THESIS

EPIDEMIOLOGICAL STUDY OF *BABESIA SPP.* INFECTIONS IN STRAY CATS IN BANGKOK METROPOLITAN AREAS BY POLYMERASE CHAIN REACTION AND GEOGRAPHIC INFORMATION SYSTEM

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GRADUATE SCHOOL, KASETSART UNIVERSITY
2007

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A Thesis Submitted in Partial Fulfillment of
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Babesiosis is a worldwide tick-borne disease infecting a variety of vertebrate hosts and caused of hemolytic anemia in domestic animals. The objective of this study was to detect and survey the distribution of babesiosis in stray cats which live in the monasteries in Bangkok metropolitan areas by the PCR assay combined with GIS technology to find out factors associated with the spreading of organism. The result of PCR method was demonstrated that only 21 from 1,490 cats (prevalence = 1.3%) in 140 monasteries from 50 districts of Bangkok metropolitan areas showed positive results. In addition, from the microscopic examination, 2 positive results (prevalence = 0.1%) which was the same positive samples by PCR assay. The *Babesia* positive samples detected by PCR assay, were found in 18 monasteries from 14 districts. The highest prevalence of babesiosis in district was found in Bueng Kum 16.5% (5/30). Furthermore, the GIS technique was shown the location of the total study monasteries and localized the infected monasteries and led to the environmental factor for disease's distribution. By the NCSS analysis, this result indicated the significant factor was sex of infected cats ($p = 0.02$, $df = 1$, $\chi^2 = 5.02$). In fact, the other important factors for disseminating of disease may be the community and the public health around the monasteries. This result will be beneficial for disease surveillance and control program in stray animals in Thailand.

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Student's signature Thesis Advisor's signature

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Pacharathon Simking

April, 2008

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LIST OF ABBREVIATIONS

°C	=	Degree(s) Celsius
µg	=	Microgram(s)
µl	=	Microliter(s)
µm	=	Micrometer(s)
bp	=	Base pair(s)
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
DW	=	Distilled water
EDTA	=	Ethylenediamine tetraacetic acid
ELISA	=	Enzyme linked-immunosorbent assay
et al	=	*et alli*
g	=	Gram(s)
kDa	=	Kilodalton(s)
kg(s)	=	Kilogram(s)
L (l)=	=	Liter(s)
M	=	Mole
mAmp	=	Milliampere(s)
mg	=	Milligram(s)
min	=	Minute(s)
ml	=	Milliliter(s)
mm	=	Millimeter(s)
mM	=	Millimolar(s)

LIST OF ABBREVIATIONS (Continued)

mRNA	=	Messenger ribonucleic acid
MW	=	Molecular weight
ng	=	Nanogram(s)
nm	=	Nanometer(s)
OD	=	Optical density(-ies)
PCR	=	Polymerase chain reaction
pH	=	Negative logarithm of hydrogen ion activity
RNA	=	Ribonucleic acid
RNase	=	Ribonuclease
rpm	=	Round(s) per minute
TBE	=	Tris – boric acid - EDTA
U/μl	=	Unit(s) per microliter
U/g	=	Unit(s) per gram
UDW	=	Ultrapure distilled water
V	=	Volts
v/v	=	Volume by volume
w/v	=	Weight by volume
w/w	=	Weight by weight

EPIDEMIOLOGICAL STUDY OF *BABESIA SPP.* INFECTIONS IN STRAY CATS IN BANGKOK METROPOLITAN AREAS BY POLYMERASE CHAIN REACTION AND GEOGRAPHIC INFORMATION SYSTEM

INTRODUCTION

The babesiosis is a metropolitan tick-borne disease distributed among in the variety of vertebrate host (Kuttler, 1988). Particularly, in domestic animals which are closely contact to human such as dog, the infection of Babesia piroplasm was transmitted by the tick bite, *Rhipecephalus* and *Dermacentor*, the vector of *Babesia canis* and *Babesia gibsoni* and commonly found as the ectoparasite in dogs (Farwell *et al*., 1982; Casapullar *et al*., 1998; Chandoga *et al*., 2001; Lippin *et al*., 2001). In feline, the first babesiosis infection in domestic cats were reported in South Africa and the main pathogenic agent was *Babesia felis,* the small babesial piroplasm, which caused of anemia and icterus (Baneth *et al*., 2003). In addition, they have been reported the unidentified the species of *Babesia* infection in domestic cats parasite in France, Germany, Thailand, and Zimbabwe (Stewart *et al*., 1980; Jittapalapong *et al*., 1993; Bourdeau, 1996; Moik *et al*., 1997). Recently, the study in Spain and Portugal have demonstrated the identification of partial DNA sequences from small subunit RNA gene of *B. canis canis* from the examination of three cat blood samples while the microscopic examination can not detect the parasite (Criaso-Formelio *et al*., 2003). Afterward of the present of *B. canis canis* infection, the initiated molecular attention was increased in term of *Babesia* infection in cats.

Different subspecies of *Babesia* resulted in the degree the different of disease' severity, the prognosis strategies, and the response to antibiotic. Therefore, it is important to determine the species, subspecies, and genotypes that cause canine babesiosis for the suitable treatment (Birkenheuer *et al*., 2003). Normally, diagnosis of the canine babesiosis is performed by visualization of the *Babesia* parasite in

Giemsa-stained thin blood smear under light microscope. However, the presentation of *Babesia* parasite is very difficult to examine, if it was in the chronic stage or the level of parasitemia is relatively low. The serological test reported the using of IFAT and ELISA for detecting *B. gibsoni* parasite (Fukumoto *et al*., 2001). However, the serological test of *Babesia* infection showed the highly sensitivity, with moderate specificity, because of antigenic cross-reaction between the species of parasite (Yamane *et al*., 1993). For this reason, the serological test may not definitively discriminate species or subspecies of *Babesia* organism (Birkenheuer *et al*., 2003). Furthermore, some studies have presented that canine babesiosis piroplasm infection were not identified under light microscope and in which serologic test yield false-negative results in dogs that were infected with *Babesia* species (Birkenheuer *et al*., 1999). On the contrary, the Polymerase Chain Reaction (PCR) technique provides the sensitivity and specificity higher than the other examination in term of differentiated species or subspecies of the infected parasite and offers more sensitive and specific than the light microscopic examination based on the limits of detection (Bose *et al*., 1995). Since infected dog may have antibodies that are unpredictable cross-reactive against other *Babesia* species or subspecies, PCR is also more specific than the serological examination (Birkenheurer *et al*., 2003).

In addition, from the specificity and sensitivity of PCR technique for detection of subclinical carriers in specific situations, this study attempts to use PCR technique to find out the infection of *Babesia* spp. in stray cat in Bangkok where most stray cats live and never been under investigation. Furthermore, we will analyze the data from the laboratory result by GIS mapping to localize the region that have incidence of babesiosis in roaming cats and study for the variety of the environmental factors in the region scale.

Actually, the epidemiology of babesiosis in domestic animals are mainly due to the laboratory examination which was far from the reality. Moreover, from the fact that ticks are the vector of the babesiosis, and animals are in close contact with humans. Therefore, stray cats are the possible carrier of tick – vector to the human environment (Inokuma *et al*., 2003). Recently, canine babesiosis is capable of

transmission to human as human babesiosis. Human babesiosis are caused by *B. microti* and *B. divergens* (Kjemtrup *et al*., 2000). Recently, in Mexico and France, several cases have been reported and caused by *B. canis.* Additionally, the pathogens of three *Babesia* species affected human and dogs in Japan (Inokuma *et al*., 2003). However, there were a few epidemiological studies on the distribution of the *Babesia* species in feline. The epidemiology of babesiosis in cats living under natural conditions in the sociobiological context is a relatively new source of knowledge in the field of epidemiology, especially when an urban stray colony is studied because it may sentinel for the circulation of new pathogens in the area. The distribution of babesiosis in stray cats may showed the risk of human babesiosis, and can establish strategies to prevent the spread of this organism among these stray animals.

For the epidemiological study, the geographic information system (GIS) is an implement that has its abilities to integrate different type of spatial data, as can be used to map available epidemiological information and relate it to influence the distribution of infectious disease, such as climate and other environment factors (Brooker *et al*., 2002).

OBJECTIVES

1. To determine the prevalence of *Babesia spp* infections in stray cats by PCR and light microscopic examination.

2. To study the spatial distribution of *Babesia spp*. in stray cats in Bangkok areas

3. To use Geographical Information System for a rapid epidemiological assessment of the status of *Babesia* - infected cats in Bangkok

LITERATURE REVIEW

Canine babesiosis is a considerable disease in dogs that caused by intraerythrocytic protozoan parasite named *Babesia spp.* The organism of disease is transmitted by the infected ixodid ticks (Boozer and Macintire, 2003; Miyama *et al*., 2005; Bourdoiseau. 2006; Dantas-Torres and Figueredo, 2006; Garcia, 2006; Kjemptrup and Conrad, 2006). The definitive diagnosis utilized by the presentation of *Babesia* organisms in the infected erythrocyte and the characterization symptoms of the canine babesiosis is hemolytic anemia (Lobetti, 1998). As a result, to recognize the species of *Babesia* organism is based on the Giemsa-stained blood smear examination for the visualization of large and small *Babesia*, *B. canis* and *B. gibsoni*, the causative agent of canine babesiosis (Aynur *et al*., 2006). Afterwards, the molecular analysis has manifested three subspecies of *B. canis* as *B. canis canis, B. canis rossi* and *B. canis vogeli* (Furlanello *et al*., 2005). The obvious recognization of the causative organism is the role in veterinary importance in the cattle, horse, and the domestic animal and gained the concentration as an emerging zoonotic disease (Vial *et al*., 2006).

Taxonomy and morphology

The *Babesia* organism is classified in the family Babesiidae, order Piroplasmida ,within the phylum Apicomplexa (Levine *et al*., 1980). The agent vary in extent from 1.0 – 0.5 µm as the small babesia (*B. bovis, B. divergens and B. microti*) to 2 – 5 µm as the large organism (such as *B. bigemina*) represented in the infected erythrocyte (Mehlhorn and Schein, 1984; Homer *et al*., 2000). In addition, canine babesiosis caused by the two global Babesia species, *B. canis* and *B. gibsoni*. *B. canis* is a large piroplasm (4 – 5 µm in length) and the small one was *B. gibsoni* (Cacció *et al*., 2002). *B. canis* usually visualizes as a single pear shaped or in pairs of merozoites dividing by binary fission within the erythrocyte.

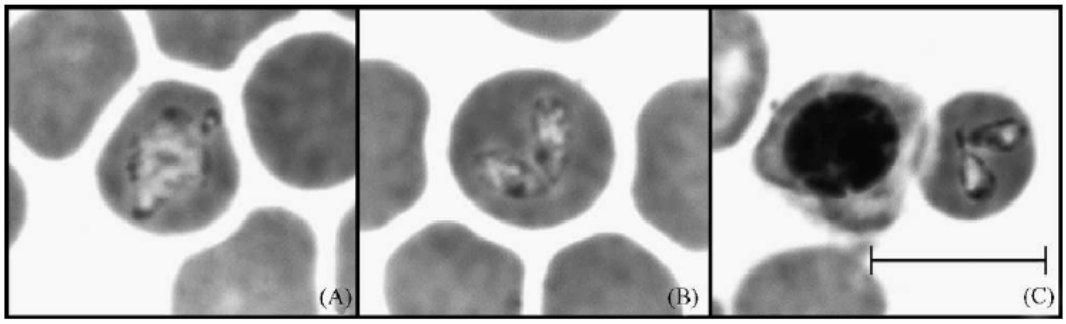


Figure 1 Photomicrograph of the large *Babesia* identified on Giemsa-stained thin blood smears. (A) Ameboid form. (B) and (C) Paired pyriform forms. Bar = 10 mm.

