A MOLECULAR SURVEY OF TICK-BORNE PATHOGENS (Anaplasma AND Ehrlichia spp.) IN ANIMAL AND TICK SAMPLES IN MALAYSIA

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KUALA LUMPUR

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A MOLECULAR SURVEY OF TICK-BORNE PATHOGENS (*Anaplasma* AND *Ehrlichia* spp.) IN ANIMAL AND TICK SAMPLES IN MALAYSIA

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Ehrlichia spp.) IN ANIMAL AND TICK SAMPLES IN MALAYSIA
Field of Study: MOLECULAR MICROBIOLOGY

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ABSTRACT

Anaplasmosis and ehrlichiosis are tick-borne diseases which are caused by Gram-negative obligate intracellular bacteria in the family *Anaplasmataceae*. The diseases have been reported in a wide variety of wild and domestic animals from different parts of the world. Little data is available on the prevalence and transmission of the diseases in Malaysia. In this study, the occurrences of *Anaplasma* spp. and *Ehrlichia* spp. in animal and tick samples derived from different sources were determined. A total of 304 blood samples collected from livestock farms (cattle, sheep and goats) and 393 various animal blood samples provided by researchers from Veterinary Research Institute, Malaysia and Department of Wildlife and National Parks (PERHILITAN) Peninsular Malaysia from 2013-2014 for health screening were included in this investigation. A wide variety of ticks collected from livestock farms, aboriginal villages and the forest areas were subjected to morphological identification and molecular analysis of tick mitochondrial 16S rRNA gene to assist tick identification. *Haemaphysalis* and *Dermacentor* ticks were the main ticks identified in this study, besides *Amblyomma* and *Rhipicephalus* ticks. Using specific polymerase chain reaction (PCR) assays, *Anaplasma* DNA was detected in 136 (60.7%) cattle and 32 (80.0%) sheep from livestock farms investigated in this study. Majority of the *Anaplasma* spp. detected from the cattle were *Anaplasma marginale*, while all goats were not infected by *Anaplasma* spp. *Anaplasma bovis* DNA was detected from both wild and domestic animals in this study. A novel *Anaplasma* sp. tentatively designated as *Candidatus* Anaplasma pangolinii was detected from three of 15 pangolins (*Manis javanica*) investigated in this study. Both *Ehrlichia canis* and *Anaplasma phagocytophilum* were detected in dog blood samples and *Rhipicephalus sanguineus* ticks. *A. phagocytophilum* was mainly detected in questing ticks from the forest areas. A total of 61.5% ticks infesting livestock animals (cattle and sheep), 28.8% ticks collected from vegetation and small animals in the forest areas and 37.0% ticks...
infesting peri-domestic animals in aboriginal villages were PCR-positive for *Anaplasmataceae* DNA. Sequence analyses of the bacterial 16S rRNA gene region (238 bp) provided the identification for *A. marginale*, *A. bovis*, *A. phagocytophilum*, *Anaplasma platys*, *Anaplasma* spp. and *Ehrlichia* spp. in ticks and animal blood samples. New sequence variants of *Anaplasma* spp., *Ehrlichia* sp. strain EBm52, *Ehrlichia mineirensis* and *Candidatus* Ehrlichia shimanensis were identified in this study. In conclusion, the high detection rates of *A. marginale* in cattle blood samples may affect the livestock production in the cattle farms. The detection of *Anaplasma* spp. and *Ehrlichia* spp. in questing ticks and ticks infesting domestic and wildlife animals may pose a risk to human who come into contact with ticks or tick-infested animals. This study reports for the first time the type and distribution of various *Anaplasma* spp. and *Ehrlichia* spp. in animals and ticks present in this region. Further investigation should be carried out on the potential role of various tick species and the transmission dynamics of anaplasmosis and ehrlichiosis. Appropriate measures should be instituted for prevention and control of tick-borne diseases in Malaysia.
ABSTRAK

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<td>B. bovis</td>
<td>Bartonella bovis</td>
</tr>
<tr>
<td>B. burgdorferi</td>
<td>Borrelia burgdorferi</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>C. ruminantium</td>
<td>Cowdria ruminantium</td>
</tr>
<tr>
<td>Candidatus A. pangolinii</td>
<td>Candidatus Anaplasma pangolinii</td>
</tr>
<tr>
<td>Candidatus E. shimanensis</td>
<td>Candidatus Ehrlichia shimanensis</td>
</tr>
</tbody>
</table>
cytB : cytochrome B
CME : canine monocytotropic ehrlichiosis
D. albipictus : Dermacentor albipictus
D. andersoni : Dermacentor andersoni
D. atrosignatus : Dermacentor atrosignatus
D. auratus : Dermacentor auratus
D. everestianus : Dermacentor everestianus
D. immitis : Dirofilaria immitis
D. marginatus : Dermacentor marginatus
D. nitens : Dermacentor nitens
D. niveus : Dermacentor niveus
D. nuttalli : Dermacentor nuttalli
D. occidentalis : Dermacentor occidentalis
D. reticulatus : Dermacentor reticulatus
D. silvarum : Dermacentor silvarum
D. variabilis : Dermacentor variabilis
DNA : deoxyribonucleic acid
dNTPs : Deoxynucleotide triphosphates
E : east
E. canis : Ehrlichia canis
E. chaffeensis : Ehrlichia chaffeensis
E. coli : Escherichia coli
E. equi : Ehrlichia equi
E. ewingii : Ehrlichia ewingii
E. kageus : Eothenomys kageus
E. mineirensis : Ehrlichia mineirensis
E. muris : Ehrlichia muris
E. phagocytophila : Ehrlichia phagocytophila
E. ruminantium : Ehrlichia ruminantium
EDTA : Ethylenediaminetetraacetic acid
ELISA : enzyme-linked immunosorbent assay
EML : Ehrlichia muris-like
et al. : et alia (Latin), and others
G+C : guanine+ cytosine
gltA : citrate synthase gene
groEL : heat shock protein
H. aborensis : Haemaphysalis aborensis
H. asiatica : Haemaphysalis asiatica
H. bispinosa : Haemaphysalis bispinosa
H. canis : Hepatozoon canis
H. concinna : Haemaphysalis concinna
H. douglasii : Haemaphysalis douglasii
H. flava : Haemaphysalis flava
H. formosensis : Haemaphysalis formosensis
H. hystricis : Haemaphysalis hystricis
HAEMAPHYSALIS LANGRANGEI
Haemaphysalis langrangei

HAEMAPHYSALIS LEPORISPALSTRIS
Haemaphysalis leporispalustris

HAEMAPHYSALIS LONGICORNIS
Haemaphysalis longicornis

HAEMAPHYSALIS MEGASPINOSA
Haemaphysalis megaspinosa

HAEMAPHYSALIS OBESA
Haemaphysalis obesa

HAEMAPHYSALIS PUNCTATA
Haemaphysalis punctata

HAEMAPHYSALIS QINGHAIENSIS
Haemaphysalis qinghaiensis

HAEMAPHYSALIS SHIMA
Haemaphysalis shimoga

HAEMAPHYSALIS SULCATA
Haemaphysalis sulcata

HAEMAPHYSALIS WELLINGTONI
Haemaphysalis wellingtoni

HAEMAPHYSALIS YENI
Haemaphysalis yeni

HYALOMMA ANATOLICUM
Hyalomma anatolicum

HYALOMMA ASIATICUM
Hyalomma asiaticum

HYALOMMA DETRITUM
Hyalomma detritum

HYALOMMA MARGINATUM
Hyalomma marginatum

HEE
human ewingii ehrlichiosis

HGA
human granulocytic anaplasmosis

HIV
human immunodeficiency virus

HME
human monocytic ehrlichiosis

IXODES DENTATUS
Ixodes dentatus

IXODES FRONTALIS
Ixodes frontalis

IXODES NIPPONENSI
Ixodes nipponensis

IXODES OVATUS
Ixodes ovatus

IXODES PACIFICUS
Ixodes pacificus

IXODES PERSULCATUS
Ixodes persulcatus

IXODES RICINUS
Ixodes ricinus

IXODES SCAPULARIS
Ixodes scapularis

IXODES SPINIPALPIS
Ixodes spinipalpis

IXODES TRIANGULICEPS
Ixodes trianguliceps

IXODES TURDUS
Ixodes turdus

IXODES VENTALLOI
Ixodes ventalloi

i.e., id est (Latin), that is

IFIA
immunofluorescent assay

IFAT
immunofluorescent antibody test

IgG
immunoglobulin G

K
guanine or thymine

kb
kilobase

kg
kilogram

L
left

LAMP
loop-mediated isothermal amplification

LB
Luria-Bertani

LPS
lipopolysaccharide

M
adenine or cytosine

Mb
megabase

MEGA
Molecular Evolutionary Genetics Analysis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>U/µl</td>
<td>unit per microlitre</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>VHE</td>
<td>Venezuela human <em>Ehrlichia</em></td>
</tr>
<tr>
<td>W</td>
<td>adenine or thymine</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>Y</td>
<td>cytosine or thymine</td>
</tr>
<tr>
<td>Appendix A</td>
<td>Source and details of samples collected from livestock farms in this study</td>
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<tr>
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<tr>
<td>Appendix B</td>
<td>Source and details of samples from PERHILITAN in this study</td>
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<tr>
<td>Appendix C</td>
<td>Source and details of tick samples collected from aboriginal villages in this study</td>
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<tr>
<td>Appendix D</td>
<td>Source and details of animal blood samples provided by VRI, Ipoh, Malaysia for this study</td>
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<td>Appendix E</td>
<td>Source and details of samples collected from urban areas (Selangor and Klang Valley) in this study</td>
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<td>Appendix F</td>
<td>Primers and PCR conditions used in this study</td>
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<td>Appendix G</td>
<td>Media preparation</td>
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<td>Appendix I</td>
<td>Papers presented</td>
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<td>Appendix J</td>
<td>GenBank submission of sequences obtained in this study</td>
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CHAPTER 1: INTRODUCTION

Anaplasmosis and ehrlichiosis are diseases that are transmitted by ticks in both animals and humans (Parola and Raoult, 2001). *Anaplasma* spp. and *Ehrlichia* spp. are small Gram-negative, obligate intracellular bacteria in the family of *Anaplasmataceae* that replicate within eukaryotic host cells (Dumler et al., 2001). According to the latest classification by Dumler et al. (2001), the genus *Anaplasma* includes *Anaplasma phagocytophilum, Anaplasma bovis, Anaplasma marginale, Anaplasma centrale, Anaplasma ovis* and *Anaplasma platys* whereas the genus *Ehrlichia* includes *Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia canis, Ehrlichia muris* and *Ehrlichia ruminantium*. To date, *A. phagocytophilum, E. chaffeensis* and *E. ewingii* are the most significant species in the family *Anaplasmataceae* that cause human infections while all the species in the family *Anaplasmataceae* can cause infection in animals. Economic loss due to *Anaplasma* and *Ehrlichia* infections in domestic animals has been reported worldwide (Atif, 2016).

Transmission of anaplasmosis and ehrlichiosis to humans are mostly through tick bites or when in contact with the fluids or secretions of infected ticks. The clinical presentation of anaplasmosis and ehrlichiosis are often nonspecific, with patients presenting with febrile illness, headache, myalgia, malaise and complications affecting the respiratory, renal and central nervous systems, especially amongst immunosuppressed patients (Bakken and Dumler, 2015; Dumler, 2005; Dumler et al., 2007; Ismail et al., 2010).

Due to the intracellular nature of the bacteria in the genera *Anaplasma* and *Ehrlichia*, conventional method of culturing on agar media cannot be applied. Hence, detection of *Anaplasma* spp. and *Ehrlichia* spp. is usually performed by examination of peripheral blood smears, *in vitro* cultivation using tissue cultures, serological or molecular diagnostic methods. Molecular techniques including conventional polymerase
chain reaction (PCR), real-time PCR (qPCR), together with direct sequencing are considered as the most rapid and sensitive methods for detection of *Anaplasma* spp. and *Ehrlichia* spp. in human and animal samples (Guillemi *et al.*, 2015). Using direct sequencing technique, amplified PCR amplicons can be sequenced and analysed for comparison with known sequences deposited in the GenBank database.

Most reports on human anaplasmosis and ehrlichiosis are documented in the United States of America (USA) and European countries (Rar and Golovljova, 2011). The recent reports of anaplasma and ehrlichiae in humans, animals and ticks in China, Japan and Korea (Kim *et al.*, 2014; Lee *et al.*, 2009; Ohashi *et al.*, 2005; Ohashi *et al.*, 2013; Zhang *et al.*, 2008b) suggest the existence of these organisms in the Asia region. The seroprevalence of canine ehrlichiosis and bovine anaplasmosis have been reported previously (Rahman *et al.*, 2012). *A. platys* and *E. canis* have also been detected in dogs using PCR assays (Mokhtar *et al.*, 2013; Nazari *et al.*, 2013).

People who live at the fringe of the forest or rural areas in Malaysia, including the indigenous community and livestock farm workers, are regarded as populations who are at high risk of acquiring tick-borne diseases (Ghane Kisomi *et al.*, 2016; Kho *et al.*, 2017). The indigenous community (also referred as Orang Asli or ‘original people’) is a minority group representing only 0.6% of the total population in Malaysia. They stay in huts or settlements surrounded by forest, and engage in activities involving agriculture, hunting and collection of forest products. Their nomadic lifestyle and close contact with peri-domestic animals have increased the risk of potential tick-borne diseases including anaplasmosis and ehrlichiosis (Masron *et al.*, 2013).

Although anaplasmosis and ehrlichiosis are emerging zoonotic diseases, little information is known in Malaysia. Few studies have investigated the extent of exposure of the local populations to these tick-borne diseases in Southeast Asia (SEA). A recent study reported high seroprevalence of ehrlichiosis in Malaysian indigenous people.
(34.3%) and livestock farm workers (29.9%) (Koh et al., 2018). In addition, little information is available on the potential vectors and maintenance hosts of Anaplasma spp. and Ehrlichia spp. in SEA countries, especially in Malaysia. The economic losses caused by anaplasmosis and ehrlichiosis have not been thoroughly investigated in Malaysia. There is a lack of extensive studies in Malaysia on these animal diseases (Rahman et al., 2012).

Hence, this study is proposed with the objectives:

i. to identify tick species using morphological and molecular identification methods.

ii. to identify Anaplasma spp. and Ehrlichia spp. from animals and ticks using molecular methods.

iii. to determine phylogenetic status of Anaplasma spp. and Ehrlichia spp. of local strains based on sequence analysis of various genes (16S rDNA, gltA, msp4 and groEL).
CHAPTER 2: LITERATURE REVIEW

2.1 Anaplasma spp.

The genus Anaplasma is consisted of a group of tick-associated bacteria belong to the family Anaplasmataceae, order Rickettsiales and class Alphaproteobacteria. The Anaplasma spp. are small Gram-negative, often pleomorphic, coccoid to ellipsoidal, obligate intracellular bacteria that replicate in membrane-bound vacuoles within the cytoplasm of an eukaryotic host (Dumler et al., 2001; Rikihisa, 2011). According to the reorganisation of genera in the family Anaplasmataceae by Dumler et al. (2001), the species included in the genus Anaplasma are A. phagocytophilum, A. bovis, A. marginale, A. centrale, A. ovis and A. platys.

2.1.1 Anaplasma marginale

A. marginale is the type species for the genus Anaplasma and the causative agent for bovine anaplasmosis (Dumler et al., 2001; Kocan et al., 2003). The bacterium was first described in 1910 by Sir Arnold Theiler who noticed the presence of small inclusions near the edge of the erythrocytes of sick cattle (Theiler, 1910). Giemsa stain is usually used for the visualisation of A. marginale where the bacteria appear as rounded and deeply stained inclusion bodies that located at the margin of the infected erythrocytes. The bacterial size is approximately 0.3 to 1.0 µm in diameter (OIE, 2012).

The genome size of A. marginale is approximately 1.2 Mb (based on St. Maries strain which was isolated from a cow with severe acute anaplasmosis) with unusual high G+C (guanine + cytosine) content of 49.8% (Brayton et al., 2005). Most of the obligate intracellular bacteria have low G+C content, for example, the G+C content of Rickettsia prowazekii (strain Madrid E), Wolbachia pipiensis (strain wMel) and E. ruminantium (Welgevonden-type strain) are 29.1%, 35.2% and 27.5%, respectively (Andersson et al., 1998; Collins et al., 2005; Wu et al., 2004). The absence of genes for lipopolysaccharide (LPS) biosynthesis in A. marginale has resulted with a lack of a cell wall in this bacterium.
Unlike other bacteria in the family *Anaplasmataceae* which are fragile due to the lack of cell wall, *A. marginale* is able to strengthen its cell wall through the major surface proteins (MSPs), homeric and heteromeric complexes which are present on the cell surface (Brayton et al., 2005; Vidotto et al., 1994).

*A. marginale* is known to infect erythrocytes of cattle and other ruminants including buffaloes, elk, deer and bison (Kocan et al., 2010). Human infection due to *A. marginale* has not been reported (OIE, 2012). *A. marginale* can be transmitted through ticks or biting flies or mechanically through blood-contaminated instruments used for veterinary practice such as needles and ear-tag applicators (Kocan et al., 2015). Intrastadial transmission (by male ticks) and transplacental transmission (which results in healthy but constantly infected calves) are also important routes that contribute to *A. marginale* transmission (Kocan et al., 2003).

### 2.1.2 Anaplasma phagocytophilum

The bacterium was initially known as the causative agent of tick-borne fever (TBF) in sheep and cattle with the given name *Rickettsia phagocytophila*. It was then renamed as *Cytoecetes phagocytophila* due to its morphological similarity to *Cytoecetes microti*, a parasite detected in the polymorphonuclear cells of the vole, *Microtus pennsylvanicus* (Tyzzer, 1938). The bacterium was then grouped in the tribe *Ehrlichieae* under the order Rickettsiales, as *Ehrlichia phagocytophila*, together with *Ehrlichia equi*, the causative agent of equine granulocytic anaplasmosis (EGA) (Woldehiwet, 2010). After the reorganisation of genera based on 16S rRNA and groEL genes by Dumler et al. (2001), *E. phagocytophila* was grouped under the same clade with *E. equi* and the human granulocytic ehrlichiosis (HGE) agent (*Ehrlichia* ‘HGE agent’) is now known as *A. phagocytophilum*.

The size of *A. phagocytophilum* is approximately 0.4 to 1.3 µm, but it can be as large as 2.0 µm (Rikihisa, 2011). Due to the lack of contrast against the host cytoplasm,
Gram staining is not suitable for the visualisation of this intracellular bacterium. Instead, Romanowsky staining is used for staining of the bacterium. Upon staining, purplish mulberry-like bacterial clumps called morulae can be observed in the bacterial smears (Rikihisa, 2011). Morulae are normally 1.5 to 2.5 µm in diameter but can be up to 6.0 µm (Rikihisa, 2011).

The genome size of *A. phagocytophilum* is about 1.47 Mb (based on strain HZ, isolated from a patient in New York State, USA) with 1,369 open reading frames (ORFs) and G+C content of 41.6% (Dunning Hotopp *et al.*, 2006). No plasmids, intact prophages or transposable elements are found in the bacterial genome (Rikihisa, 2011). In addition, the genome of *A. phagocytophilum* also lacks of genes needed for biosynthesis of LPS and peptidoglycan. As a result, *A. phagocytophilum* is tightly packed inside inclusions (Dunning Hotopp *et al.*, 2006; Lin and Rikihisa, 2003).

*A. phagocytophilum* infects granulocytes (neutrophils) and also endothelial cells of humans (Herron *et al.*, 2005) and animals such as horses, deer and wild ruminants (Atif, 2016). No transovarial transmission (from adult ticks to eggs) has been reported (Woldehiwet, 2010) except for *Dermacentor albipictus* (moose or winter tick) (Baldridge *et al.*, 2009). Hence, a reservoir vertebrate host is important for the maintenance of *A. phagocytophilum* in nature. The life cycle of *A. phagocytophilum* starts when adult ticks acquire *A. phagocytophilum* from infected mammals during blood meals. The bacterium survives in the larva, nymph and adult stage of ticks, and is then transmitted to other mammals during the next blood meal (Ogden *et al.*, 1998; Telford *et al.*, 1996; Zhi *et al.*, 2002).

### 2.1.3 *Anaplasma centrale*

*A. centrale*, first described by Sir Arnold Theiler in 1911 (Theiler, 1911), is closely related to *A. marginale*. *A. centrale*, also known as *A. marginale* subsp. *centrale*
(Dumler et al., 2001; Kocan et al., 2003), causes mild anaplasmosis in ruminants (mostly cattle) (Battilani et al., 2017).

*A. centrale* infects erythrocytes of animal hosts and the inclusion bodies are located at a more central position within the infected red blood cells when visualised under Giemsa staining. The location of inclusion bodies is a feature that distinguish *A. centrale* from *A. marginale* (OIE, 2012). The genome size of *A. centrale* is approximately 1.21 Mb (based on Israel strain) with a high G+C content of 50.0% (Herndon et al., 2010), similar to that of *A. marginale*. The transmission of *A. centrale* normally occurs through ticks as vector (Theiler, 1911). Transovarial transmission has not been reported (Palomar et al., 2015). *A. centrale* has been used as a live vaccine against *A. marginale* in Israel, Australia, Africa and South America (de la Fuente et al., 2005a).

### 2.1.4 *Anaplasma ovis*

*A. ovis* is known to infect erythrocytes of ruminants (mostly goats and sheep) and causes ovine anaplasmosis (Atif, 2016). *A. ovis* was first described in sheep in 1912 (Bevan, 1912). *A. ovis* causes mild infection which is serologically identical to *A. marginale* (Splitter et al., 1956). Unlike *A. marginale* which is only found at the edge of erythrocytes and *A. centrale* which is found at the central region of the infected erythrocytes, *A. ovis* can be found on both marginal (60.0-65.0%) and submarginal/central (35.0-40.0%) parts of the erythrocytes (Rymaszewska and Grenda, 2008; Shompole et al., 1989).

Similar to other members in *Anaplasmataceae*, *A. ovis* is not transovarially transmitted (Palomar et al., 2015) and tick is the main vector (Battilani et al., 2017). Sheep keds and biting flies may play a role in the transmission of *A. ovis* (Battilani et al., 2017; Hornok et al., 2011). *A. ovis* has not been known to infect human. However, the recent detection of a variant of *A. ovis* has been detected in a patient in Cyprus suggests the zoonotic potential of this bacterium (Chochlakis et al., 2010).
2.1.5  *Anaplasma bovis*

*A. bovis* was first described in cattle in 1936 as *Rickettsia bovis* (Donatien and Lestoquard, 1936). It was then renamed as *Ehrlichia bovis* and later, *A. bovis* (Dumler *et al.*, 2001). Based on phylogenetic analysis of 16S rRNA, *gltA* and *groEL* genes, *A. bovis* is closely related to *A. phagocytophilum* and *A. platys* (Ybañez *et al.*, 2014a).

*A. bovis* infects the monocytes of multiple animal hosts and causes monocytic anaplasmosis in cattle, deer, goats and cottontail rabbits (Atif, 2016). *A. bovis* appears in different shapes and sizes, i.e., small (1.0 µm), medium (1.0-3.0 µm) and large (3.0-6.0 µm) forms. The large form is a mature and the least common form (Sreekumar *et al.*, 1996). *A. bovis* has not been successfully cultivated *in vitro* (Rar and Golovljova, 2011). Vertebrate hosts and tick vectors are crucial in the transmission of *A. bovis* in the absence of transovarial transmission (Battilani *et al.*, 2017; Palomar *et al.*, 2015).

2.1.6  *Anaplasma platys*

*A. platys*, initially known as *Ehrlichia platys*, was first detected in a dog in Florida, USA, with the diagnosis of infectious canine cyclic thrombocytopenia (Harvey *et al.*, 1978). *A. platys* has not been cultivated *in vitro* (Rar and Golovljova, 2011).

*A. platys* is known to infect platelets of dogs. It has also been detected in animal hosts other than dogs, for example, cats, red foxes and Bactrian camels (Battilani *et al.*, 2017; Cardoso *et al.*, 2015; Y. Li *et al.*, 2015). Although human infections caused by *A. platys* have been reported (Arraga-Alvarado *et al.*, 2014; Maggi *et al.*, 2013), the role of *A. platys* as the causative agent for human infection still requires further studies. Tick is an important vector for the transmission of *A. platys* and transmission through infected blood inoculation has been reported (Battilani *et al.*, 2017).
2.2 **Ehrlichia** spp.

*Ehrlichia* spp. are tick-associated bacteria belong to the family *Anaplasmataceae*, order Rickettsiales and class Alphaproteobacteria. The small Gram-negative bacterium inhabits in an intracellular vacuole (morula) that is bounded by a membrane derived from the cytoplasm of an eukaryotic host cell. The bacterium can appear coccoid to pleomorphic in different sizes, i.e., small (0.4 µm), medium (0.7 µm) or large (1.0 µm) forms. Occasionally, bacteria with very large size (≤ 2.0 µm) can be observed (Allsopp and McBride, 2009; Dumler *et al*., 2001). According to the reorganisation of genera in the family *Anaplasmataceae* by Dumler *et al.* (2001), the genus *Ehrlichia* include *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. ruminantium* and *E. muris*.

### 2.2.1 **Ehrlichia canis**

*E. canis* is the type species for the genus *Ehrlichia* and the causative agent of canine ehrlichiosis (Rikihisa, 1991). *E. canis* was originally described in dogs in Algeria (Donatien and Lestoquard, 1935). The disease caused by *E. canis* was reported throughout the world and had gained extensive attention when a large number of American military dogs (mostly German shepherds) died from *E. canis* infection during the Vietnam War (Rikihisa, 1991). Two morphological cell types of *E. canis* have been observed under electron microscope: reticulate cells and dense-cored cells (Popov *et al*., 1998). Reticulate cells are small and oval form of bacteria, with the average diameters around 0.3 to 0.6 µm while dense-cored cells are round with diameters ranging from 0.3 to 0.4 µm (Popov *et al*., 1998). Morulae appear in different sizes and are filled with large quantities of bacteria measuring 2.0 to 4.0 µm in diameters (Popov *et al*., 1998).

The genome of *E. canis* is made up of a single circular chromosome with the size of approximately 1.32 Mb (based on strain Jake) and a G+C content of 29.0% (Mavromatis *et al*., 2006). *E. canis* is an aerobic bacterium incapable of using glucose or fructose as carbon source, therefore the transport systems and essential enzymes
associated with the use of these substrates have not been identified (Mavromatis et al., 2006). Similar to A. phagocytophilum and E. chaffeensis, enzymes related to the biosynthesis of lipid A and murein sacculus and the metabolism of peptidoglycans and amino sugars have not been reported in E. canis (Mavromatis et al., 2006).

E. canis infects monocytes (macrophages) causing monocytic ehrlichiosis (Rar and Golovljova, 2011). Although E. canis causes infection in dogs and wild canids, it has also been detected in cats and humans (Stich et al., 2008). Tick is an important vector for the maintenance of E. canis through transstadial transmission (Stich et al., 2008).

**2.2.2 Ehrlichia chaffeensis**

The first case of human infection caused by E. chaffeensis was reported in April 1986 in the USA. The bacterium, initially referred as E. canis, was present as an intracytoplasmic inclusion in the monocytes of a patient (Maeda et al., 1987). The organism which was first isolated in cell culture in 1991, has been characterised using various molecular techniques (Anderson et al., 1991; Dawson et al., 1991). E. chaffeensis is a small and nonmotile bacterium that resides and grows in the cytoplasmic vacuole of host cells forming morulae (Paddock and Childs, 2003). Two distinct cell types have been identified under electron microscope: larger reticulate cells (with uniformly dispersed nucleoid) measuring 0.4 to 0.6 µm by 0.7 to 1.9 µm and small dense-cored cells (with dense nucleoid) measuring 0.4 to 0.6 µm in diameters (Paddock and Childs, 2003). The dense-cored cell is the only cell which attaches and enters into the host cell while the reticulate cell undergoes replication by binary fission. Hence, the dense-cored cell is the mature infectious form and the reticulate cell is the multiplication form of E. chaffeensis (Zhang et al., 2007).

E. chaffeensis has a single circular chromosome genome approximately 1.18 Mb, based on strain Arkansas which was isolated from a patient in Arkansas (Dunning Hotopp et al., 2006). The G+C content of E. chaffeensis is 30.1%. The 1,115 ORFs is about one
quarter of those found in *Escherichia coli* (Dunning Hotopp *et al.*, 2006). Similar with *A. phagocytophilum*, *E. chaffeensis* also lacks of genes required for the biosynthesis of LPS and peptidoglycans (Dunning Hotopp *et al.*, 2006; Lin and Rikihisa, 2003). The bacterial cell wall may be stabilised with disulphide bonds and non-covalent bonds cross-linking the outer membrane proteins (Vidotto *et al.*, 1994).

*E. chaffeensis* infects monocytes (macrophages) causing monocytic ehrlichiosis in human and a variety of animals including white-tailed deer (primary reservoir), deer, dogs, raccoons and goats (Rar and Golovljova, 2011; Yabsley, 2010). Ticks and vertebrate hosts are important in maintaining *E. chaffeensis* in nature. Successful transovarial transmission has not been reported and the bacteria is transmitted transstadially during a blood meal (Parola *et al.*, 2005).

### 2.2.3 *Ehrlichia ewingii*

*E. ewingii* was first recognised in 1971 as a new strain of *E. canis* in a dog in Arkansas, USA (Ewing *et al.*, 1971). The bacterium is known to be the causative agent of canine granulocytic ehrlichiosis (Anderson *et al.*, 1992a). Human infections with *E. ewingii* were first described in four patients from Missouri, USA, using PCR assays (Buller *et al.*, 1999).

Unlike other *Ehrlichia* species, *E. ewingii* infects granulocytes (neutrophils) (Rar and Golovljova, 2011), similar to the genetically distinct *A. phagocytophilum* (Dumler, 2005). *E. ewingii* has not been cultivated in cell culture (Ismail *et al.*, 2010), but has recently been isolated and maintained for a short period of time (16 weeks) in human promyeloblast cell line (HL60) (Killmaster and Levin, 2016). *E. ewingii* is not studied as extensive as other *Ehrlichia* species.

### 2.2.4 *Ehrlichia ruminantium*

Heartwater was recognised as a tick-borne disease during 1900 and until 1925. *E. ruminantium* is the causative agent which was initially identified as a rickettsia (knowns
as *Rickettsia ruminantium* but later was renamed as *Cowdria ruminantium* (Cowdry, 1925a, 1925b). Based on the analysis of 16S rRNA and *groEL* genes (Dumler *et al.*, 2001), *C. ruminantium* was found in one cluster with other *Ehrlichia* species, hence it was renamed as *E. ruminantium*. The bacteria appear as purplish-blue coccoids with various sizes (small- 0.4 µm, medium- 0.76 µm, large- 1.04 µm or very large- >1.04 µm) after Giemsa staining (Allsopp, 2010). Pleomorphic forms (horseshoe, ring and bacillary shaped) have also been reported (Allsopp, 2010).

The genome size of *E. ruminantium* is approximately 1.52 Mb (based on the South African Welgevonden isolate- type strain). It has a low G+C content (27.5%) with 920 ORFs (Collins *et al.*, 2005). *E. ruminantium* genome has a large number of repetitive sequences which make up 8.3% of the chromosomes (Collins *et al.*, 2005). Just like the other members of the family *Anaplasmataceae*, *E. ruminantium* is lack of the genes needed for the biosynthesis of cell wall components, lipid A and murein sacculus (Collins *et al.*, 2005).

*E. ruminantium* is known to infect endothelial cells, neutrophils and macrophages of domestic and wild ruminants, causing heartwater cowdriosis (Rar and Golovljova, 2011). *E. ruminantium* is transmitted transstadially and maintained in *Amblyomma* ticks (Allsopp, 2010). The report of *E. ruminantium* being transmitted transovarially (Bezuidenhout and Jacobsz, 1986) suggests that *E. ruminantium* may sometimes be carried transovarially.

