Tropical Biomedicine 32(2): 390–398 (2015)

Molecular screening for *Rickettsia*, *Anaplasmataceae* and *Coxiella burnetii* in *Rhipicephalus sanguineus* ticks from Malaysia

Watanabe, M.[1], Nakao, R.[2], Amin-Babjee, S.M.[1], Maizatul, A.M.[1], Youn, J.H.[2], Qiu, Y.[2], Sugimoto, C.[2*] and Watanabe, M.[3]
[1]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[2]Division of Education and Collaboration, Research Center for Zoonosis Control, Hokkaido University, Japan
[3]Department of Companion Animal Medicine and Surgery, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[*]Corresponding authors emails: sugimoto@czc.hokudai.ac.jp; malaika@vet.upm.edu.my
Received 6 June 2013; received in revised form 17 June 2014; accepted 25 July 2014

Abstract. A total of 44 *Rhipicephalus sanguineus* ticks collected from 23 dogs from Malaysia were screened for *Rickettsia*, *Anaplasmataceae* and *Coxiella burnetii*. *Coxiella burnetii* was detected in 59% (26/44) of ticks however *Rickettsia* and *Anaplasmataceae* were not detected in any of the ticks. In order to genotype the strains of *C. burnetii*, multispacer sequence typing (MST) was carried out using three different spacers. One of the spacers; Cox2 successfully amplified a fragment for which the full length sequence of 397 bp was obtained. The sequenced product revealed only a single nucleotide difference with the Cox2.3 type sequence.

INTRODUCTION

Arthropods have a significant impact on human and animal health as they are vectors of diseases, transmitting pathogenic agents to both humans and animals. Of the blood sucking arthropods, ticks are known to transmit the widest variety of pathogens in humans and animals (Goodman *et al.*, 2005). They are able to transmit bacteria, protozoa, rickettsia, viruses and spirochetes (Kim *et al.*, 2006). One important Proteobacteria which is of zoonotic concern is *Coxiella burnetii*, the etiological agent of Q fever. *Coxiella burnetii* is an obligate intracellular gram-negative bacterium found worldwide (Raoult & Saltzman, 1994). Infections may be acute or chronic and include a wide range of clinical symptoms. Molecular screening has assisted in the detection of pathogenic bacteria in ticks from different hosts collected from different geographical locations as well as characterization of new species (Vilcins *et al.*, 2009). Molecular detection has also revealed a greater diversity of organisms in ticks than was thought possible.

Other pathogenic bacteria namely *Anaplasma*, *Ehrlichia* and *Rickettsia* are also of medical and veterinary importance. The members of genus *Rickettsia* are obligate intracellular bacteria which are divided into three biotypes, namely; the spotted fever group, the typhus group and the ancestral group which only consists of *Rickettsia bellii*. Ehrlichiosis and Anaplasmosis are emerging tick-borne diseases of both humans and animals and are distributed worldwide. Bacteria that belong to the genus *Anaplasma* and *Ehrlichia* are gram-negative obligatory intracellular bacteria. In recent years, two *Ehrlichia* species; *E. chaffeensis* and *E. ewingii* have been discovered and recognized as emerging zoonotic tick-borne pathogens (Parola *et al.*, 2005). Members of

the genus *Anaplasma* are important pathogens of their respective hosts namely; *Anaplasma phagocytophilum*, *Anaplasma centrale*, *Anaplasma marginale*, *Anaplasma bovis*, *Anaplasma ovis* and *Anaplasma platys*.

Molecular detection and epidemiological surveys of *Anaplasma*, *Ehrlichia*, *Rickettsia* and *C. burnetii* have been extensively carried out in ticks from various geographical locations (Parola *et al.*, 2000; Inokuma *et al.*, 2003; Vilcins *et al.*, 2009). However, there remains a paucity of information regarding the prevalence of these pathogens in Malaysia. Prevalence and epidemiological surveys must be carried out in order to understand and determine these organisms' potential as human pathogens. Therefore this study was carried out to detect these emerging and re-emerging tick-borne diseases in ticks collected in Malaysia through molecular techniques.

