Cat fleas (*Ctenocephalides felis* clade ‘Sydney’) are dominant fleas on dogs and cats in New South Wales, Australia: Presence of flea-borne *Rickettsia felis*, *Bartonella* spp. but absence of *Coxiella burnetii* DNA

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1. Introduction

The role of companion animal fleas in the epidemiology of *Bartonella henselae* – the causative agent of cat scratch disease – is well documented (Klotz et al., 2011). However, the exact role the flea plays in the transmission of other vector-borne zoonotic pathogens remains undefined (*Rickettsia felis*) or unknown (*Coxiella burnetii*) (Schriefer et al., 1994; Cutler et al., 2010; Klotz et al., 2011; Abdad et al., 2011; Toman et al., 2012; Njeru et al., 2016; Ng-Nguyen et al., 2020). Recently, companion dogs and cats were implicated in the transmission of both latter pathogens in Australia, including a Q fever outbreak (caused by *C. burnetii*) in a small animal veterinary hospital and a cluster of five probable cases of human infection with *R. felis* (Kopecky et al., 2013; Williams et al., 2011).

A member of the family *Rickettsiaceae*, *R. felis* causes flea-borne spotted fever (FBSF) (Abdad et al., 2011). In humans, clinical manifestations include signs of pyrexia, headaches, maculopapular rash, myalgias and eschar (Schriefer et al., 1994; Abdad et al., 2011). Over 30 arthropod species are recognised as potential vectors, but the cat flea (*Ctenocephalides felis*) is considered the main reservoir and vector. Transmission is believed to occur when an infected flea bites or contaminates open wounds with its faecal matter (Legendre and Macaluso, 2017). In Australia, *C. felis* is the dominant flea species on domestic cats and dogs (Slapeta et al., 2011), hosting *R. felis* across the east coast at a prevalence of 6.7–19.8% (Barrs et al., 2010; Teoh et al., 2018).

The role of the cat flea (*C. felis*) in the transmission of *C. burnetii* is unknown. Q fever in humans is asymptomatic in approximately 60% of cases (Toman et al., 2012). Clinical disease manifests as an acute flu-like syndrome with non-specific signs of chills, malaise, sweating or fatigue (Raoult et al., 2000). Individuals with compromised cardiovascular function or endothelial cell defects can develop persistent focal diseases...
such as endocarditis or vascular infection (Raoult et al., 2000; Kampschreur et al., 2014). In addition, a debilitating chronic fatigue syndrome is experienced by at least 20% of acute Q fever patients (Morroy et al., 2016). In adults of most other mammalian species, coxiellosis is subclinical; however, abortions, infertility, stillbirth and weak progeny have occasionally been associated with the disease, especially in domestic ruminants, the primary source of human infections (Sanford et al., 1994; Eibach et al., 2012). Infection occurs primarily through inhalation of aerosolised infected materials. Arthropod transmission is theoretically possible, although an uncommon route for C. burnetii (Porter et al., 2011; Duron et al., 2015). Little is known about the role fleas, such as C. felis, in transmission and a grooming salon.

2. Materials and methods

2.1. Flea collection

From November 2019 to May 2020 fleas were collected from around New South Wales, Australia (Table 1). Fleas were collected opportunistically from dogs and cats by veterinarians, veterinary nurses, groomers and shelter carers as part of their routine work. Fleas were placed in 70% ethanol and stored at room temperature before being transferred into the freezer (−20 °C).

2.2. Morphological and molecular identification using cytochrome c oxidase subunit 1

All fleas were examined individually under a microscope (5–200×, Olympus, Australia) and placed to a genus and species as previously described (Dunnet and Nardon, 1974; Lawrence et al., 2019). A minimum of one flea of each flea species (if more than one species was present or if the species was equivocal on morphological examination) from each animal was selected for molecular characterisation. Thirty-five fleas were incised on the dorsal caudal abdomen using a sterile scalpel blade before being placed in a microcentrifuge tube (Eppendorf, Macquarie Park, Australia) which was then incubated in a heat block set at 70 °C for 15 min to evaporate the ethanol in which they were stored (Lawrence et al., 2015). Flea DNA was extracted using the Isolate II Genomic DNA kit (BioLine, Eveleigh, Australia) according to the manufacturer’s protocol with DNA eluted in 100 μl of elution buffer and stored at −20 °C. For each batch of DNA extractions, an extraction with no DNA and the eluate served as a non-template control (negative extraction control, NEC).