Source: Birkengeuer *et al.* (2004)

The second organism of canine babesiosis, *B. gibsoni*, is display to be a small babesial agent (lass than 3 μm in length) with round or oval intraerythrocytic organism and not represented in paired form (Levine, 1985). The separating of *B. gibsoni* is demonstrated in Asia, North America, Northern and Easthern Africa, lately published in Europe (Casapulla *et al*., 1998).

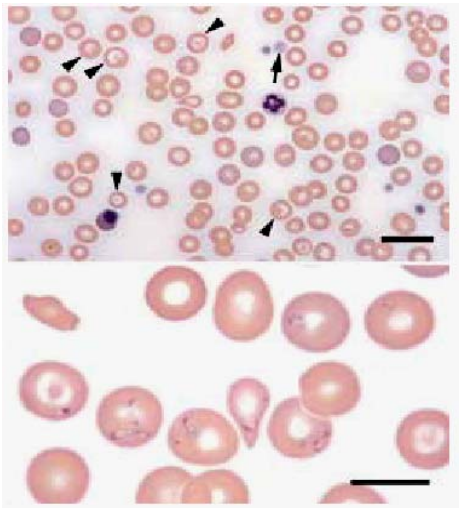


Figure 2 Peripheral blood smear from a dog infected with *Babesia gibsoni*. (Top) Hematologic abnormalities include decreased RBCdensity, increased polychromasia, leptocytes (arrowheads), large platelets (arrow), and a nucleated RBC. Small *B. gibsoni* organisms are presented within several RBCs. Wright's stain, bar = 20 µm. (Bottom) Morphologic characteristics of *B. gibsoni* organisms. Wright's stain, bar = 10 µm.

Source: Irizarry – Rocira *et al*. (2001)

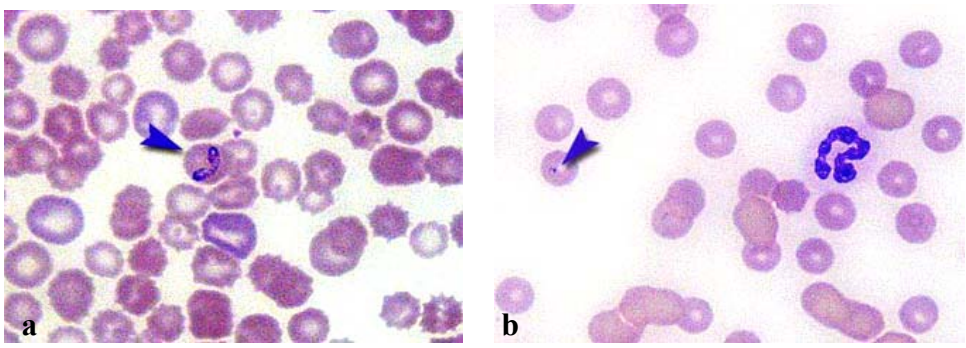


Figure 3 (a) Blood smear, dog, Wright's stain. Large, slightly irregular piroplasms of *Babesia canis* are present within erythrocytes. (b) Blood smear, dog, Wright's stain. Inclusions of *Babesia gibsoni* are smaller, ring-shaped, and more numerous than those of *B. canis*.

Source: Wyatt *et al.* (2002)

Life cycle

The life cycle of *Babesia* agent can be distributed in two periods: (i) in the vertebrate host; (ii) in the tick vector (Figure 5).

In the vertebrate host

The host is directly invaded by *Babesia* sporozoite pass through the bite by an infected tick, the sporozoite infected the erythrocytes where asexual reproduction (merogony) is occured. Sporozoites develop into piroplasm. The intraerythrocytic trophozoite multiplies and form two or four isolated merozoites, and establishes a incessant cycle of asexual reproduction. The rapid increasing of intracellular multiplication causes the eradication of host erythrocytes, with release a new parasites and subsequent infection and destruction of other erythrocytes (Vial *et al*., 2006). The less amount of merozite do not separate and turn into non – separeting large and remain shaped spherical gamont inside erythrocytes (Mackenstedt *et al*., 1990). Following the ixodid tick take blood meal from an infected host, the parasite will differentiate in the tickgut (gametogony) (Vial *et al*., 2006).

In the tick vector

On the basis of differences in geographic distributions, vector specificity, and antigenic properties (Uilenberge *et al*., 1989; Hauschild *et al*., 1995), the former species is subdivided into three subspecies , namely *Babesia canis canis* transmitted by *Dermacentor reticulates* in Europe, *B. cansi vogeli* transmitted by *Rhipicephalus sanguineus* in tropicl and subtropical regions and *B. canis rossi* transmitted by *Haemaphysalis leachi* in South Afirca (Földvári *et al*., 2005). Moreover, there are many other tick species that can be the potential vector of this parasite (table1 and 2).

Figure 4 A female cocker Spaniel dog highly infested by *R. sanguineus* ticks.

Source: Dantas – Torres *et al*. (2006)

The ticks become infected after ingesting the infected erythrocyte containing *Babesia spp*., as gametocytes (Weyon, 1926). In the tick midgut, the parasite develops into male and female gametes. The fusion of microgametes and macorgametes leads to the formation of motile zygote (Mehlhorn and Schein, 1984).

The zygote of *Babesia* can be invaded through numerous organs of ticks, including the ovaries. Therefore, the infection will pass through the ovary and ova to the next generation. This is called transovarial transmission. Usually when female ticks become infected, sporogony will take place in the salivary glands of larval, nymphal and adult ticks. When ticks attaches to a new host with a maturation of the sporozoites in the salivary gland, the host will likely be infected via saliva from the tick. Certain species of *Babesia* can persist over several tick generations, even without new infection (Uilenberg, 2006).

Table 1 Important species of *Babesia* in domestic animals.

species	Vector		Vertebrate host	size in erythrocytes (µm)	Geographic distribution
	species	stage			
Significat large Babesia species[1]					
B. bigemina	*Boophilus* spp.	Nymphs adults	Cattle, Water buffaloe, wild ruminants	5 x 2	Souther Europe, America, Africa, Asia, Australia
B. Bovis	*Boophilus* spp., *Ixodes* spp., *Rhipicephalus bursa.*	Larvae	Cattle, Water buffaloe, wild ruminants	2.5 x 1.5	Souther Europe, America, Africa, Asia, Australia
B. divergens	*Ixodes ricinus*	Larvae	Cattle, Wild ruminants, Humans	1.5 x 1.5	Europe
B. major	*Haemaphysalis punctata*	Adults	Cattle	3 x 1.5	Western and Southern Europe, Great Britain, Northwestern Africa
B. motasi	*Haemaphysalis* spp., *Rhipicephalus bursa*	Adults	Sheep, goats	4 x 2.5	Southern Europe, Middle East, Southern Russia, Africa, Asia
B. ovis	*Rhipicephalus bursa*	Adults	Sheep, goats	2 x 1	Southern Europe, Middle East, Southern Russia, Africa, Asia
B. caballi	*Hyalomma* spp., *Dermacentor* spp., *Rhipicephalus* spp.,	Adults	Horses, mules, donkeys, *Equus burcheli*	4 x 2.5	Europe, Asia, Africa, America, Australia
B. canis	*Rhipicephalus sanguineus, Haemaphysalis leachi, Dermacentor reticulatus*	Nymphs, Adults	Dog, other wild canines, fox	5 x 2.5	Europe, Asia, Africa, America, Australia
B. trautmanni	*Rhipicephalus* spp.	?	Pigs	4 x 2.5	Southern Europe, Africa
B. herpailuri	?	?	*Felis sylvestris*	3 x 2.2	Southern America
B. pantherae	?	?	*F. sylvestris, Panthera leo*	2.5 x 1.5	Africa
Small Babesia species of doubtful systematic position					
Babesia (syn. *Microbabesia*) *gibsoni*	*Haemaphysalis bispinosa, Rhipicephalus sanguineus*	All stages	Canidae including dogs, fox and other wild canines	1.2 – 2.1	Asia, Africa, India, Japan
B. microti group	*Dermacentor* spp., *Rhipicephalus* spp., *Ixodes* spp., *Haemaphysalis* spp.,	Larvae to nymphs	Rodents, humans	1.5 - 2	Europe, North America
B. felis (syn. *Achronaticus felis*)	?	?	Felidae including *Panthera leo, Felis sylvestris*	1.5 – 2	Africa

Table 1 (Continued)

species	Vector		Vertebrate host	size in erythrocytes (µm)	Geographic distribution
	species	stage			
B. (syn. *Achromaticus*) *rodhaini* = *B. quadrigemina*	?	?	Mice	1.5 – 2	Europe

[1] Babesia equi of horses was transferred to the theilerians.

Source: Mehlhorn (2004)

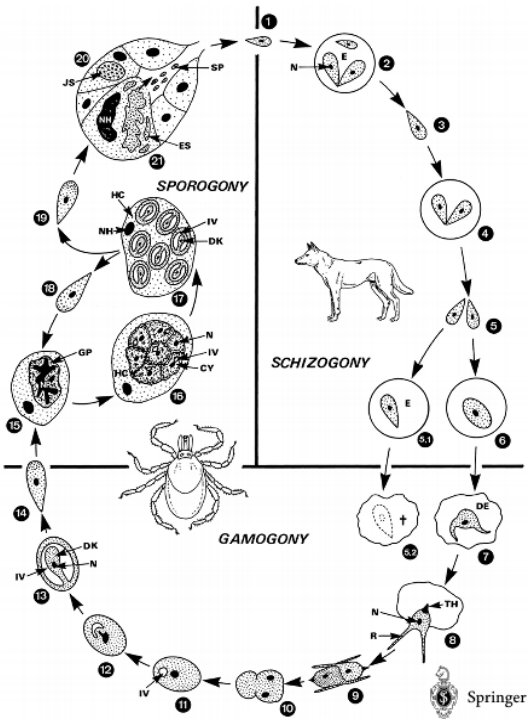


Figure 5 Life cycle of *Babesia canis*. 1 Sporozoit saliva of feeding tick. 2–5 A sexual reproduction in erythrocytes of vertebrate host (dog) by binary fission, producing merozoites (5) which enter other erythrocytes. When merozoites are ingested by a tick (5.1) they become digested inside the gut (5.2). 6 Some merozoites become ovoid gamonts. 7, 8 After ingestion into the tick's intestinal cells the ovoid gamonts form protrusions and thus appear as ray-bodies (8). Fusion of two uninucleate ray-bodies (gametes). 10 Formation of

Figure 5 (Continud)

a zygote. 11–14 Formation of a single kinete from a zygote inside the inner vacuole. The kinete leaves the intestinal cell and enters cells of various organs (including the eggs) of the vector ticks. 15–18 Formation of several kinetes (sporokinetes). This process is repeated (15–18) and also proceeds in eggs of ticks. The infection is thus transmitted to the next generation of ticks (i.e. transovarial transmission). 19–21 Some of the kinetes penetrate cells of the salivary glands, where a large multinuclear sporont (YS, ES) is formed (inside hypertrophic host cells) finally giving rise to thousands of small sporozoites (SP), which are injected during the feeding act (i.e. transstadial transmission). CY, cytomere (uninucleate); DE, digested erythrocyte; DK, developing kinete; E, erythrocyte; ES, enlarged sporont (forming sporozoites); GP, growing parasite (polymorphic stage); HC, nucleus of host cell; IV, inner vacuole; N, nucleus; NH, nucleus of host cell; R, ray - like protrusion; SP, sporozoite; T, thorn-like apial structure; YS, young sporont.

Source: Mehlhorn (2004)

Clinical signs of babesiosis

Clinical signs of canine babesiosis are fever, lethargy, anorexia and vomiting (Zahler *et al*., 2000). A non specific symptoms and feature including neurological anomalies as cerebral babesiosis, haemolytic anemia (contributed to the occurance of haemoglobinuria and bilirubinuria) and gastrointestinal disturbance have been published (Tarello, 2003). The lesions of mucosal or dermatological are only occasionally reported (Merchant and Toboada, 1991; Cappelli *et al*., 1996) and evidently of no value in the diagnostic procedure of canine babesiosis (Kraje, 2001).

