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**Molecular investigation into the presence of a *Coxiella* sp. in *Rhipicephalus sanguineus* ticks in Australia.**

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## Highlights

- 199 brown dog ticks (*R. sanguineus*) were collected from canine hosts in Australia
- Genus- and species-specific assays were used to survey the presence of *Coxiella*
- *Coxiella* DNA was detected in 100% of the *R. sanguineus* ticks
- *Coxiella* sequences in *R. sanguineus* from Australia were related to *Coxiella* endosymbionts overseas
- *Coxiella burnetii* was not observed in any *R. sanguineus* ticks

**Abstract**

Q fever is an infectious disease with a global distribution caused by the intracellular bacterium, *Coxiella burnetii*, which has been detected in a large number of tick species worldwide, including the brown dog tick, *Rhipicephalus sanguineus*. Recent reports of a high seroprevalance of *C. burnetii* in Australian dogs, along with the identification of additional *Coxiella* species within *R. sanguineus* ticks, has prompted an investigation into the presence and identification of *Coxiella* species in *R. sanguineus* ticks in Australia. Using a combination of *C. burnetii* species-specific IS1111a transposase gene and *Coxiella* genus-specific 16S rRNA PCR assays, a *Coxiella* sp. was identified in 100% (n =199) of *R. sanguineus* ticks analysed, and *C. burnetii* was not detected in any *R. sanguineus* ticks studied. Phylogenetic analysis of the 16S rRNA gene revealed the *Coxiella* sequences were closely related to *Coxiella* sp. identified previously in *R. sanguineus* and *R. turanicus* ticks overseas. This study illustrates the value of using genus specific PCR assays to detect previously unreported bacterial species. Furthermore, the presence of an additional *Coxiella* sp. in Australia requires further investigation into its potential for contributing to serological cross-reactions during Q fever testing.

**Keywords**

*Rhipicephalus sanguineus*, ticks, Australia, *Coxiella*, dogs,

## 1. Introduction

*Coxiella burnetii* is a Gram-negative obligate intracellular bacterium and the causative agent of the infectious disease known as ‘Q (query) fever’ (Maurin and Raoult, 1999). Q fever is considered to occur worldwide, except in New Zealand (Greenslade et al., 2003). Traditionally considered an occupational risk for abattoir workers and farmers, exposure to *C. burnetii* can be acquired through contact with, and inhalation of, infected tissues from domestic ruminants and companion animals (Maurin and Raoult, 1999; Kopecny et al., 2013). Diagnosis is usually undertaken through serological testing with clinical presentations of Q fever ranging from acute to chronic and can include post-Q fever fatigue syndrome; asymptomatic Q fever represents over 54-60% of infections (Maurin and Raoult, 1999). In Australia, reports of human Q fever have increased (4254 reported cases between 2007-2016 and 1085 notifications of Q fever in last 24 months (Australian Government, Department of Health, 2016)), with an increase in outbreaks unrelated to associations with domestic livestock (Tozer et al., 2014).

While a considerable number of studies have concentrated on the epidemiology of *C. burnetii*, recent research has focused on the evolution of *C. burnetii* and its phylogenetic relationship to other *Coxiella* species. Other *Coxiella* species have been reported as tick endosymbionts, that are transovarially inherited, and lack virulent characteristics (Duron et al., 2015; Raele et al., 2015; Seo et al., 2016) and have also been reported in mammals (Angelakis et al., 2016; Seo et al., 2016). Although tick endosymbionts are not known to cause infection, this principle was recently challenged when the presence of ‘*Candidatus* *Coxiella massiliensis*’ was identified in skin biopsies and ticks from patients with eschar and who had tested seropositive for Q fever but were *C. burnetii*-PCR negative (Angelakis et al., 2016).

Ticks (Acari: Ixodidae) are haematophagous ectoparasites and are considered an essential vector for maintaining the natural cycle of *C. burnetii* infection. To date over 40 tick species have been associated with *C. burnetii* and other *Coxiella* species (Parola and Raoult, 2001; Duron et al., 2015). In Australia, evidence supports a mammal-invertebrate cycle of *C. burnetii* involving

marsupials, including bandicoots and kangaroos (Cooper et al., 2012), and their associated ticks, *Haemaphysalis humerosa* (Traub et al., 2005) and *Amblyomma triguttatum* (McDiarmid et al., 2000), respectively, which act as asymptomatic reservoirs. Furthermore, *C. burnetii* has been detected in other Australian ticks, by a variety of traditional and molecular techniques (Cooper et al., 2013), however, *C. burnetii* has not been identified in *Rhipicephalus sanguineus* and the presence of other *Coxiella* species in this and other tick species in Australia is unknown.