Extracted flea DNA samples were subjected to conventional polymerase chain reaction (PCR) targeting cytochrome c oxidase subunit 1 (cox1) as previously described (Lawrence et al., 2015, 2019). Polymerase chain reaction amplification was performed in a 30 μl reaction mixture containing 15 μl MyTaq Red Mix (BioLine), with 2 μl DNA and nuclelease-free water. Assays were performed in a T100 cycler (Bio-Rad, Australia) with an initial denaturation at 95 °C for one minute followed by 25 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 10 s, and a final elongation for 5 min at 72 °C. All reactions were run with a NEC and sterile PCR water in place of DNA (nontarget control, NTC). Amplicons of cox1 were sequenced (Macrogen Ltd, Seoul, Korea) and DNA sequences were assembled using CLC Main Workbench 21 (CLC bio, Qiagen, Chadstone, Australia) and compared to reference cox1 haplotypes (h1-h90) sensu Lawrence et al. (2019) and associated with the three clades (‘Sydney’, ‘Cairns’, ‘Darwin’) sensu Crkvencic and Slapeta (2019).

2.3. Molecular detection and identification of vector-borne pathogens including Rickettsia spp., Bartonella spp. and C. burnetii

An aliquot of flea DNA underwent multiplex TaqMan qPCR targeting the C. burnetii multiplex insertion sequence gene IS1111 (146-bp amplicon), and two single copy genes: com1 (76-bp amplicon) the outer membrane protein-coding gene, and groEL (114-bp amplicon; heat-shock

Table 1: Sequence and product lengths of target gene primers for Coxiella burnetii qPCR

| Primer | Primer sequence (5’-3’) | Product length (bp) | Final concentration (nM) | Reference |
|--------|-------------------------|---------------------|--------------------------|-----------|
| IS111† | GGAGGAGCTGTTAACCCG      | 146                 | 300                      | de Bruin et al. (2011) |
| Forward primer | GGAGGAGCTGTTAACCCG   |                     |                          |           |
| Reverse primer  | TGATTTAGGAACTTGCCTAA   |                     |                          |           |
| Probe          | AAATCCTCTCATCAG         | 114                 | 300                      | Bond et al. (2016)  |
| groEL†         | GTGCTGGGCTTACATCAG      |                     |                          |           |
| Forward primer | GTGCTGGGCTTACATCAG      |                     |                          |           |
| Reverse primer  | CGATCCCTCATCAG          | 144                 | 300                      |           |
| Probe          | CCATGAGGATGCAAGAGGGCA   | 76                  | 400                      | Lockhart et al. (2011) |
| com1†          | AAAACCTCCGGGTTGTAC      | 76                  | 400                      |           |
| Forward primer | AAAACCTCCGGGTTGTAC      | 76                  | 400                      |           |
| Reverse primer  | GCAATTGACTTATTGGTATGG   |                     |                          |           |
| Probe          | Quasar670-AGAACAGGCCATTTTGGGCGCA-BHQ2 | 400                 | 200                      |           |

Note: FAM, 6-Carboxyfluorescein; BHQ1, Black Hole Quencher-1; CAF560, CAL Flour Orange 560 Amidite; Quasar670, Quasar 670 Carboxylic Acid; BHQ2, Black Hole Quencher-2.

