishuys, Jake and Kagoshima1 strains showed single nucleotide differences that were a deletion, an insertion and a substitution, respectively; (2) Gzh982, Oklahoma and Madrid strains had two positions with

polymorphisms (one deletion and one substitution at different positions); and (3) Florida and Lima strains both had three polymorphic sites, Florida with one deletion and two substitutions while Lima had three substitutions.

1.4 Molecular characterization of *A. platys* from Thai dogs

The same corresponding *16S rRNA* sequences of *A. platys*-Bangkok and eight other *A. platys* strains reported from China, France, Japan, Spain, Thailand, Venezuela and USA were aligned. *A. platys*-Bangkok was 100% identical to those from France and Okinawa, but was different from *A. platys* previously isolated from Thailand (Suksawat *et al.*, 2001a). Other closely related sequences of *A. platys* strains showed 99.60% to 99.92% sequence identity. Five sequence patterns were found among the *16S rRNA* sequence alignment of nine *A. platys* strains, with polymorphisms at 13 positions, seven of which were substitutions, three were insertions and three were deletions (Table 5). Substitutions consisted of four transitions and three transversions. Compared to *A. platys*-Bangkok, (1) Okinawa1 and Spain strains had single nucleotide additions at different positions; (2) Thailand and Venezuela strains had two nucleotide substitutions at different positions; (3) Gzh981 had two insertions and one deletion; and (4) the most polymorphisms were between Bangkok and USA strains, which were three substitutions and two deletions.

1.5 *16S rRNA* secondary structures

16S rRNA is not subject to the same selective influences on function as mRNA (i.e., *16S rR*NA function relies on structure rather than codon usage), thus the effects of nucleotide changes on predicted secondary structures could be more informative than primary sequence variation alone. The positions of *16S rRNA* sequences defined in Tables 4 and 5 were correlated with the *E. coli* J01695 numbering system (Konings and Gutell, 1995). Comparison of *E. canis*-Bangkok and *A. platys*-Bangkok *16S rRNA* predicted secondary structures to that of the *E. coli* J01695 indicated that both had conserved tetra loops that generally constrained the *rRNA* architecture (Woese *et al*., 1990). Nucleotides at positions 289, 452, 594, 888,

915, 948 and 1,200 of *E. canis*-Bangkok were common among bacteria while positions 133, 685, 783, 810, 817 and 1,174 were different from other eubacteria. At position 948, T (U) in most samples except *E. canis*-Lima that carried C, the latter of which is similar to those of alphaproteobacteria (Woese, 1987). In *A. platys*-Bangkok, nucleotide differences at positions 181, 678, 871 and 1,025 were within the common structure of eubacteria. At position 393, eubacteria generally carried A (Woese, 1987), but most of *A. platys* had a single deletion except for *A. platys*-Okinawa1 and *A. platys*-Gzh981 that carried a C. At position 1,233, T was observed in most samples except for *A. platys* USA, which had a G that is similar to those of alphaproteobacteria (Woese, 1987). Genetic polymorphisms from comparison of *16S rRNA* sequences based on the *E. coli* J01695 numbering system (Konings and Gutell, 1995) also indicated that *E. canis*-Bangkok and *A. platys*-Bangkok were structurally conserved in *16S rRNA* architecture. Therefore, all polymorphisms observed in these experiments are consistent with these two species. However, although nucleotide differences at many positions indicated that *E. canis*-Bangkok and *A. platys*-Bangkok shared some structure with other bacteria, other nucleotides were different from most eubacteria.

Table 4 Comparison of *E. canis*-Bangkok *16S rRNA* sequence to geographically dispersed *E. canis* strains

***E. canis* strain**	**GenBank accession number**	**Identity (%)[a]**	**Nucleotide differences at position[b]**												
			133	**289**	**452**	**594**	**685**	**783**	**810**	**817**	**888**	**915**	**948**	**1174**	**1200**
Bangkok	EF139458	100	**G**	**C**	**A**	**A**	**A**	**A**	**—**	**G**	**C**	**A**	**T**	**C**	**C**
VDE	AF373613	100	●	●	●	●	●	●	—	●	●	●	●	●	●
VHE	AF373612	100	●	●	●	●	●	●	—	●	●	●	●	●	●
Germishuys	U54805	99.92	●	—	●	●	●	●	—	●	●	●	●	●	●
Jake	NC_007354	99.92	●	●	●	●	●	●	**A**	●	●	●	●	●	●
Kagoshima1	AF536827	99.92	●	●	●	●	●	●	—	●	●	**C**	●	●	●
Gzh982	AF162860	99.84	●	●	●	●	●	—	—	●	●	●	●	**T**	●
Oklahoma	M73221	99.84	**A**	●	●	●	●	●	—	—	●	●	●	●	●
Madrid	AY394465	99.84	●	●	—	●	**C**	●	—	●	●	●	●	●	●
Florida	M73226	99.76	**A**	●	●	●	●	●	—	—	●	●	●	●	**T**
Lima	DQ915970	99.76	●	●	●	**G**	●	●	—	●	**T**	●	**C**	●	●

[a]The values are percentage of nucleotide sequence identities for 1,247 bp determined from pairwise alignment.

[b]Positions based on the sequence of *E. coli* J01695 numbering system. The symbols ● and — indicate conserved nucleotide and deletion, respectively.

Table 5 Comparison of *A. platys*-Bangkok *16S rRNA* sequence to other *A. platys* strains.

***A. platys* strain**	**GenBank accession number**	**Identity (%)[a]**	**Nucleotide differences at position[b]**												
			152	**181**	**393**	**678**	**766**	**818**	**820**	**871**	**961**	**1025**	**1181**	**1192**	**1233**
Bangkok	EF139459	100	**T**	**A**	**—**	**G**	**—**	**C**	**G**	**—**	**G**	**G**	**A**	**T**	**T**
Sommieres	AF303467	100	●	●	**—**	●	**—**	●	●	**—**	●	●	●	●	●
Okinawa	AY077619	100	●	●	**—**	●	**—**	●	●	**—**	●	●	●	●	●
Okinawa1	AF536828	99.92	●	●	**C**	●	**—**	●	●	**—**	●	●	●	●	●
Spain	AY530806	99.92	●	●	**—**	●	**G**	●	●	**—**	●	●	●	●	●
Thailand	AF286699	99.84	●	●	**—**	●	**—**	●	●	**—**	●	**A**	●	**C**	●
Venezuela	AF287153	99.84	**C**	●	**—**	●	**—**	●	●	**—**	●	●	**G**	●	●
Gzh981	AF156784	99.76	●	●	**C**	●	**—**	●	●	**T**	**—**	●	●	●	●
USA	M82801	99.60	●	**—**	**—**	**T**	**—**	**—**	**C**	**—**	●	●	●	●	**G**

[a]The values are percentage of nucleotide sequence identities for 1,249 bp determined from pairwise alignment.

[b]Positions based on the sequence of *E. coli* J01695 numbering system. The symbols ● and — indicate conserved nucleotide and deletion, respectively.

1.6 Phylogenetic analysis of *Ehrlichia* and *Anaplasma*

Ehrlichia and *Anaplasma 16S rRNA* sequences were used to generate a phylogenetic tree using the neighbor-joining method by MEGA software (version 3.1). In addition to *E. canis* and *A. platys* strains, closely related species included the tick-borne anaplasmal parasites *E. chaffeensis*, *E. ewingii*, *E. muris*, *E. ruminantium*, *A. bovis*, *A. centrale*, *A. marginale*, *A. ovis* and *A. phagocytophilum* were compared. A biologically divergent member of the Anaplasmataceae, *N. sennetsu*, was used as the outgroup. The resultant phylogenetic tree revealed that *E. canis*-Bangkok and *A. platys*-Bangkok were grouped tightly within the other *E. canis* and *A. platys* strains, respectively (Figure 9). This analysis revealed that (1) *Ehrlichia* and *Anaplasma* were divided into clearly defined clades; (2) *E. canis* strains from different geographic regions were always grouped in a clade independent from *E. chaffeensis*, *E. ewingii*, *E. muris* and *E. ruminantium*; (3) *E. ewingii* showed the closest relationship to *E. canis* while *E. ruminantium* was the most distant; (4) *A. platys* strains from different countries constantly grouped in a clade independent from *A. bovis*, *A. centrale*, *A. marginale*, *A. ovis* and *A. phagocytophilum*; (5) *A. phagocytophilum* had the closest relationship to *A. platys* while *A. centrale*, *A. marginale* and *A. ovis* were the most distant; and (6) *A. marginale* clustered in a branch linked to *A. centrale* and *A. ovis*.

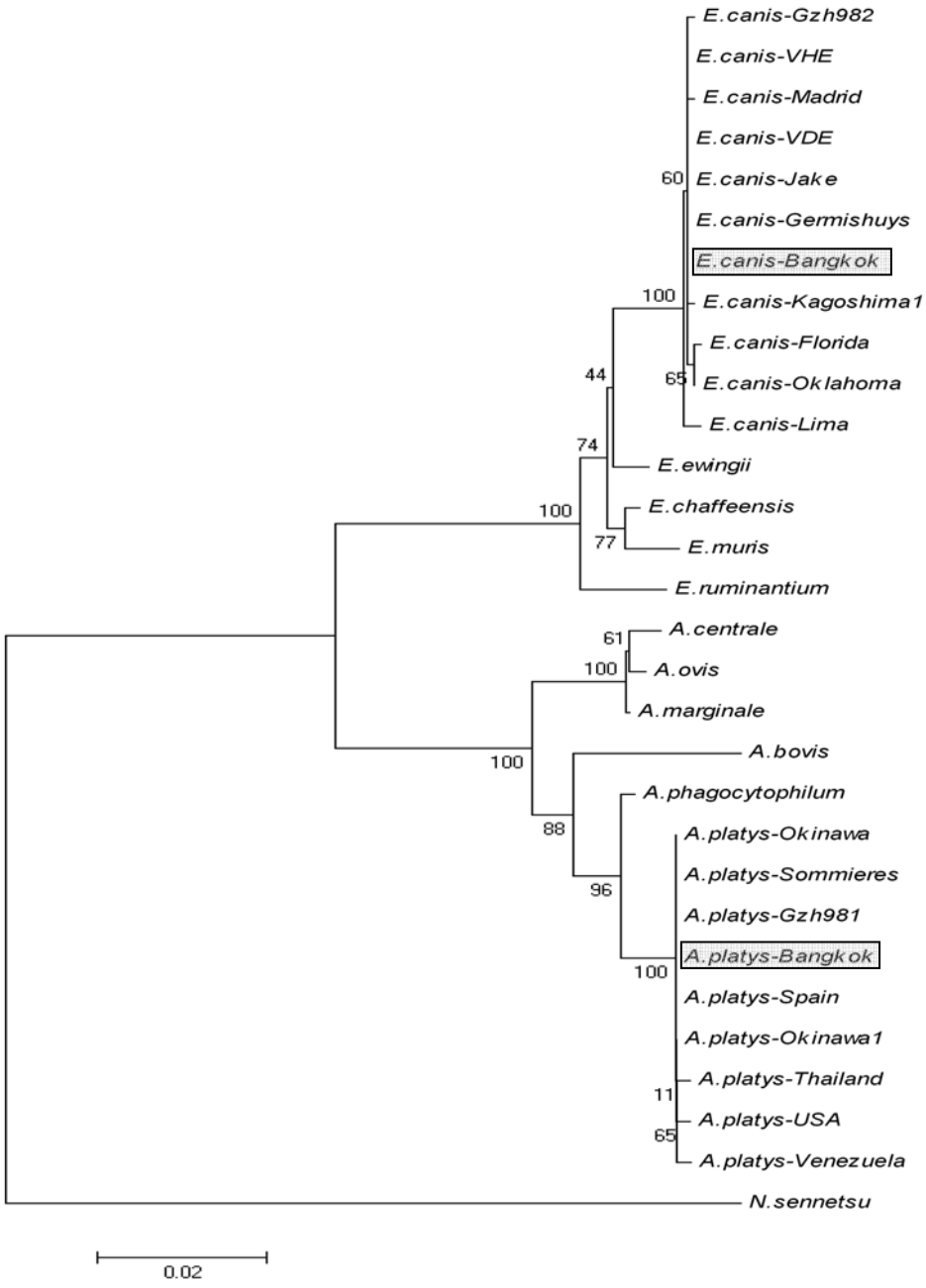


Figure 9 Phylogenetic tree based on *Ehrlichia* and *Anaplasma 16S rRNA* sequences. Sequences from the *Ehrlichia* and *Anaplasma* genera were compared using the neighbor-joining method with distance matrix calculation by Kimura-2 parameters, operated by MEGA software (version 3.1), using *N. sennetsu* as the outgroup. Scale bar indicates the number of mutations per sequence position. The numbers at the nodes represent the percentage of 1000 bootstrap re-samplings.

2. Characterization of Ricketsiales from cat blood samples

2.1 Amplification and sequencing of *16S rRNA* gene from cat blood samples

Twenty-four cat blood samples were randomly selected from sixty-seven samples from stray cats around Bangkok were then subjected to DNA extraction and subsequently amplified with primers ATT062F and ATT062R specific to *Ehrlichia* and *Anaplasma* previously used in dog blood samples (Table 2). Single band of approximately 1.5 kb PCR amplicons (Figure 10) were obtained from all of the DNA samples and were further sequenced.

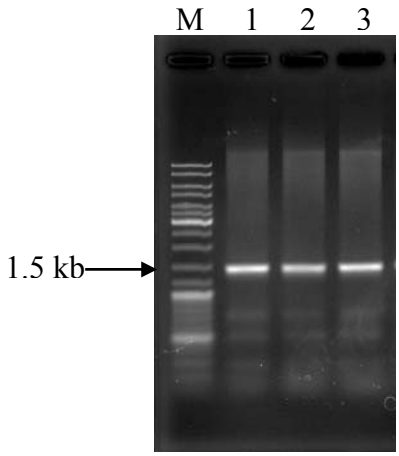


Figure 10 Samples of PCR amplicons of *16S rRNA* gene from cat blood samples. Lane M, GeneRuler^{TM} DNA ladder mix (Fermentas, USA) and Lane 1 to 3 PCR products of F1608, F1659 and F1693, respectively.

2.2 Characterization of *16S rRNA* gene amplified from cat blood samples

Most of the sequences of *16S rRNA* gene gave ambiguous results that may due to the mixture of non-specific PCR products. Only eight PCR products showed *16S rRNA* gene sequences when compared with GenBank database. F1608 sequence was similar to *16S rRNA* gene of *Bartonella*, two sequences similar to that of *Ochrobactrum* and five sequences similar to that of *Sphingomonas* spp. (Table 6).

Table 6 Cat blood samples and *16S rRNA* sequences analysis

Sample no.	Sequence results
F1519	ambiguous
F1521	ambiguous
F1593	ambiguous
F1596	ambiguous
F1599	ambiguous
F1602	ambiguous
F1606	ambiguous
F1607	ambiguous
F1608	*B. clarridgeiae*
F1621	ambiguous
F1623	*S. paucimobilis*
F1626	ambiguous
F1627	ambiguous
F1634	ambiguous
F1635	ambiguous
F1639	*S. paucimobilis*
F1641	ambiguous
F1644	*S. paucimobilis*
F1646	ambiguous
F1659	*O. intermedium*
F1684	*O. intermedium*
F1685	*S. paucimobilis*
F1693	*S. paucimobilis*
F1702	ambiguous

Three representative sequences were cloned, characterized which were F1608 [691 bp (48% AT)], F1659 [698 bp (45% AT)] and F1693 [656 bp (46% AT)] and used in further study (Figure 11).

A

```
  1 acatgcaagt cgagcgcact catttagagt gagcggcaga cgggtgagta acgcgtggga
 61 atctaccctt ttctacggaa taacacagag aaatttgtgc taataccgta tacgtcctac
121 tggagaaaga tttatcggag aaggatgagc ccgcgttgga ttagctagtt ggtgaggtaa
181 aggctcacca aggcgacgat ccatagctgg tctgagagga tgatcagcca cactgggact
241 gagacacggc ccagactcct acgggaggca gcagtgggga atattggaca atgggggcaa
301 ccctgatcca gccatgccgc gtgagtgatg aaggccctag ggttgtaaag ctctttcacc
361 ggtgaagata atgacggtaa ccggagaaga agccccggct aacttcgtgc cagcagccgc
421 ggtaatacga agggggctag cgttgttcgg atttactggg cgtaaagcgc atgtaggcgg
481 atatttaagt cagaggtgaa atcccagggc tcaaccctgg aactgccttt gatactggat
541 atcttgagtg tggaagaggt gagtggaatt ccgagtgtag aggtaaaatt cgtagatatt
601 cggaggaaca ccagtggcga aggcggctca ctggtccatt actgacgctg aggtgcgaaa
661 gcgtggggag caaacaggat tagataccct g
```

B

```
  1 cttgtgctaa taccgtatga gcccttcggg ggaaagattc atcggcaaat gatcggcccg
 61 cgttggatta gctagttggt ggggtaaagg cctaccaagg cgacgatcca tagctggtct
121 gagaggatga tcagccacac tgggactgag acacggccca gactcctacg ggaggcagca
181 gtggggaata ttggacaatg ggcgcaagcc tgatccagcc atgccgcgtg agtgatgaag
241 gccctagggt tgtaaagctc tttcaccggt gaagataatg acggtaaccg gagaagaagc
301 cccggctaac ttcgtgccag cagccgcggt aatacgaagg gggctagcgt tgttcggatt
361 tactgggcgt aaagcgcacg taggcgggct aataagtcag gggtgaaatc ccggggctca
421 accccggaac tgcctttgat actgttagtc ttgagtatgg tagaggtgag tggaattccg
481 agtgtagagg tgaaattcgt agatattcgg aggaacacca gtggcgaagg cggctcactg
541 gaccattact gacgctgagg tgcgaaagcg tggggagcaa acaggattag ataccctggt
601 agtccacgcc gtaaacgatg aatgttagcc gttggggagt ttactcttcg gtggcgcagc
661 taacgcatta aacattccgc ctggggagta cggtcgca
```

C

```
  1 gcacgggtgc gtaacgcgtg ggaatctgcc cttaggttcg gaataacagc tggaaacggc
 61 tgctaatacc ggatgatatc gcgagatcaa agatttatcg cctgaggatg agcccgcgtt
121 ggattaggta gttggtgggg taaaggccta ccaagccgac gatccatagc tggtctgaga
181 ggatgatcag ccacactggg actgagacac ggcccagact cctacgggag gcagcagtgg
241 ggaatattgg acaatgggcg aaagcctgat ccagcaatgc cgcgtgagtg atgaaggccc
301 tagggttgta aagctctttt acccgggaag ataatgactg taccgggaga ataagccccg
361 gctaactccg tgccagcagc cgcggtaata cggagggggc tagcgttgtt cggaattact
421 gggcgtaaag cgcacgtagg cggctttgta agtcagaggt gaaagcctgg agctcaactc
481 cagaactgcc tttgagactg catcgcttga atccaggaga ggtcagtgga attccgagtg
541 tagaggtgaa attcgtagat attcggaaga acaccagtgg cgaaggcggc tgactggact
601 ggtattgacg ctgaggtgcg aaagcgtggg gagcaaacag gattagatac cctgga
```

Figure 11 Nucleotide sequences of *16S rRNA* genes. (A) F1608; (B) F1659; (C) F1693.