The brown dog tick (*R. sanguineus*) is the most common tick throughout Australia to parasitize dogs (Greay et al., 2016). While the preferred host of *R. sanguineus* is the domestic dog (*Canis lupus familiaris*), it has the ability to feed opportunistically on many mammalian hosts. This generalist characteristic likely increases *R. sanguineus*' role in zoonotic transmission; *R. sanguineus* has been implicated in the epidemiology of Mediterranean Spotted Fever and Rocky Mountain Spotted Fever in Europe and the US, respectively, and is responsible for the transmission of anaplasmosis, babesiosis, and ehrlichiosis to various canids (Dantas-Torres, 2008). In addition, *R. sanguineus* is also known to host a variety of endosymbionts, including *Coxiella* (Ahantarig et al., 2013; Duron et al., 2015).

Recently, two Australian studies investigating the seroprevalence of *C. burnetii* in canine serum samples collected from dogs in New South Wales, the Northern Territory, and Queensland, estimated a 1.9-21.8% seroprevalence of *C. burnetii*. Importantly tick burden, particularly *R. sanguineus*, was highlighted as a potential factor in the epidemiology of Q fever (Cooper et al., 2011; Shapiro et al., 2016). The evidence of *C. burnetii* exposure in Australian dogs described above, growing recognition of other *Coxiella* bacteria described as tick endosymbionts, and increasing numbers of reports of *C. burnetii* infections, have prompted this present investigation. The major aims of this study were to identify and characterise the presence of *Coxiella* in *R. sanguineus* ticks in Australia using molecular methods. The presence or absence of a *Coxiella* bacterium may have implications for Q fever surveillance, and may provide potential insight into the transmission of this disease.

## 2. Material and Methods

### 2.1 Ethics statement

This research complies with the *Australian Code for the Responsible Conduct of Research*, 2007 and the *Australian Code for the Care and Use of Animals for Scientific Purposes*, 2013. The removal of ticks from canine hosts was approved by the Murdoch University Animal Ethics Committee.

### 2.2 Sample Collection

A total of 199 individual specimens of *R. sanguineus* were collected from 74 canine hosts in the New South Wales (NSW; n = 20), Northern Territory (NT; n = 108), Queensland (QLD; n = 29), and Western Australia (WA; n = 42), between 2012 and 2015 (Supplementary Table 1). The 20 NSW ticks were collected from canine hosts in a NSW North-West rural community as a proxy to investigate an atypical outbreak of Q fever affecting residents in the area (Priscilla Stanley, personal communication). The source of the outbreak was not identified. Ticks collected from dogs in six remote Aboriginal communities were also included in the present study (Walgett, NSW; Lake Nash, Mutitjulu, Tennant Creek, Yuendumu, NT; Kurrangki, WA; Supplementary Table 1). All ticks were stored in 70% ethanol until required and were identified morphologically using standard keys (Barker and Walker, 2014).

### 2.3 DNA extraction

Total genomic DNA from individual ticks (Supplementary Table 1) was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's recommendations (Qiagen Supplementary Protocol: Purification of total DNA from insects) (Gofton et al., 2015). Sterile and DNA-free equipment and tubes were used for each step and equipment was decontaminated between samples. Extraction reagent blanks were performed in parallel with all DNA extractions.



#### 2.4 Real-time PCR

A *Coxiella burnetii* specific qPCR assay targeting the IS1111a transposase gene (IS1111aF 5' GTTTCATCCGCGGTGTTAAT; IS1111aR 5'TGCAAGAATACGGACTCACG; probe IS1111aP 5' CCCACCGCTTCGCTCGCTAA) (Banazis et al., 2010) was performed in 25  $\mu$ L volumes, containing of 400 nM of each primer, 750 nM IS1111a probe (5' 6-FAM, 3' BHQ-1) (Banazis et al., 2010), 1 mM dNTPs, 1 x PCR buffer (5 Prime, Germany), 1 U of *Taq* DNA polymerase (5 Prime, Germany), 2 mM of magnesium chloride, and 2-5  $\mu$ L of undiluted DNA extract. This assay was performed using the StepOne™ Real-Time PCR machine (version 2.1, Applied Biosystems, Foster City, CA, USA) with an initial hold cycle (50°C, 2 min), then one cycle of denaturation (95°C, 5 min), followed by 40 cycles of denaturation (95°C, 20 s), and annealing and extension (60°C, 45 s). No-template and extraction reagent blank controls were included for each assay, and a positive control that was comprised of DNA ( $10^2$ ,  $10^5$  copies) extracted from the Q-Vax™ vaccine (CSL, Parkville, Australia) (Banazis et al., 2010).