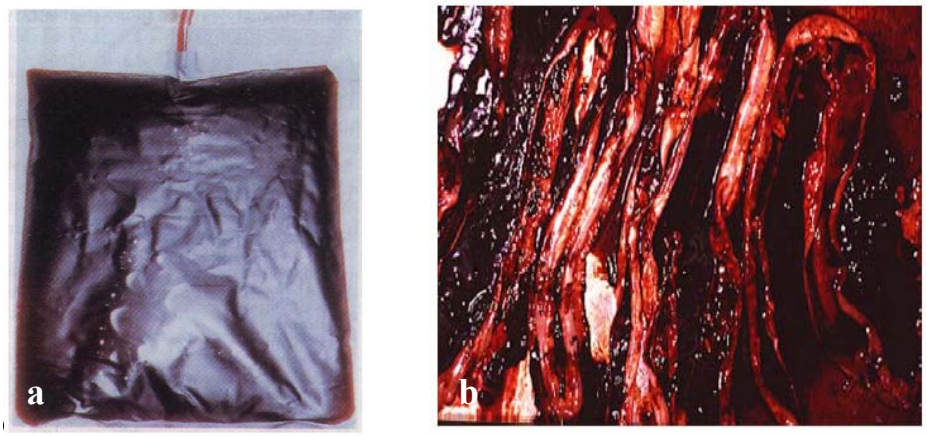


Figure 6 Clinical signs of babesiosis (a) Hemoglobunuria (b) Severe enterrorhagia in a fatal case of babesiosis.

Source: Jacobson *et al*. (2006)

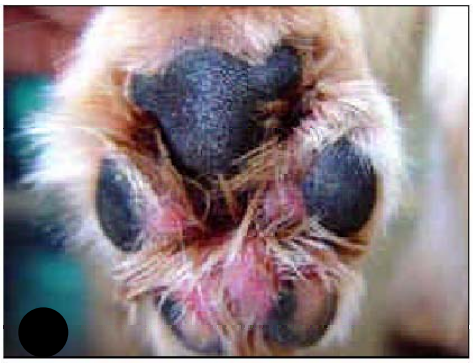


Figure 7 Severe pododermatitis in a dog infected by *B. gibsoni*-like structure

Source: Tarello (2003)

Uncomplicated babesiosis was classified by the clinical sign was characterized directly to haemolytic anemia. Sub-classified as mild if anemia was mild to moderate, and severe if anemia was life-threatening. In the other way, if the clinical sign was directly attributed to acute haemolysis, the disease was recognized as the complication babesiosis. Complication babesiosis presented in the case has the clinical sign as acute renal failure, icterus, hepatopathy, pulmonary edema, cerebral babesiosis, "red biliary" chemoconcentration, and shock (Jacobson *et al*., 2006).

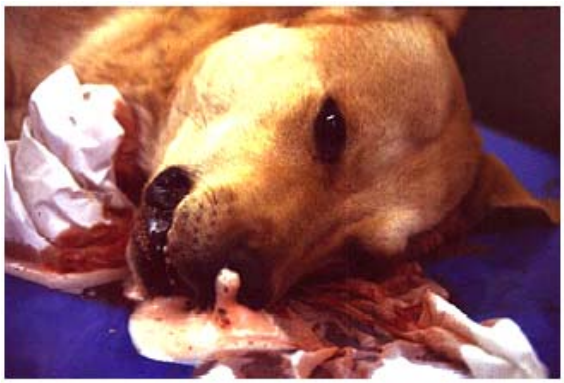


Figure 8 Agonal moments in a dog with pulmonary edema in a complicated babesiosis.

Source: Jacobson *et al*. (2006)

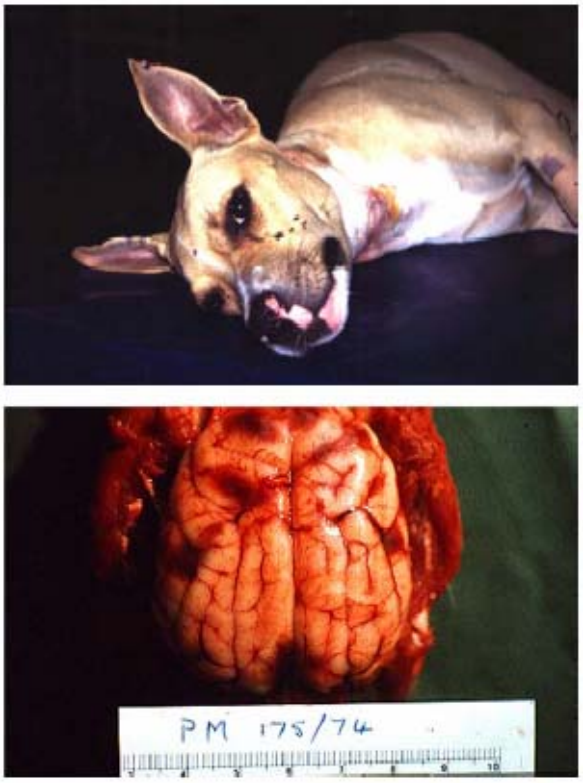


Figure 9 Clinical sings of severe canine babesiosis (a) Non-responsive dog showing rigidity and nystagmus. (b) Macroscopic brain hemorrhages.

Source: Jacobson *et al*. (2006)

The abnormalities of biochemical changing of *B. canis* infection are associated to the severity of the disease and the hypoxia degree. The general format was the increasing of the serum activity of asparate aminotransferase (AST) and alanine aminotransferase (ALT), hyperbilirubinemia, hypoalbuminemia, electrolyte and acid-base abnormalities(mostly hypokalemia, hypercloremia and metabolic acidosis) (Furlanello *et al*., 2005).

Diagnosis technique

The identification of *Babesia* infection is due to the presenting of merozoites in blood smear under light microscopic examination the sensitivity of this technique is rather low (Bose *et al*., 1995). Additionally, the light microscopic examination can not be use to recognized the genotype of *Babesia* spp. (Birkenheuer *et al*., 2003). The chronic infection or inapparent duration make the examination by the light microscopic is very difficult (Fukumoto *et al*., 2001). The serology test may not definitively identified species or subspecies because the antibodies to *Babesia spp.* are often cross-reactive. Furthermore, many studies demonstrated the detection of canine babesiosis while the piroplasms were not visualized by light microscopic examination and the serological test presented false-negative result in infected dog (Breitschwerdt *et al*.,1983; Birkenheuer *et al*., 1999). PCR assay was a practical and dependable approach to detect and identify the infection with various *Babesia* spp. and also provided as a sensitive tool for evaluating treatment consequences (Birkenheuer *et al*., 2003).

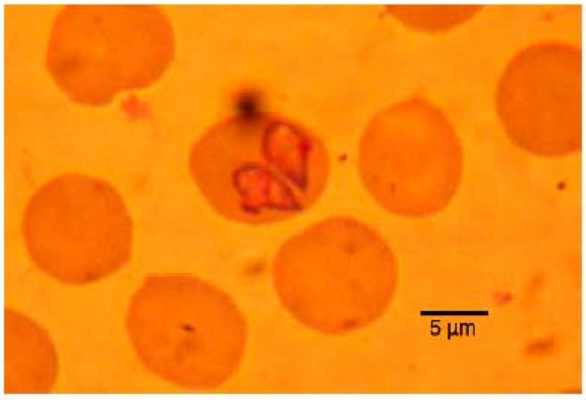


Figure 10 A large pear-shaped Babesia parasites in the peripheral blood of infected dogs from Turkey, blood smear was Giemsa-stained and observed under light microscopy (x1000).

Source: Aynur *et al.* (2006)

Babesiosis in Cats

Feline babesiosis in domestic cat caused by *B. cati* was first published India (Mudaliar *et al.,* 1950). Afterwards, the infection was mainly caused by *Babesia felis*, a small *Babesia* that due anemia and icterus (Beneth *et al.,* 2004) was reported in South Africa. The occasional case of infection by unidentified *Babesia* parasite have been published in France, Germany, Thailand, and Zimbabwe (Stewart *et al*., 1980; Jittapalapong *et al*., 1993; Bourdeau, 1996; Moik, and Gothe, 1997). The nomenclature is confused, and most parasites have been named relating to their hosts, without associated to their host-specificity. In addition, the report in Sudan demonstrated the small parasite in a wild named *Babesia felis*, and this species was also found in domestic cats of South Africa and later elsewhere (Uilenberg, 2006).

Recently, large *Babesia* has been published in Israel and recognized as a subspecies of *B. canis*: *B. canis* subsp. *presentii* (Baneth *et al.,* 2004). The occurrence of *B. canis* infected in cat has been reported in Spain and Portugal (Criado - Fornelio *et al.,* 2003). This should attracted the scientists to test the infectivity of the known (sub-) species of *B. canis* in cats.

Emerging zoonosis

Canine babesiosis has increased an attention as an emerging zoonotic disease problem (Kjemtrup, and Conrad, 2000). The first evidence of human babesiosis was reported from the patient who was detected piriform, intraerythrocytic inclusions in blood with Rocky Mountain spotted fever in the western USA (Wilson, and Chowning, 1904). In Europe, *B. divergens* infection of humans has been reported since 1957, and seemed to be increasingly (Marsaudon *et al*., 1995; Gorenflot *et al*., 1998; Dense *et al*., 1999). In USA, human babesiosis was recognized in California in 1968 (Scholtens *et al*., 1968). Since 1982, human cases of babesiosis have been reported in the eastern USA, providing the impetus to make babesiosis as a reportable disease in some states, including New York (White *et al*., 1998). In the western USA, seven human cases have been attributed to the WA1-type babesial parasite,which is distinctly different from *B. microti,* but closely related to small babesial parasites of wild life and dogs in California (Quick *et al*.,1993; Thomford *et al*.,1994; Persing *et al*.,1995; Kjemtrup *et al*., 2000 (b)). Furthermore, *B. bovis*, *B. canis*, *B. microti* and *Babesia* of unknown species have been identified in humans in Europe, based on morphological characteristic and antigenic reactivity (Jacquemin *et al*., 1980; Calvo de More *et al*., 1985; CaÑos *et al*., 1992; Marsaudon *et al*., 1995). Particularly, several cases in Mexico and France have been reported as *B. canis* infection. Therefore, it can prove that *B. canis*, the causative agent of canine and sometime feline babesiosis, can caused of human babesiosis. For this reason, the observation of canine and feline babesiosis have been recently initiated (Mandes-de-Almeida *et al*., 2004; Inokuma *et al*., 1998; Birkenheuer *et al*., 2003). Additionally, the vector of this organism was surveyed in Japan and Thailand (Inokuma *et al*., 1998; 2003; Dentrakool *et al*., 2004). As dogs and cats are closed contact with humans, they might be the potential carrier of tick vectors to the human environment. Thus, this study attempted to determine the prevalence of babesial infection in stray cats and defined the distribution of the agent with GIS system in order to evaluate the possibility of the emergence or latent existence of human babesiosis.

GIS technique

GIS is an especially horizontal technology in the sense that has wide-ranging application across the industrial and intellectual landscape. The operating of GIS technology composes of spatial data with logically linked attributed information a GIS storage database where analytical function are controlled interactively by a human operator to generate the needed information products (Tomlinson, 2003). The purpose of Geographic Information System (GIS) is to provide a spatial framework to support the decisions for the intelligent use of earth's resources and to manage the man-made environment (Zeiler, 1999).