† Insertion sequence 1111 (IS1111).
‡ Heat-shock operon (hspAB).
§ Outer membrane protein (com1).
3. Results

A total of 107 fleas were collected opportunistically from 32 dogs and cats (HH-1 to HH-32) in New South Wales, Australia including a cat shelter, grooming salon, and veterinary clinics (Table 2). Most fleas (93.5%, 102/107) were morphologically identified as the cat flea (C. felis). Three *Ctenocephalides* sp. fleas had equivocal morphological characteristics due to being damaged or having an ambiguous second notch on the hind tibia between the apical and post-median setae. One flea (HH-21-2) from a dog from north-west New South Wales, Australia, was identified as the stick fast flea (*Echidnophaga gallinacea*). Overall, there were 33 males, 72 females, one *C. felis* whose sex was not possible to determine and one male *E. gallinacea*.

At least one *C. felis* from each animal was selected for DNA isolation including *C. felis* specimens with equivocal morphology, and the single *E. gallinacea* (n = 35, Table 2, Supplementary Table S1) The cox1 gene was successfully amplified and sequenced from DNA extracts of all specimens. There were five cox1 haplotypes of *C. felis* (Cf_h1-h5) and a single *E. gallinacea* haplotype (Eg_h1). The cox1 haplotype Cf_h1 was the most dominant with 26 representatives, the remaining haplotypes had 1–4 representatives. All three *Ctenocephalides* sp. fleas with equivocal morphology had a cox1 sequence that was identical to the *C. felis* cox1 haplotype Cf_h1. Haplotypes Cf_h1 to Cf_h5 differed from each other by single variable nucleotides. The cox1 sequence of *E. gallinacea* was 100% identical to the reference cox1 sequence from Australia (KT376440, Lawrence et al., 2015). The Cf_h1 of *C. felis* was identical to “h1” sensu Lawrence et al. (2019), and together with the remaining haplotypes represent the clade ‘Sydney’ sensu Crvenkovic and Slapeta (2019).

Multiplex TaqMan qPCR targeting the *IS1111*, *Cm1* and *htpAB* genes for *C. burnetii* was negative for all examined fleas (0/35, 95% CI: 0–11.8%). Multiplex TaqMan qPCR targeting the *gltA* (*Rickettsia* spp.) and *sraA* (*Bartonella* spp.) was positive in 8 (8/35, 95% CI: 11.8–39.3%) and 4 (4/35, 95% CI: 3.9–26.6%) samples, respectively. In addition, 3 and 4 samples were considered suspect positive for amplification of *gltA* (*Rickettsia* spp.) and *sraA* (*Bartonella* spp.), respectively, based on *Cv* values > 36 (Tables 3 and 4). All NTC and NEC reactions remained negative throughout this study.

Amplification and DNA sequencing of *Rickettsia*-positive and suspect samples with conventional nested PCR targeting *gltA* and *ompA* genes revealed five fleas (HH-6-1, HH-13-1, HH-14-1, HH-15-1, HH-23-1) that sequenced as identical (100%) DNA with *R. felis* reference *gltA* (Parola et al., 2003) and *ompA* (Ogata et al., 2005). HH-18-1 amplified only using
ompA assay and its DNA sequence was identical with the R. felis reference. An additional sample (HH-16-1) that amplified only gltA yielded a sequence that was 99% identical to the R. felis reference DNA with a variation of one single nucleotide at the gltA gene (Table 4).

Sanger DNA sequencing of the ssrA product failed to unambiguously resolve the Bartonella ssrA DNA sequence. We used conventional PCR with Illumina tagged Bartonella-specific ssrA and gltA primers to amplify these regions from DNA of seven Bartonella spp. positive and suspect positive samples. Amplification was successful for all four positive samples but was unsuccessful for the three suspect positive samples. The positive amplicons were subject to Illumina DNA sequencing and on average yielded 30,811 paired-end good quality sequences for gltA and 18,102 paired-end good quality sequences for ssrA. The HH-18-1 sample showed a mixed pattern because for both gltA and ssrA, it had sequence reads matching B. henselae (strain Houston-1) and Bartonella clarridgeiae (strain 73). At ssrA, 41% of the reads belonged to B. henselae and 59% to B. clarridgeiae. At gltA, 25% of the reads belonged to B. henselae and 75% to B. clarridgeiae. The remaining three samples had only B. henselae sequences (Table 4; Fig. 1).