#### 2.5 Conventional PCR

To determine the presence of any *Coxiella* species harboured within the *R. sanguineus* ticks, a PCR assay targeting 524 bp of the 16S rRNA gene (short 16S) was performed using the primers Cox-sp434F (5' CCTTTTGAGCGTTGACGTTA) and Cox-sp1004R (5'CCAAAGGCACCAAGTCATTT) (Lalzar et al., 2012). Samples with positive PCR products (confirmed through Sanger sequencing) were subjected to another *Coxiella* genus-specific PCR targeting 1.45 kb of the 16S rRNA gene (long 16S) using the primer pairs Cox-16s-1457F (QR-F0 5'ATTGAAGAGTTTGATTCTGG) and Cox-16s-1457R (QR-R0 5' CGGCTTCCCGAAGGTTAG) (Masuzawa et al., 1997). All PCRs contained 2  $\mu$ L of DNA extracted from ticks, 1 x PCR buffer, 2.5 mM MgCl<sub>2</sub>, 1 mM dNTPs, 0.01 mg BSA (Fisher Biotech, Australia), 1.25 U Perfect *Taq* Polymerase (5 Prime, Germany), and 400 nM of each primer in a total volume of 25  $\mu$ L. All PCRs included no-template and extraction reagent blank controls. PCR reaction conditions for respective primers are listed in Supplementary Table 2.

## 2.6 Sanger sequencing

All PCR products were electrophoresed on 1-2% agarose gels stained with GelRed (Biotium, USA), and visualised under UV light. To confirm the specificity of the short 524 bp 16S PCR, representative positive bands from each geographical location were purified with the QIAquick gel extraction kit (QIAGEN, Germany), and sequenced with both forward and reverse PCR primers on an ABI 3730 96 Capillary Sequences using Big dye v3.1 terminators (Life Technologies, USA). All long 1.45 kb PCR products were purified and sequenced as above with both forward and reverse primers.

## 2.7 *Coxiella* 16S phylogenetic analysis

Short 524 bp *Coxiella* sp. 16S sequences were compared to GenBank using nucleotide BLAST with default parameters to confirm the specificity of PCR products. Phylogenetic analysis was conducted on the trimmed 16S sequences (1,210kb) obtained from the *Coxiella* PCR assay on *R. sanguineus* samples, in addition to *Coxiella burnetii* and other *Coxiella* 16S sequences retrieved from GenBank. Sequences were aligned with MAFFT (Kato et al., 2002) and the alignment was refined with the program MUSCLE (Edgar, 2004). A maximum-likelihood phylogenetic tree for *Coxiella* 16S rRNA was generated using FastTree 2 (Price et al., 2010). The unique nucleotide sequence reported in this paper is available in GenBank under the accession number KU892220.

## 3. Results

### 3.1 Tick identification

A total of 199 ticks were removed from 74 canine hosts in NSW (n = 20), NT (n = 108), QLD (n = 29), and WA (n = 42) (Supplementary Table 1). Ticks were morphologically identified as two nymphal, 102 female and 95 male *R. sanguineus*.

### 3.2 *Coxiella burnetii*-specific IS1111a qPCR assay

Amplification of the IS1111a transposase gene from *C. burnetii* was observed in both of the  $10^2$  and  $10^5$  copy number positive controls. However no amplification of this gene was observed in any *R. sanguineus* samples, the no-template or extraction reagent blank controls (Table 1; Supplementary Table 1).

### 3.3 Detection of a *Coxiella* sp. in *Rhipicephalus sanguineus* ticks in Australia

Although all *R. sanguineus* ticks failed to amplify the IS1111a gene sequence, we successfully amplified a 524 bp region of the 16S rRNA gene in 199 (100%) of *R. sanguineus* ticks, with all life-stages studied testing positive to the presence of a *Coxiella* sp. (Table 1; Supplementary Table 1). Both extraction reagent blanks and no-template controls were negative. The nucleotide sequence of the *Coxiella* sp. identified in all *R. sanguineus* ticks revealed a 100% nucleotide identity to a *Coxiella* endosymbiont observed in *R. sanguineus* on Marshall Islands (HQ116458; unpublished) and within *Rhipicephalus* sp. (KP994849; Duron *et al.* 2015).