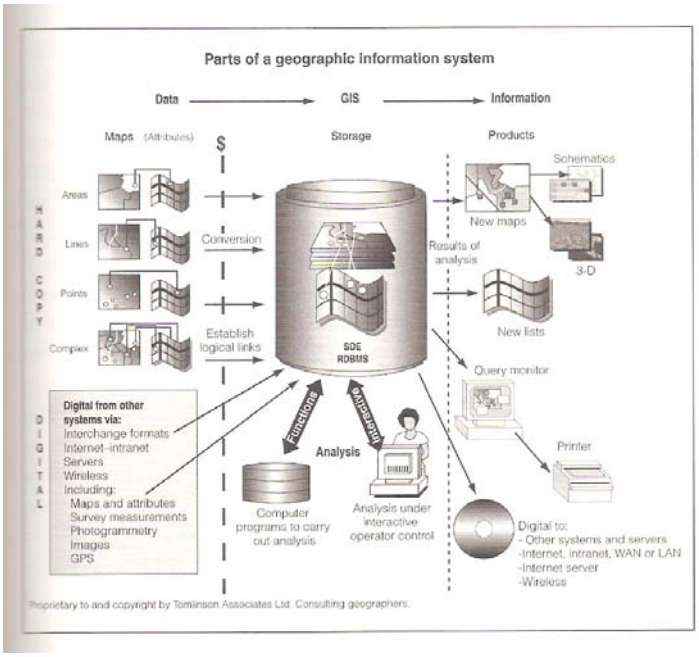


Figure 11 The GIS processing mechanism.

Source: Tomlinson (2003)

Features commonly found on a map-roads, lakes, buildings are also commonly found in GIS database as thematic layers. Most can be represented using a combination of point, lines or polygons. A crucial and related form of spatial data is data about those roads, lakes and buildings. These are called attributes in GIS

parlance, and, infact, it is the richness and depth of these attributes that make spatial data so potent in the context of GIS (Tomlinson, 2003).

Epidemiology of parasitology by GIS technique

Understanding the environmental limits of infection can also improve the knowledge of protozoa biology and epidemiology. Numerous studies have demonstrated the relationship between analytical of spatial data and disease distribution. For example, there is a report of use remote sensing and geographic information system to design and implement an epidemiological assessment of helminth in Chad by ecological zone, and the result contributed towards defining the thermal limit of geohelminth species (Brooker *et al*., 2002). Additionally, the observation of filariasis in dogs at the Mt. Vesuvius area has used the geographic information system (GIS) software to foster a better understanding of the distribution and prevalence of filarial worm in asymptomatic dogs (Cringoli *et al*., 2001). Therefore, one of the main strength of a GIS is its abilities to integrate different types of spatial data and can use to map available epidemiological information and related it to factors know to influence the distribution of infectious disease, such as climate and other environment factors (Brooker *et al*., 2002).

MATERIALS AND METHODS

1. Study area

The investigation was conducted in Bangkok metropolitan areas, Thailand. The objective areas were located in monasteries of 50 districts. The population of stray cat samples were randomly selected by the simple randomization assay. For the expectation of the low incidence and prevalence (2- 5%) of the babesiosis in cats in this study, 30 cats for each district were selected. There were 50 districts in Bangkok and at least 3 monasteries form each district was randomly selected for studying (in case of low number of monasteries district, one or two temple each district was selected). In assigned monasteries, 10 stray cats were randomized per place for blood and data collection to processing the PCR and GIS analysis. If there was only one or two temples in districts, 30 and 15 stray cats were collected as representative for districts.

2. Blood sampling

A total of 1490 blood samples were collected between March to May 2004. The animals were gently restrained by physical force. At least 3 – 5 ml of blood were drawn form jugular vein, preserved in sodium citrate vacuum tubes and stored at - 40° C until use. Each cat was thoroughly examined and recorded for age, sex, health and environmental condition, and search for ectoparasites. A questionnaire was designed to record these data.

Health condition criteria

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

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

Poor: weak, dehydration sign, purulent of ocular or nasal discharge, some clinical signs found

Environmental condition

Good: the place was clean, no filth, good administration of animal habitation

Fair: the place was clean, may be slightly filth or in the construction, but no left over of the animal feeding and habitation

Poor: the place was dirty and in disorder, poor administration of animal habitation

Figure 12 The technique to collect blood sample from jugular vein of stray cat

3. The microscopic examination

A thin blood smear was prepared in the field and was fixed with methanol for 2 mins, stained with Modified Giemsa solution for 5 mins, and washed for 10 mins in distilled water. The parasite was examined under light microscope.

4. Polymerase Chain Reaction (PCR) assay

4.1 DNA Extraction

The DNA extraction was done by the phenol – chloroform extraction technique using 100 µl of blood sample, 150 µl of phenol and 150 µl of chloroform for (Sambrook and Russell, 2001). After that, the extracted DNA was stored at -20°C until used.

4.2 Polymerase Chain Reaction

PCR method was described by Ano *et al*. (2001). The primers PIRO – F (5′ – AGTCATATGCTTGTCTTA – 3′) and PIRO – R(5′ - CCATCATTC CAATTACAA- 3′), which amplified around 500 nucleotides of the small subunit ribosomal RNA gene of *B. gibsoni* were designed according to the nucleotide sequences in DNA data bank (accession number: LI3729). The following PCR condition were used to run by MyCycler™ Thermal Cycler (BioRad Laboratories, USA). After denaturation at 90° C for 2 mins, 30 cycles of denaturation at 90°C for 30 sec, annealing at 55°C for 2 mins and extension at 72°C for 5 mins. The amplified PCR products were analyzed on 1 % agarose gel electrophoreis and stained with ethidium bromide.

A nested PCR was performed using the re - designed inner primers PIRO2 – F (5′ - ATAACCGTGCTAATTGTAGG - 3′) and PIRO2 – R (5′ - TGTTAT TTCTTGTCACTACC - 3′) which produced 327 bp products, since the first - round PCR products can not be visualized. The condition of the second - round PCR was as same as those of the first – round PCR.

4.3 Gel electrophoresis

After done with the nested PCR, the PCR products were run in 1% agarose gel at 100 volt for 50 mins in electrophoresis chamber (Mini - sub® Cell GT. Biorad, Italy) fulfilled with Tris – borate – EDTA (TBE) buffer. The positive samples presented 327 bp DNA band when stained with ethidium bromide for 10 mins, destained with water for 15 – 20 mins, and visualized under ultraviolet transluminator.

4.4 Sequencing of PCR products

After complete the gel electrophoresis, the gel extraction and purification for positive DNA fragment were proceed by gel extraction kit, QIAquick® Gel Extraction Kit (QIAGEN, Germany). The purified DNA products were submitted for sequencing at Bioservice Unit (BSU), Thailand.

5. Statistical Analysis

Chi – square and Number Cruncher Statistical System (NCSS) ver. 2000 (Kaysville, UT) program were used to assess differences in the prevalence and intensity of infection. Analysis was also undertaken to investigate environmental variable correlated with the infection patterns, as determined by the probability that an individual cats were infected. If probability (P) is less than 0.05, it indicated the significant differences.

6. Geographic Information System (GIS) technique

The mapping of parasitic distribution was created by the GIS program, ArcGIS ver.9 (USA) to find the location of the infected monasteries. The pointed monasteries indicated the nearest environment or community which may be associated with the disease dissemination.

The GIS is able to demonstrate not only the location of monastery, but also the communicative pathways as well as the agriculture part, water resource, and the construction or other environmental plans involved with the geographic or public health factors which were considerable to the distribution of organisms. In addition,

the GIS image led to the estimation of animal population, which was related to the number of stray animals. These data were beneficial for the investigation of disease surveillance.

Table 2 The study areas samples (districts and monasteries) of Bangkok metropolitan areas

District	Total number of monastery in each District	Selected study monastery (% representative)	Cats per monastery (Average)
Bangkok Noi	32	3 (9.4%)	10
Taling Chan	30	3 (10%)	10
Phasi Charoen	27	3 (11.1%)	10
Thon Buri	25	3 (12%)	10
Bang Phlat	23	3 (13%)	10
Phra Nakhon	22	3 (13.6%)	10
Chom Thong	17	3 (17.6%)	10
Dusit	17	3 (17.6%)	10
Nong Chok	16	4 (25%)	8
Bang Khun Thain	15	4 (26.7%)	8
Bangkok Yai	13	3 (23%)	10
Lat Krabang	13	3 (23%)	10
Samphanthawong	13	3 (23%)	9
Pom Prap Sattru Phai	11	4 (36.4%)	8
Bang Kho Laem	10	3 (30%)	10
Khlong Sam Wa	10	3 (30%)	10
Bang Sue	9	3 (33.3%)	10
Khlong San	8	3 (37.5%)	10
Sai Mai	8	3 (37.5%)	10
Yan Nawa	8	3 (37.5%)	10
Don Mueang	7	3 (42.9%)	10
Rat Burana	7	3 (42.9%)	10

Table 2 (Continued)

District	Total number of monastery in each District	Selected study monastery (% representative)	Cats per monastery (Average)
Bang Khae	6	3 (50%)	10
Min Buri	6	3 (50%)	10
Nong Khaem	6	3 (50%)	10
Bueng Kum	5	3 (60%)	10
Bang Kapi	4	3 (75%)	10
Bang Khen	4	3 (75%)	10
Bang Na	4	3 (75%)	10
Bang Rak	4	3 (75%)	10
Khlong Toei	4	3 (75%)	10
Lat Phrao	4	3 (75%)	10
Phra Khanong	4	3 (75%)	10
Ratchathewi	4	3 (75%)	10
Sathon	4	4 (100%)	8
Thung Khru	4	3 (75%)	10
Huai Khwang	3	3 (100%)	10
Khan Na Yao	3*	3 (100%)	10
Bang Bon	2	2 (100%)	15
Chatuchuk	2	2 (100%)	15
Din Daeng	2	2 (100%)	15
Suan Luang	2	1 (50%)	30
Thawi Watthana	2	2 (100%)	15

Table 2 (Continued)

District	Total number of monastery in each District	Selected study monastery (% representative)	Cats per monastery (Average)
Wang Thonglang	2	2 (100%)	11
Watthana	2	2 (100%)	15
Lak Si	1	1 (100%)	30
Phaya Thai	1	1 (100%)	30
Saphan Sung	1	1 (100%)	30
Total	439	140 (31.9)	11.9

Total number of monasteries in each district was referenced by the monasteries data (update 4 October 2007) from Office National Buddhism.

Remark: * In Khan Na Yao district, the data of Office National Buddhism has only 2 monasteries.

RESULT

Animal Data

The 1,490 cats form 50 districts of Bangkok metropolitan areas were classified by sex, health conditions, environmental condition, ectoparasite condition, and districts as shown in table 4 – 6.

Table 3 Factors associated with Babesia infection of stray cats in Bangkok areas

Parameters	Number of normal cats	Number of *Babesia* infected cats (%)	p value
Sex			0.02
Male	560	3 (0.5)	
Female	930	18 (1.9)	
Age (months)			
0 – 12	138	3 (2.2)	
13 – 24	405	8 (1.9)	
25 – 36	412	5 (1.2)	
37 – 48	314	4 (1.3)	
49 – 60	149	1 (0.7)	
61 – 72	72	0	
Health condition			0.77
Healthy	1396	19 (1.4)	
Fair	37	1 (2.8)	
Poor	57	1 (1.8)	
Environment condition			0.17
Good	853	8 (0.9)	
Fair	627	13 (2.1)	
Poor	10	0	
Ectoparasite (Flea)			0.62
Exposed	500	6 (1.2)	
Unexposed	990	15 (1.5)	
District	50 districts	14 (28) districtss	

Microscopic examination result

The microscopic examination found only 2 positive samples. The piroplasm was seen as pyrifrom shape (pear shape) in red blood cells (Fig 13). The size was 2 – 3 µm in diameter and identified as the large *Babesia* spp. In addition, the positive samples by microscopic examination also gave positive result in PCR assay.