### 2.2.5 *Ehrlichia muris*

*E. muris* is a murine pathogen first isolated from the spleen of a wild mouse, *Eothenomys kageus* in Japan in 1983 (Kawahara *et al.*, 1993). The bacterium is pleomorphic with sizes ranging from 0.4 to 1.5 µm long and 0.2 to 1.5 µm in diameters (Wen *et al.*, 1995). The bacterium is surrounded by cytoplasmic membrane with a rippled outer membrane but without distinct peptidoglycan layer (Wen *et al.*, 1995).
*E. muris* has a single circular chromosome with the size of approximately 1.2 Mb (based on the strain AS145\textsuperscript{T}, isolated from the spleen of a wild mouse, *E. kageus* in Japan) and G+C content of 30.0% (Thirumalapura et al., 2014). Phylogenetic analysis of 16S rRNA gene sequences shown that *E. muris* is closely related with *E. chaffeensis*, demonstrating 97.7% sequence similarity (Wen et al., 1995). *E. muris* infects monocytes (macrophages) of rodents and is transmitted by ticks (Rar and Golovljova, 2011).

### 2.3 New *Anaplasma* and *Ehrlichia* species

Due to the technological advancement in the diagnosis and intensified surveillance studies, there are increasing reports of new *Anaplasma* and *Ehrlichia* species worldwide. For instance, *Candidatus Anaplasma camelii* and *Candidatus Ehrlichia regneryi* [first detected in camel, Saudi Arabia, Bastos et al. (2015)]; *Candidatus Ehrlichia khabarensis* [previously known as *Ehrlichia* sp. Khabarovsk, detected in voles, Russia, Rar et al. (2015)]; *Anaplasma capra* [detected and isolated in goats and humans, China; H. Li et al. (2015)]; *Ehrlichia mineirensis* [first detected and isolated from *Rhipicephalus microplus* (collected from cattle), Brazil, Cabezas-Cruz et al. (2012)]; *Ehrlichia minasensis* sp. nov. [first detected and isolated from *R. microplus* (collected from cattle), Brazil, Cabezas-Cruz et al. (2016)]; *Candidatus Ehrlichia shimanensis* [previously known as *Ehrlichia* sp. TS37, first detected in *Haemaphysalis longicornis* from vegetation, Japan, Kawahara et al. (2006)] and *Anaplasma odocoilei* sp. nov. [detected in white-tailed deer, USA, Tate et al. (2013)]. Apart from *A. capra*, other new species have not been associated with human infections yet.
2.4 Anaplasma, ehrlichiae and their hosts/vectors

2.4.1 Human infections

2.4.1.1 Anaplasmosis

*A. phagocytophilum* is the only bacterium in the genus *Anaplasma* which causes human infections, also known as human granulocytic anaplasmosis (HGA) (Dumler, 2005). Most of the reported HGA cases are associated with the exposure to tick-bites. Hence, a well-established tick bite or tick exposure record is important to establish diagnosis for HGA (Bakken and Dumler, 2015). HGA can also be obtained through alternative routes, for example, perinatal transmission (Dhand et al., 2007), blood transfusion (Annen et al., 2012) and direct exposure to infected animal blood, through skin cuts or inhalation of infected aerosolised blood or infected blood splashed directly on mucous membranes (Bakken and Dumler, 2015).

Several reported cases of human infections caused by *A. ovis* and *A. platys* have been documented, however, the potential roles of these organisms in causing human infections require further investigation. Human infection by *A. ovis* was first reported in a woman with a history of fever after a tick bite in Cyprus. The organism was detected by PCR assays targeting *groEL* and *msp4* genes (Chochlakis et al., 2010). A case of human infection caused by *A. ovis* has also been reported based on detection of *A. ovis* 16S rRNA gene from an Iranian shepherd in the absence of any clinical symptoms (Hosseini-Vasoukolaei et al., 2014). Infection caused by *A. platys* was first reported in a veterinarian from Grenada using PCR assays targeting 16S rRNA and *groEL* genes (Maggi et al., 2013). *A. platys* DNA was detected from two family members who were the caretaker of a dog using PCR assays targeting 16S rRNA and *p44* genes (Breitschwerdt et al., 2014), and two women from Venezuela with the detection of intra-platelet inclusion bodies (Arraga-Alvarado et al., 2014). In addition, a newly identified
Anaplasma species known as A. capra has been reported to cause human infection in China (H. Li et al., 2015).

2.4.1.2 Ehrlichiosis

Human ehrlichiosis is mostly caused by E. chaffeensis (Human monocytic ehrlichiosis, HME) and E. ewingii (Human ewingii ehrlichiosis, HEE) (Dumler et al., 2007). E. chaffeensis infections are often associated with exposure to ticks and the geographical expansion of animal reservoirs and vectors. Severe HME cases have been reported in organ transplants or immunocompromised patients (Doudier et al., 2010; Safdar et al., 2002). HEE is understudied due to the lack of a specific diagnostic assay and reporting system for E. ewingii (Ismail et al., 2010). Most of the reported HEE cases occurred in HIV-infected patients or patients with immunosuppression after organ transplantation (Ismail et al., 2010).

Additionally, there are reported cases of human infections caused by E. canis, E. ruminantium, E. muris and E. muris-like (EML) organism, but their roles as the causative agents remain unknown. E. canis was first isolated from a man in Venezuela in 1996 (Perez et al., 1996). The organism was genetically and antigenically most closely related with E. canis strain Oklahoma and was thus designated as Venezuelan human Ehrlichia (VHE). Later on, E. canis VHE strain was detected in patients in Venezuela with clinical signs of HME (Perez et al., 2006). Recently, E. canis was detected by molecular and serological techniques in the blood donors in Costa Rica (Bouza-Mora et al., 2017). Three patients in South Africa were tested positive for E. ruminantium using PCR assay targeting the 16S rRNA V1 loop region and pCS20 genes (Allsopp et al., 2005).

E. muris was first detected in the spleen of a wild mouse in Japan (Kawahara et al., 1999). An unknown Ehrlichia species was detected in four patients and Ixodes scapularis ticks, from Wisconsin and Minnesota, USA in 2009 (Pritt et al., 2011). Genetic analysis showed that the unknown Ehrlichia species was closely related to E. muris and
it was thus referred as EML agent (Pritt et al., 2011). A recent study reported 69 patients from five different states in USA who were seropositive to the EML pathogen by using PCR assays targeting groEL gene, thus suggesting human exposure to EML pathogen in USA (Johnson et al., 2015).

In general, several human populations including farm workers, forestry workers, pet owners (especially cats and dogs), people with exposure to ticks or tick-bites, people infected with Borrelia burgdorferi (causative agent for Lyme disease), campers and hikers have been identified to have higher risk of contracting anaplasmosis and ehrlichiosis (Dinc et al., 2017; Dumler, 2005).

2.4.2 Animal infections

Different species of Anaplasma and Ehrlichia have been shown to infect certain animal hosts (Zobba et al., 2014). A. phagocytophilum has been reported in a wide range of hosts including horses, cats, canids (dogs and foxes), ruminants (cattle, sheep and goats), birds, rodents, wild boars and reptiles while the known reservoir is white-tailed deer, Odocoileus virginianus (Stuen et al., 2013). A. marginale and A. centrale are best known to cause infection in cattle, but other ruminants such as water buffaloes, American bison, elk and deer can also be infected with A. marginale (Kocan et al., 2010). A. ovis is a tick-borne bacterium that infects goats, sheep, deer and Mongolian gazelles (Rar and Golovljova, 2011). Similar to A. phagocytophilum, A. bovis also infects diverse animal hosts including ruminants (cattle, sheep, goats, buffaloes and deer), cottontail rabbits, dogs, raccoons and cats (Atif, 2016). Dogs are a common host for A. platys, however, A. platys infections have also been reported on deer, cats, camels and red foxes (Atif, 2016). Table 2.1 shows various animals that have been associated with infections caused by Anaplasma spp.

Dogs have been reported to acquire E. chaffeensis, E. ewingii and E. canis infections, and are considered as one of the most susceptible animals for Ehrlichia
infection (Chomel, 2011). E. chaffeensis infects most animals including canids (dogs, foxes and coyotes), deer, goats, rodents, raccoons and birds (Yabsley, 2010). E. ewingii infections in dogs, deer and rodents have been reported (Chae et al., 2003; Yu et al., 2007). E. canis is known to infect dogs, as well as other animals such as cats, wild cats, deer, rodents and wild canids (Li et al., 2016; Stich et al., 2008; Tateno et al., 2013). Wild ruminants and other domestic ruminants such as cattle, sheep, goats and water buffaloes are susceptible to infection caused by E. ruminantium (Allsopp, 2010). E. muris is known to infect rodents including Japanese field mouse, voles, mice and Siberian chipmunks; deer and common shrews (Rar and Golovljova, 2011). Further information on the animals infected by Ehrlichia spp. are summarised in Table 2.1.

2.4.3 Anaplasma and ehrlichiae in ticks

Ticks are obligate hematophagous arthropods grouped under the family Ixodidae (hard tick) or Argasidae (soft tick), order Parasitiformes, subclass Acari and class Arachnida (Parola and Raoult, 2001). Ixodid tick has three basic life stages (larva, nymph and adult), with each stage feeding on a different or the same host (Parola and Raoult, 2001). The transmission of blood-borne pathogens may occur from the ticks to either human or animal hosts during blood meals (Fritz, 2009). The major genera of ixodid ticks are Amblyomma spp., Ixodes spp., Dermacentor spp., Haemaphysalis spp., Hyalomma spp. and Rhipicephalus spp. (Parola and Raoult, 2001).

Ticks can be identified morphologically under a stereomicroscope, by referring to specific taxonomic keys, for example, taxonomic keys as reported by Burridge (2001), Kohls (1957), Walker et al. (2003), Geevarghese and Mishra (2011), Tanskul and Inlao (1989) and Wassef and Hoogstraal (1984). However morphological identification of ticks to species level may be difficult due to the lack of expertise in tick identification or when a tick has not fully developed (i.e., larva or nymph), or is engorged with blood or physically damaged. Hence, molecular technique (PCR incorporated with sequence
Table 2.1: Animals and ticks associated with various species of *Anaplasma* spp. and *Ehrlichia* spp. (Allsopp, 2010; Atif, 2016; Chae et al., 2003; Chastagner et al., 2013; Kocan et al., 2010; Li et al., 2014; Li et al., 2016; Noaman, 2012; Palomar et al., 2015; Rar and Golovljova, 2011; Silaghi et al., 2017; Stuen et al., 2013; Tateno et al., 2013; Yabsley, 2010; Yang et al., 2015b; Yu et al., 2007; Yu et al., 2015).

<table>
<thead>
<tr>
<th>Species</th>
<th>Animal hosts</th>
<th>Ticks</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. centrale</em></td>
<td>- Cattle, goats</td>
<td>- Rhipicephalus spp. (<em>R. simus</em>)&lt;br&gt;- Haemaphysalis spp. (<em>H. punctata</em>)</td>
</tr>
</tbody>
</table>
### Table 2.1, continued

<table>
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<tbody>
<tr>
<td></td>
<td>Canids (dog; red fox, <em>V. vulpes</em>; timber wolf, <em>C. l. occidentalis</em>)</td>
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<td></td>
<td>Cats</td>
</tr>
<tr>
<td></td>
<td>Common shrew (<em>S. araneus</em>)</td>
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<tr>
<td></td>
<td>Deer (white-tailed deer, <em>O. virginianus</em>; roe deer, <em>C. capreolus</em>; red deer, <em>C. elaphus</em>; sika deer, <em>C. nippon</em>; fallow deer, <em>D. dama</em>; mule deer, <em>O. hemionus</em>)</td>
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<tr>
<td></td>
<td>Donkeys</td>
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<td></td>
<td>European brown bear (<em>U. arctos</em>)</td>
</tr>
<tr>
<td></td>
<td>Horses</td>
</tr>
<tr>
<td></td>
<td>Mongolian gazelle (<em>P. guturosa</em>)</td>
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<tr>
<td></td>
<td>Ruminants (cattle, sheep, goat, elk, yak, bison)</td>
</tr>
<tr>
<td></td>
<td>White-toothed shrew (<em>C. lasiura</em>)</td>
</tr>
<tr>
<td></td>
<td>Wild boar (<em>S. scrofa</em>)</td>
</tr>
<tr>
<td></td>
<td><em>Amblyomma</em> spp. (<em>A. americanum</em>)</td>
</tr>
<tr>
<td></td>
<td>Dermacentor spp. (<em>D. variabilis</em>, <em>D. occidentalis</em>, <em>D. silvarum</em>, <em>D. reticulatus</em>, <em>D. marginatus</em>, <em>D. albipictus</em>, <em>D. niveus</em>)</td>
</tr>
<tr>
<td></td>
<td>Hyalomma spp. (<em>H. marginatum</em>, <em>H. detritum</em>)</td>
</tr>
<tr>
<td></td>
<td>Rhipicephalus spp. (<em>R. bursa</em>, <em>R. sanguineus</em>, <em>R. turanicus</em>)</td>
</tr>
</tbody>
</table>
Table 2.1; continued

|----------------|------------------------|----------------------------------------------------|---------------------------------------------------------------------------------|-------|------------------------|-------------------------------------------------|-----------------------------|------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
analysis) targeting genes such as cytochrome C oxidase subunit I (COI), internal transcribed spacer 2 (ITS2), 16S rDNA, 12S rDNA and 28S rDNA (Black and Piesman, 1994; Inokuma et al., 2003; Lv et al., 2014a) is a useful tool for tick identification.

The details of ticks associated with *Anaplasma* spp. and *Ehrlichia* spp. are shown in Table 2.1. Five genera of ixodid ticks including *Amblyomma* spp., *Ixodes* spp., *Haemaphysalis* spp., *Dermacentor* spp., and *Rhipicephalus* spp. have been implicated as potential vectors. *A. phagocytophilum* has been detected in *Ixodes* ticks (*I. scapularis*, *I. persulcatus*, *I. ricinus* and *I. pacificus*) and other tick species including *H. longicornis*, *Haemaphysalis concinna*, *Haemaphysalis megaspinosa*, *Dermacentor nuttalli*, *Dermacentor variabilis*, *Dermacentor silvarum*, *Rhipicephalus sanguineus* and *Rhipicephalus bursa* (Chastagner et al., 2013; Stuen et al., 2013). Approximately 20 tick species have been reported as potential vectors of *A. marginale*, of which *D. variabilis*, *R. microplus* and *Dermacentor andersoni* are most frequently reported (Kocan et al., 2010).

Transmission of *A. centrale* to animals through *Rhipicephalus simus* and *Haemaphysalis punctata* has been reported (Palomar et al., 2015; Rar and Golovljova, 2011). *A. ovis* has been detected in various tick species including *D. andersoni*, *R. bursa*, *H. punctata*, *H. concinna*, *I. ricinus* and *Dermacentor marginatus* (Silaghi et al., 2017). *A. bovis* can be transmitted through different tick species including *Haemaphysalis* spp. (*H. longicornis*, *H. megaspinosa* and *Haemaphysalis shimoga*), *Amblyomma* spp. (*A. variegatum*), *Rhipicephalus* spp (*R. sanguineus*) and *Dermacentor* spp. (*D. andersoni* and *Dermacentor occidentalis*) (Silaghi et al., 2017). *A. platys* has been detected from *R. sanguineus*, the brown dog tick, as well as other tick species including *H. longicornis*, *Dermacentor auratus*, *Rhipicephalus turanicus* and *Rhipicephalus evertsi* (Rar and Golovljova, 2011; Silaghi et al., 2017).
Amblyomma americanum has been recognised as the main vector for E. chaffeensis and E. ewingii (Parola et al., 2005). Other tick species harbouring E. chaffeensis include R. sanguineus, I. persulcatus, H. longicornis, D. variabilis and Amblyomma testudinarium. E. ewingii has also been reported in R. sanguineus and H. longicornis (Rar and Golovljova, 2011). R. sanguineus is the primary vector for E. canis, however, transmission of E. canis by D. variabilis, H. longicornis and Ixodes turdus has been reported (Rar and Golovljova, 2011). E. ruminantium has only been reported in ticks from the genus Amblyomma with A. variegatum and Amblyomma hebraeum as the most important vectors (Allsopp, 2010). Meanwhile, E. muris has been reported in I. ricinus, I. persulcatus and Haemaphysalis flava (Rar and Golovljova, 2011) (Table 2.1).

Even though Anaplasma spp. and Ehrlichia spp. are found mainly in ticks, some species have been detected in other arthropods, for example, A. marginale has been described in biting flies including stable flies (Stomoxys calicitrans) and horse flies (Tabanidae) (Aubry and Geale, 2011); A. ovis has been detected in sheep keds (Melophagus ovinus) (Hornok et al., 2011) and E. ruminantium in tsetse flies (Glossina pallidipes) (Hornok et al., 2016).

2.5 Infections caused by anaplasma and ehrlichiae

2.5.1 Clinical symptoms in humans

The clinical presentations of patients diagnosed with anaplasmosis and ehrlichiosis are often nonspecific. Patients presenting with febrile illness, headache, myalgia and malaise have been reported (Dumler, 2005). Even though the diseases fatality rates are low (less than 1.0% for HGA and 3.0% for HME), complications such as septic or toxic shock-like syndrome, acute respiratory distress syndrome (ARDS), acute renal failure, meningoencephalitis, brachial plexopathy, demyelinating polyneuropathy, opportunistic infections with both viral and fungal agents, haemorrhage
and acute abdominal syndrome have been documented (Bakken and Dumler, 2015). The respiratory and gastrointestinal systems are often affected but skin rash is generally not often observed in patients with HME and is rare in patients with HGA (Dumler, 2005). Other clinical symptoms observed in children and pregnant women having HME are altered mental status and abdominal pain that can mimic acute appendicitis (Ismail et al., 2010).

Only very few HEE cases have been reported. The cases reported in the USA were associated with increasing age, human immunodeficiency virus (HIV) infection and immunocompromised status (undergoing immunosuppression therapy or organ transplantation) (Dumler et al., 2007; Ismail et al., 2010).

The laboratory findings of infections caused by *A. phagocytophilum*, *E. chaffeensis* and *E. ewingii* are almost similar, i.e., thrombocytopenia, leukopenia, anaemia, increased serum transaminase activity (proposing mild to moderate liver injury) and lymphopenia (Dumler, 2005).

### 2.5.2 Clinical signs in animals

In general, animals infected with *Anaplasma* spp. and *Ehrlichia* spp. demonstrate fever, anorexia, apathy, lethargy, reduction in milk production (mostly for ruminants), anaemia, weight loss, pallor mucous membrane and sometimes death (Atif, 2016). *A. phagocytophilum* is the causative agent of TBF or pasture fever in ruminants (sheep, goats and cattle) (Woldehiwet, 2006). But the fever may vary depending on the age of the infected animals, the variant of *A. phagocytophilum* involved, the host species and immunological status (Stuen et al., 2013). Besides that, there have been cases of abortion and reduced fertility in *A. phagocytophilum*-infected ewes and rams, respectively (Stuen et al., 2013). Interestingly, dogs naturally infected with *A. phagocytophilum* often remain healthy and this has result in widespread serological evidence of canine granulocytic anaplasmosis but a lack of history of clinical illness (Carrade et al., 2009).
*A. marginale* is the major causative agent of bovine anaplasmosis. *A. marginale* can infect cattle of all ages and the disease severity varies according to the age of cattle. Calves are usually more resistant to the infection, hence, animals between the age of 6 months and one year develop mild disease while animals between one to two years of age suffer non-fatal but acute disease, and animals more than two years of age suffer from acute disease with mortality risks (Aubry and Geale, 2011). Once cattle infected with *A. marginale*, they will persistently remain as infected carriers for their whole life (Aubry and Geale, 2011).

Dogs infected by *E. canis* (canine monocytotropic ehrlichiosis, CME) can be demonstrated in acute, subclinical or chronic forms (Harrus and Waner, 2011). All dog breeds are susceptible to *E. canis* infection with German shepherd dogs seem to be more susceptible with high morbidity and mortality rates (Harrus and Waner, 2011). Acute disease normally develops within two to four weeks following tick transmission with disease manifestations differ depending on the virulence of *E. canis* strains and co-infections with other tick-borne pathogens (for example *Hepatozoon canis, Babesia canis*) (Gal et al., 2007; Little, 2010). Some dogs may enter subclinical phase of infection with the dogs stay chronically infected for months to years without showing much clinical signs (normally only show mild thrombocytopenia) (Little, 2010).

### 2.6 Anaplasma and ehrlichiae infections in Asian countries

Asia is the largest continent on Earth that is bounded by the Pacific Ocean on the east, the Indian Ocean on the south, the Arctic Ocean on the north and the European countries on the west. Southeast Asia (SEA) lies between the Pacific Ocean and Indian Ocean and falls within the equatorial and sub-equatorial zones. Both Asia and SEA provide different habitats and microclimates which are conductive for the survival of
various haematophagous arthropods including ticks, fleas, mosquitoes, mites, lice and tabanids (Irwin and Jefferies, 2004).

Tick-borne diseases such as anaplasmosis and ehrlichiosis are reported in many parts of Asian and SEA countries. In Asia, human cases have been reported in China, Japan, Korea and Mongolia, while ruminants (cattle, sheep, goats and deer), pet animals (dogs), *H. longicornis*, *R. microplus*, *R. sanguineus* and *I. persulcatus* are among the most reported animal hosts and ticks associated with anaplasmosis and ehrlichiosis, as shown in Table 2.2. In SEA, limited human cases have been reported (only one publication from Indonesia and Thailand, respectively) while both ruminant (cattle) and pet animal (dogs) and *R. sanguineus* ticks are the most reported animal hosts and tick vector for anaplasmosis and ehrlichiosis, respectively.

2.7 **Current status of anaplasmosis and ehrlichiosis in Malaysia**

The tropical rainforest of Malaysia is the habitat for various animals and haematophagous arthropods including ticks. There has been no report of human cases of anaplasmosis and ehrlichiosis in Malaysia. However, anaplasmosis and ehrlichiosis have been reported in dogs, cattle and *R. microplus* ticks, using either serology or molecular methods. Table 2.3 shows the anaplasma and ehrlichiae infections that have been reported from animal samples in Malaysia.
Table 2.2: Prevalence of anaplasma and ehrlichiae infections reported in Asian region, and the method used for detection. Reports involved human subjects are highlighted in bold.

<table>
<thead>
<tr>
<th>Country</th>
<th>Hosts or vectors</th>
<th>Anaplasmataceae</th>
<th>Prevalence (%)</th>
<th>Detection method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh</td>
<td>Cattle</td>
<td>Anaplasma spp.</td>
<td>18.5</td>
<td>Blood smear</td>
<td>Kispotta et al. (2016)</td>
</tr>
<tr>
<td>Dogs</td>
<td></td>
<td>Anaplasma spp.</td>
<td>28.0</td>
<td>PCR</td>
<td>Qiu et al. (2016)</td>
</tr>
<tr>
<td>R. sanguineus, host: dogs</td>
<td>Anaplasma spp.</td>
<td>40.0</td>
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<tr>
<td>H. bispinosa, host: cattle</td>
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<td></td>
<td></td>
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<tr>
<td>Cattle</td>
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<td>Anaplasma spp.</td>
<td>3.9</td>
<td>Blood smear</td>
<td>Alim et al. (2012)</td>
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<tr>
<td>China</td>
<td>Human- patients</td>
<td>A. phagocytophilum</td>
<td>22.7</td>
<td>qPCR</td>
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<td></td>
<td>E. chaffeensis</td>
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<td>A. capra</td>
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<td>5.9</td>
<td>nPCR</td>
<td>H. Li et al. (2015)</td>
</tr>
<tr>
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<td>Zhang et al. (2014)</td>
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<td>33.7</td>
<td>IFA</td>
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<tr>
<td></td>
<td>A. phagocytophilum</td>
<td></td>
<td>9 clinical cases</td>
<td>nPCR</td>
<td>Zhang et al. (2008a)</td>
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<td>Cattle</td>
<td><em>A. marginale</em></td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>North Korea</strong></td>
<td><em>H. longicornis</em>, host: goats</td>
<td><em>A. phagocytophilum</em></td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. bovis</em></td>
<td>26.4</td>
</tr>
<tr>
<td>Region</td>
<td>Hosts</td>
<td>Species</td>
<td>Prevalence</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------------------------</td>
<td>------------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Pakistan</strong></td>
<td>Horses</td>
<td><em>A. phagocytophilum</em></td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td><em>Anaplasma</em> spp.</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. marginale</em></td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td>Buffaloes</td>
<td><em>Anaplasma</em> spp.</td>
<td>7.5</td>
</tr>
<tr>
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<td></td>
<td><em>A. marginale</em></td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>Buffaloes</td>
<td><em>A. marginale</em></td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td><em>A. marginale</em></td>
<td>31.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. centrale</em></td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. marginale</em></td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. centrale</em></td>
<td>13.6</td>
</tr>
<tr>
<td><strong>Taiwan</strong></td>
<td><em>H. hystricis</em>, host: pangolins</td>
<td><em>Anaplasma</em> spp.</td>
<td>1 tick</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ehrlichia</em> spp.</td>
<td>1 tick</td>
</tr>
<tr>
<td></td>
<td>Rodents</td>
<td><em>A. phagocytophilum</em></td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. bovis</em></td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>Dogs</td>
<td><em>E. canis</em></td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Anaplasma</em> spp.</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. canis</em></td>
<td>20.8</td>
</tr>
<tr>
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<td><em>A. platys</em></td>
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<td><strong>Southeast Asia region</strong></td>
<td>Cambodia Dogs</td>
<td><em>E. canis</em></td>
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<tr>
<td></td>
<td><strong>Indonesia</strong> Human- residents</td>
<td><em>E. chaffeensis</em></td>
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<tr>
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<td>Laos</td>
<td><em>A. testudinarium</em></td>
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<tr>
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<td></td>
<td><em>Haemaphysalis</em> spp.</td>
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</tr>
<tr>
<td></td>
<td></td>
<td><em>H. aborensis</em></td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td>Philippines Dogs</td>
<td><em>A. platys</em></td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td><em>A. marginale</em></td>
<td>95.5</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td><em>A. marginale</em></td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>Dogs</td>
<td><em>E. canis</em></td>
<td>2.9</td>
</tr>
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</table>
Table 2.2, continued

<table>
<thead>
<tr>
<th>Country</th>
<th>Animal Type</th>
<th>Pathogen</th>
<th>Prevalence (%)</th>
<th>Detection Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Philippines (Cebu)</td>
<td>Cattle</td>
<td>Anaplasma spp.</td>
<td>54.7</td>
<td>PCR</td>
<td>Ybañez et al. (2013c)</td>
</tr>
<tr>
<td></td>
<td>A. marginale</td>
<td>8 cattle</td>
<td>nPCR</td>
<td>Ybañez et al. (2013b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R. microplus, host: cattle</td>
<td>A. marginale</td>
<td>8 ticks</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. centrale</td>
<td>1 tick</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R. sanguineus, host: dogs</td>
<td>E. canis</td>
<td>4.3</td>
<td>nPCR</td>
<td>Ybañez et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>A. platys</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Philippines</td>
<td>Military working dogs</td>
<td>E. canis</td>
<td>59.0</td>
<td>ELISA</td>
<td>Baticados and Baticados (2011)</td>
</tr>
<tr>
<td>Thailand</td>
<td>Human- patients</td>
<td>A. phagocytophilum</td>
<td>4.5</td>
<td>IFA</td>
<td>Blacksell et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>E. chaffeensis</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human- volunteers</td>
<td>E. chaffeensis</td>
<td>36.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dogs with anaemia</td>
<td>E. canis</td>
<td>9.9</td>
<td>nPCR</td>
<td>Kaewmongkol et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>A. platys</td>
<td>3.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beef cattle</td>
<td>A. marginale</td>
<td>14.5</td>
<td>PCR</td>
<td>Jirapattharasate et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>D. auratus, host: wild boar</td>
<td>A. platys</td>
<td>9.0</td>
<td>PCR</td>
<td>Sumrandee et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>H. langrangei, host: deer</td>
<td>A. bovis</td>
<td>16.7</td>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. platys</td>
<td>20.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. obesa, host: deer</td>
<td>A. bovis</td>
<td>66.7</td>
<td>PCR</td>
<td>Liu et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>A. platys</td>
<td>4.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stray dogs</td>
<td>E. canis</td>
<td>3.9</td>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. platys</td>
<td>4.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water buffaloes</td>
<td>A. marginale</td>
<td>8.0</td>
<td>PCR</td>
<td>Saetiew et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>H. shimoga, host: vegetation</td>
<td>A. bovis</td>
<td>1 tick</td>
<td>nPCR</td>
<td>Malaisri et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>Domestic dogs</td>
<td>E. canis</td>
<td>3.0</td>
<td>PCR</td>
<td>Laummaunwai et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Domestic cat</td>
<td>A. platys</td>
<td>1 cat</td>
<td>PCR</td>
<td>Salakij et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>R. sanguineus, host: dogs</td>
<td>E. canis</td>
<td>3.3</td>
<td>PCR</td>
<td>Foongladda et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>A. platys</td>
<td>2.3</td>
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<td></td>
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<tr>
<td></td>
<td>Rats</td>
<td>Anaplasma spp.</td>
<td>46.5</td>
<td>Blood smear</td>
<td>Thane et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>Tree shrews</td>
<td>Anaplasma spp.</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. auratus, host: dogs</td>
<td>Anaplasma spp.</td>
<td>15.0</td>
<td>PCR</td>
<td>Parola et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>A. javanense, host: pangolins</td>
<td>Anaplasma spp.</td>
<td>29.6</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>H. langrangei, host: bears</td>
<td>Anaplasma spp.</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R. microplus, host: cattle</td>
<td>Ehrlichia spp.</td>
<td>22.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.2, continued

<table>
<thead>
<tr>
<th>Country</th>
<th>Host</th>
<th>Species</th>
<th>Prevalence (%)</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand</td>
<td>Dogs</td>
<td><em>E. chaffeensis</em></td>
<td>74.0</td>
<td>IFA</td>
<td>Suksawat <em>et al.</em> (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. canis</em></td>
<td>71.0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. phagocytophilum</em></td>
<td>58.0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. canis</em></td>
<td>20.4</td>
<td>nPCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. platys</em></td>
<td>10.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR: Polymerase chain reaction; qPCR: Real-time polymerase chain reaction; nPCR: Nested polymerase chain reaction; IFA: Immunofluorescent assay; ELISA: Enzyme-linked immunosorbent assay; PCR-RFLP: Polymerase chain reaction- restriction fragment length polymorphism; PCR-RLB: Polymerase chain reaction- reverse line blot hybridisation

### Table 2.3: Animal host for Anaplasmataceae, prevalence (%) and detection methods for infections reported in Malaysia.

<table>
<thead>
<tr>
<th>Animal hosts or vectors</th>
<th>Anaplasmataceae</th>
<th>Prevalence (%)</th>
<th>Detection method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. varanense</em>, host: snakes</td>
<td><em>A. phagocytophilum</em></td>
<td>1 tick</td>
<td>PCR</td>
<td>Kho <em>et al.</em> (2015b)</td>
</tr>
<tr>
<td></td>
<td><em>A. bovis</em></td>
<td>2 ticks</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>A. platys</em></td>
<td>2 ticks</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Ehrlichia spp.</em></td>
<td>2 ticks</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. helvolum</em>, host: snakes</td>
<td><em>A. phagocytophilum</em></td>
<td>2 ticks</td>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td>Monkeys</td>
<td><em>A. bovis</em></td>
<td>10.0</td>
<td>PCR</td>
<td>Tay <em>et al.</em> (2015)</td>
</tr>
<tr>
<td>Cattle</td>
<td>Anaplasma spp.</td>
<td>84.4</td>
<td>PCR</td>
<td>Tay <em>et al.</em> (2014)</td>
</tr>
<tr>
<td><em>R. microplus</em>, host: cattle</td>
<td>Anaplasma spp.</td>
<td>5.5</td>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td>Dogs</td>
<td><em>A. platys</em></td>
<td>13.3</td>
<td>nPCR</td>
<td>Mokhtar <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>Cattle and buffaloes</td>
<td><em>A. marginale</em></td>
<td>77.6</td>
<td>c-ELISA</td>
<td>Premaalatha <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>Dogs</td>
<td><em>E. canis</em></td>
<td>2.0</td>
<td>PCR</td>
<td>Nazari <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>Cattle and buffaloes</td>
<td><em>A. marginale</em></td>
<td>77.6</td>
<td>c-ELISA</td>
<td>Rahman <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>Dogs</td>
<td><em>E. canis</em></td>
<td>15.0</td>
<td>IFAT</td>
<td>Rahman <em>et al.</em> (2010)</td>
</tr>
</tbody>
</table>

PCR: Polymerase chain reaction; nPCR: Nested polymerase chain reaction; c-ELISA: Competitive enzyme-linked immunosorbent assay; IFAT: Immunofluorescent antibody test
2.8 Laboratory diagnosis of anaplasmosis and ehrlichiosis

2.8.1 Histochemistry and immunochemistry

Direct examination of peripheral blood is one of the methods for diagnosis of anaplasmosis and ehrlichiosis. A diagnosis can be established by the identification of morulae in the eosin-azure (Romanowsky)-type stained (including Giemsa’s, Wright’s, Leishman’s and Diff-Quik) blood smear using light microscopy during the acute phase of the disease (Ganguly and Mukhopadhayay, 2008; Thomas et al., 2009). Even though this method gives fast result, it is relatively insensitive as compared to other available diagnostic assays and an experienced microscopist is required to perform and analyse the result (Dumler et al., 2007; Thomas et al., 2009).