Although 155 (78%) samples amplified the longer 1.45kb 16S amplicon as visualised by gel electrophoresis, the majority produced mixed chromatograms and only 52 generated clean sequences for phylogenetic analysis. All 52 sequences were identical and the designated “*Coxiella* sp. in *R. sanguineus*, Australia” sequence (accession number KU892220) was used for further analysis. Consistent with the shorter 16S rRNA gene amplicon, the longer sequence revealed a *Coxiella* bacterium with high nucleotide identity to previously described *Coxiella* endosymbionts of ticks (KP994843 and KP994849, 100%, Duron *et al.* 2015; CP011126, 99%, Gottlieb *et al.* 2015; JQ480823.1, 99%, Lalar *et al.* 2012; D84559.1, 99%, Noda *et al.* 1997).

### 3.4 *Coxiella* phylogenetic analysis

The genus *Coxiella* has been traditionally classified based on the 16S gene (Zhong, 2012). Here, a maximum-likelihood phylogenetic tree was constructed using the 16S “*Coxiella* sp. in *R. sanguineus*, Australia” sequence, along with available *Coxiella* bacterial sequences, with *Legionella*

*pneumophila* as an outgroup (Figure 1). The *Coxiella* sequence from this study clustered with known *Coxiella* endosymbionts previously sequenced from *R. sanguineus* (reported as Clade C by Duron et al. 2015) with a high consensus and support value confidence (90%), and did not cluster with *Coxiella* sequenced from other tick species. The *Coxiella* sp. identified in our Australian *R. sanguineus* ticks was 3-4% distinct from *C. burnetii*, which clustered with *Coxiella* species associated with argasid (soft) ticks (Figure 1) (reported as Clade A by Duron et al. 2015).

#### 4. Discussion

*Coxiella burnetii* is a zoonotic infectious disease with a worldwide distribution. Most individual cases and outbreaks of Q fever in people have been associated with close interactions between humans and domesticated ruminants or companion animals, and the role that ticks may play, if any, in the transmission of *C. burnetii* to people is largely unexplored. The brown dog tick, *R. sanguineus*, is highly endophilic and is closely associated with human activity because of its preferred canine hosts. *Rhipicephalus sanguineus* has been shown to harbour *C. burnetii*, albeit only at extremely low prevalence rates (Mantovani and Benazzi, 1953; Toledo et al., 2009; Watanabe et al., 2015; Noda et al., 2016), and is also known to host other related *Coxiella* bacteria (Noda et al., 1997; Duron et al., 2015). This study was conducted following reports indicating a surprisingly high seroprevalence of *C. burnetii* in dogs living in tropical north Queensland (Cooper et al., 2011) and more recently, in New South Wales and the Northern Territory (Shapiro et al., 2016), regions within the normal geographical distribution of *R. sanguineus* ticks.

Our initial studies revealed *R. sanguineus* ticks were negative through real-time PCR analysis for *C. burnetii*. The failure to amplify IS1111 is consistent with the absence of IS1111 in other *Coxiella* previously observed in *R. sanguineus* and *Rhipicephalus bursa* ticks (Duron, 2015; Raelle et al., 2015) and further confirms the absence of *C. burnetii* in this present study. However, we show here for the first time in Australia, detection of a *Coxiella* sp. in *R. sanguineus* ticks analysed from four geographical regions and with a 100% prevalence, consistent with previous studies

overseas (Duron et al., 2015). A reduced PCR efficiency, usually detected when amplifying longer gene fragments, was observed in this study when *Coxiella* DNA was amplified in the long 16S PCR assay (78%), compared to the short 16S PCR assay (100%). The high sequence homology between the *Coxiella* sequences observed in this study with those observed in *R. sanguineus* and *R. turanicus* ticks overseas (Clade C; Duron et al. 2015), indicates a low genetic diversity of the endosymbiont within this tick genus. Despite the high diversity observed within the genus *Coxiella*, the low diversity of the *Coxiella* species observed within *R. sanguineus* aligns with the maternal-inheritance model of these endosymbionts in ticks (Duron et al., 2015). While Angelakis et al. (2016) only amplified the *GroEL* gene, the phylogenetic analysis and high sequence homology of ‘*Candidatus* C. massiliensis’ isolated from ticks and human skin biopsies is consistent with the 16S clustering observed in *Rhipicephalus* ticks, specifically *R. sanguineus* and *R. turanicus*, and necessitates further consideration of the pathogenicity of this bacterium.