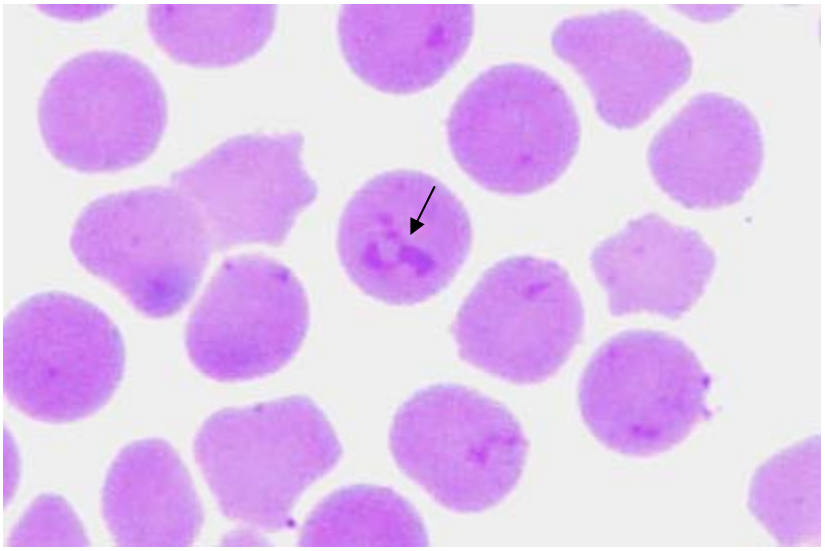


Figure 13 The pear – shaped intraerythrocytic protozoan (arrow) in cat blood smear (1000x) (number F1312)

Table 4 Prevalence of *Babesia* infection in stray cats classified by districts.

District	Number of Cats	Infected Cats (%)
Lak Si	30	1 (3.33)
Saphan Sung	30	0
Suan Luang	30	0
Phaya Thai	30	0
Samphanthawong	28	0
Sathon	30	0
Lat Phrao	30	0
Chatuchuk	30	0
Ratchathewi	30	0
Phra Khanong	30	0
Bang Khen	30	0
Bang Kapi	30	0
Bang Na	30	0
Pom Prap Sattru Phai	30	0
Pathum Wan	30	0
Don Mueang	30	0
Sai Mai	30	1 (3.33)
Nong Khaem	30	1 (3.33)
Bang Rak	30	1 (3.33)
Prawet	30	0
Min Buri	30	0
Bang Sue	30	0
Bang Khae	30	0
Taling Chan	30	0
Rat Burana	30	0
Khlong San	30	0
Khlong Toei	30	0
Khlong Sam Wa	30	0
Bang Kho Laem	30	1 (3.33)
Lat Krabang	30	0
Bang Khun Thain	30	0
Bangkok Noi	30	0
Bangkok Yai	30	1 (3.33)
Bang Bon	30	0
Chom Thong	30	0
Thung Khru	30	0
Phasi Charoen	30	0
Thawi Watthana	30	1 (3.33)
Bang Phlat	30	0
Thon Buri	30	0
Yan Nawa	30	0
Watthana	30	0
Huai Khwang	30	0
Bueng Kum	30	5 (16.67)
Khan Na Yao	30	1 (3.33)
Din Daeng	30	2 (6.67)
Wang Thonglang	22	2 (9.09)
Dusit	29	2 (6.89)
Phra Nakhon	29	1 (3.45)
Nong Chok	30	1 (3.33)
Total	**1,490**	**21 (1.41)**

PCR results

The presence of Babeisa spp. In cat blood samples were analyzed by nested – PCR. Twenty one blood samples were PCR positive (21/1490:1.4%). Five positive samples came form two monasteries areas in Bueng Kum district and 16 from 21 samples covered 26% of study areas. The PCR products were sequenced, compared the similarity to the reported sequences in GenBank and shown 98% homology was observed with 3 – 4 nucleotide as minor variations. Blast search collated GeneBanK® demonstrated the 98% similar to *B. canis vogeli* (clone 10 18s ribosomal RNA gene, partial sequence from dog in Salvador, Bahia, Brazil) (GeneBank® accession number EU436752). (Figure. 14)

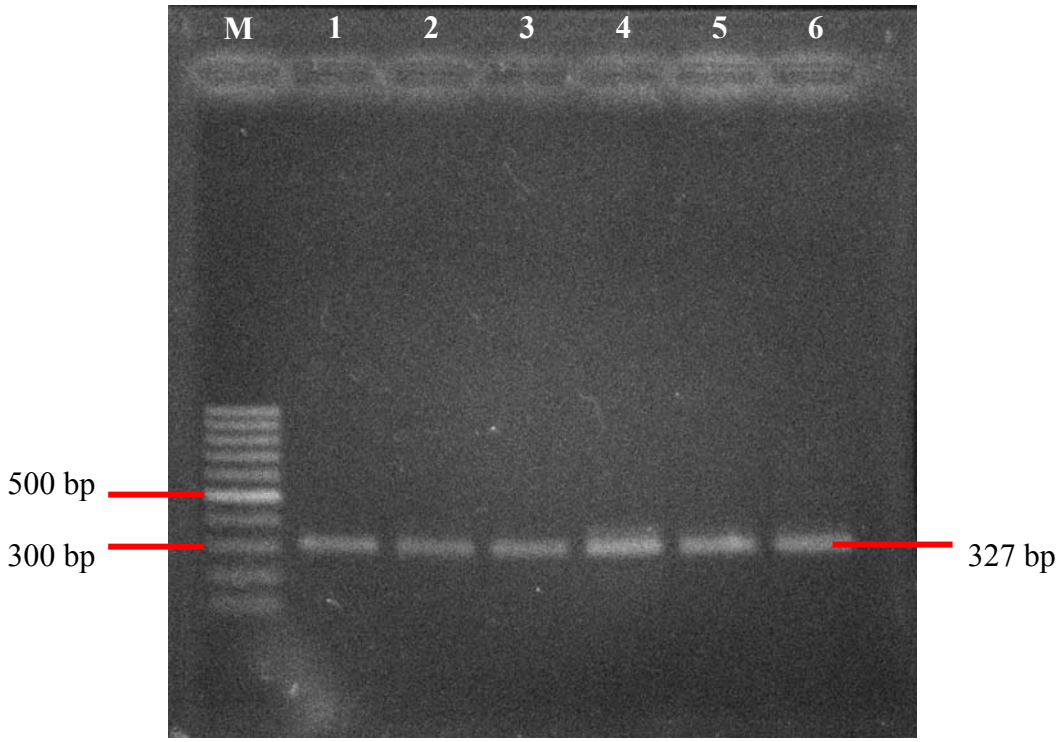
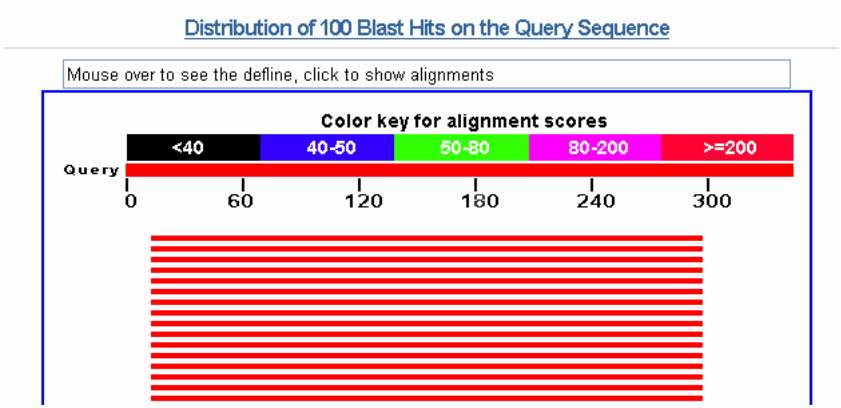


Figure 14 The PCR products (327 bp) of *B. canis vogeli* in ethidium bromide stained 1% agarose gel electrophoresis. M: marker 100 bp, Lane 1: Positive control, (dog's blood positive with *Babesia* spp by light microscope.); lane 2 - 6: cat blood sample (F544, F844, F1312, F1318, and F1320).



Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
EU436752.1	Babesia canis vogeli clone 10 18S ribosomal RNA gene, partial sequer	491	491	82%	6e-136	98%	
DQ297390.1	Babesia canis vogeli from Venezuela 18S ribosomal RNA gene, partial	491	491	82%	6e-136	98%	
DQ439545.1	Babesia canis vogeli isolate Spain 1 18S ribosomal RNA gene, partial	491	491	82%	6e-136	98%	
EF052633.1	Babesia canis vogeli isolate RP12 18S ribosomal RNA gene, partial se	491	491	82%	6e-136	98%	
EF052632.1	Babesia canis vogeli isolate RP11 18S ribosomal RNA gene, partial se	491	491	82%	6e-136	98%	
EF052631.1	Babesia canis vogeli isolate RP10 18S ribosomal RNA gene, partial se	491	491	82%	6e-136	98%	
EF052630.1	Babesia canis vogeli isolate RP9 18S ribosomal RNA gene, partial seq	491	491	82%	6e-136	98%	
EF052629.1	Babesia canis vogeli isolate RP8 18S ribosomal RNA gene, partial seq	491	491	82%	6e-136	98%	
EF052628.1	Babesia canis vogeli isolate RP7 18S ribosomal RNA gene, partial seq	491	491	82%	6e-136	98%	
EF052627.1	Babesia canis vogeli isolate RP5 18S ribosomal RNA gene, partial seq	491	491	82%	6e-136	98%	
EF052625.1	Babesia canis vogeli isolate RP3 18S ribosomal RNA gene, partial seq	491	491	82%	6e-136	98%	
EF052624.1	Babesia canis vogeli isolate RP2 18S ribosomal RNA gene, partial seq	491	491	82%	6e-136	98%	
EF052623.1	Babesia canis vogeli isolate RP1 18S ribosomal RNA gene, partial seq	491	491	82%	6e-136	98%	

Figure 15 BLAST search across multiple DNA databases by using BLASTN software showed the homology of *B. canis vogeli* clone 10 for rRNA with *Babesia* PCR product at 98% identity (EU436752)

Table 5 Prevalence of *Babesia* infection in stray cats classified by the monasteries and districts

District	Monastery	Total	PCR positive (%)	Microscopic Examination positive (%)
Bangkok Noi	Bangkhunnon	10	0	0
	Mai Yaipan	10	0	0
	Plengwipassana	10	0	0
Taling Chan	Pho	10	0	0
	Makok	6	0	0
	Noi Nai	14	0	0
Phasi Charoen	Pradu Bangchak	10	0	0
	Chaichimphli	10	0	0
	Tanot	10	0	0
Thon Buri	Buppharam Worawihan	10	0	0
	Bang Saikai	10	0	0
	Waramarttayapansararam	10	0	0
Bang Phlat	Bowon Mongkhon Ratchaworawihan	10	0	0
	Chaturamit Pradittharam	10	0	0
	Phanu Rangsi	10	0	0
Phra Nakhon	Makutkasattriyaram	10	0	0
	Parinayok	10	1 (10)	0
	Mahannapharam	10	0	0
Chom Thong	Sai	10	0	0
	Pho Kaeo	10	0	0
	Bang Khun Thain Nai	10	0	0
Dusit	Prasatbunyawat	10	1 (10)	0
	Sawatwarisimaram	10	0	0
	Thewaratchakunchon	10	1 (10)	0

Table 5 (Continued)