Immunohistochemical (IHC) staining is another method to document the presence of anaplasma or ehrlichiae in patients before the beginning of antibiotic therapy or within the first 48 hours after antibiotic therapy has been started (Biggs et al., 2016). The method requires the staining of antigens in paraffin-embedded, formalin-fixed biopsy or autopsy tissues (bone marrow, spleen, liver, lymph node and lung).

2.8.2 Serology methods

Several serological tests including indirect immunofluorescent assay (IFA), enzyme-linked immunosorbent assay (ELISA), complement fixation test (CF) and card agglutination test (CAT) have been employed for the diagnosis of anaplasmosis and ehrlichiosis. IFA is the gold standard used for serological diagnosis of human anaplasmosis and ehrlichiosis (Ismail et al., 2010). Using IFA, antibodies present in the patient serum samples bind to the anaplasma or ehrlichia antigens (A. phagocytophilum or E. chaffeensis) fixed on a slide and are detected using fluorescein labelled conjugates (Biggs et al., 2016). In most cases, since antibody has not reached to a detectable level during the first week of infection, paired sera collected 2-3 weeks after illness onset are preferred samples for serological examination (Biggs et al., 2016).
A confirmed case of *A. phagocytophilum* or *E. chaffeensis* infection is determined by a fourfold increase in the antibody titers between acute and convalescent sera or a seroconversion to a titer of 128 or higher (Bakken et al., 2002; Thomas et al., 2009). Due to the possibility that antibody titer can last for months or years after initial exposure, a confirmed case should be based on both the evidence of antibody titers and other clinical evidence of infection (Thomas et al., 2009). In addition, non-specificity may occur due to cross-reactive immune responses to antigens which are typically group-specific (Biggs et al., 2016).

Several serological methods are also employed for the detection of exposure to *Anaplasma* spp. and *Ehrlichia* spp. in animals. An ELISA commercial kit that utilizes a monoclonal antibody specific for MSP5 [*Anaplasma* Antibody Test Kit (cELISA); VMRD Inc., Pullman, WA, USA] was used to identify *Anaplasma*-infected cattle. In addition, commercially available SNAP 4Dx® test kit (IDEXX Laboratories, Westbrook, ME, USA) that detect antibodies to specific peptides of *Ehrlichia* spp. (*E. canis*) and *Anaplasma* spp. (*A. phagocytophilum*) is used for the diagnosis of canine anaplasmosis and ehrlichiosis and other animals (including cats and horses) (Little, 2010). In addition, an ELISA kit based on the recombinant major antigenic protein 2 (rMAP2) and the Immunocomb dot-ELISA test which uses a crude ehrlichial extract (Biogal, Galed Laboratories, Israel) are also used for the detection of *E. canis* in dogs (causing CME) (Harrus and Waner, 2011). These serological assays are facing the same limitations as other serological tests: low sensitivity for early infection, false negative results (negative serologic results does not exclude diagnosis) and cross-reactivity among *Anaplasma* spp. and *Ehrlichia* spp. (Aubry and Geale, 2011).

### 2.8.3 Isolation of anaplasma and ehrlichiae

Due to the obligate intracellular nature of the bacteria, isolation of *Anaplasma* spp. and *Ehrlichia* spp. requires cell culture technique. Members in the genera *Anaplasma*
and *Ehrlichia* (except *A. bovis* and *A. platys*) have been isolated using different cell lines (Table 2.4). *In vitro* cultivation of these pathogens provides an opportunity for better understanding of the pathogens, development of vaccines and serological tests and investigation of pathogen-host cell interaction in a controlled environment (Bell-Sakyi *et al.*, 2000; Bell-Sakyi *et al.*, 2007). This method is more likely to be employed in research laboratories rather than serving as a diagnostic tool in clinical laboratories (Harrus and Waner, 2011).

Table 2.4: Cell lines used for successful isolation of *Anaplasma* spp. and *Ehrlichia* spp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell lines for successful isolation</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. phagocytophilum</em></td>
<td>• Human promyelocytic leukemia cell line (HL-60)</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td></td>
<td>• <em>I. scapularis</em> embryo-derived cell lines IDE8 and ISE6</td>
<td>Goodman <em>et al.</em> (1996)</td>
</tr>
<tr>
<td></td>
<td>• <em>I. ricius</em> embryo-derived cell line IRE/CTVM20</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td><em>A. marginale</em></td>
<td>• <em>I. scapularis</em> embryo-derived cell lines IDE8 and ISE6</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td></td>
<td>• <em>I. ricius</em> embryo-derived cell line IRE/CTVM18</td>
<td>Bell-Sakyi <em>et al.</em> (2015)</td>
</tr>
<tr>
<td><em>A. centrale</em></td>
<td>• <em>R. appendiculatus</em> embryo-derived cell line RAE25</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td><em>A. ovis</em></td>
<td>• <em>I. scapularis</em> embryo-derived cell line IDE8</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td><em>A. bovis</em></td>
<td>Not yet been isolated</td>
<td></td>
</tr>
<tr>
<td><em>A. platys</em></td>
<td>Not yet been isolated</td>
<td></td>
</tr>
<tr>
<td><em>E. chaffeensis</em></td>
<td>• Canine peritoneal macrophage (DH82 cell line)</td>
<td>Killmaster and Levin (2016)</td>
</tr>
<tr>
<td></td>
<td>• <em>I. scapularis</em> embryo-derived cell line ISE6</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td><em>E. ewingii</em></td>
<td>• Human promyelocytic leukemia cell line (HL-60)</td>
<td>Killmaster and Levin (2016)</td>
</tr>
<tr>
<td>(for only about 16 weeks)</td>
<td>• Canine peritoneal macrophage (DH82 cell line)</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td><em>E. canis</em></td>
<td>• <em>I. scapularis</em> embryo-derived cell lines IDE8 and ISE6</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td></td>
<td>• <em>I. ricius</em> embryo-derived cell line IRE/CTVM18</td>
<td>Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td><em>E. ruminantium</em></td>
<td>• <em>A. variegatum</em> larva-derived cell lines AVL/CTVM13, 17</td>
<td>Bell-Sakyi <em>et al.</em> (2000); Bell-Sakyi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td></td>
<td>• <em>I. scapularis</em> embryo-derived cell line IDE8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• <em>R. appendiculatus</em> embryo-derived cell lines RAE25 and RAE/CTVM1</td>
<td></td>
</tr>
<tr>
<td><em>E. muris</em></td>
<td>• Canine peritoneal macrophage (DH82 cell line)</td>
<td>Wen <em>et al.</em> (1995)</td>
</tr>
</tbody>
</table>

### 2.8.4 Molecular detection of *Anaplasma* spp. and *Ehrlichia* spp.

As compared to other laboratory diagnostic methods, molecular detection of *Anaplasma* spp. and *Ehrlichia* spp. (mostly by PCR) is more sensitive, specific and rapid
Molecular methods are able to give information on the epidemiology of the pathogens and allow the detection of co-infections in the clinical samples (Guillemi et al., 2015). Unlike serological methods which rely on the time taken for antibodies to build to a detectable level, PCR amplification of *Anaplasmataceae* DNA from the whole blood samples of patients during acute stage of illness (where antibody level is still very low or undetectable) is particularly useful for early confirmation of the causative pathogens (Biggs et al., 2016). Additionally, PCR allows the detection of *Anaplasma* spp. and *Ehrlichia* spp. in various sample types including ticks and organ tissues (Silaghi et al., 2017).

Conventional PCR assay is the most widely used PCR format for detection of *Anaplasmataceae* DNA but other PCR formats have also been developed. For example, qPCR allows quantification of bacterial load in the samples (Harrus and Waner, 2011) and eliminate the time-consuming gel electrophoresis step by using intercalating fluorescent dyes. Nested PCR (nPCR) is a more sensitive approach than conventional PCR where two rounds of amplifications take place by using two different primer sets (inner and outer primer pairs with different annealing temperatures) (Hunt, 2011). Multiplex PCR enables the detection of multiple pathogens simultaneously (Hunt, 2011; Thomas et al., 2009). Loop-mediated isothermal amplification (LAMP) is an isothermal DNA amplification method that is used for rapid detection of infectious disease especially in poor or underdeveloped countries because of its low cost and easy to operate procedure (Kuleš et al., 2017; Ma et al., 2011).

PCR incorporated with other techniques such as PCR-RFLP (restriction fragment length polymorphism) which uses specific restriction endonucleases for genotyping purpose (Alberti et al., 2005) and PCR-RLB (reverse line blot hybridisation) which allows simultaneous detection and identification of *Anaplasma* spp. and *Ehrlichia* spp. in the samples by using primers (PCR part) and probes (RLB part) have been developed...
(Bekker et al., 2002). FRET (fluorescence resonance energy transfer)-qPCR is an advanced method which uses one set of primers and probes to detect and differentiate different Anaplasma spp. and Ehrlichia spp. present in the samples in a single reaction and melting curve is used for quantification analysis of the detected organisms (Zhang et al., 2015). PCR-HRM (high resolution melt analysis) is a rapid and sensitive diagnostic method that allows differentiation of organisms in a sample based on different melting temperatures and fluorescent dyes for real-time monitoring of the process (Krücken et al., 2013). Fluorescence in situ hybridisation (FISH) method uses a DNA probe (with the length between 15-30 nucleotides) which is linked to a fluorescent molecule hybridised to its matching target DNA sequence in situ (Kuleš et al., 2017).

2.8.4.1 Molecular characterisation of Anaplasma spp. and Ehrlichia spp.

Many PCR protocols have been designed to target the 16S rRNA gene of Anaplasma spp. and Ehrlichia spp. (Guillemi et al., 2015). However, since 16S rRNA gene is highly conserved among the family members of the same genus, gltA and groEL genes have been proposed as an alternative to 16S rRNA gene for phylogenetic analysis and identification of Anaplasma spp. and Ehrlichia spp. as both genes exhibit high variation, and thus provide better discrimination power among closely related species (Inokuma et al., 2001a; Sumner et al., 1997). In addition, other genes that have been used as targets for molecular diagnosis include msp1β (major surface protein 1β), msp2 (major surface protein 2), msp4 (major surface protein 4), ankA (ankyrin protein), p28 (28kDa outer membrane protein) and p30 (30kDa outer membrane protein) (Guillemi et al., 2015; Silaghi et al., 2017; Strašek Smrdel et al., 2015).

Using direct sequencing technique, amplified PCR amplicons can be sequenced and analysed. Multilocus sequence typing (MLST) allows genotypic characterisation of isolates and population studies by analysing DNA sequences of multiple housekeeping genes (Huhn et al., 2014). Phylogenetic analysis enables sequence comparison and
determines the phylogenetic relationships of bacteria (Wen et al., 2002). For example, Dumler et al. (2001) reorganised the genera in the family *Anaplasmataceae* based on the sequences of 16S rRNA and *groEL* genes. In addition, next generation sequencing (NGS) is a reliable DNA sequencing approach that plays an important role for microbial discovery and provides an estimation of the pathogenicity of organisms through sequencing of certain pathogenicity-related genes (Kuleš et al., 2017; Tijssse-Klasen et al., 2014).

### 2.9 Treatment for anaplasmosis and ehrlichiosis

Most patients respond well to tetracycline (bacteriostatic agent) if treatment is given during the early onset of the disease (Ismail et al., 2010). However, doxycycline (bacteriostatic agent) is preferred over tetracycline because of its twice-daily oral dosage, better patient tolerance and relatively lower risk of adverse drug effects for children under the age of eight. The recommended doxycycline dosage for an adult is 100 mg given orally or intravenously every 12 hours; while the dosage for children aged eight years or older is 2.2 mg/kg every 12 hours with a maximum dosage of 100 mg. The recommended tetracycline dosage for adult is 500 mg for every six hours and for children aged eight years or older it is 25-50 mg/kg in four divided doses (Dumler et al., 2007). Despite having the risk of causing dental discoloration in children, doxycycline remains the main treatment of choice for paediatric patients (Ismail et al., 2010). For patients having allergy issue with tetracycline and children under eight years old or pregnant patients, rifampin is recommended (Dumler et al., 2007; Jin et al., 2012). Treatment for anaplasmosis and ehrlichiosis are normally continued for 5 to 14 days (Dumler et al., 2007).

Oxytetracycline and doxycycline are the two most common antibiotics for horses and other ruminants (including cattle). Doxycycline is effective for cats and dogs (Atif,
Chlortetracycline is proved to be an effective drug for persistent bovine anaplasmosis caused by *A. marginale* (Reinbold *et al*., 2010).

### 2.10 Prevention and control of anaplasmosis and ehrlichiosis

The most effective method of prevention is to avoid exposure to tick and immediate removal of ticks (Ismail *et al*., 2010). Other methods such as wearing protecting or light coloured clothes and applying repellent sprays [N, N-diethyl-m-toluamide (DEET), permethrin, natural compounds (citriodiol, p-menthane-3,8-diol)] can help in prevent tick attachment (Jin *et al*., 2012; Piesman and Eisen, 2008). For controlling bovine anaplasmosis, maintenance of *Anaplasma*-free herd and quarantine of any *Anaplasma*-positive animals is required as they are the source of infection for other uninfected animals (Aubry and Geale, 2011).

The universal vaccine for any *Anaplasma* or *Ehrlichia* species is not yet available. Vaccines have been developed for bovine anaplasmosis caused by *A. marginale*. Attenuated strains of *A. marginale* and live *A. centrale* are used as vaccine candidates (Kocan *et al*., 2010). Besides that, the use of tick vaccines containing tick proteins (Q38, SUB, SILK, TROSPA, BM86/BM95 and 64P) which interfere with tick vector competence may result in reduced tick infestation (Atif, 2016).
CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection

The samples processed in this study were ticks and animal blood samples obtained from various sources. Animal blood samples were mainly provided by collaborators from the Veterinary Research Institute, Ipoh and Department of Wildlife and National Parks (PERHILITAN) Peninsular Malaysia from 2013 to 2014 for health screening purpose. Tick samples were collected from livestock farms (cattle and sheep), the forest areas (questing ticks and ticks collected from small animals) as well as from peri-domestic animals in aboriginal villages (Appendix A-C). Due to the limited information about the animal hosts and tick vectors for anaplasmosis and ehrlichiosis in Malaysia, samples were collected whenever possible. Ticks were collected from livestock subjected to blood sampling.

3.1.1 Samples obtained from livestock farms

Sampling of animal blood samples and ticks were conducted in eight livestock farms (six cattle, one goat and one sheep farms) located in different regions of Peninsular Malaysia (Table 3.1) from February to September 2013. Approval (Reference no: JPV/PSTT/100-8/1) for the sampling has been obtained from the Director, Department of Veterinary Services, Ministry of Agriculture and Agro-Based Industry, Malaysia, prior to the commencement of the study. A total of 304 blood samples (224 cattle, 40 sheep and 40 goats) were collected in this study (Table 3.1) (Appendix A). Blood sampling was performed by veterinarians in each farm. Briefly, approximately 1 to 3 ml whole blood sample was collected from each animal via jugular vein in EDTA-coated tube. The samples were then transported in ice to the laboratory. A volume of 200 µl was aliquoted from each blood sample and stored at -20 °C for DNA extraction. The remaining of the blood samples were stored at -80 °C for future work. Ticks were collected from livestock
whenever possible. The ear, eyes, abdomen, tail and perineal regions of the animals were examined for ticks. The collected ticks were preserved in -80 °C prior to processing.

Table 3.1: Sampling sites, date of sampling and number of samples collected from livestock farms in this study.

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Date of sampling</th>
<th>No. blood sample collected (Animal breed, n)</th>
<th>No. of animals infested with ticks (No. of ticks subjected to molecular analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jelai Gemas, Negeri Sembilan</td>
<td>February 2013</td>
<td>27 cattle bloods (Nellore, 11; YKK [Yellow cattle cross with Kedah Kelantan], 15)</td>
<td>27 (47 ticks)</td>
</tr>
<tr>
<td>Jerantut, Pahang</td>
<td>August 2013</td>
<td>38 cattle bloods (Kedah-Kelantan [Zebu], 38)</td>
<td>9 (14 ticks)</td>
</tr>
<tr>
<td>Ulu Lepar, Pahang</td>
<td>August 2013</td>
<td>40 cattle bloods (Nellore, 32; Brahman, 8)</td>
<td>38 (94 ticks)</td>
</tr>
<tr>
<td>Pokok Sena, Kedah</td>
<td>September 2013</td>
<td>40 sheep bloods (Damara, 40)</td>
<td>21 (44 ticks)</td>
</tr>
<tr>
<td>Tanah Merah, Kelantan</td>
<td>September 2013</td>
<td>40 cattle bloods (Kedah-Kelantan, 40)</td>
<td>Not collected</td>
</tr>
<tr>
<td>Kuala Berang, Terengganu</td>
<td>September 2013</td>
<td>40 cattle bloods (YKK, 40)</td>
<td>39 (71 ticks)</td>
</tr>
<tr>
<td>Kuala Pah, Negeri Sembilan</td>
<td>September 2013</td>
<td>40 goat bloods (Boer, 32; African dwarf, 5; Savannah, 2; Cashmere, 1)</td>
<td>Not collected</td>
</tr>
<tr>
<td>Air Hitam, Johore</td>
<td>September 2013</td>
<td>40 cattle bloods (Mafriwal, 36; Jersey, 4)</td>
<td>Not collected</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>304</td>
<td>134 (270 ticks)</td>
</tr>
</tbody>
</table>
3.1.2 Samples obtained from Veterinary Research Institute (VRI), Ipoh

A total of 302 animal blood samples (78 deer, 75 goats, 55 buffaloes, 46 horses, 22 cows, 15 pangolins, seven dogs and four rats) collected in EDTA tubes were provided by the Parasitology department of the Veterinary Research Institute (VRI), Ipoh, from January to October 2013 (Appendix D). Approval to use the sample for molecular analysis has been obtained from the Director of VRI prior to the commencement of the study [Reference no.: JPV: VRI/197/PA/141 Jid.11(48)].

Additionally, 11 animal blood samples obtained from ten dogs and one cat (Appendix D) which were collected by VRI staff during a field trip at Ipoh, Perak (from 10-13 December 2012) were also included in this investigation. Animal ethical approval from the VRI Animal Care and Use Committee (VRI ACUCVRIpara/1/2014) was obtained prior to the sampling. A volume of 200 µl of each blood sample has been pipetted to a sterile microcentrifuge and was kept at -20 °C prior to DNA extraction.

3.1.3 Samples obtained from Wildlife Department (PERHILITAN)

Approval was obtained from PERHILITAN [Reference no.: JPHL&TN (IP):80-4/2 Jilid 15(51)] prior to the commencement of the study. A total of 139 ticks (Appendix B) were collected from vegetation (n=117) and small animals (n=22) from two sampling trips organised by the Department of Wildlife and National Parks (PERHILITAN), Peninsular Malaysia in Kuala Lompat, Krau Wildlife Reserve (N 03º50’ E 102º06’), Pahang (June 2013) and a forest at Sungai Deka Elephant Sanctuary, (N 05º28’ E 102º44’) Terengganu (October 2012).

A total of 36 organ samples (liver, kidney and spleen) from small mammals (i.e., one squirrel, four rats and seven bats) collected from the field trips were also included (Appendix B). A total of 80 animal blood samples (70 monkeys and ten wild boars) and 71 Whatman® FTA® cards collected from a variety of animals were provided by PERHILITAN (Appendix B) on March 2013 and April 2015. Each blood sample (200
µl) was aliquoted to a sterile microcentrifuge tube and kept at -20 °C prior to DNA extraction. For the FTA cards, ten discs (2.0 mm in diameter) were excised from each card using a puncher and kept in -20 °C prior to processing.

3.1.4 Tick sampling in aboriginal villages

Sampling for ticks from peri-domestic animals in 12 aboriginal villages located in six states of Peninsular Malaysia was conducted in between September 2012 to January 2013 by research students working under “Tick-borne Emerging Infectious Disease Program (funded by RP013-2012A)”. Approval has been granted from the Department of Orang Asli Development (JAKAO), Malaysia prior to the study. Of a total of 246 animals (106 dogs, 105 cats, 20 chickens, 11 goats, three cows and one snake) examined, only 88 animals (31 dogs, 31 cats, 13 chickens, nine goats, three cows and one snake) were infected with ticks. All the ticks collected were preserved in -80 °C prior to processing.

A total of 192 ticks collected from 47 animals (16 cats, 13 dogs, 12 chickens, three goats, two cows and one snake) were grouped into 65 pools (one to ten individuals according to geographical location, tick species and animal host) prior to DNA extraction and molecular analysis (Table 3.2) (Appendix C).

3.1.5 Other samples

Other samples that were investigated in this study include:

i. Twenty-one adult ticks (11 *Amblyomma varanense* and ten *Amblyomma helvolum*) collected from seven *Python molurus* snakes from Sepang, Selangor during September 2012 (Appendix E).

ii. Thirty stray dog blood samples collected from an animal shelter in Klang Valley (Mokhtar *et al.*, 2013) and 33 *R. sanguineus* ticks collected from 13 dogs in Klang Valley (Appendix E).
Table 3.2: Sampling sites and samples collected from aboriginal villages in this study.

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Date of sampling</th>
<th>No. of animal host examined (n)</th>
<th>No. of ticks subjected to molecular analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dog</td>
<td>Cat</td>
</tr>
<tr>
<td>Negeri Sembilan</td>
<td>September 2012</td>
<td>40</td>
<td>12</td>
</tr>
<tr>
<td>Pahang</td>
<td>October-November 2012</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Johore</td>
<td>November 2012</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>Kedah</td>
<td>January 2013</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Perak</td>
<td>January 2013</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Kelantan</td>
<td>January 2013</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>106</td>
<td>105</td>
</tr>
</tbody>
</table>
3.2 Identification of tick species using morphological identification methods

Ticks collected in this study were identified morphologically based on the taxonomic keys of Burridge (2001), Kohls (1957), Walker et al. (2003) or Geevarghese and Mishra (2011). The tick images were viewed and captured using a stereomicroscope (Olympus, Japan). Representative ticks of different species were placed individually in microcentrifuge tubes containing 70% ethanol at room temperature as voucher specimens.

3.3 Identification of tick species using molecular identification methods

Molecular identification of ticks was also carried out based on sequence analysis of the tick 16S rRNA or 28S rRNA genes (Black and Piesman, 1994; Inokuma et al., 2003), whenever the species identification was in doubt.

3.4 DNA extraction

The DNA extraction procedures were carried out in a Class II, A2 biological safety cabinet, (NuAire Inc, USA). A QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used for extracting DNA from each sample. The protocols were slightly adjusted for different sample types.

3.4.1 Tick samples

Ticks which had been stored at -80 °C freezer were first thawed, washed in 5% sodium hypochlorite and 70% ethanol for 3 min each and rinsed twice in sterile distilled water for 3 min. The ticks were homogenised in 180 µl Buffer ATL (tissue lysis buffer) using surgical blades in a microcentrifuge tube. After adding 20 µl Proteinase K, the sample was mixed thoroughly by vortexing for 10 s before incubated at 56 °C for overnight. A volume of 200 µl Buffer AL (lysis buffer) was then added to the sample. After vortexing for 10 s, the sample was incubated at 70 °C for 10 min before added with 230 µl ethanol (molecular grade). The mixture was transferred to a spin column (provided
by the kit) and centrifuged at 8000 rpm for 1 min. After the centrifugation step, the spin column was placed in a new, clean 2 ml collection tube. A volume of 500 µl Buffer AW1 (wash buffer) was added and the column was centrifuged at 8,000 rpm for 1 min. The spin column was later placed in a new, clean collection tube and added with 500 µl Buffer AW2 (wash buffer). After centrifugation at 14,000 rpm for 3 min, an additional step of centrifugation (14,000 rpm for 1 min) was added to prevent carryover of Buffer AW2. The column was placed in a sterile 1.5 ml microcentrifuge tube, added with 60 µl Buffer AE (elution buffer) and incubated at room temperature for 5 min before centrifuging at 8,000 rpm for 1 min to elute the DNA. Lastly, another 60 µl Buffer AE were added into the column. The column was incubated again at room temperature for 5 min and centrifuged at 8,000 rpm for 1 min. The eluted DNA was collected and stored at -20 °C prior to PCR amplification.

3.4.2 Whole blood samples

A volume of 200 µl whole blood sample was added to a 1.5 ml microcentrifuge tube containing 20 µl proteinase K. The mixture was then added with 200 µl Buffer AL and vortexed for 10 s prior to incubation at 56 °C for 10 min. This was then followed by the addition of a volume of 200 µl ethanol (molecular grade) to the mixture. Subsequent extraction steps were performed as described in section 3.4.1. A total of 100 µl DNA solution was eluted from each sample and stored at -20 °C prior to PCR amplification.

3.4.3 Organ samples

The organ samples were thawed and cut into tiny pieces using sterile surgical blades, prior to DNA extraction. The organ samples (25 mg for liver and kidney; 10 mg for spleen) were homogenised using a hand-held homogeniser in a 1.5 ml microcentrifuge tube containing 80 µl phosphate buffered saline (PBS). A volume of 100 µl Buffer ATL and 20 µl proteinase K were then added to the homogenate. The sample was mixed thoroughly by vortexing for 10 s and incubated at 56 °C for approximately 3 hours. During
incubation, the sample was vortexed occasionally. DNA extraction for the subsequent steps was performed as described in section 3.4.1. A total of 200 µl DNA solution was eluted and stored at -20 °C prior to PCR amplification.

3.4.4 Whatman® FTA® card

Ten discs excised from each FTA card were placed in a sterile 1.5 ml microcentrifuge tube. The discs were washed thrice with 200 µl FTA Purification Reagent (GE Healthcare, UK) at room temperature for 5 min to remove cell debris and PCR inhibitors. The discs were then washed twice with 200 µl 1X TE buffer at room temperature for 5 min. Lastly, the discs were placed in a sterile 1.5 ml microcentrifuge tube and dried at 50 °C for 15 min. The purified discs were stored at -20 °C. A single disc was used as the DNA template for amplification.

3.5 Identification of Anaplasma spp. and Ehrlichia spp. from animals and ticks using molecular methods

3.5.1 Integrity checking of samples for amplification

3.5.1.1 Amplification of the 16S rRNA or 28S rRNA gene from tick DNA samples

The amplification of either 16S rRNA or 28S rRNA gene from tick DNA samples was used as an indicator to show that DNA extraction from ticks was successful and there was no PCR inhibitors in the samples. In this study, primers 16S+2 and 16S-1 or primers 28SF and 28SR (Black and Piesman, 1994; Inokuma et al., 2003) (Appendix F) were used in the PCR assays. These PCR assays amplified a portion of tick 16S rRNA gene (approximately 300 bp amplicon) and tick 28S rRNA gene (approximately 490 bp amplicon). The thermal cycling profile for amplification of tick 16S rRNA gene consisted of an initial denaturation step at 94 °C for 2 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. The PCR conditions for amplification of tick 28S rRNA
gene included an initial denaturation step at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min.

3.5.1.2 Amplification of the cytochrome B (cytB) gene of animal blood and organ samples

A PCR assay using primers cytBFor and cytBRev (Oshaghi et al., 2006) (Appendix F) was used to check the integrity of the DNA template for animal blood and organ samples. The thermal cycling condition consisted of an initial denaturation step at 95 °C for 3.5 min, 36 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 50 s and extension at 72 °C for 40 s, followed by a final extension step at 72 °C for 5 min. The PCR assay is expected to amplify a fragment of cytB gene (approximately 358 bp) from animal blood and organ samples.

3.5.2 Polymerase chain reaction

All PCR assays were performed in a reaction volume of 25 µl containing two microliters of DNA template, 1U GoTaq® Flexi DNA Polymerase (Promega, WI, USA), 1X Green GoTaq® Flexi Buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.2 µM of forward and reverse primers and 15.4 µl miliQ water. The composition of PCR reaction mixture is shown in Table 3.3. For nested PCR assays, two microliters of the products from the first amplification was used as the DNA template for the nested amplification. Positive (A. phagocytophilum and E. chaffeensis genomic DNA extracted from IFA slides or plasmid DNA) and negative (miliQ water) controls were included in each PCR assay. Amplification was performed in a MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA).
Table 3.3: PCR reaction mixture used for amplification of *Anaplasmataceae* DNA.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Initial concentration</th>
<th>Final concentration</th>
<th>Volume (µl per reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miliQ water</td>
<td>-</td>
<td>-</td>
<td>15.4</td>
</tr>
<tr>
<td>Green GoTaq® Flexi Buffer</td>
<td>5X</td>
<td>1X</td>
<td>5.0</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>1.5 mM</td>
<td>1.5</td>
</tr>
<tr>
<td>Forward primer</td>
<td>25 µM</td>
<td>0.2 µM</td>
<td>0.2</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>25 µM</td>
<td>0.2 µM</td>
<td>0.2</td>
</tr>
<tr>
<td>GoTaq® Flexi DNA Polymerase</td>
<td>5 U/µl</td>
<td>1 U</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA template</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

### 3.5.3 Preliminary screening of *Anaplasmataceae* DNA

Ticks, animal bloods, organ samples and FTA cards were subjected to preliminary screening using a PCR assay targeting a partial fragment (345 bp) of the 16S rRNA gene of *Anaplasmataceae*. The composition of the PCR master mix used in this study is shown in Table 3.3. Two primers, i.e., EHR16SD and EHR16SR (Parola *et al.*, 2000) (Appendix F) were used in the PCR assay. The amplification procedure was performed with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s, and a final extension step at 72 °C for 5 min.

### 3.5.4 Further characterisation of *Anaplasma* spp. and *Ehrlichia* spp.

Samples that were positive in the screening assays (section 3.4.3) were randomly selected for further characterisation using different PCR approaches. The selection was based on sample type, sample source, and the availability of DNA template.

#### 3.5.4.1 Amplification of the full length 16S rRNA, *gltA* and *groEL* genes of anaplasma and ehrlichiae

a. Amplification of the full length 16S rRNA gene of *Anaplasma* spp. and *Ehrlichia* spp. was performed using the following primers and PCR conditions:
i. Primers fD1 and Rp2 (Weisburg et al., 1991) (Appendix F). The thermal cycling condition consisted of an initial denaturation step at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 2 min, annealing at 58 °C for 30 s and extension at 72 °C for 4 min, followed by a final extension step at 72 °C for 20 min. The PCR assay is expected to amplify a nearly full length fragment of the 16S rRNA gene (1500 bp).

ii. Primers ATT062F and ATT062R (Pinyoowong et al., 2008) (Appendix F). The thermal cycling condition consisted of an initial denaturation step at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 4 min. The PCR assay is expected to amplify a nearly full length fragment of the 16S rRNA gene (1500 bp).

iii. Primers fD1 with EHR16SR and primers Rp2 with EHR16SD. The thermal cycling condition consisted of an initial denaturation step at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 90 s, followed by a final extension step at 72 °C for 5 min. Each PCR assay is expected to amplify a partial fragment of the 16S rRNA gene (760 bp).

b. For amplification of the gltA gene of Anaplasma spp. and Ehrlichia spp., a PCR assay was performed using primers EHR-CS136F and EHR-CS778R as previously described by Inokuma et al. (2001a) (Appendix F). The thermal cycling condition consisted of an initial denaturation step at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s, followed by a final extension step at 72 °C for 5 min. A high primer concentration (final concentration: 0.5 µM) was used for the PCR assay. The PCR assay is expected to amplify a fragment of 643 bp.

c. A nested PCR assay was used to amplify a 1320 bp fragment of the groEL gene of Anaplasma spp. and Ehrlichia spp. Primers HS1-f and HS6-r (Rar et al., 2010; Sumner et al., 1997) (Appendix F) were used for the first amplification. The amplification procedure was performed with an initial denaturation step at 94 °C for 3 min followed by
35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min. Primers HS3-f and HSVR (Liz et al., 2000; Rar et al., 2010) (Appendix F) were used for nested amplification. Two microliters of the first PCR product was used for the nested amplification. The amplification procedure was performed with an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1.5 min. For the PCR assay, a higher primer concentration (final concentration: 0.5 µM) was used.