Numerous studies were conducted to genotype strains of *C. burnetii* using various techniques such as restriction endonuclease analysis (REA) of chromosomal DNA separated by SDS-PAGE (Vodkin *et al.*, 1986; Hendrix *et al.*, 1991), pulsed field gel electrophoresis (PFGE) (Heinzen *et al.*, 1990; Jager *et al.*, 1998), random amplified polymorphic DNA (RAPD) fingerprinting (Sidi-Boumedine *et al.*, 2009), PCR-based IS1111 fingerprinting (Denison *et al.*, 2007), variable number tandem repeats-PCR (VNTR-PCR) (Arricau-Bouvery *et al.*, 2006; Svraka *et al.*, 2006) and real time PCR SNP typing (Huijsmans *et al.*, 2011). Sequence analysis of certain genes such as *com*1, *muc*Z and *icd* have also been used to differentiate *C. burnetii* isolates (Zhang *et al.*, 1997; Nguyen & Hirai, 1999; Sekeyova *et al.*, 1999). However, the more recent methods such a multiple locus variable number tandem repeats (MLVA) (Arricau-Bouvery *et al.*, 2006) and multispacer sequence typing (MST) (Glazunova *et al.*, 2005; Tilburg *et al.*, 2012) have shown to have high discriminatory power as well as high reliability and reproducibility. MST is based on sequencing of several intergenic regions that are potentially variable as they are subject to lower selection pressure than the coding genes. Studies using the MST approach have been able to discriminate between isolates of *C. burnetii* in humans (Tilburg *et al.*, 2012), domestic ruminants (Astobiza *et al.*, 2012) and ticks (Mediannikov *et al.*, 2010). We conducted a preliminary run in an attempt to determine if the MST approach could be used to genotype our *C. burnetii* strains from samples found in Malaysia.

MATERIALS AND METHODS

Sample collection

A total of 53 ticks were collected from 23 dogs that visited the University Veterinary Hospital, Universiti Putra Malaysia between July 2010 and July 2011. On average four to five ticks were collected from each dog. Ticks were placed in sterile microcentrifuge tubes and stored at room temperature. Ticks were identified morphologically using the classification keys of Soulsby (1982) and taxonomic keys as described by Kohls (1957). After species identification, engorged females (n=30), males (n=15) as well as nymphs (n= 8) were selected for screening.

DNA extraction

DNA was extracted from ticks using the phenol-chloroform extraction method and then ethanol precipitated (Sambrook & Russell, 2001). Ticks were initially washed twice with 75% ethanol followed by a wash step with sterile distilled water. The ticks were then homogenized by Micro Smash MS-100R (TOMY, Tokyo, Japan) for 30 s at 2,000 rpm, followed by DNA extraction with DNAzol (Invitrogen, Carlsbad, CA). Extracted DNA was ethanol precipitated and reconstituted in 200 µl of 8mMNaOH supplemented with 2 µl of 1M HEPES.

PCR for the detection of 28SrRNA gene of ticks

In order to determine whether DNA was successfully extracted from ticks, PCR was carried out to amplify the 28S rRNA gene of ticks. The primer sequences of 28SF and 28SR (Inokuma *et al.*, 2003) are shown in Table 1. PCR amplification was performed

in a 20 µl reaction mixture containing 5.0 µl of template DNA, 400 µM of each dNTP, 0.5 µM of each primer, 1.5 U GoTaq DNA polymerase (Promega, Madison, WI) in 2X Green GoTaq reaction buffer. The cycling conditions consisted of an initial denaturation step at 95ºC for 2 min followed by 35 cycles at 95ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s and a final extension step at 72ºC for 5 min. The resulting amplified products were electrophoresed on a 1.5% agarose gel at 100 V for 30-35 min, stained using GelRed dye (Biotium, Hayward, CA) and subsequently visualized under a UV transilluminator.

Real-time PCR for the detection of *Rickettsia*

Real-time PCR was performed using primers; CS-F and CS-R and a probe CS-P (Stenos *et al.*, 2005) to amplify the citrate synthase, *gltA*, gene of spotted fever and typhus group rickettsiae. The primer and probe sequences are shown in Table 1. The reaction was performed with THUNDERBIRD™ Probe qPCR 2X Mix (Toyobo, Osaka, Japan), 0.3 µM of each primer, 0.2 µM of probe, 2.0 µl of template DNA in a total reaction volume of 20 µl. Standard controls were prepared from plasmids containing the *gltA* gene fragment of *Rickettsia japonica* YH strain (10^6, 10^4 and 10^2 copies/reaction). PCR was performed using CFX96 Real-Time System (BioRad, USA) with an initial holding temperature of 50ºC for 3 min followed by 95ºC for 1 min and 40 cycles at 95ºC for 15 s and 60ºC for 60 s.