The percentage values of nucleotide sequence similarity for 598 bp were determined from pairwise alignment. *16S rRNA* sequence of F1608 showed sequence similarity to those of *Bartonella* spp (97.5-99.7% similarity) from GenBank database including *B. bacilliformis* (DQ179108), *B. clarridgeiae* (EU571939), *B. henselae* (Z11684), *B. koehlerae* (AF076237), *B. rochalimae* (DQ683196), *B. tamiae* (EF672728) and *B. vinsonii* (AF214558) that can cause disease of human and animals.

16S rRNA sequence of F1659 and F1684 showed 94.0-98.7% similarity to *Ochrobactrum* spp. including *O. anthropi* (U70978), *O.intermedium* (EU434428) and *O. pseudintermedium* (DQ365923)].

16S rRNA sequence of F1623, F1639, F1644, F1685 and F1693 showed 93.8-100% similarity to *Sphingomonas* spp. including *S. parapaucimobilis* (D84525), *S. paucimobilis* (AM237364), *S. sanguinis* (D84529) and *S. yanoikuyae* (D13728).

Sequence similalarity of the three different genera were compared to other related genera such as *Ehrlichia* spp. [*E. canis*-Bangkok (EF139458), *E. chaffeensis* (U23503), *E. ewingii* (U96436), *E. muris* (AB196302) and *E. ruminantium* (CR767821)], and *Anaplasma* spp. [*A. bovis* (AB211163), *A. centrale* (AF414869), *A. marginale* (AF414873), *A. ovis* (AF414870), *A. phagocytophilum* (DQ342324), and *A. platys*-Bangkok (EF139459)], values of sequence similarity were fill in Table 7.

Table 7 The sequence similarity of *16S rRNA* cat blood samples compared with *Bartonella* spp., *Ochrobactrum* spp., *Sphingomonas* spp., *Ehrlichia* spp. and *Anaplasma* spp.

	%identity		
Organisms	**F1608**	**F1659**	**F1693**
Bartonella spp.	**97.5-100**	93.5-95.2	87.7-88.8
Ochrobactrum spp.	88.5-95.2	**94.0-98.7**	84.8-89.5
Sphingomonas spp.	87.7-88.9	89.4-90.0	**93.8-100**
Ehrlichia spp.	83.3-84.2	84.0-84.8	86.2-87.5
Anaplasma spp.	86.1-86.3	86.0-87.1	85.0-85.8

The sequence similarity revealed that F1608, F1659 and F1693 were closely related to *Bartonella* spp., *Ochrobactrum* spp. and *Sphingomonas* spp. more than *Ehrlichia* spp. and *Anaplasma* spp.

2.3 Phylogenetic analysis of *16S rRNA* genes from cat blood samples

Seven species of *Bartonella*, 3 species of *Ochrobactrum*, 4 species of *Sphingomonas*, 5 species of *Ehrlichia* and 6 species of *Anaplasma 16S rRNA* sequences corresponding to the data used in 2.2 were used to generate a phylogenetic tree using neighbor-joining method by MEGA software (version 3.1). The alpha-proteobacteria, *Caulobacter crescentus*, was used as the outgroup. The resultant phylogenetic tree (Figure 12) revealed that (1) the phylogenetic tree devided into 2 groups, group 1 composed of *Bartonella, Ochrobactrum* and *Sphingomonas*, while group 2 composed of *Ehrlichia* and *Anaplasma*; (2) F1608, F1659 and F1693 were clearly grouped into different clades that belonging to *Bartonella, Ochrobactrum* and *Sphingomonas*, respectively; (3) *Bartonella* and *Ochrobactrum* were closely related since they are in the Order Rhizobiales; (4) *Bartonella* was close to *Ochrobactrum* and *Sphingomonas* as they are in the Class alpha-proteobacteria.

Figure 12 Phylogenetic tree based on *16S rRNA* genes of *Anaplasma* spp., *Bartonella spp., Ehrlichia* spp., *Ochrobactrum* spp. and *Sphingomonas* spp. Sequences from these genera were compared with the neighbor-joining method with distance matrix calculation by Kimura-2 parameters, operated by MEGA software (version 3.1), using *C. crescentus* as the outgroup. Scale bar indicates the number of mutations per sequence position. The numbers at the nodes represent the percentage of 1000 bootstrap re-samplings. The text in boxes ( ) are the sequences from this study.

3. Cloning and sequence analysis of bicyclomycin gene

3.1 Cloning and sequencing of *bcr* gene from *E. canis*-Bangkok

To study and identify antibiotic resistance gene from uncultured or difficult to culture bacteria such as *Ehrlichia* spp. are problematical worked and to date culture of *Ehrlichia* is limited (Forbes *et al*., 1998). However, the technique that may use to identify these genes is polymerase chain reaction (PCR). Putative bicyclomycin resistance gene (*bcr*) from *E. canis*-Bangkok was amplified by PCR using specific primers, ATT069F and ATT069R. The 1.1 kb PCR product was obtained (Figure 13) and successfully cloned into pGEM-T Easy vector. Sequence analysis showed that this gene consists of 1,161 bases encoding a protein of 386 amino acids (Figure 14). The protein was predicted a mass of 42.45 kDa. This sequence was deposited under accession number EU880584 in the GenBank database as a new bicyclomycin resistance gene.

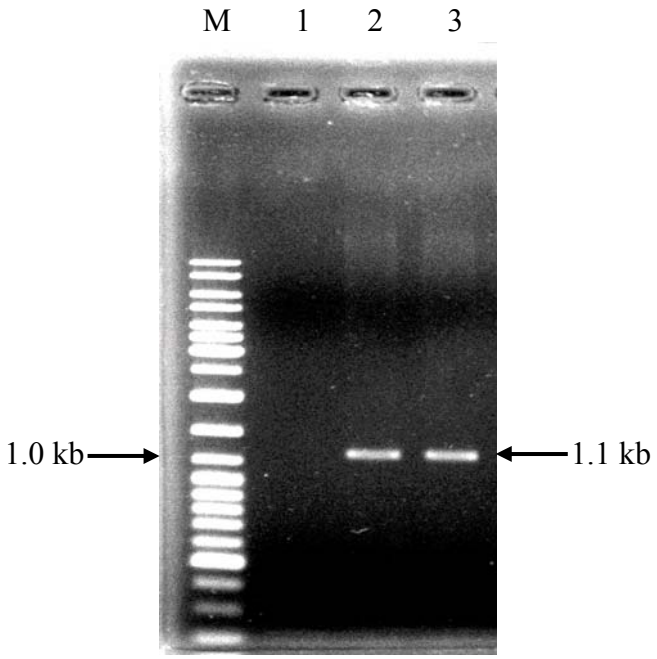


Figure 13 PCR product of *Bcr* gene from *E. canis*-Bangkok. Lane M, DNA ladder mix (Fermentas, USA); Lane 1, negative control; Lane 2 and 3, PCR amplicons of *E. canis*-Bangkok.

```
Start codon
   1 ATGTGTGATATGTCTTCTGATATATATTTGCCTGCATTGCCAAAAATTAGTGAATTTTTT
   1  M  C  D  M  S  S  D  I  Y  L  P  A  L  P  K  I  S  E  F  F

  61 AATGTAGACCATACTATTGTTCAGTTAACAGTTAGTTTAAATTTGGTGGGAATCTCTGTC
  21  N  V  D  H  T  I  V  Q  L  T  V  S  L  N  L  V  G  I  S  V

 121 TCCGGATTATTATATGGTCCATTATCTGATTATTATGGACGTAGGCCTATTATCCTGTTA
  41  S  G  L  L  Y  G  P  L  S  D  Y  Y  G  R  R  P  I  I  L  L

 181 GGTGTAGGAATATTTATGGTAGCAAGCATAATGTGTTGTTTTTCTAGTAATATTATTATG
  61  G  V  G  I  F  M  V  A  S  I  M  C  C  F  S  S  N  I  I  M

 241 TTAATTATCATGCGTTTTATTCAGGGATTTGGTGCAGGTGTAGCTGGAGTTGTTGAATAT
  81  L  I  I  M  R  F  I  Q  G  F  G  A  G  V  A  G  V  V  E  Y

 301 GCTATTATTAAAGATATGTATTCAGGAAATGAATGTGCTAAAAATATCTCTATAGTAAAT
 101  A  I  I  K  D  M  Y  S  G  N  E  C  A  K  N  I  S  I  V  N

 361 ATAGCAGTAGCATTTGCTCCTGTGGTAGCTCCAATTTTAGGTAGTGCTATTATAGCACAT
 121  I  A  V  A  F  A  P  V  V  A  P  I  L  G  S  A  I  I  A  H

 421 GGTTATCATTGGAACATGTTGTTTGTTACAATATCTATTATGGCAGTATTGGTTTTGGCT
 141  G  Y  H  W  N  M  L  F  V  T  I  S  I  M  A  V  L  V  L  A

 481 AGTTTGTTTATGTTTTTGCAAGAAACTATTTCTTTTGATAATGTTGATAGTAGTATATCT
 161  S  L  F  M  F  L  Q  E  T  I  S  F  D  N  V  D  S  S  I  S

 541 TTTTTGTCTATTATACGAAAATATAAAGAACTTGTTTTAAATGTTAGATTTTGTGGTTTT
 181  F  L  S  I  I  R  K  Y  K  E  L  V  L  N  V  R  F  C  G  F

 601 GCTTTAATTCAAAGTTTTACTATTATGTGGATTTGGTCATGTGTAGCTAACTTACCATTT
 201  A  L  I  Q  S  F  T  I  M  W  I  W  S  C  V  A  N  L  P  F

 661 ATTTTTGTTAATGATATGGGTGTTCCTGTTGGCTATTATGGGTATTTTGTTGCTATTAAT
 221  I  F  V  N  D  M  G  V  P  V  G  Y  Y  G  Y  F  V  A  I  N

 721 GTAGCTGCATATATTGTTGGTGCAATTATTAATCAAAGGTTTGTTGAGAGATTTGGAATC
 241  V  A  A  Y  I  V  G  A  I  I  N  Q  R  F  V  E  R  F  G  I

 781 AATAATATGTTGCTTATAGGATTAATTTTAACTACTTTATCAGATTTGGTAGTACTTGTA
 261  N  N  M  L  L  I  G  L  I  L  T  T  L  S  D  L  V  V  L  V

 841 CTTTATCAAATAATTGAAATTAGTCCTTTATTAGCAGAAATTATGTGGATGCCGTCTGGC
 281  L  Y  Q  I  I  E  I  S  P  L  L  A  E  I  M  W  M  P  S  G

 901 ATGGGAATTGCTTTTATTCTTGGTAATAATATGACAGCTGCATTTTCTGAAATTAAGGAA
 301  M  G  I  A  F  I  L  G  N  N  M  T  A  A  F  S  E  I  K  E

 961 CCAGGTATAGGTTCCGCATTTATTCTGTTTTTACAAACAATTTTTGGTGCACTTGGTATT
 321  P  G  I  G  S  A  F  I  L  F  L  Q  T  I  F  G  A  L  G  I

1021 TATATTTTAGGATGTTTTTATGATGGTACTCTAATTCCAGTAATACTGTTTCCAATAATT
 341  Y  I  L  G  C  F  Y  D  G  T  L  I  P  V  I  L  F  P  I  I

1081 TGTTCTGTAATTTGCCTTGTAATATATGCTTTTTTACAAGTGACTAATAAGAGTAATAGA
 361  C  S  V  I  C  L  V  I  Y  A  F  L  Q  V  T  N  K  S  N  R
                Stop codon
1141 TCTAAGTTTGTAGATGTATAA
 381  S  K  F  V  D  V  *
```

Figure 14 Nucleotide and amino acid sequence of *Bcr* gene. This sequence showed *bcr* complete gene product of 1,161 nucleotides and Bcr amino acid sequence of 386 amino acid of *E. canis*-Bangkok.

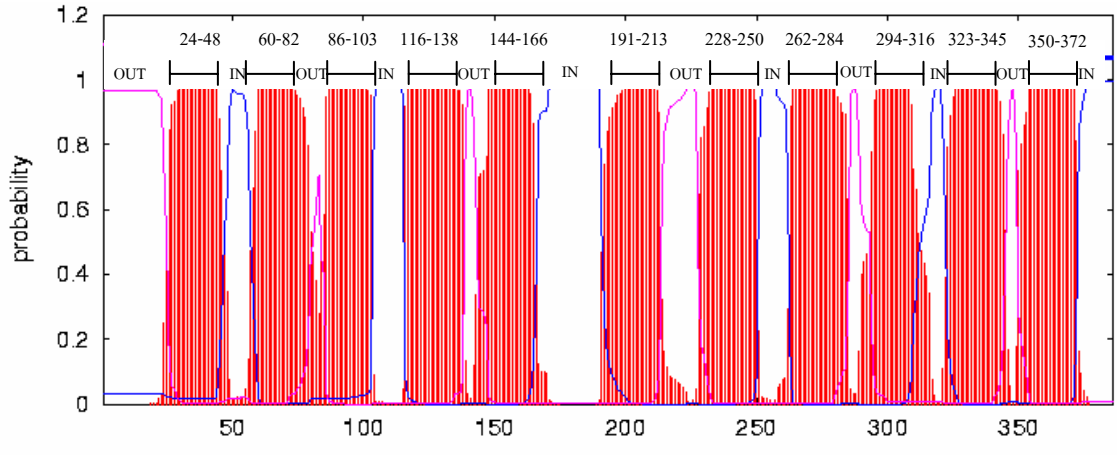
3.2 Topology prediction of the Bcr protein

Bcr amino acid sequence of *E. canis*-Bangkok was analysed by BlastP and revealed sequence similarity to those of drug resistance transporter Bcr/CflA subfamily sequences from organisms in order Rickettsiales. The results indicated that Bcr amino acid sequence of *E. canis*-Bangkok was very close to that of *E. canis* strain Jake (YP303315) with 99 % identity. It showed close relationship with 84-89 % similarity to those of genus *Ehrlichia* including *E. chaffeensis* strain Sapulpa (ZP00544976), *E. ruminantium* strain Welgevonden (YP180541) and *E. ruminantium* strain Gardel (YP19663). It shared 70 % and 56 % similarity with those of *Anaplasma phagocytophilum* HZ (YP504788) and *Anaplasma marginale* strain Maries (YP153562), respectively. Similarity values of 67-70 % were observed with those of genus *Wolbachia* including *Wolbachia pipientis* (CAQ55329), *Wolbachia* endosymbiont of *Drosophilla willistoni* (ZP01314869), *Wolbachia* endosymbiont of *Drosophilla melanogaster* (NP966176) and *Wolbachia* endosymbiont strain TRS of *Brugia malayi* (YP198034). It shared 59 % similarity with that of *Neorickettsia sennetsu* strain Miyayama (YP505965) and 53 % with *Orientia tsutsugamushi* strain Boryong (YP505965), and *Orientia tsutsugamushi* strain Ikeda (YP001937329).

To determine the *E. canis*-Bangkok Bcr protein belonging to MFS, the amino acid sequence was first scanned for the presence of signal peptide sequences using SignalP program. The result revealed that there was no such leader peptide sequence located at the N-terminal of the Bcr protein. The Bcr amino acid sequence of *E. canis*-Bangkok was then predicted the hydrophobic profiles and TMS using five different computer programs; SOSUI, ConPredII, HMMTOP, TMHMM, and TMMOD. The results obtained from ConPredII, HMMTOP, TMHMM and TMMOD identified the *E. canis*-Bangkok Bcr protein as an 11-TMS with N-terminal out of cell and C-terminal in the cytoplasm (Figure 15A and B). The *E. canis*-Bangkok Bcr protein contained 59.84% hydrophobic amino acids, 13 charged residues inside, 2 charged residue outside. Eleven TMSs from TMHMM were found between residues 26-48, 60-82, 86-103, 116-138, 144-166, 191-213, 228-250, 262-284, 294-316, 323-345 and 350-372 (Figure 15A). Each position of 11 TMSs was in approximately the

same positions as predicted by the four programs. In contrast, SOSUI predicted this protein as 12-TMS, with the N and C termini of the protein located in the cytoplasm.

