Tropical Biomedicine 34(3): 675–680 (2017)

Molecular Detection of *Coxiella burnetii* from Farm Animals and Ticks in Malaysia

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Received 20 June 2016; received in revised form 29 June 2017; accepted 30 June 2017

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. As the organism is difficult to culture, this study was aimed to detect *C. burnetii* DNA in animal (mainly blood and vaginal samples of cattle, goats and sheep) and tick samples obtained from farm animals, wild rodents and vegetation. Two polymerase chain reaction (PCR) assays targeting *IS1111* transposon-like gene (Trans-PCR) and *com1* gene (OMP-PCR) were used for *C. burnetii* detection. Sequence determination of the amplified fragments and a real-time PCR assay were used to confirm PCR findings. *C. burnetii* DNA was detected from 9.1% of cattle blood and 4.2% vaginal samples, respectively. A small percentage (5.8%) of ticks (including *Amblyomma*, *Dermacentor*, *Rhipicephalus* and *Haemaphysalis* spp.) haboring *C. burnetii* were identified in this study. This study provides molecular evidence on the presence of *C. burnetii* in cattle and ticks. The possible zoonotic transmission of *C. burnetii* is yet to be investigated.

INTRODUCTION

Coxiella burnetii is the causative agent of Q fever in humans and coxiellosis in animals. The organism is an obligate intracellular bacterium in the γ subdivision of the *Proteobacteria* phylum (Weisburg *et al.*, 1989). A broad range of hosts including domestic/wild animals and ticks, have been associated with *C. burnetii* (van Schaik *et al.*, 2013). Q fever is a zoonotic disease and may pose an occupational hazard, especially to veterinarians and farm workers. Cattle and goats are believed to be the main reservoir for human infections (Guatteo *et al.*, 2011). The shedding route of *C. burnetii* is via birth products (birth fluids, placenta etc.). Ticks are considered a natural reservoir of *C. burnetii* as more than 40 tick species have been documented to be infected with *C. burnetii* (Thompson & Dasch, 2005). Possible tick-borne transmission of *C. burnetii* to humans has been reported (Beaman & Hung, 1989; Eklund *et al.*, 1947).

Data on the prevalence and ecology of *C. burnetii* infection is scarce in Malaysia. The first case of suspected Q fever in Peninsular Malaysia was probably caused by contaminated milk samples (Bush, 1952). Serological evidence of *C. burnetii* infection

in the communities has been reported in two subsequent studies (Kaplan & Bertagna, 1955; Tay *et al.*, 1998). A recent study documented a zoonotic case of Q fever with high seropositivity (42.8%) in farm workers, veterinary and laboratory staff towards *C. burnettii* in the northern region of Malaysia (Bina *et al.*, 2011). In this study, detection of *C. burnetii* in the animal and tick samples (collected from wild rodents, livestock and forest) was performed using two polymerase chain reaction (PCR) assays targeting transposon-like IS1111 (Trans-PCR) and *com1* genes (OMP-PCR), respectively. A real-time PCR assay was used to confirm the PCR findings.

MATERIALS AND METHODS

Processing of animal samples

Table 1 summarises the origin and details of the samples investigated in this study. Blood and vaginal swabs were collected from several cattle, goat and sheep farms in Selangor (Serdang), Perak (Ipoh), Negeri Sembilan (Jelai Gemas), Pahang (Kuantan and Jerantut), Kelantan (Tanah Merah), Kedah (Pokok Sena), and Johore (Air Hitam) in Peninsular Malaysia (Kho *et al.*, 2015). Approval for animal and tick 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). 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).

Collection and identification of ticks

Ticks were collected from vegetation and wild rodents from a forest area (Krau Wildlife Reserve, N 03º50' E 102º06'), several goat and cattle farms and two shelters for dogs in the Klang Valley from March to May 2013 (Table 1). The ticks were examined using a stereo microscope (Olympus, Japan) for morphological identification to genus level.

DNA extraction

Animal blood and vaginal samples were extracted using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) and Favor Prep Tissue Genomic DNA Extraction Mini kit (Favorgen Biotech Corp., Taiwan), respectively, as recommended by the manufacturers. All extracted DNA was stored at -20ºC prior to PCR amplification. Ticks were washed in 5% (v/v) hypochlorite, followed by a dip in 70% (v/v) ethanol and lastly with distilled water. The ticks were then crushed with the tip of a pipette or disrupted mechanically using a Kontes Pellet Pestle and a cordless motor (Thermo Fisher Scientific, USA). The lysate was then processed using a QIAamp® DNA Mini kit (Qiagen, Hilden, Germany), in accordance to the manufacturer's instruction. The integrity of the tick DNA samples was examined using a PCR assay targeting the tick 28S gene region, as described by Inokuma *et al.* (2003).

