MOLECULAR SURVEY OF *COXIELLA BURNETII* IN VETERINARY SAMPLE AND TICKS IN MALAYSIA

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ABSTRACT

Coxiella burnetii, the causative agent of Q fever, is an intracellular bacterium of medical and veterinary importance. The reservoirs of *C. burnetii* are extensive which include mammals and arthropods, particularly ticks. The incidence of Q fever in Malaysian population is rarely reported due to the lack of diagnostic facilities. As the organism is difficult to culture, the objective of this study is to use molecular methods, i.e., polymerase chain reaction (PCR) and sequence analysis to determine whether *C. burnetii* is present in the veterinary samples (milk, vaginal swab and blood samples of domestic livestock) and ticks collected from wildlife, livestock and those from the vegetation. Screening for *C. burnetii* DNA was conducted using two conventional PCR methods, targeting the transposon like gene *IS1111* (Trans-PCR) and the *com1* gene (OMP-PCR). The PCR findings were confirmed based on sequence analysis of the amplified fragments or by using a real-time PCR assay. In this study, a total of 173 ticks were examined for the presence of *C. burnetii* DNA. Ten tick samples (5.8%) were tested positive using Trans-PCR assays and five tick samples (2.9%) were tested positive using OMP-PCR assays. Positive amplification results were obtained from *Amblyomma* spp.*, Dermacentor* spp.*, Rhipicephalus* spp. and *Haemaphysalis* spp. ticks. Of 59 milk samples collected from cattle, 17 samples (28.8%) were tested positive by Trans-PCR assays, but none by the OMP-PCR assays. Of 180 vaginal swabs collected from cattle, sheep and goats, 22 samples (12.2%) were positive by Trans-PCR assays and 12 (6.7%) were positive by OMP-PCR assays. Of 103 blood samples collected from cattle, five samples (4.9%) were tested positive by Trans-PCR assays, but none by the OMP-PCR assays. Of the animal samples, the highest percentage of *C. burnetii* DNA-positive samples from domestic livestock was derived from milk and vaginal samples whereas the lowest percentage was detected in blood samples. The assay targeting the transposon-like gene *IS1111* was more sensitive in detecting *C. burnetii* DNA.

Sequence determination of the amplified products confirmed the PCR findings. In view of the detection of *C. burnetii* DNA in the veterinary and tick samples, awareness for prevention and control of the possible transmission of *C. burnetii* infection to the local population is necessary.

ABSTRAK

Coxiella burnetii, agen penyebab demam Q adalah bakteria intrasel yang mempunyai kepentingan perubatan dan veterinar. Reservoir *C. burnetii* adalah luas termasuk mamalia dan arthropod, khasnya sengkenit. Kes demam Q di Malaysia jarang dikesan kerana kekurangan fasiliti diagnostik. Oleh kerana organisma ini adalah sukar untuk dikultur, objektif kajian ini adalah untuk menggunakan kaedah molekular seperti asai "Polymerase Chain Reaction" (PCR) dan analisis jujukan untuk menentukan sama ada *C. burnetii* hadir dalam sampel veterinar seperti darah, calitan vagina, susu dan juga sengkenit dari hidupan liar, ternakan, anjing dan tumbuhan. Kajian ini telah dijalankan dengan menggunakan kaedah PCR konvensional. Asai PCR yang digunakan menyasarkan gen "Transposon-like" *IS1111* (Trans-PCR) dan gen *com 1* (OMP-PCR) daripada *C. burnetii.* Dalam kajian ini, sebanyak 173 sengkenit telah diperolehi dan sepuluh sampel (5.8%) telah diuji positif dengan menggunakan asai Trans-PCR dan lima sampel (2.9%) telah diuji positif dengan menggunakan asai OMP–PCR. Hasil yang positif telah diperolehi daripada sengkenit yang dikenalpasti sebagai *Amblyomma* spp., *Dermacentor* spp., *Rhipicephalus* spp. dan *Haemaphysalis* spp.. Di antara 59 sampel susu lembu yang diuji, 17 sampel (28.8%) didapati positif dengan asai Trans-PCR. Daripada 180 calitan vagina lembu, biri-biri dan kambing, 22 (12.2%) sampel adalah positif dengan asai Trans-PCR dan 12 (6.7%) adalah positif dengan asai OMP-PCR. Daripada 103 sampel darah lembu dan kambing, lima sampel (4.9%) telah didapati positif dengan asai Trans-PCR. Bagi sampel veterinar, peratusan tertinggi *C. burnetii* DNA dikesan dalam susu dan calitan vagina manakala peratusan terendah telah dikesan dalam sampel darah. Asai penyasaran terus gen transposon *IS1111* adalah lebih sensitif dalam mengesan DNA *C. burnetii* kerana ia wujud dalam berbilang salinan berbanding dengan gen *com 1* yang hanya mempunyai salinan tunggal. Penentuan jujukan daripada produk yang telah diamplifikasi mengesahkan hasil penemuan PCR. Pengesanan *C.*

burnetii dalam sampel veterinar dan sengkenit dalam kajian ini menunjukkan perlu adanya kesedaran di kalangan masyarakat tempatan untuk pencegahan dan kawalan penularan jangkitan *C. burnetii*.

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List of Symbols and Abbreviations

-	Negative
%	Percentage
+	Positive
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
17kD	17 kilodaltons antigenic protein gene
ACCM	Acidified Citrate Cysteine Medium
BLAST	Basic Local Alignment Search Tool
C. burnetii	*Coxiella burnetii*
CFS	Chronic Fatigue syndrome
Ct	Cycling threshold
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
EDTA	Ethylene-Diamine-Tetra-Acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ID	Infectious Dose
IFA	IF assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IS	Insertion Sequence
LCV	Large Cell Variant
LD	Lethal Dose
LPS	Lipopolysaccharide
min	Minute
MLVA	Multiple-Locus VNTR Analysis
MST	Multi-Spacer Sequence Typing
MVKBT	Makmal Veterinar Kawasan Bukit Tengah
NTC	No Template Control
OD	Optical Density
OMP	Outer Membrane Protein
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
QFS	Post Q Fever Fatigue Syndrome
qPCR	Quantitative Real-Time PCR
RBC	Red Blood Cell
RBCL	RBC Lysis buffer
RFLP	Restriction Fragment Length Polymorphism
s	Seconds
SCID	Severe Combined Immuno-deficient mice
SCV	Small Cell Variant
SDS	Sodium Dodecyl Sulfate
spp.	Species
UK	United Kingdom
USA	United States of America
VNTR	Variable Number Tandem Repeat

CHAPTER 1: INTRODUCTION

Coxiella burnetii is the causative agent of Q fever in humans and coxiellosis in animals. It is an obligate intracellular bacteria which belonged to the γ-subdivision of the *Proteobacteria* phylum (Weisburg *et al*., 1989). Q fever exhibits a broad range of clinical presentations in humans, ranging from mild to fatal. It is known to infect a wide range of hosts including ticks, wildlife, domestic pets and ruminants. Coxiellosis in animals are usually asymptomatic but pneumonia, abortion, still birth and delivery of weak offspring in ruminants has been widely reported (Angelakis & Raoult, 2010). Q fever is a zoonotic disease. The causative agent can be transmitted to humans via aerosolized contaminants in farms from birth fluids, thus posing an occupational hazard. *C. burnetii* is also considered a potential bioterrorist agent and is classified as a group B agent by the Centers for Disease Control and Prevention, USA (Madariaga *et al.*, 2003).

Although the true extent of the reservoirs for *C. burnetii* is unknown, the main reservoir for human infection is said to be from the domestic livestock, for example cattle and goats (Babudieri, 1959; Guatteo *et al*., 2007). The main shedding route of *C. burnetii* is via birth products (birth fluids and placenta). It is also shed via vaginal mucus, milk and faeces, (Berri *et al*., 2000), urine (Heinzen *et al*., 1999), and semen (Kruszewska & Tylewska-Wierzbanowska, 1997).

Traditional methods that are used for detection of *C. burnetii* include culturing and serological tests. Isolation of *C. burnetii* is time consuming because it is an obligate intracellular bacterium which requires host cells to replicate. The organism is also hazardous because infection can occur through inhalation of contaminated aerosols. Serological tests are not suitable for investigation of possible routes of dissemination and transmission of the disease as the antibody can persist for several months or years. PCR is a highly sensitive and specific detection method which has been widely used to trace *C. burnetii* in clinical samples.

Polymerase chain reaction (PCR) assay with primers targeting *IS1111*, the repetitive, transposon-like gene has been found to be very specific and sensitive for the detection of *C. burnetii* (Vaidya *et al*., 2008). The gene is a preferred target for PCR assays as it is present in multiple copies (about 7 to 110 copies) within the bacterial genome (Klee *et al*., 2006).

1.1 Objectives

The objectives of this study are:

a. to identify the ticks collected
b. to use molecular methods such as PCR and real-time PCR to determine the occurrence of *C. burnetii* in:
    i. ticks collected from wildlife and domestic livestock
    ii. milk, vaginal swab and blood samples of domestic livestock (cattle and goat), and dogs.

CHAPTER 2: LITERATURE REVIEW

2.1 *Coxiella burnetii*

Coxiella burnetii was initially known as *Rickettsia burnetii* because of its morphological similarity to the *Rickettsia* spp. It is now classified under the phylum *Proteobacteria*, class γ-*Proteobacteria*, order *Legionellales*, family *Coxiellaceae* based on the 16S rRNA gene sequence analysis (Weisburg *et al*., 1989; Waag & Thompson, 2005).

Figure 2.1 is a dendrogram which is constructed based on the 16S rRNA gene sequences of *C. burnetii* and its closest members in the phylum *Proteobacteria*. The complete genome of *C. burnetii* type strain, Nine Mile phase I RSA493, has been determined. It has a 1,995,275- base pair circular chromosome and a single 37,393 base-pair QpH1 circular plasmid (Seshadri *et al*., 2003). The Nine Mile strain also possesses a resident plasmid (QpH1) of 37,393 base pair. The chromosome contains 29 insertion sequences, of which 21 is the unique transposon *IS1111* (Miller *et al*., 2006).

C. burnetii, the etiological agent of Q fever, is a small, obligate intracellular bacterium which replicates exclusively in an acidified, lysosome-like vacuole. The bacterium is a pleomorphic rod-shaped organism with a diameter of approximately 0.2-0.4 μm and 0.4-1.0 μm in length (Drancourt & Raoult, 2005). Although *C. burnetii* has a cell wall structure that resembles other Gram-negative bacteria (Amano *et al.*, 1984), it does not stain well with Gram stain (Baca & Paretsky, 1983).

The unique characteristic of *C. burnetii* is its antigenic phase variation (Stoker & Fiset, 1956). Phase I variant is found in naturally infected animals, whereas Phase II variant is maintained in the laboratories (Maurin & Raoult, 1999). Only phase I bacteria have a complete lipopolysaccharide (LPS) on their surface and this makes them a more virulent bacteria (Moos & Hackstadt, 1987). Phase II bacteria occur during serial

passage in immunologically incompetent hosts, such as cell cultures or fertilized eggs (Setiyono *et al*., 2005). They exist in two structural forms: the metabolically active large cell variant (LCV) and the metabolically dormant small cell variant (SCV) (Heinzen *et al*., 1999). Figure 2.2 shows the transmission electron microscopy of the SCV and LCV variants of *C. burnetii*. While the SCV variant is resistant to extracellular stresses, the LCV is sensitive to environmental stresses (Howe & Mallavia, 2000).

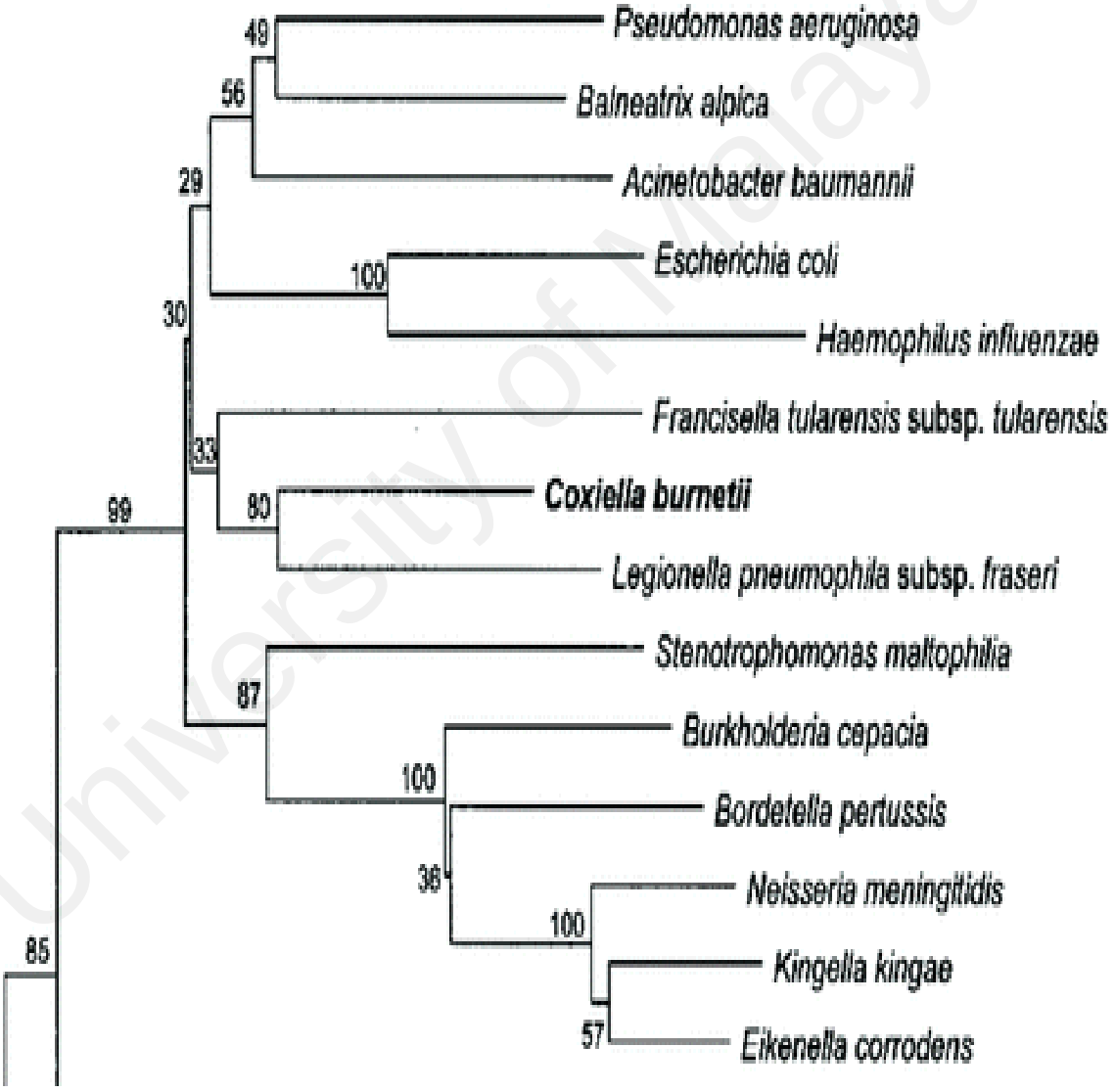

Figure 2.1 : Phylogenetic relationship of bacteria within the phylum proteobacteria. The dendrogram was constructed based on 16S rRNA gene sequences for comparison of *C. burnetii* with its closest member of the proteobacteria phylum (Drancourt & Raoult, 2005)

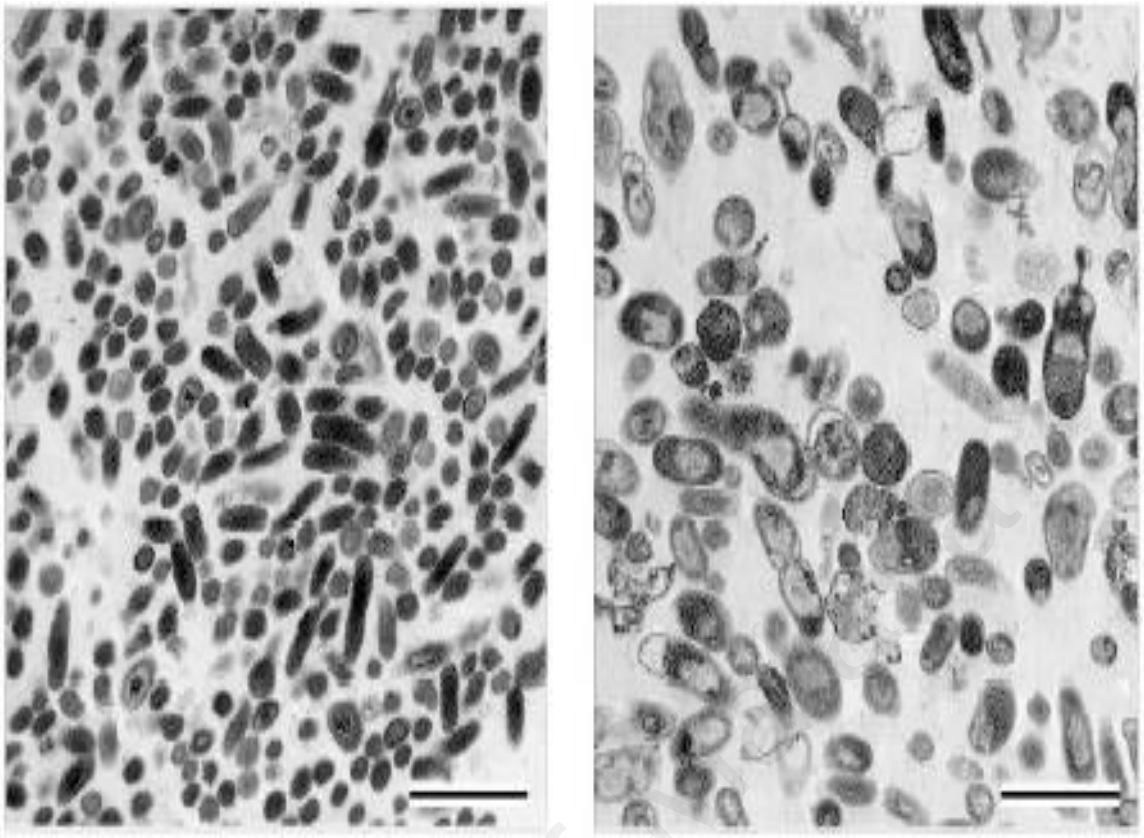

Figure 2.2: The SCV (left image) and the LCV (right image) of *C. burnetii* photographed by transmission electron microscopy (Coleman *et al.*, 2007).

C. burnetii has the ability to withstand harsh condition and is easily transmitted from an host to environmental reservoirs (van Schaik *et al.*, 2013.). The bacterium is able to resist physical and chemical stresses (Ormsbee, 1969). Compared with vegetative bacteria and other rickettsiae, *C. burnetii* is able to resist higher temperature, osmotic shock, ultraviolet light and chemical disinfectant (Scott & Williams, 1990). *C. burnetii is* able to resist heating, drying and sunlight in order to survive outside a host cell. It has been reported that the organism dried on wool can remain infectious for months and tick faeces which are infected with the bacteria has the ability to remain infectious for two years (Marmion, 2009). *C. burnetii* has been recognized as a potential agent of bioterrorism because of its accessibility, low infectious dose, resistance to environmental degradation, and aerosol route of transmission (Groseclose *et al*., 2000).

2.2 Background of Q fever

Q fever was first discovered in a slaughter house in Brisbane, Australia when workers were falling ill from a mysterious disease (Derrick, 1983). As the source of the disease was unknown, the illness was named query (Q) fever. The first description of clinical Q fever ("query fever") was reported in Queensland. Burnet and Freeman (1937) isolated a bacterium with viral and rickettsia-like properties from samples of infected tissue from Australian abattoir workers. In 1936, Cox and Beli (1939) isolated a microorganism from ticks and named it as the Nine Mile agent. The organism investigated in these two independent discoveries was then identified as the same pathogen when Dyer (1939) acquired a Q fever infection in the Rocky Mountain Laboratory in 1938. The febrile illness was reproduced in guinea pigs by inoculating them with Dyer's blood, and rickettsiae were identified in the spleen samples of the infected animals. He then established a definitive link between the Nine Mile agent and the Australian Q fever agent (Maurin & Raoult, 1999).

2.3 Epidemiology

C. burnetii has been reported worldwide. The largest outbreak of Q fever was reported in the Netherland (Schimmer *et al*., 2009) where 982 and 2305 confirmed cases have been reported in 2008 and 2009, respectively. However, the factors leading to outbreaks are not fully understood (Lahuerta *et al*., 2011). From 1999 to 2004, there were 18 reported outbreaks of Q fever from 12 different countries involving two to 289 people (Arricau-Bouvery & Rodolakis, 2005).

Large outbreaks of Q fever have also been reported in Australia (Garner *et al*., 1997), Spain (Errasti *et al*., 1984), Switzerland (Dupuis *et al.*, 1987), Great Britain (Marmion & Stoker, 1950), Germany (Schneider *et al*., 1993) and France (Dupont *et al*., 1992).

Q fever is an occupational disease. Farmers, abattoir workers, meat-packing workers, and laboratory workers in contact with livestock are at high risk of infection (Thomas *et al*., 1995; Casolin, 1999). It has been reported that military personnel who used contaminated hay or stables previously occupied by sheep were at risk of contracting the disease (Spicer, 1978).

Cattle, sheep, and goats are usually the source of human infections. However, cats, dogs, and rabbits are also important (Jacob *et al*., 2013; Brom *et al*., 2015) in this regard. *C. burnetii* localises the uterus and mammary glands in female mammals. During pregnancy, reactivation occurs, and the organism multiplies in the placenta. These organisms are shed in the environment at the time of parturition. Humans become infected after inhaling organisms aerosolized at the time of parturition or later when organisms in the dust are stirred up on a windy day. Infected animals can shed *C. burnetii* in milk or faeces for months (Rodolakis *et al*., 2007).

Many wild mammals and birds have been found to be hosts to the infectious organism (Enright *et al*., 1971; To, Sakai, *et al*., 1998; Astobiza *et al*., 2011). A few cases of transmission of *C. burnetii* from wild animals to humans have been reported but further experimental research is needed to validate this findings (Syrucek & Raska, 1956; To, Sakai, *et al*., 1998).