A



B

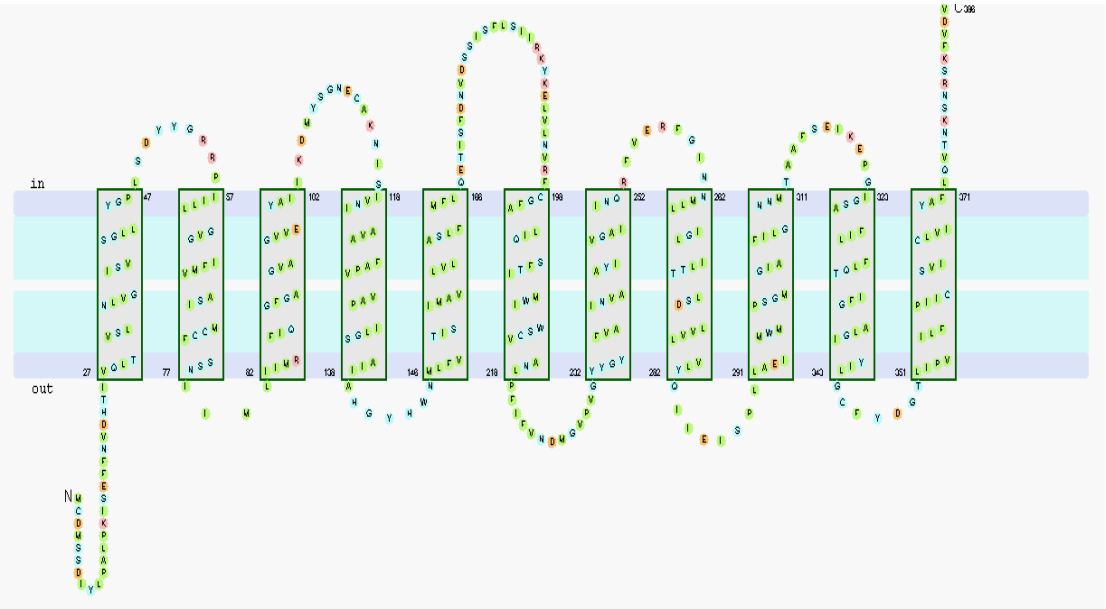
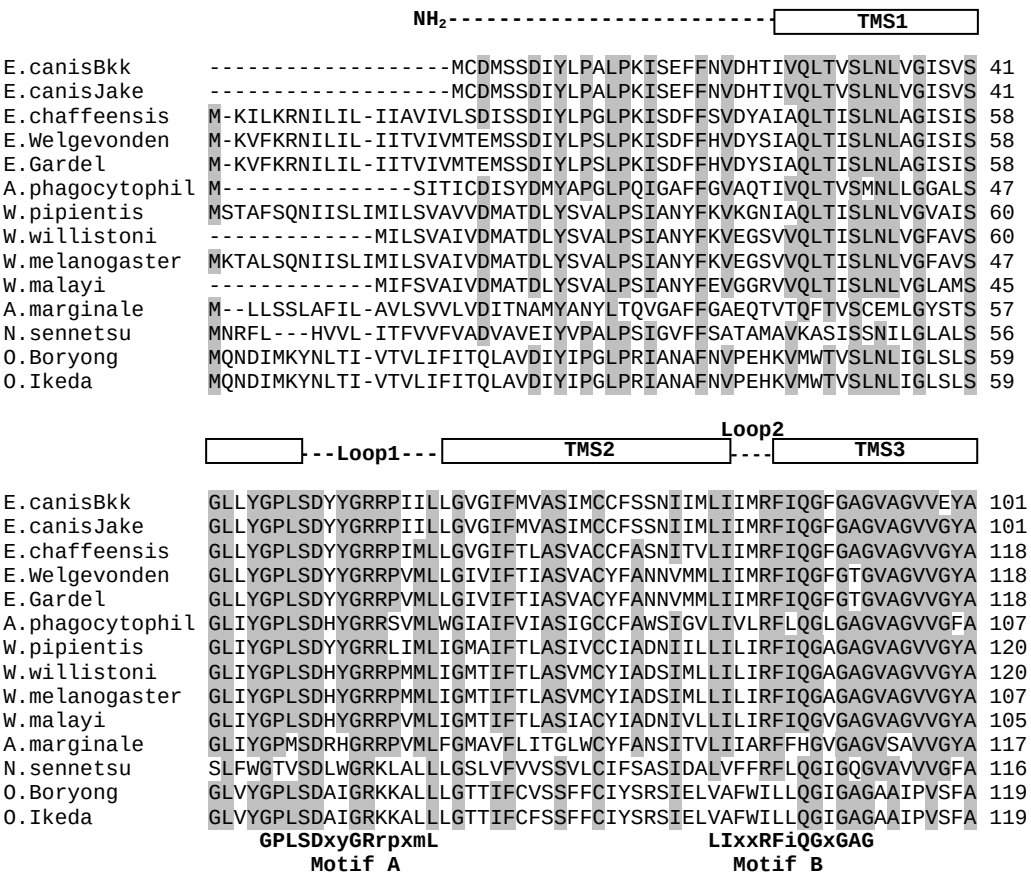
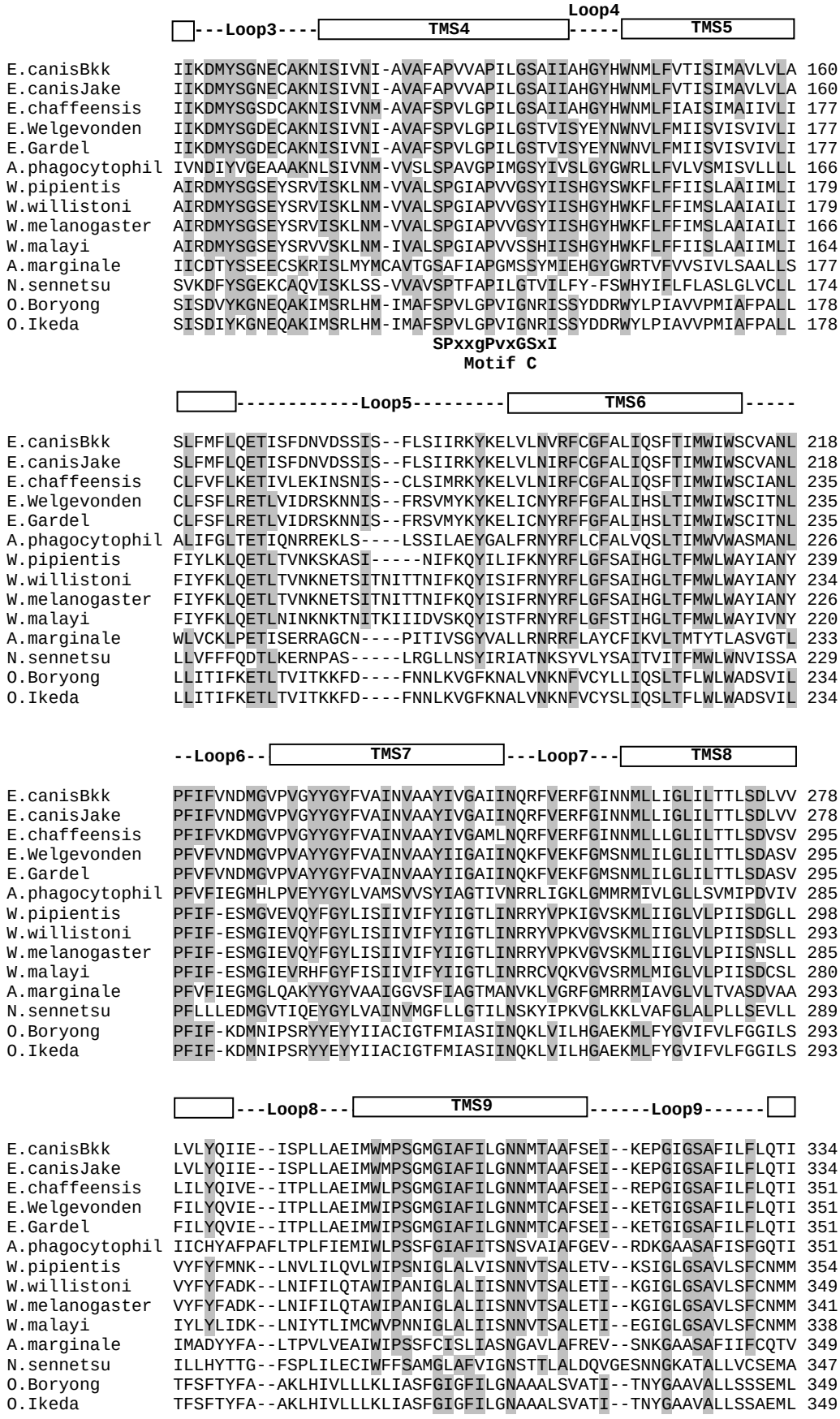


Figure 15 The elucidation of the topology of the *E. canis*-Bangkok Bcr protein. (A) Hydropathy analysis by TMHMM. The probability score for transmembrane helical regions are shown as vertical lines with the amino acid position of the ends of the predicted helices given above the bars at the top. (B) Topological model by ConPredII. Hypothetical transmembrane segments are indicated in boxes.

3.3 Signature motif prediction and phylogenetic relationship

Since the *E. canis*-Bangkok Bcr protein was predicted as 11-TMS, all of those 13 drug resistance transporters in order Rickettsiales were then subjected to HMMTOP, TMHMM and TMMOD to analyse the transmembrane profiles. The results indicated that they were 11-TMS (data not shown). 11-TMS proteins from 14 species in order Rickettsiales were multiple aligned (Figure 16). The sequences were scanned for the presence of MFS transporter family signature motifs A (GxLaDrxGrkxxxl), B (lxxxRxxqGxgaa) and C (gxxxGPxxGGxl) (Paulsen *et al*., 1996; Putman *et al*., 2000) using the program MAFFT. Motif A was found as **G**P**L**S**D**xy**GR**rpxm**L** located in the cytoplasmic loop between TMS 1 and 2, motif B was found as **L**Ixx**R**Fi**QG**x**GA**G located within TMS 3, and motif C was conserved as SPxx[**G**A]**P**[VI]x**G**SxI found within TMS 4 of all 14 proteins (Figure 16; residues in bold indicate those conserved in the MFS signature motifs).




Loop4
---Loop3----
TMS4

TMS5
SPxxgPvxGSxI
Motif C
-----------Loop5---------
TMS6

--Loop6--
TMS7
---Loop7---
TMS8
---Loop8---
TMS9
------Loop9------
E.canisBkk IIKDMYSGNECAKNISIVNI-AVAFAPVVAPILGSAIIAHGYHWNMLFVTISIMAVLVLA 160
E.canisJake IIKDMYSGNECAKNISIVNI-AVAFAPVVAPILGSAIIAHGYHWNMLFVTISIMAVLVLA 160
E.chaffeensis IIKDMYSGSDCAKNISIVNM-AVAFSPVLGPILGSAIIAHGYHWNMLFIAISIMAIIVLI 177
E.Welgevonden IIKDMYSGDECAKNISIVNI-AVAFSPVLGPILGSTVISYEYNWNVLFMIISVISVIVLI 177
E.Gardel IIKDMYSGDECAKNISIVNI-AVAFSPVLGPILGSTVISYEYNWNVLFMIISVISVIVLI 177
A.phagocytophil IVNDIYVGEAAAKNLSIVNM-VVSLSPAVGPIMGSYIVSLGYGWRLLFVLVSMISVLLLL 166
W.pipientis AIRDMYSGSEYSRVISKLNM-VVALSPGIAPVVGSYIISHGYSWKFLFFIISLAAIIMLI 179
W.willistoni AIRDMYSGSEYSRVISKLNM-VVALSPGIAPVVGSYIISHGYHWKFLFFIMSLAAIAILI 179
W.melanogaster AIRDMYSGSEYSRVISKLNM-VVALSPGIAPVVGSYIISHGYHWKFLFFIMSLAAIAILI 166
W.malayi AIRDMYSGSEYSRVVSKLNM-IVALSPGIAPVVSSHIISHGYHWKFLFFIISLAAIIMLI 164
A.marginale IICDTYSSEECSKRISLMYMCAVTGSAFIAPGMSSYMIEHGYGWRTVFVVSIVLSAALLS 177
N.sennetsu SVKDFYSGEKCAQVISKLSS-VVAVSPTFAPILGTVILFY-FSWHYIFLFLASLGLVCLL 174
O.Boryong SISDVYKGNEQAKIMSRLHM-IMAFSPVLGPVIGNRISSYDDRWYLPIAVVPMIAFPALL 178
O.Ikeda SISDIYKGNEQAKIMSRLHM-IMAFSPVLGPVIGNRISSYDDRWYLPIAVVPMIAFPALL 178
E.canisBkk SLFMFLQETISFDNVDSSIS--FLSIIRKYKELVLNVRFCGFALIQSFTIMWIWSCVANL 218
E.canisJake SLFMFLQETISFDNVDSSIS--FLSIIRKYKELVLNIRFCGFALIQSFTIMWIWSCVANL 218
E.chaffeensis CLFVFLKETIVLEKINSNIS--CLSIMRKYKELVLNIRFCGFALIQSFTIMWIWSCIANL 235
E.Welgevonden CLFSFLRETLVIDRSKNNIS--FRSVMYKYKELICNYRFFGFALIHSLTIMWIWSCITNL 235
E.Gardel CLFSFLRETLVIDRSKNNIS--FRSVMYKYKELICNYRFFGFALIHSLTIMWIWSCITNL 235
A.phagocytophil ALIFGLTETIQNRREKLS----LSSILAEYGALFRNYRFLCFALVQSLTIMWVWASMANL 226
W.pipientis FIYLKLQETLTVNKSKASI-----NIFKQYILIFKNYRFLGFSAIHGLTFMWLWAYIANY 239
W.willistoni FIYFKLQETLTVNKNETSITNITTNIFKQYISIFRNYRFLGFSAIHGLTFMWLWAYIANY 234
W.melanogaster FIYFKLQETLTVNKNETSITNITTNIFKQYISIFRNYRFLGFSAIHGLTFMWLWAYIANY 226
W.malayi FIYFKLQETLNINKNKTNITKIIIDVSKQYISTFRNYRFLGFSTIHGLTFMWLWAYIVNY 220
A.marginale WLVCKLPETISERRAGCN----PITIVSGYVALLRNRRFLAYCFIKVLTMTYTLASVGTL 233
N.sennetsu LLVFFFQDTLKERNPAS-----LRGLLNSYIRIATNKSYVLYSAITVITFMWLWNVISSA 229
O.Boryong LLITIFKETLTVITKKFD----FNNLKVGFKNALVNKNFVCYLLIQSLTFLWLWADSVIL 234
O.Ikeda LLITIFKETLTVITKKFD----FNNLKVGFKNALVNKNFVCYSLIQSLTFLWLWADSVIL 234
E.canisBkk PFIFVNDMGVPVGYYGYFVAINVAAYIVGAIINQRFVERFGINNMLLIGLILTTLSDLVV 278
E.canisJake PFIFVNDMGVPVGYYGYFVAINVAAYIVGAIINQRFVERFGINNMLLIGLILTTLSDLVV 278
E.chaffeensis PFIFVKDMGVPVGYYGYFVAINVAAYIVGAMLNQRFVERFGINNMLLLGLILTTLSDVSV 295
E.Welgevonden PFVFVNDMGVPVAYYGYFVAINVAAYIIGAIINQKFVEKFGMSNMLILGLILTTLSDASV 295
E.Gardel PFVFVNDMGVPVAYYGYFVAINVAAYIIGAIINQKFVEKFGMSNMLILGLILTTLSDASV 295
A.phagocytophil PFVFIEGMHLPVEYYGYLVAMSVVSYIAGTIVNRRLIGKLGMMRMIVLGLLSVMIPDVIV 285
W.pipientis PFIF-ESMGVEVQYFGYLISIIVIFYIIGTLINRRYVPKIGVSKMLIIGLVLPIISDGLL 298
W.willistoni PFIF-ESMGIEVQYFGYLISIIVIFYIIGTLINRRYVPKVGVSKMLIIGLVLPIISDSLL 293
W.melanogaster PFIF-ESMGIEVQYFGYLISIIVIFYIIGTLINRRYVPKVGVSKMLIIGLVLPIISNSLL 285
W.malayi PFIF-ESMGIEVRHFGYFISIIVIFYIIGTLINRRCVQKVGVSRMLMIGLVLPIISDCSL 280
A.marginale PFVFIEGMGLQAKYYGYVAAIGGVSFIAGTMANVKLVGRFGMRRMIAVGLVLTVASDVAA 293
N.sennetsu PFLLLEDMGVTIQEYGYLVAINVMGFLLGTILNSKYIPKVGLKKLVAFGLALPLLSEVLL 289
O.Boryong PFIF-KDMNIPSRYYEYYIIACIGTFMIASIINQKLVILHGAEKMLFYGVIFVLFGGILS 293
O.Ikeda PFIF-KDMNIPSRYYEYYIIACIGTFMIASIINQKLVILHGAEKMLFYGVIFVLFGGILS 293
E.canisBkk LVLYQIIE--ISPLLAEIMWMPSGMGIAFILGNNMTAAFSEI--KEPGIGSAFILFLQTI 334
E.canisJake LVLYQIIE--ISPLLAEIMWMPSGMGIAFILGNNMTAAFSEI--KEPGIGSAFILFLQTI 334
E.chaffeensis LILYQIVE--ITPLLAEIMWLPSGMGIAFILGNNMTAAFSEI--REPGIGSAFILFLQTI 351
E.Welgevonden FILYQVIE--ITPLLAEIMWIPSGMGIAFILGNNMTCAFSEI--KETGIGSAFILFLQTI 351
E.Gardel FILYQVIE--ITPLLAEIMWIPSGMGIAFILGNNMTCAFSEI--KETGIGSAFILFLQTI 351
A.phagocytophil IICHYAFPAFLTPLFIEMIWLPSSFGIAFITSNSVAIAFGEV--RDKGAASAFISFGQTI 351
W.pipientis VYFYFMNK--LNVLILQVLWIPSNIGLALVISNNVTSALETV--KSIGLGSAVLSFCNMM 354
W.willistoni VYFYFADK--LNIFILQTAWIPANIGLALIISNNVTSALETI--KGIGLGSAVLSFCNMM 349
W.melanogaster VYFYFADK--LNIFILQTAWIPANIGLALIISNNVTSALETI--KGIGLGSAVLSFCNMM 341
W.malayi IYLYLIDK--LNIYTLIMCWVPNNIGLALIISNNVTSALETI--EGIGLGSAVLSFCNMM 338
A.marginale IMADYYFA--LTPVLVEAIWIPSSFCISLIASNGAVLAFREV--SNKGAASAFIIFCQTV 349
N.sennetsu ILLHYTTG--FSPLILECIWFFSAMGLAFVIGNSTTLALDQVGESNNGKATALLVCSEMA 347
O.Boryong TFSFTYFA--AKLHIVLLLKLIASFGIGFILGNAAALSVATI--TNYGAAVALLSSSEML 349
O.Ikeda TFSFTYFA--AKLHIVLLLKLIASFGIGFILGNAAALSVATI--TNYGAAVALLSSAEML 349

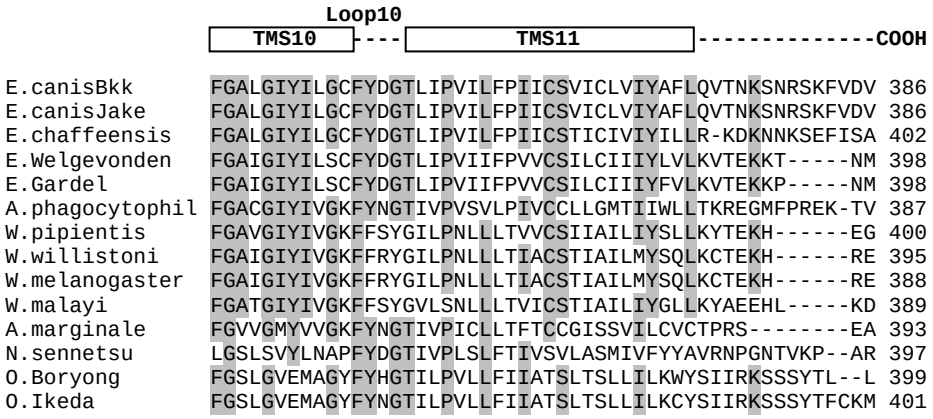


Figure 16 Multiple-sequence alignments of the drug resistance transporter Bcr/CflA subfamily from the order Rickettsiales**.** Grey background for identity 70-100 %. The consensus of motifs A, B and C were defined from conserved amino acid with an identity over 80 %. The predicted transmembrane seqments (TMS) are squared.