Twenty ticks in the present study were collected from dogs housed in a canine shelter and from a local veterinary clinic in a rural town in North West NSW, a community that had experienced an atypical Q fever outbreak with an increase in disease incidence significantly above baseline rates. Given the 22% seroprevalence of *C. burnetii* in dogs in Queensland (Cooper et al., 2011), the absence of *C. burnetii* was unexpected. Furthermore, in response to an increased seroprevalence of *C. burnetii* in dogs within Aboriginal communities (Shapiro et al., 2016), *R. sanguineus* ticks were collected from dogs in six communities in the present study (Walgett, NSW; Lake Nash, Mutitjulu, Tennant Creek, Yuendumu, NT; Kurrangki, WA; Supplementary Table 1). Two such communities, Walgett (NSW) and Yuendumu (NT), which were previously reported to have a 6.5% seroprevalence to *C. burnetii* possibly attributed to *R. sanguineus* infestation (Shapiro et al., 2016), were only positive for the *Coxiella* sp.; the remaining ticks from Aboriginal communities tested in our study were also negative for *C. burnetii*. Moreover, no molecular techniques were employed to verify the *Coxiella* species observed by Cooper *et al.* (2011) or Shapiro *et al.* (2016). Similarly in a Sydney veterinary clinic in 2010, where a number of clinical

staff contracted a *C. burnetii* infection following caesarean of a breeding cat (Kopečný et al., 2013), despite positive serological tests from the cat in question and clinical staff to *C. burnetii*, parturient material did not show any histological evidence of sepsis and molecular analyses of *C. burnetii* failed to amplify the diagnostic targeted gene, *IS1111*.

Here we revealed the presence of a *Coxiella* sp. within *R. sanguineus* ticks in Australia and the absence of the Q fever causative agent, *C. burnetii*. This finding is intriguing as this *Coxiella* sp. exposes potential cross reactions with *C. burnetii* serological tests, previously reported by Angelakis et al. (2015), and questions previous reports of high seroprevalence in companion animals. The presence of a *Coxiella* sp., in addition to positive serological tests, might further explain the observed high level of observed asymptomatic Q fever cases. Moreover, the use of the *Coxiella*-genus specific PCR assay was fundamental in the identification of this novel bacterium in Australia and is therefore recommended following a seropositive test that has also failed the traditional *C. burnetii* specific PCR (*IS1111*) in order to confirm the organism present. Lastly, the finding of another *Coxiella* sp. in Australia with the potential to cross-react with Q fever serological tests warrants further investigation.

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**Authorship**

CLO conceived, designed, coordinated the study, and contributed to the data analyses and wrote the first and final version of the manuscript. AWG and TLG performed tick identification, DNA extractions, PCR assays, and contributed to data analysis and manuscript preparation. SG contributed to the tick collection and identification and preparation of manuscript. UMR and PJI contributed to the design of the study and manuscript preparation. All authors read and approved the final version of the manuscript.

**Conflict of interest statement**

The authors declare that there is no conflict of interest.

**Ethics approval**

This study was conducted under the compliance of the *Australian Code for the Responsible Conduct of Research, 2007* and *Australian Code for the Care and Use of Animals for Scientific Purposes, 2013*. Tick collection was carried out opportunistically with approval from the Murdoch University Animal Ethics Committee.

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**Figures caption**

Figure 1. Phylogenetic analysis of *Coxiella*, including *Coxiella* endosymbionts of ticks, *Coxiella* sp. from *R. sanguineus* ticks in Australia, and *C. burnetii*, based on 1,210 bp 16S rRNA gene sequences. The maximum likelihood tree was constructed using FastTree2 (Price et al., 2010) with support values displayed at each node. *Legionella pneumophila* is the cropped outgroup. Bold type indicates the consensus sequence from this study.

Table 1. Summary of 199 *Rhipicephalus sanguineus* tick specimens from three states (New South Wales, NSW; Queensland, QLD; Western Australia, WA) and one territory (Northern Territory, NT) in Australia and the number of positive amplifications at each locus.

<b>Geographical location</b>	<b>n</b>	<b>IS1111a</b>	<b>Short 16S</b>	<b>Long 16S*</b>
<b>NSW</b>	20	0	20	16 (10)
<b>NT</b>	108	0	108	82 (24)
<b>QLD</b>	29	0	29	21 (7)
<b>WA</b>	42	0	42	36 (11)
<b>TOTAL</b>	199	0	199	155 (52)

\* Values in parentheses indicates how many specimens generated clean sequences