3.5.4.2 PCR assays targeting specific organism

a. Amplification of A. phagocytophilum DNA

i. The 16S rRNA gene of A. phagocytophilum was amplified by using a nested PCR assay as described by Kawahara et al. (2006). The first amplification was performed by using primers EC9 and EC12A (Appendix F). Each of the 40 amplification cycles was started with denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min. The PCR assay produced an approximately 1462 bp amplicon. The nested amplification was performed by using primers SSAP2f and SSAP2r (Appendix F). Each of the 40 amplification cycles was started with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The PCR assay amplifies approximately 641 bp of the 16S rRNA gene of A. phagocytophilum.

ii. Additionally, a nested PCR was also used to amplify the msp4 gene of A. phagocytophilum. The first amplification was performed by using primers MSP4AP5 and MSP4AP3 (de la Fuente et al., 2005b; Silaghi et al., 2011) (Appendix F). The PCR thermal cycling condition consisted of an initial denaturation step at 95 °C for 10 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. A higher primer concentration (final concentration: 0.8 µM) was used in this assay. The PCR produced an approximately 849 bp amplicon. Primers msp4f and msp4r (Bown et al., 2007; Silaghi et
al., 2011) (Appendix F) were used in the second amplification. The thermal cycling condition consisted of an initial denaturation step at 95 °C for 10 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. Similarly, a higher primer concentration (final concentration: 0.8 µM) was used in this assay. The PCR assay produces an approximately 343 bp amplicon.

iii. A heminested PCR assay was performed to amplify a 573 bp of the groEL gene of *A. phagocytophilum*, using primers EphplgroEL(569)F and EphplgroEL(1193)R (Alberti et al., 2005; Reye et al., 2010) (Appendix F) for first amplification. The PCR thermal cycling condition consisted of an initial denaturation step at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension step at 72 °C for 10 min. A higher primer concentration (final concentration: 0.8 µM) was used in this assay. The PCR assay produced an approximately 624 bp amplicon. The heminested amplification was performed using primers EphplgroEL(569)F and EphgroEL(1142)R (Alberti et al., 2005; Reye et al., 2010) (Appendix F). The PCR thermal cycling condition consisted of an initial denaturation step at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension step at 72 °C for 10 min. A high primer concentration (final concentration: 0.8 µM) was used in this assay. The PCR assay produces an approximately 573 bp amplicon.

b. Amplification of *A. bovis* DNA

The 16S rRNA gene of *A. bovis* was amplified by using a nested PCR assay as described by Kawahara et al. (2006). The first amplification was performed by using primers EC9 and EC12A (Appendix F). Each of the 40 amplification cycles started with denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min. The PCR assay produced an approximately 1462 bp amplicon. The following nested
amplification was performed by using primers AB1f and AB1r (Appendix F). Each of the 40 amplification cycles started with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. This PCR amplifies approximately 551 bp of the 16S rRNA gene of A. bovis.

c. Amplification of E. chaffeensis DNA

A nested PCR assay was used to amplify a 389 bp of the 16S rRNA gene of E. chaffeensis. Primers ECB and ECC (Anderson et al., 1992b; Dawson et al., 1994) (Appendix F) were used for the first amplification. Each of the 35 amplification cycles was started with denaturation at 94 °C for 1 min, annealing at 45 °C for 2 min and extension at 72 °C for 1 min. The PCR assay produces an approximately 450 bp amplicon. The following nested amplification was performed using primers HE1 and HE3 (Anderson et al., 1992b; Dawson et al., 1994) (Appendix F). Each of the 35 amplification cycles was started with denaturation at 94 °C for 1 min, followed by annealing at 55 °C for 2 min and extension at 72 °C for 1 min.

d. Amplification of E. canis DNA

The 16S rRNA gene of E. canis was amplified by using a nested PCR assay. Primers ECB and ECC (Anderson et al., 1992b; Dawson et al., 1994) (Appendix F) were used for the first amplification. Each of the 35 amplification cycles was started with denaturation at 94 °C for 1 min, followed by annealing at 45 °C for 2 min and extension at 72 °C for 1 min. The PCR assay produced an approximately 450 bp amplicon. The nested amplification was performed using primers ECA and HE3 (Wen et al., 1997) (Appendix F). The thermal cycling condition consisted of an initial denaturation step at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. The PCR assay amplifies an approximately 389 bp of the 16S rRNA gene of E. canis.
3.5.5 Detection of amplified PCR products generated in the PCR assays

After amplification, agarose gel electrophoresis was used to analyse the amplified products obtained from the PCR assays. The amplified products were electrophoresed in a 1.5 % agarose gel prestained with GelRed (Biotium, CA, USA). The electrophoresis was performed in Tris-Borate-EDTA (TBE) buffer (Vivantis, CA, USA) (Appendix G) at a constant voltage of 100 V for 50 min. 1 µl DNA ladder (100 bp or 1 kb, depending on the expected size of the amplicon) (Solis BioDyne, Estonia) was run in parallel with 5 µl PCR products added with 1 µl of gel loading buffer. The gels were then visualised and photographed under UV light (G-Box, Syngene, UK). Positive results were indicated by the presence of a band corresponding with the expected size of each PCR assay.

3.6 Generation of positive control for PCR assays

Positive controls are essential to be included in each PCR assay to avoid false positive or false negative results. In this study amplified DNA fragments (Table 3.4) were cloned into pCR4-TOPO vector (Invitrogen, CA, USA). One microliter of the purified plasmids was used as positive controls in each PCR run.

Table 3.4: Details of the DNA fragments cloned as positive control.

<table>
<thead>
<tr>
<th>Plasmid Label</th>
<th>Target gene, organism</th>
<th>Length of gene insert (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0088 EHR-1</td>
<td>16S rRNA gene (short), <em>A. bovis</em></td>
<td>345 bp</td>
</tr>
<tr>
<td>0088 ATT-1</td>
<td>16S rRNA gene, <em>A. bovis</em></td>
<td>1500 bp</td>
</tr>
</tbody>
</table>

3.6.1 Setting up cloning reaction and transformation of plasmid into competent *E. coli*

Cloning was performed using TOPO TA Cloning® Kit for Sequencing (Invitrogen, CA, USA) according to the manufacturer’s instructions. Table 3.5 shows the reagents used in the TOPO® cloning reaction. All the reagents were mixed thoroughly and
incubated for 30 min at room temperature. Transformation was performed by adding 4 µl of the TOPO® cloning reaction to a vial of One Shot® *E. coli* competent cells and mixed gently. The reaction was then incubated on ice for 30 min. The cells were subjected to heat-shock for 30 s at 42 °C without shaking and immediately transferred to ice after 30 s of incubation. A volume of 250 µl S.O.C. medium was then added and the culture was incubated horizontally in a shaker (200 rpm) at 37 °C for 1 hour (or until a cloudy solution was observed). The cell suspension (50 µl) was spread on a pre-warmed Luria-Bertani (LB) agar plate containing 50 µg/ml ampicillin (Appendix G) and incubated overnight at 37 °C. Any colonies growing on the agar plate were considered positive for the cloning reaction. At least one loop full of colonies was preserved in glycerol stock for long term storage at -80 °C.

Table 3.5: Reagents and the volumes required in the TOPO® cloning reaction.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh PCR product</td>
<td>4</td>
</tr>
<tr>
<td>Salt solution</td>
<td>1</td>
</tr>
<tr>
<td>TOPO® vector</td>
<td>1</td>
</tr>
<tr>
<td>Final volume</td>
<td>6</td>
</tr>
</tbody>
</table>

3.6.2 Plasmid extraction

Five single *E. coli* colonies were picked from an LB-ampicillin agar plate and inoculated into a tube containing 5 ml LB-ampicillin broth (Appendix G). The inoculated broth was incubated horizontally in a shaker (150 rpm) overnight at 37 °C. Plasmid extraction was performed by using GeneAll Hybrid-Q Plasmid Rapidprep (GeneAll, South Korea). Briefly, the bacterial culture was harvested in a 15 ml sterile centrifuge tube by centrifugation at 14,000 rpm for 5 min. The supernatant was discarded as much as possible. The bacterial pellet was then resuspended in 250 µl Buffer S1 (provided by the kit and added with RNase) and transferred to a new 1.5 ml microcentrifuge tube. A
volume of 250 µl Buffer S2 was added and mixed by inverting the microcentrifuge tube for 4 times. After the cell suspension had become viscous, 350 µl Buffer G3 was added to the microcentrifuge tube. The mixture was mixed by inverting the microcentrifuge tube for 4 to 6 times and centrifuged at 14,000 rpm for 10 min. The supernatant was then transferred to a spin column (provided by the kit) and centrifuged at 14,000 rpm for 1 min. After the pass-through had been discarded, 500 µl Buffer AW was added to the column. This was followed by a centrifugation step at 14,000 rpm for 1 min. The step was repeated with the addition of 700 µl Buffer PW. Any residual wash buffer was removed by centrifugation at 14,000 rpm for 1 min. The column was then transferred to a new 1.5 ml microcentrifuge tube. Lastly, 50 µl Buffer EB was added to the column and incubated for 1 min before centrifugation at 14,000 rpm. The eluted plasmid DNA was stored at -20 °C prior to use.

3.6.3 Analysing positive clones

To ensure that the gene of interest was successfully inserted into the plasmid as described in section 3.6.1, purified plasmid was used as DNA template for PCR assays targeting the corresponding gene by using respective forward and reverse PCR primers (sections 3.5.3 and 3.5.4).

3.7 Sequence determination and analysis of selected amplified products

Sequence determination of amplified products was performed to confirm the specificity of each PCR assay and to identify the organisms amplified from PCR assays. Only selected amplified products (representing different sample types) were subjected to sequence determination.

3.7.1 Purification of PCR products

Amplified PCR products were purified using a GeneAll Expin™ Combo GP kit (GeneAll, South Korea) prior to sequencing. For PCR product with a single band on
agarose gel, 5 volumes of Buffer PB was added to 1 volume of the PCR product. The mixture was then transferred to a SV column (provided by the kit) prior to centrifugation at 13,000 rpm for 1 min. The pass-through was discarded and the SV column was reinserted back into the same collection tube. A volume of 700 µl Buffer NW was added to the SV column and centrifuged at 13,000 rpm for 1 min. The pass-through was discarded and the SV column was reinserted back into the same collection tube. The SV column was centrifuged for an additional 1 min at 13,000 rpm to remove any residual wash buffer. The SV column was then transferred to a sterile 1.5 ml microcentrifuge tube. Lastly, 30 µl Buffer EB was added to the column and incubated at room temperature for 1 min prior to centrifugation at 13,000 rpm for 1 min. The eluted purified PCR product was then subjected to sequence determination.

For PCR product generating multiple bands on the agarose gel, the band of interest was excised using a sterile surgical blade on a UV transilluminator (Vilber Lourmat, Germany). The gel slice was weighed in a 1.5 ml microcentrifuge tube and added with 3 volumes of Buffer GB. The agarose gel was incubated at 50 ºC until it was completely melted (required 5-10 min depends on the size of the gel). The mixture was transferred to a SV column (provided by the kit) and centrifuged at 13,000 rpm for 1 min. The pass-through was discarded and the SV column was reinserted back into the same collection tube. A volume of 700 µl Buffer NW was then added to the SV column and centrifuged at 13,000 rpm for 1 min. The pass-through was discarded and the SV column was reinserted back into the same collection tube. The SV column was centrifuged for an additional 1 min at 13,000 rpm to remove any residual wash buffer. Lastly, the SV column was added with 30 µl Buffer EB and incubated at room temperature for 1 min prior to centrifugation at 13,000 rpm for 1 min. The purified PCR product was subjected to sequence determination as described above, using primers M13 Forward (-20) and M13 Reverse, provided by the TOPO TA Cloning® Kit for Sequencing (Invitrogen, CA, USA).
3.7.2 Sequence determination and analysis

Sequence determination of amplified products was performed by a service provider (First BASE Laboratories, Malaysia) using a Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, CA, USA). Both forward and reverse PCR primers were used as the primers for sequencing. To determine the sequence of the full length 16S rRNA gene of Anaplasma spp. and Ehrlichia spp., primer pairs ATT062F/ATT062R, ATT066F (5’- CCC TGG TAG TCC ACG CTG -3’) and ATT067R (5’- CAG CGT GGA CTA CCA GGG -3’) (Pinyoowong et al., 2008) were used. For amplicons derived from nested PCR assays, the primers used in the nested amplification were used for sequencing.

The obtained sequences were aligned with BioEdit Sequence Alignment Editor Software (version 7.0.5.3) or Geneious version R6.1 (Biomatters, New Zealand). The quality of the sequences were checked manually. Sequences with noisy data or ambiguous nucleotide positions were discarded. The samples were either re-sequenced or cloned into a plasmid to generate better sequence data. All the aligned sequences were compared for similarity with sequences deposited in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (National Center for Biotechnology Information, United States National Library of Medicine, MD, USA). The phylogenetic status of the anaplasma and ehrlichiae identified in this study were analysed using the neighbour-joining method of the MEGA (Molecular Evolutionary Genetics Analysis) 6.0 software (Tamura et al., 2013).

3.7.3 Sequence with ambiguous nucleotide positions

Amplified fragments with low sequence quality, for instance, noisy data or with ambiguous nucleotides were cloned into pCR4-TOPO vector (Invitrogen, CA, USA) according to sections 3.6.1-3.6.3. Plasmids with the amplified PCR products were
subjected to sequence determination, using primers M13 Forward (-20) and M13 Reverse, provided by the TOPO TA Cloning® Kit for Sequencing (Invitrogen, CA, USA).

3.8 Serological detection of selective tick-borne pathogens from dog samples

3.8.1 SNAP 4Dx® kit

A total of 43 dog blood samples (30 blood samples from Klang Valley while 13 blood samples from VRI, Ipoh) were tested for serological response against *A. phagocytophilum*, *E. canis*, *A. platys* and *B. burgdorferi* using SNAP 4Dx® test kits (IDEXX Laboratories, ME, USA) in accordance with the manufacturer’s protocol (the kit also includes testing against *Dirofilaria immitis*, which is a mosquito-borne organism). Briefly, three drops of each blood sample were added to the provided sample tube together with four drops of conjugate. The mixture was mixed thoroughly by inverting 3 to 5 times before pipetting into a sample well of a SNAP device. The test result was read after 8 min. A positive reaction was indicated by the appearance of color in the activation circle. The result of the test is valid only when the positive control develops colour.
CHAPTER 4: RESULTS

4.1 Collection and identification of tick species using morphological identification method

4.1.1 Livestock farms

During the sampling of animal ticks from eight livestock farms (six cattle, one goat and one sheep farms) located at different regions of Peninsular Malaysia (Table 3.1) from February to September 2013, a total of 226 ticks were collected from four cattle farms and 44 ticks were collected from a sheep farm (Table 4.1). Based on morphological identification, 70 of the cattle ticks were identified as *R. microplus* while 200 ticks (156 ticks collected from cattle and 44 ticks collected from sheep) were identified as *Haemaphysalis bispinosa* (Figure 4.1), as reported by Kho *et al.* (2015a).

The identification of *Rhipicephalus (Boophilus)* were based on the previous report by Brahma *et al.* (2014). The ticks were identified based on small palpal segments, hexagonal basis capitulum, presence of indistinct eyes, coxa I with paired spur and coxa II to IV without spurs. Festoons were absent in the male *Rhipicephalus (Boophilus)* ticks. The caudal appendage in a male tick is the most visible feature used to differentiate *R. microplus* from *R. annulatus* (Brahma *et al.*, 2014).

*Haemaphysalis* ticks were identified based on the presence of small palpal segments, rectangular basis capitulum (straight lateral margins), absence of eyes and presence of festoons (Brahma *et al.*, 2014; Geevarghese and Mishra, 2011). *Haemaphysalis* ticks are also characterised by short mouthparts with the anal groove forming a loop posterior to the anus (Walker *et al.*, 2003). Male ticks were also characterised by the absence of ventral plates (Brahma *et al.*, 2014).

The identity for some of the ticks were confirmed using molecular identification method, as described in section 4.2.
Table 4.1: Total number of tick samples collected from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Animal hosts (n)</th>
<th>Tick species (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Livestock farms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Jelai Gemas, Negeri Sembilan</td>
<td>Cattle (27)</td>
<td><em>H. bispinosa</em> (41), <em>R. microplus</em> (6)</td>
</tr>
<tr>
<td>• Jerantut, Pahang</td>
<td>Cattle (38)</td>
<td><em>R. microplus</em> (14)</td>
</tr>
<tr>
<td>• Ulu Lepar, Pahang</td>
<td>Cattle (40)</td>
<td><em>H. bispinosa</em> (57), <em>R. microplus</em> (37)</td>
</tr>
<tr>
<td>• Pokok Sena, Kedah</td>
<td>Sheep (40)</td>
<td><em>H. bispinosa</em> (44)</td>
</tr>
<tr>
<td>• Tanah Merah, Kelantan</td>
<td>Cattle (40)</td>
<td>0</td>
</tr>
<tr>
<td>• Kuala Berang, Terengganu</td>
<td>Cattle (40)</td>
<td><em>H. bispinosa</em> (58), <em>R. microplus</em> (13)</td>
</tr>
<tr>
<td>• Kuala Pah, Negeri Sembilan</td>
<td>Goat (40)</td>
<td>0</td>
</tr>
<tr>
<td>• Air Hitam, Johore</td>
<td>Cattle (40)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>270 (200 <em>H. bispinosa</em> and 70 <em>R. microplus</em>)</td>
</tr>
<tr>
<td><strong>Forest areas (PERHILITAN)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Sg. Deka, Terengganu</td>
<td>Vegetation areas</td>
<td><em>Dermacentor</em> spp. (29), <em>Haemaphysalis</em> spp. (9), <em>Amblyomma</em> spp. (3)</td>
</tr>
<tr>
<td>• Kuala Lompat, Pahang</td>
<td>Vegetation areas</td>
<td><em>Dermacentor</em> spp. (48), <em>Haemaphysalis</em> spp. (26), <em>Amblyomma</em> spp. (2)</td>
</tr>
<tr>
<td></td>
<td>Rat (10), bat (1), squirrel (1), skink (1)</td>
<td><em>Haemaphysalis</em> spp. (15), <em>Dermacentor</em> spp. (6), <em>Amblyomma</em> sp. (1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>139 (83 <em>Dermacentor</em> spp., 50 <em>Haemaphysalis</em> spp. and 6 <em>Amblyomma</em> spp.)</td>
</tr>
<tr>
<td><strong>Aboriginal villages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Negeri Sembilan</td>
<td>Dog (2), chicken (1), goat (3)</td>
<td><em>R. sanguineus</em> (3 individuals), <em>Haemaphysalis</em> spp. (4 pools)</td>
</tr>
<tr>
<td>• Pahang</td>
<td>Dog (1), cat (1), chicken (3)</td>
<td><em>Haemaphysalis</em> spp. (7 pools)</td>
</tr>
<tr>
<td>• Johore</td>
<td>Snake (1), dog (1), chicken (2), cat (6)</td>
<td><em>Amblyomma</em> spp. (1 pool), <em>Haemaphysalis</em> spp. (1 individual and 8 pools)</td>
</tr>
<tr>
<td>• Kedah</td>
<td>Cow (2)</td>
<td><em>R. microplus</em> (3 individuals)</td>
</tr>
<tr>
<td>• Perak</td>
<td>Dog (9), chicken (3), cat (6)</td>
<td><em>R. sanguineus</em> (1 individual), <em>Dermacentor</em> spp. (2 individuals), <em>Haemaphysalis</em> spp. (13 individuals and 11 pools)</td>
</tr>
<tr>
<td>• Kelantan</td>
<td>Cat (3), chicken (3)</td>
<td><em>Haemaphysalis</em> spp. (10 individuals and 1 pool)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>33 individuals and 32 pools</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Sepang, Selangor</td>
<td>Snake (7)</td>
<td><em>A. varanense</em> (11), <em>A. helvolum</em> (10)</td>
</tr>
<tr>
<td>• Klang Valley</td>
<td>Dog (13)</td>
<td><em>R. sanguineus</em> (33)</td>
</tr>
</tbody>
</table>
4.1.2 Forest areas

A total of 117 questing ticks were collected from vegetation from two sampling sites at Kuala Lompat, Krau Wildlife Reserve (N 03°50’ E 102°06’), Pahang and a forest at Sungai Deka Elephant Sanctuary, (N 05°28’ E 102°44’), Terengganu. Based on morphological identification, five, 35 and 77 ticks collected from vegetation (Table 4.1) were identified as *Amblyomma* spp., *Haemaphysalis* spp. and *Dermacentor* spp., respectively (Figure 4.2). Additionally, 22 ticks were collected from one reptile (*Eutropis multifasciata*) and 12 small mammals (one *Lariscus insignis*, one *Rhinolophus lepidus*, one *Leopoldamys sabanus* and nine *Maxomys rajah*) from a field trip in a forest area (Kuala Lompat, Pahang) (Table 4.1), through a collaborative effort with Department of Wildlife Peninsular Malaysia (PERHILITAN), Malaysia. The tick identified from the reptile was identified as *Amblyomma* sp., while six and 15 ticks collected from small mammals were morphologically identified as *Dermacentor* spp. and *Haemaphysalis* spp., respectively.
The *Dermacentor* ticks were identified based on the presence of basis capitulum which is rectangular dorsally, coxa I have large and equal paired spurs, mouthpart is anterior and anal groove is posterior to the anus (Walker et al., 2003). For male *Dermacentor* ticks, festoons are present, coxa IV is enlarged and ventral plates are absence (Geevarghese and Mishra, 2011).

The *Amblyomma* ticks were characterised by the long and anterior mouthparts, basis capitulum have straight lateral margins, palp articles 2 are longer than article 1 and 3, coxa 1 have unequal paired spurs while coxa 4 are of normal size, the presence of festoons and anal groove is posterior to the anus (Walker et al., 2003). The ventral plates are absent in the male ticks (Geevarghese and Mishra, 2011).

Figure 4.2: Ticks collected from the forest areas in this study. A: Questing ticks (L: Female, R: Male), *Dermacentor* spp.; B: Questing tick, *Amblyomma* sp.; C: Questing tick, *Haemaphysalis* sp. (The female tick was characterised by the small scutum size as compared to the male tick where the scutum covers the entire dorsum of the male idiosoma).
4.1.3 Aboriginal villages

A total of 192 ticks were collected from 12 aboriginal villages located in six states in Peninsular Malaysia (Kho et al., 2017). The ticks were identified based on morphology to genus level and segregated into 65 pools (one to ten individuals of the similar species) (Table 4.1). As the size of most of the ticks were small, the ticks collected from the same location, genus and animal host were pooled. A total of 55 pools of *Haemaphysalis* ticks were collected from three goats, 11 dogs, 12 chickens and 16 cats. One pool of *Amblyomma* ticks was collected from a snake. Three pools of *R. microplus* ticks were collected from two cows while four pools of *Rhipicephalus* ticks were collected from three dogs. Two pools of ticks collected from a dog were not able to be identified morphologically because the ticks were engorged with blood. Hence, they were identified using molecular method (Figure 4.3).
Figure 4.3: Ticks collected from aboriginal villages in this study.
A: Chicken tick, *Haemaphysalis* sp.;
B: Dog tick, *Rhipicephalus* sp.; C: Goat tick, *Haemaphysalis* sp.; D: Cat tick, *Haemaphysalis* sp.; E: Dog ticks (L: Male, R: Female), *Haemaphysalis* spp. (The female tick was characterised by the small scutum size as compared to the male tick where the scutum covers the entire dorsum of the male idiosoma).

4.1.4 Other samples

Of the 21 ticks collected from seven *P. molurus* snakes from Sepang, 11 and ten ticks were morphologically identified as *A. varanense* and *A. helvolum*, respectively.
(Figure 4.4) according to Chao et al. (2013) and Burridge (2001), as reported by Kho et al. (2015b).

All the 33 ticks collected from 13 strayed dogs in Klang Valley were morphologically identified as *Rhipicephalus* spp. (Figure 4.5).

![Figure 4.4: Snake ticks collected from Sepang, Selangor. A: *A. helvolum*; B: *A. varanense*](image)

![Figure 4.5: *Rhipicephalus* sp. collected from a dog in Klang Valley.](image)

### 4.2 Identification of tick species using molecular identification methods

A total of 117 ticks collected from cattle (n=25), sheep (n=5), vegetation (n=49), and other animals (n=38) were subjected to sequence analysis of the 16S rRNA gene region in this study. Table 4.2 shows the results of BLAST analyses of the 16S rRNA
gene sequences obtained from 117 ticks collected from different sources and locations in this study. Of the 73 sequences demonstrating 99-100% similarity with the sequences available in the GenBank database, *H. bispinosa* (n=25), *Haemaphysalis wellingtoni* (n=13), *H. shimoga* (n=2), *Haemaphysalis hystricis* (n=4), *Dermacentor atrosignatus* (n=10), *D. auratus* (n=2), *R. microplus* (n=12), *R. sanguineus* (n=3) and *A. helvolum* (n=2) were identified. Additionally, four sequence types (STs) of *Dermacentor* ticks (n=25), four sequence types (STs) of *Haemaphysalis* ticks (n=17) and one sequence type (ST) of *Amblyomma* ticks (n=2) which did not have any full match (< 99%) with sequences deposited in the GenBank database were identified only up to the genus level.

4.2.1 Livestock farms

The 16S rDNA sequences obtained from 12 representative cattle ticks demonstrated 100% (186 nt/186 nt) similarity to that of *R. microplus* collected from cattle in a previous study conducted in this laboratory (isolate A1-A4, GenBank accession nos.: KM246878-KM246882) (Low et al., 2015). The sequences obtained from 18 representative *Haemaphysalis* ticks (from 13 cattle and five sheep) demonstrated 100% (193 nt/193 nt) similarity to several *H. bispinosa* strains (vouchers TUTEZ-G135, TUTEZ-R1324 and TUTEZ-R1320; GenBank accession nos.: KC853418-KC853420) (Brahma et al., 2014) collected from goat and cattle in India. The details of the BLAST results are shown in Table 4.2.
Table 4.2: Results of BLAST analyses of the 16S rRNA gene sequences obtained from 117 ticks collected from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Animal host</th>
<th>Tick species (n) (morphological identification)</th>
<th>BLAST result (nearest match)</th>
<th>Sequence type (ST)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Livestock farms</strong></td>
<td>Cattle</td>
<td>Haemaphysalis spp. (2)</td>
<td><em>H. bispinosa</em> voucher TUTEZ-G135 [KC853420; 193 nt/193 nt (100% identity); goat, India]</td>
<td><em>H. bispinosa</em></td>
</tr>
<tr>
<td>• Jelai Gemas, Negeri Sembilan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Ulu Lepar, Pahang</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Pokok Sena, Kedah</td>
<td>Sheep</td>
<td>Haemaphysalis spp. (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Kuala Berang, Terengganu</td>
<td>Cattle</td>
<td>Haemaphysalis spp. (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Jerantut, Pahang</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Ulu Lepar, Pahang</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Kuala Berang, Terengganu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Forest areas</strong></td>
<td>Bat</td>
<td>Haemaphysalis sp. (1)</td>
<td><em>H. bispinosa</em> voucher TUTEZ-R1324 [KC853419; 233 nt/233 nt (100% identity); cattle, India]</td>
<td><em>H. bispinosa</em></td>
</tr>
<tr>
<td>• Kuala Lompat, Pahang</td>
<td>Rat</td>
<td>Haemaphysalis sp. (1)</td>
<td><em>H. bispinosa</em> voucher TUTEZ-R1324 [KC853419; 230 nt/231 nt (99% identity); cattle, India]</td>
<td></td>
</tr>
<tr>
<td>• Vegetation</td>
<td></td>
<td>Haemaphysalis sp. (2)</td>
<td><em>H. hystrix</em> [KC170733; 237 nt/239 nt (99% identity); vegetation, Thailand]</td>
<td><em>H. hystrix</em></td>
</tr>
<tr>
<td>• Sg. Deka, Terengganu</td>
<td></td>
<td>Haemaphysalis sp. (1)</td>
<td><em>H. shimoga</em> [KC170730; 239 nt/239 nt (100% identity); vegetation, Thailand]</td>
<td><em>H. shimoga</em></td>
</tr>
<tr>
<td>• Vegetation</td>
<td></td>
<td>Haemaphysalis sp. (1)</td>
<td><em>H. shimoga</em> [KC170730; 238 nt/239 nt (99% identity); vegetation, Thailand]</td>
<td></td>
</tr>
<tr>
<td>• Kuala Lompat, Pahang</td>
<td>Vegetation</td>
<td>Haemaphysalis sp. (5)</td>
<td><em>H. obesa</em> [KC170732; 223 nt/236 nt (94% identity); vegetation, Thailand]</td>
<td><em>Haemaphysalis</em></td>
</tr>
<tr>
<td>• Rat</td>
<td></td>
<td>Haemaphysalis sp. (1)</td>
<td></td>
<td>ST1</td>
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### Table 4.2, continued

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<tr>
<th>Location</th>
<th>Taxa</th>
<th>Species</th>
<th>Accession Numbers</th>
<th>Identity</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuala Lompat, Pahang</td>
<td>Vegetation</td>
<td>Haemaphysalis spp. (3)</td>
<td>H. obesa [KC170732; 222 nt /236 nt (94% identity); vegetation, Thailand]</td>
<td>Haemaphysalis ST1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Haemaphysalis sp. (1)</td>
<td>H. obesa [KC170732; 222 nt /237 nt (94% identity); vegetation, Thailand]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squirrel</td>
<td>Vegetation</td>
<td>Haemaphysalis sp. (1)</td>
<td>H. asiatica [KC170734; 223 nt/239 nt (93% identity); vegetation, Thailand]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sg. Deka, Terengganu</td>
<td>Rat</td>
<td>Dermacentor spp. (2)</td>
<td>D. atrosignatus [KC170745; 239 nt /239 nt (100% identity); wild boar, Thailand]</td>
<td>Dermacentor ST4</td>
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</tr>
<tr>
<td>Kuala Lompat, Pahang</td>
<td>Vegetation</td>
<td>Dermacentor sp. (1)</td>
<td>D. atrosignatus [KC170745; 238 nt /239 nt (99% identity); wild boar, Thailand]</td>
<td>Dermacentor ST3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Dermacentor sp. (1)</td>
<td>D. atrosignatus [KC170745; 231 nt /232 nt (99% identity); wild boar, Thailand]</td>
<td>Dermacentor ST3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vegetation</td>
<td>Dermacentor sp. (2)</td>
<td>D. atrosignatus [KC170745; 230 nt /231 nt (99% identity); wild boar, Thailand]</td>
<td>Dermacentor ST3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dermacentor spp. (11)</td>
<td>D. andersoni haplotype SSCP Van_269 [AF309032; 218 nt /233 nt (94% identity); vegetation, Canada]</td>
<td>Dermacentor ST1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Dermacentor spp. (6)</td>
<td>D. andersoni haplotype SSCP Van_269 [AF309032; 221 nt /234 nt (94% identity); vegetation, Canada]</td>
<td>Dermacentor ST2</td>
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<tr>
<td></td>
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<td>Dermacentor sp. (1)</td>
<td>D. andersoni haplotype SSCP Van_269 [AF309032; 220 nt /234 nt (94% identity); vegetation, Canada]</td>
<td>Dermacentor ST2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dermacentor sp. (3)</td>
<td>D. andersoni haplotype SSCP Van_269 [AF309032; 220 nt /235 nt (94% identity); vegetation, Canada]</td>
<td>Dermacentor ST3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dermacentor sp. (1)</td>
<td>D. nuttalli isolate XJ088 [JX051114; 222 nt/239 nt (93% identity); China]</td>
<td>Dermacentor ST3</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2, continued

<table>
<thead>
<tr>
<th>Location</th>
<th>Host</th>
<th>Species</th>
<th>GenBank Accession Numbers</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuala Lompat, Pahang</td>
<td>Vegetation</td>
<td><em>Dermacentor</em> sp. (1)</td>
<td>- <em>D. nuttalli</em> [KT764942; 210 nt/227 nt (93% identity); China]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- <em>D. silvarum</em> [KP258209; 210 nt/227 nt (93% identity); China]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Skink</td>
<td><em>Amblyomma</em> sp. (1)</td>
<td><em>A. helvolum</em> [KC170738; 239 nt/239 nt (100% identity); snake, Thailand]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vegetation</td>
<td><em>Amblyomma</em> spp. (2)</td>
<td><em>A. testudinarium</em> [KC170737; 232 nt/239 nt (97% identity); deer, Thailand]</td>
<td></td>
</tr>
<tr>
<td>Aboriginal villages</td>
<td>Chicken</td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td><em>H. bispinosa</em> voucher TUTEZ-R1324 [KC853419; 248 nt/248 nt (100% identity); cattle, India]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td><em>H. bispinosa</em> voucher TUTEZ-R1324 [KC853419; 182 nt/182 nt (100% identity); cattle, India]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td><em>Haemaphysalis</em> spp. (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td><em>H. hystricis</em> [KC170733; 232 nt/233 nt (99% identity); vegetation, Thailand]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cat</td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chicken</td>
<td><em>Haemaphysalis</em> spp. (3)</td>
<td><em>H. wellingtoni</em> isolate: HW-A [AB819221; 228 nt/228 nt (100% identity); grey bunting, Japan]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cat</td>
<td><em>Haemaphysalis</em> spp. (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td><em>H. wellingtoni</em> isolate: HW-A [AB819221; 288 nt/289 nt (99% identity); grey bunting, Japan]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td><em>Haemaphysalis</em> spp. (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td><em>H. hystricis</em> [KC170733; 234 nt/242 nt (97% identity); vegetation, Thailand]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td><em>Haemaphysalis</em> spp. (4)</td>
<td><em>H. hystricis</em> [KC170733; 234 nt/242 nt (97% identity); vegetation, Thailand]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td><em>Haemaphysalis</em> spp. (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td><em>H. wellingtoni</em> isolate: HW-A [AB819221; 288 nt/289 nt (99% identity); grey bunting, Japan]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td><em>Haemaphysalis</em> spp. (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td><em>Rhipicephalus</em> spp. (3)</td>
<td><em>R. sanguineus</em> [KC170744; 255 nt/255 nt (100% identity); dog, Thailand]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td><em>Amblyomma</em> sp. (1)</td>
<td><em>A. helvolum</em> [KC170738; 235 nt/235 nt (100% identity); snake, Thailand]</td>
<td></td>
</tr>
</tbody>
</table>
4.2.2 Ticks collected from the forest areas

4.2.2.1 Vegetation

i. *Dermacentor* spp.