3.4 Phylogenetic tree analysis

From BlastP analysis, the *E. canis*-Bangkok Bcr protein showed higher similarity to those of 12-TMS family than 14-TMS family of MFS. The amino acid sequence of the *E. canis*-Bangkok Bcr protein was then compared with those of 10 proteins of 12-TMS family including Bcr from *Escherichia coli* (X63703), Blt from *Bacillus subtilis* (L32599), Bmr from *B. subtilis* (M33768), Cmr from *Corynebacterium glutamicum* (U43535), EmrD from *E. coli* (P31442), LmrP from *Lactococcus lactis* (X89779), MdfA (Cmr/CmlA) from *E. coli* (Y08743), NorA from *Staphylococcus aureus* (D90119), PmrA from *Streptococcus pneumoniae* (AJ007367) and Tap from *Mycobacterium fortuitum* (AJ000283). The *E. canis*-Bangkok Bcr protein shared much lower similarity (27-48 %) with these 12-TMS family than those of rickettsia species. An unrooted phylogenetic tree was generated from 14 drug transporters of 11-TMS of rickettsia species and 10 proteins of 12-TMS family. The members of our 11-TMS family were segregated from the 12-TMS family and grouped relatively tight in their own branch (Figure 17).

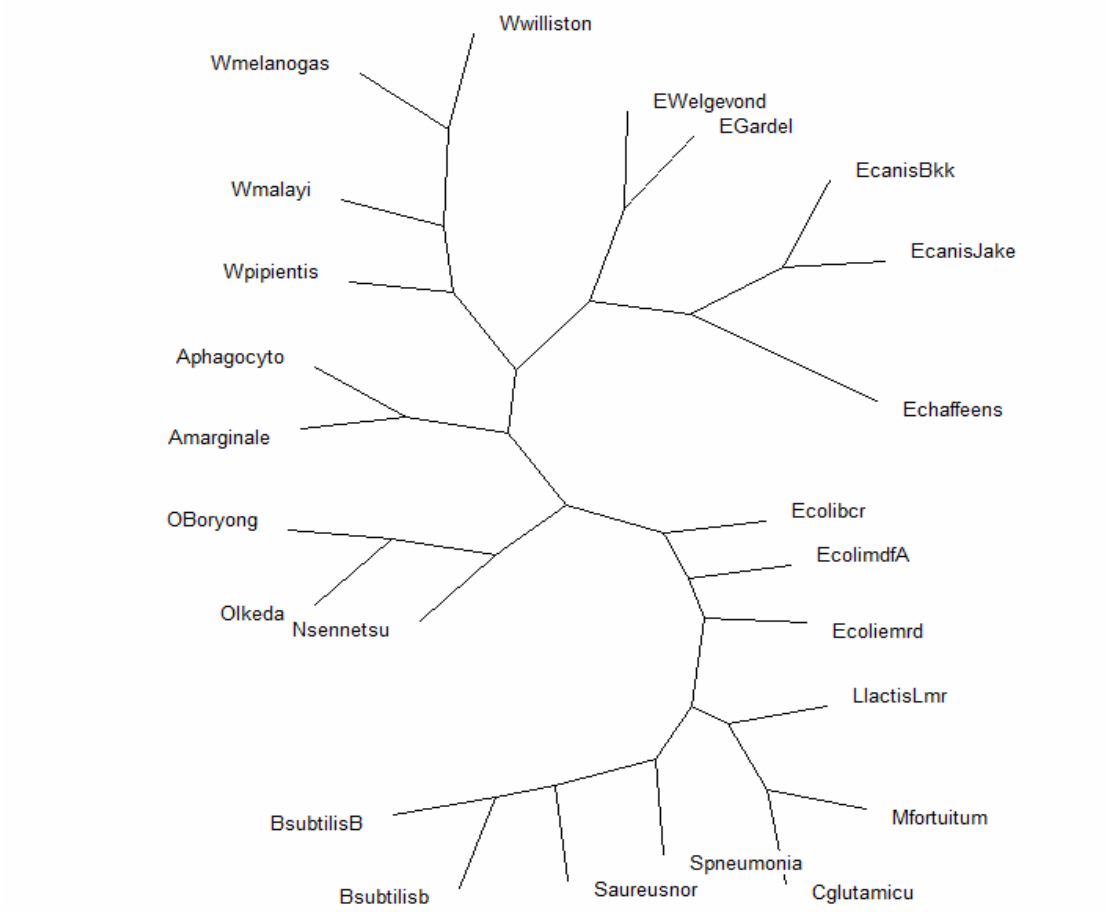


Figure 17 Unrooted phylogenetic tree of the drug resistance transporter Bcr/CflA subfamily from PHYLIP package program version 3.61 of 14 rickettsia species and 10 of 12-transmembrane proteins of major facilitator superfamil

4. Patatin (*pat*) gene expression and characterization

4.1 Amplification and sequencing of *pat* gene

The patatin coding region (*pat* gene) was amplified by PCR using *E. canis* positive DNA of dog blood sample as a template and using ATT068F and ATT068R as specific primers (Table 2). The gradient PCR condition was optimized at 50.0, 50.3, 50.9, 51.7, 52.8, 54.3, 56.0, 57.7, 58.5, 59.3, 59.8 and 60.0 °C (lane 1-12 respectively) and showed optimal annealing temperature at 56°C. A 948 bp PCR product of *pat* gene is shown in Figure 18.

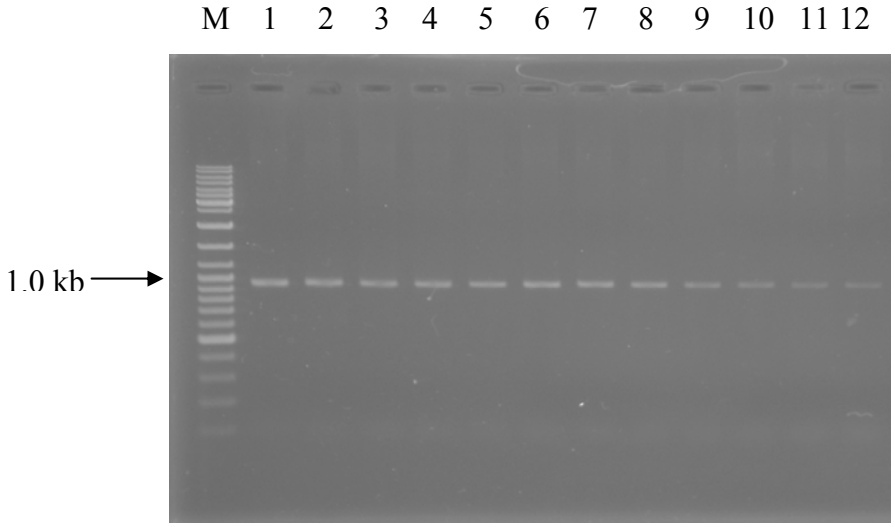


Figure 18 Gradient PCR of *patatin* gene from *E. canis*-Bangkok using primers ATT068F and ATT068R. Lane M, GeneRuler^{TM} DNA ladder mix; Lane 1-12, 50.0, 50.3, 50.9, 51.7, 52.8, 54.3, 56.0, 57.7, 58.5, 59.3, 59.8 and 60.0 °C, respectively.

4.2 Cloning and characterization of *pat* gene

The 948 bp PCR products was purified and ligated to pGEM-T Easy vector. Ligated product was transformed into *E. coli* DH5α competent cells by heat shock method. Ten white colonies on selected plate containing 100 µg/ml amplicilin, X-gal and IPTG were randomly picked for plasmid extraction and PCR analysis. The

recombinant plasmids were verified by digested with *Eco*RI (data not shown). The recombinant plasmid was named pGEM-T/*pat* and was verified by digested with *Nde*I/*Bam*HI (Figure 19). The pGEM-T/*pat* was then sequenced using SP6 forward and T7 reverse primers. The nucleotide sequence showed 100% identity with *pat* gene of *E. canis* strain Jake in GenBank database (GenBank accession number: NC007354). The sequence included 5´*Nde*I restriction site at start ATG codon and 3´*Bam*HI restriction site at stop codon (data not show).

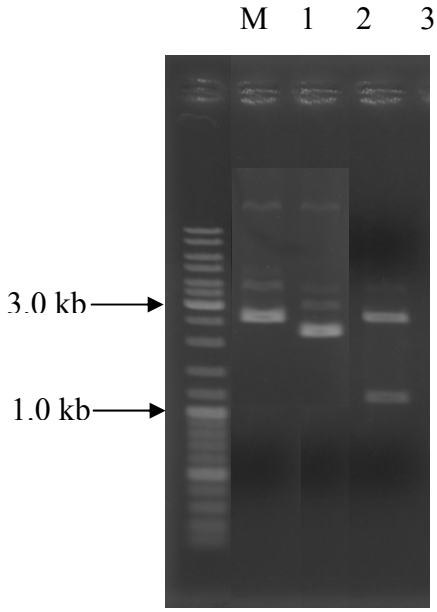


Figure 19 Determination of pGEM-T/*pat*. Lane M, GeneRuler^{TM} DNA ladder mix; Lane 1, pGEM-T/*pat*; Lane 2, pGEM-T; Lane 3 pGEM-T/*pat*/*Nde*I/ *Bam*HI.

4.3 Expression of *pat* gene of *E. canis*-Bangkok in *E. coli*.

4.3.1 Cloning of *pat* gene in expression vector

A 948 bp *pat* gene from pGEM-T/*pat* was subcloned into pET-15b expression plasmid at *Nde*I and *Bam*HI sites. The ligation product was transformed into *E. coli* DH5α competent cells. The colonies were randomly selected and analyzed. Recombinant plasmid was confirmed by *Nde*I and *Bam*HI digest and was

designated as pET-15b/*pat* (Figure 20). The recombinant plasmid was then transformed into *E.coli* BL21 (DE3), a strain for protein expression.

For further analysis, plasmid pET-15b/*pat* was sequenced using T7 promoter and T7 terminator primers to confirm the correction of the sequence of gene encoding for patatin. The fragment contained 948 nucleotide composing of 5´*Nde*I restriction site, start ATG codon, 6x His tag, thrombin site and 3´ *Bam*HI (Figure 21). The patatin gene completed sequence encoded 316 amino acids. The predicted molecular mass of deduced amino acids was 34.63 kDa and expected theoretical pI was 5.12 (predicted by using program at http://br.expasy.org/tools/pi_tool.htm).

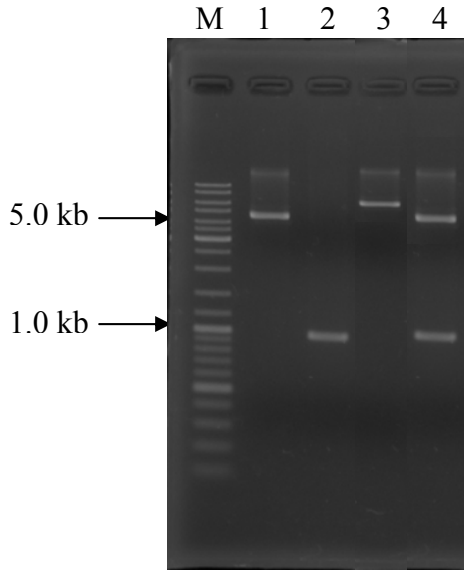


Figure 20 Determination of pET-15b/*pat*. Lane M, GeneRuler^{TM} DNA ladder mix; Lane 1, pET-15b; Lane 2, PCR product of *pat* gene; Lane 3 pET-15b/*pat*; Lane 4, pET-15b/*pat*/*Nde*I/*Bam*HI.

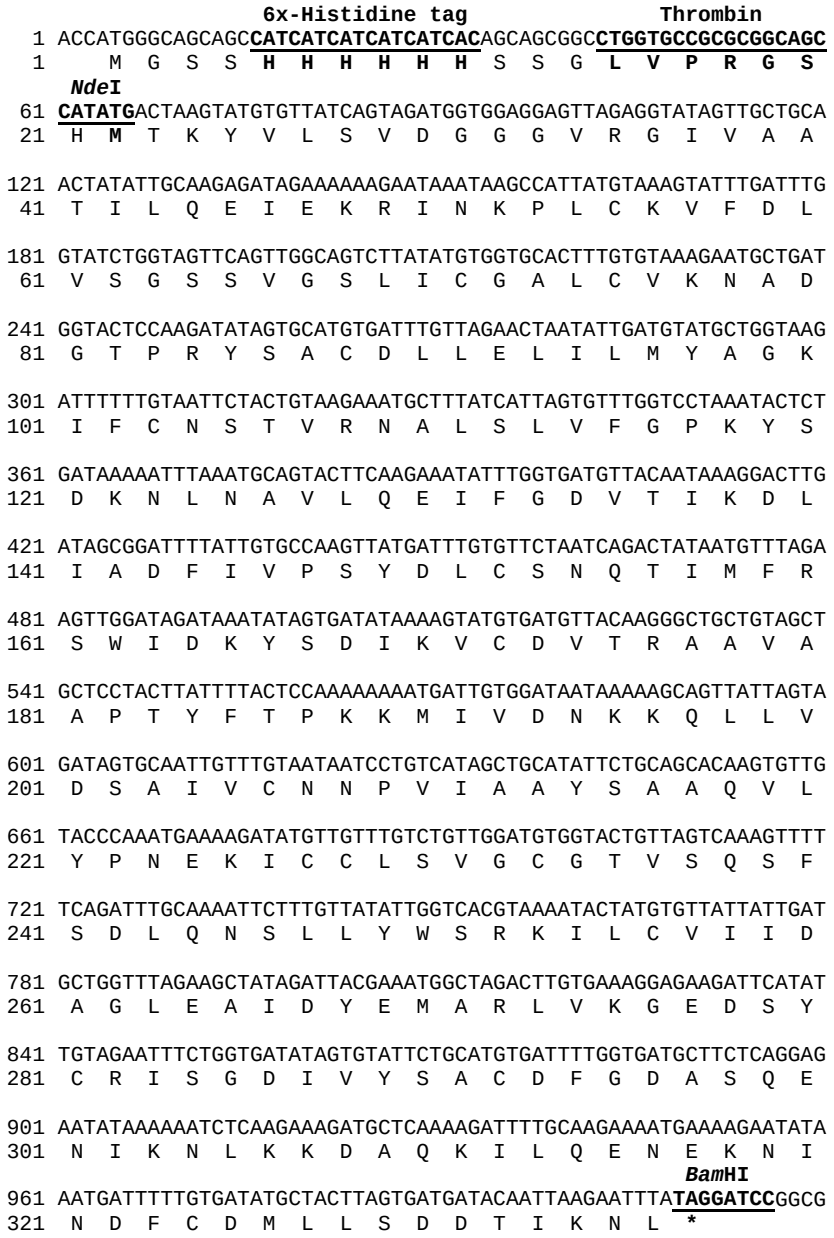


Figure 21 Nucleotide and amino acid sequences of patatin of *E. canis*-Bangkok. This sequence showed *pat* complete gene product of 948 nucleotides with 316 residues.

4.3.2 Optimum condition for *Ehrlichia* pat gene expression in *E. coli*

To confirm the expression of pET-15b/*pat*, the proteins were detected by Western blot hybridization with anti-histidine probe. The results showed overexpression band of patatin on nitrocellulose membrane when detected with

bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) as specific substrate for alkaline phosphatase (AP)-conjugated antibody. AP-conjugated antibody is an Anti-His(C-term)-AP Antibody prepared by crosslinking the primary antibody (Anti-His) with alkaline phosphatase using glutaraldehyde (Harlow and Lane, 1988).

4.3.3 Induction of pET-15b/*pat* in *E.coli*

The expression of the target DNA could be induced by adding lactose. The inducible protein was analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue dye. The recombinant protein was confirmed the expression in whole cell lysate induction with 1.0 mM lactose at 4 h and overnight by western blot using His-tag antibody (Figure 22). When lysed cell with lysis buffer recombinant protein expressed in supernatant not in pellet (Figure 23).

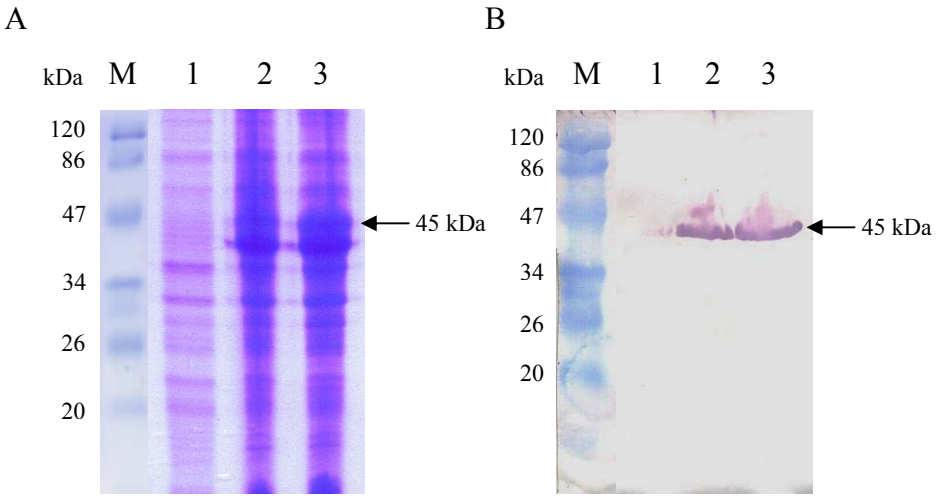


Figure 22 Whole cell lysate of pET-15b/*pat***.** (A) SDS-PAGE (B) Western blot. Lane M, protein marker; Lane 1, non-induction; Lane 2, 4 h incubation after induction; Lane 3, overnight incubation after induction.