Molecular detection of C. burnetii

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

C. burnetii DNA was amplified using primers targeting the IS1111 gene, Trans3/F (5'-CAA CTG TGT GGA ATT GAT GA-3') and Trans5/R (5'-TTT ACA TGA CGC AAT AGC GC-3'), as described previously (Greub *et al.*, 2005). PCR master mix containing 2 µL DNA sample, 19.4 µL sterile distilled water, 2.5 µL 10x DreamTaq™ buffer (Fermentas, Lithuana), 0.5 µL dNTPs (10µM), 0.2 µL of each primer (25µM), and 0.2 µL DreamTaq™ DNA Polymerase (5U/µL) (Fermentas, Lithuana), was prepared. Amplification was carried out in a MyCycler Thermalcycler (BioRad, USA). The PCR program included an initial denaturation 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 control derived from *C. burnetii* antigen slides (Fuller Laboratories, Fullerton, California, USA) and negative control (sterile

distilled water) were included in each PCR run.

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'-AGTAG AAGCATCCCAAGCATTG-3') and OMP2 (5'-TGCCTGCTAGCTGTAACGATTG-3') have been described by Zhang *et al.* (1998). The PCR program included denaturation 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 501 bp fragment after amplification. The amplicons were analysed in a 2% (v/v) agarose gel (pre-stained with ethidium bromide) after electrophoresis in 1x TBE buffer at 100 V for 45 min. The image of the agarose gel was visualized using a UV transilluminator (G-Box, Syngene, England).

ii. Purification of PCR products and sequence analysis

Sequence determination of the amplified products was carried out to confirm the specificity of each PCR assay, 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 program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

iii. Real-time PCR assay

A real-time PCR assay was performed to ascertain the positive findings obtained from Trans-PCR and OMP-PCR assays. The real-time PCR assay was performed as described by Loftis *et al.* (2006). Primers IS1111F (5'-CGG CGG TGT TTA GGC-3') and IS1111R (5'-CGG CGG TGT TTA GGC-3') and probe IS1111Pa (5'-TTA ACA CGC CAA GAA ACG TAT CGC TGT G-3') labelled with FAM at the 5' end and MGB at the 3' end were used. Real-time PCR assays were conducted in triplicate per DNA sample. Each reaction consisted of a total volume of 20µl containing 10 µL TaqMan® Fast Advanced Master Mix, 1 µL Primer/Probe Mix, 7 µL nuclease-free water and 2 µL DNA template. The assays were performed in Applied Biosystems StepOnePlus™ thermal cycler (USA). Data of the fluorescence signals detected during the thermal cycling was viewed and analysed using StepOne™ Software version v2.3 (Applied Biosystems, USA).

RESULTS AND DISCUSSION

Table 1 summarizes the results of PCR detection in this study. *C. burnettii* DNA was amplified from five each of the cattle blood samples and vaginal swabs using Trans-PCR assays. The results from vaginal swabs were confirmed by real-time PCR as the level of PCR products were insufficient for direct sequencing. None of the animal samples were positive using OMP-PCR assays.

C. burnetii DNA was detected in 10(5.8%) of tick samples using Trans-PCR assays (Table 1). The positive samples included one *Dermacentor* and two *Amblyomma* ticks collected from different wild rodents (*Maxomys rajah*, *Callosciurus notatus*, *Tupaia glis*), three questing ticks (*Haemaphysalis* spp.) from the forest area. The OMP-PCR assay amplified *C. burnetii* DNA from three *Dermacentor* questing ticks and a *R. sanguineus* dog tick in Klang valley. BLAST analysis of the amplified *ISIII* gene fragment from an *Amblyomma* tick shows 100% similarity to *C. burnetii* strain Cb175 (GenBank accession no.: HG825990) and RSA 493 (GenBank accession no.: AE016828). None of the ticks collected from cattle and goats were positive using Trans-PCR and OMP-PCR assays.