Five sequence types of mitochondrial 16S rRNA amplified gene fragments were obtained from 32 representative *Dermacentor* ticks collected from vegetation. One sequence type (from seven ticks) demonstrated 99-100% sequence similarity to *D. atrosignatus* from a wild boar in Thailand (GenBank accession no.: KC170745, unpublished). Hence, these ticks were referred as *D. atrosignatus* in this study. The amplified 16S rRNA gene fragments from 23 ticks (*Dermacentor* ST1 and ST2) exhibited the highest 94% sequence similarity with *D. andersoni* from vegetation in Canada (haplotype SSCP Van_269; GenBank accession no.: AF309032) (Qiu et al., 2002). One sequence type (*Dermacentor* ST3) obtained from a single tick demonstrated the highest 93% sequence similarity with *D. nuttalli* from China (isolate XJ088; GenBank accession no.: JX051114) (Lv et al., 2014b). Additionally, the 16S rDNA sequence of a single tick showed the highest 93% sequence similarity with *D. nuttalli* (GenBank accession no.: KT764942, unpublished) and *D. silvarum* from China (GenBank accession no.: KP258209, unpublished) (*Dermacentor* ST4). Due to the low sequence similarity with known deposited tick sequences, these ticks (*Dermacentor* ST1-ST4) were referred as *Dermacentor* spp. throughout the study. The details of the BLAST results are shown in Table 4.2.

ii. *Haemaphysalis* spp.

The mitochondrial 16S rRNA gene fragments amplified from two ticks demonstrated 99% sequence similarity with *H. hystricis* from vegetation in Thailand (GenBank accession no.: KC170733, unpublished). Two ticks revealed 99-100% similarity with *H. shimoga* from vegetation in Thailand (GenBank accession no.: KC170730, unpublished). The mitochondrial 16S rRNA gene amplified from nine
representative ticks from the vegetation showed the highest 94% sequence similarity to *Haemaphysalis obesa* collected from vegetation in Thailand (GenBank accession no.: KC170732, unpublished) (*Haemaphysalis* ST1). The mitochondrial 16S rDNA sequence of a single tick demonstrated the highest 93% sequence similarity with *Haemaphysalis qinghaiensis* from China (GenBank accession no.: KJ609201, unpublished) (*Haemaphysalis* ST4). The mitochondrial 16S rDNA sequence of another tick showed the highest 93% sequence similarity to *Haemaphysalis asiatica* from vegetation in Thailand (GenBank accession no.: KC170734, unpublished) (*Haemaphysalis* ST3). Due to low sequence similarity with known tick species, these ticks (*Haemaphysalis* ST1, ST3, and ST4) are referred as *Haemaphysalis* spp. The details of the BLAST results are shown in Table 4.2.

iii. *Amblyomma* spp.

This study identified two ticks with mitochondrial 16S rDNA sequences demonstrating the highest 97% sequence similarity with those of *A. testudinarium* from deer in Thailand (GenBank accession no.: KC170737, unpublished). The details of the BLAST results are shown in Table 4.2.

**4.2.2.2 Small animals**

i. *Dermacentor* spp.

The mitochondrial 16S rRNA gene sequences amplified from three ticks infesting rats demonstrated 99-100% sequence similarity to *D. atrosignatus* from wild boar in Thailand (GenBank accession no.: KC170745, unpublished). The details of the BLAST results are shown in Table 4.2.

ii. *Haemaphysalis* spp.

The mitochondrial 16S rDNA sequences of a bat tick and a rat tick showed 99-100% sequence similarity to *H. bispinosa* collected from goats in India (voucher TUTEZ-R1324; GenBank accession no.: KC853419) (Brahma *et al.*, 2014). The mitochondrial
16S rDNA sequence of a rat tick demonstrated the highest 94% sequence similarity to *H. obesa* from vegetation in Thailand (GenBank accession no.: KC170732, unpublished) (*Haemaphysalis* ST1). A tick collected from squirrel demonstrated the highest 93% sequence similarity to *H. asiatica* from vegetation in Thailand (GenBank accession no.: KC170734, unpublished) (*Haemaphysalis* ST3). Due to the low sequence similarity to known tick species, these ticks (*Haemaphysalis* ST1 and ST3) were referred as *Haemaphysalis* spp. The details of the BLAST results are shown in Table 4.2.

iii. *Amblyomma* spp.

The mitochondrial 16S rDNA sequence of a tick collected from a skink from Kuala Lompat showed 100% sequence similarity with that of *A. helvolum* from snake in Thailand (GenBank accession no.: KC170738, unpublished). The detail of the BLAST result is shown in Table 4.2.

4.2.3 Ticks collected from peri-domestic animals in aboriginal villages

The 28S rRNA gene amplified from six pools of chicken ticks and eight pools of cat ticks was not discriminative as the sequences demonstrated the highest 99% (396 nt/399 nt) sequences similarity with several *Haemaphysalis* spp., including *H. flava*, *H. hystricis*, *Haemaphysalis sulcata*, *Haemaphysalis formosensis* and *Haemaphysalis leporispalustris* from Australia (GenBank accession nos.: JX573128-JX573130, JX573132, JX573134) (Burger et al., 2013).

Hence, most of the time, ticks were identified based on sequence analyses of the mitochondrial 16S rDNA sequences. The mitochondrial 16S rDNA sequences of seven pools of chicken ticks, four pools of cat ticks, one pool of dog ticks and one pool of goat ticks exhibited 99-100% sequences similarity to *H. wellingtoni* collected from grey bunting in Japan (isolate HWA; GenBank accession no.: AB819221) (Takano et al., 2014). The mitochondrial 16S rDNA sequences obtained from another pool of chicken ticks, one pool of goat ticks and two pools of dog ticks were 100% identical to *H.*
bispinosa collected from goats in India (voucher TUTEZ-R1324; GenBank accession no.: KC853419) (Brahma et al., 2014). While the mitochondrial 16S rRNA gene amplified from a pool of cat ticks demonstrated 99% sequence similarity to H. hystricis collected from vegetation in Thailand (GenBank accession no.: KC170733, unpublished).

Of the 13 representative mitochondrial 16S rDNA sequences obtained from 13 pools of dog ticks, five demonstrated the highest (97-99%) sequences similarity to H. hystricis collected from vegetation in Thailand (GenBank accession no.: KC170733, unpublished) (Haemaphysalis ST2). The mitochondrial 16S rDNA sequences of two pools of dog ticks showed 99% similarity to D. auratus collected from a wild boar in Thailand (GenBank accession no.: KC170746, unpublished), while the sequences obtained from two pools of dog ticks demonstrated 100% similarity to H. bispinosa collected from goats in India (voucher TUTEZ-R1324; GenBank accession no.: KC853419) (Brahma et al., 2014). Another pool of ticks demonstrated 100% identity in the 16S rDNA sequence to H. wellingtoni collected from grey bunting in Japan (isolate HW-A; GenBank accession no.: AB819221) (Takano et al., 2014). Three pools of ticks had 100% matching sequences with that of R. sanguineus collected from a dog in Thailand (GenBank accession no.: KC170744, unpublished). In addition, the mitochondrial 16S rDNA sequence of a pool of snake ticks was 100% identical to A. helvolum collected from a snake in Thailand (GenBank accession no.: KC170738, unpublished). The details of the BLAST results are shown in Table 4.2.

4.2.4 Ticks collected from other sources

The 28S rRNA gene sequences amplified from 23 dog ticks collected from dogs in Klang Valley were 100% (386 nt/386 nt) matching with R. sanguineus from USA (GenBank accession no.: AF120312) (Klompen et al., 2000) while four dog ticks were only 99% (392 nt/393 nt) matching with R. sanguineus from USA (GenBank accession no.: AF120312) (Klompen et al., 2000). Besides that, the 28S rRNA gene amplified from
a dog tick collected from Klang Valley demonstrated 100% (400 nt/400 nt) sequence similarity with several *Haemaphysalis* spp., including *H. flava*, *H. formosensis*, *H. hystricis*, *H. leporispalustris* and *H. sulcata* from Australia (GenBank accession nos.: JX573128-JX573130, JX573132, JX573134) (Burger et al., 2013).

4.3 Integrity checking of extracted samples prior to amplification for *Anaplasmataceae* DNA

i. Tick DNA samples

The integrity of the tick DNA samples used in this study were assessed using PCR assays targeting the tick mitochondrial 16S rRNA gene fragment or 28S rRNA gene fragment (refer to section 4.2). The amplification of a ~300 bp of tick 16S rRNA gene fragment (Figure 4.6) or a ~490 bp of tick 28S rRNA gene fragment (Figure 4.7) was suggestive of the absence of PCR inhibitors in a tick DNA sample.

ii. Animal blood DNA samples

The integrity of the DNA extracted from animal blood and organ samples were assessed using PCR assay targeting *cytB* gene. The amplification of a 358 bp amplicon of *cytB* gene (Figure 4.8) was suggestive of the absence of PCR inhibitors.

The *cytB* sequences were also determined for each animal species. Table 4.3 shows the results of the sequence analysis. The *cytB* sequences for all animals matched 99-100% with their reference strains except for deer (94%) and sheep (90%). This may be due to the sequences for these animals are still not available in the GenBank database.
Figure 4.6: Amplification of approximately 300 bp of the 16S rRNA gene fragments from tick samples using 16S-1/16S+2 primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-9: tick DNA samples.

Figure 4.7: Amplification of approximately 490 bp of the 28S rRNA gene fragments from tick samples using 28SF/28SR primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-7: tick DNA samples.

Figure 4.8: Amplification of approximately 358 bp of the cytB gene fragments from animal blood samples using cytBFor/cytBRev primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-8: animal blood DNA samples.
Table 4.3: Sequence analyses of the cytB sequences from one sample of each animal type.

<table>
<thead>
<tr>
<th>Animal blood sample</th>
<th>Animal with the closest sequence match, country (Genbank accession no.)</th>
<th>Percentage similarity (Nucleotide of the sample/nucleotide of reference strain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey</td>
<td><em>Macaca fascicularis</em>, Malaysia (KJ592589)</td>
<td>99% (261 nt/262 nt)</td>
</tr>
<tr>
<td>Buffalo</td>
<td><em>Bubalus bubalis</em>, Japan (AB529514)</td>
<td>100% (273 nt/273 nt)</td>
</tr>
<tr>
<td>Cow</td>
<td><em>Bos indicus</em>, Malaysia (GU367347)</td>
<td>100% (258 nt/258 nt)</td>
</tr>
<tr>
<td>Deer</td>
<td><em>Cervus unicolour</em>, India (EU882027)</td>
<td>94% (238 nt/253 nt)</td>
</tr>
<tr>
<td>Horse</td>
<td><em>Equus caballus</em>, USA (AY819737)</td>
<td>100% (227 nt/227 nt)</td>
</tr>
<tr>
<td>Dog</td>
<td><em>Canis lupus familiaris</em>, Kenya (KP858493)</td>
<td>100% (244 nt/244 nt)</td>
</tr>
<tr>
<td>Rat</td>
<td><em>Rattus rattus</em>, Malaysia (JF437010)</td>
<td>100% (271 nt/271 nt)</td>
</tr>
<tr>
<td>Goat</td>
<td><em>Capra hircus</em>, Switzerland (DQ514548)</td>
<td>100% (249 nt/249 nt)</td>
</tr>
<tr>
<td>Pangolin</td>
<td><em>Manis javanica</em>, South-East Asia (KP261030)</td>
<td>100% (247 nt/247 nt)</td>
</tr>
<tr>
<td>Sheep</td>
<td><em>Ovis aries</em>, Japan (AB006800)</td>
<td>90% (292 nt/323 nt)</td>
</tr>
</tbody>
</table>

4.4 Seroprevalence of selective tick-borne pathogens in dog blood samples

A total of 17 (39.5%) of the 43 dog blood samples, of which 14 (46.7%) from Klang Valley and three (23.1%) obtained from VRI, Ipoh, were serologically positive for *E. canis*. *A. phagocytophilum* antibodies were only detected from four (9.3%) dogs from Klang Valley. Antibodies to both *A. phagocytophilum* and *E. canis* were detected in four (9.3%) dogs from Klang Valley. None of the dogs were seropositive to *B. burgdorferi* and *D. immitis*.

4.5 Identification of *Anaplasma* spp. and *Ehrlichia* spp. from ticks using molecular methods

4.5.1 Preliminary screening

Tick samples were subjected to preliminary screening by using a PCR assay targeting a short fragment (345 bp) of the 16S rRNA gene of *Anaplasmataceae* (Figure 4.9). A high detection rate (61.5%) was obtained from ticks collected from livestock farms, whereby 166 ticks out of 270 ticks (including 103 *H. bispinosa* from cattle; 22 *H. bispinosa* from sheep and 41 *R. microplus* from cattle) collected from five farms (four cattle and one sheep) were positive.
A total of 40 (28.8%) of 139 ticks (including 18 Dermacentor spp., two D. atrosignatus, nine Haemaphysalis spp., two H. hystricis, one H. shimoga and two Amblyomma spp. from vegetation; two Dermacentor spp., one D. atrosignatus and three Haemaphysalis spp. from small animals) from the forest areas were positive for Anaplasmataceae DNA.

Of the ticks collected from 12 aboriginal villages, 37.0% (24 pools/65 pools) of the ticks were positive for Anaplasmataceae DNA. Positive ticks were derived from 13 pools of Haemaphysalis spp. from cats, chickens and dogs; five pools of H. wellingtoni from chickens, cat and goat; three pools of H. bispinosa from goats and chicken; two pools of R. sanguineus from dogs and one pool of R. microplus from a cow. Of the 21 ticks collected from snakes in Sepang, Selangor, 15 snake ticks (nine A. varanense and six A. helvolum) were positive for Anaplasmataceae DNA.

BLAST analyses were performed on 53 sequences successfully obtained from 19 H. bispinosa (18 individuals and one pool), nine Haemaphysalis spp. (eight individuals and one pool), one H. hystricis, five Dermacentor spp., two D. atrosignatus, six A. varanense, three A. helvolum, seven R. microplus and one pool of R. sanguineus. BLAST analyses reveal the presence of A. marginale, A. bovis (two sequence variants), A. platys, A. phagocytophilum (two sequence variants), Anaplasma spp. (two sequence variants) and Ehrlichia spp. (three sequence variants). The results of the BLAST analyses are shown in Table 4.4.

Fifteen representative sequences were selected to build a dendrogram (Figure 4.10). Phylogenetic analysis reveals the clustering of A. platys detected from ticks in this study with A. platys detected in dog from a previous study in Malaysia (strain Sarawak 41 UPM; GenBank accession no.: KU500914; unpublished) and Thailand (GenBank accession no.: EF139459) (Pinyoowong et al., 2008). The A. phagocytophilum identified in this study was placed in the same group with A. phagocytophilum detected in a mouse’s...
spleen from Korea (strain AAIK4; GenBank accession no.: KR611719; unpublished) and
*D. silvarum* from China (strain Jilin 5; GenBank accession no.: DQ449948) (Cao *et al.*, 2006).

![Figure 4.9: Amplification of approximately 345 bp of the 16S rRNA gene fragments from samples using EHR16SD/EHR16SR primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-7: EHRPCR-positive samples; 8: positive control (cloned plasmid contain *A. bovis*).](image)

Phylogenetic analysis also reveal the presence of two clusters of *A. bovis*, of which one is grouped with *H. longicornis* from Japan (strain NR07; GenBank accession no.: AB196475) (Kawahara *et al.*, 2006) and *A. bovis* detected in a wild boar from Malaysia (this study, section 4.6.2.1) while another cluster is grouped with *A. bovis* detected in a raccoon from Japan (strain raccoon499; GenBank accession no.: GU937020) (Sashika *et al.*, 2011) and *A. bovis* detected in monkeys from Malaysia (this study, section 4.6.2.1). The short sequences of *A. marginale* detected in cattle ticks are clustered in one group with *A. marginale* from Philippines (isolate C6A; GenBank accession no.: JQ839012) (Ybañez *et al.*, 2013b), *A. centrale* strain vaccine (GenBank accession no.: AF318944) (Bekker *et al.*, 2002) and *A. ovis* from South Africa (isolate OVI; GenBank accession no.: AF414870) (Lew *et al.*, 2003). The *Anaplasma* sp. detected from a snake tick is grouped with an *Anaplasma* sp. most closely related to *A. phagocytophilum*, which has been reported from a dog from South Africa (isolate 1076; GenBank accession no.: AY570539) (Inokuma *et al.*, 2005).
Table 4.4: BLAST analyses of 53 short 16S rRNA gene sequences amplified from ticks from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Animal host</th>
<th>Tick species (n)</th>
<th>BLAST result (nearest match)</th>
<th>Representative samples for construction of dendrogram</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Livestock farms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jelai Gemas, Negeri Sembilan</td>
<td>Cattle</td>
<td><em>H. bispinosa</em> (6)</td>
<td><em>A. marginale</em> strain Uganda MT27 [KU686794; 234 nt/234 nt (100% identity); cattle, Uganda]</td>
<td>• X102-1 (KY046278)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• EKY4766-F (KY046277)</td>
</tr>
<tr>
<td>Jelai Gemas, Negeri Sembilan</td>
<td>Cattle</td>
<td><em>H. bispinosa</em> (1)</td>
<td><em>A. bovis</em> clone 85 [KM114612; 241 nt/241 nt (100% identity); monkey, Malaysia] (A. bovis type I)</td>
<td></td>
</tr>
<tr>
<td>Kuala Berang, Terengganu</td>
<td>Sheep</td>
<td><em>H. bispinosa</em> (2)</td>
<td><em>Anaplasma</em> sp. clone WBM1 [KU189194; 243 nt/243 nt (100% identity); wild boar, Malaysia] (A. bovis type II)</td>
<td>• KM40-F2 (KY046276)</td>
</tr>
<tr>
<td>Pokok Sena, Kedah</td>
<td>Cattle</td>
<td><em>H. bispinosa</em> (1)</td>
<td><em>A. platys</em> strain Sarawak41 UPM (KU500914; 237 nt/237 nt (100% identity); dog, Malaysia)</td>
<td></td>
</tr>
<tr>
<td>Pokok Sena, Kedah</td>
<td>Sheep</td>
<td><em>H. bispinosa</em> (1)</td>
<td><em>A. phagocytophilum</em> isolate HB-G5-goat-China [KR002112; 238 nt/239 nt (99% identity); goat, China]</td>
<td>• WYY1292 (KY046285)</td>
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<tr>
<td></td>
<td>Cattle</td>
<td><em>R. microplus</em> (2)</td>
<td><em>Ehrlichia</em> sp. clone 2-3 [HM486685; 243 nt/243 nt (100% identity); cattle, Canada]</td>
<td>• VKAA024-F (KY046290)</td>
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<tr>
<td></td>
<td>Cattle</td>
<td><em>R. microplus</em> (1)</td>
<td><em>Ehrlichia</em> sp. isolate BL157-9 [KJ410257; 240 nt/240 nt (100% identity); <em>H. asiaticum</em>, China]</td>
<td>• UN2-100-F (KY046291)</td>
</tr>
<tr>
<td>Jelai Gemas, Negeri Sembilan</td>
<td>Cattle</td>
<td><em>H. bispinosa</em> (2)</td>
<td><em>Ehrlichia</em> sp. isolate TC251-2 [KJ410253; 243 nt/243 nt (100% identity); <em>D. nuttalli</em>, China]</td>
<td>• UN2-19-M (KY046295)</td>
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<tr>
<td>Jelai Gemas, Negeri Sembilan</td>
<td>Cattle</td>
<td><em>H. bispinosa</em> (1)</td>
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<td></td>
</tr>
<tr>
<td>Ulu Lepar, Pahang</td>
<td>Cattle</td>
<td><em>R. microplus</em> (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jelai Gemas, Negeri Sembilan</td>
<td>Cattle</td>
<td><em>H. bispinosa</em> (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulu Lepar, Pahang</td>
<td>Cattle</td>
<td><em>H. bispinosa</em> (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forest areas</td>
<td>Vegetation</td>
<td>A. platys strain Sarawak41 UPM (KU500914; 237 nt/237 nt (100% identity); dog, Malaysia)</td>
<td>• KLV077 (KY046283)</td>
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<tr>
<td>Kuala Lompat, Pahang</td>
<td>D. atrosignatus (1)</td>
<td>Haemaphysalis spp. (2) H. hystricus (1)</td>
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<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Haemaphysalis sp. (1)</td>
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<td></td>
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</tr>
<tr>
<td>Vegetation</td>
<td>Dermacentor spp. (5)</td>
<td>A. phagocytophilum strain AAIK4 [KR611719; 238 nt/238 nt (100% identity); mouse spleen, South Korea]</td>
<td>• KLV086</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. atrosignatus (1)</td>
<td>Haemaphysalis spp. (5)</td>
<td></td>
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</tr>
<tr>
<td>Aboriginal villages</td>
<td>Dog</td>
<td>R. sanguineus (1 pool)</td>
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<tr>
<td>Negeri Sembilan</td>
<td>Goat</td>
<td>H. bispinosa (1 pool)</td>
<td></td>
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<tr>
<td></td>
<td>Cat</td>
<td>Haemaphysalis sp. (1 pool)</td>
<td>• SP002-F (KY046288)</td>
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<tr>
<td>Perak</td>
<td>Snake</td>
<td>Anaplasma sp. isolate 1076 [AY570539; 225 nt/225 nt (100% identity); dog, South Africa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td>A. varanense (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sepang, Selangor</td>
<td></td>
<td>A. varanense (2) A. phagocytophilum strain AAIK4 [KR6111719; 238 nt/238 nt (100% identity); mouse spleen, South Korea]</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>A. helvolum (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. helvolum (1)</td>
<td>• S4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anaplasma sp. isolate 1076 [AY570539; 225 nt/225 nt (100% identity); dog, South Africa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ehrlichia sp. isolate BL157-9 [KJ410257; 249 nt/250 nt (99% identity); H. asiaticum, China]</td>
<td>• S3</td>
<td></td>
</tr>
</tbody>
</table>
Phylogenetic analysis reveals the clustering of *Ehrlichia* sp. investigated in this study to three groups (Figure 4.10). One group (represented by one tick sample) is closely related to *Ehrlichia* sp. strain EBm52 from Thailand (GenBank accession no.: AF497581) (Parola *et al*., 2003). The other group (represented by one tick sample) is clustered with *Ehrlichia* sp. TC251-2 isolate from China (GenBank accession no.: KJ410253) (Kang *et al*., 2014) and *Ehrlichia* sp. clone 2-3 from Canada (GenBank accession no.: HM486685) (Gajadhar *et al*., 2010) (Figure 4.10). Sequences obtained from two tick samples [SP002-F and S3] are not clustered with any reference sequences deposited in GenBank, suggesting the existence of potential new strains of *Anaplasmataceae* in ticks investigated in this study.
Figure 4.10: Phylogenetic relationships among various *Anaplasma* spp. and *Ehrlichia* spp. based on partial sequences of the 16S rRNA gene (222 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. *R. rickettsii* (U11021) was used as an outgroup.

*: Representative sequences amplified from ticks in this study.
Further characterisation of *Anaplasma* spp. and *Ehrlichia* spp.

Amplification of nearly full length 16S rRNA gene of *Anaplasma* spp. and *Ehrlichia* spp. from ticks

*Anaplasma* or *Ehrlichia*-positive tick samples were subjected to further amplification to obtain the full length sequences of 16S rRNA gene for analysis. The samples included 118 ticks [85 ticks (57 *H. bispinosa* and 28 *R. microplus*) from livestock farms, 21 ticks (eight *Dermacentor* spp., two *D. atrosignatus*, nine *Haemaphysalis* spp., one *H. hystricis* and one *Amblyomma* sp.) collected from the forest areas, 11 pools of ticks (four pools of *H. wellingtoni*, three pools of *H. bispinosa*, one pool of *Haemaphysalis* spp., two pools of *R. sanguineus* and one pool of *R. microplus*) from aboriginal villages and a snake tick (*A. varanense*) from Sepang, Selangor].

DNA fragments of approximately 1500 bp were obtained from amplification using either primer pairs of fD1/RP2 or ATT062F/ATT062R or fD1/EHR16SR paired with Rp2/EHR16SD in this study (Figure 4.11). The amplicons were successfully obtained from 46 (38.0%) tick samples [(including 13 *R. microplus* from cattle, six *H. bispinosa* from sheep and 16 *H. bispinosa* from cattle) ticks from livestock farms, nine questing ticks (three *Haemaphysalis* spp., one *H. hystricis* and five *Dermacentor* spp.) collected from the forest areas, one pool of ticks (*H. bispinosa* from chicken) from aboriginal village and a snake tick (*A. varanense*) from Sepang, Selangor].

A total of 17 amplicons were selected for sequence determination, of which only ten sequences were successfully obtained. These amplicons were derived from four *R. microplus*, two *H. bispinosa*, one *Haemaphysalis* sp., one *H. hystricis*, one *A. varanense* and one *Dermacentor* sp. BLAST analyses reveal the identification of *Ehrlichia* spp. (in five ticks), *A. marginale* (in one tick), *Candidatus* E. shimanensis (in one tick) and *A. bovis* (in three ticks). The results of the BLAST analyses are shown in Table 4.5.
Figure 4.11: Amplification of approximately 1500 bp of the 16S rRNA gene fragments from samples using fD1/Rp2 primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-5: fD1/Rp2-positive samples; 6: positive control (cloned plasmid contain *A. bovis*).