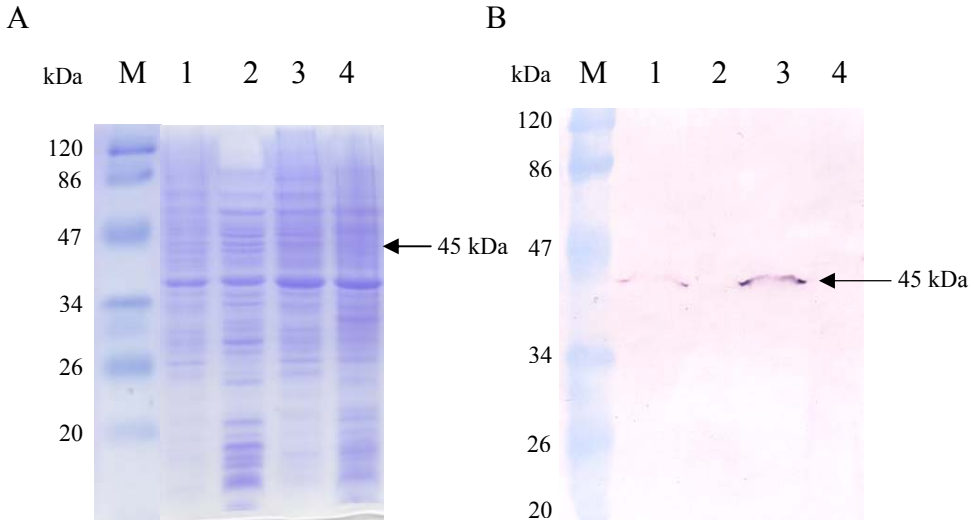


Figure 23 Supernatant and pellet of pET-15b/*pat* after induction with 1 mM lactose. (A) SDS-PAGE (B) Western blot. Lane M, protein marker; Lane 1, Supernatant of 4 h incubation; Lane 2, pellet of 4 h incubation; Lane 3, Supernatant of overnight incubation; Lane 4, pellet of overnight incubation.

The optimization of induced condition to gain high amount of recombinant protein was done by varied induction time, lactose concentration, temperature for expression and OD of cell culture at initial induction.

4.3.3.1 Incubation time after induction

E. coli BL21 (DE3) pET-15/*pat* was cultured as previously described. The inducing time was varied and the culture was harvested at 4 h and overnight, respectively. After induced by 1 mM lactose, the expressed protein was submitted to 12% SDS–PAGE. Figure 24 showed that the recombinant patatin expression could be observed after induced with 1 mM lactose for 4 h and overnight at equal amount.

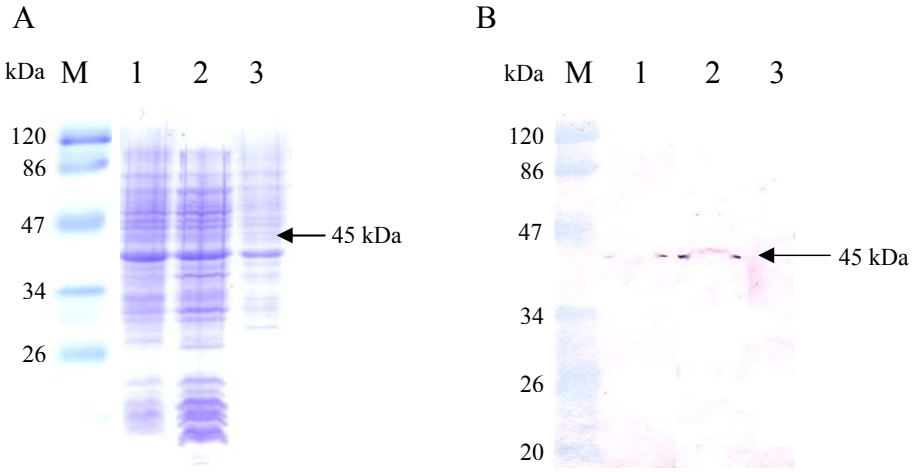


Figure 24 Induction of pET-15b/*pat* expression by 1 mM lactose. (A) SDS-PAGE (B) Western blot. Lane M, protein marker Lane1, 4 h incubation; Lane 2, overnight incubation; Lane 3, no induction.

4.3.3.2 Lactose concentration

E. coli BL21 (DE3) pET-15/*pat* was cultured and induced with lactose to a final concentration of 1.0, 2.0, 3.0 and 4.0 mM, respectively. SDS-PAGE analysis showed that the recombinant patatin protein was expressed when induced with 1.0-4.0 mM lactose (Figure 25).

4.3.3.3 Temperature for expression

In this experiment, the inducing temperature was varied. After induced with 1 mM lactose, cells were induced at 25, 30 and 37 °C, respectively. The expressed protein was then submitted to 12% SDS–PAGE. The Figure 26 showed that the recombinant patatin protein mainly appeared at 37 °C.

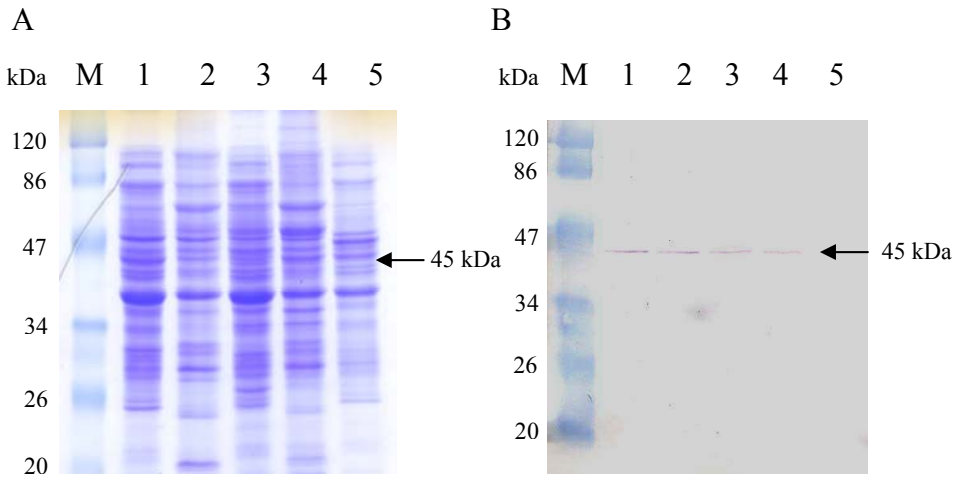


Figure 25 Induction of pET15-b/*pat* expression by various lactose concentrations. A) SDS-PAGE (B) Western blot. Lane M, protein marker; Lane 1, 1.0 mM; Lane 2, 2.0 mM; Lane 3, 3.0 mM, Lane 4, 4.0 mM; Lane 5, no induction.

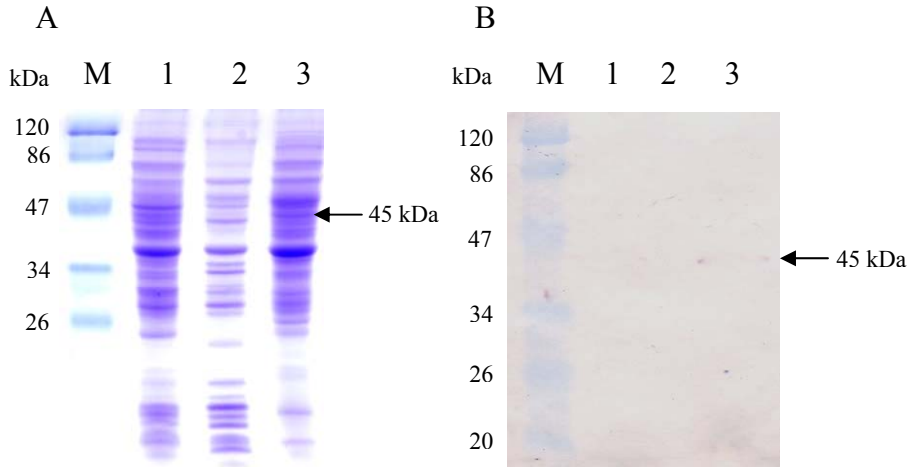


Figure 26 Temperature for induction of pET-15b/*pat* expression after induced by 1 mM lactose. A) SDS-PAGE (B) Western blot. Lane M, protein marker; Lane 1, 25 °C; Lane 2, 30 °C; Lane 3, 37 °C.

4.3.3.4 OD of cell culture at initial induction

E. coli BL21 (DE3) pET-15/*pat* was cultured at 37 °C and 1 mM lactose was added when OD_{600} reached at 0.4, 0.5, 0.6 and 1.0 respectively. The culture was harvested after 4 h and overnight. The expressed protein was submitted to 12% SDS–PAGE. The Figure 27 showed that the recombinant patatin protein was found to be induced at OD_{600} of 0.4-0.6.

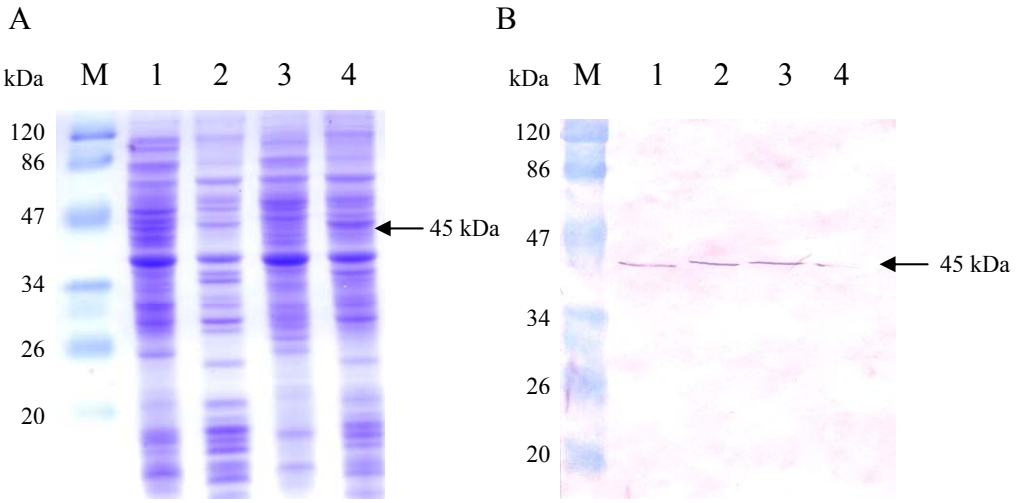


Figure 27 Various OD_{600} of cell cultures in initial induction. A) SDS-PAGE (B) Western blot. Lane M, protein marker; Lane 1, 0.4; Lane 2, 0.5; Lane 3, 0.6; Lane 4, 1.0.

The optimal expressed condition to achieve high amount of recombinant protein was 1.0 mM lactose at OD_{600} of 0.4-0.6 for overnight at 37 °C. When induced cell by optimal condition, the recombinant protein was mostly expressed in whole cell lysate and supernatant. The result in Figure 28B showed that, the recombinant protein fragment was observed at about 45 kDa in induced recombinant cell but not in cell containing pET-15b plasmid and non-induced recombinant cell.

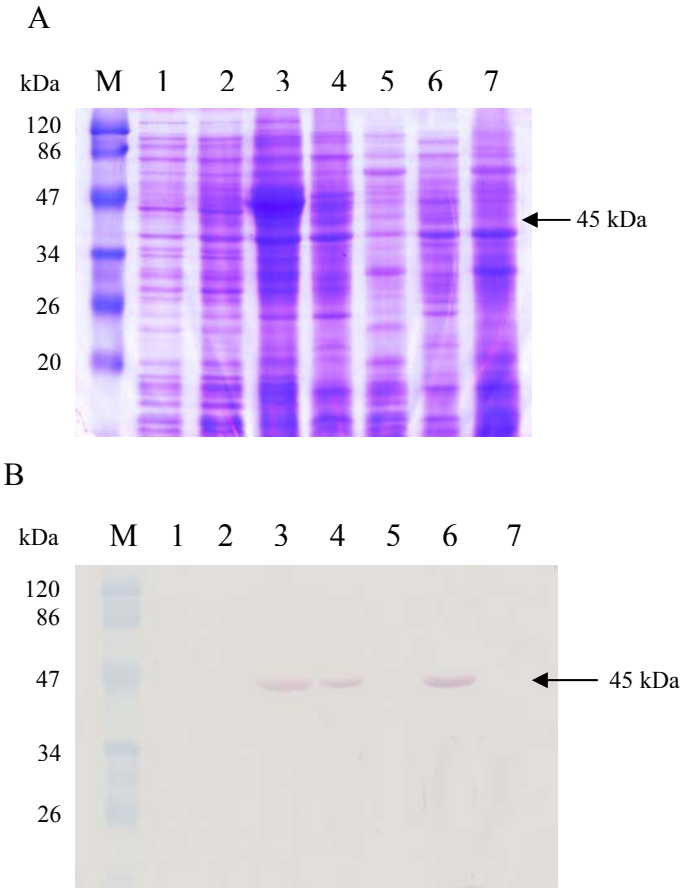


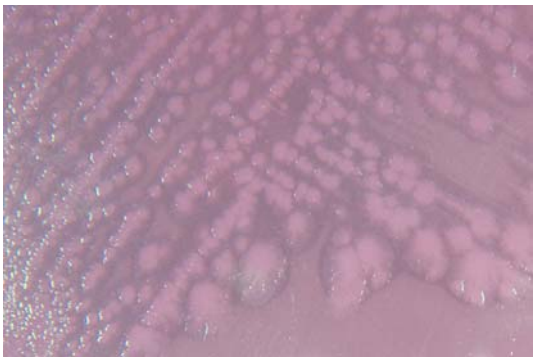
Figure 28 Over expression of patatin gene by SDS-PAGE and western blot analysis. *E. coli* BL21 (DE3) pET-15/*pat* induced by lactose for 4 h and overnight was submitted to 12% SDS–PAGE. (A) SDS-PAGE. (B) Western blot analysis. Lane M, protein marker; Lane 1, pET -15b; Lane 2, pET-15b/*pat*; Lane 3, Whole cell lysate; Lane4, supernatant from pET-15/*pat* induced for 4 h; Lane 5, pellet from pET-15/*pat* induced for 4 h; Lane 6, supernatant from pET-15/*pat* induced for overnight; Lane 8, pellet from pET-15/*pat* induced for overnight.

4.4 Determination of lipolytic activity

The lipolytic activities of *E. coli* BL21 (DE3)/pET-15b/*pat* were tested by plating the recombinant cells onto LB agar containing 1% trioleoylglycerol or 1% tributylglycerol and incubated at 37°C for 1 day, and incubated further at room temperature for another 3 days. After incubation, clear halo zone was detected on

tributylglycerol agar plate (Figure 29A) but not on trioleoylglycerol agar plate (Figure 29B). The result suggested that *E. canis* patatin is able to digest an acyl chain length of four carbon atoms but not digest an acyl chain length of eighteen carbon atoms.

A



B

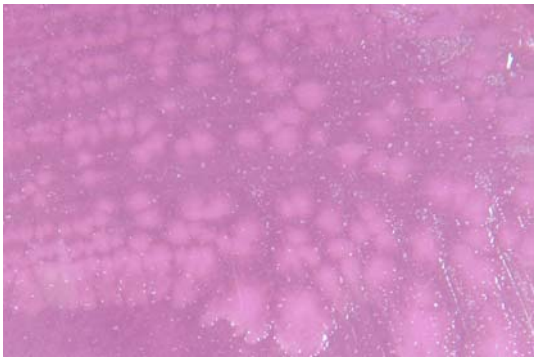


Figure 29 Lipolytic activity tests. (A) Tributylglycerol plate. (B) Trioleoylglycerol plate.

4.5 Purification of *E. canis* patatin

For protein purification, *E. coli* BL21 (DE3)/pET-15b/*pat* was induced by 1.0 mM lactose when OD_{600} reached 0.4-0.6 at 37 °C for overnight. This was the optimal condition to yield the highest amount of patatin protein from supernatant.

E. coli BL21 (DE3)/pET-15b/*pat* was grown to the OD_{600} 0.4-0.6 before induction of 1 mM lactose to express the patatin gene. Cell samples of overnight post-

induction were harvested in the equal volume. The overnight induced cell were harvested and resuspended in BugBuster™ buffer. The cell supernatant after centrifugation was mixed with binding buffer and loaded into HiTrap™ affinity column (Amersham Biosciences, UK). Proteins were washed off with binding buffer containing 20 mM immidazole, and the bound proteins were eluted with elution buffer containing 100-400 mM immidazole. The purities of each purification step were analysed by 12% SDS-PAGE. With many attempts of purification, the patatin was however failed to be purified (data not shown).

4.6 Characterization of *E. canis* patatin

Since attempt to purify *E. canis* patatin was unsuccessful, the protein was then characterized using crude extract.

4.6.1 The optimal pH

The optimal pH of patatin activity from crude extract was determined at various pH values (pH 3.0-12.0). The buffers used for variation of pH were 50 mM sodium acetate (pH 3.0-5.0); 50 mM MES (pH 5.0-7.0); 50 mM HEPES (pH 7.0-8.0) and 50 mM Tris-HCl (pH 7.0-10.0). The production of *p*-nitrophonol products from *p*-nitrophenyl butyrate were monitored at 405 nm. Using *p*-nitrophenyl butyrate as the substrate, patatin exhibited more than 50% of its maximal activity in the pH range of 8.0-9.0, with the highest activity at pH 8.0 (Figure 30).