District	Monastery	Total	PCR positive (%)	Microscopic Examination positive (%)
Nong Chok	Mai Charoen Rat	6	0	0
	Saen Kasem	4	0	0
	Si Chomphu	10	0	0
	Krathumrai	10	1 (10)	0
Bang Khun Thain	Suthamwadi	7	0	0
	Prommarangsi	11	0	0
	Bua Pan	7	0	0
	Kampang	5	0	0
Bangkok Yai	Deedoud	10	1 (10)	0
	Tha Phra	10	0	0
	Khruea Wan Worawihan	10	0	0
Lat Krabang	Sutthaphot	10	0	0
	Uthai Thammaram	10	0	0
	Bueng Bua	10	0	0
Samphanthawong	Trimit	10	0	0
	Chakrawat	5	0	0
	Samphanthawong	13	0	0
Pom Prap Sattru Phai	Disanukaram	10	0	0
	Sitaram	10	0	0
	Khanikaphon	5	0	0
	Sa Ket	5	0	0
Bang Kho Laem	Chan Nok	8	1 (12.5)	1 (12.5)
	Ratchasingkhon	12	0	0
	Phai Ngoen Chotanaram	10	0	0

Table 5 (Continued)

District	Monastery	Total	PCR positive (%)	Microscopic Examination positive (%)
Khlong Sam Wa	Phraya Suren	10	0	0
	Chinditwihan	10	0	0
	Lam Kradan	10	0	0
Bang Sue	Liapratbamrung	10	0	0
	Thongsuttharam	10	0	0
	Soi Thong	10	0	0
Khlong San	Thongplang	10	0	0
	Thongthummachat	10	0	0
	Phichiyatikaram	10	0	0
Sai Mai	Yu Di Bamrung Tham	10	0	0
	Ko Suwannaram	10	0	0
	Rat Niyom Thom	10	1 (10)	0
Yan Nawa	Dokmai	10	0	0
	Dan	10	0	0
	Pariwat	10	0	0
Don Mueang	Thep Nimit	10	0	0
	Prommarangsi	10	0	0
	Khlong Ban Mai	10	0	0
Rat Burana	Bangpakok	10	0	0
	Prasertsutthawas	10	0	0
	Rat Burana	10	0	0
Bang Khae	Muang	10	0	0
	Ratbumrung	10	0	0
	Promsuwansamakki	10	0	0

Table 5 (Continued)

District	Monastery	Total	PCR positive (%)	Microscopic Examination positive (%)
Khlong San	Thongplang	10	0	0
	Thongthummachat	10	0	0
	Phichiyatikaram	10	0	0
Yan Nawa	Dokmai	10	0	0
	Dan	10	0	0
	Pariwat	10	0	0
Bang Kapi	Thep Lila	10	0	0
	Phra Kraisi	10	0	0
	Si Bunrueang	10	0	0
Bang Sue	Liapratbamrung	10	0	0
	Thongsuttharam	10	0	0
	Soi Thong	10	0	0
Khlong Toei	Khlong Toei Nok	10	0	0
	Khlong Toei Nai	10	0	0
	Saphanphrakhanong	10	0	0
Pathum Wan	Pathumwanaram	10	0	0
	Chai Mongkhon	10	0	0
	Duang Khae	10	0	0
Bueng Kum	Nuanchan	10	2 (20)	0
	Bangtaey	10	0	0
	Suwan Prasit	10	3 (30)	1 (10)
Bang Khae	Muang	10	0	0
	Ratbumrung	10	0	0
	Promsuwansamakki	10	0	0

Table 5 (Continued)

District	Monastery	Total	PCR positive (%)	Microscopic Examination positive (%)
Min Buri	Bang Pheng Tai	10	0	0
	Lumnok Khwaek	10	0	0
	Thong Samrit	10	0	0
Nong Khaem	Si Nuan Thammawimon	8	1 (12.5)	0
	Wong Lapharam	12	0	0
	Phai Lieang	10	0	0
Pathum Wan	Pathumwanaram	10	0	0
	Chai Mongkhon	10	0	0
	Duang Khae	10	0	0
Prawet	Kaeo Phithak Charoen Tham	10	0	0
	Thung Lanna	10	0	0
	Krathum Suea Pla	10	0	0
Bueng Kum	Nuanchan	10	2 (20)	0
	Bangtaey	10	0	0
	Suwan Prasit	10	3 (30)	1 (10)
Bang Kapi	Thep Lila	10	0	0
	Phra Kraisi	10	0	0
	Si Bunrueang	10	0	0
Bang Khen	Bang Bua	10	0	0
	Siri Phong Thamma Nimit	10	0	0
	Prasi Mahadhat	10	0	0
Bang Na	Si-Iam	10	0	0
	Phong Phloi Wittayaram	10	0	0
	Bang Na Nok	10	0	0

Table 5 (Continued)

District	Monastery	Total	PCR positive (%)	Microscopic Examination positive (%)
Bang Rak	Mahaphruettharam	10	0	0
	Muang Khae	10	1 (10)	0
	Hua Lumphong	10	0	0
Khlong Toei	Khlong Toei Nok	10	0	0
	Khlong Toei Nai	10	0	0
	Saphanphrakhanong	10	0	0
Lat Phrao	Ladplakhao	10	0	0
	Sirikamalawad	10	0	0
	Sakhon Sun Pracha San	10	0	0
Phra Khanong	Tham Mongkhon Thao Bunrot Nonthawihan	10	0	0
	Bunrot Thammaram	10	0	0
	Ratsatthatham	10	0	0
Ratchathewi	Payayang	10	0	0
	Thasanaroon Suntrikaram	10	0	0
	Dishongsaram	10	0	0
Sathon	Suthiwararam	5	0	0
	Lum Charoen Sattha	10	0	0
	Barom Sadhol (Don)	5	0	0
	Yan Nawa	10	0	0
Thung Khru	Phutthabucha	10	0	0
	Luang Pho O-Phasi	10	0	0
	Thung Khru	10	0	0
Huai Khwang	Phraram Kao Kanchanaphisek	5	0	0
	Maichonglom	15	0	0
	Uthaitharam	10	0	0

Table 5 (Continued)

District	Monastery	Total	PCR positive (%)	Microscopic Examination positive (%)
Khan Na Yao	Ratsatthatham	15	0	0
	Khlong Khru	4	1 (25)	0
	Bunsimunikon	11	0	0
Bang Bon	Ninsukharam	15	0	0
	Bang Bon	15	0	0
Chatuchuk	Sameiennaree	15	0	0
	Tewasoontorn	15	0	0
Din Dang	Kunnatiruttharam	15	1 (6.67)	0
	Phrom Wongsaram	15	1 (6.67)	0
Suan Luang	Mahabud	30	0	0
Thawi Watthana	Komut Phuttha Ransi	15	0	0
	Puranawas	15	1 (6.67)	0
Wang Thonglang	Bueng Thonglang	15	1 (6.67)	0
	Samakkhitham	7	1 (14.29)	0
Watthana	Thatthong	15	0	0
	Pasee	15	0	0
Lak Si	Lak Si	30	1 (3.33)	0
Phaya Thai	Paiton	30	0	0
Saphan Sung	Lad Buakao	30	0	0
Total		1490	21 (1.40)	2 (0.13)

Table 6 The summary of *Babesia* infection in stray cats detected by PCR and microscopic examination classified by monasteries, districts, sex and age group

District	Monastery	Total	PCR positive (%)	Microscope positive (%)	Sex	Age (month)
Lak Si	Lak Si	30	1 (3.33)	0	F	24
Sai Mai	Rat Niyom Thom	10	1 (10)	0	F	30
Nong Khaem	Si Nuan Thammawimon	8	1 (12.5)	0	F	48
Bang Rak	Muang Khae	10	1 (10)	0	F	48
Bang Kho Laem	Chan Nok	8	1 (12.5)	1 (12.5)	M	24
Bangkok Yai	Deedoud	10	1 (10)	0	F	12
Thawi Watthana	Puranawas	15	1 (6.67)	0	F	12
Bueng Kum	Nuanchan	10	2 (20)	0	F	36/24
	Suwan Prasit	10	3 (30)	1* (10)	F	24*/36/24
Khan Na Yao	Khlong Khru	4	1 (25)	0	F	24
Din Daeng	Kunnatiruttharam	15	1 (6.67)	0	F	48
	Phrom Wongsaram	15	1 (6.67)	0	M	60
Wang Thonglang	Bueng Thonglang	15	1 (6.67)	0	F	36
	Samakkhitham	7	1 (14.29)	0	F	12
Dusit	Prasatbunyawat	10	1 (10)	0	F	48
	Thewaratchakunchon	10	1 (10)	0	M	24
Phra Nakhon	Parinayok	10	1 (10)	0	F	24
Nong Chok	Krathumrai	10	1 (10)	0	F	36
Total		207	21(21.10)	2(0.97)		

Remark: F = female

M = male

GIS data analysis

The distribution mapping was constructed by the cooperation of the geographic programmer from Geo – Informatic and Space technology development Agency (Public Organization ,Thailand). The map was created by ArcGis ver. 9and shown the location of study areas in 140 monasteries of Bangkok (Figure 16 and Figure 17). Additionally, the 18 locations in satellite images showed positive *Babesia* infection analyzed by PCR method. The highest number of infected cats was found in Bueng Kum district (Figure 20).

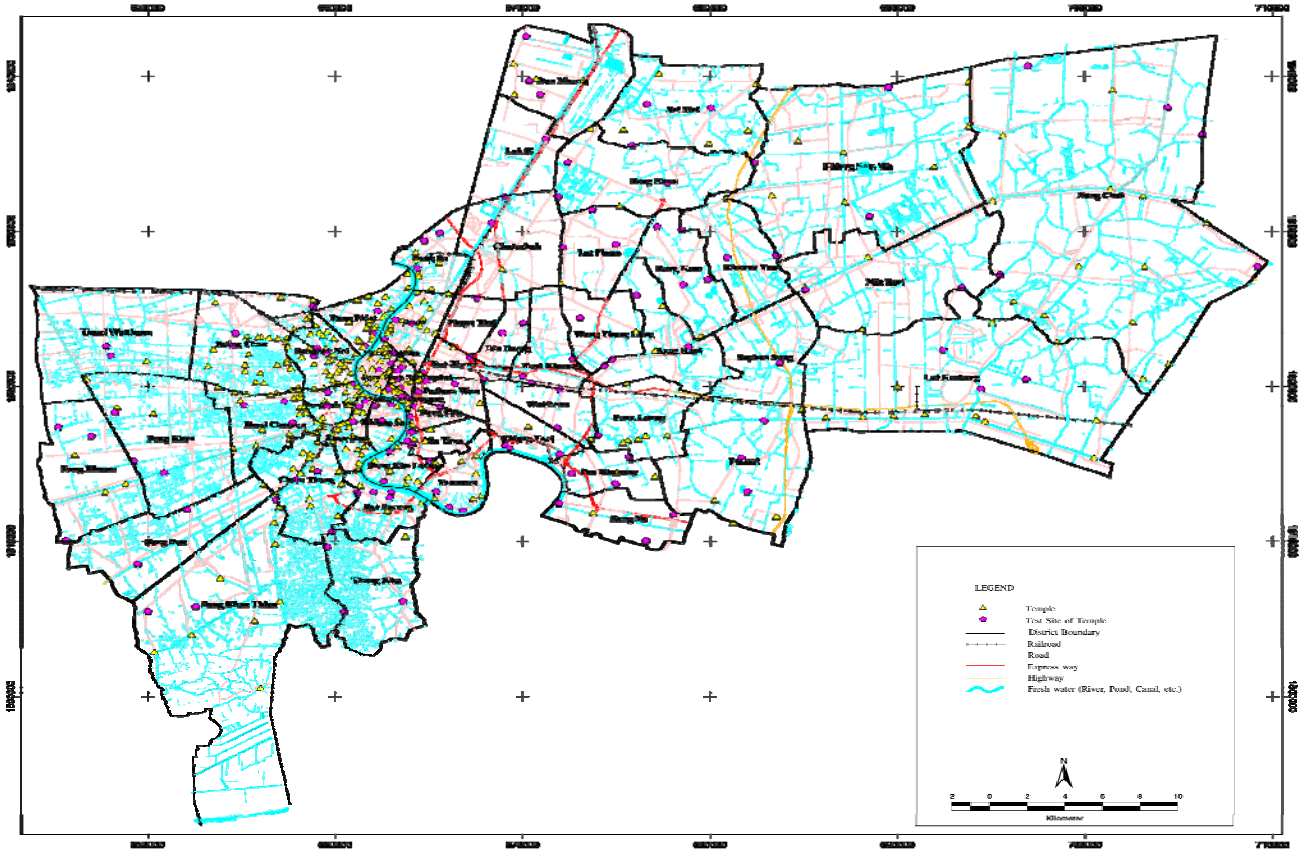


Figure 16 The location of the infected *Babesia* monasteries (●) and all monasteries in Bangkok metropolitan (▲). The selected monasteries was randomized by the simple randomizatio