A phylogenetic tree was constructed to show the genetic relationship between the *Anaplasma* spp. and *Ehrlichia* spp., using sequences derived in this investigation and those sequences of the reference strains (Figure 4.12). The analysis exhibits that *A. marginale* detected in ticks in this study were clustered with *A. marginale* reported from *R. microplus* from Philippines (isolate C6A; GenBank accession no.: JQ839012) (Ybañez *et al.*, 2013b) and cattle from China (isolate ZJ02/2009; GenBank accession no.: HM439433; unpublished). The phylogenetic analysis in this study also reveals the clustering of *Ehrlichia* spp. in cattle ticks with *Candidatus* E. shimanensis from Japan (GenBank accession no.: AB074459) (Kawahara *et al.*, 2006); *Ehrlichia* sp. from Japan (isolate Yonaguni138; GenBank accession no.: HQ697588) (Matsumoto *et al.*, 2011) and *Ehrlichia* sp. from Brazil (*E. mineirensis*) (strain UFMG-EV; GenBank accession no.: JX629805) (Cabezas-Cruz *et al.*, 2012). Additionally, the *Ehrlichia* sp. from a *R. microplus* tick (collected from a cow) is clustered with a few *Ehrlichia* spp. including *Ehrlichia* sp. from Thailand (strain EBm52; GenBank accession no.: AF497581) (Parola *et al.*, 2003); *Ehrlichia* sp. from China (Fujian; GenBank accession no.: DQ324547; unpublished) and *Ehrlichia* sp. from China (isolate BL157-9; GenBank accession no.: KJ410257) (Kang *et al.*, 2014). The 16S rRNA gene sequence obtained from a snake tick...
Table 4.5: BLAST analyses of 23 nearly full length 16S rRNA, *gltA* and *groEL* gene sequences amplified from ticks from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Animal host</th>
<th>Tick species (n)</th>
<th>Gene used</th>
<th>BLAST result (nearest match)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Livestock farms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Jerantut, Pahang</td>
<td>Cattle</td>
<td><em>R. microplus</em> (2)</td>
<td>16S rRNA</td>
<td><em>Ehrlichia</em> sp. strain UFMG-EV [JX629805; 1294 nt/1308 nt (99% identity); <em>R. microplus</em>, Brazil]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>gltA</em> (only one tick)</td>
<td><em>Ehrlichia</em> sp. strain UFMG-EV [JX629807; 544 nt/545 nt (99% identity); <em>R. microplus</em>, Brazil]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>groEL</em></td>
<td><em>Ehrlichia</em> sp. strain UFMG-EV [JX629806; 1207 nt/1223 nt (99% identity); <em>R. microplus</em>, Brazil]</td>
</tr>
<tr>
<td>• Ulu Lepar, Pahang</td>
<td><em>H. bispinosa</em> (1)</td>
<td>- H5T056-F</td>
<td>16S rRNA</td>
<td><em>Ehrlichia</em> sp. Tibet [AF414399; 1457 nt/1469 nt (99% identity); <em>R. microplus</em>, China]</td>
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<tr>
<td></td>
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<td></td>
<td><em>gltA</em></td>
<td><em>Ehrlichia</em> sp. clone SY36 [KF728364; 497 nt/544 nt (91% identity); <em>H. longicornis</em>, China]</td>
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<tr>
<td></td>
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<td></td>
<td><em>groEL</em></td>
<td><em>Candidatus</em> E. shimanensis [AB074462; 933 nt/992 nt (94% identity); <em>H. longicornis</em>, Japan]</td>
</tr>
<tr>
<td>• Ulu Lepar, Pahang</td>
<td><em>R. microplus</em> (1)</td>
<td>- UN2-100-F</td>
<td>16S rRNA</td>
<td><em>Ehrlichia</em> sp. clone EBm52 [AF497581; 1335 nt/1335 nt (100% identity); <em>R. microplus</em>, Thailand]</td>
</tr>
<tr>
<td></td>
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<td></td>
<td><em>gltA</em></td>
<td><em>Ehrlichia</em> sp. isolate BL157-9(a) [KJ410273; 545 nt/545 nt (100% identity); <em>H. asiaticum</em>, China]</td>
</tr>
<tr>
<td>• Ulu Lepar, Pahang</td>
<td><em>R. microplus</em> (1)</td>
<td>- UN2-100-F</td>
<td><em>groEL</em></td>
<td><em>Ehrlichia</em> sp. clone Tajikistan-1 [KJ930191; 1249 nt/1254 nt (99% identity); <em>H. anatolicum</em>, Tajikistan]</td>
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<td>- UN2-19-M</td>
<td>16S rRNA</td>
<td><em>Candidatus</em> E. shimanensis [AB074459; 1326 nt/1326 nt (100% identity); <em>H. longicornis</em>, Japan]</td>
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<td></td>
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<td><em>gltA</em></td>
<td><em>Ehrlichia</em> sp. clone SY130 [KF728365; 517 nt/537 nt (96% identity); <em>H. longicornis</em>, China]</td>
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<td></td>
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<td></td>
<td><em>groEL</em></td>
<td><em>Candidatus</em> E. shimanensis [AB074462; 953 nt/1013 nt (94% identity); <em>H. longicornis</em>, Japan]</td>
</tr>
<tr>
<td>Location</td>
<td>Species</td>
<td>16S rRNA</td>
<td>Sequence Information</td>
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</tr>
<tr>
<td>Kuala Berang, Terengganu</td>
<td><em>R. microplus</em> (1)</td>
<td>16S rRNA</td>
<td>A. <em>marginale</em> strain Uganda MT40 [KU686785; 1364 nt/1366 nt (100% identity); cattle, Uganda]</td>
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<td></td>
<td><strong>gltA</strong></td>
<td>A. <em>marginale</em> strain Dawn [CP006847; 542 nt/542 nt (100% identity); cattle, USA]</td>
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<td><strong>groEL</strong></td>
<td><em>Ehrlichia</em> sp. isolate Yonaguni206 [HQ697591; 1143 nt/1187 nt (96% identity); <em>H. longicornis</em>, Japan]</td>
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<tr>
<td><strong>H. bispinosa</strong> (1)</td>
<td></td>
<td><strong>16S rRNA</strong></td>
<td>Noisy data (not able to analyse)</td>
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<td>- 2023-M1</td>
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<td><strong>gltA</strong></td>
<td><em>Ehrlichia</em> sp. clone SY130 [KF728365; 516 nt/534 nt (96% identity); <em>H. longicornis</em>, China]</td>
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<td><strong>groEL</strong></td>
<td><em>Candidatus</em> E. shimanensis [AB074462; 1129 nt/1196 nt (94% identity); <em>H. longicornis</em>, Japan]</td>
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<tr>
<td>Forest areas</td>
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<tr>
<td>Kuala Lompat, Pahang</td>
<td><em>Dermacentor</em> sp. (1)</td>
<td>16S rRNA</td>
<td>A. <em>bovis</em> clone 85 [KM114612; 934 nt/934 nt (100% identity); monkey, Malaysia] (A. <em>bovis</em> type I)</td>
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<td>- KL086</td>
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<tr>
<td></td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td>16S rRNA</td>
<td>A. <em>bovis</em> clone 85 [KM114612; 1008 nt/1010 nt (99% identity); monkey, Malaysia] (A. <em>bovis</em> type I)</td>
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<td>- KL032</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>H. hystrix</em> (1)</td>
<td>16S rRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- KL023</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td><em>A. varanense</em> (1)</td>
<td>16S rRNA</td>
<td>E. <em>ruminantium</em> isolate Ball3 [U03777; 1303 nt/1325 nt (98% identity), South Africa]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- S3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
is most genetically (98% sequence similarity) related to that of *E. ruminantium* (isolate Ball3; GenBank accession no.: U03777) (Allsopp *et al.*, 1996), as shown in Figure 4.12.

![Phylogenetic relationships among various *Anaplasma* spp. and *Ehrlichia* spp. based on partial sequences of the 16S rRNA gene (887 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. *R. rickettsii* (U11021) was used as an outgroup. *: Representative sequences amplified from ticks in this study.](image-url)
4.5.2.2 Amplification of gltA gene of anaplasma and ehrlichiae

The same 85 ticks (57 *H. bispinosa* and 28 *R. microplus*) from livestock farms (cattle and sheep) as mentioned in section 4.5.2.1 were further subjected to amplification of gltA gene in this study. Amplicons of approximately 641 bp were obtained by using primer pair EHR-CS136F/EHR-CS778R (Figure 4.13). Only 32.9% (14 *R. microplus* and *H. bispinosa*, respectively) ticks were positive for gltA-PCR assays. The gltA sequences were successfully obtained from three *R. microplus* and *H. bispinosa*, respectively. BLAST analyses reveal the presence of *Ehrlichia* spp. (in five ticks) and *A. marginale* (in one tick). The results of the BLAST analyses are shown in Table 4.5.

All the six sequences were used to build a phylogenetic tree (Figure 4.14), together with sequences from the reference strains. The analysis shows the clustering of *A. marginale* detected in this study with that of *A. marginale* from USA (strain Dawn; GenBank accession no.: CP006847) (Aguilar Pierlé et al., 2014). The five *Ehrlichia* spp. were clustered separately with those of *Ehrlichia* sp. clone SY130 from China (GenBank accession no.: KF728365) (Dong et al., 2014); *Ehrlichia* sp. strain UFMG-EV (*E. mineirensis*) from Brazil (GenBank accession no.: JX629807) (Cabezas-Cruz et al., 2012); *Ehrlichia* sp. isolate BL157-9(a) from China (GenBank accession no.: KJ410273) (Kang et al., 2014) and *Ehrlichia* sp. clone SY36 from China (GenBank accession no.: KF728364) (Dong et al., 2014), respectively.
Figure 4.13: Amplification of approximately 641 bp of the gltA gene fragments from samples using EHR-CS136F/EHR-CS778R primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-5: EHR-CS136F/EHR-CS778R-positive samples; 6: Positive control (A. phagocytophylum genomic DNA extracted from IFA slides); 7: Positive control (E. chaffeensis genomic DNA extracted from IFA slides).

Figure 4.14: Phylogenetic relationships among various Anaplasma spp. and Ehrlichia spp. based on partial sequences of the gltA gene (533 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. Neorickettsia risticii (AF304147) was used as an outgroup.
* : Representative sequences amplified from ticks in this study.
4.5.2.3 Amplification of groEL gene of anaplasma and ehrlichiae

The same 85 ticks (57 H. bispinosa and 28 R. microplus) from livestock farms as mentioned in section 4.5.2.1 were further subjected to amplification of groEL gene using established primers. Approximately 1320 bp amplicons were obtained by using nested amplification primer pair HS3-f/HSV (Figure 4.15). A total of 20 ticks (23.5%; including 11 R. microplus and nine H. bispinosa) were positive for groEL-PCR assays. As direct sequencing of the amplicons produced noisy sequence data, seven randomly selected amplicons were cloned into pCR4-TOPO vector (Invitrogen, CA, USA) and sequenced. BLAST analyses of seven groEL sequences obtained from four R. microplus and three H. bispinosa ticks confirms the identification of Ehrlichia spp. in all seven ticks in this study. The results of the BLAST analyses are shown in Table 4.5.

All the seven groEL sequences were used to build a phylogenetic tree (Figure 4.16). Phylogenetic analysis shows the clustering of Ehrlichia spp. detected in this study with those of Ehrlichia sp. isolate Yonaguni206 from Japan (GenBank accession no.: HQ697591) (Matsumoto et al., 2011); Ehrlichia sp. strain LCT20 from China (GenBank accession no.: KF977220; unpublished); Candidatus E. shimanensis from Japan (GenBank accession no.: AB074462) (Kawahara et al., 2006) and Ehrlichia sp. strain UFMG-EV (E. mineirensis) from Brazil (Genbank accession no.: JX629806) (Cabezas-Cruz et al., 2012), respectively.
Figure 4.15: Amplification of approximately 1320 bp of the \textit{groEL} gene fragments from samples using HS3-f/HSVR nested amplification primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-3: HS3-f/HSVR-positive samples; 4: Positive control (\textit{A. phagocytophilum} genomic DNA extracted from IFA slides); 5: Positive control (\textit{E. chaffeensis} genomic DNA extracted from IFA slides).

Figure 4.16: Phylogenetic relationships among various \textit{Ehrlichia} spp. based on partial sequences of the \textit{groEL} gene (943 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. \textit{R. rickettsii} (CP003318) was used as an outgroup.

*: Representative sequences amplified from ticks in this study.
4.5.2.4 Molecular characterisation of *A. phagocytophilum* based on three genes

(*msp4*, 16S rRNA and *groEL*)

Nested PCR assays targeting on three *A. phagocytophilum*-genes (16S rRNA, *msp4* and *groEL*) were used for detection of *A. phagocytophilum* in this study. A total of 46 ticks [27 ticks from the forest areas (one *Amblyomma* sp., eight *Haemaphysalis* spp., two *H. hystricis*, one *H. shimoga*, 13 *Dermacentor* spp., and two *D. atrosignatus*) and 19 ticks from aboriginal villages (one *R. microplus*, two *R. sanguineus*, eight *Haemaphysalis* spp., five *H. wellingtoni* and three *H. bispinosa*)] were subjected to amplification of *msp4* gene of *A. phagocytophilum*. The nested amplification produced 343 bp amplicon by using primer pair *msp4f/*msp4r (Figure 4.17). A total of 17 (37.0%) ticks (including two *Dermacentor* spp. and two *D. atrosignatus* from the forest areas; one *R. microplus*, two *H. bispinosa*, five *H. wellingtoni* and five *Haemaphysalis* spp. from aboriginal villages) were successfully amplified. BLAST analyses from seven representative sequences show the presence of two sequence variants of *A. phagocytophilum*. The results of the BLAST analyses are shown in Table 4.6.

All the seven sequences were used to build a phylogenetic tree, as shown in Figure 4.18. Phylogenetic analysis reveals the differentiation of *A. phagocytophilum* into two groups. One group was clustered with *A. phagocytophilum* detected in patient from USA (strain Webster; GenBank accession no.: EU857674; unpublished) and *A. phagocytophilum* detected in ticks from France (clone cam3, GenBank accession no.: JX197225; clone cam5, GenBank accession no.: JX197227) (Chastagner et al., 2013). Another group is clustered with *A. phagocytophilum* detected in a rat from China (strain ZJ-China; GenBank accession no.: EU008082) (Zhan et al., 2008).

Of 13 ticks (six *Dermacentor* spp., four *Haemaphysalis* spp. and one *H. shimoga* from the forest areas; one *H. bispinosa* and one *R. sanguineus* from aboriginal villages) subjected to amplification targeting 16S rRNA gene using nested PCR assay, only three
ticks (two *Haemaphysalis* spp. and one *Dermacentor* sp.) were positive for *A. phagocytophilum*. However, sequence determination of the 16S rRNA partial gene fragment of *A. phagocytophilum*-positive samples was not successful due to noisy data generated from the sequencing. All the 13 ticks were negative for the *groEL*-PCR assay.

![Amplification of approximately 343 bp of the msp4 gene fragments of *A. phagocytophilum* from samples using msp4f/msp4r nested amplification primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-5: msp4f/msp4r-positive samples; 6: Positive control (*A. phagocytophilum* genomic DNA extracted from IFA slides).]
Table 4.6: BLAST analyses of the partial 16S rRNA gene sequences of *A. bovis*, *E. chaffeensis* and the *msp4* gene sequences of *A. phagocytophilum* amplified from ticks from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Animal host</th>
<th>Tick species (n)</th>
<th>Gene used</th>
<th>BLAST result (nearest match)</th>
<th>Representative samples for construction of dendrogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forest areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Kuala Lompat, Pahang</td>
<td>Vegetation</td>
<td><em>D. atrosignatus</em> (2)</td>
<td><em>msp4</em></td>
<td><em>A. phagocytophilum</em> clone cam5 [JX197227; 258 nt/258 nt (100% identities); <em>R. bursa</em>, France]</td>
<td>• KLV077 • KLV046 • KLV039</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Dermacentor</em> sp. (1)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td>16S rRNA</td>
<td><em>A. bovis</em> clone 115 [KM114613; 450 nt/450 nt (100% identity); <em>monkey</em>, Malaysia (<em>A. bovis</em> type I)]</td>
<td>• KL004</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Dermacentor</em> sp. (1)</td>
<td></td>
<td><em>A. bovis</em> strain SG175_HL 16S [EU181142; 450 nt/450 nt (100% identity); <em>H. longicornis</em>, South Korea]</td>
<td>• T020</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td></td>
<td><em>E. chaffeensis</em> isolate ES 113 [KM009066; 261 nt/261 nt (100% identity), <em>R. microplus</em>, Colombia]</td>
<td>• KLV004</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. atrosignatus</em> (1)</td>
<td></td>
<td><em>E. ewingii</em> isolate PB91 [KM009067; 261 nt/261 nt (100% identity), <em>R. microplus</em>, Colombia]</td>
<td>• KLV046</td>
</tr>
<tr>
<td>Aboriginal villages</td>
<td>Chicken</td>
<td><em>Haemaphysalis</em> spp. (2)</td>
<td><em>msp4</em></td>
<td><em>A. phagocytophilum</em> clone cam5 [JX197227; 258 nt/258 nt (100% identities); <em>R. bursa</em>, France]</td>
<td>• SW003 • SW004-C • UK020</td>
</tr>
<tr>
<td>• Kelantan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Negeri Sembilan</td>
<td>Goat</td>
<td><em>H. wellingtoni</em> (1)</td>
<td></td>
<td></td>
<td>• SW003</td>
</tr>
<tr>
<td>• Perak</td>
<td></td>
<td><em>Haemaphysalis</em> sp. (1)</td>
<td></td>
<td><em>A. phagocytophilum</em> strain ZJ-China [EU008082; 272 nt/273 nt (99% identities); rat, China]</td>
<td>• SP002-F</td>
</tr>
</tbody>
</table>
Figure 4.18: Phylogenetic relationships among various *A. phagocytophilum* based on partial sequences of the *msp4* gene (252 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates.

* : Representative sequences amplified from ticks in this study.

4.5.2.5 Molecular characterisation of *A. bovis* in tick samples

The DNA of 13 ticks (including six *Dermacentor* spp. and five *Haemaphysalis* spp. from the forest areas, one *H. bispinosa* and one *R. sanguineus* from aboriginal villages) were subjected to amplification using nested PCR assays targeting on 16S rRNA gene of *A. bovis*. The nested amplification produced a 551 bp DNA fragment, by using primer pair AB1f/AB1r (Figure 4.19). Five ticks (three *Haemaphysalis* spp. and two *Dermacentor* spp.) were positive while all the two ticks from aboriginal villages were negative for the nested PCR. BLAST analyses of two amplified 16S rDNA sequences
reveal the presence of two genetic variants of *A. bovis*. The results of the BLAST analyses are shown in Table 4.6. All the two sequences were used to build a phylogenetic tree (Figure 4.20). Phylogenetic analysis shows the separation of the sequences, with one grouped with *A. bovis* detected in monkey from Malaysia (this study, section 4.6.2.1, GenBank accession no.: KM114613) and another sequence grouped with *A. bovis* detected in a wild boar in Malaysia (this study, section 4.6.2.1, GenBank accession no.: KU189194) and *A. bovis* detected in a *H. longicornis* tick in Korea (strain SG175_HL; GenBank accession no.: EU181142) (Lee and Chae, 2010).

Figure 4.19: Amplification of approximately 551 bp of the 16S rRNA gene fragments of *A. bovis* from samples using AB1f/AB1r nested amplification primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-5: AB1f/AB1r-positive samples; 6: Positive control.
4.5.2.6 Molecular characterisation of E. chaffeensis in tick samples

A total of 51 tick DNA samples (11 Haemaphysalis spp., two H. hystricis, one H. shimoga, 15 Dermacentor spp., three D. atrosignatus and one Amblyomma sp. from the forest areas; eight Haemaphysalis spp., four H. wellingtoni, three H. bispinosa, two R. sanguineus and one R. microplus from aboriginal villages) were subjected to amplification by using a nested PCR assay targeting the 16S rRNA gene of E. chaffeensis (Figure 4.21). A total of 15 ticks were positive, with the generation of a DNA fragment of 389 bp. Amplified fragments were derived from one R. sanguineus tick from an aboriginal village; five Dermacentor spp., two D. atrosignatus, six Haemaphysalis spp. and one Amblyomma sp. from the forest areas.
BLAST analyses of two representative sequences reveal high similarity (100%, 261 nt/261 nt) to those of the reference strains of *E. chaffeensis* detected in *R. microplus* from Colombia (isolate ES 113; GenBank accession no.: KM009066) (Miranda and Mattar, 2015) and *E. ewingii* detected in *R. microplus* from Colombia (isolate PB91; GenBank accession no.: KM009067) (Miranda and Mattar, 2015) (Table 4.6). The phylogenetic tree (Figure 4.22) shows the grouping of the sequences with those of *Ehrlichia* sp. detected in *Hyalomma anatolicum* from Tajikistan (clone Tajikistan-5; GenBank accession no.: KP059122; unpublished), and *R. microplus* from Thailand (strain EBm52; GenBank accession no.: AF497581) (Parola *et al*., 2003) and Tibet, China (strain Tibet; GenBank accession: AF414399) (Wen *et al*., 2002).

Figure 4.21: Amplification of approximately 389 bp of the 16S rRNA gene fragments of *E. chaffeensis* from samples using HE1/HE3 nested amplification primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-5: HE1/HE3-positive samples; 6: Positive control (*E. chaffeensis* genomic DNA extracted from IFA slides).
Figure 4.22: Phylogenetic relationships among various *E. chaffeensis* based on partial sequences of the 16S rRNA gene (261 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. *R. rickettsii* (U11021) was used as an outgroup. *: Representative sequences amplified from ticks in this study.

4.6 Identification of *Anaplasmataceae* from animal samples using molecular methods

4.6.1 Preliminary screening

DNA extracts from animal samples (in bloods, organ samples and FTA cards) were subjected to preliminary screening by using PCR assays targeting a short fragment (345 bp) of the 16S rRNA gene of *Anaplasmataceae* (Figure 4.9). High detection rates of *Anaplasmataceae* DNA were obtained in cattle (136/224, 60.7%) and sheep (32/40, 80.0%) from livestock farms. None of the 40 goats from livestock farm investigated in this study were positive for *Anaplasmataceae* DNA.

Of the 296 animal blood samples provided by VRI, 27.7% (82/296) animal blood samples were positive for the EHR-PCR assays. Positive blood samples were derived
from the blood of deer (46/78, 59.0%), goats (2/75, 2.7%), buffaloes (12/55, 21.8%), cattle (14/22, 63.6%) and pangolins (8/15, 53.3%) while negative blood samples were derived from 46 horses, four rats and a cat. Of the samples collected from the trapped animals from the forest areas, 8.6% (6/70) monkeys, 70.0% (7/10) wild boars and 28.2% blood samples stored in FTA cards (20/71; ten from monitor lizards, eight from bats, one from primate and one from cat) were positive. Besides that, 22 organ samples (eight kidneys, seven liver and spleen, respectively) from six bats, two rats and a squirrel were positive for *Anaplasmataceae* DNA.

BLAST analyses were performed on 84 sequences successfully obtained from 40 cattle, 20 deer, five bats, five monkeys, four pangolins, two buffaloes, two goats and a squirrel. The details of the BLAST analyses are shown in Table 4.7. BLAST analyses reveal the presence of *A. marginale*, *A. phagocytophilum* (three sequence variants), *A. bovis* (two sequence variants), *A. platys* (two sequence variants) and one uncharacterised *Anaplasma* sp.

The findings obtained from the phylogenetic analysis (Figure 4.23) are summarised as below:-

i. The *Anaplasma* spp. from the pangolins is grouped in the same cluster with the *Anaplasma* sp. detected in *Amblyomma javanense* ticks from Thailand (strain AnAj360; GenBank accession no.: AF497580) (Parola *et al.*, 2003).

ii. *A. phagocytophilum* DNA that was amplified from animal blood samples can be grouped into three clusters, of which one is closely related to *A. phagocytophilum* strain Webster (GenBank accession no.: U02521) (Chen *et al.*, 1994) and *A. phagocytophilum* in mouse’s spleen from Korea (strain AAIK4; GenBank accession no.: KR611719; unpublished); one is grouped with *A. phagocytophilum* in goat from Korea (strain Hubei E4; GenBank accession no.: KF569909; unpublished), and another is grouped with *A.
phagocytophilum reported from a goat in China (isolate HB-G5-goat-China; GenBank accession no.: KR002112; unpublished).

iii. *A. bovis* detected in this study grouped into two clusters based on minor sequence variation, of which one is grouped with *A. bovis* detected in *H. longicornis* from Japan (strain NR07; GenBank accession no.: AB196475) (Kawahara et al., 2006) and another cluster was grouped with *A. bovis* detected in *H. concinna* from China (strain hc-hlj209; GenBank accession no.: KU921422; unpublished).

iv. Two clusters of *A. platys* were observed, with one closely related to *A. platys* detected in deer from China (isolate 2ax1; GenBank accession no.: KJ659044; unpublished) and another with *A. platys* detected in dog from Malaysia (strain Sarawak41 UPM; GenBank accession no.: KU500914; unpublished).

v. *A. marginale* in cattle is clustered with *A. marginale* from Philippines (isolate C7D; GenBank accession no.: JQ839011) (Ybañez et al., 2013b), *A. centrale* from Philippines (isolate C4B; GenBank accession no.: JQ839010) (Ybañez et al., 2013b) and *A. ovis* from South Africa (isolate OVI; GenBank accession no.: AF414870) (Lew et al., 2003).

vi. One sequence (*Anaplasma* sp. 0105) derived from the blood sample of a monkey is placed on a single branch. Further investigation is warranted to determine whether it is genetically related to other *Anaplasma* species.
Table 4.7: BLAST analyses of 84 short 16S rRNA gene sequences amplified from animal samples from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Animal host (n)</th>
<th>BLAST result (nearest)</th>
<th>Representative samples for construction of dendrogram</th>
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<tbody>
<tr>
<td><strong>Livestock farms</strong></td>
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<tr>
<td>• Jelai Gemas, Negeri Sembilan Cattle (2)</td>
<td>A. <em>platys</em> strain Sarawak41 [KU500914; 237 nt/237 nt (100% identity); dog, Malaysia]</td>
<td>Not selected</td>
<td></td>
</tr>
<tr>
<td>• Jelai Gemas, Negeri Sembilan Cattle (3)</td>
<td>A. <em>phagocytophilum</em> strain AAIAK4 [KR611719; 237 nt/237 nt (100% identity); mouse spleen, South Korea]</td>
<td>● EKP1246</td>
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<tr>
<td>• Ulu Lepar, Pahang Cattle (3)</td>
<td>A. <em>marginale</em> strain Uganda MT27 [KU686794; 225 nt/225 nt (100% identity); cattle, Uganda]</td>
<td>● WYY1474</td>
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</tr>
<tr>
<td>• Ulu Lepar, Pahang Cattle (3)</td>
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<td>Not selected</td>
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<tr>
<td>• Jelai Gemas, Negeri Sembilan Cattle (17)</td>
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<td>● WYYX1096</td>
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<tr>
<td>• Jerantut, Pahang Cattle (1)</td>
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<td>Not selected</td>
<td></td>
</tr>
<tr>
<td>• Jerantut, Pahang Ulu Lepar, Pahang Cattle (3)</td>
<td></td>
<td>Not selected</td>
<td></td>
</tr>
<tr>
<td>• Ulu Lepar, Pahang Cattle (3)</td>
<td>A. <em>marginale</em> strain Uganda MT27 [KU686794; 234 nt/237 nt (99% identity); cattle, Uganda]</td>
<td>● WYYX1096</td>
<td></td>
</tr>
<tr>
<td>• Jelai Gemas, Negeri Sembilan Cattle (3)</td>
<td></td>
<td>Not selected</td>
<td></td>
</tr>
<tr>
<td>• Ulu Lepar, Pahang Cattle (3)</td>
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<td>Not selected</td>
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</tr>
<tr>
<td>• Jelai Gemas, Negeri Sembilan Cattle (3)</td>
<td>A. <em>marginale</em> strain Uganda MT27 [KU686794; 234 nt/237 nt (99% identity); cattle, Uganda]</td>
<td>● WYYX1096</td>
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<tr>
<td>• VRI, Ipoh Deer (16)</td>
<td>A. <em>platys</em> strain Sarawak41 [KU500914; 237 nt/237 nt (100% identity); dog, Malaysia]</td>
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</tr>
<tr>
<td>• Deer (3)</td>
<td>A. <em>platys</em> isolate 2ax1 [KJ659044; 255 nt/255 nt (100% identity); deer, China]</td>
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<td>• Goat (1)</td>
<td>A. <em>phagocytophilum</em> isolate HB-G5-goat-China [KR002112; 223/226 (99% identity); goat, China]</td>
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<td>• Cattle (6)</td>
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<tr>
<td>• Pangolin (4)</td>
<td>Anaplasma sp. AnAj360 [AF497580; 251 nt/251 nt (100% identity); A. javanense, Thailand]</td>
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<td>• Deer (3)</td>
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<td>● DE25230</td>
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<tr>
<td>• Kuala Lompat, Pahang</td>
<td>Bat’s liver (3)</td>
<td><em>A. phagocytophilum</em> strain AAIK4 [KR611719; 237 nt/237 nt (100% identity)]; mouse spleen, South Korea</td>
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<td>Squirrel’s liver (1)</td>
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<td>Bat’s kidney (3)</td>
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<td>Bat’s spleen (3)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Bat’s kidney (1)</td>
<td><em>A. phagocytophilum</em> strain Hubei E4 [KF569909; 242 nt/242 nt (100% identity)]; goat, China</td>
<td>B0037-K</td>
</tr>
<tr>
<td>• Perhilitan provided</td>
<td>Monkey (1)</td>
<td><em>A. phagocytophilum</em> strain Hubei E4 [KF569909; 235 nt/238 nt (99% identity)]; goat, China</td>
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<tr>
<td></td>
<td>Monkey (4)</td>
<td><em>A. bovis</em> strain hc-hlj209 [KU921422; 238 nt/238 nt (100% identity); <em>H. concinna</em>, China]</td>
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</tbody>
</table>
Figure 4.23: Phylogenetic relationships among various *Anaplasma* spp. based on partial sequences of the 16S rRNA gene (224 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. *Rickettsia rickettsii* (U11021) was used as an outgroup.

*: Representative sequences amplified in this study.
4.6.2  Further characterisation of *Anaplasmataceae*

4.6.2.1  Amplification of the nearly full length 16S rRNA gene of *Anaplasma* spp. and *Ehrlichia* spp.

A total of 150 animal samples [78 cattle and 16 sheep from livestock farms; 17 deer, eight pangolins, six cows, six buffaloes and two goats from VRI, Ipoh; 12 organ samples (from five bats and a squirrel), three wild boars and two monitor lizards blood samples stored in FTA cards] were subjected to amplification to determine the full length 16S rRNA gene of *Anaplasma* spp. or *Ehrlichia* spp. The 1500 bp amplicon was obtained by PCR assays using primer pairs of fD1/Rp2 or ATT062F/ATT062R or fD1/EHR16SR paired with Rp2/EHR16SD (Figure 4.11). All the 94 cattle and sheep bloods selected from the livestock farms were positive while only 33 animal blood samples provided by VRI (13 deer, eight pangolins, five cows, five buffaloes and two goats) were positive. The nearly full length 16S rRNA genes were also amplified from six samples [three wild boars (provided by PERHILITAN) and three organ samples from three bats (from field trips)].

Of 39 amplicons selected for sequencing, only 28 sequences were successfully obtained from 15 cattle, five monkeys, three pangolins, two deer, two wild boars and a goat (Table 4.8). BLAST analyses reveal the identification of *A. marginale*, *A. centrale*, *A. platys* and *A. bovis* (three sequence variants). Referring to the phylogenetic tree (Figure 4.24), the findings are summarised as below:

i. *A. centrale* (represented by a sample labelled as Y9) obtained from three cattle in this study was grouped in one cluster with *A. centrale* from Japan (GenBank accession no.: AF283007) (Inokuma et al., 2001c). *A. marginale* obtained from cattle from different sources (represented by samples labelled as WYY1474, 1958, EKV4995 and B90046) was clustered with *A. marginale* from USA (GenBank accession no.: AF311303; unpublished) and *A. marginale* detected in *R. microplus* from Philippines (isolate C7D;
Table 4.8: BLAST analyses of nearly full length 16S rRNA and *gltA* gene sequences amplified from animal samples from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
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<th>Gene used</th>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>• Jelai Gemas, Negeri Sembilan</td>
<td>Cattle (1)</td>
<td>16S rRNA</td>
<td><em>A. platys</em> 16S ribosomal RNA gene, [EF139459; 1064 nt/1066 nt (99% identity); dog, Thailand]</td>
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<td>• VKAB001</td>
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<tr>
<td>• Jelai Gemas, Negeri Sembilan</td>
<td>Cattle (3)</td>
<td><em>A. centrale</em> 16S ribosomal RNA gene [AF283007; 1313 nt/1313 nt (100% identity); cattle, Japan]</td>
<td>• Y9</td>
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<tr>
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<td>• EKV4995</td>
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<td></td>
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<td></td>
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<td>• Air Hitam, Johore</td>
<td>Cattle (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Jelai Gemas, Negeri Sembilan</td>
<td>Cattle (1)</td>
<td><em>gltA</em></td>
<td><em>A. marginale</em> strain Dawn [CP006847; 493 nt/493 nt (100% identity); cattle, USA]</td>
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<td>• Air Hitam, Johore</td>
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Not selected
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<th>Gene Region</th>
<th>Description</th>
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<tbody>
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<td>16S rRNA</td>
<td><em>A. platys</em> 16S ribosomal RNA gene, [EF139459; 1064 nt/1066 nt (99% identity); dog, Thailand]</td>
<td></td>
<td>DE5576</td>
<td></td>
</tr>
<tr>
<td>Cow (1)</td>
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<td><em>A. marginale</em> strain Uganda MT40 [KU686785; 1167 nt/1172 nt (99% identity), cattle, Uganda]</td>
<td></td>
<td>BPK2-6219</td>
<td></td>
</tr>
<tr>
<td>Goat (1)</td>
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<td><em>A. bovis</em> isolate tick 17/China/2013 [KP314250; 1312 nt/1314 nt (99% identity); <em>H. longicornis</em>, China]</td>
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<td>G4699</td>
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</tr>
<tr>
<td>Pangolin (3)</td>
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<td><em>A. bovis</em> isolate tick 17/China/2013 [KP314250; 1284 nt/1314 nt (98% identity); <em>H. longicornis</em>, China]</td>
<td></td>
<td>T7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T15</td>
</tr>
<tr>
<td><strong>Perhilitan provided</strong></td>
<td></td>
<td>16S rRNA</td>
<td><em>A. bovis</em> strain NR07 [AB196475; 1313/1313 (100% identity); <em>H. longicornis</em>, Japan]</td>
<td></td>
<td>WBM1</td>
</tr>
<tr>
<td>Wild boar (2)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey (5)</td>
<td></td>
<td><em>E. bovis</em> 16S rRNA gene [U037775; 1305 nt/1315 nt (99% identity)]</td>
<td></td>
<td>0115</td>
<td>0088</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0085</td>
</tr>
</tbody>
</table>
GenBank accession no.: JQ839011) (Ybañez et al., 2013b).

ii. *A. platys* detected in animal from this study (represented by samples labelled as Y28, VKAB001, DE5576 and BPK2-6219) was grouped in one cluster with *A. platys* in dog from Thailand (GenBank accession no.: EF139459) (Pinyoowong et al., 2008) and sika deer from China (isolate 2ax1; GenBank accession no.: KJ659044; unpublished).

iii. *A. bovis* is separated into three clusters: *A. bovis* detected in wild boars (represented by a sample labelled as WBM1) was in one cluster with *A. bovis* detected in *H. longicornis* from Japan (strain NR07; GenBank accession no.: AB196475) (Kawahara et al., 2006); *A. bovis* detected in a goat (represented by sample labelled as G4699) was in one cluster with *Anaplasma* sp. detected in *R. sanguineus* from Bangladesh (closely related to *A. bovis*) (isolate tick6; GenBank accession no.: LC066136) (Qiu et al., 2016); *A. bovis* detected in three monkeys (represented by samples labelled as 0115, 0085 and 0088) was clustered with *A. bovis* type strain (GenBank accession no.: U03775; unpublished).

iv. The *Anaplasma* spp. detected from three pangolins (represented by samples labelled as T7, T14 and T15) were segregated on a distinct branch amongst members of *Anaplasmataceae*, with a high bootstrap value (98.0%). This finding suggests that the organisms could belong to a new species of *Anaplasma*. The pangolin-associated *Anaplasma* was proposed as “*Candidatus Anaplasma pangolinii*” in accordance with the host where it was derived.