Figure 2.3 demonstrates the possible routes of *C. burnetii* transmission. The main animal to human transmission is believed to be from goats, sheep and cattle. Pet animals such as dogs, cats and horses pose a moderate risk. While there have been reports of *C. burnetii* in wild animals as previously described, the route to human transmission is not well documented. Although ticks are known to be a natural reservoir, tick-borne transmission to human is considered rare (Dumler, 2002).

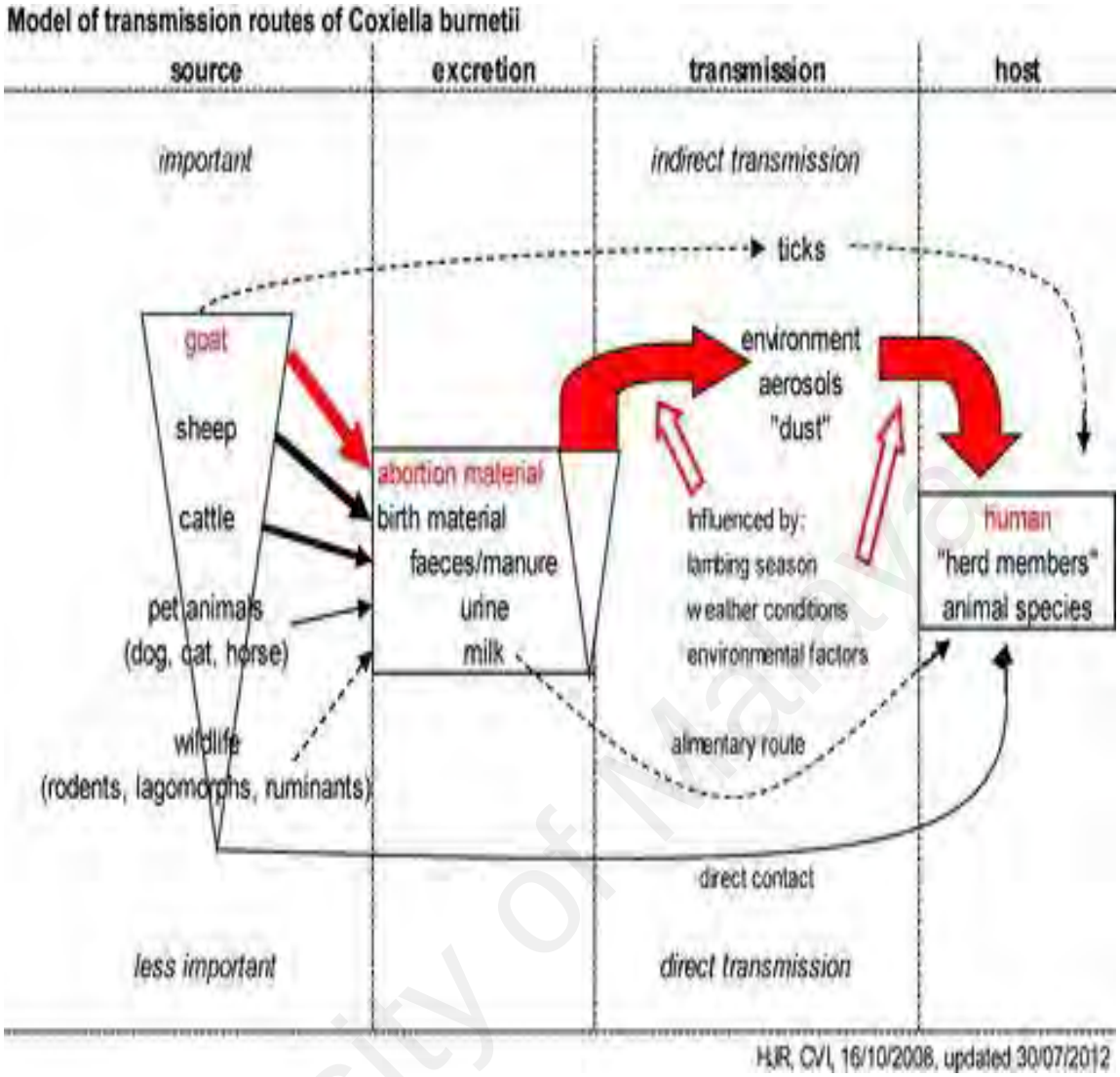

Figure 2.3: Overview of the possible transmission routes of *C. burnetii* from the animal reservoir to the human and animal hosts. The boldness of the arrows indicate the importance of the route, dotted lines indicate possible contributions (Roest *et al*., 2009)

2.4 Q fever

2.4.1 Q fever in humans

Q fever results from the inhalation of contaminated aerosols from infected amniotic fluid or placenta of farm animals such as cattle, goats, and sheep (Woldehiwet, 2004). According to Maurin and Raoult (1999) following exposure to *C. burnetii*, almost 60% of the Q fever cases are asymptomatic. *C burnetii* infections manifest as either acute Q fever, chronic Q fever and post Q fever fatigue syndrome (QFS) (Marmion *et al*., 1996). Alveolar macrophages and other mononuclear phagocytes are

thought to be the primary target cells of this bacterium (Shannon & Heinzen, 2009). A bacteraemia will lead to systemic infection in the humans with the involvement of liver, spleen, lungs and bone marrow (Maurin & Raoult, 1999).

Acute Q fever patients are associated with a wide spectrum of clinical manifestations. The most frequent clinical manifestation is probably a self-limited febrile illness (91%) which is associated with severe headaches (51%), myalgias (37%), arthralgias (27%) and cough (34%) (Dupont & Raoult, 2007). More severe clinical symptoms include fever, headache, chills, atypical pneumonia and hepatitis (Derrick, 1983; Maurin & Raoult, 1999; Raoult *et al.*, 2005)

Chronic Q fever can develop from a primary infection in a small number of patients and the disease manifests years after the initial infection. The most common clinical symptoms of chronic Q fever is endocarditis (Figure 2.4) which may take 10-15 years to develop (Brouqui & Raoult, 2001). Endocarditis is more frequently observed in men over 40 years of age (Brouqui *et al*., 1993). Some patients have been reported to experience non-specific fatigue, fever, weight loss, night sweats and hepato-splenomegaly (Raoult *et al*., 2005; Wegdam-Blans *et al*., 2012). A higher risk to develop chronic infection is reported in pregnant women and patients with heart valve disorders, vascular prosthesis and impaired immunity (Wilson *et al.*, 1976; Karakousis *et al.*, 2006).

QFS is another long-term presentation of Q fever. Contrary to chronic Q fever, *C. burnetii* is not detectable in QFS patients and antibody levels against the bacteria are low or negligible. Symptoms of QFS include prolonged fatigue, arthralgia, myalgia, blurred vision and enlarged painful lymph nodes (Maurin & Raoult, 1999). Acute Q fever with hospitalization was found to be a risk factor for QFS (Morroy *et al*., 2011). The cause for the development of chronic Q fever or QFS in certain individuals is still unknown.

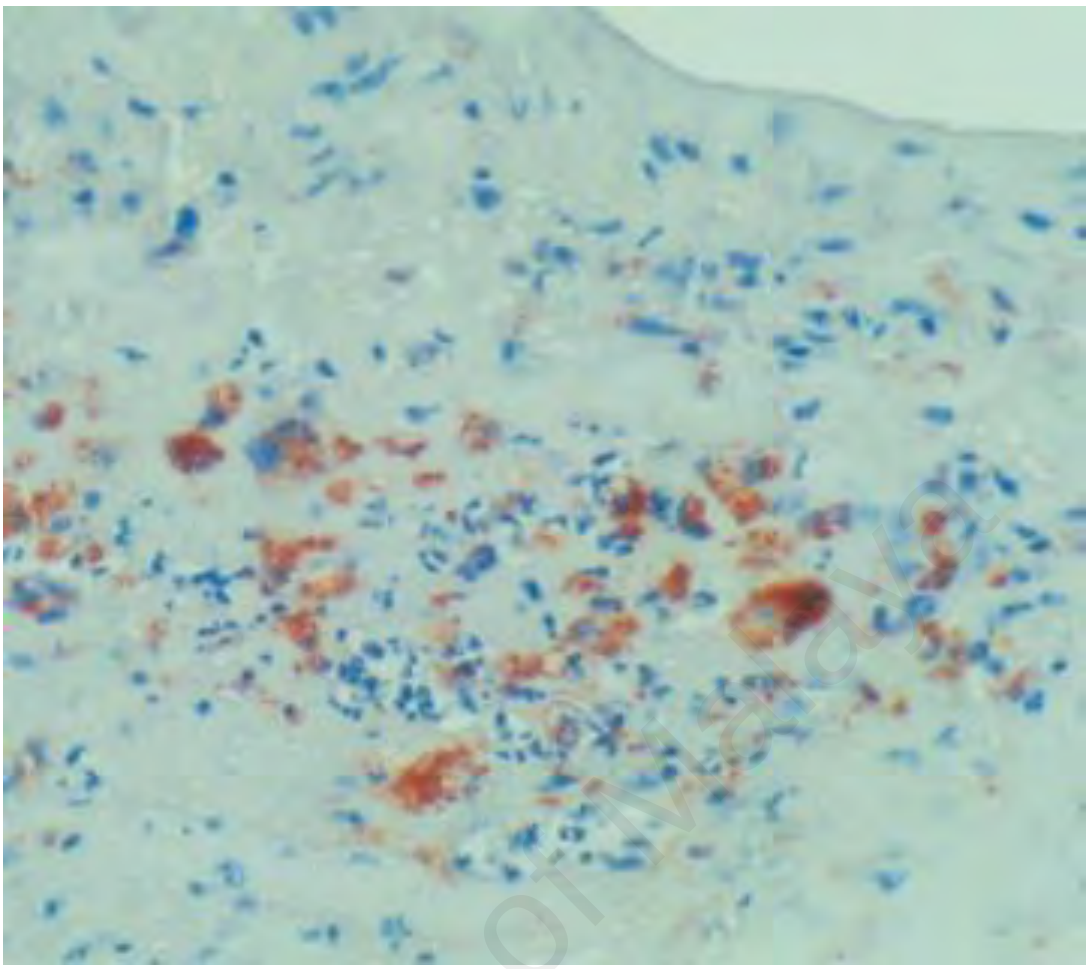

Figure 2.4: Endocarditis caused by *C burnetii. C burnetii* (in red) was detected by immunochemistry in the valve removed from a patient with chronic Q fever. *Retrieved from Maltezou and Raoult (2002).*

2.4.2 Coxiellosis in animals

Unlike acute Q fever in humans, *C. burnetii* infection in animals are usually asymptomatic and is more appropriately termed coxiellosis (Psaroulaki *et al*., 2014; Egberink *et al*., 2014; Brom *et al*., 2015; Jacob *et al*., 2015). The reservoirs for *C. burnetii* are extensive and include mammals, birds, arthropods (Fournier *et al*., 1998), wild animals (Marrie *et al*., 1993) and ruminants (Behymer *et al*., 1976; To *et al*., 1998). Pets, including cats (Higgins & Marrie, 1990; Egberink *et al*., 2013), rabbits (González-Barrio *et al*., 2015), and dogs (Jacob *et al*., 2015) , have also been documented to be the potential sources.

Cats and dogs are suspected as important reservoirs of *C. burnetii* in urban areas (Langley *et al.*, 1988; Buhariwalla *et al.*, 1996). All these mammals, when infected, shed the desiccation-resistant organisms in urine, faeces, milk, and birth products (Bernard *et al*., 1982).

The well-known manifestations of coxiellosis in ruminants are abortion, stillbirth, premature delivery and delivery of weak offspring (Angelakis & Raoult, 2010), particularly in sheep and goats. In cattle, Q fever is frequently asymptomatic, however; it has been reported that some infected cows may develop infertility and mastitis *(To et al*., 1998).

2.5 Ticks as the potential vector and reservoir host for C. burnetii

Ticks are known to be the second most common vector to transmit infectious diseases after mosquitoes (Parola & Raoult, 2001). Ticks are obligate hematophagous arthropods that parasitize every class of vertebrates in almost every region of the world (Sonenshine & Roe, 2013). There are two major tick families: the *Ixodidae*, or hard ticks and the *Argasidae*, or soft ticks (Sonenshine & Roe, 2013). A total of 869 species or subspecies of ticks have been recorded (Camicas *et al*., 1998). Ticks transmits a variety of pathogens affecting livestock, humans and companion animals (Jongejan *et al*., 2004). Ticks may be important in the spreading of *C. burnetii* in the environment because of the high concentration of *C. burnetii* in tick faeces (Parola & Raoult, 2001). More than 40 tick species are naturally infected with *C. burnetii* (Thompson and Dasch, 2005).

Direct transmission of *C. burnetii* to humans from arthropods has never been documented (Thompson *et al*., 2005). In contrast, ticks may play a significant role in the transmission of *C. burnetii* among wild vertebrates (Maurin & Raoult, 1999; Kazan,

2005). Both hard ticks (*Ixodidae*) and soft ticks (*Argasidae*) can be infected with *C. burnetii* (Guglielmone *et al*., 2010).

The significance of ticks in causing human disease in Europe, North America and Africa is well known and a great deal of effort has gone into characterizing the diseases which they transmit. This is not true for Southeast Asia where comparatively little has been published on the medical and veterinary significance of tick-borne diseases (Tanskul & Inlao, 1989). In South-east Asia, *Rhipicephalus haemaphysaloides, Haemaphysalis nadchatrami, Haemaphysalis semermis* and *Boophilus microplus* have been implicated as vectors for Q fever to wild animals (Marchette, 1966; Hoogstraal *et al.,* 1972).

2.6 Methods for detection of *C. burnetii* in clinical and environmental samples

2.6.1 Culture

Isolation of *C. burnetii* is time consuming because it is an obligate intracellular bacterium which requires host cells to replicate. The organism is also hazardous because infection can occur through inhalation of contaminated aerosols. Culture is rarely performed because the isolation of *C. burnetii* must be done only in biosafety level 3 laboratories due to its extreme infectivity (Chosewood & Wilson, 2007).

C. burnetii can be isolated by inoculation of suspected specimens *in vitro* in conventional cell cultures including Vero cells (African green monkey kidney cells) (Baca & Paretsky, 1983; Baca *et al.*, 1985), and mouse L cell (Burton *et al.*, 1978) or into embryonated hen yolk sacs. It also can be inoculated into laboratory animals, such as mice or guinea pigs (Russell-Lodrigue *et al*., 2009). Recently, the successful growth of the organism in a growth medium called acidified citrate cysteine medium (ACCM) has been reported (Omsland, 2012).

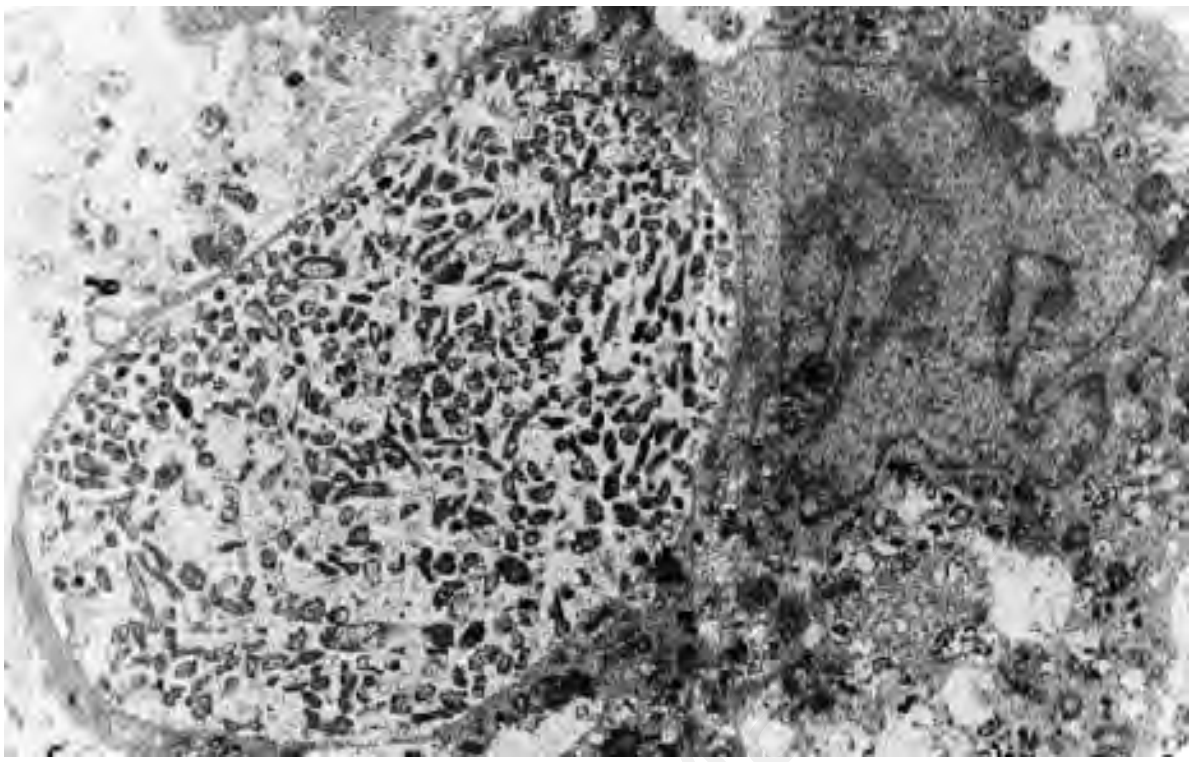

Figure 2.5: Electron micrograph of a Buffalo green monkey cell heavily infected by *C. burnetii* (Baca & Paretsky, 1983*)*.

2.6.2 Serology

Serology is the most commonly used diagnostic test in the clinical microbiology laboratories. As previously mentioned, *C. burnetii* undergoes phase variation characterized by the development of phase I and II antigens. Several methods have been described but the most widely used ones are complement-fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA).

Serodiagnosis of acute Q fever is made by detecting the IgG titres and IgM titres in serum specimens. IgG antibodies are present in serum for more than one year in 90% of the patients, whereas IgM antibodies are present only for 2 weeks and become negative after 2 weeks (Hunt *et al.*, 1983). Serodiagnosis of chronic Q fever in a patient is made by demonstration of high antibody titres against phase I and II antigens. Antibodies against phase I antigen is always higher than phase II (Soriano *et al.*, 1993).

Table 2.1 shows the comparison of the sensitivity and specificity of various serology tests from various studies.

The drawback about serological tests is that the tests are not suitable for investigation of possible routes of dissemination and transmission of the disease. Additionally, serological tests are not able to determine whether the patient has acute or chronic disease because they do not detect differences in *C. burnetii* isolates (Zhang *et al*., 1998). A potential for serological cross-reactivity, with other pathogens such as *Legionella* sp. and *Bartonella* sp. has been reported (Santos *et al*., 2012).

Table 2.1: Sensitivity and specificity of various serology tests

Test	Sensitivity	Specificity	References
Microaggutination	81.6%	98.6%	Nguyen *et al*., 1996
CFT	77.8%	99%	Peter *et al*, 1985
IFA	58.4%	92.2%	Dupont *et al.* , 1994
ELISA	84%	99%	Waag *et al.*, 1995

2.6.3 Immunohistochemistry (IHC) for detection of *C. burnetii*

Immunohistochemistry can be used to detect the presence of *C. burnetii* antigens in formalin-fixed, paraffin-embedded tissues. It is particularly valuable for examining cardiac valve specimens excised from patients with culture-negative endocarditis for whom chronic Q fever is suspected (Lepidi *et al.*, 2003). This assay is useful because it can stain *C. burnetii* bacteria in tissues from patients even after they have received antibiotic therapy.

2.6.4 Molecular methods for detection and genotyping of *C. burnetii*

Early detection of *C. burnetii* is one of the critical points for the control of its spread among animals and transmission from animals to humans. Several molecular methods have been developed to facilitate the detection and genotyping of *C. burnetii.* Polymerase chain reaction (PCR) is a rapid, sensitive and specific molecular detection method that can detect even small numbers of *C. burnetii.* This method can be applied for different matrices such as: milk, semen, urine, faeces, placenta of animals, and aerosols (Brom *et al*., 2015).

The characteristics of PCR (high sensitivity and specificity) has made it very useful for early diagnosis of infection during the period when antibodies are not present (Guatteo *et al*., 2006). The PCR detects not only infectious agents but non-viable agents as well. It is more sensitive than capture ELISA and is much more rapid and convenient than cell culture, in which at least six days of examination is required for diagnostic results (Lorenz *et al*., 1998). Several PCR-based diagnostic methods, such as conventional PCR, nested PCR, or real-time PCR, have successfully been applied for the direct detection of *C. burnetii* DNA in clinical samples.

2.6.4.1 Polymerase chain reaction (PCR)

PCR assays targeting plasmid sequences (QpH1 or QpRS) (Minnick *et al.*, 1990; Minnick *et al.*, 1991) or chromosomal genes such as isocitrate-dehydrogenase (*icd*) (Nguyen & Hirai, 1999), the outer membrane protein-coding gene *com1* (Zhang *et al.*, 1997), the superoxide dismutase gene (*sod*) (Stein & Raoult, 1992), or the insertion element *IS1111* (Greub *et al*., 2005) have been developed. PCR assay with primers targeting insertion element *IS1111* has been found to be very specific and sensitive for the detection of *C. burnetii* (Vaidya *et al*., 2008). The gene is a preferred target for PCR assay because it is present in multiple copies (about 7 to 110 copies) within the bacterial

genome (Klee *et al*., 2006). The PCR assay has been reported to be sensitive and specific using primers Trans3/F (5'-CAA CTG TGT GGA ATT GAT GA-3') and Trans5/R (5'-TTT ACA TGA CGC AAT AGC GC-3') when comparing the results obtained from histological examination and culture on valve samples taken from patients with and without endocarditis (Greub *et al*. 2005).

Additionally, *com 1* gene was also used to differentiate different genotypes of *C. burnetii* (Zhang *et al*., 1998). Specific primers have been designed from a conserved region of the *com1* gene of *C. burnetii* on the basis of the gene sequences of 21 strains (Zhang *et al*., 1997). The sequence specificities of these primers have been checked by using the sequences in the GenBank database, and no homology with the sequences of other viral or bacterial organisms was detected using a search with the BLAST program (Zhang *et al.,* 1997).