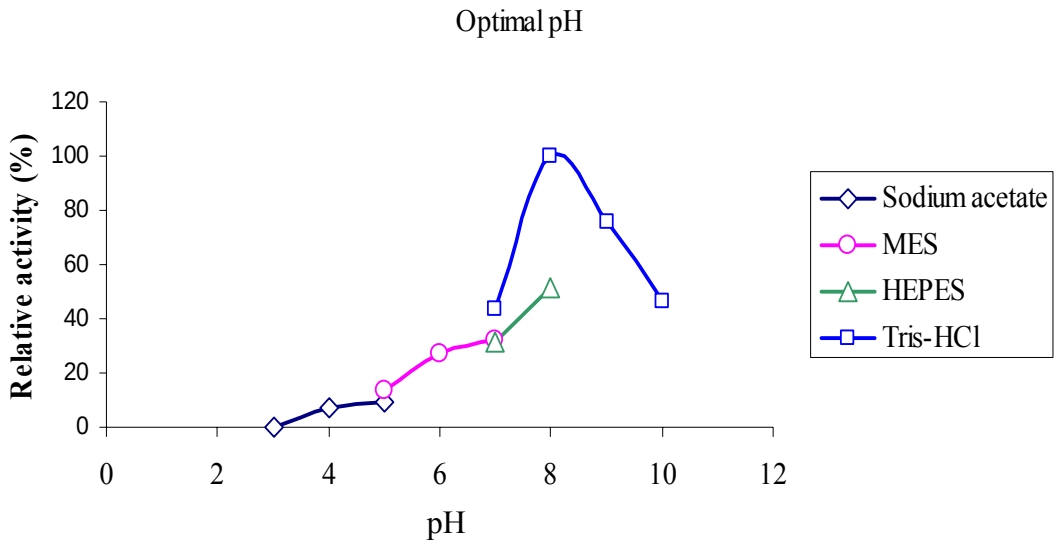


Figure 30 Effect of pH on patatin. The enzymatic assay was performed at pH ranging from 3.0 to 10.0 under standard assay conditions.

4.6.2 The optimal temperature

The effect of temperature on patatin activity was determinated by mornitoring the hydrolysis of *p*-nitrophenyl butyrate at various temperatures ranging from 30-60 °C (Figure 31). The optimal temperature for enzyme activity was determined by assaying enzyme reaction at temperature ranging from 30-60 °C for 15 min in 50 mM Tris-HCl buffer (pH 8.0) containing 0.4% Triton-X and 0.2% gum Arabic and *p*-nitrophenyl butyrate as a substrate. The production of *p*-nitrophenol products from *p*-nitrophenyl butyrate was monitored at 405 nm. Under the conditions used, the enzyme show highest activity at 40 °C. Fifty percent maximal activity was observed between 35-45 °C.

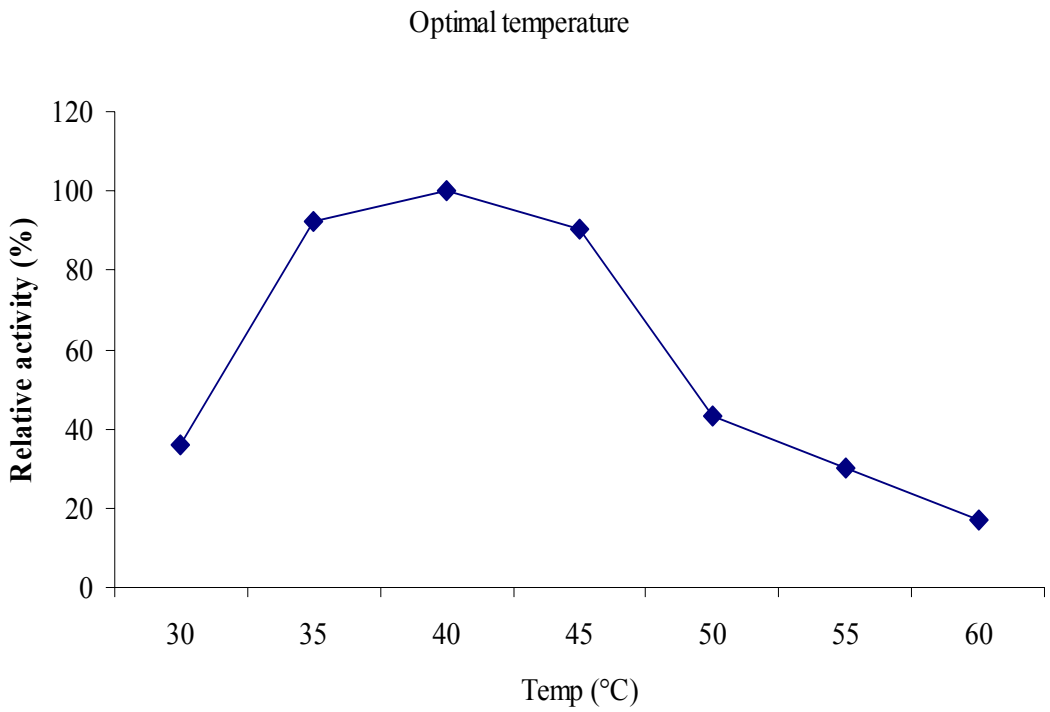


Figure 31 Effect of the temperature on patatin activity.

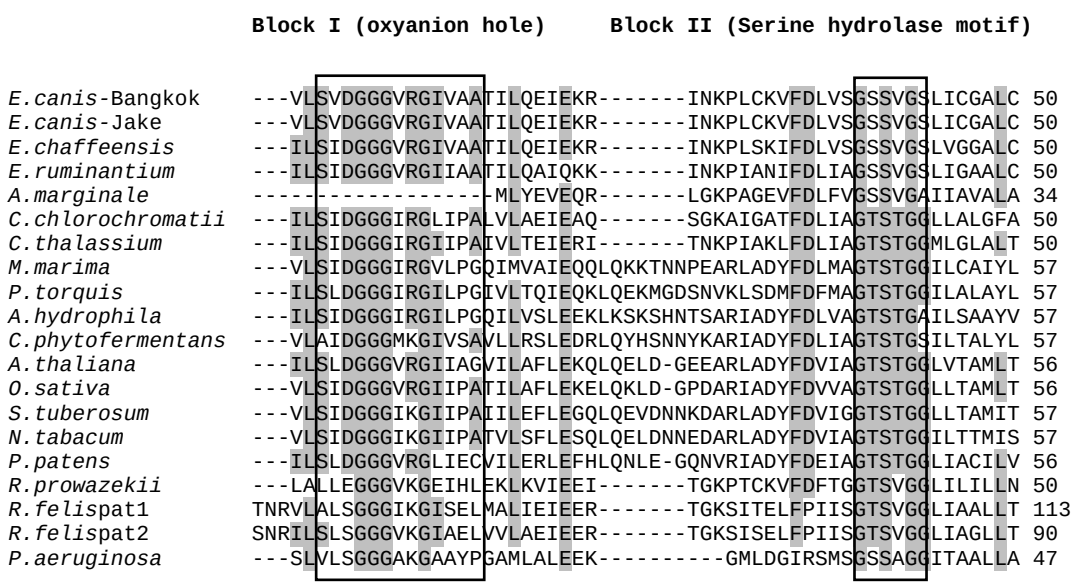
4.7 Patatin sequence analysis

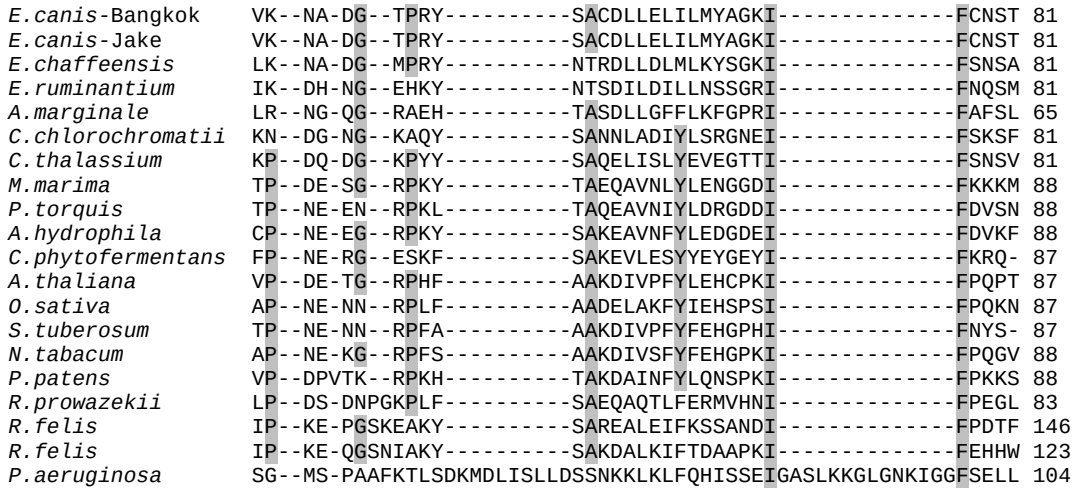
Sequence analysis of *E. canis* patatin gene revealed 948 bp that encoded 361 amino acids with calculated molecular weight of 34.63 kDa. BlastP analysis of patatin revealed a potential patatin-like phospholipase containing the conserved motif of lipolytic enzyme (GXSXG) (Figure 32, Block II). BlastP analysis revealed a similarity between patatin and phospholipase from Rickettsiales including *Anaplasma marginale* str. St. Maries (YP_153631), *Ehrlichia canis* str. Jake (YP_302831), *Ehrlichia chaffeensis* str. Sapulpa (ZP_00544882), *Ehrlichia ruminantium* str. Welgevonden (YP_197059), *Rickettsia felis* (YP_246376) pat1, *Rickettsia felis* (YP_247427) pat2, *Rickettsia prowazekii* (Q9ZCV7); various bacteria including *Aeromonas hydrophila* (YP_857433), *Chlorobium chlorochromatii* (YP_378344), *Chloroherpeton thalassium* (YP_001997190), *Clostridium phytofermentans* (YP_001557258), *Microscilla marina* (ZP_01690449), *Pseudomonas aeruginosa* (O34208), *Psychroflexus torques* (ZP_01254029); and plants including *Arabidopsis*

thaliana (CAA05628), *Nicotiana tabacum* (O24152), *Oryza sativa* (NP_001062044), *Physcomitrella patens* (XP_001771140), and *Solanum tuberosum* (P15477).

Multiple sequence alignments comparision of the *E. canis* patatin to patatin-like phospholipases from other bacteria and plants were adjusted to the length approximately 300 amino acid created by MAFFT program (Katoh *et al*., 2002). *E. ca*nis-Bangkok patatin was 100% identical to *E. canis* str. Jake from USA. *E. canis* patatin sequence showed similarity from 31.1% to 91.5% comparing to patatins from database. It revealed four conserved domain (Block I-IV) similar to those presented in other patatin-like phospholipases from various bacteria (Figure 32).

The alignments of patatin from *E. canis*-Bangkok with other organisms showed that all enzymes possessed the four blocks of conserved amino acid sequences which are characteristics for patatin-like phospholipase group (Benerji and Flieger, 2004). Block I consisted of a glycine-rich region with a coserved arginine residue. Block II located adjacent to Block I and comprised of the hydrolase motif, G-X-**S**-X-G, containing a serine residue located at the putative active-site. Block III contained an A-S-X-X-X-P sequence which conserved in other bacterial patatin-like phospholipase. Block IV contained the putative active site aspartate (lx**D**GgxxaNxnP).


Block I (oxyanion hole)
Block II (Serine hydrolase motif)
E.canis-Bangkok ---VLSVDGGGVRGIVAATILQEIEKR-------INKPLCKVFDLVSGSSVGSLICGALC 50
E.canis-Jake ---VLSVDGGGVRGIVAATILQEIEKR-------INKPLCKVFDLVSGSSVGSLICGALC 50
E.chaffeensis ---ILSIDGGGVRGIVAATILQEIEKR-------INKPLSKIFDLVSGSSVGSLVGGALC 50
E.ruminantium ---ILSIDGGGVRGIIAATILQAIQKK-------INKPIANIFDLIAGSSVGSLIGAALC 50
A.marginale -----------------MLYEVEQR-------LGKPAGEVFDLFVGSSVGAIIAVALA 34
C.chlorochromatii ---ILSIDGGGIRGLIPALVLAEIEAQ-------SGKAIGATFDLIAGTSTGGLLALGFA 50
C.thalassium ---ILSIDGGGIRGIIPAIVLTEIERI-------TNKPIAKLFDLIAGTSTGGMLGLALT 50
M.marima ---VLSIDGGGIRGVLPGQIMVAIEQQLQKKTNNPEARLADYFDLMAGTSTGGILCAIYL 57
P.torquis ---ILSLDGGGIRGILPGIVLTQIEQKLQEKMGDSNVKLSDMFDFMAGTSTGGILALAYL 57
A.hydrophila ---ILSIDGGGIRGILPGQILVSLEEKLKSKSHNTSARIADYFDLVAGTSTGAILSAAYV 57
C.phytofermentans ---VLAIDGGGMKGIVSAVLLRSLEDRLQYHSNNYKARIADYFDLIAGTSTGSILTALYL 57
A.thaliana ---ILSLDGGGVRGIIAGVILAFLEKQLQELD-GEEARLADYFDVIAGTSTGGLVTAMLT 56
O.sativa ---VLSIDGGGVRGIIPATILAFLEKELQKLD-GPDARIADYFDVVAGTSTGGLLTAMLT 56
S.tuberosum ---VLSIDGGGIKGIIPAIILEFLEGQLQEVDNNKDARLADYFDVIGGTSTGGLLTAMIT 57
N.tabacum ---VLSIDGGGIKGIIPATVLSFLESQLQELDNNEDARLADYFDVIAGTSTGGILTTMIS 57
P.patens ---ILSLDGGGVRGLIECVILERLEFHLQNLE-GQNVRIADYFDEIAGTSTGGLIACILV 56
R.prowazekii ---LALLEGGGVKGEIHLEKLKVIEEI-------TGKPTCKVFDFTGGTSVGGLILILLN 50
R.felispat1 TNRVLALSGGGIKGISELMALIEIEER-------TGKSITELFPIISGTSVGGLIAALLT 113
R.felispat2 SNRILSLSGGGVKGIAELVVLAEIEER-------TGKSISELFPIISGTSVGGLIAGLLT 90
P.aeruginosa ---SLVLSGGGAKGAAYPGAMLALEEK----------GMLDGIRSMSGSSAGGITAALLA 47
Conserved motif SxDGGGxrGixxa GtstG


E.canis-Bangkok VK--NA-DG--TPRY----------SACDLLELILMYAGKI--------------FCNST 81
E.canis-Jake VK--NA-DG--TPRY----------SACDLLELILMYAGKI--------------FCNST 81
E.chaffeensis LK--NA-DG--MPRY----------NTRDLLDLMLKYSGKI--------------FSNSA 81
E.ruminantium IK--DH-NG--EHKY----------NTSDILDILLNSSGRI--------------FNQSM 81
A.marginale LR--NG-QG--RAEH----------TASDLLGFFLKFGPRI--------------FAFSL 65
C.chlorochromatii KN--DG-NG--KAQY----------SANNLADIYLSRGNEI--------------FSKSF 81
C.thalassium KP--DQ-DG--KPYY----------SAQELISLYEVEGTTI--------------FSNSV 81
M.marima TP--DE-SG--RPKY----------TAEQAVNLYLENGGDI--------------FKKKM 88
P.torquis TP--NE-EN--RPKL----------TAQEAVNIYLDRGDDI--------------FDVSN 88
A.hydrophila CP--NE-EG--RPKY----------SAKEAVNFYLEDGDEI--------------FDVKF 88
C.phytofermentans FP--NE-RG--ESKF----------SAKEVLESYYEYGEYI--------------FKRQ- 87
A.thaliana VP--DE-TG--RPHF----------AAKDIVPFYLEHCPKI--------------FPQPT 87
O.sativa AP--NE-NN--RPLF----------AADELAKFYIEHSPSI--------------FPQKN 87
S.tuberosum TP--NE-NN--RPFA----------AAKDIVPFYFEHGPHI--------------FNYS- 87
N.tabacum AP--NE-KG--RPFS----------AAKDIVSFYFEHGPKI--------------FPQGV 88
P.patens VP--DPVTK--RPKH----------TAKDAINFYLQNSPKI--------------FPKKS 88
R.prowazekii LP--DS-DNPGKPLF----------SAEQAQTLFERMVHNI--------------FPEGL 83
R.felis IP--KE-PGSKEAKY----------SAREALEIFKSSANDI--------------FPDTF 146
R.felis IP--KE-QGSNIAKY----------SAKDALKIFTDAAPKI--------------FEHHW 123
P.aeruginosa SG--MS-PAAFKTLSDKMDLISLLDSSNKKLKLFQHISSEIGASLKKGLGNKIGGFSELL 104


E.canis-Bangkok V-----RNALSLVFGPKYSDKNLNAVLQEIFGDVTIKDL---------- 115
E.canis-Jake V-----RNALSLVFGPKYSDKNLNAVLQEIFGDVTIKDL---------- 115
E.chaffeensis A-----RNAFALIFGPKYSDKNLNSVLKEIFGDVAMKDL---------- 115
E.ruminantium I-----NKVISVVVGPMYSDKNLNAVLKEVFGDSTMNDL---------- 115
A.marginale V-----RQALSVVVGTRFSPKNLENTLSGFFSNLKMGNV---------- 99
C.chlorochromatii L---KSVASVEGLRDELYSANGIEHVLDDYFGDDPLSSC---------- 117
C.thalassium W---YRIPAIGNLTEEKYKVQGLEHVLNEYLGETMLSEA---------- 117
M.marima F-------SFGGITNEKYPSAPMEEALEKYLGNAKLSEM---------- 120
P.torquis W---QKIKSLNGLADEKYNASELEEALEDTFGELKLSNL---------- 124
A.hydrophila W---RSIGTLGGTSDEKYSAKELERVLMEAFGETKLSEL---------- 124
C.phytofermentans --------KFYPFWGPKYTNKYLEEMLLKYFGDATLGSL---------- 118
A.thaliana GVLALLPKLPKLLSGPKYSGKYLRNLLSKLLGETRLHQT---------- 126
O.sativa WVLSKIAGTLRMVSGPKYDGKYLHSLLREKLGDTRLDKA---------- 126
S.tuberosum ----------GSILGPMYDGKYLLQVLQEKLGETRVHQA---------- 116
N.tabacum W---------PPILGPKYDGKYLHKVLEDKLGETRLHQT---------- 118
P.patens L-----RSLITRLTGPKYKSAPLETILKEVVGDLKLTET---------- 122
R.prowazekii T--FRKLWSCNGLFSYKFSPDPLVKLLKKYCKDYTLKDL---------- 120
R.felis L------GSVKQLFTHKYSQKPLKELLEKYLGDNRMDNT---------- 179
R.felis Y------DGIKQIFTHKHSQGPLKEILEQHLAGLRLDDT---------- 156
P.aeruginosa L---------NVLPRIDSRAEPLERLLRDETRKAVLGQIATHPEVARQPTVAAIASRLQS 155