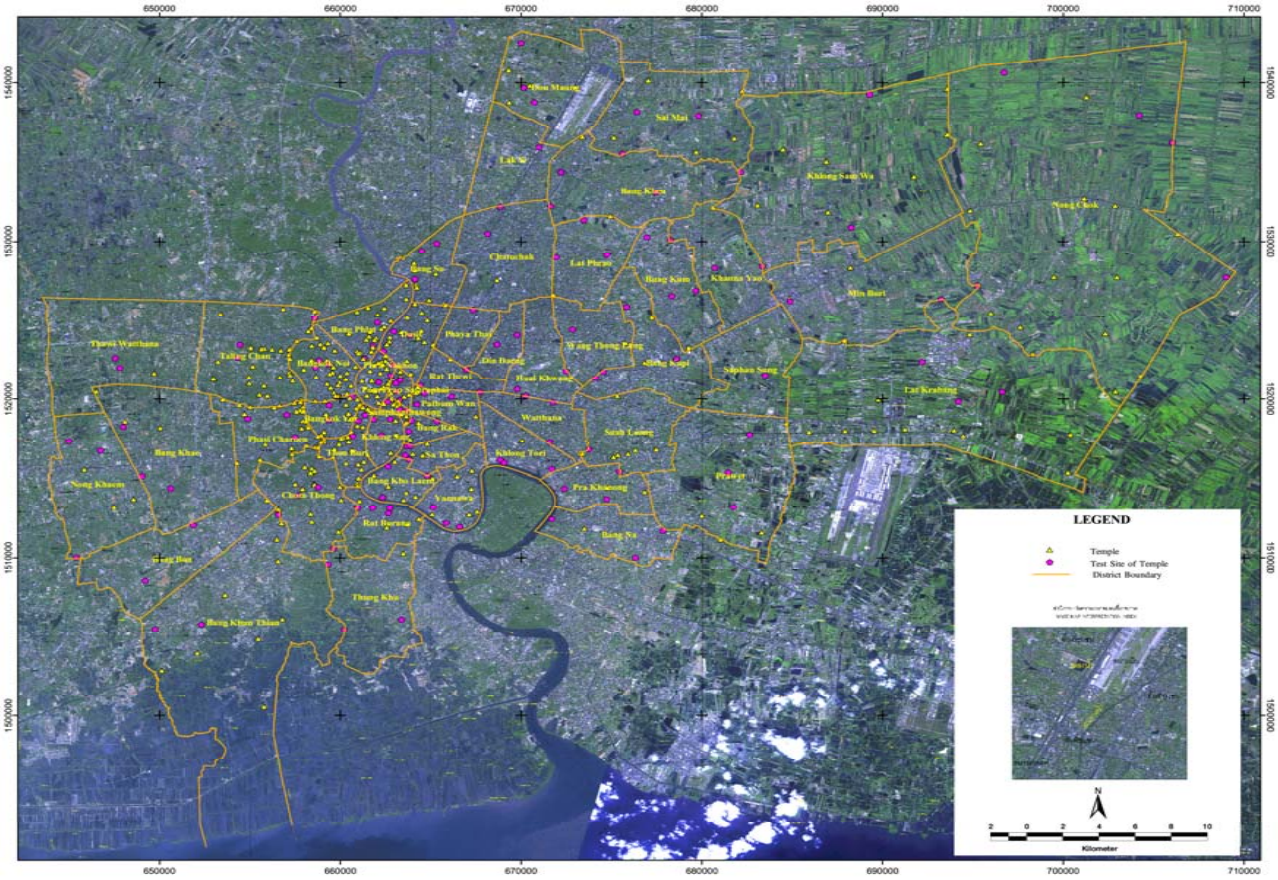


Figure 17 The satellite image shows the realistic condition of study areas as studied monasteries (●) and total monasteries in Bangkok (▲)

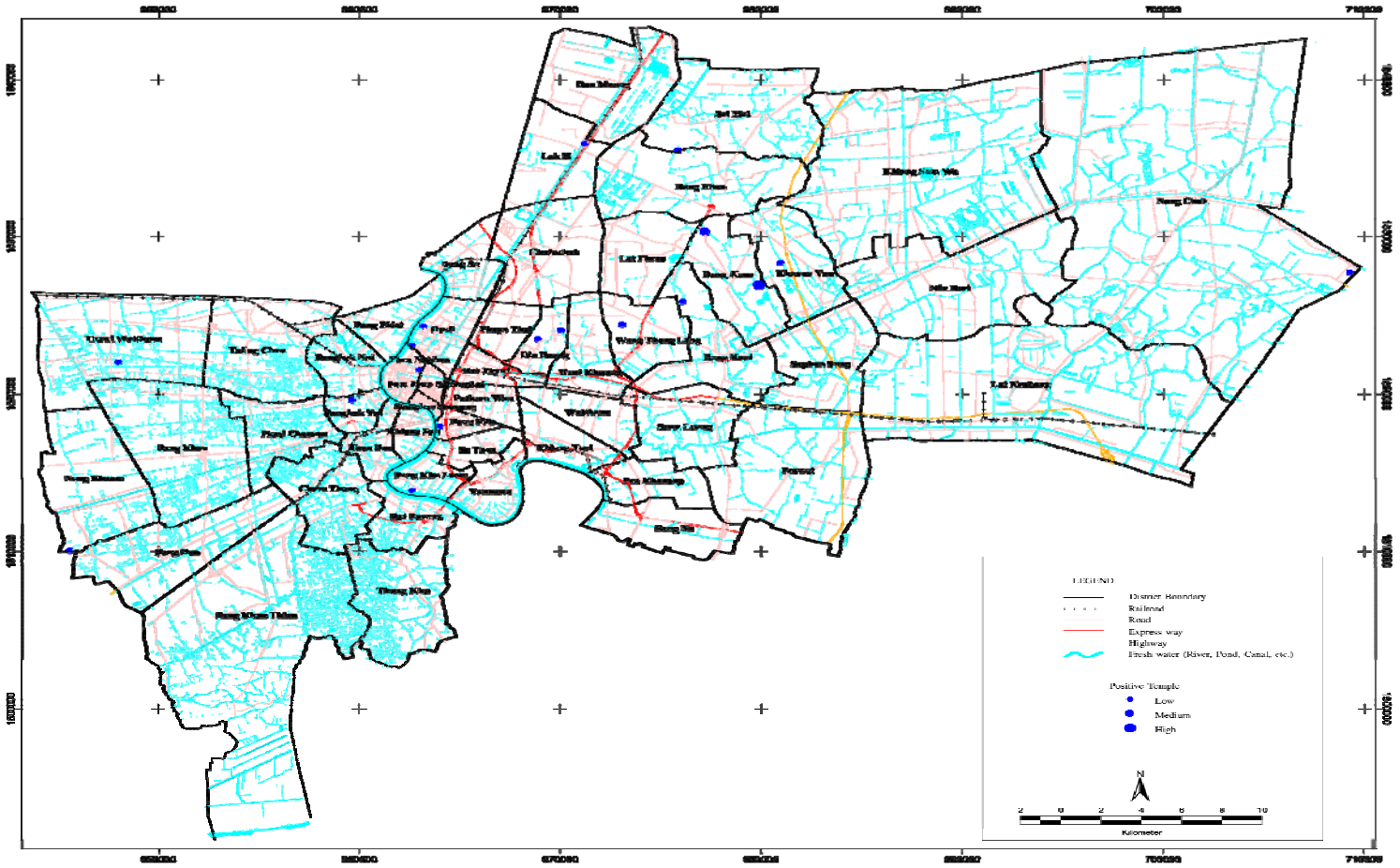


Figure 18 The location of the *Babesia* positive monasteries (18 places) (●) line on the map for identify the distribution of the infected monasteries.

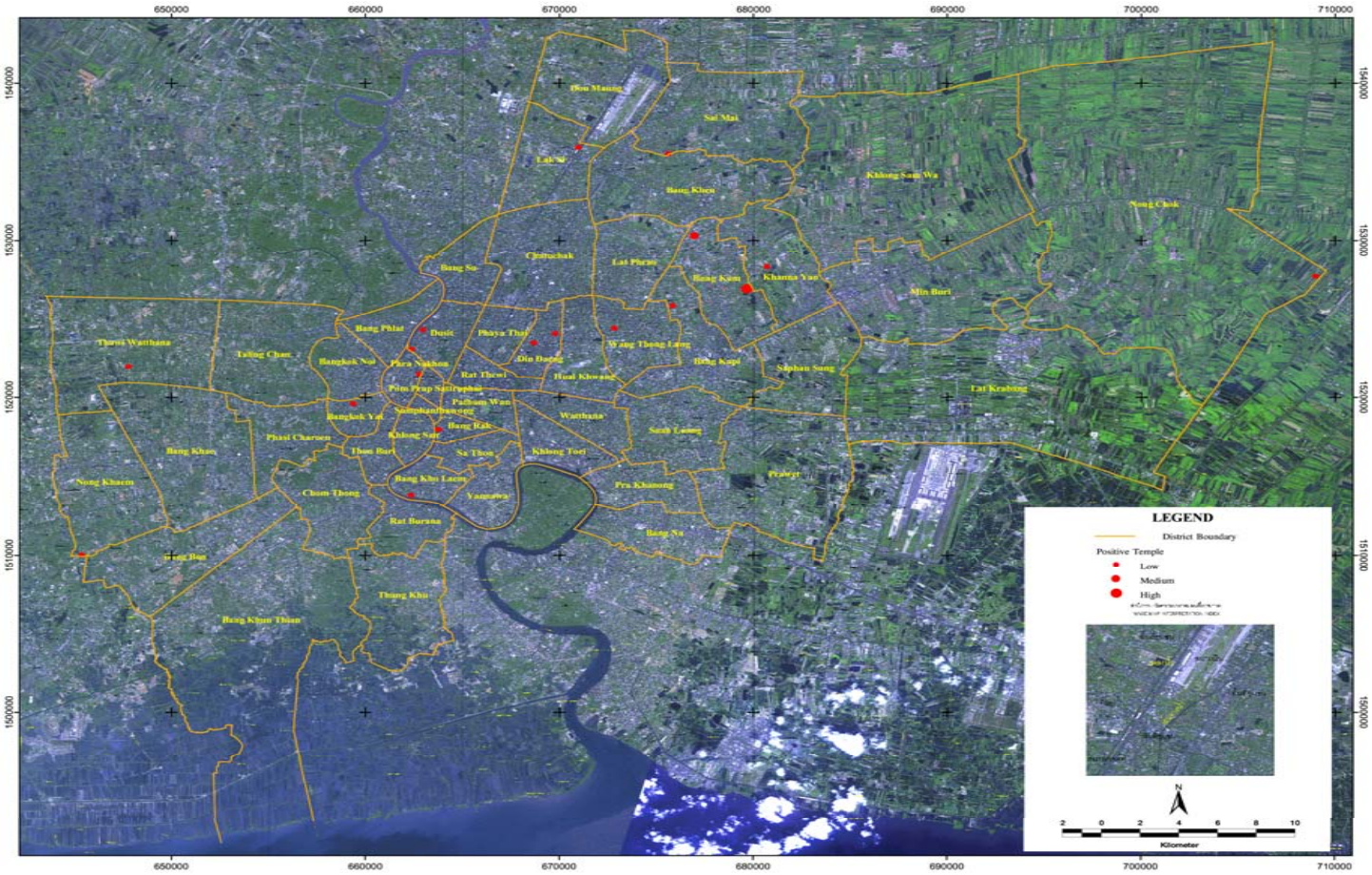


Figure 19 The satellite image shows the location of *Babesia* positive monasteries (18 places) (●) line on the realistic image to identify the environment and instruction nearest the place