Real-time PCR assay provides an additional means of detection and quantification for bacterial DNA (Kim *et al.*, 2005; Klee *et al.*, 2006). As with the conventional PCR, various target genes have been used for detection of *C. burnetii* using real-time PCR approach (Brom *et al*., 2015). Real-time PCR assays offer additional advantage for its ability to provide information on bacterial loads in a sample. Real-time PCR assays can be automated and thus can be used in large scale studies.

In this study a real-time PCR assay was performed as described by Loftis *et al.* (2006) who investigated the presence of *C. burnetii* in Egyptian ticks. The assay which was designed for detection of the *IS1111* insertion sequence in *C. burnetii* has demonstrated superior sensitivity compared to conventional PCR by the detection of one genome of *C. burnetii* in a sample.

2.7 *C. burnetii* infections in Malaysia

The first probable case of human *C. burnetii* infection was reported in 1952 in Selangor, Malaysia (Bush, 1952), and it was thought to be caused by *C. burnetii* contaminated milk samples. Q fever was also reported among local population in Malaysia during a World Health Organization-assisted survey in 1955 (Kaplan & Bertagna, 1955). Antibody towards the organism among villagers in patients with fevers in a health center in East Malaysia (Sarawak) has been reported (Tay *et al*., 1998). Most recently, a zoonotic case of Q fever and a high seropositivity (42.8%) of farm workers, veterinary staff and laboratory staff towards *C. burnetii* in Penang, a northern region of Malaysia have been reported (Bina *et al.*, 2011).

Q fever is not amongst notifiable diseases under the Malaysia Prevention and Control of Infectious Diseases Act (1988). Although domestic livestock (cattle and sheep) in Malaysia are subjected to stringent screening measures by the Veterinary Department, however; the screening program has not included Q fever as one of the target organism (Bina *et al*., 2011). This could be due to the lack of appropriate laboratory tests and facility as the organism is difficult to be cultured. Despite of the cases reported, so far, there has been no intensive study investigating the occurrence of *C. burnetii* in domestic animals and ticks.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection and DNA extraction

3.1.1 Collection of ticks from wildlife and vegetation

Ticks were collected from Krau Wildlife Reserve (KWR), Pahang (N 03º50' E 102º06'), with the help from the staff of the Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN), Kuala Lumpur, Malaysia from March to May 2013. The Krau Wildlife Reserve covers an area of 624 km^2, which makes it the third largest protected area in Peninsular Malaysia (Nurulhuda *et al*., 2014). The forest reserve is located about 66 km from Kuala Lumpur and is drained by three rivers, i.e., Sungai Krau, Sungai Lompat and Sungai Teris. Tick samples were collected from areas adjacent to Sungai Lompat (from 22nd-25th March 2013) and Sungai Teris (from 23rd - 27th May 2013).

A total of 66 ticks were collected from Krau Wildlife Reserve, Pahang, of which 24 ticks were collected mainly from the body of the rodents (Table 3.1). Small mammals such as rodents were first anesthetized using Zoletil, a general anesthesia with minimal side effect to the wildlife. The animals were then checked thoroughly for ticks particularly on the soft tissues. Flea combs were used to brush the animals, while forceps were used to pick ticks from the ears and the paws. A total of 36 vegetation ticks (questing ticks) were collected from the tip of the vegetation by hand. Trekking along forest trails to look for ticks was performed for two hours once in the morning and once in the evening for 6 days. Six ticks found on the bodies of several forest rangers were also included in this investigation.

3.1.2 Collection of ticks from domestic livestock (cattle and goats)

A total of 34 ticks collected from a Department of Veterinary Services (DVS) cattle farm in Negeri Sembilan were included for investigation in this study. The study was carried out with the assistance provided by veterinarian and farmers. Briefly, the cattle were restrained using a head crush, and ticks were then collected using forceps. All the ticks were identified as *Rhipicephalus* (formerly *Boophilus*) *microplus*. A total of 30 ticks given by Makmal Veterinary Kawasan Bukit Tengah (MVKBT) were also used in this study. These ticks were collected by MVKBT staff from 10 goats (*Haemaphysalis* spp.) and 20 cattle (*Haemaphysalis* spp.) from farms suspected of *C. burnetii* infections. The ticks were stored in alcohol tubes and identified by the DVS staff.

Table 3.1: Tick collected from Krau Wildlife Reserve, Pahang

Host	**No of ticks collected**
Rodent	21
Bat	1
Bird	1
Monitor Lizard	1
Human	6
Vegetation	36
Total	**66**

3.1.3 Collection of ticks from strayed dogs

A total of 43 ticks were collected from strayed dogs from two animal shelters (SPCA and Second Chance) (Table 3.2) in Klang Valley. The ticks were removed using forceps from the ears, paws and abdomen of the dogs.

3.1.4 Tick identification and processing

Ticks were either kept in ziplock bags or stored in -80ºC until use. The ticks were individually placed in single tubes containing 70% ethanol. Ticks were observed using a stereo microscope (Olympus, Japan). The dorsal and ventral images of the ticks were taken for documentation purposes.

The ticks were identified morphologically up to genus level based on the taxonomic keys of Walker *et al*. (2003). Table 3.2 summarizes the sampling sites and the source of ticks collected in this study.

Table 3.2: Sampling sites and the sources of ticks collected in this study

Sampling site	Source	No of ticks
Kuala Krau Forest Reserve, Pahang	Small mammals	24
	Vegetation	36
	Forest ranger	6
Negeri Sembilan Cattle Farm	Cattle	34
Makmal Kawasan Bukit Tengah (MVKBT)	Goat	10
	Cattle	20
Dog shelter 1 (SPCA, Ampang)	Dog	13
Dog shelter 2 (Second Chance, Kuala Lumpur)	Dog	30
Total		**173**

3.1.5 Tick DNA extraction

Once the ticks have been identified morphologically, DNA extraction was carried out. The ticks were first washed in 20% (v/v) sodium hypochlorite (Clorox), followed by a dip in 70% (v/v) ethanol and lastly with distilled water to clean up any host tissues as well as any dirt from the surface of the ticks. All the ticks were treated individually except for the ticks from MVKBT which were pooled. 10 ticks were pooled and kept in Eppendorf tubes containing 70% alcohol.

The ticks were then processed using a QIAamp® DNA Mini Kit (Qiagen, Germany). The whole tick was first moistened with 180 µl of ATL buffer and then crushed with the tip of a pipette or disrupted mechanically using a Kontes Pellet Pestle and a cordless motor (Thermo Fisher Scientific, USA). A volume of 20 µl of proteinase K (provided by the kit) was added to the sample and incubated at 56ºC for about an hour until the tissue had been digested, leaving only the exoskeleton. The sample was intermittently vortexed. A total of 200 µl Buffer AL (lysis buffer) was then added to the sample, and vortexed. The sample was incubated at 70°C for 10 min and added with 230 µl of absolute ethanol.

The mixture was carefully pipetted into a spin column that was provided in the kit and centrifuged at 8000 rpm for 1 min. The flow-through was discarded and 500 µl Buffer AW1 (wash buffer) was added prior to centrifugation at 8000 rpm for 1 min. The flow-through was discarded and 500 µl Buffer AW2 (wash buffer) was added. The column was centrifuged at a maximum speed (13400 rpm) for 3 min. Finally, a volume of 30 µl of Buffer AE was added directly onto the spin column. After incubation at room temperature for 1 min, the column was centrifuged at 8000 rpm for 1 min to elute the DNA. All extracted DNA was stored at -20ºC prior to PCR amplification.

3.1.6 PCR assay for amplification of 28S rRNA gene of ticks

The PCR assay was carried out for two purposes. First, it was used to confirm that the DNA extraction was successful; and second, to identify ticks based on the sequence analysis of the amplified fragments. As identification of immature ticks or damaged mouthparts based on morphology can be difficult, analysis of the sequence obtained through BLAST analysis will be useful for tick identification.

Table 3.3 shows the composition of the PCR reagents used in this study. PCR assay was prepared by adding 2 µl of tick DNA sample to 19.4 µl sterile distilled water, 2.5 µl 10x DreamTaq™ buffer (Fermentas, Lithuana), 0.5 µl dNTPs (10 µM), 0.2 µl of each primer (25 µM), and 0.2 µl DreamTaq™ DNA Polymerase (5 U/µl) (Fermentas, Lithuana).

Primers 28SF (5'-GAC-TCT-AGT-CTG-ACT-CTG-TG-3') and 28SR (5'-GCC-ACA-AGC-CAG-TTA-TCC-C-3') were used in the PCR assay. These primers had been designed based on the alignment data of the 28S rRNA gene sequences of *Haemaphysalis* spp., *Rhipicephalus* spp., and *Ixodes* spp. ticks (Inokuma *et al*., 2003).

Amplification was performed in a MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA) with an initial denaturation step at 95º for 5 min, followed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 30 seconds. The PCR program was ended with a final extension at 72ºC for 5 min.

The expected size of the amplicon is about 490 base pair in length. Confirmation of the genus of the ticks was through sequence determination of the 28S rRNA gene region of the ticks. The gene sequence was searched for similarity with those available in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information, National Institute of Health).

Table 3.3: PCR reaction mixture used for amplification of the 28S rRNA gene of ticks

Reagents	Concentration	Volume (µl per reaction)
DNA template		2
DreamTaq™ buffer	10X	2.5
dNTPs	10 µM	0.5
28SF Primer	25 µM	0.2
28SR Primer	25 µM	0.2
DreamTaq™ DNA Polymerase	5 U/µl	0.2
Sterile distilled water		19.4
Total		**25.0**

3.2 Collection and processing of animal samples

3.2.1 Animal blood samples

The animal samples collected were blood, vaginal swabs and milk samples from domestic livestock (cattle and goat/sheep) in 13 farms across Peninsular Malaysia (Table 3.4). Ethical approval for animal care and use was obtained from Faculty of Veterinary Medicine, Universiti Putra Malaysia (Reference no. UPM/FPV/PS/3.2.1.551/AUPR164). Approval for animal sampling was obtained from the Director, Department of Veterinary Services, Ministry of Agriculture and Agro-Based Industry, Malaysia (Reference no. JPV/PSTT/100-8/1). The samples were collected with the help of a veterinarian or an assistant veterinarian managing the farms. Malaysia. Animals were first restrained and blood samples were then drawn from either the jugular veins or the tail veins. Whole blood samples were collected in EDTA tube to avoid the blood from clotting. The samples were placed in a cooling box and brought to the laboratory as soon as possible. The blood samples were stored at 4°C prior to processing. DNA extraction of blood samples were carried out as soon as possible to avoid the heamolysis of the cells.

DNA of the whole blood samples were extracted using Qiagen Blood Kits (QIAGEN, Germany) according to the manufacturer's protocol. This process was carried out in a Class II, A2 biological safety cabinet (NuAire Inc, USA). All extracted DNA was stored at -20ºC prior to PCR amplification.

3.2.2 Vaginal Swab

Vaginal swabs were collected from nine farms across Peninsular Malaysia (Table 3.4). A total of 180 vaginal swab were collected from cattle (n=120) and goats (n=60). The animals were restrained and the vulvas of the animals were thoroughly cleaned with a tissue paper. Vaginal swabs were collected using sterile cotton swabs. The cotton swabs were rotated against the vaginal areas of the animals for a few times in order to collect cells onto the cotton tip. The swabs were then stored in the transport medium provided and sent to the laboratory for further processing.

In the laboratory, the swabs were first suspended in 1 ml of PBS for 1 min at room temperature. The suspension was vortexed for 15 seconds at room temperature. DNA was extracted using a Favor Prep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan). A volume of 200 µl of the suspension was pipetted into a 1.5 ml microcentrifuge tube. The sample was added with 20 µl proteinase K and incubated at 56ºC for about an hour until the tissue had been digested. After adding 200 µl of FATG2 (lysis buffer), the sample was mixed thoroughly by pulse-vortexing followed by incubation at 70 °C for 10 min. The tube was spinned briefly to remove drops from the inside of the lid before adding with 200 µl ethanol (96 ~ 100%). The mixture was then carefully transferred into the column and it was then centrifuged for 1 min. The FATG Column was washed with 500 µl W1 Buffer (wash buffer), followed by 750 µl W2 Buffer (wash buffer). Lastly, the column was centrifuged for an additional 3 min to dry the column. The column was placed in a sterile microcentrifuge tube and

DNA was eluted using 60 µl elution buffer after incubation for 3 min. All extracted DNA was stored at -20ºC prior to PCR amplification.

3.2.3 Milk Samples

Milk samples were collected from three dairy cattle farms in Malaysia (Table 3.4). A total of 59 milk samples were collected. The udders of the cattle were first cleaned and the milk was then collected into a 50 ml falcon tube. Samples were kept in ice and transferred to a freezer as soon as possible to avoid milk protein from denaturation and to inhibit the growth of other bacteria in the milk.

A volume of 200 µl milk samples was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer (as described in section 3.1.2). All extracted DNA was stored at -20ºC prior to PCR amplification.

Table 3.4: Sampling site and the source of animal samples collected in this study.

Sampling site	Sample Type	Source	No of samples
Behrang private farm	Vaginal swab	Cattle	12
	Milk		30
Makmal Veterinar Kawasan Bukit Tengah	Vaginal swab	Goat	20
Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	Vaginal swab	Cattle	18
Pusat Ternakan Haiwan Pantai Timor, Tanah Merah, Kelantan	Vaginal swab	Cattle	23
Pusat Pembiakan Kambing Kampung Kuala Pah	Vaginal swab	Goat	20
Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	Vaginal swab	Goat	21
Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	Vaginal swab	Cattle	18

Pusat Ternakan Haiwan Air Hitam, Johor	Vaginal swab	Cattle	18
	Milk		20
Pusat Ternakan Haiwan Tersat, Kuala Berang, Terengganu	Vaginal swab	Cattle	19
Jelai Gemas	Blood	Cattle	25
TPU	Blood	Cattle	30
	Blood	Goat	20
	Vaginal swab	Cattle	10
	Milk	Cattle	9
VRI	Blood	Goat	16
Sungai Siput	Blood	Goat	12

3.3 Collection and processing of animal organ tissue samples

The animal tissues were collected during field trips which were conducted at two locations:

i. The University of Malaya Field Studies Centre/Ulu Gombak Biodiversity Centre which is located on a 120-hectares of secondary and primary forests. A total of 30 wire traps were set up in UM Field Studies Center, Gombak. Mist traps were also set up to capture bats. A total of five animals were caught and sacrified. Heart, liver and kidney samples were harvested and kept in 1.5 ml microcentrifuge tubes at -80°C prior to processing.

ii. Krau Forest Reserve, Pahang, as described in section 2.1. The samples were collected with the help of the staff of the Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN), Kuala Lumpur, Malaysia from March till May, 2013. The tissue samples collected in Krau Forest Reserve forest were stored in 80% alcohol.

Other animal tissues, including eight samples were obtained from cattle which were kindly provided by MVKBT. The samples include placenta, uterus, stomach content, spleen and liver samples (Table 3.5). The tissues were disrupted using a rotor-stator before being processed for DNA extraction.

DNA extraction was carried out in a Class II, A2 biological safety cabinet. The animal tissue samples were processed using a QIAamp® DNA Mini Kit (Qiagen, Germany), as described above. The tissues from small rodents were pooled prior to processing. The tissue samples (approximately 25 mg) were placed in a 1.5 ml microcentrifuge tube. A volume of 180 µl Buffer ATL was added to the tissue sample. The tissue samples were homogenized using a hand held motor homogenizer (Kimble Chase, USA). The sample was then added with 20 µl of proteinase K (provided by the kit) to each sample to aid protein digestion. After vortexing, the sample was incubated at 56°C overnight until the tissues had completely lysed. The rest of the steps were carried out as described by the manufacturer (as described in section 3.3)

Table 3.5: Sampling site and the source of animal tissue samples collected in this study

Sampling site	Source	No of samples
UM Field Studies Center, Ulu Gombak	Rodents	2
	Bats	3
Krau Forest Reserve.	Rodents	5
Sg Teris	Bats	6
	Frog	4
	Bird	2
MVKBT	Cattle	8
Total		**30**

3.4 Molecular detection of *C. burnetii* from DNA extracts

3.4.1 Amplification of the *IS1111* gene of *C. burnetii* (Trans-PCR)

DNA of *C. burnetii* were amplified using primers targeting the *IS1111* transposase gene of the organism (Trans3/F-5'-CAA CTG TGT GGA ATT GAT GA and Trans5/R-TTT ACA TGA CGC AAT AGC GC-3'), as described previously (Greub *et al*., 2005). PCR master mix (Table 3.6) containing 2 µl tick DNA sample, 19.4 µl sterile distilled water, 2.5 µl 10x DreamTaq™ buffer (Fermentas, Lithuana), 0.5 µl dNTPs (10 µM), 0.2 µl of each primer (25 µM), and 0.2 µl DreamTaq™ DNA Polymerase (5 U/µl) (Fermentas, Lithuana), was prepared. Table 3.6 shows the composition of the PCR reagents. Amplification was carried out using a MyCycler Thermalcycler (BioRad, USA).

The PCR program included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C (30 s), 52 °C (30 s) and 72 °C (90 s), and a final elongation step at 72 °C for 5 min. The PCR assay is expected to generate an 832 bp fragment after amplification. Positive controls were derived from DNA extracted from *C. burnetii*-positive goat vaginal samples (obtained from Makmal Veterinar Kawasan Bukit Tengah, Penang, Malaysia) or *C. burnetii* antigen slides (Fuller Laboratories, Fullerton, California, USA). Negative control (sterile distilled water) was included in each PCR run.

Table 3.6: PCR reaction mixture used for the amplification *IS1111* gene of *C. burnetii* from animal samples

Reagents	Concentration	Volume (µl per reaction)
DNA template		2
DreamTaq™ buffer	10x	2.5
dNTPs	10 µM	0.5

28SF Primer	25 µM	0.2
28SR Primer	25 µM	0.2
DreamTaq™ DNA Polymerase	5 U/µl	0.2
Sterile distilled water		19.4
Total		**25.0**

3.4.2 Amplification of the *com1* gene of *C. burnetii* (OMP-PCR)

In order to confirm the validity of the PCR results as well as for genotyping purpose, a second set of primers targeting the *com1* gene (encoding the 27-kDa outer membrane protein of *C. burnetii*) was used in this study. The PCR primers (OMP1 5′-AGTAGAAGCATCCCAAGCATTG-3′ and OMP2 5′-TGCCTGCTAGCTGTAACGATTG-3′) have been described by Zhang *et al.* (1998).

PCR Master Mix (Table 3.7) containing 2 µl tick DNA sample, 19.4 µl sterile distilled water, 2.5 µl 10x DreamTaq™ buffer (Fermentas, Lithuana), 0.5 µl dNTPs (10 µM), 0.2 µl of each primer (25 µM), and 0.2 µl DreamTaq™ DNA Polymerase(5 U/µl) (Fermentas, Lithuana) was prepared. Table 3.7 shows the composition of the PCR master mix. PCR was run on a MyCycler Thermalcycler (BioRad, USA). The PCR program included an initial denaturation step at 95 °C for 3 min, followed by 36 cycles of 95 °C (1 min), 54 °C (1 min) and 72 °C (1 min), and a final elongation step at 72 °C for 5 min. The PCR assay is expected to generate a 501bp fragment after amplification. Positive controls were derived from DNA extracted from *C. burnetii*-positive goat vaginal samples (obtained from Makmal Veterinar Kawasan Bukit Tengah, Penang, Malaysia) or *C. burnetii* antigen slides (Fuller Laboratories, Fullerton, California, USA). Negative control (sterile distilled water) was included in each PCR run.

Table 3.7: PCR reaction mixture used for amplification of the *com1* gene of *C. burnetii*

Reagents	Concentration	Volume (µl per reaction)
DNA template		2
DreamTaq™ buffer	10x	2.5
dNTPs	10 µM	0.5
28SF Primer	25 µM	0.2
28SR Primer	25 µM	0.2
DreamTaq™ DNA Polymerase	5 U/µl	0.2
Sterile distilled water		19.4
Total		**25.0**

3.4.3 Analysis of PCR products

After amplification, 5 µl of each PCR product were mixed with 2 µl loading dye (Fermentas, Lithuania) and loaded into a well in an agarose gel (1% in Tris-borate-EDTA [TBE] buffer) pre-stained with ethidium bromide (0.5 µg/ml).A DNA Ladder VC 100bp Plus (Vivantis, USA) was included in every run for size estimation of the PCR products. The electrophoresis was performed in 1X Tris-Base-EDTA (TBE) buffer at 90V for 60 min. The DNA bands were visualized and photographed under ultraviolet light using InGenius Gel Documentation System (Syngene, United Kingdom).

3.4.4 Purification of PCR products and sequence analysis

Sequence determination of the amplified products was carried out to confirm the specificity of each PCR assay. Purification of PCR products was carried out using a LaboPass PCR Purification Kit (Cosmo Genetech, Korea). Briefly, 5 volumes of binding buffer were added and mixed with 1 volume of PCR product. The mixture was

then added with 700 µl wash buffer and centrifuged for 1 min at full speed. DNA was eluted by adding 30 µl of elution buffer.

Sequencing was performed by a service provider (Firstbase Laboratory, Malaysia) using a Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems). Both forward and reverse PCR primers were used as the primers for sequencing. The obtained sequences were aligned with BioEdit Sequence Alignment Editor Software (version 7.0.5.3) and compared for similarity with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information, National Institute of Health).

3.5 Quantitative Real Time PCR

As not all of the amplified products generated from Trans-PCR and OMP-PCR assays generated sufficient amount of amplified products for sequence determination, a real-time PCR assay was performed to ascertain the positive findings obtained from the two PCR assays.

3.5.1 Primers and probes for detection of *C. burnetii*

The real-time PCR assay was performed as described by Loftis *et al.* (2006). Primers *IS1111*F (5'-CGG CGG TGT TTA GGC-3' or 5'- CCGATCATTTGGGCGCT-3') and *IS1111*R (5'-CGG CGG TGT TTA GGC-3') and probe *IS1111*Pa (5'-TTA ACA CGC CAA GAA ACG TAT CGC TGT G-3') labeled with FAM at the 5' end and MGB at the 3' end were used. The primers and probe target at the *IS1111* transposase gene which exists in multiple copies in *C. burnetii*.