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E.canis-Bangkok     ------------------IADFIVPSYDLCSNQT--IMFRSWI---DKYSDIKVCDVTRA 152
E.canis-Jake        ------------------IADFIVPSYDLCSNQT--IMFRSWI---DKYSDIKVCDVTRA 152
E.chaffeensis       ------------------MTNFIVPSYDLCSNQT--VMFRSWV---DKYHDIKVSDVTRG 152
E.ruminantium       ------------------MVNFIVPSYNLYSNQT--VMFRSWV---KKYQNIKIRDVARA 152
A.marginale         ------------------TANIMIPSYDLHTGYT--FMMRNWE---PKFQDLKLVDVLLA 136
C.chlorochromatii   ------------------ITKSLVTCYDIQNREP--LFLKSWR---EEYQSVLMKHAARA 154
C.thalassium        ------------------MTNLLVSSYEIERRIP--FFFKSVRAKEFVDYDFPMKIVARA 157
M.marima            ------------------IKECLITSYDIERSNP--HFFKRHKAIDNKGYDFYMRDVARS 160
P.torquis           ------------------LKPCIISSYDIRNGKP--HFFKQHKSN-NDIYNFKIKDVARA 163
A.hydrophila        ------------------LKPTCFVSYDVDRREP--RIFKQHTAI-KNQKDFLVRELLRG 163
C.phytofermentans   ------------------RKPCLMTSYDTTTRSA--VFFNSVTGRKDENRNYLLRDAILA 158
A.thaliana          ------------------LTNIVIPTFDIKKLQP--TIFSSYQLLVDPSLDVKVSDICIG 166
O.sativa            ------------------LTNVVIPTFDIANLQP--TIFSKFELKYKPLKNALLSDISIS 166
S.tuberosum         ------------------LTEVAISSFDIKTNKP--VIFTKSNLAKSPELDAKMYDICYS 156
N.tabacum           ------------------LTNVVIPTFDMKKFQP--IIFTKSEIANSPHLDAKMSDICYG 158
P.patens            ------------------VKPIIIPSYDINYQSS--VLFSTTQAISKQIPDCFIRDVCRA 162
R.prowazekii        ------------------IGEVIVTGYDLNNQQNPLITFSTIEARKSQDNNYYLSDIIQG 162
R.felis             ------------------TSRLVIPVNDLTTNGGELEIFDSFHGYS---PHVRVKDVLLA 218
R.felis             ------------------TSRLIIPVTDLASKDKEVKIFDSHDSYS---PHIRVQDVLLA 195
P.aeruginosa        GSGVTFGDLDRLSAYIPQIKTLNITGTAMFEGRPQLVVFNA-----SHTPDLEVAQAAHI 210

Conserved motif                                                              A
```

Block III (conserved proline) **Block IV (active site aspartic acid)**

```
E.canis-Bangkok     AVAAPTYFTPKKMIVD--------NKKQLLVDSAIVCN-NPVIAAYSAAQ-----VL--- 195
E.canis-Jake        AVAAPTYFTPKKMIVD--------NKKQLLVDSAIVCN-NPVIAAYSAAQ-----VL--- 195
E.chaffeensis       AVAAPTYFTPKKIIVE--------GKKTLLIDSSIVCN-NPIIAAYAGAQ-----VL--- 195
E.ruminantium       AVAAPTYFTPYELVID--------NKKELLIDSSLVSN-NPIIEAYAAAQ-----VL--- 195
A.marginale         ASAAPTIFPPRNVVIQ--------NTKCCMIDSGLVAN-NPSICGYAASS-----VL--- 179
C.chlorochromatii   TSAAPTYFEPALIPIG--------GATKALVDGAVYIN-TPSVSAYAEAL-----KL--- 197
C.thalassium        TSAAPTYFKPLKLHTQ------GLQEYYALVDGSVFAN-NPAMCAFVEAK-----SM--- 202
M.marima            TSAAPTYFEPNHATSF-------AEVKYALIDGGVYVN-NPTLCAYAATR-----KL--- 204
P.torquis           TSAAPTYFEPARVKND-------LGTPYPLIDGGVFVN-NPSLVAYSEVR-----SM--- 207
A.hydrophila        STAAPTYFEAARIYST-----SPLKQKFVLVDGGVVAN-DPALCAYSEAV-----SM--- 209
C.phytofermentans   STAAPTYFPPSCFHAK-----DNCYN--QLIDGGVFAN-NPTLCALIEAM-----KL--- 201
A.thaliana          TSAAPTFFPPHYFSNE---DSQGNKTEFNLVDGAVTAN-NPTLVAMTAVS-----KQIV- 216
O.sativa            TSAAPTFFPAHYFETK---DDNGQTREFNLVDGGVAAN-NPTLCAMSQVS-----KYIIL 217
S.tuberosum         TAAAPIYFPPHHFVTH---TSNGARYEFNLVDGAVATVGDPALLSLSVAT-----RLA-- 206
N.tabacum           TAAAPTYFPPYYFEND---DGKGNQHEFNLIDGGVVAV-NPALIAVSTVT-----K---- 205
P.patens            TTAAPTYFAPYYYTSI---TDEGERIEFNLVDGGVAAN-NPCLRGKVTRT---------- 208
R.prowazekii        ITAAPGYFPSHHFSNI------TNTKVHTIIDGGIYAN-DPTLQTWQLLK---------- 205
R.felis             TTAAPTYFKPIMDKAAVQGHEYASGTPYTYADGGLDAN-RPANEVLKLLKKGYIHKEQSH 277
R.felis             TTAAPTYFKPVTNQEHIKGYENQEDALYAYADGGLGAN-RPAYEALKILKNGH------- 247
P.aeruginosa        SGSFPGVFQKVSLSDQ---PYQAGVEWTEFQDGGVMIN-VPVPEMIDKNFDSGPLRR--- 262

Conserved motif     xxAAP                       lxDGgxxaNxnP
```

```
E.canis-Bangkok     ------------YPN-EKICCLSVGCGTV---SQSFSD--------LQNSLLYWSR---- 227
E.canis-Jake        ------------YPN-EKICCLSVGCGTV---SQSFSD--------LQNSLLYWSR---- 227
E.chaffeensis       ------------YPN-EKLCCLSVGCGTV---NKDFSD--------LQNSLLYWSS---- 227
E.ruminantium       ------------YPS-DTIYCLSIGCGTV---NMDFSH--------VQNSLLYWGS---- 227
A.marginale         ------------YPG-EEVYFLSVGSGER---SKPVLR--------VRDSLAFWAL---- 211
C.chlorochromatii   ------------FEDEQDFFVLSLGTGEL---IRPISY-----DKSKNWGKAEWVV---- 233
C.thalassium        ------------FPDAEDFLMVSLGTGDV---NFVQTY-----QDDKGWGLIQWAE---- 238
M.marima            -----DFGEDKIKPTASEMMMVSIGTGST---KYSYEY-----EKAKDWGAIGWIK---- 247
P.torquis           -----TFENMENFPSAKNMMIVSIGTGSV---SKGYEY-----KKAKDWGAIGWIK---- 250
A.hydrophila        -----------GVSGIKDMIIVSIGTGKK---LKNYSY-----SDVKDWGPLGWAK---- 246
C.phytofermentans   ----------PGCDGIGDTICLSVGNVKN---TKSYTY-----QKVRRFGLLQWAV---- 239
A.thaliana          --KNNPDMGKLKPLGFDRFLVISIGTGSTKR-EEKYSA-----KKAAKWGIISWLYDDGS 268
O.sativa            EDKEDCDFFPVKPTEYGKFMVISIGCGSNH--DQKYKA-----KDAAKWGIFNWLIKGSS 270
S.tuberosum         --QEDPAFSSIKSLDYKQMLLLSLGTGTNSEFDKTYTA-----EEAAKWGPLRWML---- 255
N.tabacum           --SVDPSVASIKPLDVKQVLLLSLGTGTTADFAGTYTA-----KEADNWGLVSWLFHNNS 258
P.patens            -----PEETVKRLQKYEDLLVLSLGTGRT---TVSYAA-----KNAAKWGTLSWIYNNGN 255
R.prowazekii        ----------ENKCIIDNALYLSLKE------ENNADY-----KTVCCGGGILELLKN-- 242
R.felis             IREEKTLTREEQKEILDNTMVCAFNFSNDI--EPTSSI-----PKIGFDGVIGWLVKG-- 328
R.felis             -------SREENAKILDDTMVLSLNFDNDH--KASSSI-----PKIGFDGVIGWLVKG-- 291
P.aeruginosa        ----------------NDNLILEF-EGEAGEVAPD--------RGTRGGALKGWVVGV-- 295
```

```
E.canis-Bangkok     -KILCVIIDAGLEAIDYEMARLVK---GEDS--YCRISGD-IVYSACDFGDASQENIKNL 280
E.canis-Jake        -KILCVIIDAGLEAIDYEMARLVK---GEDS--YCRISGD-IVYSACDFGDASQENIKNL 280
E.chaffeensis       -KILFVIIDAGLDAIDYQMARLVK---GEDT--YCRISGD-IIYSTCDFSDASPGNIQNV 280
E.ruminantium       -KIIFVIIDAGLDAVDYKMERLVK---EGDK--YFRMSGD-VKYATHDFSDATPMNIKNV 280
A.marginale         -NVANVFLDAGMDAVDYQMTRMV----GNYR--YTRITGF-LNRASHNFTDASRKNMQAL 263
C.chlorochromatii   -PLLSCMFDGMADAANYQMKMLLD-----DK--YVRLQTN-LSVASDDLDNVTANNLENL 284
C.thalassium        -PLLDIIVHGSDLSVNYQMSQLLTNTDGFKR--YYRFQPK-LSERHAEIDNTSKTNIRML 294
M.marima            -PLIDIMMKGVSQTVDYQLKQIFDAVGKPDQ--YYRIEPK-LVHAVSGMDNAGKENLINL 303
P.torquis           -PIIEIMMSGNSKTVHHHLKQIFGTLEEQDQKDYHRLEPE-IITADTEMDNASLENLQKL 307
A.hydrophila        -PVIDITLEGGPQMTEYYLKQIASTV-PNSK--YFRIQPE-LYGADPALDNATRENLEKL 300
C.phytofermentans   -PIFDILMDASEQTVDYQLKKIYKSVNHQQY--YYRMVLN-TEEEIPKMDDCSKEAVHKL 295
A.thaliana          TPILDITMESSRDMIHYHSSVVFKALQSEDK--YLRIDDDTLEGDVSTMDLATKSNLENL 326
O.sativa            APIIDMFTSASADMVDIHLGVLFSALQCEKN--YLRIQYDQLTGSAGSIDDCSKENMDNL 328
S.tuberosum         -AIQQMTNAASSYMTDYYISTVFQARHSQNN--YLRVQENALNGTTTEMDDASEANMELL 312
N.tabacum           NPLIEMSSEASVIMNDYYIATIYRALGAETN--YLRIEENALTGTTTQMDNATEANMNLL 316
P.patens            VPLLDMLLLASQDIVDYNMSNTFYEQCSHDN--YLRIQTEELEESQMQLDNSSQSNLQSL 313
R.prowazekii        -NMPNKILAATQQADEITAQRIFG-----NK--LIEIPTY-IPEQHAEMSNSSIENLQAL 293
R.felis             -KLVSRLMNNMENSSTIEVKN---DLSGEDE--FFEIGLP-ITKETESLDDASPKNIEKL 381
R.felis             -KLVSRLMQSSEDSSTAEVRV---GLPGEDE--FVEVKLP-ITKETSSLDNSSLKNIEAL 344
P.aeruginosa        -PALQAREMLQLEGLEELREQTVVVPLKSERGDFSGMLGGTLNFTMP--DEIKAHLQERL 352
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Figure 32 Multiple sequence alignments of the amino acid sequences of patatin-like phospholipase enzymes. Multiple sequence alignments comparing the isolated *E. canis*-Bangkok patatin to patatin-like phospholipase gene from BlastP analysis revealed a similarity between patatin and phospholipase from Rickettsiales including *Anaplasma marginale* str. St. Maries, *Ehrlichia canis* str. Jake, *Ehrlichia chaffeensis* str. Sapulpa, *Ehrlichia ruminantium* str. Welgevonden, *Rickettsia felis* pat1, *Rickettsia felis* pat2, *Rickettsia prowazekii*, various bacteria including *Aeromonas hydrophil*, *Chlorobium chlorochromatii*, *Chloroherpeton thalassium*, *Clostridium phytofermentans*, *Microscilla marina*, *Pseudomonas aeruginosa*, *Psychroflexus torques* and plants including *Arabidopsis thaliana*, *Nicotiana tabacum*, *Oryza sativa*, *Physcomitrella patens* and *Solanum tuberosum*.

4.8 Phylogenetic tree analysis

E. canis-Bangkok patatin amino acid sequence and other patatin were used to generate a phylogenetic tree using the neighbor-joining method by MEGA software (version 3.1). Phylogenetic tree analysis revealed relationship between patatin from various bacteria and plants. The result showed that patatin divided into three groups composed of order Rickettsiales, bacteria and plants. Patatin of *E. canis*-Bangkok was the most closely related to *E. canis* str. Jake. The resultant phylogenetic

tree revealed that *E. canis*-Bangkok was grouped tightly within genus *Ehrlichia* included the tick-borne anaplasmal parasites *E. chaffeensis*, *E. ruminantium* and *A. marginale*, and was clustered in Rickettsiales group (Figure 33). Interestingly, patatins from other bacteria closely grouped to those of plants more than those of bacteria in order Rickettsiales (Figure 33).

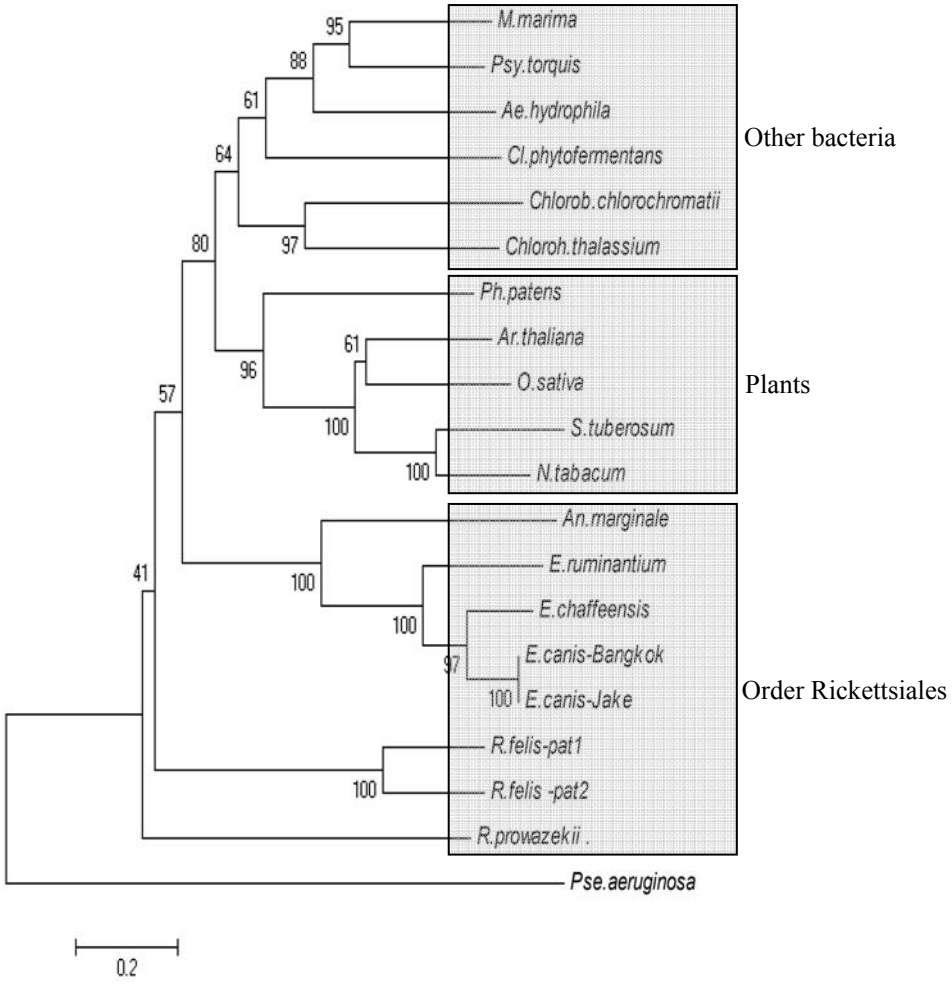

Figure 33 Phylogenetic tree based on patatin amino acid sequences. Sequences from the *Ehrlichia* and other genera (see text for details) were compared with the neighbor-joining method operated by MEGA software (version 3.1). Scale bar indicates the number of mutations per sequence position. The numbers at the nodes represent the percentage of 1000 bootstrap re-samplings.

DISCUSSION

Characterization of *E. canis* and related genera from canine blood

Understanding the epidemiology of the diseases and infectious cycles of anaplasmal organisms is expected to facilitate understanding of similar diseases, including zoonoses that may share similar etiologic agents or vectors. These results support previous studies that *E. canis* and *A. platys* are uniform worldwide, suggesting that biological observations with different strains and from different regions could be laterally applicable. However, it is important to note that these observations are based on the highly conserved *16S rRNA* gene. Therefore, further work with more divergent sequences form other genes such as heat shock protein gene (*groESL*) (Sumner *et al*., 2000), citrate synthase (*gltA*) (Inokuma *et al*., 2001) and outer membrane protein (Felek *et al*., 2003) is needed to confirm whether these strains are indeed uniform. Moreover, it is important to remember that potential heterogeneity of vertebrate and invertebrate hosts from different locations is likely to influence the biology of these organisms. Thus global analyses of evolutionary patterns among *E. canis* and *A. platys* will likely require parallel analyses of such patterns among their reservoirs and vectors. *E. canis* and *A. platys* are distributed globally, and it is generally assumed that all strains primarily utilize dogs and rhipicephaline ticks as vertebrate and invertebrate hosts (Breitschwerdt, 1990). However, closely related anaplasmal pathogens, including other monocytotropic species, utilize much different mammalian hosts and tick vectors. For example, *R. sanguineus*, which is considered an important vector of both pathogens described in this report, is considered highly host-specific in some regions but reportedly feeds on diverse medium to large mammalian hosts (including humans) in other areas. Susceptibility of these tick strains to various pathogens, including *E. canis* and *A. platys*, could explain reports suggesting *E. canis* infections of ruminants and humans (Yu *et al*., 2007). Alternatively, similarities among geographically diverse *E. canis* strains could be reflective of relatively recent global spread of this pathogen with its vertebrate and invertebrate hosts (Yu *et al*., 2007).