The prevalence rate of *C. burnetii* in farm animals has been reported to vary in different geographical regions. Based on a review of Guatteo *et al.* (2011), *C. burnetii* infection has been higher in cattle than in small ruminants. Only 9.1% of cattle blood samples were positive in this study, while

Table 1. Molecular detection of *C. burnetii* DNA from animals and ticks in this study

Samples	Location	No. (%) sample positive	
		Trans-PCR	OMP-PCR
Tick samples (n=173)			
Ticks from vegetation (n=36)	Krau Wildlife Reserve	3 (8.3)	3(8.3)
Ticks from wild rodents (n=30)	Krau Wildlife Reserve	3 (10.0)	0(0)
Ticks from cattle (n=54)	Serdang, Ipoh	0(0)	0(0)
Ticks from goats (n=10)	Pulau Pinang (MVKBT)	0(0)	0(0)
Ticks from dogs (n=43)	Klang Valley	0(0)	1(2.3)
Subtotal		**6 (3.5)**	**4 (2.3)**
Animal samples (n=263)			
Vaginal swab, cattle (n=120)	Serdang, Ipoh, Kuantan, Tanah Merah,	5(4.2)	0(0)
Vaginal swab, goats/sheep (n=40)	Kuala Pah, Pokok Sena, Pokok Sena,	0(0)	0(0)
Blood, cattle (n=55)	Jerantut and Air Hitam	5(9.1)	0(0)
Blood, goat (n=48)		0(0)	0(0)
Subtotal		**10 (3.8)**	**0(0)**

none was detected from goats. Comparatively, 9.5% of the blood samples obtained from goats were positive for *C. burnetii* in Korea (Jung *et al.*, 2014). Although animal vagina has been reported as the easiest route of shedding for the bacteria (Welsh *et al.*, 1958), only 4.2% of cattle vaginal samples investigated in this study were positive.

C. burnetii DNA was not detected from 54 cattle ticks in this study. Similar finding (zero prevalence) has been reported for the cattle at the Reunion Island (Cardinale *et al.*, 2014). *Heamaphysalis* spp. was the main tick species collected from the forest area in this study, followed by *Dermacentor* spp. Three (10.0%) ticks collected from wild rodents and a number of questing ticks from the forest area were positive for *C. burnetii*. Some of these ticks including *Dermacentor* spp. and *Haemaphysalis* spp. have been shown to involve in the cycles of tick typhus and Q-fever in Malaysian forests (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 may pose a risk to domestic/wild animals and humans. In other parts of Asia, *C. burnetii* has been detected from *H. longicornis* ticks from Korea (Lee *et al.*, 2004). Ahantarig *et al.* (2011) reported for the first time *Coxiella*-like symbionts in *Haemaphysalis* ticks in Thailand. A subsequent study (Arthan *et al.*, 2015) reported a high prevalence (approximately 40%) of *Coxiella*-like endosymbionts in *Haemaphysalis* tick species in Thailand. Only 2.3% of *R. sanguineus* ticks collected from dogs in this study were positive for *C. burnetii*. Dogs are competent hosts for *R. sanguineus* tick, a known vector for *C. burnetii* (Smith, 1941).

Taken together, the Trans-PCR assay demonstrated a higher detection rate for *C. burnetii*, as compared to the *com 1* gene PCR assay (Table 1). The target of Trans-PCR assay, *IS1111* transposase gene is present in multiple copies within the *Coxiella* genome (Klee *et al.*, 2006). The assay has been applied for detection of *C. burnetii* in clinical samples and ticks (Greub *et al.*, 2005; Vilcins *et al.*, 2009). 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 (Tozer *et al.*, 2014). Although the assay has been used to differentiate different genotypes of *C. burnetii* through sequence determination of the amplicon (Zhang *et al.*, 1998), it was not feasible to determine the sequence of the amplified fragments in this study as the amount of amplicon generated from the PCR assay was insufficient for direct sequencing. Hence, real-time PCR analysis was found to be more useful in providing an additional means for confirmation of PCR results.

CONCLUSIONS

This study documented for the first time the molecular evidence of *C. burnetii* in farm animals and ticks in Malaysia. Further studies are necessary to characterize the genotype of *C. burnetii* and to identify the potential risk of transmission between human and animals.

Acknowledgements. The authors would like to thank Department of Veterinary Services, Ministry of Agriculture and Agro-Based Industry, Malaysia, managers and workers from each farm, and staff of PERHILITAN for their collaborative efforts. Our appreciation is also extended to Mr. Chai Koh Shin and Ms Asha Devi for their assistance in sample collection. This project was funded by grants HIR-MOHE E000013-20001 (sub programme 4), UMRG RP013-2012A and SGF120014-P0009-2013A from University of Malaya, Kuala Lumpur, Malaysia.

Author Disclosure Statement
No competing financial interests exist.

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