3.5.2 Eukaryotic 18S rRNA endogenous control

The eukaryotic 18S rRNA endogenous control (4319143E, Applied Biosystems, USA) was included in each PCR reaction. The gene of target is the 18S ribosomal rRNA which is present in all eukaryotes. The probe used was labeled with VIC at the 5' end and MGB at the 3' end.

3.5.3 Real-time PCR reaction setup and analysis

Real-time PCR assays were conducted in triplicate per DNA sample. Table 3.8 shows the composition of the PCR master mix used in this study. Each reaction consisted of a total volume of 20 µl containing 10 µl TaqMan® Fast Advanced Master Mix, 1 µl Primer/Probe Mix, 7 µl nuclease-free water and 2 µl DNA template. Applied Biosystems StepOnePlus ™ thermal cycler (USA) was used to run the assays. Data of the fluorescence signals detected during the thermal cycling was viewed and analysed with StepOne™ Software version v2.3.

Table 3.8: Preparation of the reaction mixture targeting the *IS1111* gene using real-time PCR assay

Reagent	**Concentration**	**Volume (µl per reaction)**
TaqMan® Fast Advanced Master Mix	10 x	10
Primer/Probe Mix		1
18S endogenous control		1
Nuclease free water		6
DNA Template		2
Total		20

3.5.4 Standard Curve

A standard calibration curve was designed using one of the positive samples (KB 8). This sample was obtained from a goat vaginal swab sample (provided by MVKBT). The stock concentration was 50 ng/µl and a 5x serial dilution was made as shown in Table 3.9. To make a concentration of 10 ng/µl, 2 µl of the stock DNA was added to 8 µl distilled water.

Table 3.9: Five points of 5-fold serial dilution of *C. burnetii* DNA

Dilution	Concentration (ng/µl)
Stock	50
Dilution 1	10
Dilution 2	2
Dilution 3	0.4
Dilution 4	0.08

CHAPTER 4: RESULTS

4.1 Ticks

4.1.1 Tick Collection and identification

The origin and the details of the ticks are shown in Appendix 1. Out of the 173 ticks collected, 172 were hard ticks. Table 4.1 shows the different genera of ticks collected in this study. The hard ticks are mainly from the genus *Dermacentor* spp., *Amblyomma* spp., *Rhipicephalus* spp., and *Haemaphysalis* spp.. The identities for some of these ticks were confirmed by BLAST analysis (Figure 4.2). One soft tick that was collected from a bat was identified as *Carios mimon* (based on blast analysis of the 28S sequence).

Table 4.1: Identification of ticks collected in this study

Host	Tick genera	No. ticks
Bat	*Carios mimon*	1
Rodent	*Amblyomma* spp.	13
	Haemaphysalis spp.	4
	Dermacentor spp.	4
Bird	*Amblyomma* spp.	1
Human	*Amblyomma* spp.	6
Monitor Lizard	*Amblyomma* spp.	1
Vegetation	*Haemaphysalis* spp.	26
	Rhipicephalus spp.	5
	Dermacentor spp.	5
Cattle	*Boophilus* spp.	34
Goat	*Haemaphysalis* spp..	20
	Heamophysalis spp.	10
Dog	*Rhipicephalus sanguineus*	43
Total		173

Ticks collected from the Krau Forest Reserve (Kuala Lompat and Sungai Teris) were consisted of a mixture of the genera *Dermacentor* spp., *Amblyomma* spp., *Rhipicephalus* spp., and *Haemaphysalis* spp.. The ticks collected from the dogs at the Society for the Prevention of Cruelty to Animals (SPCA) Ampang and Second Chance Dog Shelter, Kuala Lumpur were mainly *Rhipicephalus sanguineus* (Appendix 1). The cattle ticks collected from Jelai, Gemas, Negeri Sembilan were mainly *Rhipicephalus* (formerly *Boophilus microplus*) whereas the ticks collected from goats were *Haemaphysalis* spp.. Due to financial constraints, sequence determinations of the amplified fragments were only performed for very few ticks (Figure 4.2).

Table 4.2: Identities for some ticks that were confirmed by BLAST analysis

Tick label	Blast results	Nucleotide (percentage similarity
B022	*Carios mimon* 28S ribosomal RNA gene, partial sequence	324/334(97%)
R008	*Amblyomma maculatum* 28S ribosomal RNA gene, partial sequence	427/452(94%)
S002	*Rhipicephalus sanguineus* 28S ribosomal RNA gene, partial sequence	439/446(98%)
S003	*Dermacentor* sp. 28S ribosomal RNA gene, partial sequence	443/447(99%)
S007	*Haemaphysalis sulcata* 28S ribosomal RNA gene, partial sequence	420/452(93%)

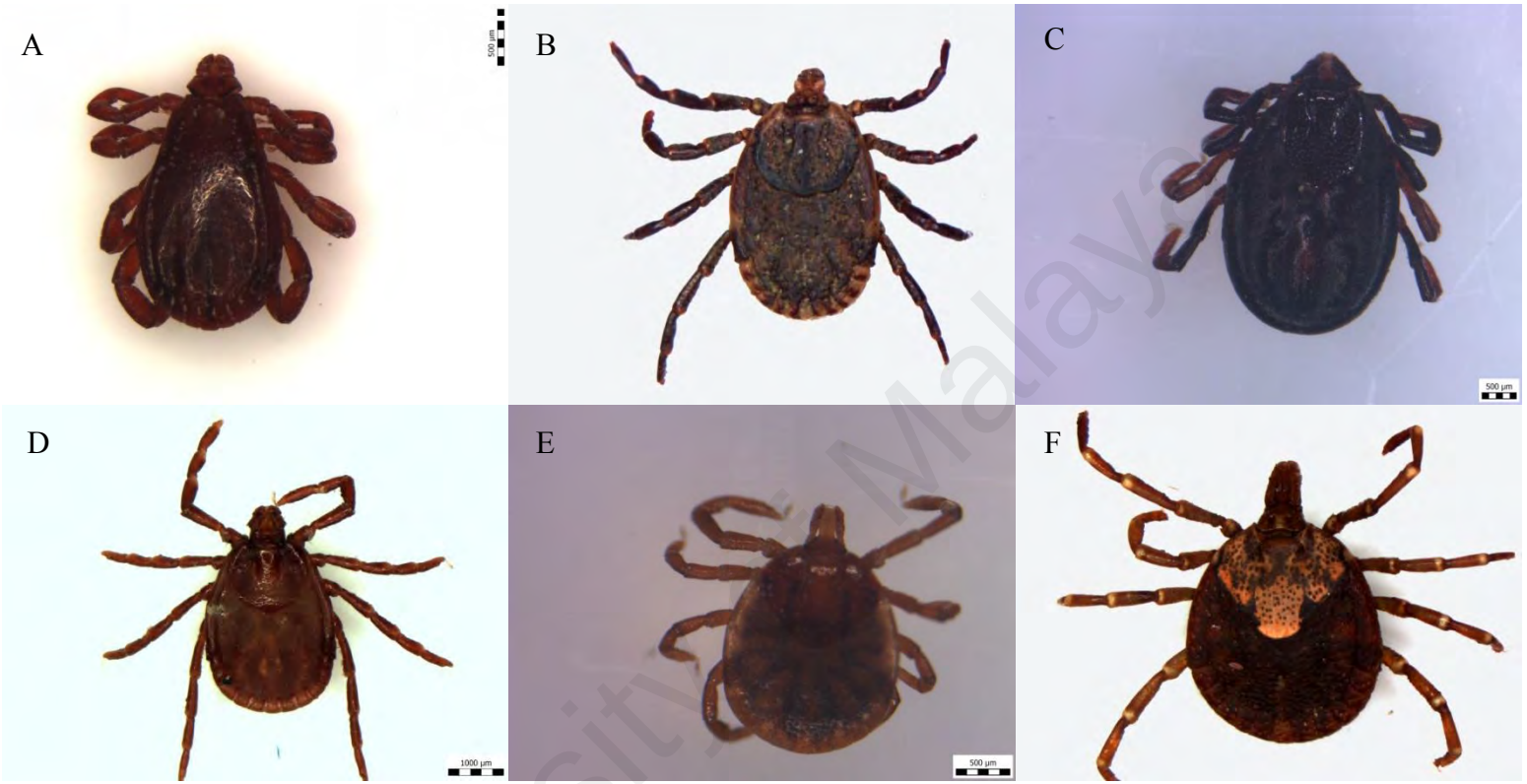

Figure 4.1: Images for some of the ticks collected in this study. A: Brown dog tick, *Rhipicephalus sanguineus* collected from a dog shelter in Kuala Lumpur. B: *Dermacentor* spp. collected from vegetation in Sungai Teris. C: *Heamaphysalis* spp. collected from vegetation in Sungai Teris. D:*Heamaphysalis* spp. collected from a crab-eating mongoose from Kuala Lompat. E: *Amblyomma* spp. collected from vegetation in Sungai Teris. F: *Amblyomma* spp. collected from a forest ranger in Kuala Lompat.

4.1.2 Amplification of 28S rRNA gene of ticks

A total of 173 tick DNA were amplified using primers 28SF and 28SR as described by Inokuma *et al.* (2003). The amplification of the gene from all the tick DNA samples indicates that the DNA extraction was successful and there was no PCR inhibitor in the sample. This step was important to validate the negative results to rule out false negative results due to sample preparations. Figure 4.2 shows the image of the agarose gel for some PCR products obtained from the amplification of the 28S rDNA.

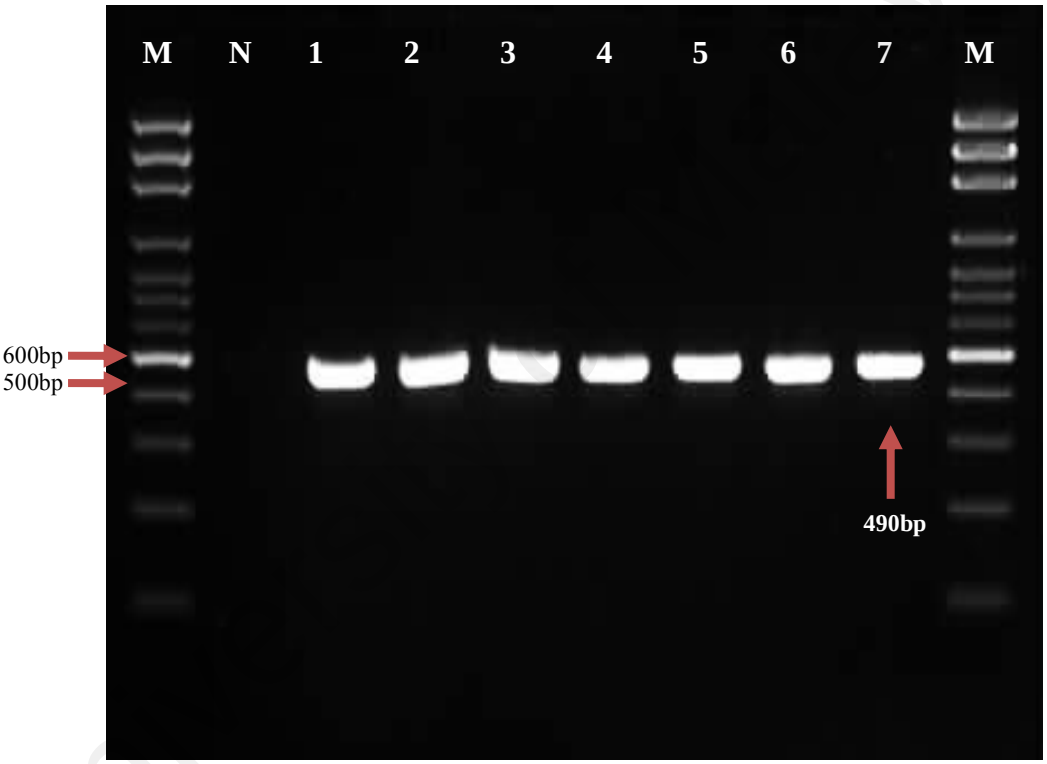

Figure 4.2: Amplification of 490bp fragment of the 28S rRNA gene of tick DNA samples. M: 100 bp ladder; N: negative control (sterile distilled water); 1-7: tick DNA samples.

4.2 Molecular detection of *C. burnetii* DNA using conventional PCR assays

4.2.1 Amplification of the *IS1111* gene of *C. burnetii* (Trans-PCR)

A total of 545 samples as in Table 4.3 were subjected to amplification using primers Trans3/F and Trans5/R targeting the *IS1111* gene, as described by Greub *et al*. (2005).

Table 4.3: List of samples amplified using primers Trans3/F and Trans5/R

Sample type	No of samples
Ticks	173
Blood	103
Milk	59
Vaginal swab	180
Organ tissues	30
Total	545

4.2.1.1 Tick DNA

C. burnetii DNA was detected in 10 (5.8%) of 173 tick DNA samples. The size of the amplicons generated was 832bp as shown in Figure 4.3. *C. burnetii* was detected in various genera of ticks including *Amblyomma* spp. (n=3), *Dermacentor* sp. (n=1), *Haemaphysalis* spp. (n=5) and *R. sanguineus* (n=1). None of the DNA samples from ticks collected from cattle and goat farms were positive. Table 4.4 shows the genera, source and the location of the PCR-positive ticks.

Table 4.4: Tick DNA samples that were tested positive for *C. burnetii* DNA using primers targeting the *IS1111* gene. Genera, source and location of tick samples are listed

Sample	Tick Genera	Source	Location
R002	*Amblyomma* sp.	Rodent (*Maxomys rajah*)	Kuala Lompat
S002	*Amblyomma* sp.	Rodent (*Callasciuras notatus*)	Kuala Lompat
S003	*Dermacentor* sp.	Rodent (*Tupaia glis*)	Kuala Lompat
KL06	*Amblyomma* sp.	Human	Kuala Lompat
ST19	*Heamaphysalis* sp.	Vegetation	Sungai Teris
ST25	*Heamaphysalis* sp.	Vegetation	Sungai Teris
ST37	*Heamaphysalis* sp.	Vegetation	Sungai Teris
ST51	*Heamaphysalis* sp.	Vegetation	Sungai Teris
ST77	*Heamaphysalis* sp.	Vegetation	Sungai Teris
SP001	*Rhipicephalus sanguineus*	Dog	Second Chance, Kuala Lumpur

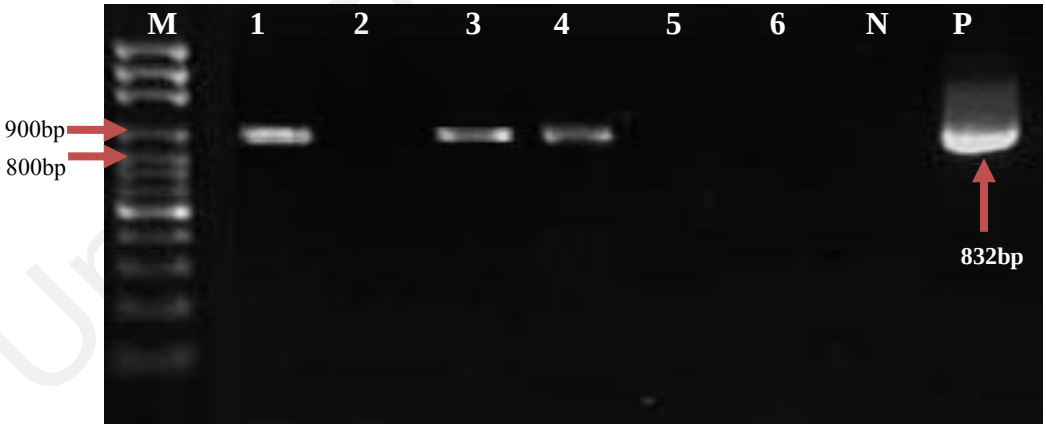

Figure 4.3: Amplification of 832bp fragment of *IS1111* gene from tick DNA samples. M: 100 bp ladder; 1,3,4: positive tick DNA sample. 2,5,6: Negative tick DNA samples. N: negative control (sterile distilled water); P: positive control (*C. burnetii* antigen).

4.2.1.2 Animal blood DNA samples

Out of the 103 blood samples (Refer to Appendix 2) from cattle and goats, 5 (4.7%) were tested positive. The positive blood samples were derived from beef cattle (YKK breed that were free range and grass fed) in a farm in Negeri Sembilan.

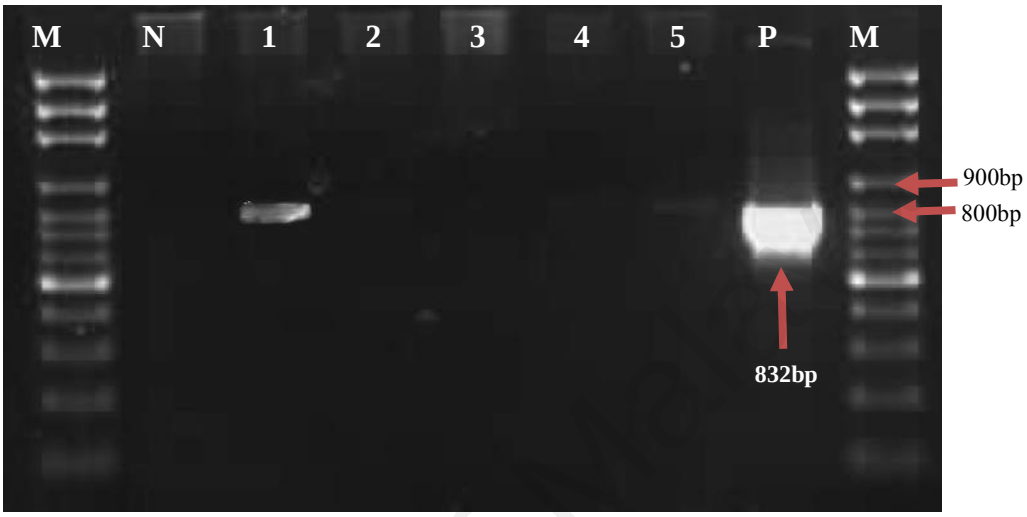

Figure 4.4: Amplification of 832bp fragment of *IS1111* gene of *C. burnetii* from cattle blood samples. M: 100 bp ladder; Lane 1: positive blood samples. Lane 2,3,4 & 5: negative blood samples. N: negative control (sterile distilled water); P: positive control (*C. burnetii* antigens from IFA kit)

4.2.1.3 Vaginal Swab DNA

Of the 180 vaginal swabs (Refer to Appendix 3) investigated in this study, 22 (12.2%) were tested positive. Out of the 20 vaginal swab samples collected from a goat farm (provided by the Makmal Veterinary Kawasan Bukit Tengah) and suspected of having *coxiella* infections in Penang, 17 (85%) were tested positive. The positive samples were obtained from Boer goats.

4.2.1.4 Milk DNA samples

Of 59 cow milk samples (Refer to Appendix 4) obtained from three farms, 17 (28.8%) of the samples were tested positive in which one was sequenced. All 17 positive samples were collected from a private dairy farm in Behrang, Selangor.

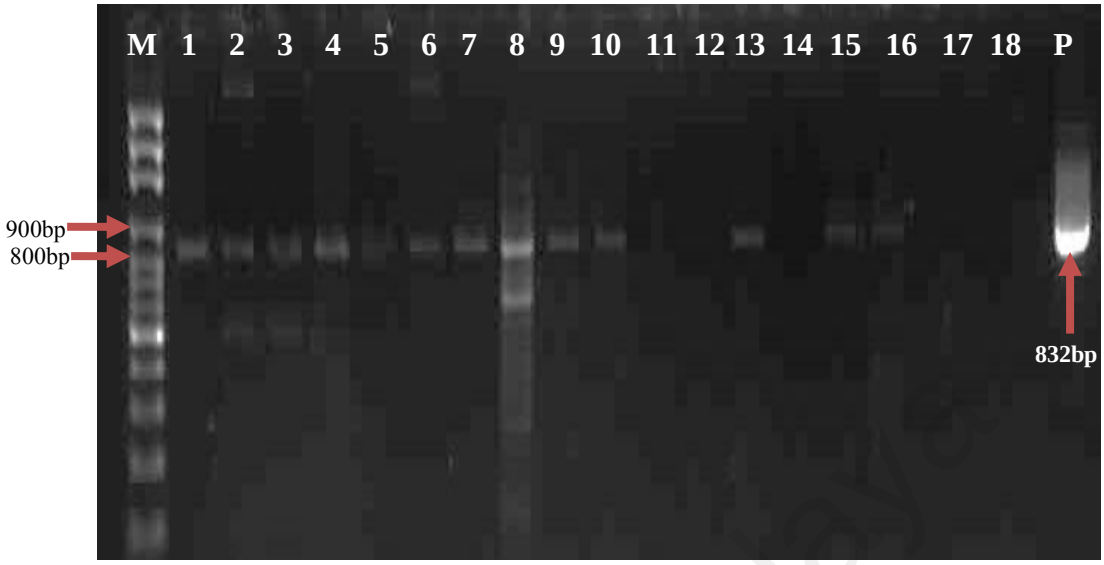

Figure 4.5: Amplification of 832bp fragment of *IS1111* gene of *C. burnetii* from vaginal swab. M: 100 bp ladder; Lane 1-10,13,15,16: Positive vaginal swab DNA samples from goats; Lane 11,12,14,17,18: Negative vaginal swab DNA sample from goats. N: negative control (sterile distilled water); P: positive control (*C. burnetii* antigens from IFA kit)

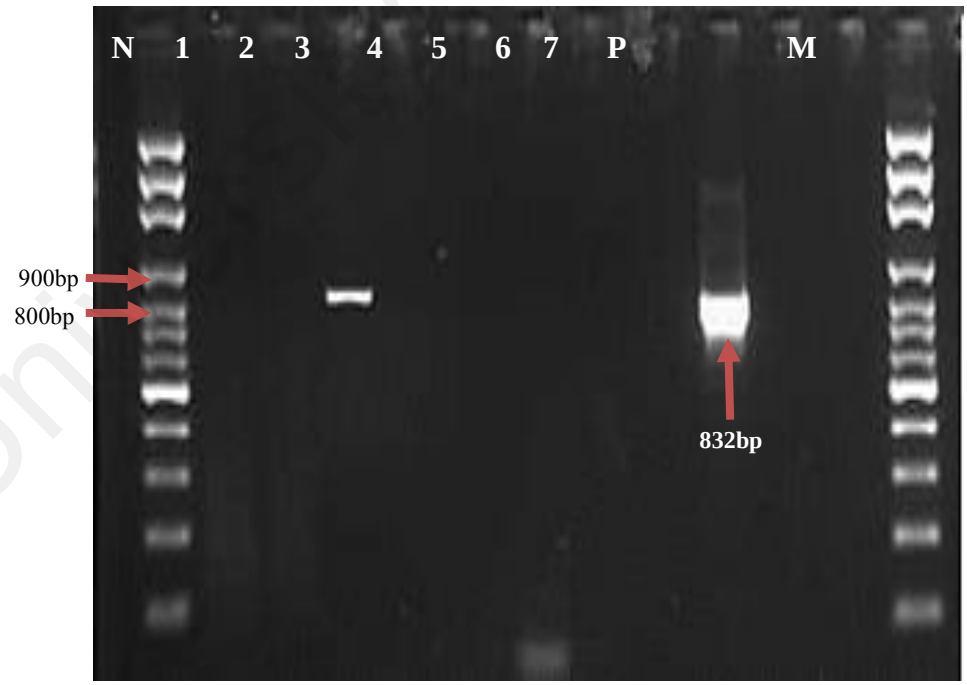

Figure 4.6: Amplification of 832bp fragment of *IS1111* gene of *C. burnetii* in milk samples. M: 100 bp ladder; N: negative control (sterile distilled water); Lane 2: positive milk sample; Lane1,3-6: negative milk sample. P: positive control (*C. burnetii* antigens from IFA kit)

4.2.1.5 DNA extracted from animal tissues samples

Of 30 tissue samples (Refer to Appendix 5) collected from various sources (section 3.21), none of the samples were tested positive (Figure 4.7), despite of the positive control showing a fragment size of 832 bp.