Several almost full length 16S rDNA sequences, i.e., KU189194 (*A. bovis*, wild boar, clone WBM1); KM114611-KM114613 (*A. bovis*, monkey, clone 88, 85 and 115, respectively); KU189193 (*Candidatus A. pangolinii*, pangolin, clone T7) have been deposited in the GenBank database.
Figure 4.24: Phylogenetic relationships among various *Anaplasma* spp. based on partial sequences of the 16S rRNA gene (1068 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. *R. rickettsii* (U11021) was used as an outgroup.

* : Representative sequences amplified from animal samples in this study.

### 4.6.2.2 Amplification of gltA gene of *Anaplasma* spp. and *Ehrlichia* spp.

A total of 86 animals (70 cattle and 16 sheep) from livestock farms were subjected to further characterisation by amplification of *gltA* gene, using primer pair EHR-CS136F/EHR-CS778R (Figure 4.13). An approximately 641 bp amplicon was obtained from 70 (81.4%; 69 cattle and a sheep) blood samples.
The sequences for ten amplicons were sequenced, of which eight were successfully obtained from (all cattle) for analysis. BLAST analyses reveal the identification of *A. marginale* (strain Dawn; GenBank accession no.: CP006847) (Aguilar Pierlé *et al.*, 2014) (Table 4.8), based on the clustering of the sequences with the type strain (strain Dawn) detected in cattle from USA (GenBank accession no.: CP006847) (Aguilar Pierlé *et al.*, 2014).

Figure 4.25: Phylogenetic relationships among various *Anaplasma* spp. based on partial sequences of the *gltA* gene (469 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates.

* : Representative sequences amplified from animal samples in this study.

### 4.6.2.3 Molecular characterisation of *A. phagocytophilum* from animal blood DNA samples

Several nested PCR assays targeting on three genes (16S rRNA, *msp4* and *groEL*) of *A. phagocytophilum* were used for detection of *A. phagocytophilum* from animal blood DNA samples in this study. A total of 35 animal samples from VRI (23 deer, six
buffaloes, four cows and two goats) and 17 animal samples from the forest areas [12 organ samples (four kidneys, three livers and three spleens from five bats; one liver and one spleen from a squirrel), three wild boars and two monitor lizard blood samples stored in FTA cards] were subjected to amplification of the 16S rRNA, \textit{msp4} and \textit{groEL} genes of \textit{A. phagocytophilum}. The highest detection rate (68.6\%) was observed with the 16S rRNA gene PCR assay, followed by the PCR assays targeting \textit{msp4} gene (55.6\%) and \textit{groEL} gene (34.3\%).

A total of 24 animal blood samples from VRI (19 deer, three buffaloes and two cows) and 13 animal samples from the forest areas [11 organ samples (three kidneys, three livers and three spleens from five bats; one liver and one spleen from a squirrel) and two wild boars] were positive for the nested PCR assays which amplified the 641 bp of the 16S rRNA gene of \textit{A. phagocytophilum}, using primer pair SSAP2f/SSAP2r (Figure 4.26). A total of 13 amplicons were sequenced, however; only 11 sequences were successfully determined. BLAST analyses show that six sequences obtained from one cow, two buffaloes and three deer (GenBank accession nos: MG988297-MG988299) demonstrated 100\% (520 nt/520 nt) similarity with that of \textit{Candidatus Anaplasma boleense} (strain WHBMXZ-139; GenBank accession no.: KX987335). Additionally, another five sequences matched 99-100\% (520 nt/520 nt or 519 nt/520 nt) with an \textit{Anaplasma} sp. detected in a goat from China (clone Ap20-5a; GenBank accession no.: KX272643; unpublished). The sequences demonstrated only 96.9-97.6\% similarity with \textit{A. phagocytophilum} type strain (strain Webster; GenBank accession no.: U02521) (Chen \textit{et al.}, 1994). The results of the BLAST analyses for \textit{A. phagocytophilum} are shown in Table 4.9.
Figure 4.26: Amplification of approximately 641 bp of the 16S rRNA gene fragments of *A. phagocytophilum* from samples using SSAP2f/SSAP2r nested amplification primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-5: SSAP2f/SSAP2r-positive samples; 6: Positive control (*A. phagocytophilum* genomic DNA extracted from IFA slides).

The dendrogram (Figure 4.27) reveals the clustering of the sequences with *Anaplasma* sp. detected in goat from China (clone Ap20-5a; GenBank accession no.: KX272643; unpublished) and *A. phagocytophilum* detected in sheep from China (isolate YC38; GenBank accession no.: KJ782381) (Yang *et al.*, 2015a) but not in one cluster with the *A. phagocytophilum* type strain (strain Webster; GenBank accession no.: U02521) (Chen *et al.*, 1994).

For *msp4*-PCR assay, 20 animal samples obtained from VRI (14 deer, four buffaloes, a goat and a cow) and nine animal samples from the forest areas [four organ samples (two kidney and one spleen from bats; one liver from squirrel), three wild boars and two monitor lizard blood samples stored in FTA cards] were positive, producing approximately 343 bp amplicons (Figure 4.17). Eight amplicons were selected for sequence determination but only five sequences were successfully obtained. BLAST analyses of all five sequences show the identification of two sequence variants of *A. phagocytophilum* (Table 4.9) demonstrating 100% and 99% identity with *A. phagocytophilum* clone cam5 from France and *A. phagocytophilum* strain ZJ-China, respectively.
Table 4.9: BLAST analyses of the partial 16S rRNA, \( msp4 \) and \( groEL \) gene sequences of \( A. \ phagocytophilum \) amplified from animal samples from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Animal host (n)</th>
<th>Gene used</th>
<th>BLAST result (nearest match)</th>
<th>Representative samples for construction of dendrogram</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VRI, Ipoh</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Deer (2)                  | 16S rRNA       | \( Candidatus \) A. boleense strain WHBMXZ-139 [KX987335; 520 nt/520 nt (100% identity); \( R. \ microplus \), China] | • DE2064  
• DE25241  
• BG538  
• BU10698-12  
• BU10698-28  
• DE0345 |
| Cow (1)                   |                |           |                                              |                                                       |
| Buffalo (2)               |                |           |                                              |                                                       |
| Deer (1)                  | \( groEL \)    | Anaplasma sp. clone SY49 [KF728361; 464 nt/480 nt (97% identity); \( H. \ longicornis \), China] | • DE25206  
• DE25208  
• DE5576 |
| Deer (1)                  | \( msp4 \)     | \( A. \ phagocytophilum \) strain ZJ-China [EU008082; 267 nt/268 nt (99% identity); rat, China] | • DE25206  
• DE25208  
• DE5576 |
| Deer (2)                  | \( groEL \)    | Anaplasma sp. clone SY49 [KF728361; 447 nt/463 nt (97% identity); \( H. \ longicornis \), China] | • DE25206  
• DE25208  
• DE5576 |
| **Forest areas**          |                |           |                                              |                                                       |
| Kuala Lompat, Pahang      |                |           |                                              |                                                       |
| Bat’s kidney (1)          | 16S rRNA       | Anaplasma sp. clone Ap20-5a [KX272643; 520 nt/520 nt (100% identity); goat, China] | • B0014-K  
• S0040-L  |
| Squirrel’s liver (1)      |                | Anaplasma sp. clone Ap20-5a [KX272643; 519 nt/520 nt (99% identity); goat, China] | • B0037-K  |
| Bat’s kidney (1)          | \( msp4 \)     | \( A. \ phagocytophilum \) clone cam5 [JX197227; 261 nt/261 nt (100% identity); \( R. \ bursa \), France] | • B0014-K  
• S0040-L  |
| Squirrel’s liver (1)      |                | \( A. \ phagocytophilum \) strain ZJ-China [EU008082; 267 nt/268 nt (99% identity); rat, China] | • B0037-K  |
| Bat’s kidney (1)          |                | \( A. \ phagocytophilum \) strain ZJ-China [EU008082; 267 nt/268 nt (99% identity); rat, China] | • B0037-K  |
| **Perhilitan provided**   |                |           |                                              |                                                       |
| Wild boar (2)             | 16S rRNA       | Anaplasma sp. clone Ap20-5a [KX272643; 520 nt/520 nt (100% identity); goat, China] | • WBM3  
• WBK21 |
| Monitor lizard (FTA card) (1) | \( msp4 \) | \( A. \ phagocytophilum \) strain ZJ-China [EU008082; 267 nt/268 nt (99% identity); rat, China] | • 0284 |
Figure 4.27: Phylogenetic relationships among various *Anaplasma* spp. based on partial sequences of the 16S rRNA gene (434 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. *R. rickettsii* (U11021) was used as an outgroup.

*: Representative sequences amplified from animal samples in this study.

The phylogenetic tree (Figure 4.28) reveals the grouping of the short gene fragments of *msp4* detected in this study into two clusters whereby one group (dog, bat and squirrel) is clustered with *A. phagocytophilum* type strain (strain Webster; GenBank accession no.: EU857674; unpublished) and another group (deer, monitor lizard and bat) with *A. phagocytophilum* detected in a rat from China (strain ZJ-China; GenBank accession no: EU008082) (Zhan *et al.*, 2008).
Figure 4.28: Phylogenetic relationships among various *A. phagocytophilum* based on partial sequences of the *msp4* gene (259 bp). The dendrogram was constructed using the neighbor-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates.

* : Representative sequences amplified from animal samples in this study.

For *groEL*-PCR assay, 12 deer from VRI and seven animal samples from the forest areas [five organ samples (two kidneys, one spleen and one liver from three bats; one spleen from a squirrel), one monitor lizard blood sample stored in FTA card and a wild boar] were positive, producing approximately 573 bp amplicons by using heminested primer pair EphplgroEL(569)F/ EphgroEL(1142)R (Figure 4.29). Five amplicons were selected for sequencing but only three sequences were successfully obtained for analysis. BLAST analyses of three sequences show the identification of
Anaplasma sp. (clone SY49; GenBank accession no.: KF728361) (Dong et al., 2014) (Table 4.9). All three sequences were used to construct a dendrogram (Figure 4.30). All the short fragments were in one cluster with *Anaplasma* spp. detected in ticks from China (isolate BL126-13; GenBank accession no.: KJ410303) (Kang et al., 2014); (isolate TC250-2; GenBank accession no.: KJ410304) (Kang et al., 2014); (clone SY49; GenBank accession no.: KF728361) (Dong et al., 2014).

Figure 4.29: Amplification of approximately 573 bp of the *groEL* gene fragments of *A. phagocytophilum* from samples using EphplgroEL(569)F/EphgroEL(1142)R nested amplification primer pair. M: 100 bp DNA markers (Solis BioDyne, Estonia); 1: Negative control (miliQ water); 2-3: EphplgroEL(569)F/EphgroEL(1142)R-positive samples; 4: Positive control (*A. phagocytophilum* genomic DNA extracted from IFA slides).
Figure 4.30: Phylogenetic relationships among various *Anaplasma* spp. based on partial sequences of the *groEL* gene (463 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates.

* : Representative sequences amplified from animal samples in this study.

### 4.6.2.4 Molecular characterisation of *A. bovis* from animal blood DNA samples

A total of 36 animal samples from VRI (24 deer, six buffaloes, four cows and two goats) and 17 animal samples from the forest areas [12 organ samples (four kidneys, three livers and three spleens from five bats; one liver and one spleen from a squirrel), three wild boars and two monitor lizard blood samples stored in FTA cards] were subjected to amplification of 551 bp of the 16S rRNA gene of *A. bovis* by using primer pair AB1f/AB1r (Figure 4.19). A total of 32 animal samples from VRI (24 deer, five buffaloes, two goats and a cow) and 16 animal samples from the forest areas [12 organ samples (four kidneys, three livers and three spleens from five bats; one liver and one spleen from a squirrel), three wild boars and a monitor lizard blood sample stored in FTA...
card] were positive. BLAST analyses of 12 sequences reveal the presence of three sequence variants of *A. bovis*. The details of the BLAST analyses are shown in Table 4.10. All the sequences were used to build a dendrogram (Figure 4.31). The phylogenetic analysis shows that the *A. bovis* detected can be grouped into three clusters: *A. bovis* detected in monkey from Malaysia (this study, section 4.6.2.1), *A. bovis* detected in wild boar from Malaysia (this study, section 4.6.2.1) and *A. bovis* detected in *H. longicornis* from China (isolate tick 17/China/2013; GenBank accession no.: KP314250) (Sun *et al.*, 2015).

Table 4.10: BLAST analyses of partial 16S rRNA gene sequences of *A. bovis* amplified from animal samples from different sources in this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Animal host (n)</th>
<th>BLAST result (nearest match)</th>
<th>Representative samples for construct of dendrogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>VRI, Ipoh</td>
<td>Goat (1)</td>
<td><em>A. bovis</em> isolate Ab-YN219 [KU509996; 437 nt/437 nt (100% identity); goat, China]</td>
<td>G4699, BU10698-2, DE25211, DE25230, DE0345</td>
</tr>
<tr>
<td></td>
<td>Buffalo (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deer (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cow (1)</td>
<td><em>Anaplasma</em> sp. clone WBM1 [KU189194; 450 nt/450 nt (100% identity); wild boar, Malaysia]</td>
<td>BPK2-6219</td>
</tr>
<tr>
<td>Forest areas</td>
<td>Bat’s kidney (2)</td>
<td><em>A. bovis</em> clone 115 [KM114613; 447 nt/447 nt (100% identity); monkey, Malaysia] (A. bovis type I)</td>
<td>B0014-K, B0037-K, B0032-S, S0040-L, 0257</td>
</tr>
<tr>
<td></td>
<td>Bat’s spleen (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Squirrel’s liver (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perhilitan provided</td>
<td>Monitor lizard (FTA card) (1)</td>
<td><em>A. bovis</em> clone 115 [KM114613; 447 nt/447 nt (100% identity); monkey, Malaysia] (A. bovis type I)</td>
<td>WBM3</td>
</tr>
<tr>
<td></td>
<td>Wild boar (1)</td>
<td><em>Anaplasma</em> sp. clone WBM1 [KU189194; 450 nt/450 nt (100% identity); wild boar, Malaysia] (A. bovis type II)</td>
<td>WBM3</td>
</tr>
</tbody>
</table>
Figure 4.31: Phylogenetic relationships among various A. bovis based on partial sequences of the 16S rRNA gene (433 bp). The dendrogram was constructed using the neighbour-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. R. rickettsii (U11021) was used as an outgroup.
* : Representative sequences amplified from animals in this study.
4.6.2.5 Molecular characterisation of *E. chaffeensis* from animal blood DNA samples

A total of 35 animal samples from VRI (23 deer, six buffaloes, four cows and two goats), 17 animal samples from the forest areas [12 organ samples (four kidneys, three livers and three spleens from five bats; one liver and one spleen from a squirrel), three wild boars and two monitor lizard blood samples stored in FTA cards] were subjected to PCR assays targeting the approximately 389 bp partial fragment of 16S rRNA of *E. chaffeensis*, using primer pair HE1/HE3 (Figure 4.21). Only three samples (two cattle and a goat) from VRI and two animal samples from the forest areas (one kidney and one liver from a bat) were positive. Since very faint band was generated from the positive samples, sequence determination of these positive samples were not able to be performed.

4.7 Molecular detection of *Anaplasma* spp. and *Ehrlichia* spp. in stray dogs and dog ticks

A total of 47 dog samples (30 from Klang Valley and 17 from VRI, Ipoh) and 33 *R. sanguineus* collected from 13 dogs in Klang Valley were subjected to amplification by nested PCR assays targeting the 16S rRNA genes of *E. canis* (389 bp), *E. chaffeensis* (389 bp) and *A. phagocytophilum* (641 bp).

DNA of *E. canis* was amplified from 12 (25.5%, 10 from Klang Valley and 2 from VRI, Ipoh) dog blood samples and 17 (51.5%) *R. sanguineus*. Sequence analysis of 305 nucleotides of five positive samples (three dog blood samples and two *R. sanguineus*) confirmed 100% similarity with *E. canis* (strain Jake; GenBank accession no.: CP000107; unpublished) and many other *E. canis* sequences deposited in the GenBank database. Furthermore, the 1433 bp full length sequence of the 16S rRNA gene amplified from one of the positive samples (designated as 002, deposited at GenBank as KR920044) demonstrated 99% similarity (three nucleotides difference) with that of the *E. canis* type
strain (strain Oklahoma; GenBank accession no.: M73221) (Anderson et al., 1991) and one nucleotide difference with E. canis (strain ECAN_Bkk_07; GenBank accession no.: EU263991; unpublished). E. chaffeensis 16S rRNA gene was amplified from two (6.0%) R. sanguineus ticks (deposited at GenBank as KR920047). Sequence analysis revealed 100% (318 nt/318 nt) similarity of the amplified fragments with E. chaffeensis (strain Arkansas; GenBank accession no.: CP000236) (Dunning Hotopp et al., 2006). No amplification was obtained using the nested PCR assay targeting E. chaffeensis for dog blood samples.

The 16S rRNA genes of A. phagocytophilum were amplified from six (12.8%) dog blood samples and one (3.0%) R. sanguineus. Sequence analysis of the partial fragment of the positive tick sample (deposited at GenBank as KR920046) revealed 99% (562 nt/566 nt) similarity with A. phagocytophilum (strain Dog2; GenBank accession no.: CP006618; unpublished) but sequence determination of the 16S rRNA gene from A. phagocytophilum-positive dog blood samples was not successful due to generation of noisy data. Amplification and sequence analyses of msp4 gene (270 nucleotides) from two 16S rRNA positive-dog blood samples (deposited at GenBank as KR920045) demonstrated 100% similarity to A. phagocytophilum (strain Dog2; GenBank accession no.: CP006618; unpublished) while msp4 gene of A. phagocytophilum was not amplified from 16S rRNA A. phagocytophilum-positive tick.

Table 4.11 summarises the results obtained from the molecular detection of Anaplasma spp. and Ehrlichia spp. in animal and tick samples in this study.
Table 4.11: Summary of the identification of *Anaplasma* spp. and *Ehrlichia* spp. DNA in different animal and tick samples collected in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Animal hosts</th>
<th>Ticks (hosts)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. marginale</em> (<em>A. centrale</em> and <em>A. ovis</em>)</td>
<td>Cattle</td>
<td><em>R. microplus</em> (cattle)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>H. bispinosa</em> (cattle)</td>
</tr>
<tr>
<td><em>A. phagocytophilum</em></td>
<td>Bat</td>
<td><em>H. bispinosa</em> (sheep)</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td><em>H. wellingtoni</em> (goat)</td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td><em>D. atrosignatus</em> (vegetation)</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td><em>R. sanguineus</em> (dog)</td>
</tr>
<tr>
<td></td>
<td>Monitor lizard</td>
<td><em>Dermacentor</em> spp. (vegetation)</td>
</tr>
<tr>
<td></td>
<td>Squirrel</td>
<td><em>Haemaphysalis</em> spp. (vegetation, chicken, cat)</td>
</tr>
<tr>
<td><em>A. bovis</em></td>
<td>Bat</td>
<td><em>H. bispinosa</em> (cattle, goat, sheep)</td>
</tr>
<tr>
<td></td>
<td>Buffalo</td>
<td><em>H. hystricis</em> (vegetation)</td>
</tr>
<tr>
<td></td>
<td>Deer</td>
<td><em>R. sanguineus</em> (dog)</td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td><em>A. varanense</em> (snakes)</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td><em>Dermacentor</em> spp. (vegetation)</td>
</tr>
<tr>
<td></td>
<td>Wild boar</td>
<td><em>Haemaphysalis</em> spp. (vegetation)</td>
</tr>
<tr>
<td></td>
<td>Squirrel</td>
<td></td>
</tr>
<tr>
<td><em>A. platys</em></td>
<td>Cattle</td>
<td><em>H. bispinosa</em> (cattle)</td>
</tr>
<tr>
<td></td>
<td>Deer</td>
<td><em>H. hystricis</em> (vegetation)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. atrosignatus</em> (vegetation)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. varanense</em> (snake)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. helvolum</em> (snake)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Haemaphysalis</em> spp. (vegetation)</td>
</tr>
<tr>
<td><em>Candidatus A. pangolinii</em></td>
<td>Pangolin</td>
<td>Not detected</td>
</tr>
<tr>
<td><em>Candidatus A. boleense</em></td>
<td>Cattle</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Deer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Buffalo</td>
<td></td>
</tr>
<tr>
<td><em>E. canis</em></td>
<td>Dog</td>
<td><em>R. sanguineus</em> (dog)</td>
</tr>
</tbody>
</table>

**New sequence variants:**

<table>
<thead>
<tr>
<th><em>Anaplasma</em> spp. (closely related to <em>A. phagocytophilum</em>)</th>
<th>Bat</th>
<th>Buffalo</th>
<th>Cow</th>
<th>Deer</th>
<th>Squirrel</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. helvolum</em> (snake)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemaphysalis sp. (cat)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| *Ehrlichia* spp. (undifferentiated between *E. chaffeensis* and *E. ewingii*) | Not detected | *D. atrosignatus* (vegetation) |
|                                                                              |               | *Haemaphysalis* spp. (vegetation) |
|                                                                              |               | *R. sanguineus* (dog)           |

<table>
<thead>
<tr>
<th><em>Ehrlichia</em> sp. (closely related to <em>E. ruminantium</em>)</th>
<th>Not detected</th>
<th><em>A. varanense</em> (snake)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><em>Ehrlichia</em> sp. (closely related to <em>E. mineirensis</em>)</th>
<th>Not detected</th>
<th><em>R. microplus</em> (cattle)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><em>Ehrlichia</em> sp. (closely related to <em>Ehrlichia</em> sp. clone EBm52)</th>
<th>Not detected</th>
<th><em>R. microplus</em> (cattle)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><em>Ehrlichia</em> sp. (closely related to <em>Candidatus E. shimanensis</em>)</th>
<th>Not detected</th>
<th><em>H. bispinosa</em> (cattle)</th>
</tr>
</thead>
</table>
CHAPTER 5: DISCUSSION

Limited data is available on the disease burden of anaplasmosis and ehrlichiosis in Malaysia and most SEA countries. Previous investigations showed the presence of antibodies to *E. chaffeensis* in 44.0% and 14.6% of healthy individuals from Thailand and Indonesia, respectively (Heppner *et al.*, 1997; Richards *et al.*, 2003).

In our recent investigation, IgG antibodies against *E. chaffeensis* were detected in 34.3%, 29.9% and 9.8% of Malaysian indigenous people, farm workers and blood donors, respectively (Koh *et al.*, 2018). This serological data was consistent with the findings reported in Thailand and Indonesia whereby high antibody prevalences against *E. chaffeensis* were reported. Comparatively, the seropositivity to *A. phagocytophilum* was relatively low in the indigenous people (6.9%) and none of the farm workers investigated was seropositive in the recent Malaysian study (Koh *et al.*, 2018). This observation was different from those reported in China whereby higher antibody prevalences against *A. phagocytophilum* have been reported in farmers (8.8-33.7%), rural residents (15.4%) and forest area residents (7.1%) have been reported (Hao *et al.*, 2013; Zhang *et al.*, 2008b; Zhang *et al.*, 2014; X. C. Zhang *et al.*, 2012; Y. Zhang *et al.*, 2012). The variation observed in the seropositivity to *A. phagocytophilum* could be due to many reasons, for instance, climate and geographical differences can influence the type and distribution of ticks and animal hosts for transmission of *A. phagocytophilum* (Stuen *et al.*, 2013).

5.1 Identification of ticks

5.1.1 *Haemaphysalis* spp.

*Haemaphysalis* was the main genus of ticks identified in a variety of sources, including sheep, cattle, goat, dog, rat, bat, cat and vegetation in this study (Table 4.1). This finding is consistent with a study carried out by Petney *et al.* (2007), who described the identification of about 104 species of ticks from 12 genera in SEA countries. In that
study, *Haemaphysalis* was the most species-abundant genus comprising 52 species. In this study, BLAST analyses show that tick mitochondrial 16S rRNA gene is a better DNA marker than 28S rRNA gene for differentiation amongst the species of *Haemaphysalis* ticks, as sequence analysis of the gene allows a better discrimination of ticks to the species level (Table 4.2). Comparatively, the gene encoding the 28S rRNA is more conservative than 16S rRNA gene and BLAST analyses of the sequences obtained only enable the identification of ticks up to genus level (Sections 4.2.3 and 4.2.4).

The main *Haemaphysalis* species identified in ticks collected from cattle and sheep investigated in this study was *H. bispinosa*. Besides that, *H. bispinosa* has also been detected in ticks collected from bat (n=1), rat (n=1), goats (n=2), chicken (n=1) and dogs (n=2) from aboriginal villages and Kuala Lompat reserve forest in this study (Table 4.2). *H. bispinosa* was previously reported in Peninsular Malaysia in ticks collected from wild animals (bearcat, dhole, Asian leopard cat, Malayan weasel, Malayan civet, toddy cat and Indian boar) and domestic animals (sheep, goat and dog) (Hoogstraal *et al.*, 1969). Recently, *H. bispinosa* has also been detected in ticks collected from dogs in Sabah, Malaysia (Wells *et al.*, 2012) and various sources (including vegetation, goats, cattle and dogs) in Thailand, India and Bangladesh (Brahma *et al.*, 2014; Fuehrer *et al.*, 2012; Malaisri *et al.*, 2015). *H. bispinosa* has a three host life cycle which requires a single host during each feeding stage (larva, nymph and adult) (Parola and Raoult, 2001) and is associated with tick-borne pathogens such as *Bartonella bovis* in Malaysia (Kho *et al.*, 2015a); *Rickettsia* spp. in Thailand (Malaisri *et al.*, 2015); *B. burgdoferi*, *Babesia bigemina*, *Rickettsia* spp. and *Theileria sergenti* in China (Yu *et al.*, 2016; Yu *et al.*, 2015) and *A. bovis* in Bangladesh (Qiu *et al.*, 2016). In this study, *H. bispinosa* has been shown to harbour *A. marginale*, *A. bovis*, *A. platys*, *A. phagocytophilum* and *Ehrlichia* sp. (closely related to *Candidatus E. shimanensis*) (Tables 4.4 and 4.5).
*H. wellingtoni* was the main species detected from the peri-domestic animals (dogs, cats and chickens) in aboriginal villages in this study (Table 4.2). *H. wellingtoni* has been previously reported in ticks collected from jungle fowls in Peninsular Malaysia (Konto *et al.*, 2015), chickens from Thailand (Parola *et al.*, 2003) and chickens, rats and birds from Indonesia (Durden *et al.*, 2008). The free-range system of animals rearing in the aboriginal villages might increase the susceptibility of animals to tick infestation. *H. wellingtoni* has been reported to be associated with *Coxiella* spp. and *Rickettsia* spp. in Malaysia (Khoo *et al.*, 2016) and Kyasanur forest disease virus in China (Yu *et al.*, 2015).

In this study, *A. phagocytophilum* has been detected in *H. wellingtoni* ticks (Table 4.6).

In this study, some of the ticks collected from vegetation and peri-domestic animals (dog and cat) were identified as *H. hystricis* (Table 4.2). *H. hystricis* has been previously collected from dogs, rats and tree shrews in Peninsular Malaysia (Ernieenor *et al.*, 2017), vegetation in Thailand (Malaisri *et al.*, 2015); cattle, dogs and vegetation in Laos (Vongphayloth *et al.*, 2016) and dogs, deer, rats and wild boars in Indonesia (Durden *et al.*, 2008). *H. hystricis* has been associated as a vector for diseases including ehrlichiosis (*Ehrlichia* spp.), human granulocytic anaplasmosis (*A. phagocytophilum*) and Japanese spotted fever (*R. japonica*) (Parola *et al.*, 2003; Yu *et al.*, 2015). In this study, *H. hystricis* ticks harbouring *A. platys* and *A. bovis* have been detected (Tables 4.4 and 4.5).

Two questing ticks collected from the vegetation of a forest at Sg. Deka Elephant Sanctuary, Terengganu were identified as *H. shimoga* in this study (Table 4.2). There is no previous report on distribution of *H. shimoga* in Malaysia. *H. shimoga* has been reported in questing ticks from Thailand (Malaisri *et al.*, 2015). *H. shimoga* harbouring *Rickettsia* spp., *Anaplasma* spp. and *Coxiella*-like endosymbiont has been reported in Thailand (Ahantarig *et al.*, 2011; Arthan *et al.*, 2015; Malaisri *et al.*, 2015). The distribution of *H. shimoga* and its capability in carrying different pathogens have not been
widely reported in Malaysia. *Anaplasma* spp. and *Ehrlichia* spp. have not been detected from two *H. shimoga* identified in this study.

A number of ticks collected from vegetation (n=10), rat (n=1), squirrel (n=1) and dog (n=4) in aboriginal villages and the forest areas were only recognised as *Haemaphysalis* spp. as there are no matching sequences of the mitochondrial 16S rRNA gene in the GenBank database (Table 4.2). Based on sequence analyses of tick DNA, four sequence types demonstrating the highest sequence similarity to either *H. obesa* (94%), *H. hystricis* (97%), *H. asiatica* (93%) or *H. qinghaiensis* (93%) to their closest relatives were recognised in this study. The *Haemaphysalis* ticks were found to carry various *Anaplasma* spp. (*A. phagocytophilum*, *A. bovis*, *A. platys* and uncharacterised *Anaplasma* sp.) and *Ehrlichia* spp. (*E. chaffeensis*/*E. ewingii*) (Tables 4.4-4.6). Further identification of these ticks to the species level should be carried out to provide a better understanding on the distribution and the role of *Haemaphysalis* ticks in disease transmission.

### 5.1.2 *Dermacentor* spp.

A number of ticks from the vegetation (n=7) and ticks collected from rat (n=3) in the forest areas were identified as *D. atrosignatus* in this study (Table 4.2). The distribution of *D. atrosignatus* has been previously reported in various sources including vegetation, pigs, dogs, pangolins, monitor lizards, buffaloes and snakes in Malaysia (both Peninsular Malaysia and Sarawak) (Hoogstraal and Wassef, 1985a; Mariana *et al.*, 2011). Besides that, *D. atrosignatus* has also been reported in a few SEA countries including Thailand (vegetation), Indonesia (vegetation, pigs, bats, pangolins and shrews) and Philippines (vegetation and pigs) (Durden *et al.*, 2008; Hoogstraal and Wassef, 1985a; Malaisri *et al.*, 2015). To the best of our knowledge, there is no report of *D. atrosignatus* harbouring pathogens in Malaysia or other Asian countries. However in this study, *D. atrosignatus* ticks were found to carry *A. platys*, *A. phagocytophilum* and *Ehrlichia* spp. (*E. chaffeensis*/*E. ewingii*) (Tables 4.4 and 4.6).
Two ticks collected from a dog in an aboriginal village were identified as *D. auratus* in this study (Table 4.2). The ticks have previously been identified from various sources including vegetation, snakes, civets and pigs in Peninsular Malaysia and Sarawak (Hoogstraal and Wassef, 1985b). The distribution of *D. auratus* has also been recorded in a few Asian countries including Thailand, Myanmar, Vietnam, Laos, Indonesia, Sri Lanka, India, Nepal and Bangladesh from the vegetation and a variety of animal hosts including dogs, bears, pigs, rats, deer, squirrels toddy cats and tree shrews (Hoogstraal and Wassef, 1985b; Malaisri *et al.*, 2015; Parola *et al.*, 2003; Vongphayloth *et al.*, 2016). *D. auratus* has been reported as a vector harbouring pathogens such as *Anaplasma* spp., *Rickettsia* spp., *Francisella* spp. and *Hepatozoon* spp. (Parola *et al.*, 2003; Sumrandee *et al.*, 2015, 2016). *Anaplasma* spp. and *Ehrlichia* spp. have not been detected from two *D. auratus* identified in this study. Since *D. auratus* is widely distributed in Asian countries and is capable to transmit potential human pathogens, more attention should be paid on the distribution of this tick in Malaysia and understanding its role in spreading diseases.