PCR for the detection of Anaplasmataceae

PCR amplification of Anaplasmataceae was performed using primers; EHR16SD and EHR16SR (Parola *et al.*, 2000) as listed in Table 1. This primer set amplifies a 345 bp fragment of the 16S rRNA gene of the members of the Anaplasmataceae family. The positive control was *Anaplasma phagocytophilum* DNA isolated from in vitro cultures. PCR amplification was performed as described above for the detection of 28S rRNA gene of ticks. PCR cycling conditions were also the same except an annealing temperature of 53ºC for 30 s was used.

PCR for the detection of *Coxiella burnetii*

PCR amplification was performed using primers; IS111F1 and IS111R1 (Fournier and Raoult, 2003) which flank a 484 bp fragment of the *htp*AB-associated repetitive element listed in Table 1. A nested PCR was carried out with primers IS111F2 and IS111R2 flanking a 260 bp fragment. PCR amplification for the first and nested PCR was carried out as described above. The first PCR product was diluted ten times and used as template for nested PCR. The positive control was *C. burnetii* Nine Mile strain DNA isolated from Vero cells. The first PCR cycling conditions consisted of an initial denaturation step at 95ºC for 2 min followed by 40 cycles at 95ºC for 30 s, 52ºC for 30 s, 72ºC for 1 min and a final extension step at 72ºC for 5 min. Cycling conditions for the nested PCR was carried out using the same parameters except that the number of cycles was reduced to 35 and an annealing temperature of 48ºC was used. PCR products were electrophoresed and visualized as described above.

Characterization of *Coxiella burnetii*: Multispacer sequence typing (MST)

MST was carried out using three previously designed primers; Cox2F, Cox5F and Cox18F, and three newly designed reverse primers; Cox2R2, Cox5R2 and Cox18R2 listed in Table 1 for first PCR. PCR amplification for the first PCR was performed in a 20 µl reaction mixture containing 5.0 µl of template DNA, 200 µM of each dNTP, 0.5 µM of each primer, 0.4U KAPA2G Robust HotStart DNA polymerase (Kapabiosystems, Boston, MA) in 1X KAPA 2G Buffer A and filled to 20 µl with sterile distilled water. The first PCR cycling conditions consisted of an initial denaturation step of 95ºC for 2 min followed by 45 cycles at 95ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min and a final extension step at 72ºC for 5 min. The second semi-nested PCR was carried out with the same forward primers and previously designed reverse primers; Cox2R, Cox5R and Cox18R (Glazunova *et al.*, 2005) listed in Table 1. PCR cycling conditions for the semi-nested PCR consisted of an initial denaturation step of

Table 1. Oligonucleotide primers and probe used in this study

Target sequence	Primer sequences 5'-3'	Amplicon size (bp)	Reference
Tick 28S rRNA	28SF- GACTCTAGTCTGACTCTGTG 28SR–GCCACAAGCCAGTTATCCC	484	(Inokuma *et al.*, 2003)
Rickettsial *gltA*	CS-F - TCGCAAATGTTCACGGTACTTT CS-R TCGTGCATTTCTTTCCATTGTG CS-P 6-FAM-TGCAATAGCAAGAACCGT AGGCTGGATG-BHQ-1-3	74	(Stenos *et al.*, 2005)
Anaplasmataceae 16S rRNA	EHR16SD -GGTACCYACAGAAGAAGTCC EHR16SR- TAGCACTCATCGTTTACAGC	345	(Parola *et al.*, 2000)
C. burnetii *htp*AB-associated repetitive element	IS111F1 - TACTGGGTGTTGATATTGC IS111R1 - CCGTTTCATCCGCGGTG IS111F2 - GTAAAGTGATCTACACGA IS111R2– TTAACAGCGCTTGAACGT	485 260	(Fournier and Raoult, 2003)
C. burnetii MST	Cox2F- CAACCCTGAATACCCAAGGA Cox2R- GAAGCTTCTGATAGGCGGGA *Cox2R2- GTCTTTCCAATTCGTCGAAATAA Cox5F -CAGGAGCAAGCTTGAATGCG Cox5R- TGGTATGACAACCCGTCATG *Cox5R2- CAAGGGAAAACGAGGATCAA Cox18F- CGCAGACGAATTAGCCAATC Cox18R- TTCGATGATCCGATGGCCTT *Cox18R2–GGCGATTAACCATTTCCAGT	397 459 395 547 557 584	(Glazunova *et al.*, 2005)

Note: *primers designed in this study

95ºC for 2 min followed by 45 cycles at 95ºC for 30 s, 57ºC for 30 s, 72ºC for 40 s and a final extension step at 72ºC for 5 min.