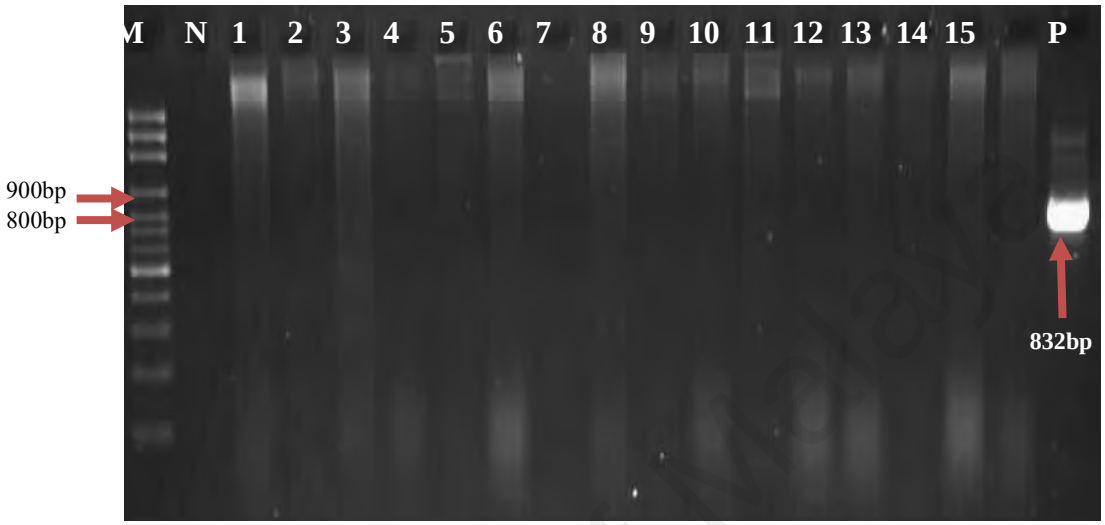

Figure 4.7: Amplification of 832bp fragment of *IS1111* gene of *C. burnetii* in animal tissue samples. M: 100 bp ladder; 1-15: negative tissue DNA sample. N: negative control (sterile distilled water); P: positive control (*C. burnetii* antigens from IFA kit).

4.2.2 Amplification of the *com1* gene of *C. burnetii* (OMP-PCR)

All the samples in this study were then amplified using a second set of primers targeting the *com1* gene which encodes the 27-kDa outer membrane protein of *C. burnetii* (Zhang *et al*., 1998).

4.2.2.1 Tick DNA samples

The c*om 1* gene of *C. burnetii* was amplified in five (2.9%) of 173 tick DNA samples. Four were vegetation ticks collected in Sungai Teris, Pahang and one was collected on the body of a dog from an animal shelter in Kuala Lumpur. The genera and

the source of the ticks are summarised in Table 4.5. Figure 4.8 shows the amplification of the 501bp amplicon from the positive control and some of the tick DNA samples.

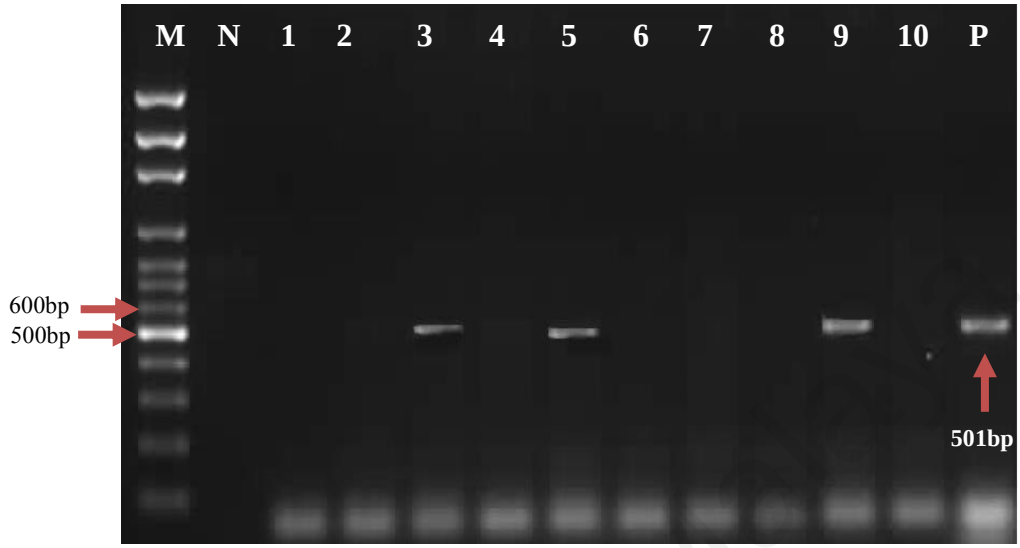

Figure 4.8: Amplification of 501bp fragment of *com* 1 gene of *C. burnetii* from tick DNA samples. M: 100 bp ladder; Lane 3, 5 & 9: positive tick DNA samples; Lane 1,2 4,6,7,8: negative tick DNA samples; N: negative control (sterile distilled water); 8: positive control (*C. burnetii* antigens from IFA kit)

Table 4.5: Tick DNA samples that were tested positive for *C. burnetii* using primers targeting the *com 1* gene. The genera, source and the location of the samples are listed.

Sample	Genera	Source	Location
KL09	*Dermacentor* spp.	Vegetation	Kuala Lompat
KL22	*Rhipicephalus* spp	Vegetation	Kuala Lompat
KL25	*Rhipicephalus* spp	Vegetation	Kuala Lompat
KL31	*Rhipicephalus* spp	Vegetation	Kuala Lompat
SP001	*Rhipicephalus sanguineus*	Dog	Second Chance dog shelter, KL

4.2.2.2 Animal blood Samples

None out of the 103 blood sample from cattle and goats tested positive by PCR assays targeting the *com 1* gene. Figure 4.9 is the image of an agarose gel loaded with the PCR products for some of the blood samples.

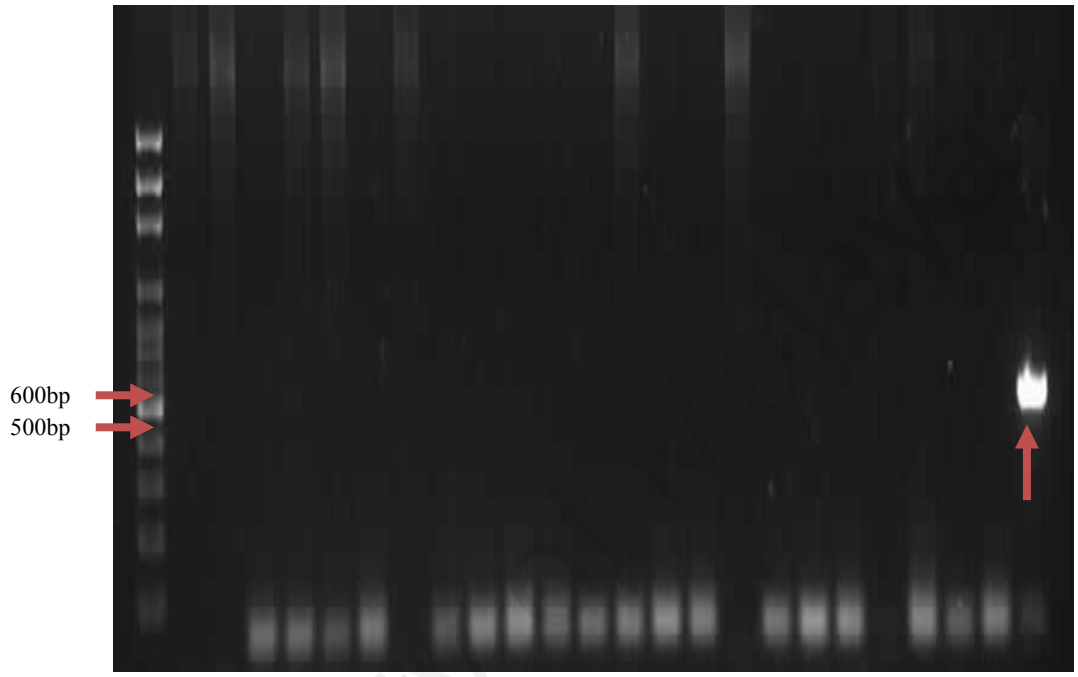

Figure 4.9: Amplification of 501bp fragment of *com 1* gene of *C. burnetii* in blood samples. M: 100 bp ladder; 1-22: negative blood sample; N: negative control (sterile distilled water); P: positive control (*C. burnetii* antigens from IFA kit).

4.2.2.3 Vaginal DNA samples

Out of the 180 swabs, 12 (6.7%) were tested positive. Twelve (60%) of the 20 vaginal swabs provided by MVKBT from goats suspected of having coxiellosis were positive. Figure 4.10 is the image of an agarose gel loaded with the PCR products for some of the vaginal samples.

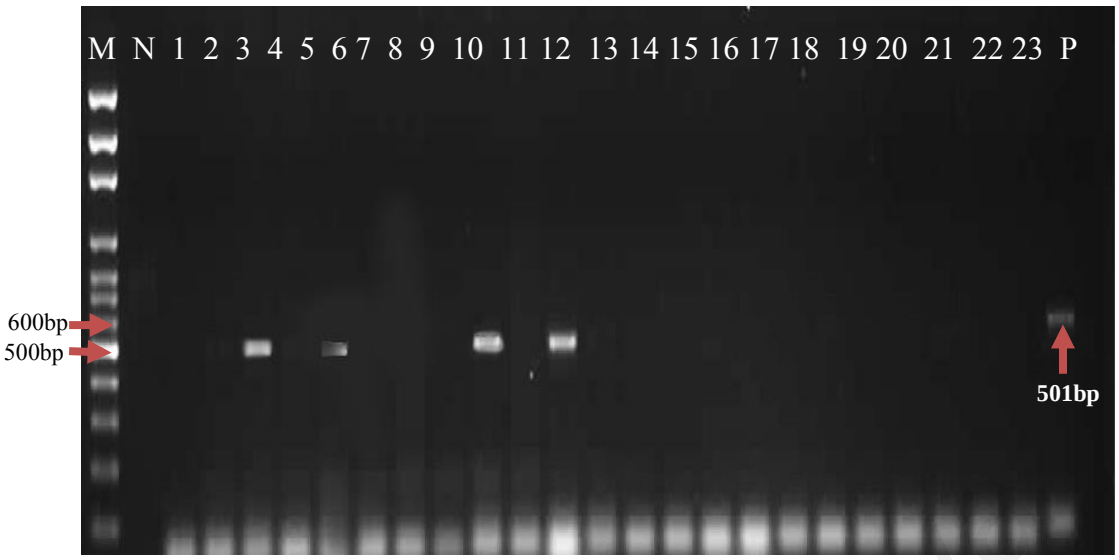

Figure 4.10: Amplification of 501bp fragment of the *com 1* gene of *C. burnetii* in vaginal. M: 100 bp ladder; N: negative control (sterile distilled water). Lane 3, 5, 9 & 11:positive vaginal; Lane 1,2,4, 6, 7, 8, 10, 12-23: negative; P: positive control (*C. burnetii* antigens from IFA kit)

4.2.2.4 Milk samples

Of 59 cow milk samples obtained from three different farms, the *com 1* gene of the *C. burnetii* was not amplified from any of the milk samples. Figure 4.11 is the image of an agarose gel loaded with the PCR products for some of the milk samples.

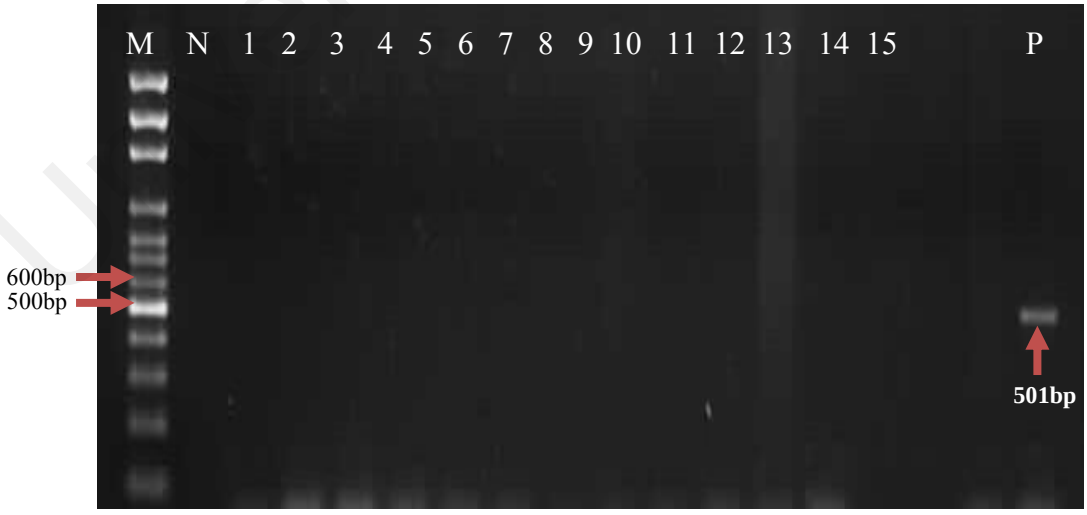

Figure 4.11: Amplification of 501bp fragment of the *com 1 C. burnetii* gene in milk samples. M: 100 bp ladder; 1-15: milk sample. N: negative control (sterile distilled water); P: positive control (*C. burnetii* antigens from IFA kit.

4.2.2.5 Animal tissue samples

Of the 30 tissue samples collected from various sources (Table 3.4), none of the samples were tested positive, as shown in Figure 4.12. is the image of an agarose gel loaded with the PCR products for some of the tissue samples.

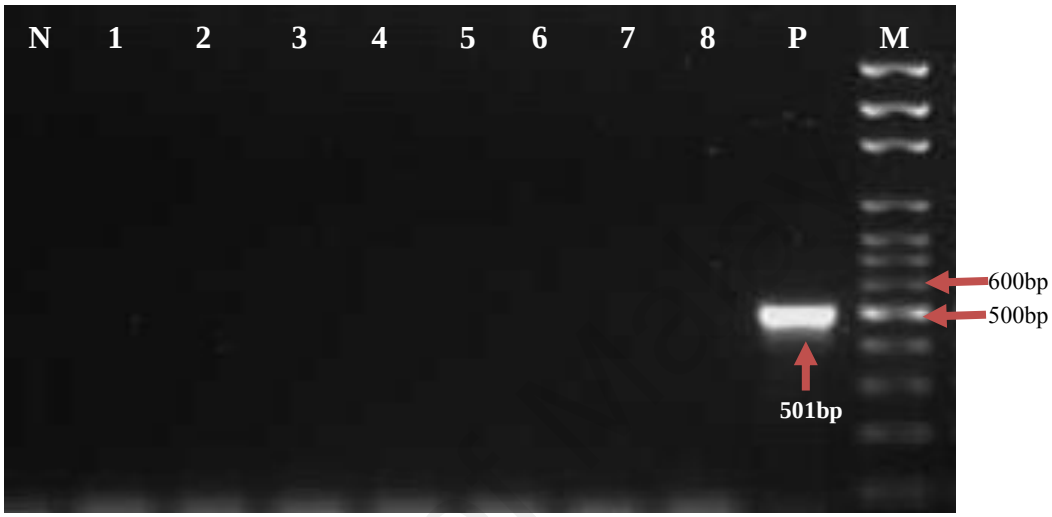

Figure 4.12: Amplification of 501bp fragment of *com 1* gene of *C. burnetii* in animal tissue samples. M: 100 bp ladder; Lane 1-8: Negative tissue sample. N: negative control (sterile distilled water); P: positive control (*C. burnetii* antigens from IFA kit).

4.2.3 Comparison between the results obtained from Trans-PCR and OMP-PCR assays

Table 4.6 summarizes the PCR findings obtained from this study. Trans-PCR assay detected 54 positive samples (4 mammalian ticks, 1 dog tick, 5 vegetation ticks, 17 milk samples, 22 vaginal swabs and 5 cattle blood) as compared to 17 samples (4 vegetation ticks, 1 dog tick and 12 vaginal samples) by the OMP-PCR assays.

Table 4.6: Detection of *C. burnetii* DNA using Trans-PCR and OMP-PCR assay

Sample	No. tested	No. (%) samples positive by Trans-PCR	No. (%) samples positive by OMP-PCR	No. samples confirmed by real-time PCR assay
Ticks				
Wildlife	30	4 (13.3)	0 (0)	**3**
Vegetation	36	5 (13.9)	4 (11.1)	**6**
Cattle	54	0 (0)	0 (0)	**0**
Goat	10	0 (0)	0 (0)	**0**
Urban Dogs	43	1 (2.3)	1 (2.3)	**1**
Subtotal	**173**	**10 (5.7)**	**5 (2.9)**	**10**
Milk				
Cattle	59	17 (28.8)	0 (0)	**12**
Subtotal	**59**	**17 (28.8)**	**0 (0)**	**12**
Vaginal Swab				
Cattle	120	5 (4.1)	0 (0)	5
Goat	60	17 (28.3)	12 (20)	17
Subtotal	**180**	**22 (12.2)**	**12 (6.7)**	**22**
Blood				
Cattle	60	5 (8.3)	0 (0)	5
Goat	43	0 (0)	0 (0)	0
Subtotal	**103**	**5 (4.9)**	**0 (0)**	**5**
Animal Tissues				
Rodents	16	0 (0)	0 (0)	0
Frog	4	0 (0)	0 (0)	0
Bird	2	0 (0)	0 (0)	0
Cattle	8	0 (0)	0 (0)	0
Subtotal	**30**	**0 (0)**	**0 (0)**	**0**
Total	**545**	**54 (9.9)**	**17(3.1)**	**49**

4.3 Sequence analysis of amplicons derived from Trans-PCR assay

To confirm the specificity of the PCR assays, amplified fragments were sequenced in both directions. The positive controls and two amplicons (ROO2 & M025) of the *IS1111* gene were sequenced in this study. Sample R002 was originated form a tick sample collected on the body of a rodent (*Maxomys rajah)* from Kuala Lompat, Kuala Krau Forest Reserve, Pahang. Sample M025 was originated from a cow milk sample obtained from Behrang. BLAST analysis of 646 nucleotides from both samples shows a 100% similarity to *C. burnetii* strain Z3055 (Raoult *et al*., unpublished, Genbank accession no. LK937696), and *C. burnetii* RSA 493 (Seshadri *et al*., 2003, GenBank accession no. AE016828) and phase I Nine Mile clone 7 strains (Hoover *et al*., 1992, Genbank accession no: M80806). Figure 4.13 show the alignment of the sequences analysed in this study.