Additionally, a high number of questing ticks (n=25) collected from the Kuala Lompat reserve forest and a forest at Sg. Deka Elephant Sanctuary, Terengganu were recognised as *Dermacentor* spp. only in this study (Table 4.2) due to the lack of matching sequences to the mitochondrial 16S rRNA gene in the GenBank database. Sequence analyses of the tick 16S rRNA gene reveal the presence of four sequence types demonstrating the highest similarity with *D. andersoni* (94%), *D. nuttalli* (93%) and *D. silvarum* (93%), with their closest relatives in the GenBank database, respectively. *A. bovis* and *A. phagocytophilum* were detected in *Dermacentor* spp. ticks in this study (Tables 4.4-4.6). Further identification of these ticks to the species level should be carried out.
5.1.3 *Amblyomma* spp.

In this study, three types of *Amblyomma* ticks have been identified from ticks collected from vegetation (*Amblyomma* spp.), a skink (*A. helvolum*) and snakes (*A. helvolum* and *A. varanense*) (Table 4.2). *Amblyomma* ticks have been reported infesting reptilian hosts, for example, monitor lizards (*A. helvolum* and *A. varanense*), skinks (*A. helvolum*), snakes (*A. helvolum*, *A. varanense* and *Amblyomma cordiferum*) as reported by previous investigation from various Asian countries (Auffenberg, 1988; Chao et al., 2013; Durden et al., 2008; Sumrandee et al., 2014; Vongphayloth et al., 2016). In addition, *Amblyomma* ticks have also been identified from vegetation (*A. americanum*, *A. helvolum*, *A. testudinarium*, *Amblyomma* spp. and *Amblyomma integrum*) and animal hosts other than reptiles, such as bats (*A. cordiferum*), rats (*Amblyomma* spp.), squirrels (*Amblyomma* spp.), tree shrews (*Amblyomma* spp.), deer (*A. americanum*), cattle (*A. testudinarium*), dogs (*A. testudinarium*) and pigs (*A. helvolum* and *A. testudinarium*) (Ahamad et al., 2013; Durden et al., 2008; Malaisri et al., 2015; Mariana et al., 2011; Rudenko et al., 2016; Vongphayloth et al., 2016). *Amblyomma* ticks have been reported as a vector for tick-borne pathogens including *E. ruminantium*, *A. phagocytophilum*, *B. burgdorferi*, *Rickettsia rickettsii* and *Rickettsia* spp. (Malaisri et al., 2015; Oliveira et al., 2010; Rudenko et al., 2016; Teshale et al., 2015). The distribution of *Amblyomma* ticks in other animal species in Malaysia is yet to be explored. In this study, *A. bovis*, *A. platys*, uncharacterised *Anaplasma* sp. and *Ehrlichia* sp. (closely related to *E. ruminantium*) were detected in *Amblyomma* ticks (*A. helvolum* and *A. varanense*) (Tables 4.4 and 4.5).

5.1.4 *Rhipicephalus* spp.

*R. microplus* is another species of ticks besides *H. bispinosa*, which was collected from cattle in livestock farms in this study (Table 4.2). The tick has been reported in ruminants (mainly cattle) from SEA countries including Thailand, Philippines, Laos, Indonesia and Malaysia (Changbunjong et al., 2009; Durden et al., 2008; Tay et al., 2014;
Vongphayloth et al., 2016; Ybañez et al., 2013b). R. microplus is known to transmit some bovine diseases including anaplasmosis, babesiosis and theileriosis (Kocan et al., 2010; Yu et al., 2015). In this study, A. marginale, Ehrlichia sp. (closely related to E. mineirensis) and Ehrlichia sp. (closely related to Ehrlichia sp. clone EBm52) were detected in R. microplus (Tables 4.4 and 4.5).

R. sanguineus is a common tick vector found in dogs from many parts of SEA countries including Thailand, Philippines, Laos and Indonesia (Changbunjong et al., 2009; Durden et al., 2008; Vongphayloth et al., 2016; Ybañez et al., 2012). In addition, R. sanguineus had been reported to harbour pathogens such as E. canis, A. platys, H. canis, R. rickettsii and Rickettsia conorii (Latrofa et al., 2014). It is the main species of ticks collected from dogs in this study. A. bovis, A. phagocytophilum, E. canis and Ehrlichia spp. (E. chaffeensis/ E. ewingii) were detected in R. sanguineus in this study (Table 4.4 and Section 4.7).

5.2 Detection and identification of Anaplasmataceae DNA in ticks

This study shows that a total of 61.5% ticks infesting livestock (cattle and sheep), 28.8% ticks collected from vegetation and small animals in the forest areas and 37.0% ticks infesting peri-domestic animals in aboriginal villages were PCR-positive for Anaplasmataceae DNA. Of the 17 amplified fragments obtained from 14 cattle ticks (12 H. bispinosa and two R. microplus) and three H. bispinosa sheep ticks, sequences matching to those of A. marginale, A. bovis, A. platys and A. phagocytophilum were obtained. Additionally, a representative of one, two and one 16S rDNA sequences obtained from cattle ticks were found to match with those of Ehrlichia sp. strain EBm52 (Parola et al., 2003), E. mineirensis (Cabezas-Cruz et al., 2012) and Candidatus E. shimanensis (Kawahara et al., 2006), respectively. A. bovis was identified from ticks infesting peri-domestic animals in aboriginal villages, while both A. phagocytophilum
and *A. platys* were identified from questing ticks and a tick collected from a rat in the forest areas (Table 4.4). The significance of the detection of each *Anaplasma* spp. and *Ehrlichia* spp. in ticks and animals is discussed in the following sections.

### 5.3 Molecular detection of *A. marginale* in tick and animal samples

*A. marginale* is known to infect cattle and *R. microplus* ticks from various parts of the world (Aubry and Geale, 2011; Kocan et al., 2010). In this study, sequence analyses of representative PCR-positive samples confirmed the presence of *A. marginale* in cattle, *R. microplus* and *H. bispinosa* ticks (Tables 4.4 and 4.7). However, the presence of other *Anaplasma* spp. closely related with *A. marginale* such as *A. centrale* and *A. ovis* cannot be ruled out as these organisms share similar 16S rRNA sequences (Figures 4.10 and 4.23). This result is in agreement with a pilot study carried out previously whereby cattle blood samples and *R. microplus* ticks collected from an livestock farm were tested positive for *A. marginale* and other closely related species (Tay et al., 2014). In addition, the presence of *A. marginale* (in cattle and *R. microplus* tick) and *A. centrale* (in cattle only) are further confirmed by the sequence analyses of the long 16S rDNA fragments (Figures 4.12 and 4.24). The findings in this study are thus consistent with reported cases of *A. marginale* in ruminants (cattle and water buffaloes) and *R. microplus* ticks from Philippines (Ochirkhuu et al., 2015; Ybañez et al., 2013b) and Thailand (Saetiew et al., 2015).

For the first time in Malaysia, *A. marginale* (potentially *A. centrale* and *A. ovis*) has been detected in *H. bispinosa* ticks collected from cattle. Previously, *B. bovis* (another tick-borne pathogen) had been reported in *H. bispinosa* collected from cattle in Malaysia (Kho et al., 2015a). But the role of *H. bispinosa* as a vector requires further investigation as the DNA detected might have come from host blood ingested by the ticks. Experimental transmission studies are needed to confirm the vector competent of *H.*
bispinosa for transmission of A. marginale. Besides that, phylogenetic analyses (Figures 4.10, 4.12 and 4.23-4.24) based on 16S rRNA gene also showed that only one sequence type of A. marginale was detected in this study and the organism was grouped in one cluster with A. marginale in the R. microplus ticks from Philippines. This suggests that a similar strain of A. marginale is circulating in SEA countries.

Cattle is known to develop persistent A. marginale infections which may explain for the high detection rate of Anaplasmataceae DNA (60.7%) in the blood samples of apparently healthy cattle examined in this study (Table 4.7). Persistently infected cattle (also known as carrier cattle) are characterised by a low level of bacteraemia in blood, life-long immunity and the cattle usually do not develop clinical disease except under stress or immunosuppressed (Kocan et al., 2010). Due to the high prevalence of anaplasmosis detected in cattle and ticks, implementation of tick control or vaccination for cattle is important as the animal health and production can be affected by bovine anaplasmosis.

5.4 Molecular detection of A. bovis in tick and animal samples

A. bovis has been reported to infect a wide variety of mammalian hosts and tick vectors in various parts of the world (Table 2.1) (Atif, 2016; Battilani et al., 2017). In Asia, A. bovis has been detected in animals (including dogs, deer, goats, cattle, cats, rodents and wild boars) and ticks (including H. longicornis, H. shimoga, H. megaspinosa, H. bispinosa, Haemaphysalis langrangei, Dermacentor niveus and R. sanguineus) from China, Bangladesh, Japan, Korea, India, Taiwan and Thailand (Table 2.2). Based on sequence analyses of the short 16S rDNA sequences in this study, A. bovis DNA has been detected in a variety of animals (including goats, deer, monkeys, wild boar, cow, monitor lizard, buffalo, bats and squirrel (Tables 4.7 and 4.10); ticks [H. bispinosa (collected from cattle, goat and sheep), R. sanguineus (collected from dog) and A. varanense (collected
from snakes]) and questing ticks (H. hystricis, Haemaphysalis spp. and Dermacentor spp.) (Tables 4.4-4.6). These findings imply that A. bovis is a common species spreading across animals and ticks in Malaysia.

In recent years, A. bovis DNA has been detected in various Haemaphysalis species especially in Asian countries, suggesting the potential role of Haemaphysalis ticks as vector for A. bovis. A. bovis has been detected in H. bispinosa cattle tick and R. sanguineus dog tick in Bangladesh (Qiu et al., 2016). In this study, a pool of R. sanguineus dog ticks collected from an aboriginal village was positive for A. bovis (Table 4.4). R. sanguineus ticks are commonly known as the vector for E. canis (and potentially A. platys) which cause infection in dogs (Dantas-Torres, 2008). Their role in transmitting A. bovis is unknown. As dogs have been reported to be infected by A. bovis in Japan (Sakamoto et al., 2010), it is postulated that R. sanguineus ticks might acquire the organism during blood feeding on infected dog hosts.

Another interesting finding in this study is the presence of A. bovis DNA in the A. varanense snake ticks. This represents the first detection of A. bovis in Amblyomma ticks in Malaysia and Asia. A. bovis has been previously reported to infect Amblyomma ticks in Africa (Dumler et al., 2001). As Anaplasma spp. have been reported in reptiles including sand lizard, Northern alligator lizard, Pacific gopher snake and common garter snake (Ekner et al., 2011; Nieto et al., 2009), it is speculated that reptiles may play a role in the transmission cycle of A. bovis. Sequence analysis of Anaplasma 16S rRNA gene also confirmed the presence of A. bovis in several species of questing ticks (H. hystricis, Haemaphysalis spp. and Dermacentor spp.) collected from the forest areas, thus providing evidence on the existence of Anaplasma spp. in ticks collected in our forest. It is possible for livestock and wildlife to get infected upon exposure to these Amblyomma ticks.
Based on the analysis of the amplified 16S rRNA partial gene fragments from different animal and tick samples, *A. bovis* can be differentiated into two sequence types (*A. bovis* type I and *A. bovis* type II), based on one nucleotide difference in the sequences (Figures 4.20 and 4.31). *A. bovis* type I had a sequence variation (A →T) in the amplified fragments of 16S rRNA gene region as compared to *A. bovis* type II. Preliminary results showed that *A. bovis* type I was detected in mostly monkeys, monitor lizard, organ tissues of small mammals collected from the forest areas and ticks collected from the rural and the forest areas while *A. bovis* type II was more prevalent in domestic animals and ticks collected from domestic animals (goat, deer, cow) and ticks collected from sheep and cattle (livestock farms) (Tables 4.4-4.7, 4.10). The significance noted in the strain differentiation of *A. bovis* (two sequence types) is yet to be determined.

As *A. bovis* has not been successfully cultivated *in vitro*, little is known about its biology and virulence properties. Hence, further investigation on the zoonotic potential (despite no human infections has been reported yet) of *A. bovis* is needed.

5.5 Molecular detection of *A. platys* in tick and animal samples

*A. platys* has been known to show unique tropism for dog platelets, and hence, is the aetiologic agent for infectious canine cyclic thrombocytopenia (Rar and Golovljova, 2011). In this study, *A. platys* was detected from cattle, deer and ticks (*H. bispinosa, A. helvolum, A. varanense, D. atrosignatus, H. hystricis* and *Haemaphysalis* spp. collected from cattle, snake, rat and questing ticks in the forest areas) (Tables 4.4 and 4.7). *A. platys* has been previously reported in Bactrian camels and red deer in China; red foxes in Portugal; cat in Thailand (Cardoso *et al.*, 2015; Y. Li *et al.*, 2015; Li *et al.*, 2016; Salakij *et al.*, 2012) while a number of *Anaplasma* species closely related to *A. platys* have been reported in ruminants (cattle, goats and sheep) from Italy and China (Liu *et al.*, 2012; Zobba *et al.*, 2014). These findings suggest that *A. platys* has the ability to infect a wider...
range of hosts than is initially thought. The role of vertebrate species either as accidental hosts or maintenance hosts requires future investigation through experimental transmission studies.

Apart from the report in *R. sanguineus* ticks, *A. platys* has also been reported in *Haemaphysalis* ticks (*H. longicornis* and *H. flava*), *D. auratus* and *Ixodes nipponensis* ticks from Thailand and Korea (Chae et al., 2008; Parola et al., 2003). As human infection caused by *A. platys* has been reported in two women from Venezuela (Arraga-Alvarado et al., 2014), further studies are required to determine the zoonotic potential of *A. platys*.

### 5.6 Molecular detection of *A. phagocytophilum* in tick and animal samples

*A. phagocytophilum* has been documented as the causative agent for human granulocytic anaplasmosis (HGA) (Dumler, 2005). Previously, a low seroprevalence (6.9%) of *A. phagocytophilum* has been detected in the indigenous people in Malaysia (Koh et al., 2018). At the moment there is no report on the molecular detection of *A. phagocytophilum* in Malaysia.

In this study, *A. phagocytophilum* DNA was detected in various animal blood samples (cattle, deer, dog, monkey, goat and monitor lizard and organ samples of small mammals collected from the forest areas including bats and a squirrel using PCR assays targeting the short 16S rRNA and *msp4* genes of *Anaplasma* spp. (Tables 4.7 and 4.9; Section 4.7). *A. phagocytophilum* was also detected from ticks infesting dog, chicken, sheep, goat and cat (*R. sanguineus, H. bispinosa, H. wellingtoni* and *Haemaphysalis* spp.) and questing ticks (*D. atrosignatus, Dermacentor* spp. and *Haemaphysalis* spp.) (Tables 4.4 and 4.6).

This study presents the first report of *A. phagocytophilum* in animal and tick samples in Malaysia. Although the presence of *A. phagocytophilum* has not been reported in other SEA countries, evidences of its presence are available in animals and ticks.
collected from China, Japan, Korea and Taiwan (Table 2.2). Previously, Anaplasma spp. (undifferentiated between A. platys and A. phagocytophilum) have been detected from cattle and ticks in a pilot study in Malaysia (Tay et al., 2014) and cattle from Philippines (Ybañez et al., 2013c). Interestingly, this study also confirms the presence of A. phagocytophilum in questing ticks collected from the forest areas and ticks collected in animals from livestock farms and aboriginal villages (Table 4.4 and 4.6). Ixodes ticks (especially I. ricinus, I. pacificus, I. scapularis and I. persulcatus) have been shown to be competent vectors of A. phagocytophilum and play a role in the transmission of the organism to humans and animals (Rar and Golovljova, 2011). In this study, A. phagocytophilum has been detected in Dermacentor and Haemaphysalis ticks collected from vegetation and animals (Table 4.4), however the role of these ticks in carrying pathogenic A. phagocytophilum strains requires further investigation.

In recent years, A. phagocytophilum has been detected in ruminants including cattle, sheep and goats (Rar and Golovljova, 2011). In this study, only one H. bispinosa (collected from sheep), one H. wellingtoni (collected from goat), a cattle and a goat were positive for A. phagocytophilum DNA (Tables 4.4, 4.6-4.7). Majority of the Anaplasma spp. detected in ruminants (mostly cattle) was A. marginale. This might be due to PCR bias as only the abundant species in a mixed DNA sample would be amplified.

It was unfortunate that only short fragments of 16S rRNA and msp4 genes were available for the identification of A. phagocytophilum in this study, as the attempts of amplifying longer 16S rRNA and msp4 genes from A. phagocytophilum-positive samples were not successful. Troubleshooting of the relevant PCR assays should be carried out to solve this problem, especially for further conformation and characterisation of A. phagocytophilum detected in this study.

Previous studies reported that A. phagocytophilum strains might have preferential hosts for multiplication and survival. For example, A. phagocytophilum strain (Ap-
Variant 1) detected in white-tailed deer has not been associated with human disease and it cannot cause an infection in mice, while human pathogenic variant (Ap-ha) can cause infection in human, cattle, sheep, horses, dogs, *I. ricinus*, *I. scapularis*, *I. persulcatus* and *Ixodes ovatus* (Jin et al., 2012; Rar and Golovljova, 2011; Stuen et al., 2013). This information will be beneficial for public health authority and epidemiologist in the investigations of outbreaks caused by *A. phagocytophilum*.

5.7 Molecular detection of *Candidatus A. pangolinii* in pangolins

To the best of our knowledge, this is the first evidence of the detection of *Anaplasma* DNA in pangolins (*M. javanica*). *Anaplasma* spp. have previously been detected in both *A. javanense* ticks from Thailand (Parola et al., 2003) and *H. hystricis* ticks from Taiwan (Khatri Chhetri et al., 2016) collected from pangolins. The nearly full length 16S rDNA sequences (1439 bp; GenBank accession no.: KU189193) obtained from the pangolin blood samples in this study demonstrated the closest similarity with *A. bovis* (97.7%) and next closest to *A. phagocytophilum* (97.6%) and *A. platys* (97.5%). The sequence divergence of 2.3% (in comparison with *A. bovis*) is greater than that 1.0% of the cut off value required for delineation of a new species based on 16S rRNA gene (Janda and Abbott, 2007). In addition, phylogenetic analysis also showed that the pangolin-associated *Anaplasma* sp. is segregated on a distinct branch amongst members of the genus *Anaplasma* and *Ehrlichia* (Figure 4.24). It is believed that it could be a potential novel *Anaplasma* sp. and the species was tentatively named as “*Candidatus Anaplasma* pangolinii” in accordance with the host where it was derived.

Interestingly, a portion of the 16S rRNA gene of *Candidatus A. pangolinii* (GenBank accession no.: KU189193) was found to be 99.9% matching (954 nt/955 nt) with the 16S rRNA gene of *Anaplasma* sp. strain AnAj360 (amplified from *A. javanense* collected from a pangolin in Thailand, a neighbouring country of Malaysia) (Parola et al.,
2003). *A. javanense* has been reported to infest three Asian pangolins (*M. javanica*, *Manis pentadactyla* and *Manis crassicaudata*) (Mohapatra et al., 2015). Hence, there is a high possibility that *A. javanense* tick is the maintenance vector for *Candidatus* A. pangolinii detected in this study. However, there are also possibility that the organism was merely present in the blood of the infected pangolins. Further investigation is necessary to establish the potential host-vector relationship of *A. javanense* ticks, pangolins and *Candidatus* A. pangolinii. Further studies on the pathogenicity and zoonotic potential of this organism merits further investigation.

5.8 **Detection of *A. phagocytophilum* and *E. canis* in ticks and dog blood samples**

Little is known about anaplasma and ehrlichial infections in animals in Malaysia. Serological detection of *E. canis* in dog samples in Malaysia was reported by Rahman et al. (2010) where 15.0% of dogs tested were serologically positive by using indirect fluorescent antibody test (IFAT). A recent study reported that 56.7% of dogs from East Malaysia investigated were seropositive to *A. platys* and *E. canis* by using a commercial test kit (SNAP 4Dx) (Mohammed et al., 2017). In this current study, serological analysis of dog blood samples was carried out by using a commercial test kit (SNAP 4Dx, IDEXX, Laboratories, ME, USA). Antibodies against *E. canis* were detected in 39.5% of the dogs (Section 4.7). This finding suggested that *E. canis* is a common vector-borne pathogen found in dogs investigated in this study. *E. canis* has also been investigated in other Asian countries including Thailand (IFA; 71.0%), Philippines (ELISA; 59.0%), Taiwan (SNAP 4Dx; 9.9%), Hong Kong (SNAP 4Dx; 4.0%), India (dot-ELISA; 86.9%), China (SNAP 4Dx; 2.2%) and Russia (SNAP 4Dx; 2.4%) with variable seropositivities (Baticados and Baticados, 2011; Kottadamane et al., 2017; Suksawat et al., 2001; Volgina et al., 2013; Wong et al., 2011; Xia et al., 2012; Yuasa et al., 2012).
A low seroprevalence of *A. phagocytophilum* was detected in four (9.3%) dogs which were also co-infected with *E. canis* (Section 4.7). This is the first report on the seroprevalence of *A. phagocytophilum* in Malaysian dogs. Previously, serological evidences of *A. phagocytophilum* have been reported in dogs from Thailand (IFA; 58.0%), Korea (SNAP 4Dx; 15.6%), China (SNAP 4Dx; 0.5%) and Russia (SNAP 4Dx; 0.6%) (Suh et al., 2017; Suksawat et al., 2001; Volgina et al., 2013; Xia et al., 2012). The detection of *A. phagocytophilum* and *E. canis* DNA in this study suggests the need for appropriate measures for prevention and control of tick-borne diseases in dogs as *A. phagocytophilum* and *E. canis* may potentially cause human infections (Perez et al., 2006).

Canine ehrlichiosis is one of the most reported tick-borne diseases in SEA countries. *E. canis* has been reported in dogs and ticks from Malaysia, Thailand, Philippines and Cambodia (Table 2.3). The high detection rate of *E. canis* DNA in dog blood samples suggests that canine ehrlichiosis might be prevalent in this region. *E. canis* DNA was obtained in dogs from this study with a higher detection rate (25.5%) as compared to a previous study by Nazari et al. (2013) whereby only 2.0% of the tested dogs from private veterinary clinics and a veterinary teaching hospital were positive. Additionally, *E. canis* has been detected in *R. sanguineus* ticks collected from dogs in this study for the first time in Malaysia (Section 4.7). This finding provides evidence that dogs can act as a host and *R. sanguineus* is a tick vector for *E. canis* in Malaysia.

*E. canis* has been reported in patients and blood bank donors from Venezuela and Costa Rica, respectively (Bouza-Mora et al., 2017; Perez et al., 2006). Hence, the zoonotic potential of *E. canis* should not be overlooked as *R. sanguineus* can bite human and play a role in the transmission of tick-borne pathogens to human (Gray et al., 2013). As dogs are pet animals in most family in Malaysia and have close contact with their
owners, a nationwide investigation of ehrlichiosis should be carried out in order to assess the impact of the disease to general population.

5.9 Molecular detection of new variants of *Anaplasma* spp. and *Ehrlichia* spp. in tick and animal samples

Due to the advancement in molecular techniques, new genetic variants of *Anaplasma* and *Ehrlichia* have been detected in ticks and animal samples recently (Table 2.2). A few new genetic variants or potentially new species have been detected in ticks (*R. microplus, H. bispinosa, A. varanense* and *Haemaphysalis* sp.) and animal bloods and organ samples (deer, cow, buffaloes, wild boars, bat and squirrel) based on sequence analysis of the 16S rRNA and *groEL* genes in this study. Interestingly, BLAST analysis of sequence obtained from a *Haemaphysalis* cat tick (labelled as SP002-F) in an aboriginal village shows low sequence similarity to the *Anaplasma* reference strains. The amplified 16S rDNA sequence of the *Anaplasma* sp. demonstrated 98% (250 nt/255 nt; GenBank accession no.: KY046288) similarity with *Anaplasma* sp. detected in *H. longicornis* tick from China (GenBank accession no.: JN715833) (Table 4.4 and Figure 4.10). Another interesting finding is the identification of an *Anaplasma* sp. from an *A. helvolum* snake tick (labelled as S4). BLAST analysis of this amplified 16S rDNA sequence showed 100% identity (225 nt/225 nt) with *Anaplasma* sp. detected in a dog from South Africa (GenBank accession no.: AY570539) but only 99% similarity with the *A. phagocytophilum* type strain (GenBank accession no.: U02521) (Table 4.4 and Figure 4.10).

Phylogenetic analysis based on partial *Anaplasma* sp. 16S rRNA gene sequences (approximately 520 bp) amplified from various wild animals (wild boar, bat and squirrel) (Section 4.6.2.3) (Table 4.9) in this study showed clustering of the sequences with *Anaplasma* sp. detected in goat from China (clone Ap20-5a; GenBank accession no.:
KX272643) and *A. phagocytophilum* detected in sheep from China (isolate YC38; GenBank accession no.: KJ782381) (Figure 4.27) but only 96.9-97.6% similarity with *A. phagocytophilum* type strain (GenBank accession no.: U02521). Analyses of the 16S rDNA sequences (approximately 530 bp) from three deer, a cow and two buffaloes (GenBank accession nos.: MG988297-MG988299) in this study reveal the identification of *Candidatus* A. boleense which was first identified in ticks from Bole in the Xinjiang Uygur Autonomous Region, China (Kang et al., 2014) and later in the mosquitoes in China (Guo et al., 2016). To the best of our knowledge, this is the first report of *Candidatus* A. boleense infection in animals. Besides cattle, matching sequence of *Candidatus* A. boleense was also found in buffalo and deer samples in our sequence database (Table 4.9). This finding suggests that *Candidatus* A. boleense is able to infect a variety of domestic animals in the tropics. The clinical relevance of this strain in animals merits further investigation.

Through sequence analysis, the *Ehrlichia* species obtained from cattle ticks in this study can be grouped into three groups with each group demonstrating close genetic relatedness with *Ehrlichia* sp. strain EBm52 (amplified from *R. microplus*), *E. mineirensis* (amplified from *R. microplus*) and *Candidatus* E. shimanensis (amplified from *H. longicornis*, respectively (Table 4.5). To the best of our knowledge, this is the first report of three *Ehrlichia* species in cattle ticks (*R. microplus* and *H. bispinosa*) in Malaysia based on analyses of 16S rRNA, *gltA* and *groEL* gene sequences.

In the past, there were reports on new *Ehrlichia* spp. strains detected in *R. microplus* ticks from Thailand (*Ehrlichia* sp. strain EBm52), China (*Ehrlichia* sp. Fujian) and Tibet (*Ehrlichia* sp. Tibet) (Parola et al., 2003; Wen et al., 2002). New *Ehrlichia* spp. strains have also been reported in different *Haemaphysalis* ticks, for example, *H. longicornis, H. hystricis* and *Haemaphysalis japonica* (Rar and Golovljova, 2011). These findings proved that *R. microplus* and *Haemaphysalis* ticks might be able to harbour
different *Ehrlichia* spp. Additionally, a nearly full length (1325 bp) sequence of 16S rDNA fragment obtained from an *A. varanense* tick collected from a snake (labelled as S3) demonstrated the highest sequence identity of 98.5% (1303 nt/1325 nt) with an *E. ruminantium* isolate Ball3 from South Africa (Table 4.5 and Figure 4.12). The < 99% sequence similarity suggests that the *Ehrlichia* strain could be a new genetic variants of *E. ruminantium*. The zoonotic or veterinary potential of these *Ehrlichia* spp. is yet to be explored. The presence of various *Anaplasma* spp. and *Ehrlichia* spp. in various ticks may pose a potential health threat to domestic animals and possibly humans as well.

5.10 Limitations of this study and future investigation

Ticks were not collected from all livestock farms as some farms had undergone deticking program prior to tick sampling. Only a small subset of blood samples from stray dogs were subjected for screening of *Anaplasma* spp. and *Ehrlichia* spp. in this study (Section 3.1.5), hence, the true disease prevalences of anaplasmosis and ehrlichiosis in stray dogs are still not known.

The primer sets used for PCR assays in this study were not specific enough to differentiate between species of *Anaplasma* spp. and *Ehrlichia* spp., for example *A. marginale*, *A. centrale* and *A. ovis*; *E. chaffeensis* and *E. ewingii* are closely related species that shared almost similar sequences in the 16S rDNA fragments. Additionally, due to potential PCR bias, PCR assays tend to amplify the most abundant organism present in a mixed sample. As a result, some other organisms can become undetectable in the samples (those with lower abundance). A PCR incorporated with DNA hybridisation assay, for example PCR-RLB (Bekker *et al*., 2002) will be useful in this case where more than one *Anaplasma* spp. or *Ehrlichia* spp. in the tick or animal blood samples can be detected simultaneously. Besides that, morphological identification of ticks to the species level is difficult and challenging without the aid of molecular tools.
Hence, there is a need to build expertise in this area to provide better morphological identification of ticks in the future.

*A. bovis* has been detected in a wide range of animals and tick samples collected in this study. Future work in cultivation of *A. bovis* strain is needed for a better understanding of the pathogenicity of the bacterium. In view of the high detection rate (60.7%) of *A. marginale* in cattle blood samples, development of vaccines (which is important to reduce anaplasmosis in livestock) will be desirable. In addition, next generation sequencing approach can be applied for whole genome determination of *Anaplasma* spp. and *Ehrlichia* spp. This will provide further insight on the biology and pathogenicity of these interesting tick-borne pathogens.
CHAPTER 6: CONCLUSION

The molecular investigation in this study show that *Anaplasma* spp. and *Ehrlichia* spp. are potential tick-borne pathogens circulating in Malaysia as the organisms were detected in various animal and tick samples from different sources (livestock farms, the forest areas and aboriginal villages). The use of molecular techniques (PCR followed by sequencing approach) has enabled the identification of various *Anaplasma* spp. and *Ehrlichia* spp. in animal and tick samples in this study. With the detection of *Ehrlichia* spp. from various ticks (*R. microplus* and *H. bispinosa* from cattle ticks; *D. atrosignatus* and *Haemaphysalis* sp. from vegetation), this study provides some explanation on the high seropositivity to *Ehrlichia* spp. noted amongst indigenous villagers and livestock farm workers in a recent serological study (Koh *et al.*, 2018).

Based on literature review, *A. phagocytophilum* is the most important human pathogen amongst *Anaplasma* spp. The detection of *A. phagocytophilum* in the infected dog bloods and various tick species collected from the forest areas and aboriginal villages suggest possible risk to people who are exposed to tick bites. *A. platys* is another emerging human pathogen which could be transmitted through ticks infesting dogs and other animal hosts. The detection of *A. bovis* from a wide variety of animal and tick samples also raises concern on its potential role as a human or animal pathogen. *A. phagocytophilum, E. canis* and *Ehrlichia* spp. (*E. chaffeensis/ E. ewingii*) are detected from infected dogs in this study. Additionally, findings are also obtained in this study regarding new sequence variants of *Ehrlichia* spp. in cattle ticks (*H. bispinosa* and *R. microplus*), a novel *Anaplasma* sp. in the blood samples of pangolins and a closely related species of *E. ruminantium* in snake tick. The high infection rates of *A. marginale* in cattle and ticks may affect the meat and milk production in livestock farms. As ticks play an important role as a maintenance host for anaplasmosis and ehrlichiosis, appropriate measures should be instituted to reduce tick populations and to prevent exposure to ticks.
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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of published papers:


List of papers presented:


- Koh Fui Xian, Ong Bee Lee, Chandrawathani Panchadcharam and Tay Sun Tee. (2013) Molecular detection of *Ehrlichia* and *Anaplasma* species in domestic animals and tick vectors in Malaysia. 24th International Conference of the World Association for the Advancement of Veterinary Parasitology, Australia, pg. 644 (International).