Gel purification

PCR products prepared in total volumes of 50 µl were used for gel purification. PCR products were electrophoresed on 1.5% low melt agarose gels containing SYBR safe DNA gel stain at 100V for 30-35 min. The fragment of interest was excised from the gel and purified using Wizard® SV Gel and PCR clean-up system (Promega, USA) according to the manufacturer's instructions.

Cloning and Transformation

The purified product was ligated into pGEM-T vector (Promega, Madison, WI) and subsequently transformed into chemically competent *Escherichia coli* cells. Transformation was carried out by subjecting cells to heat shock at 42ºC for 90 s and then immediately placing on ice. The transformed cells were then incubated in SOC medium shaken at 200 rpm at 37ºC for 1 hour. LB agar plates containing ampicillin (50µg/µl) were plated with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (Isopropyl β-D-1-thiogalactopyranoside) for blue white colony screening. The plates were left to dry for 10 min followed by plating of transformation culture. The plates were placed in an incubator at 37°C overnight for approximately16-24 hours.

Screening transformants for inserts/ colony PCR

Colony direct PCR was performed using universal primers SP6 and T7 in a 10 µl reaction mixture containing 1.0 µl of colony template, 400 µM of each dNTP, 0.5 µM of each primer, 1.5 U GoTaq DNA polymerase

(Promega, USA) in 2X Green GoTaq reaction buffer. The cycling conditions consisted of an initial denaturation step at 95ºC for 2 min followed by 30 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 2 min and a final extension step at 72ºC for 5 min.

Sequencing

The sequencing reactions were carried out using SP6 and T7 promoter primers for plasmid clones and specific PCR primers for purified PCR products. Reactions were carried out in 10.5 µl reaction mixtures containing 2.5 µl of template, 1.85 µl 5X sequencing buffer (Applied Biosystems), 0.5 µl (0.48 µM) of primer, 0.25 µl BigDye (BigDye Terminator version 3.1, Applied Biosystems) and 5.40 µl nuclease free water. Cycling conditions consisted of an initial denaturation step at 96ºC for 1 min followed by 25 cycles at 96ºC for 10 s, 50ºC for 5 s and 60ºC for 1.25 min. Sequencing products were resolved with an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

RESULTS

All the ticks collected were identified as *R. sanguineus* through morphological examination. A total of 44 ticks (24 females, 12 males and 8 nymphs) out of the 53 tested positive by PCR amplification of the 28S rRNA gene of ticks.

Rickettsia* and *Anaplasma

All the 44 ticks that were screened for members of the genus *Rickettsia* by real-time PCR were negative. Screening for members of the family Anaplasmataceae using primers to amplify a partial fragment of the 16S rRNA gene revealed negative results for all 44 tick samples.

Coxiella burnetii

Screening of the 44 ticks detected the presence of *C. burnetii* DNA in 59% (26/44) of the ticks with primers targeting the *htp*AB-associated repetitive element. Ticks from 13 of the 23 dogs were positive for *C. burnetii*. Though *C. burnetii* DNA was detected in only four ticks by first PCR with primers IS111F1 and IS111R1 (Fig. 1), the presence of *C. burnetii* DNA was detected in an additional 22 ticks using nested primers IS111F2 and IS111R2 (Fig. 2). Five randomly selected positive samples were sequenced, one PCR product of primers IS111F1 and IS111R1 and four nested PCR products. A BLAST search of the sequences against GenBank revealed a 99% identity with the *C. burnetii* genome (GenBank accession number AE016828).