4.4 Sequence analysis of OMP-PCR

The amplified *com* 1 gene from six of the vaginal swab (KB4, KB5, KB6, KI3, KI6 and KI8) were sequenced. BLAST analysis of KB4, KB5, KI3, KI6 and KI8 sequences demonstrated a 100% similarity while KB6 showed a 99% similarity to *C. burnetii* CbuK_Q154 (GenBank accession no. CP001020), *C. burnetii* CbuG_Q212 (GenBank accession no. CP001019) and *Haemaphysalis longicornis* symbiont 66 (GenBank accession no. AY342039)

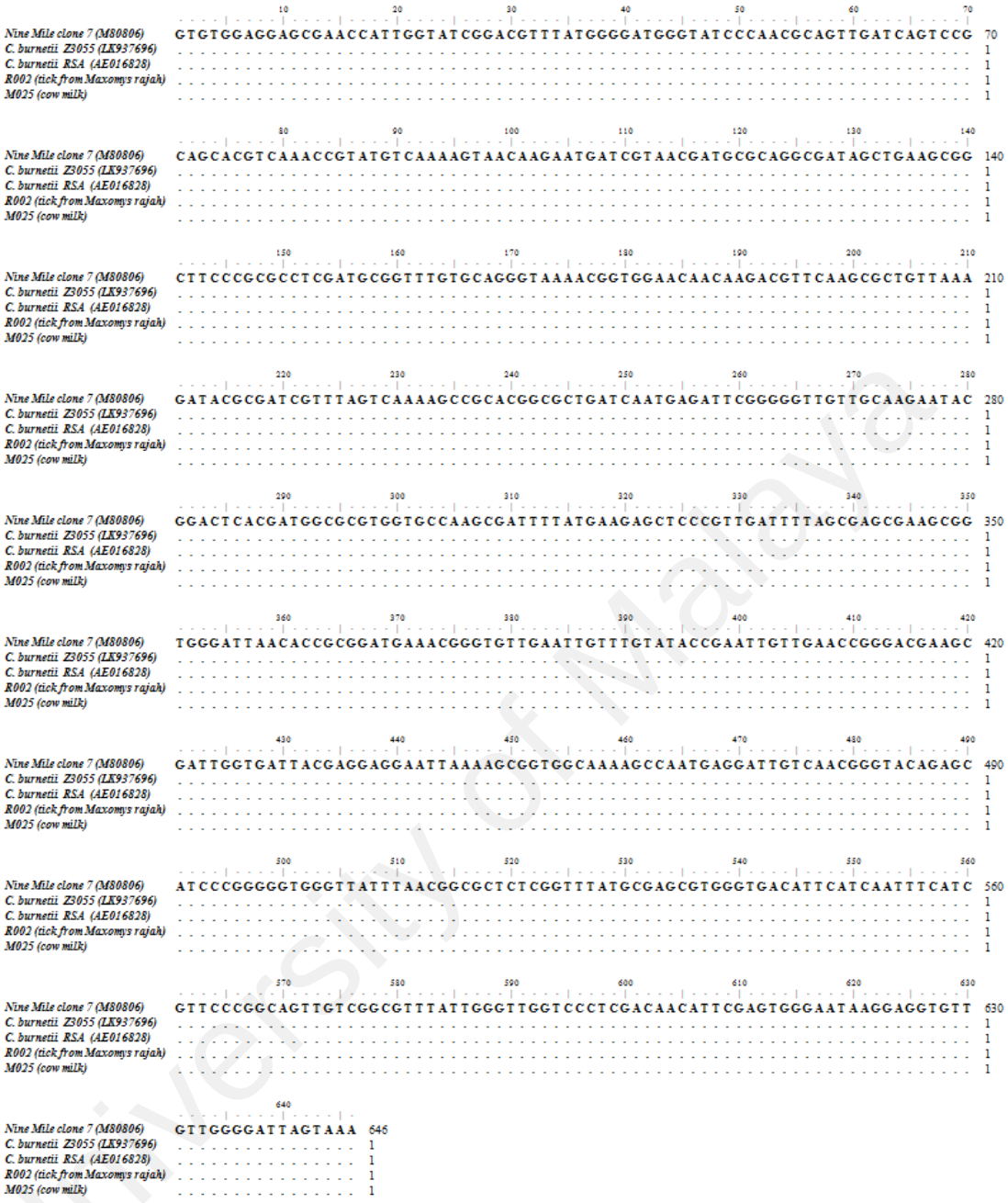

Figure 4.13: Sequence alignment of *C. burnetii* IS1111 gene amplified from sample R002 and M025 with *C. burnetii* Nine Mile clone 7 transposase (*IS1111*) gene (GenBank accession no. M80806), *C. burnetii* Z3055 (GenBank accession no. LK937696) and *C. burnetii* RSA(GenBank accession no. AE016828)

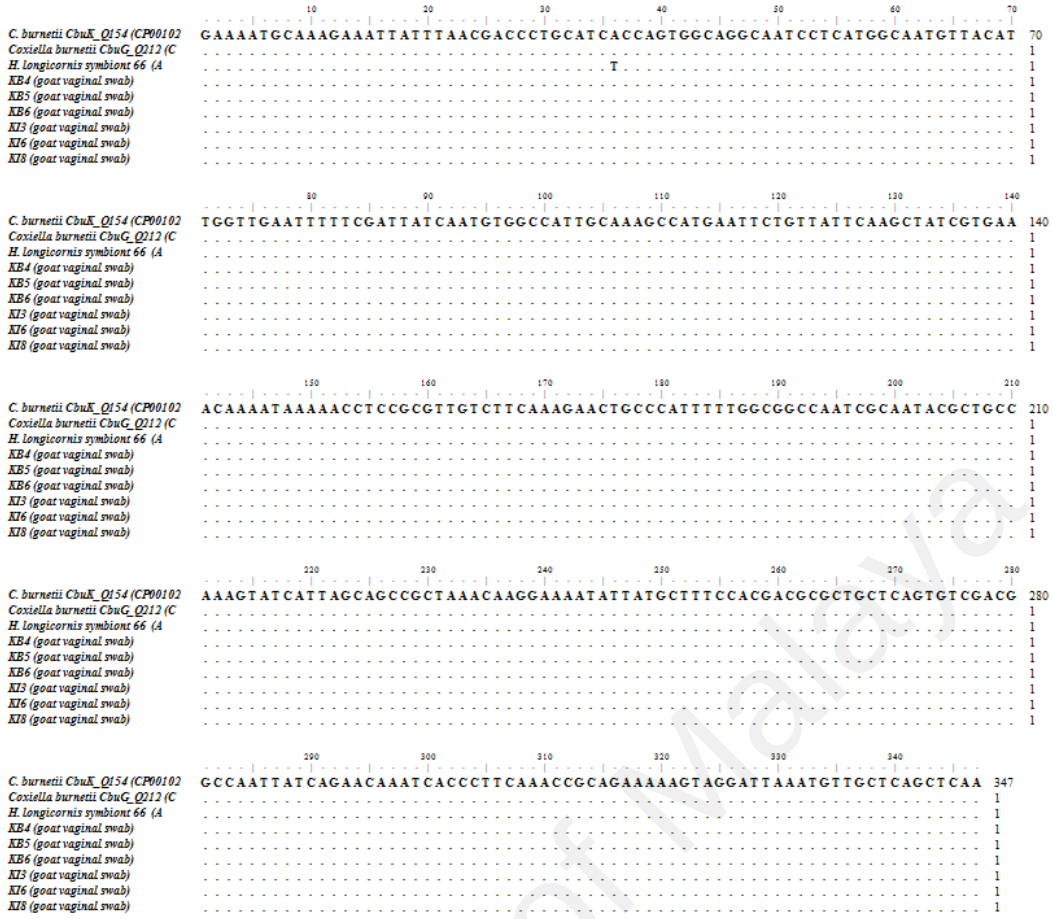

Figure 4.14: Sequence Alignment of *C. burnetii com 1* gene amplified from sample KB4, KB5, KB6, KI3, KI6 and KI8 with CbuK_Q154 (GenBank accession no. CP001020), *C. burnetii* CbuG_Q212 (GenBank accession no. CP001019) and *Haemaphysalis longicornis* symbiont 66 (GenBank accession no. AY342039

4.5 Molecular detection of *C. burnetii* using Real-Time PCR

4.5.1 Standard Curve

Sample KB 8 which was a positive sample from a goat vaginal swab was used to build a standard curve. A five-fold dilution made from a 50 µg/ml stock sample was used to construct the standard curve. The curve gave a R^2 value of 0.991 and 103.07 efficiency (within the acceptable range: 90-105%).

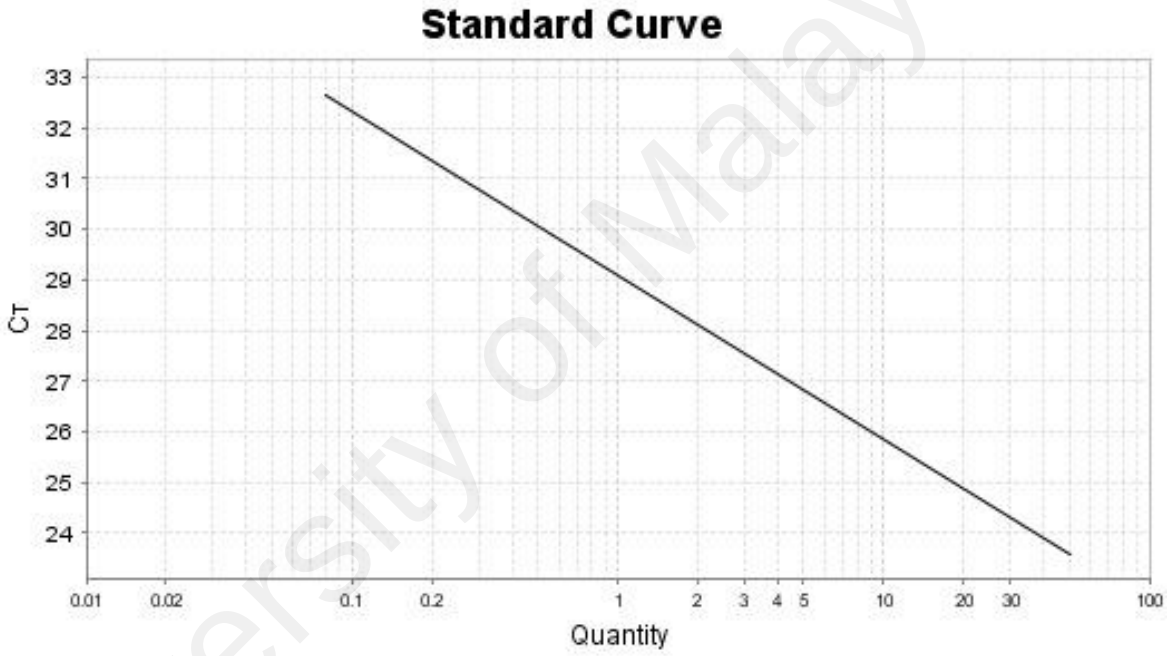

Figure 4.15: Standard curve constructed from a five-fold dilution.

4.5.2 Real Time PCR Result Analysis

4.5.2.1 Tick samples

Out of the 14 positive tick samples (detected by Trans-PCR and OMP-PCR assays, refer to Table 4.6), nine of them gave a Ct values ranging between 30 and 39

(Table 4.7) as shown in Figure 4.16. The remaining five samples gave an undetermined Ct value.

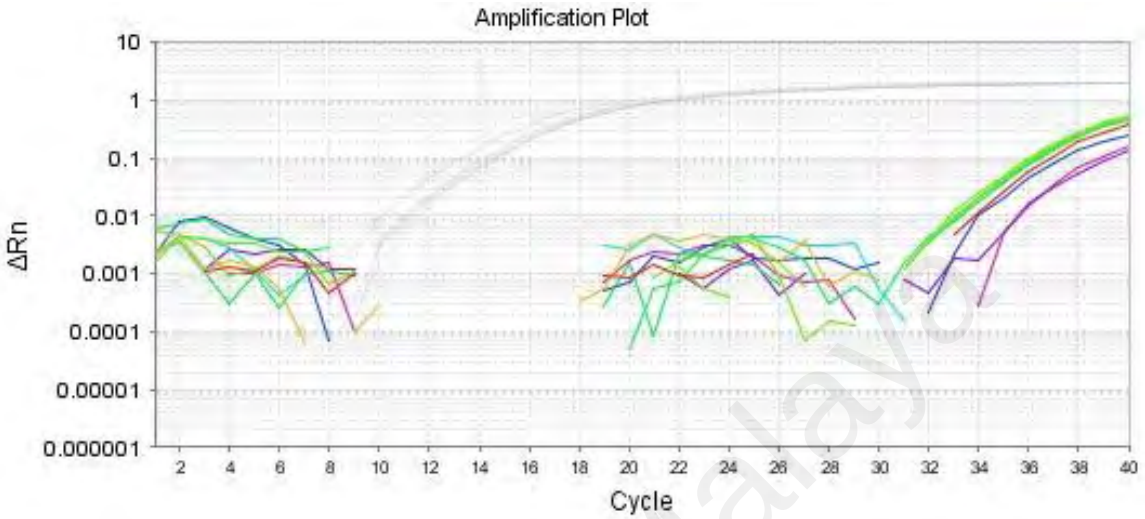

Figure 4.16: Amplification plot of tick samples

4.5.2.2 Vaginal swab

Out of the 22 positive vaginal swab detected by Trans-PCR assay, all of them gave a Ct value ranging from 18 to 37 as shown in Figure 4.17.

4.5.2.3 Milk samples

Out of the 17 milk samples that tested positive by Trans-PCR assay, 12 of them gave a Ct value ranging between 20 and 27, as shown in Figure 4.18. The remaining five milk samples gave an undetermined Ct value.

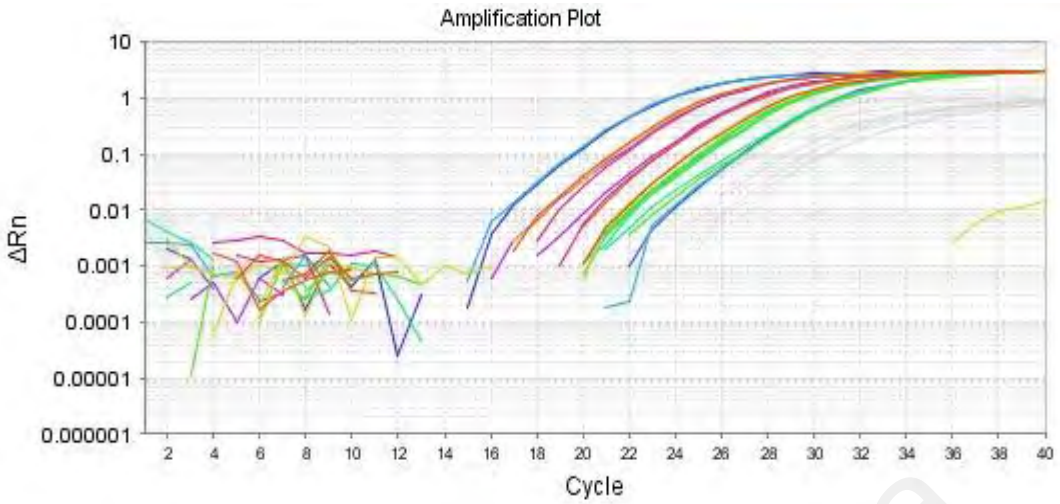

Figure 4.17: Amplification plot of vaginal swab

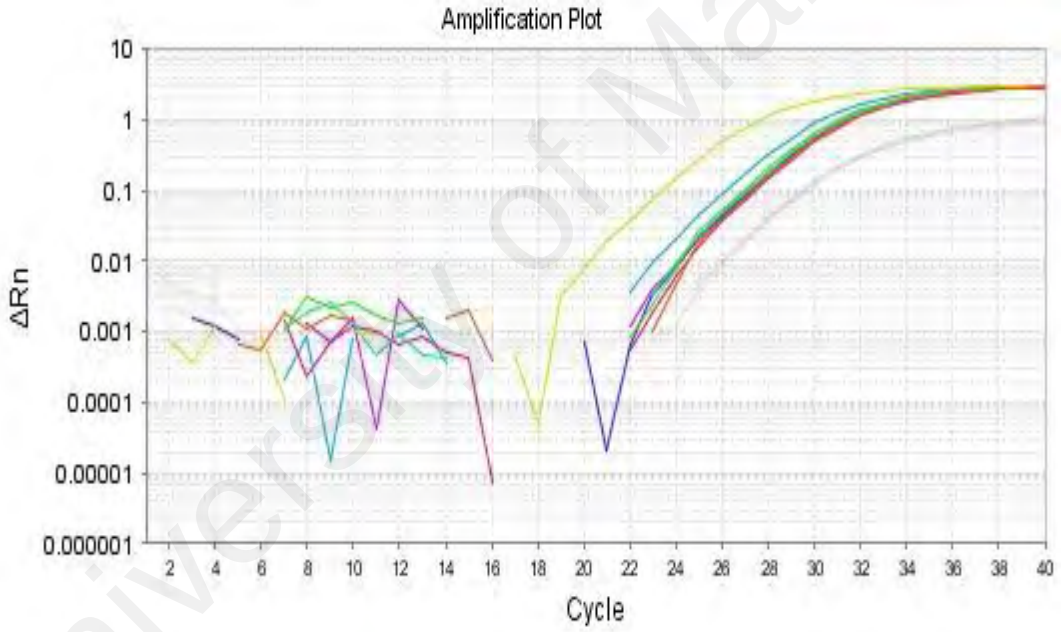

Figure 4.18: Amplification plot of milk samples

4.5.2.4 Blood Sample

Out of the 5 positive blood detected by Trans-PCR assay, all of them gave a Ct value ranging from 29 to 31 as shown in Figure 4.19. Table 4.7 summarizes the CT values obtained from real-time PCR assays for tick, vaginal, and milk samples.

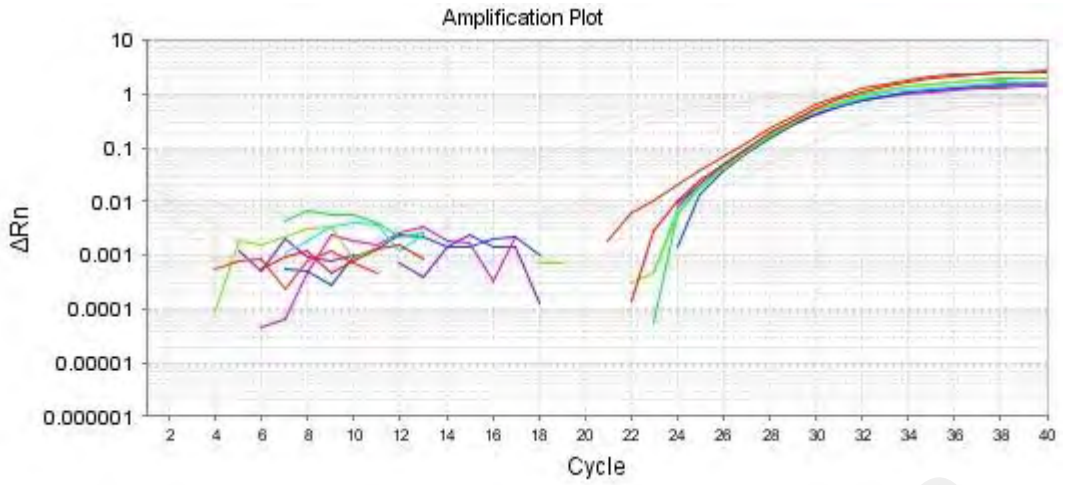

Figure 4.19: Amplification plot of blood samples.

Table 4.7: Real-time PCR analysis of positive samples detected by Trans-PCR and OMP-PCR assays (Appendix 6)

Sample source	No. of sample tested	No. of samples positive by real-time PCR	Range of Ct value
Tick	15	9	30 - 39
Vaginal Swab	22	22	18 - 37
Milk	17	12	20 - 27
Blood	5	5	29 -31

CHAPTER 5: DISCUSSION

Q fever has been regarded as an emerging zoonosis and a major public health concern affecting animal and human health worldwide. There has been limited investigation on Q fever in Malaysia. Besides serological data from two recent studies and a report of a veterinarian contracting Q fever in Penang (Tay *et al*., 1998; Bina *et al*., 2011), the extent of *coxiella* infections in humans and animals in Malaysia is largely unknown.

This study has taken a molecular approach to investigate *C. burnetii* infection in domestic ruminants as many studies have documented the animals (sheep, goats and cattle) as the reservoirs for *C. burnetii* (Brom *et al*., 2015) and the main sources of human infections (Heinzen *et al*., 1999; Berri *et al*., 2005). It has been reported that humans are infected via inhalation of aerosols contaminated with parturient products from the urines or faeces of infected animals (Tissot-Dupont & Raoult, 2008). In livestock, *C. burnetii* is shed mainly via birth products (birth fluids and placenta), but may also be shed by ruminants via vaginal mucus, milk, faeces (Berri *et al.*, 2002; Guatteo *et al*., 2007), urine (Heinzen *et al*., 1999) and semen (Kruszewska & Tylewska-Wierzbanowska, 1997). Using Trans-PCR assay, this study detected *C. burnetii* infection in 10 (5.7%) of 176 cattle (5 vaginal swabs and 5 blood samples) and 17 (19.3%) of 88 goats (mainly from vaginal swabs). The PCR findings were confirmed using either sequence analysis or a real-time PCR assay in this study.

Based on the review of Guatteo *et al* (2011), *C. burnetii* infection has been higher in cattle (20.0% and 37.7% of mean apparent prevalence) than in small ruminants (around 15.0% and 25% respectively for animal and herd level in sheep and goat (Guatteo *et al*., 2011). The prevalence rate of *C. burnetii* in domestic animals has been reported to vary in different geographical regions. In Asia, the herd prevalence have been reported to range from 16.7% to 35.4% (Guatteo *et al*., 2011).

The detection rate for the cattle understudied was higher as compared to that reported for cattle in Thailand, a neighboring country at the northern region of Malaysia (Muramatsu *et al*., 2014). Only nine of the 130 serum samples from the Thailand cattle tested were positive for antibodies against *C. burnetii* and no *C. burnetii*-specific DNA was detected using restriction fragment length polymorphism-nested PCR in the spleens of cattle. In contrast, in a Japanese survey, the presence of Q fever was 25.4% in healthy cattle and 23.5% in goats (Htwe *et al*., 1992).

The detection of *C. burnetii* from blood and vaginal samples from domestic animals in this study are not surprising as similar findings have also been reported in other geographical regions. Detection of *C. burnetii* in cattle blood samples or coxiellaemia in this study was in concurrence with an earlier report where the pathogen was detected in 6.0% of bovine samples including blood by Trans-PCR assay (Lorenz *et al*., 1998). Jung *et al*. (2014) reported that the blood samples obtained from 57 (9.5%) goats in their study were positive in the PCR-based screening for *C. burnetii*. Guatteo *et al*. (2007) reported that vaginal mucus shedding in almost 50% of the cows studied. The vaginal swabs gave the highest percentage of positive PCR results in this study, with 22 (12.1%) of 181 samples from both cattle and goats were positive. As reported by previous investigators (Welsh *et al.,* (1958), *C. burnetii* favors the placenta and exists in higher concentration. Hence, this makes the vagina the easiest route of shedding for the bacteria.

It has been reported that almost 40% of cows investigated in a previous study (Guatteo *et al*., 2007) were milk shedders. *C. burnetii* shedding in milk has also been reported in 1% of cows, and 4.7% in goats in a study carried out at the Reunion Island (Cardinale *et al*., 2014). In this study, 17% of the milk samples tested were positive and the samples came solely from a private cattle farm (Table 4.6). According to ESPA (2010), milk sample can be contaminated with *C. burnetii* through faecal materials or

from infected sites of the lactating animal. The detection of *C. burnetii* from milk samples suggests that *C. burnetii* is a food-borne pathogen as several studies reported seroconversion when volunteers were fed with contaminated milk (Benson *et al*., 1963; Krumbiegel & Wisniewski, 1970; Cerf & Condron, 2006;). However, it is not conclusive that consumption of contaminated milk would result in Q fever in humans.