This report describes molecular analysis of nearly complete *16S rRNA* sequences of *E. canis* and *A. platys* from Bangkok, Thailand. The sequence alignments and phylogenetic tree suggested low diversity within *E. canis* and *A. platys* strains based on the close similarity amongst their *16S rRNA* sequences from all geographic regions tested, and these conclusions are consistent with other reports (de la Fuente *et al*., 2006; Aguirre, *et al*., 2006; Yu *et al*., 2007). Genetic polymorphisms from comparison of *16S rRNA* sequences based on the *E. coli* J01695 numbering system (Konings and Gutell, 1995) also indicated that *E. canis*-Bangkok and *A. platys*-Bangkok were structurally conserved in *16S rRNA* architecture. Therefore, all polymorphisms observed in these experiments are consistent with these two species. However, although nucleotide differences at many positions indicated that *E. canis*-Bangkok and *A. platys*-Bangkok shared some structure with other bacteria, other nucleotides were different from most eubacteria.

Close similarity among *16S rRNA* of *A. platys* from different locations worldwide supported the hypothesis that *A. platys* strains are not geographically segregated (Huang *et al*., 2005). Interestingly, the sequence of *A. platys*-Bangkok reported from this study had two nucleotide substitutions compared to a previously characterized *A. platys* strain from Thailand (Suksawat *et al*., 2001a) at positions 1,025 and 1,192 (Table 5) that appeared through G/A and T/C transitions, respectively, suggesting that there might be at least two *A. platys* strains enzootic to Thailand.

The phylogenetic tree separated two major clusters of *Ehrlichia* spp. and *Anaplasma* spp. As expected, *E. canis*-Bangkok was within the *Ehrlichia* clade and *A. platys*-Bangkok was within its respective clade. Within these two clusters, *E. canis* and *A. platys* strains grouped mostly in multiple connected branches. Although they were from geographically diverse regions, little genetic diversity was observed, suggesting slow and homogeneous evolution (Keysary *et al*., 1996). These results were in concordance with previous reports of slight genetic variation among *16S rRNA* from different *E. canis* strains (Parola *et al*., 2003; Unver *et al*., 2003; Aguirre *et al*., 2004) and *A. platys* strains (Unver *et al*., 2003; Huang *et al*., 2005; Martin *et*

al., 2005; de la Fuente *et al*., 2006). Notably, the *E. canis*-Bangkok *16S rRNA* was identical to VDE and VHE that were respectively isolated from a dog and a human in Venezuela (Unver *et al*., 2001a), which suggests little differentiation among *E. canis* between these geographic locations and tempts speculation about potential similarities in the epidemiology of these strains, but further analyses of less conserved sequences are needed to test this idea. This phylogenetic analysis also suggested that the *E. canis* was more closely related to *E. ewingii* than to *E. chaffeensis*, *E. muris* and *E. ruminantium*, and that *A. platys* was more closely related to *A. phagocytophilum* than to *A. bovis*, *A. centrale*, *A. marginale* and *A. ovis*, which both corroborate an earlier report (Yu *et al*., 2001).

Characterization of *E. canis* and related genera in feline blood

Bloodstream infections are an important cause of morbidity and mortality in animal. Blood culture is clearly the most important diagnostic procedure for identifying micro-organisms involved in blood stream infections. Ideally, blood samples should be taken immediately prior to the start of empirical antimicrobial treatment. However the blood culture is low and insufficiently sensitive when the patient has previously received antibiotics or in the presence of slow-growing or intracellular micro-organism (Peters *et al*., 2004). Detection of micro-organisms, mainly in blood using pathogen-specific or broad-range PCR assay is promising, however, it is very important to emphasize that the interpretation of this molecular tool is critical because of the risk of interfering contamination, understanding the necessity to interpret the results obtained with caution (Fenollar and Raoult, 2007). In uncultured bacteria, PCR assay can be very useful because infection can be detected before seroconversion or positive culture has occurred (Fenollar and Raoult, 2007). Several PCR techniques have been used for the diagnosis of rickettsioses. Nested-PCR techniques have been described in order to increase the analytical sensitivity of the test (Apfalter *et al*., 2005). Recently, quantitative real-time PCR assays have been developed for the diagnosis of infection caused by *Orientia tsutsugamishi*, SFG and TG rickettsiae (Jiang *et al*., 2004; Singhsilarak *et al*., 2005; Stenos *et al*., 2005). In addition, quantitative real-time PCR assays using species-specific probes targeting

Rickettsia prowazekii, *R. typhi* and *R. felis* are also available (Svraka *et al*., 2006; Henry *et al*., 2007).

In this study, amplified *16S rRNA* genes were from feline blood samples by primers designed specific to *Ehrlichia* and *Anaplasma*, the results showed PCR sequences of *Bartonella, Ochrobactrum and Sphingomonas*. *Bartonella* species cause a wide spectrum of cat diseases (Koehler *et al*., 1994; Chomel *et al*., 1995) especially *B. henselae* and *B. clarridgeiae* that can cause disease in cat and human (Heller *et al*., 1997). *Ochrobactrum* is member of the soil microbial (Lebuhn *et al*., 2000) and increasing number of studies has reported the isolation of *O. anthropi* and *O. intermedium* from clinical specimen (Moller *et al*., 1999). The natural habitat of *Sphingomonas* has not been totally defined, but it is widely distributed in the natural environment especially in water and soil (Hsueh *et al*., 1998) and also been recovered from hospital environments including tap water, distilled water, neubulizers, respirators, dialysis fluid, and other equipments (Glupczynski *et al*., 1984).

Bartonella species are Gram-negative, fastidious bacteria, belonging to the alpha-proteobacteria. Three species, *B. henselae* (formerly *Rochalimaea henselae*), *B. koehlerae* and *B. clarridgeiae* have been isolated from domestic cat (*Felis catus*) which is the only known reservoir of the organisms (Spach and Koehler, 1998; Jensen *et al*., 2000; Yamamoto *et al*., 2002). These three species may cause disease in people after being transmitted by scratches or bites from infected cats or by the cat flea, *Ctenocephalides felis* (Chomel *et al*., 1996). In human, *B. henselae* is an agent of cat-scratch disease (CDS) in which there is inflammation of the lymph nodes draining the site of a cat scratch or bite (Regnery *et al*., 1992) and now *B. henselae* can be detected in the dental pulp of stray cats (Aboudharam *et al*., 2005). Our study showed that the molecular detection of DNA of *Bartonella* spp. is possible in cats. Other investigators have also reported the recovery of unique *Bartonella* isolates (Koehler *et al*., 1994) or DNA (Bergmans *et al*., 1996) from cat blood. Bergmans and coworkers submitted a partial *16S rRNA* sequence (GenBank accession no. Z69039) derived from bacterial DNA extracted from cat blood (Bergmans *et al*., 1996).

Ochrobactrum belonging to Family Brucellaceae (de Ley *et al*., 1987). Normally, *Ochrobactrum* spp. are members of the soil bacteria (Lebuhn *et al*., 2000), and an increasing number of studies have reported the isolation of *O. anthropi* and *O. intermedium* from clinical specimen. Using highly conserved sequences such as the RNA gene or housekeeping protein such as the GroEL chaperonin and RecA phylogenetic trees have been constructed which have allowed the definition of the alpha subgroup of the proteobacteria (Woese, 1987; Eisen, 1995).In this study, suggested that, *Ochrobactrum* spp. infection might be contaminated from medical device or opportunistic infection in cats.

Sphingomonas paucimobilis (formerly *Pseudomonas paucimobilis*) (Yabuuchi *et al*., 1990) is a yellow-pigmented, aerobic, motile with polar flagellum, non-fermentative, Gram-negative bacteria (Morrison and Shulman, 1986). *S. paucimobilis* is an opportunistic pathogen, which has been isolated from hospital infections and is considered to originate from contaminated hospital equipment or manipulation of some medical devices (Hajiroussou *et al*., 1979; Reina *et al*., 1991). However, *S. paucimobilis* has since been isolated in pure culture from a leg ulcer (Peel *et al*., 1979), from the blood of a septicemia man and from the cerebrospinal fluid of a man with acute meningitis (Hajiroussou *et al*., 1979). Bacteria belonging to the genus *Sphingomonas* have been intensively studied because many members of this genus are known to be decomposers of aromatic compounds such as pentachlorophenol (Karlson *et al*., 1995; Nohynek *et al*., 1996), hexachlorocyclohexane (Imai *et al*., 1991), chlorinated biphenyl, and dibenzo-*p*-dioxin (Wittich *et al*., 1992), These bacteria are therefore expected to be used for bioremediation of the environment (Nohynek *et al*., 1996; Yrjala *et al*., 1998). However, there have been rare reports of endemic and epidemic animal infections due to this organism. To our knowledge, this is the first time that *S. paucimobilis* bacteremia has been epidemiologically linked cat blood stream using molecular typing methods.

Topology prediction of drug resistance gene

In *E. canis* strain Jake genome showed a protein with transmembrane helices, Bicyclomycin resistance gene (*Bcr*), which associated with drug resistance transporter. *Bcr* from *E. canis*-Bangkok was isolated by polymerase chain reaction using primers designed from the genome sequence of *E. canis* strain Jake. 386 amino acids sequence of Bcr was first scanned for signal peptide. It is important to identify the signal peptide prior to analysis of transmembrane type of proteins because these leader peptides that target a protein for export contain a hydrophobic region that can easily be mistaken for a transmembrane region by a prediction program (Krogh *et al*., 2001).

Bcr is a drug efflux proteins belonged to major facilitator superfamily (MSF) (Paulsen *et al*., 1996; Putman *et al*., 2000; Paulsen, 2003). Hydropathy and phylogenetic analyses of several MSF revealed that they shared a common structure and could be divided into 12 and 14 transmembrane segments (TMS) (Paulsen *et al*., 1996; Putman *et al*., 2000; Paulsen, 2003). Topology of Bcr were predicted with five different computer program. SOSUI has been originally developed to predict TM alpha-helices by using hydropathy plot model to distinguish between transmembrane and soluble proteins (Arai *et al*., 2003). However, HMMTOP, TMHMM and TMMOD employed hidden Markov models (HMMs) for more accurate topology prediction. Like hydropathy plot and neural network methods, they compute both sequence to structure and structure to structure relationships. The differences are that in HMMs the two steps are joining together into one integrated model (Punta *et al*., 2007). On the basis of the HMMs analysis we, therefore, regard the eleven membrane-span model as the most likely for the *E. canis*-Bangkok Bcr protein.

E. canis-Bangkok showed motif A, B and C that conserved in MFS family. These signature motifs are typical characters of both 12-TMS and 14-TMS families of the MSF (Paulsen *et al*., 1996; Putman *et al*., 2000) indicated that *E. canis*-Bangkok Bcr protein and those of rickettsia species were membrane transporters belonging to MSF. However, motifs D2 and G specific for 12-TMS family, and motifs D1, H, E

and F specific for 14-TMS family, could not be identified in 11-TMS family of drug transporters of rickettsias. It could, therefore, be suggested that transporters with a number of TMS smaller than 12 may be also involved in drug transport and these 14 proteins may represent a new 11-TMS family. With highly conserved sequences of motifs A, B and C has allowed us to define more precisely the sequence motif A (GPLSDxyGRrpxmL), motif B (LIxxRFiQGxGAG) and motif C (SPxx[GA]P[VI]xGSxI) specifically for the drug efflux proteins of *Ehrlichia* and the closely related genera *Anaplasma*, *Neorickettsia*, *Orientia* and *Wolbachia*. These signatures will be very useful for identification of a new member of the drug transporters of the 11-TMS family from other rickettsia species. The conserved of motifs A, B and C were also detected around N-terminal half of the proteins but lower similarity was observed at the C-terminal half (data not shown). It was previously proposed that N-terminal region of MFS proteins involved primarily in proton translocation, whereas, C-terminal half involved primarily in substrate recognition (Paulsen *et al*., 1996). The results supported that the proposed 11-TMS family of *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Orientia* and *Wolbachia* were likely to represent a new family of MSF.

Characterization of gene involved in pathogenicity

Patatin is the major storage glycoprotein found in potato tubers, but also exhibits phospholipase A_2 activity for protection from infection. Proteins containing patatin-like domains are more frequently found in pathogenic than in non pathogenic bacteria (Ogata *et al*., 2005). Phospholipase from prokaryotes share a group of conserved amino acids, including a serine in a highly conserved G-X-S-X-G pentapeptide and an aspartate or glutamate residue that is hydrogen bonded to a histidine to form a catalytic triad. Based on comparison of amino acid sequences and biological properties, prokaryote-derived lipases have been classified into eight different families composed of true lipase, GDSL family, family III, Hormone-sensitive lipase (HSL) family, family V, family VI, family VII and family VIII (Arpigny and Jaeger, 1999). Phospholipases are considered as potential bacterial virulence factors which might promote the establishment of the severe pneumonia due

to their capability of destroying lung surfactant, a phospholipid monolayer in the alveoli, essential for the stability of the lung. Furthermore, phospholipases could be involved in the remodeling and lysis of phagosomal membrane and might generate reaction product capable of interfering with the signal transduction of the host (Fischer *et al*., 2001). Thus, it was of interest to investigate this enzyme in *E. canis*.

In this study, *E. canis* patatin showed phospholipase activity that found in other intracellular bacteria usefulness to escape from phagocytosis. Wisseman and Waddell showed that escape from the phagosome is dependent on the virulence of the strain. Although virulent and avirulent strains of *Rickettsia prowazekii* multiply equally well in irradiated chicken embryo cells (Wisseman and Waddell, 1975), only the virulent strain escapes the phagosome in human macrophages. A phospholipase appeared to be involved in the mechanism of escape (Winkler and Miller, 1980). Patatin-like proteins might be responsible for the phospholipase A_2 activity identified in *Rickettsiae* (Silverman *et al*., 1992; McLeod *et al*., 2004). If the rickettsia is to survive, it must quickly escape from the phogosome into cytoplasm. This phenomenon is the best illustrated in those instances where escape is efficient (Weiss, 1982). In addition, phospholipase A is also involved in entry of *R. rickettsii* into host cells (Walker *et al*., 1983). In *R. felis* patatin, *pat*2 exhibits a significant homology to patatin-like phospholipase whereas its paralog (*pat*1) was also identified in the chromosome (Ogata *et al*., 2005).

Normal of pH in dog blood stream is approximately 7.4. The highest activity of phospholipase A in this study was found at pH 8.0 and 40°C. Increasing of pH inhibits fusion of phagosomes and endosome with decrease lysosomal activity (Gordon *et al*., 1980; Mellman *et al*., 1986; Tapper and Sundler, 1995). For example, *Listeria monocytogenes* escapes from the phagosome (Lm vacuole) by alkalinization of the phagosome (Shaughnessy *et al*., 2006). In addition, clinical signs of canine ehrlichiosis have the febrile phase last from two days to several weeks (Farrow and Love, 1983) that assumed the high temperature (fever, 39.8°C) in dogs infected with *E. canis* (Ewing *et al*., 1971) can cause high activity of patatin. Results propose that the high pH and temperature is excellent for phospholipase activity.

The phylogenetic tree was constructed by using amino acid sequences of reported patatins. There is a general agreement that comparison of primary amino acid sequences is a more reliable indicator of phylogenetic relationships than nucleotide based comparison, since the function of protein is closely coupled to their primary sequences (Scholz *et al*., 2008). In this study, phylogentic tree showed surprisingly that plants patatin clustered with the bacterial ones which may refer to the story of host-pathogen evolution. For example, the Rickettsiales are clearly separated from the Chlamydiales (Moulder, 1984), a normally defined group of energy-parasitizing, obligately intracellular bacteria. Certain fastidious parasites of plant vacuolar tissues and arthropods, sometimes reffered to as rickettsia-like (Davis *et al*., 1981).

CONCLUSION

The major findings of this study are nucleotide-based comparative *16S rRNA* analysis providing higher resolution at the different genus level. In this study our primers detected both *Ehrlichia* and *Anaplasma* in canine blood, which resulted in new *16S rRNA* sequences from *E. canis* and *A. platys* infections of Thai dogs. Although the *16S rRNA* sequences were highly conserved among geographically diverse strains of these organisms, additional analyses of genes more subject to selective pressure from host environments (e.g., outer membrane protein gene families) could help elucidate the diversity and evolution of strains from different geographic areas. Current efforts include examination of additional canine blood samples to determine the presence and genetic diversity of *Ehrlichia* and *Anaplasma* found in Thailand. In addition, characterization of organisms in feline blood samples by using the same primers could be amplified bacteria in class alpha-proteobacteria, *Bartonella, Ochrobactrum and Sphingomonas* that can cause disease in human and animals. Furthermore, it revealed normal bacteria found in feline blood samples, *Bartonella* spp, and novel bacterial infection ever reported in cat bloodstream included *Ochrobactrum* and *Sphingomonas*.

The phylogenetic tree analysis and topological data of drug resistance gene, Bcr, from *E. canis* reported in this study will be particularly useful in defining the relationship and function of Bcr. This discovery has encouraged several pharmaceutical companies to develop therapeutic agents, which could inhibit the transport activities of multidrug transporters and increase the operative of an antibiotic. Our understanding of drug resistance topology can suggest novel therapeutic strategies. In particular, the structure, number and specificity of drug-binding sites/domains for many bacterial transporters remain unknown.

Gene involved in pathogenicity of *E. canis* such as patatin showed phopholipase activity. Bacterial phospholipase is a key factor in bacterial-induced cell lysis. The *patatin* gene is highly conserved in Gram-negative bacteria, suggesting that

it has significant role in bacterial survival. However, the role of phospholipase in bacterial virulence is to be elucidated. In *E. canis* optimal pH and temperature propose this organism can servive and escape from phagocytosis. Sequence analysis of patatin showed four conserved sequence of phospholipase activity and phylogenetic tree showed patatin from plants are closely related to bacterial ones than bacteria in Order Rickettsiales.

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