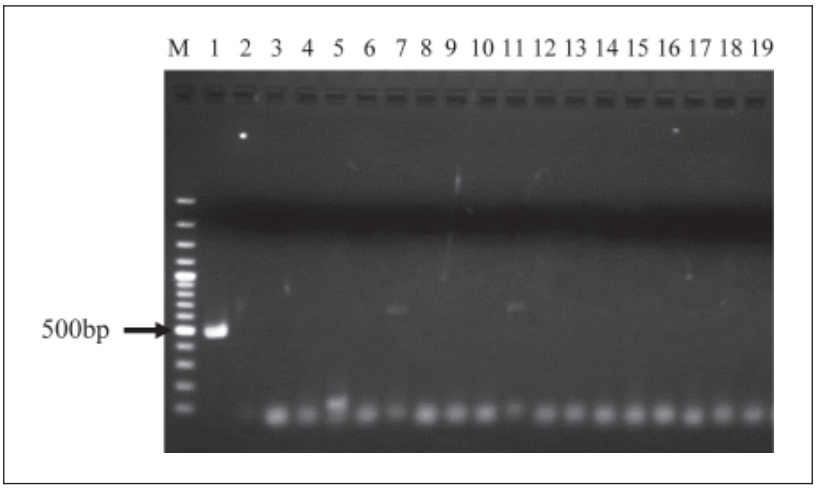

Figure 1. PCR amplification of the *htp*AB-associated repetitive element of *Coxiella burnetii* with primers, IS111F1 and IS111R1.
Lane M, 100 bp DNA ladder; Lane 1, positive control (DNA from *C. burnetii* Nine Mile strain cultured in Vero cells); Lane 2, negative control; Lane 3-19 tested samples.

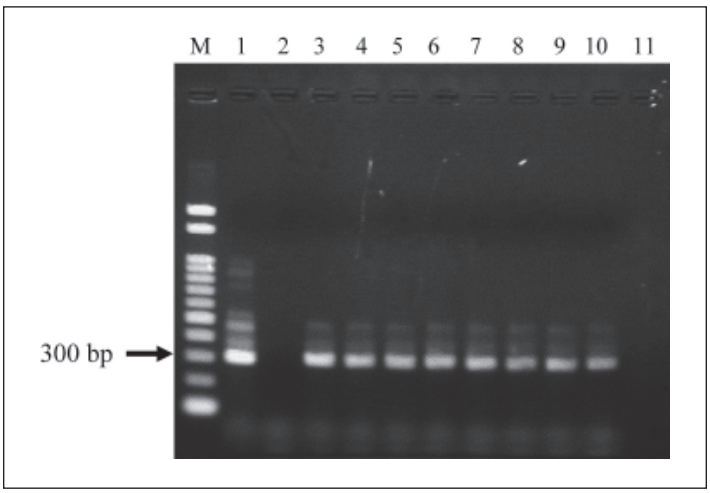

Figure 2. Nested PCR amplification of the *htp*AB-associated repetitive element of *Coxiella burnetii* with primers, IS111F2 and IS111R2.
Lane M, 100 bp DNA ladder; Lane 1, positive control (*C. burnetii* DNA from cell culture); Lane 2, negative control; Lane 3-11, tested samples.

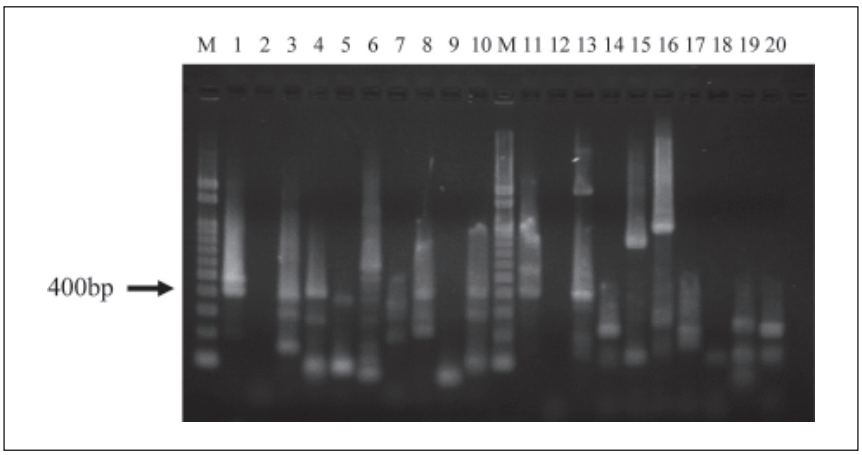

Figure 3. Semi-nested PCR amplification of the multispacer sequence, Cox2/R and Cox5F/R of *Coxiella burnetii*.
Lane M, 100 bp DNA ladder; Lane 1, positive control (*C.burnetii* DNA from cell culture), Lane 2, negative control; Lane, 3-10 tested samples with primers Cox2F and Cox2R, Lane M, 100 bp DNA ladder; Lane 11, positive control; Lane 12, negative control; Lane, 13-20 tested samples with primers Cox5F and Cox5R.