Ticks are considered a natural reservoir for *C. burnetii* as more than 40 tick species have been documented to be infected with *C. burnetii* (Maurin & Raoult, 1999). In fact, the reference strain Nine Mile was isolated from a *Dermacentor andersoni* (Davis *et al*., 1938). Laboratory-reared and field-collected *Amblyomma americanum* ticks were hosts of a *Coxiella* sp. (Jasinskas *et al.*, 2007).

Coxiella-like bacteria have been reported to have been present in *Rhipicephalus sanguineus* (Bernasconi *et al*., 2002). Although the direct transmission of *C. burnetii* to human from infected ticks has never been documented, crushing an infected tick between the fingers has resulted in Q fever (Eklund *et al.*, 1947). Ticks are responsible for the spread of infection in domestic animals (de Bruin *et al*., 2012) and wild vertebrates (Maurin & Raoult, 1999). So far, a number of studies have demonstrated varying prevalence of infected ticks in different parts of the world.

A PCR assay targeting tick 28S rRNA gene was used in this study to demonstrate the presence of amplifiable DNA and the absence of PCR inhibitors in the tick samples. The method has been used for tick identification. However due to cost constraint, only a limited number of ticks in this study were identified using this method.

None of the 34 cattle ticks collected from a cattle farm in this study were positive. Similar result was also reported by Cardinale *et al.* (2014) for cattle at the Reunion island as all cattle ticks in that study were also negative for *C. burnetii*. Although the specific reason is not known yet, Cardinale *et al.* (2014) postulated that

the use of deltamethrin for tick control and management might reduce tick population and alter their ability to carry *C. burnetii*.

Three (13.3%) of 30 mammalian ticks and four (13.3%) of 35 questing ticks from a forest reserve in this study were positive for *C. burnetii*. Although soft ticks such as *Ornithodoros* and *Carios* have been reported to have a high prevalence of *Coxiella*-like organisms (Reeves *et al*., 2006; Almeida *et al.*, 2012; Mediannikov *et al*., 2010), no *C. burnetii* was detected from the only soft tick in this study. The *Amblyomma* spp. was the predominant ticks infesting small mammals as the ticks were collected from several animal hosts such as small rodents (*Maxomys rajah*, *Callosciurus notatus* and *Tupaia glis)* in this study. The *Heamaphysalis* spp. was the main tick genus collected from the forest vegetation, followed by *Dermacentor* spp. Some of these ticks including *Dermacentor* spp. and *Haemaphysalis* spp. have been shown to be involved in the cycles of tick typhus and Q-fever in the forests of Malaysia (Marchette, 1966). Although there is no data about the affinity of the ticks to bite humans yet, the detection of *C. burnetii* in these ticks poses a risk to wildlife, domestic animal and human.

The involvement of different genera of hard ticks, including *Amblyomma* spp. (Jasinskas *et al*., 2007), *Rhipicephalus* spp. (Bernasconi *et al.,* 2002), *Dermacentor* spp. (Psaroulaki et al., 2006), *Ixodes* spp. (Špitalská & Kocianova, 2003), *Hyalomma* spp. (Spyridaki *et al.*, 2002) and *Haemaphysalis* spp. (Machado-Ferreira *et al*., 2011) have been documented as potential vectors for *C. burnetii* (Tanskul & Inlao, 1989).

In the Asia region, *C. burnetii* has been detected from 2 of 100 *Haemaphysalis longicornis* ticks from Korea, demonstrating high nucleotide sequence similarity (99.5% and 100%) when compared to the *com1* gene of *C. burnetii* Derrick reference strains (Lee *et al.*, 2004). In another investigation, *Haemaphysalis coccinna* from Far-east Russia was found to be positive for *Coxiella*-like bacteria based on a technique using

specific primers for 16S rRNA gene sequence. Ahantarig *et al.* (2011) reported for the first time *Coxiella*-like symbionts in *Haemaphysalis* spp. ticks in Thailand.

A subsequent study (Arthan *et al.*, 2015) reported a high prevalence of *Coxiella*-like endosymbionts in four *Haemaphysalis* tick species (i.e., *Haemaphysalis hystricis*, *Haemaphysalis lagrangei, Haemaphysalis obesa*, and *Haemaphysalis shimoga)* in Thailand. Ticks may acquire *C. burnetii* from a variety of sources. Female ticks harbouring *Coxiella*-like bacteria may pass the bacteria to the next generation in a process called transovarial transmission (Klyachko *et al*., 2007). All the ticks were adult in this study, however; almost every stage (larva, nymph, and adult) of ticks could be positive for *Coxiella*-like bacteria (Arthan *et al*., 2015). Interestingly, only one of the 30 *R. sanguineus* ticks removed from dogs were positive for *C. burnetii*. Dogs are competent hosts for *C. burnetii* and *R. sanguineus* has been known as a vector for this host (Greay, 2014). The presence of *Coxiella*-infected *R. sanguineus* ticks can pose a risk to pet owners in the urban area.

Overall, the Trans-PCR assay with primers targeting *IS1111* gene demonstrated higher detection rates for *C. burnetii*, as compared to the *com 1* gene PCR assay. In this study, only 17 positive samples were detected using *com 1* gene PCR assay, as compared to 54 positive samples detected by Trans-PCR. The Trans-PCR assay has been found to be specific and sensitive for the detection of *C. burnetii* in clinical samples (Berri *et al*., 2000; Vaidya *et al*., 2008; Vaidya *et al*., 2010). The gene is present in multiple copies (about 7 to 110 copies) within the bacterial genome (Klee *et al.,* 2006). The OMP-PCR assay has also been used for specific detection of *C. burnetii* DNA from animal and environmental sources of Q fever infection for humans in Queensland (Tozer *et al*., 2014). The advantage of the assay was its ability to differentiate different genotypes of *C. burnetii* through sequence determination of the amplicon (Zhang *et al*., 1998). However, when the amount of amplicon generated from

the PCR assay is low, sequence determination of the amplicon may not be feasible. Trans-PCR assay has a higher sensitivity due to the assay's ability to detect more cases and higher specificity and thus it is suggested be used for detection of *Coxiella* spp. However there is no perfect test, therefore continued effort to develop and validate PCR assays is necessary. In this study, real-time PCR analysis was used to provide an additional means for confirmation of PCR results in the event when sequence analysis was not successful for the PCR-positive samples.

To improve surveillance of Q fever, new investigative tools are required for detection of *C. burnetii* with high sensitivity and specificity. Due to the high specificity of PCR assay, the detection of novel species or variants of a known species may not be possible, as the organism cannot be amplified during the initial PCR screening step (McLaughlin & Castrodale, 2011). Hence, several approaches such as microarrays and reverse-line hybridization methods have been introduced, not only for detection of *C. burnetii*, but also for detecting a wide variety of tick-borne pathogens from various samples. The use of these new methods should be used for future investigation or surveillance of *C. burnetii*.

The genetic diversity of *Coxiella* has been proven through the identification of 20 genotypes within this species (Stein & Raoult, 1993). Many studies have shown the presence of *Coxiella*-like bacteria in the ticks. For instance, comparison of *Coxiella*-like partial DNA sequences from different species of *Haemaphysalis* spp. ticks in Thailand demonstrated 1% to 3% dissimilarities (Arthan *et al*., 2015). Hence, sequence determination plays an important role for accurate identification of *C. burnetii*. However, sequence analysis of *Coxiella* can be hampered by the low quality and quantity of amplicons generated from a PCR assay. In this study, only very few of the samples were confirmed as *C. burnetii* through direct sequencing of the amplicons. Some of the samples have too low intensity of DNA for direct sequencing approach and

some generated noisy data which were not able to be analysed. To improve sequence determination, the amplicon should be cloned into suitable plasmids prior to sequencing as this approach is known to produce better sequences for analysis.

5.1 Limitation and further Investigations

For future investigation, more systematic studies combining serology and DNA testing should be conducted for epidemiological surveillance and monitoring of *C. burnetii* infections in different animal populations and tick samples in Malaysia. Further studies are needed to characterize the genotypes of the *C. burnetii* strains and to identify the potential transmission risks of these organisms to human and animals. More sensitive detection techniques can be performed to provide a better insight into the epidemiology of *C. burnetii* infections in Malaysia. Additionally, more extensive study should be carried out to determine the circulation of this organism in the natural environment of Malaysia. As ticks play an important role in the transmission of *C. burnetii* infection, accurate species identification of ticks is necessary. Continuous surveillance programs in wild and domestic animals are necessary to identify endemic areas, and to monitor risk of zoonotic infections.

CHAPTER 6: CONCLUSION

This study documented for the first time the molecular evidence of *C. burnetii* in ticks and animal samples in Malaysia. There is a low prevalence of *Coxiella* infection reported in this study, as compared to those published in other geographical regions (Guatteo *et al*. 2007). The information derived in this study shows the presence of C. *burnetii* in various samples from the domestic animals. The detection of *C. burnetii* from a number of ticks suggests that some of these ticks may play an essential role in the enzootic cycle of coxiellosis at low incidence.

Further work is required to assess the prevalence of *C. burnetii* on a larger scale. In view of the detection of *C. burnetii* DNA in the veterinary and tick samples, awareness for prevention and control of the possible transmission of *C. burnetii* infection to the local population is necessary.

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APPENDIX 1

Tick samples

No	ID	Tick Species	Host	Location	28S	Trans	OMP1
1	R001	*Amblyomma* sp.	*Maxomys rajah*	Kuala Lompat 1	√	x	x
2	B022	*Ornithodoros turicata*	*Rhinolophus sedulus*	Kuala Lompat 1	√	x	x
3	S002	*Dermacentor* sp.	*Tupaia glis*	Kuala Lompat 1	√	x	x
4	R004	*Amblyomma* sp.	*Maxomys rajah*	Kuala Lompat 1	√	√	x
5	KL01	*Amblyomma* sp.	Human	Kuala Lompat 1	√	x	x
6	R006	*Haemaphysalis* sp.	*Maxomys rajah*	Kuala Lompat 1	√	x	x
7	S003	*Dermacentor* sp.	*Tupaia glis*	Kuala Lompat 1	√	√	x
8	S007	*Dermacentor* sp.	*Sundasciurus tenuis*	Kuala Lompat 1	√	x	x
9	R007	*Amblyomma* sp.	*Leopoldamys sabanus*	Kuala Lompat 1	√	x	x
10	R008	*Dermacentor* sp.	*Leopoldamys sabanus*	Kuala Lompat 1	√	x	x
11	S011	*Heamaphysalis* sp.	*Sundasciurus tenuis*	Kuala Lompat 1	√	x	x
12	M001	*Heamaphysalis* sp	*Herpestes urva*	Kuala Lompat 2	√	x	x
13	R002	*Amblyomma* sp.	*Leopoldamys sabanus*	Kuala lompat 2	√	x	x
14	R003	*Amblyomma* sp.	*Maxomys rajah*	Kuala Lompat 2	√	x	x
15	R006	*Haemaphysalis* sp.	*Maxomys whiteheadii*	Kuala Lompat 2	√	x	x
16	S002	nymph *Amblyomma* sp.	*Callasciuras notatus*	Kuala Lompat 2	√	√	x
17	S003	*Amblyomma sp.*	*Callasciuras notatus*	Kuala Lompat 2	√	x	x
18	KL05	female *Amblyomma* sp.	*Varanus nebulosus*	Kuala Lompat 2	√	x	x
19	KLO6	*Amblyomma* sp.	Human	Kuala Lompat 2	√	√	x
20	KL13	*Amblyomma* sp.	Human	Kuala Lompat 2	√	x	x
21	S008	nymph *Heamaphysalis* sp	*Lariscus insignis*	Kuala Lompat 2	√	x	x
22	S011	*Amblyomma* sp.	*Tupaia glis*	Kuala Lompat 2	√	x	x
23	KL20	larvae *Amblyomma* sp.	human	Kuala Lompat 2	√	x	x
24	KL35	*Amblyomma* sp.	human	Kuala Lompat 2	√	x	x
25	KL36	nymph *Amblyomma* sp.	Human	Kuala Lompat 2	√	x	x
26	DOG1	*Amblyomma* sp.	Orang Asli Dog	Kuala Lompat 2	√	x	x
27	S014	nymph *Amblyomma* sp.	*Lariscus insignis*	Kuala Lompat 2	√	x	x
28	BIRD1	nymph *Amblyomma* sp.	*Alophoixus bres*	Kuala Lompat 2	√	x	x
29	R021	*Amblyomma* sp.	*Maxomys whiteheadii*	Kuala Lompat 2	√	x	x
30	S017	*Amblyomma* sp.	*Tupaia glis*	Kuala Lompat 2	√	x	x

No	ID	Tick Species	Host	Location	28S	Trans	OMP1
31	KL09	*Dermacentor* sp.	Vegetation	Kuala Lompat 2	√	x	√
32	KL22	*Rhipicephalus* sp.	Vegetation	Kuala Lompat 2	√	x	√
33	KL25	*Rhipicephalus* sp.	Vegetation	Kuala Lompat 2	√	x	√
34	KL31	*Rhipicephalus* sp.	Vegetation	Kuala Lompat 2	√	x	√
35	KL39	*Dermacentor* sp.	Vegetation	Kuala Lompat 2	√	x	x
36	KL42	*Dermacentor* sp.	Vegetation	Kuala Lompat 2	√	x	x
37	KL45	*Rhipicephalus* sp.	Vegetation	Kuala Lompat 2	√	x	x
38	KL48	*Rhipicephalus* sp.	Vegetation	Kuala Lompat 2	√	x	√
39	KL53	*Dermacentor* sp.	Vegetation	Kuala Lompat 2	√	x	x
40	ST07	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
41	ST12	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
42	ST15	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
43	ST17	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
44	ST19	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	√	x
45	ST20	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
46	ST22	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
47	ST23	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
48	ST25	*Heamaphysalis sulcata*	Vegetation	Sungai Teris	√	√	x
49	ST26	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
50	ST35	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
51	ST36	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
52	ST37	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	√	x
53	ST44	*Heamaphysalis sulcata*	Vegetation	Sungai Teris	√	x	x
54	ST51	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	√	x
55	ST52	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
56	ST53	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
57	ST54	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
58	ST57	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
59	ST63	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
60	ST65	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
61	ST67	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
62	ST70	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
63	ST71	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
64	ST77	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	√	x
65	ST79	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
66	ST81	*Heamaphysalis* sp.	Vegetation	Sungai Teris	√	x	x
67	Y9-2	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
68	X108-1	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
69	X108-2	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
70	X62-1	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
71	X62-2	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
72	X102-1	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x

No	ID	Tick Species	Host	Location	28S	Trans	OMP1
73	X102-2	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
74	X110-1	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
75	X110-2	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
76	Y57-1	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
77	Y57-2	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
78	Y40-1	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
79	Y40-2	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
80	Y75-1	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
81	Y75-2	*Boophilus* sp.	Nellow	Jelai Gemas	√	x	x
82	WXY1167-1	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
83	WXY1167-2	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
84	WYY1392-1	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
85	WYY1392-2	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
86	WYX786	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
87	WYY1470	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
88	WYY1474	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
89	WYX672-1	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
90	WYX672-2	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
91	WYY1362	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
92	WYX1096-1	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
93	WYX1096-2	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
94	WYY1292	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
95	WYX1180-1	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
96	WYX1180-2	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
97	WYX1204	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
98	WYY1356	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
99	WYY1301	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
100	WYX1199	*Boophilus* sp.	YKK	Jelai Gemas	√	x	x
101	SP01a	*R.sanguines*	Dog	SPCA	√	x	√
102	SP01b	*R.sanguines*	Dog	SPCA	√	x	x
103	SP02a	*R.sanguines*	Dog	SPCA	√	x	x
104	SP02b	*R.sanguines*	Dog	SPCA	√	x	x
105	SP03a	*R.sanguines*	Dog	SPCA	√	x	x
106	SP03b	*R.sanguines*	Dog	SPCA	√	x	x
107	SP04a	*R.sanguines*	Dog	SPCA	√	x	x
108	SP04b	*R.sanguines*	Dog	SPCA	√	x	x
109	SP05a	*R.sanguines*	Dog	SPCA	√	x	x
110	SP05b	*R.sanguines*	Dog	SPCA	√	x	x
111	SP05c	*R.sanguines*	Dog	SPCA	√	x	x
112	SP06a	*R.sanguines*	Dog	SPCA	√	x	x
113	SP06b	*R.sanguines*	Dog	SPCA	√	x	x

No	ID	Tick Species	Host	Location	28S	Trans	OMP1
114	SC01a	*R.sanguines*	Dog	Second Chance	√	x	x
115	SC01b	*R.sanguines*	Dog	Second Chance	√	x	x
116	SC01c	*R.sanguines*	Dog	Second Chance	√	x	x
117	SC02a	*R.sanguines*	Dog	Second Chance	√	x	x
118	SC02b	*R.sanguines*	Dog	Second Chance	√	x	x
119	SC02c	*R.sanguines*	Dog	Second Chance	√	x	x
120	SC03a	*R.sanguines*	Dog	Second Chance	√	x	x
121	SC03b	*R.sanguines*	Dog	Second Chance	√	x	x
122	SC03c	*R.sanguines*	Dog	Second Chance	√	x	x
123	SC03d	*R.sanguines*	Dog	Second Chance	√	x	x
124	SC03e	*R.sanguines*	Dog	Second Chance	√	x	x
125	SC03f	*R.sanguines*	Dog	Second Chance	√	x	x
126	SC03g	*R.sanguines*	Dog	Second Chance	√	x	x
127	SC03h	*R.sanguines*	Dog	Second Chance	√	x	x
128	SC03i	*R.sanguines*	Dog	Second Chance	√	x	x
129	SC03j	*R.sanguines*	Dog	Second Chance	√	x	x
130	SC04a	*R.sanguines*	Dog	Second Chance	√	x	x
131	SC04b	*R.sanguines*	Dog	Second Chance	√	x	x
132	SC05	*R.sanguines*	Dog	Second Chance	√	x	x
133	SC06a	*R.sanguines*	Dog	Second Chance	√	x	x
134	SC06b	*R.sanguines*	Dog	Second Chance	√	x	x
135	SC07	*R.sanguines*	Dog	Second Chance	√	x	x
136	SC08	*R.sanguines*	Dog	Second Chance	√	x	x
137	SC09a	*R.sanguines*	Dog	Second Chance	√	x	x
138	SC09b	*R.sanguines*	Dog	Second Chance	√	x	x
139	SC10a	*R.sanguines*	Dog	Second Chance	√	x	x
140	SC10b	*R.sanguines*	Dog	Second Chance	√	x	x
141	SC10c	*R.sanguines*	Dog	Second Chance	√	x	x
142	SC11	*R.sanguines*	Dog	Second Chance	√	x	x
143	SC12a	*R.sanguines*	Dog	Second Chance	√	x	x
144	MVKBT01	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
145	MVKBT02	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
146	MVKBT03	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
147	MVKBT04	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
148	MVKBT05	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
149	MVKBT06	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
150	MVKBT07	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
151	MVKBT08	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x

No	ID	Tick Species	Host	Location	28S	Trans	OMP1
152	MVKBT09	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
153	MVKBT10	*Heamaphysalis* sp.	Cattle	MVKBT	√	x	x
154	MVKBT11	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
155	MVKBT12	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
156	MVKBT13	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
157	MVKBT14	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
158	MVKBT15	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
159	MVKBT16	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
160	MVKBT17	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
161	MVKBT18	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
162	MVKBT19	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
163	MVKBT20	*Heamaphysalis sp.*	Goat	MVKBT	√	x	x
164	MVKBT21	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
165	MVKBT22	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
166	MVKBT23	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
167	MVKBT24	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
168	MVKBT25	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
169	MVKBT26	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
170	MVKBT27	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
171	MVKBT28	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
172	MVKBT29	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x
173	MVKBT30	*Heamaphysalis* sp.	Goat	MVKBT	√	x	x

APPENDIX 2

Blood Samples

No	ID	Species	Breed	Location	Trans	OMP
1	T018	Cattle	Unknown	TPU	x	x
2	T009	Cattle	Unknown	TPU	x	x
3	T121	Cattle	Unknown	TPU	x	x
4	T111	Cattle	Unknown	TPU	x	x
5	T119	Cattle	Unknown	TPU	x	x
6	T902	Cattle	Unknown	TPU	x	x
7	T107	Cattle	Unknown	TPU	x	x
8	T113	Cattle	Unknown	TPU	x	x
9	T104	Cattle	Unknown	TPU	x	x
10	T109	Cattle	Unknown	TPU	x	x
11	T077	Cattle	Unknown	TPU	x	x
12	T087	Cattle	Unknown	TPU	x	x
13	T004	Cattle	Unknown	TPU	x	x
14	T083	Cattle	Unknown	TPU	x	x
15	T092	Cattle	Unknown	TPU	x	x
16	T039	Cattle	Unknown	TPU	x	x
17	T052	Cattle	Unknown	TPU	x	x
18	T068	Cattle	Unknown	TPU	x	x
19	T088	Cattle	Unknown	TPU	x	x
20	5455	Cattle	Unknown	TPU	x	x
21	T007	Cattle	Unknown	TPU	x	x
22	T010	Cattle	Unknown	TPU	x	x
23	T099	Cattle	Unknown	TPU	x	x
24	ZO20	Cattle	Unknown	TPU	x	x
25	Z013	Cattle	Unknown	TPU	x	x
26	Y032	Cattle	Unknown	TPU	x	x
27	T068	Cattle	Unknown	TPU	x	x
28	T024	Cattle	Unknown	TPU	x	x
29	T907	Cattle	Unknown	TPU	x	x
30	50410	Cattle	Unknown	TPU	x	x
31	WYX1167	Cattle	YKK	Jelai Gemas	x	x
32	WYY1392	Cattle	YKK	Jelai Gemas	x	x
33	WYX786	Cattle	YKK	Jelai Gemas	√	x
34	WYY1470	Cattle	YKK	Jelai Gemas	x	x
35	WYY1474	Cattle	YKK	Jelai Gemas	√	x