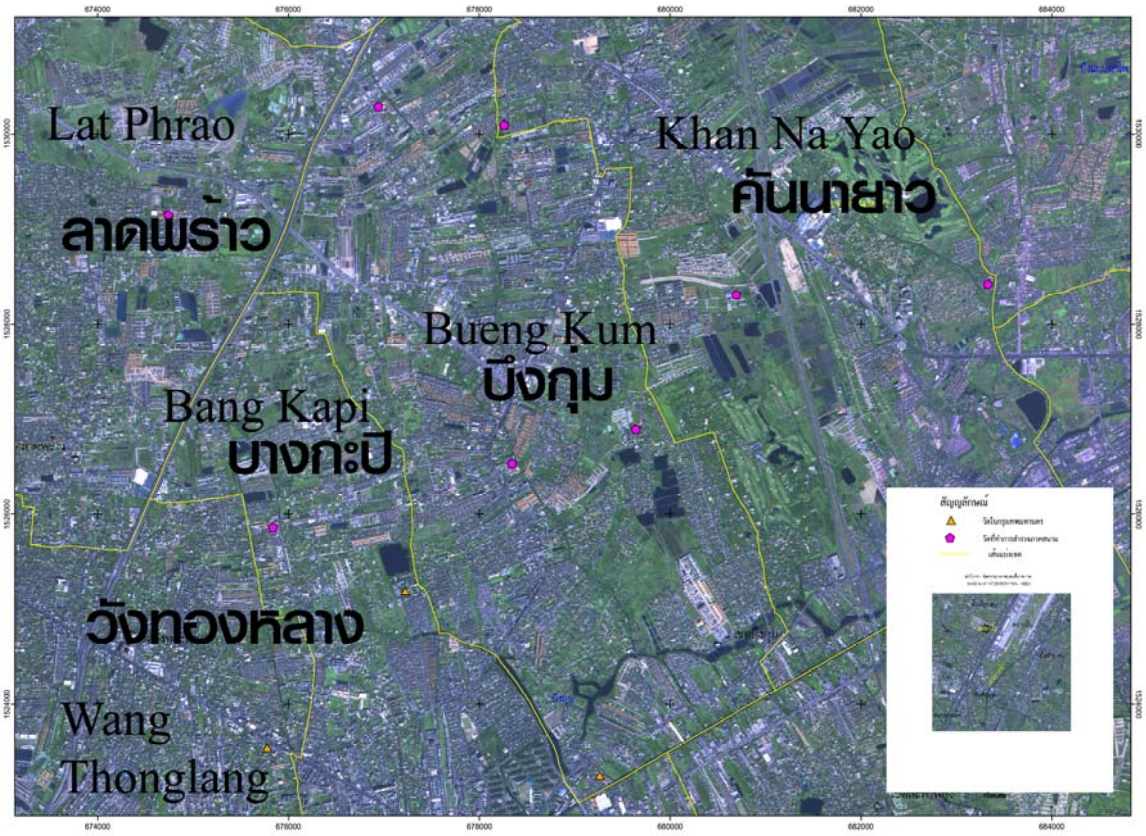


Figure 20 The satellite image shows the location of the *Babesia* positive monastery (●) in Bueng Kum district , and other position districts.

Statistic Analysis

From 1,490 cat blood samples, only 21 samples (1.4%) yielded the positive *Babesia* infection by PCR method. The factor associated with parasitic distribution was shown in table 7. However, the only one factor that has the statistic significant for the spreading of the organism is sex ($p = 0.02$, $\chi 2 = 5.02$, $df = 1$) but other factors such as age, health condition, environment condition, ectoparasite, and the distance to the water resource such as river or canal were not significant ($p > 0.05$).

Table 7 Statistical analysis of factors associated with *Babesia* infection of stray cats in Bangkok areas

Statistic analysis	**Statistic value**		
	Chi-square	**Degree of freedom**	**Probability (p value)**
Sex	5.02	1	0.02
Age	The analysis of Variance and found no different between group of age		
Health condition	0.51	2	0.77
Environment condition.	3.49	2	0.17
Ectoparasite	0.23	1	0.62
River (distance)	0.26	1	0.60

DISCUSSION

Diagnosis of *Babesia* infection.

Babesiosis caused the hemolytic anemia in animals. The degree of severity and therapeutic response depended on the species or subspecies of *Babesia* piroplasm (Birkenheuer *et al.,* 2003). Generally, the diagnosis of *Babesia* organism in the vertebrate host is mainly due to the identification of *Babesia* by microscopic examination. However this method required expertise since the piroplasm has the closely morphological character. Therefore, it may confuse when mixed infections occur (Aktaş *et al.,* 2005).

The serological tests were reported using Indirect fluorescent antibody test (IFAT) and Enzyme linked – immunosorbent assay (ELISA) for detecting the *B. gibsoni* parasite, but there are some difficulties with specificity and sensitivity since the cross reaction among the species happened. Moreover, many reports have shown the false-negative and false – positive results by the microscopic and serologic examination, respectively in infected dogs (Breishwerdt *et al.,* 1983; Birkenheuer *et al.,* 1999). However, the detection of *Babesia* infection in carrier animals by DNA amplification was a prestige tool for epidemiological investigation and more sensitivity and specificity than detection by conventional method (Aktaş *et al.,* 2005). In the negatives samples analyzed by microscopic examination, they turned out to be positive by PCR method. For this reason, this nested PCR technique is useful to detect low parasitemia or in the carrier animals because of its high sensitivity. Therefore, the results showed higher specificity and sensitivity of PCR assay (1.4%) to detect *B. canis vogeli* in stray cats compared to the microscopic examination (0.2%).

The distribution of the parasite

The babesiosis is generally distributed among a wide range of wild and domestic animals (Kuttler, 1988). There are many studies about the epidemiology of *Babesia* agent throughout the world. In Hungary, the occurrence of canine babesiosis was in accordance with the geographic distribution of the vector, *D. reticulatus* and suggested that large part of the country can be considered to be endemic for this disease (Földvári *et al.,* 2005) by detected the 39 positive (88.6%) form 44 blood samples form dogs showing clinical signs. In South Africa, *B. canis* were serologically found varied from strains transmitted by *Hyalomma leachi* and suggested that these strains may be transmissible by *R. sanguineus* (Matjila *et al.,* 2004). Many preliminary epidemiological survey of *H. leachi*-transmitted *B. canis* strains have been reported in various regions of South Africa (Lewis *et al.,* 1996).

In thailand, most common dog tick was *R. sanguineus* (brown dog tick), the vector of *B. canis vogeli* (Földvári *et al.,* 2005), and this tick is spreading in tropical and subtropical region. In fact, stray animals in the metropolitan areas are always contact to the human and domestic dogs, so they would be a potential carrier of some pathogens to the public community. As the result of cat localized in 18 monasteries were infected by *B. canis vogeli*, this indicated that the distribution of *Babesia* organism in stray cats might be due to the condense number of cats in each monastery or the density of cats nearby monasteries. However, there was no correlated with the humidity, or the agriculture land.

The factor that should get more attention is the density of stray dogs (natural host) nearby or sharing the environment. Distribution of canine babesiosis is extremely correlated with the habitat of stray animals. The statistical analysis showed that sex was also a significant factor. Normally, the life – span of female cats are often longer than male cats and always stay in their familiarized place where can make them easier to infest by the parasite.

The GIS technique

Recently, Geographic Information System (GIS) have been used to be another dimension to epidemiology of infectious disease (Zanilman *et al.,* 2005). GIS technology provides the integration of data from a wide variety sources, with the combined the benefit of computer graphic assisted display. Therefore, GIS have been widely proposed as a public health tool for the investigations of chronic diseases, zoonoses, injuries and accident patterns, allocation of health resources, occupational morbidity and environmental exposure (Zanilman *et al.,* 2005).

GIS is beneficial for case reports associated with time, place and person from the traditional framework of infectious disease surveillance program. In this study, mapping distribution of *Babesia* infections in Bangkok was created by ArcGis Ver. 9 That revealed the location and the infrastructure nearby the infected location and indicated the factor involved with parasitic distribution.

By the useful of GIS technology, it is possible to provide the strategies to identify the relation of the environment for prognosis the transmission of vector borne diseases (Melone *et al.,* 2001). For current result, it was shown that babesiosis might be mainly related to the density of the house cats nearby the monasteries that will lead to the increasing of babesiosis incidence of the stray animals or domestic animals within endemic areas. In addition, GIS will give some realistic visualization to initiate the preventive program for other infectious disease in the further study of epizoonotic disease. The model may be expanded to enable the development of other investigations on a national scale.

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APPENDICES

Appendix A
The Standard Method

- **Phenol – Chloroform Extraction of DNA and Ethanol precipitation (Sambrook and Russel, 2001)**

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

- **Agarose Gel Electrophoresis**

1. Prepare an agarose gel by combining the agarose and water in a 500 ml flask
2. Pour the gel onto an electrophoresis plate with casing comb in place. Allow 30 – 45 minutes for solidification.
3. Carefully remove the gel casting combs and place the gel in a horizontal electrophoresis apparatus. Add 1x TBE electrophoresis buffer to reservoirs until the buffer just cover the agarose gel.
4. Add 2 µl of 6x agarose loading dye (Fermentus®) with 5 – 7 µl of DNA sample, mix and load into the wells. Electrophoresis the gel at 100 V/cm for 45 – 50 minutes or until the required separation has been achieved.
5. Incubate the agarose gel in Ethidium Bromide (EtBr) tank for 10 – 15 minutes.

6. Visualize the DNA fragments on a long wave UV light box.

- **QIAquick Gel Extraction Kit Protocol**: using a microcentrifuge

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

14. If the purified DNA is to be analyzed on a gel =, add 1 volume of loading dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix B
Chemical reagent and Substance

- **Buffer for dissolve DNA pallet**: TE buffer (Tris - EDTA)

  - 10x TE buffer (1 liter)

    Tris HCL (MW 157.6 g) 15.76 g

    EDTA (MW 372.24 g) 3.72 g

    Distilled water 1 liter

    Adjust pH to 8.0

  - 1x TE buffer (TE pH 8.0)

    10mM Tris HCL

    1mM EDTA

    Adjust pH to 8.0

- **Buffer for Agarose Gel Electrophoresis**

  - 10x TBE stock solution (1 liter) pH 8.0

    Tris base (MW 121.14 g) 108 g

    Boric acid (MW 61.83 g) 55 g

    EDTA (MW 372.24) 9.3 g

    Distilled water (DW) 1 liter

    Adjusted pH to 8.0

  - 1x TBE working solution (1 liter)

    10x TBE working solution 100 ml

    DW 900 ml

    Mix by shaken and adjusted pH to 8.0

  - 6x Loading Dye

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

Appendix C

The picture from two infected monastery

Appendix Figure C1 Wat Suwan Prasit, the most infected monastery of *Babesia* spp (3/1490, 0.2%)

Appendix Figure C2 Wat Suwan Prasit, the small park in the monastery, in the long distance is the monk's house.

Appendix Figure C3 Wat Parinayok, Phra Nakhon District, where found one female stray cat (0.06%) was infected with *Babesia* spp.

Appendix Figure C4 The habitat of the stray cats leaving with a nun in the monastery

Appendix Figure C5 The feeding place in nun's house for the stray cat

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