Multispacer sequence typing of *C. burnetii*

Eight positive samples identified from the *htp*AB-associated repetitive element PCR assays were subjected to multispacer sequence typing. Out of the eight samples tested only five revealed the expected 397 bp fragment with spacer, Cox2F/R, only one produced an expected 395 bp fragment with spacer Cox5F/R (Fig. 3) and none of the samples amplified the expected 557 bp fragment with spacer Cox18F/R. The sequences were compared with entries in the MST database (http://ifr48.timone.univ-mrs.fr/MST_Coxiella/mst) by BLAST. Four out of the five Cox2F/R products showed highest identity to Cox2.3 and the sequence of one sample revealed only a single nucleotide difference with Cox2.3 type sequence.

DISCUSSION

All ticks collected from dogs that visited the University Veterinary Hospital, Universiti Putra Malaysia (UPM) were identified as *R. sanguineus*. Although Rickettsiae and Anaplasmataceae were not detected in any of the ticks, the prevalence of *C. burnetii* in the Malaysian ticks was startlingly high at 59%. Though there have been sporadic reports of Q fever in humans in Malaysia (Bush, 1952; Kaplan & Bertagna, 1955; Tay *et al.*, 1998; Saraswathi *et al.*, 2011), there are no reports of Q fever in companion animals. Studies to detect *C. burnetii* in ticks by PCR has not been carried out before in Malaysia, however based on the findings of this study it is evident that humans and animals are both at risk of contracting Q fever in Malaysia. The high prevalence of *C. burnetii* in ticks collected from pet dogs poses a significant risk to humans due to the close contact between pet owners and their dogs.

Primers amplifying the *htp*AB-associated repetitive element appeared to be more efficient and sensitive compared to other primers (data not shown). The *htp*AB-associated repetitive element exists in 20 copies in the genome of *C. burnetii* (GenBank accession number AE016828) (Seshadri *et al.*, 2003) and therefore even a small number of bacterial copies can be detected by targeting this element. However, with only the outer primer set; IS111F1 and IS111R1, most of the *C. burnetii* DNA could not be amplified, but when a nested PCR was carried out more positive samples were detected. This nested PCR approach was highly sensitive and could detect relatively low *C. burnetii* burdens in the tested samples. This would be an effective approach for the detection of *C. burnetii* in field samples with low bacterial burdens such as in milk and blood.

Reverse primers designed in this study along with forward primers designed previously to amplify the Cox2F/R and Cox5F/R proved to be useful in amplifying these specific spacers. The first PCR product when used as a template for the semi-nested PCR appeared to be effective and potentially useful for genotyping strains of *C. burnetii.* Out of the few samples that were subjected to MST in this preliminary study, Cox2F/R was able to amplify the full 356 bp fragment of one of the samples which was successfully sequenced. When the sequence was compared with those on the MST database it was most similar to a Cox2.3 with one single nucleotide polymorphism of G instead of A which could possibly be another genotype but this cannot be ascertained at this point. Among the samples tested, spacer Cox18F/R was unable to amplify the expected 557 bp fragment. The difficulties encountered using the MST approach in this study may have been due to low levels of target DNA in the ticks as compared to other studies where bacterial cultures were used (Glazunova *et al.*, 2005; Mediannikov *et al.*, 2010). In order to genotype the strains of *C. burnetii* a more thorough study should be conducted using a number of spacers for typing and analysis by MST.

It was unusual that neither Rickettsiae nor Anaplasmataceae were detected in any of the ticks as *E. canis* and *A. platys* have been detected by PCR in blood from dogs that visited the University Veterinary Hospital, UPM (Nazari *et al.*, 2013). The inability to detect these pathogens could have been due to the small sample size used in this preliminary study. A larger sample size would be required for a more representative prevalence study.

The primer sets used for genotyping *C. burnetii* in this study can be used for further characterization of *C. burnetii* strains. More extensive prevalence studies should be carried out on bacteria in Malaysian ticks, to determine what types of pathogens they harbor and the possible role they may play in the transmission and spread of zoonotic diseases. Furthermore, the prevalence of *C. burnetii* in dogs in Malaysia should be investigated to determine if they are reservoirs of infection especially in pet dogs.

Acknowledgements. This work was supported by the Global Center of Excellence (COE) Program, "Establishment of International Collaboration Centers for Zoonosis Control" from the Ministry of Education, Culture, Sports, Science and

Technology of Japan (MEXT) and in part by the Research University Grant Scheme, University Putra Malaysia (05-03-11-1446RU). DNA from *Rickettsia japonica* YH strain was kindly provided by Dr. T. Uchiyama (The University of Tokushima Graduate School, Tokushima, Japan).