No	ID	Species	Breed	Location	Trans	OMP
36	WYY1518	Cattle	YKK	Jelai Gemas	x	x
37	WYX672	Cattle	YKK	Jelai Gemas	x	x
38	WYX1362	Cattle	YKK	Jelai Gemas	x	x
39	WYX1096	Cattle	YKK	Jelai Gemas	x	x
40	WYY1292	Cattle	YKK	Jelai Gemas	x	x
41	WYX1680	Cattle	YKK	Jelai Gemas	√	x
42	WYX1204	Cattle	YKK	Jelai Gemas	x	x
43	WYY1356	Cattle	YKK	Jelai Gemas	x	x
44	WYY1302	Cattle	YKK	Jelai Gemas	x	x
45	WYX1199	Cattle	YKK	Jelai Gemas	x	x
46	X70	Cattle	Nellore	Jelai Gemas	x	x
47	Y71	Cattle	Nellore	Jelai Gemas	x	x
48	Y28	Cattle	Nellore	Jelai Gemas	x	x
49	X59	Cattle	Nellore	Jelai Gemas	x	x
50	Y9	Cattle	Nellore	Jelai Gemas	√	x
51	X61	Cattle	Nellore	Jelai Gemas	x	x
52	X108	Cattle	Nellore	Jelai Gemas	√	x
53	X110	Cattle	Nellore	Jelai Gemas	x	x
54	X62	Cattle	Nellore	Jelai Gemas	x	x
55	X57	Cattle	Nellore	Jelai Gemas	x	x
56	BP03	Caprine	Jamnapari, Boer	TPU	x	x
57	BP04	Caprine	Jamnapari, Boer	TPU	x	x
58	BP01	Caprine	Jamnapari, Boer	TPU	x	x
59	V007	Caprine	Jamnapari, Boer	TPU	x	x
60	NoID(black,white)	Caprine	Jamnapari, Boer	TPU	x	x

No	ID	Species	Breed	Location	Trans	OMP
61	BP02	Caprine	Jamnapari, Boer	TPU	x	x
62	NS104	Caprine	Jamnapari, Boer	TPU	x	x
63	V0607	Caprine	Jamnapari, Boer	TPU	x	x
64	BP101	Caprine	Jamnapari, Boer	TPU	x	x
65	NS105	Caprine	Jamnapari, Boer	TPU	x	x
66	V003	Caprine	Jamnapari, Boer	TPU	x	x
67	101	Caprine	Jamnapari, Boer	TPU	x	x
68	103	Caprine	Jamnapari, Boer	TPU	x	x
69	102	Caprine	Jamnapari, Boer	TPU	x	x
70	No ID (White)	Caprine	Jamnapari, Boer	TPU	x	x
71	No ID(Brown)	Caprine	Jamnapari, Boer	TPU	x	x
72	4752	Caprine	Jamnapari, Boer	TPU	x	x
73	4752 (kid)	Caprine	Jamnapari, Boer	TPU	x	x
74	NS101	Caprine	Jamnapari, Boer	TPU	x	x
75	V001	Caprine	Jamnapari, Boer	TPU	x	x
76	00-9	Caprine	Unknown	VRI	x	x
77	0-10	Caprine	Unknown	VRI	x	x
78	V004	Caprine	Unknown	VRI	x	x
79	BG1	Caprine	Unknown	VRI	x	x
80	BG2	Caprine	Unknown	VRI	x	x
81	BG3	Caprine	Unknown	VRI	x	x
82	BG4	Caprine	Unknown	VRI	x	x
83	BG5	Caprine	Unknown	VRI	x	x
84	BG6	Caprine	Unknown	VRI	x	x
85	BG7	Caprine	Unknown	VRI	x	x
86	BG8	Caprine	Unknown	VRI	x	x
87	BG9	Caprine	Unknown	VRI	x	x
88	BG10	Caprine	Unknown	VRI	x	x
89	4KB 2	Caprine	Unknown	VRI	x	x
90	4KB 2937	Caprine	Unknown	VRI	x	x
91	KB 1	Caprine	Unknown	VRI	x	x
92	2447	Caprine	Unknown	Sg Siput	x	x
93	2596	Caprine	Unknown	Sg Siput	x	x
94	3545	Caprine	Unknown	Sg Siput	x	x
95	2536	Caprine	Unknown	Sg Siput	x	x

No	ID	Species	Breed	Location	Trans	OMP
96	2124	Caprine	Unknown	Sg Siput	x	x
97	2111	Caprine	Unknown	Sg Siput	x	x
98	2128	Caprine	Unknown	Sg Siput	x	x
99	2590	Caprine	Unknown	Sg Siput	x	x
100	2508	Caprine	Unknown	Sg Siput	x	x
101	3110	Caprine	Unknown	Sg Siput	x	x
102	2440	Caprine	Unknown	Sg Siput	x	x
103	572	Caprine	Unknown	Sg Siput	x	x

APPENDIX 3

Vaginal Swabs

NO	ID	Source	Location	Trans	OMP
1	KB1	Boer Goat	Bukit Tengah, Penang	√	√
2	KB2	Boer Goat	Bukit Tengah, Penang	√	√
3	KB3	Boer Goat	Bukit Tengah, Penang	√	x
4	KB4	Boer Goat	Bukit Tengah, Penang	√	√
5	KB5	Boer Goat	Bukit Tengah, Penang	√	√
6	KB6	Boer Goat	Bukit Tengah, Penang	√	√
7	KB7	Boer Goat	Bukit Tengah, Penang	x	x
8	KB8	Boer Goat	Bukit Tengah, Penang	√	√
9	KB9	Boer Goat	Bukit Tengah, Penang	√	x
10	KB10	Boer Goat	Bukit Tengah, Penang	√	x
11	KI1	Boer Goat	Bukit Tengah, Penang	√	x
12	KI2	Boer Goat	Bukit Tengah, Penang	√	√
13	KI3	Boer Goat	Bukit Tengah, Penang	√	√
14	KI4	Boer Goat	Bukit Tengah, Penang	√	x
15	KI5	Boer Goat	Bukit Tengah, Penang	√	x
16	KI6	Boer Goat	Bukit Tengah, Penang	√	√
17	KI7	Boer Goat	Bukit Tengah, Penang	√	√
18	KI8	Boer Goat	Bukit Tengah, Penang	√	√
19	KI9	Boer Goat	Bukit Tengah, Penang	√	√
20	KI10	Boer Goat	Bukit Tengah, Penang	√	x
21	T083	Cattle	TPU	x	x
22	T009	Cattle	TPU	x	x
23	T010	Cattle	TPU	√	x
24	T024	Cattle	TPU	x	x
25	T077	Cattle	TPU	√	x
26	50410	Cattle	TPU	√	x
27	5401	Cattle	TPU	√	x
28	T018	Cattle	TPU	x	x
29	50410	Cattle	TPU	√	x
30	T068	Cattle	TPU	x	x
31	B20	Cattle	Behrang	x	x
32	B21	Cattle	Behrang	x	x
33	B22	Cattle	Behrang	x	x
34	B23	Cattle	Behrang	x	x
35	B24	Cattle	Behrang	x	x
36	B25	Cattle	Behrang	x	x
37	B26	Cattle	Behrang	x	x
38	B27	Cattle	Behrang	x	x
39	B28	Cattle	Behrang	x	x

NO	ID	Source	Location	Trans	OMP
40	B29	Cattle	Behrang	x	x
41	B30	Cattle	Behrang	x	x
42	B31	Cattle	Behrang	x	x
43	WYAA1732	YKK	Kuala Berang, Terengganu	x	x
44	WYAA1699	YKK	Kuala Berang, Terengganu	x	x
45	WYAA1714	YKK	Kuala Berang, Terengganu	x	x
46	WYAA1797	YKK	Kuala Berang, Terengganu	x	x
47	WYAA1930	YKK	Kuala Berang, Terengganu	x	x
48	WYAA1630	YKK	Kuala Berang, Terengganu	x	x
49	WYAA1646	YKK	Kuala Berang, Terengganu	x	x
50	WYAA1675	YKK	Kuala Berang, Terengganu	x	x
51	WYAA1789	YKK	Kuala Berang, Terengganu	x	x
52	WYAA1850	YKK	Kuala Berang, Terengganu	x	x
53	WYAA1986	YKK	Kuala Berang, Terengganu	x	x
54	WYAA1771	YKK	Kuala Berang, Terengganu	x	x
55	WYAA1834	YKK	Kuala Berang, Terengganu	x	x
56	WYAA1731	YKK	Kuala Berang, Terengganu	x	x
57	WYAA1980	YKK	Kuala Berang, Terengganu	x	x
58	WYAA1730	YKK	Kuala Berang, Terengganu	x	x
59	WYAA1795	YKK	Kuala Berang, Terengganu	x	x
60	1725	YKK	Kuala Berang, Terengganu	x	x
61	WYAA1794	YKK	Kuala Berang, Terengganu	x	x
62	EKV3742	KK breed	Tanah Merah, Kelantan	x	x
63	EKV4995	KK breed	Tanah Merah, Kelantan	x	x
64	EKY4957	KK breed	Tanah Merah, Kelantan	x	x
65	EKP1406	KK breed	Tanah Merah, Kelantan	x	x
66	EPX4676	KK breed	Tanah Merah, Kelantan	x	x
67	V3923	KK breed	Tanah Merah, Kelantan	x	x
68	EKY4785	KK breed	Tanah Merah, Kelantan	x	x
69	EKX4510	KK breed	Tanah Merah, Kelantan	x	x
70	EKW4166	KK breed	Tanah Merah, Kelantan	x	x
71	EKY4940	KK breed	Tanah Merah, Kelantan	x	x
72	EKY5004	KK breed	Tanah Merah, Kelantan	x	x
73	EKY4880	KK breed	Tanah Merah, Kelantan	x	x
74	EKX4604	KK breed	Tanah Merah, Kelantan	x	x
75	WKL1354	KK breed	Tanah Merah, Kelantan	x	x
76	EKY4928	KK breed	Tanah Merah, Kelantan	x	x
77	EKY4868	KK breed	Tanah Merah, Kelantan	x	x
78	EKY4985	KK breed	Tanah Merah, Kelantan	x	x
79	EKY4872	KK breed	Tanah Merah, Kelantan	x	x
80	EKY4747	KK breed	Tanah Merah, Kelantan	x	x
81	Y4885	KK breed	Tanah Merah, Kelantan	x	x

NO	ID	Source	Location	Trans	OMP
82	EKX4528	KK breed	Tanah Merah, Kelantan	x	x
83	EKY4907	KK breed	Tanah Merah, Kelantan	x	x
84	EKY4884	KK breed	Tanah Merah, Kelantan	x	x
85	I7X 4359	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
86	F5X 4371	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
87	I6S 3714	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
88	I5L 1547	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
89	I5N 2540	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
90	I6T 3760	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
91	F5L 1776	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
92	I92 4712	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
93	IJ2 4706	Jersey	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
94	I82 4615	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
95	F72 4628	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
96	I82 4644	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
97	I7Y 4591	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
98	I72 4626	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
99	I82 4685	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
100	I82 4662	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
101	I72 4648	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
102	I72 4681	Mafriwal	Pusat Ternakan Haiwan Air Hitam, Johor	x	x
103	HM194	Savannah	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
104	HM59	African dwarf	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
105	HN56	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
106	HM234	African dwarf	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
107	HN203	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x

NO	ID	Source	Location	Trans	OMP
108	HN79	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
109	HM149	African dwarf	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
110	HN132	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
111	HN186	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
112	HM112	Cashmere	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
113	HN182	African dwarf	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
114	HN122	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
115	HN187	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
116	HN135	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
117	HM256	African dwarf	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
118	HL432	Savannah	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
119	HN95	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
120	HN202	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
121	HN173	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
122	HN180	Boer	Pusat Pembiakan Kambing Kampung Kuala Pah	x	x
123	WKM 1558	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
124	EKY 4774	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
125	WKK 907-1	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
126	EKL 071	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
127	WKM 1374	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
128	EKJ 084	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
129	EKR 2162	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
130	EKJ 371	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
131	EKY 4712	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
132	EKM 737	KK breed	Institute Bioteknologi Haiwan	x	x

NO	ID	Source	Location	Trans	OMP
			Kebangsaan, Jerantut, Pahang		
133	WKK 907-2	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
134	EKY 4757	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
135	WKK 1033	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
136	EKY 4806	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
137	EKP 1246	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
138	WKK 849	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
139	WLM 1035	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
140	EKS 2728	KK breed	Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang	x	x
141	KN95	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
142	1103	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
143	KN188	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
144	KN127	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
145	KN214	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
146	KN234	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
147	KN221	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
148	KN216	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
149	KN43	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
150	KN54	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
151	KN109	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
152	KN125	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
153	KN190	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
154	KN73	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
155	KN136	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
156	KN232	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x

NO	ID	Source	Location	Trans	OMP
157	KN223	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
158	KN50	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
159	KN231	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
160	KN159	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
161	KM79	Damara	Pusat Pembiakan Kambing Bebiri Gajah Mati, Pokok Sena, Kedah	x	x
162	UN2-88	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
163	UN2-92	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
164	UB2-28	Brahman	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
165	UN2-21	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
166	UN2-46	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
167	UN2-37	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
168	UN2-19	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
169	UB2-38	Brahman	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
170	UN2-07	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
171	UN2-56	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
172	UN2-95	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
173	UN2-17	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
174	UNY-137	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
175	UN2-28	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
176	UB2-32	Brahman	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
177	UN2-32	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
178	UN2-101	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
179	UN2-05	Nellore	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x
180	UB2-41	Brahman	Pusat Ternakan Haiwan Ulu Lepar, Kuantan Pahang	x	x

APPENDIX 4

Milk Samples

No	ID	Cattle Sp	Location	Trans	OMP
1	B01	Unknown	Behrang	x	x
2	B02	Unknown	Behrang	√	x
3	B03	Unknown	Behrang	√	x
4	B04	Unknown	Behrang	√	x
5	B05	Unknown	Behrang	√	x
6	B06	Unknown	Behrang	√	x
7	B07	Unknown	Behrang	√	x
8	B08	Unknown	Behrang	√	x
9	B09	Unknown	Behrang	x	x
10	B10	Unknown	Behrang	√	x
11	B11	Unknown	Behrang	√	x
12	B12	Unknown	Behrang	√	x
13	B13	Unknown	Behrang	√	x
14	B14	Unknown	Behrang	x	x
15	B15	Unknown	Behrang	x	x
16	B16	Unknown	Behrang	√	x
17	B17	Unknown	Behrang	√	x
18	B18	Unknown	Behrang	√	x
19	B19	Unknown	Behrang	x	x
20	B20	Unknown	Behrang	x	x
21	B21	Unknown	Behrang	√	x
22	B22	Unknown	Behrang	√	x
23	B23	Unknown	Behrang	x	x
24	B24	Unknown	Behrang	x	x
25	B25	Unknown	Behrang	√	x
26	B26	Unknown	Behrang	x	x
27	B27	Unknown	Behrang	x	x
28	B28	Unknown	Behrang	x	x
29	B29	Unknown	Behrang	x	x
30	B30	Unknown	Behrang	x	x
31	T058	Unknown	TPU	x	x
32	T052	Unknown	TPU	x	x
33	50410	Unknown	TPU	x	x
34	T024	Unknown	TPU	x	x
35	T907	Unknown	TPU	x	x
36	Y032	Unknown	TPU	x	x
37	T083	Unknown	TPU	x	x
38	Z013	Unknown	TPU	x	x
39	5545	Unknown	TPU	x	x

No	ID	Cattle Sp	Location	Trans	OMP
40	I6W 4288	Mafriwal	PTH Air Hitam	x	x
41	I6W 4244	Mafriwal	PTH Air Hitam	x	x
42	F8W 4266	Mafriwal	PTH Air Hitam	x	x
43	I7M 2182	Mafriwal	PTH Air Hitam	x	x
44	I8W 4289	Mafriwal	PTH Air Hitam	x	x
45	I6X 4330	Mafriwal	PTH Air Hitam	x	x
46	I6N 2338	Mafriwal	PTH Air Hitam	x	x
47	I8W 4240	Mafriwal	PTH Air Hitam	x	x
48	I8X 4345	Mafriwal	PTH Air Hitam	x	x
49	I6T 3863	Mafriwal	PTH Air Hitam	x	x
50	I6W 4203	Mafriwal	PTH Air Hitam	x	x
51	I7X 4441	Mafriwal	PTH Air Hitam	x	x
52	I8X 4444	Mafriwal	PTH Air Hitam	x	x
53	I6V 4104	Mafriwal	PTH Air Hitam	x	x
54	I8V 4119	Mafriwal	PTH Air Hitam	x	x
55	I6S 3518	Mafriwal	PTH Air Hitam	x	x
56	I7V 4158	Mafriwal	PTH Air Hitam	x	x
57	No tag	Mafriwal	PTH Air Hitam	x	x
58	I6V 4086	Mafriwal	PTH Air Hitam	x	x
59	F7T 3790	Mafriwal	PTH Air Hitam	x	x

APPENDIX 5

Tissues Samples

No	Sample ID	Source	Location	Trans	OMP
1	1062	Cattle	MVKBT	x	x
2	1058	Cattle	MVKBT	x	x
3	2745	Cattle	MVKBT	x	x
4	2758	Cattle	MVKBT	x	x
5	2744	Cattle	MVKBT	x	x
6	2748	Cattle	MVKBT	x	x
7	2741	Cattle	MVKBT	x	x
8	2548	Cattle	MVKBT	x	x
9	UMG05a	Rodent	UM Research Center, Gombak	x	x
10	UMG05b	Rodent	UM Research Center, Gombak	x	x
11	UMG05c	Bat	UM Research Center, Gombak	x	x
12	UMG06a	Bat	UM Research Center, Gombak	x	x
13	UMG06b	Bat	UM Research Center, Gombak	x	x
14	R002	Rodent	Kuala Krau Forest Reserve	x	x
15	R021	Rodent	Kuala Krau Forest Reserve	x	x
16	S005	Rodent	Kuala Krau Forest Reserve	x	x
17	S012	Rodent	Kuala Krau Forest Reserve	x	x
18	Sawit1	Rodent	Kuala Krau Forest Reserve	x	x
19	B76	Bat	Sg Teris	x	x
20	B165	Bat	Sg Teris	x	x
21	B167	Bat	Sg Teris	x	x
22	B168	Bat	Sg Teris	x	x
23	B166	Bat	Sg Teris	x	x
24	B169	Bat	Sg Teris	x	x
25	041	Frog	Sg Teris	x	x
26	F02	Frog	Sg Teris	x	x
27	F03	Frog	Sg Teris	x	x
28	F09	Frog	Sg Teris	x	x
29	B36	Bird	Sg Teris	x	x
30	WRS	Bird	Sg Teris	x	x

APPENDIX 6

Real-time PCR Ct Value

No	Sample	Sample Type	Ct value 1	Ct value 2	Ct value 3	Average
1	B02	Milk	25.15	25.01	25.47	25.21
2	B03	Milk	36.24	36.49	36.26	36.33
3	B04	Milk	24.76	24.63	25.13	24.84
4	B05	Milk	24.56	24.53	24.75	24.61
5	B06	Milk	UD	UD	UD	UD
6	B07	Milk	24.36	24.29	25.3	24.65
7	B08	Milk	UD	UD	UD	UD
8	B10	Milk	25.95	25.31	30.11	27.12
9	B11	Milk	UD	UD	UD	UD
10	B12	Milk	UD	UD	UD	UD
11	B13	Milk	30.21	24.4	30.36	28.32
12	B16	Milk	23.45	23.77	24.04	23.75
13	B17	Milk	24.81	24.48	23.76	24.35
14	B18	Milk	38.44	37.27	37.63	37.78
15	B21	Milk	UD	UD	UD	UD
16	B22	Milk	25.83	26.09	25.97	25.96
17	B25	Milk	20.87	21.41	20.67	20.98
18	T010	Vaginal swab	30.87	30.23	31.03	30.71
19	T1111	Vaginal swab	29.49	29.01	28.64	29.05
20	T077	Vaginal swab	34.47	35.05	34.12	34.55
21	50410	Vaginal swab	37.02	36.71	37.19	36.97
22	5401	Vaginal swab	35.83	35.29	34.91	35.34

No	Sample	Sample Type	Ct value 1	Ct value 2	Ct value 3	Average
23	KI01	Vaginal swab	23.23	23.17	23.32	23.24
24	KI02	Vaginal swab	23.55	23.59	23.41	23.52
25	KI03	Vaginal swab	25.38	25.31	25.57	25.42
26	KI05	Vaginal swab	19.75	19.9	20.18	19.94
27	KI06	Vaginal swab	23.79	23.71	23.45	23.65
28	KI07	Vaginal swab	18.23	18.5	19.03	18.59
29	KI08	Vaginal swab	19.96	20.23	20.14	20.11
30	KI10	Vaginal swab	21.94	21.9	21.77	21.87
31	KI11	Vaginal swab	24.95	24.64	24.26	24.62
32	KB1	Vaginal swab	35.25	33.87	30.42	33.18
33	KB2	Vaginal swab	28.96	27.54	28.11	28.20
34	KB3	Vaginal swab	38.73	37.64	38.93	38.43
35	KB4	Vaginal swab	32.46	33.83	36.63	34.31
36	KB5	Vaginal swab	33.24	35.46	35.37	34.69
37	KB6	Vaginal swab	37.11	33.65	UD	35.38
38	KB8	Vaginal swab	25.53	27.93	27.14	26.87
39	KB9	Vaginal swab	27.84	26.38	26.93	27.05
40	T010	Blood	30.15	29.82	29.51	31.49
41	T111	Blood	31.42	31.21	30.31	30.65
42	T077	Blood	29.82	28.83	27.61	26.87
43	50410	Blood	29.43	25.54	25.11	25.36
44	5545	Blood	30.24	32.51	32.61	31.79
45	R004	Tick	29.51	28.72	29.03	28.75
46	S002	Tick	30.69	30.55	30.83	30.69

No	Sample	Sample Type	Ct value 1	Ct value 2	Ct value 3	Average
47	S003	Tick	34.25	34.61	34.29	34.38
48	KL06	Tick	UD	UD	UD	UD
49	ST19	Tick	34.35	34.62	34.11	34.36
50	ST25	Tick	35.48	35.19	35.82	35.50
51	ST37	Tick	UD	UD	UD	UD
52	ST51	Tick	UD	UD	UD	UD
53	ST77	Tick	34.79	34.35	34.81	34.65
54	KL09	Tick	UD	UD	UD	UD
55	KL22	Tick	32.48	32.12	32.56	32.39
56	KL25	Tick	34.98	35.02	35.14	35.05
57	KL31	Tick	34.67	33.92	34.37	34.32
59	KL48	Tick	UD	UD	UD	UD
60	SP01a	Tick	32.03	32.45	31.77	32.03