REFERENCES

Arricau-Bouvery, N., Hauck, Y., Bejaoui, A., Frangoulidis, D., Bodier, C.C., Souriau, A., Meyer, H., Neubauer, H., Rodolakis, A. & Vergnaud, G. (2006). Molecular characterization of *Coxiella burnetii* isolates by infrequent restriction site-PCR and MLVA typing. *BMC Microbiology* **6**: 38.

Astobiza, I., Tilburg, J., Piñero, A., Hurtado, A., García-Pérez, A., Nabuurs-Franssen, M. & Klaassen, C. (2012). Genotyping of *Coxiella burnetii* from domestic ruminants in northern Spain. *BMC Veterinary Research*, **8**(1): 1-8.

Bush, F.K. (1952). *Proceedings of the Alumni Association of King Edward VII College of* Medicine, **5**: 84. (Abstracted in Excerpta Med (Amst) Sect IV) 1953.**6**: 536.

Denison, A.M., Thompson, H.A. & Massung, R.F. (2007). IS1111 insertion sequences of *C. burnetii*: characterization and use for repetitive element PCR-based differentiation of *C. burnetii* isolates. *BMC Microbiology* **7**: 91.

Fournier, P.E. & Raoult, D. (2003). Comparison of PCR and Serology Assays for Early Diagnosis of Acute Q Fever. *Journal of Clinical Microbiology* **41**: 5094-5098.

Glazunova, O., Roux, V., Freylikman, O., Sekeyova, Z., Fournous, G., Tyczka, J., Tokarevich, N., Kovacava, E., Marrie, T. & Raoult, D. (2005). *Coxiella burnetii* genotyping. *Emergining Infecious Diseases* **11:** 1211-1217.

Goodman, J.L., Dennis, D.T. & Soneshine, D.E. (2005). Tick-Borne Disease of Humans. Washington, DC: ASM Press.

Heinzen, R., Stiegler, G.L., Whiting, L.L., Schmitt, S.A., Mallavia, L.P. & Frazier, M.E. (1990). Use of pulsed field gel electrophoresis to differentiate *C. burnetii* strains. *Annals of the New York Academy of Sciences* **590**: 504-513.

Hendrix, L.R., Samuel, J.E. & Mallavia, L.P. (1991). Differentiation of *Coxiella burnetii* isolates by analysis of restriction-endonuclease digested DNA separated by SDS-PAGE. *Journal of General Microbiology* **137**: 269-276.

Huijsmans, C.J., Schellekens, J.J., Wever, P.C., Toman, R., Savelkoul, P.H., Janse, I. & Hermans, M.H. (2011). SNP-genotyping of a *Coxiella burnetii* outbreak in the Netherlands. *Applied Environmental Microbiology* **77**: 2051-2057.

Inokuma, H., Beppu, T., Okuda, M., Shimada, Y. & Sakata Y. (2003). Epidemiological survey of *Anaplasma platys* and *Ehrlichia canis* using ticks collected from dogs in Japan. *Veterinary Parasitology* **115:** 343-348.

Jager, C., Willems, H., Thiele, D. & Baljer, G. (1998). Molecular characterization of *Coxiella burnetii* isolates. *Epidemiology & Infection* **120**: 157-164.

Kaplan, M.M. & Bertagna, P. (1955). The geographical distribution of Q fever. *Bulletin of the World Health Organization* **13**: 829-860.

Kim, C., Yi, Y., Yu, D., Lee, M., Cho, M., Desai, A., Shringi, S., Klein, T., Kim, H., Song, J., Baek, L., Chong, S., O'Guinn, M.L., Lee, J.S., Lee, I., Park, J., Foley, J. & Chae, J. (2006). Tick-Borne Rickettsial Pathogens in Ticks and Small Mammals in Korea. *Applied Environmental Microbiology* **72:** 5766-5776.

Kohls, G.M. (1957). Malaysian parasites XVII. Ticks (Ixodoidea) of Borneo and Malaya. *Studies from the Institute for Medical Research*, Fedeartion of Malaya **28**: 65-94.

Mediannikov, O., Fenollar, F., Socolovschi, C., Diatta, G., Bassene, H., Molez, J.F., Sokhna, C., Trape, J.F. & Raoult, D. (2010). *Coxiella burnetii* in Humans and Ticks in Rural Senegal. *PLoS Neglected Tropical Diseases*, **4**(4): e654.

Nazari, M., Lim, S.Y., Watanabe, M., Sharma, R.S.K., Cheng, N.A.B.Y. & Watanabe, M. (2013). Molecular Detection of *Ehrlichia*

canis in Dogs in Malaysia. *PLoS Neglected Tropical Diseases* **7**(1): e1982.

Nguyen, S.V. & Hirai, K. (1999). Differentiation of *Coxiella burnetii* isolates by sequence determination and PCR-restriction fragment length polymorphism analysis of isocitrate dehydrogenase gene. *FEMS Microbiology Letters* **180**: 249-254.

Parola, P., Davoust, B. & Raoult, D. (2005). Tick- and flea-borne rickettsial emerging zoonoses. *Veterinary Research* **36:** 469-492.

Parola, P., Roux, V., Camicas, J.L., Baradji, I., Brouqui, P. & Raoult, D. (2000). Detection of ehrlichiae in African ticks by polymerase chain reaction. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94:** 707-708.

Raoult, D. & Saltzman, R.L. (1994). Q fever. In: Infectious Diseases, Hoeprich, P.D., Jordan, M.C. and Ronald, A.R. (editors). Philadelphia: Lippincott Company, pp. 417-420.

Sambrook, J.R. & Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd edition. New York: Cold Spring Harbor Laboratory Press.

Saraswathi, B.R., Kamaludin, F., Chow, T. & Yoon, C. (2011). First documented zoonotic case of Q fever in Penang, Malaysia. *Outbreak, Surveillance and Investigation Reports* **4:** 1-4.

Sekeyova, Z., Roux, V. & Raoult, D. (1999). Intraspecies diversity of *Coxiella burnetii* as revealed by com1 and mucZ sequence comparison. *FEMS Microbiology Letters* **180**: 61-67.

Seshadri, R., Paulsen, I.T., Eisen, J.A., Read, T.D., Nelson, K.E., Nelson, W.C., Ward, N.L., Tettelin, H., Davidsen, T.M., Beanan, M.J., Deboy, R.T., Daugherty, S.C., Brinkac, L.M., Madupu, R., Dodson, R.J., Khouri, H.M., Lee, K.H., Carty, H.A., Scanlan, D., Heinzen, R.A., Thompson, H.A., Samuel, J.E., Fraser, C.M. & Heidelberg, J.F. (2003). Complete genome of *Coxiella burnetii*. *Proceedings of the National Academy of Sciences* **100**: 5455-5460.

Sidi-Boumedine, K., Duquesne, V., Fernandes, I., Marro, S. & Thiery, R. (2009). Evaluation of randomly amplified polymorphic DNA (RAPD) for discrimination of *Coxiella burnetii* ruminant strains isolated in France. *Clinical Microbiology and Infection* **15**: 194-195.

Soulsby, E.J.L. (1982). *Helminthes, Arthropods and Protozoa of Domesticated Animals*, 7th edition. London: Bailliere Tindall.

Stenos, J., Graves, S.R. & Unsworth, N.B. (2005). A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group Rickettsiae. *American Journal of Tropical Medicine and Hygiene* **73:** 1083-1085.

Svraka, S., Toman, R., Skultety, L., Slaba, K. & Homan, W.L. (2006). Establishment of a genotyping scheme for *Coxiella burnetii*. *FEMS Microbiolgy Letters* **254**: 268-274.

Tay, S.T., Ho, T.M. & Rohani, M.Y. (1998). Serological findings of *Coxiella burnetii* infection among patients with fevers in a health centre in Sarawak, Malaysia. *The Southeast Asian Journal of Tropical Medicine and Public Health* **29:** 94-95.

Tilburg, J.J., Roest, H.J., Buffet, S., Nabuurs-Franssen, M.H., Horrevorts, A.M., Raoult, D. & Klaassen, C.H. (2012). Epidemic genotype of *Coxiella burnetii* among goats, sheep, and humans in the Netherlands. *Emerging Infectious Diseases* **18**: 887-889.

Vilcins, I.M., Old, J. & Deane, E. (2009). Molecular detection of Rickettsia, Coxiella and Rickettsiella DNA in three native Australian tick species. *Experimental and Applied Acarology* **49:** 229-242.

Vodkin, M.H., Williams, J.C. & Stephenson, E.H. (1986). Genetic heterogeneity among isolates of *Coxiella burnetii*. *Journal of General Microbiology* **132**: 455-463.

Zhang, G.Q., To, H., Yamaguchi, T., Fukushi, H. & Hirai, K. (1997). Differentiation of *Coxiella burnetii* by sequence analysis of the gene (com1) encoding a 27-kDa outer membrane protein. *Microbiology and Immunology* **41**: 871-877.