4. Discussion

Arthropods are known vectors of zoonotic pathogens worldwide, the most common of which is the cat flea, C. felis (Clark et al., 2018; Lawrence et al., 2019), a well-documented carrier of Rickettsia and Bartonella species (Barrs et al., 2010; Slapeta and Slapeta, 2016). In this study the presence of Rickettsia and Bartonella species DNA and that of C. burnetii, the causative agent of Q fever, in fleas of cats and dogs were investigated in Greater Sydney and rural communities in New South Wales. All but one flea were identified to be C. felis, consistent with previous studies in Australian companion animals (Slapeta et al., 2011; Lawrence et al., 2014). The only other flea species identified in this study was the stick-fast flea (E. gallinaceae), which is rarely found on dogs and cats, since it traditionally favours residence on avian species (Slapeta et al., 2011). This study confirmed that only the clade ‘Sydney’ of C. felis is present in New South Wales (Slapeta et al., 2011; Lawrence et al., 2015; Chandra et al., 2017; Crkvencic and Slapeta, 2019). In neighbouring New Zealand, only the clade ‘Sydney’ of C. felis has been documented and a study by Chandra et al. (2017) found similar percentages of fleas positive for Bartonella and Rickettsia using multiplexed TaqMan qPCR on 38 C. felis DNA samples, with 5.3% (n = 2) positive for Bartonella and 18.4% (n = 7) positive for Rickettsia. The identification of co-infection of B. henselae and B. clarridgeiae in a single flea was demonstrated using recently developed multi-locus Illumina next-generation amplicon sequencing, demonstrating the advantages that this technology and

### Table 3

| Sample Location | Animal Category | Negative | Positive | Suspect | Grand total |
|-----------------|----------------|----------|----------|---------|-------------|
| **Bartonella sp. qPCR** | | | | | |
| Greater Sydney | Owned | 20 | 4 | 2 | 26 |
| North-west New South Wales | Unknown | 11 | 3 | 1 | 15 |
| | Stray | 7 | 2 | 9 | 16 |
| **Rickettsia spp. qPCR** | | | | | |
| Greater Sydney | Owned | 17 | 7 | 2 | 26 |
| North-west New South Wales | Unknown | 6 | 1 | 2 | 9 |
| | Stray | 8 | 1 | 1 | 10 |

| Sample | Flea species | qPCR Bartonella | Sanger gltA/SSR | C. value | Result |
|--------|--------------|-----------------|-----------------|---------|--------|
| HH-2-1 | R. felis | Negative | Positive | 30.62 | Suspect |
| HH-5-1 | R. felis | Negative | Positive | 28.98 | Suspect |
| HH-6-1 | R. felis | Negative | Positive | 33.68 | Suspect |
| HH-13-1 | R. felis | Suspect | Negative | 24.21 | Suspect |
| HH-15-1 | R. felis | Suspect | Negative | 24.92 | Suspect |
| HH-16-1 | R. felis | Suspect | Negative | 26.05 | Suspect |

All C. felis were typed using COX1 gene sequencing, percent identity against reference genome of R. felis (CP000053).

a DNA amplification and sequencing percent identity against reference genome of R. felis (CP000053).

b DNA sequencing and percent identity against reference genome of R. felis (CP000053).

c DNA sequencing and percent identity against reference genome of R. felis (CP000053).

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**Table 4**

| Sample | Flea species | qPCR Bartonella | Sanger gltA/SSR | C. value | Result |
|--------|--------------|-----------------|-----------------|---------|--------|
| HH-2-1 | R. felis | Negative | Positive | 30.62 | Suspect |
| HH-5-1 | R. felis | Negative | Positive | 28.98 | Suspect |
| HH-6-1 | R. felis | Negative | Positive | 33.68 | Suspect |
| HH-13-1 | R. felis | Suspect | Negative | 24.21 | Suspect |
| HH-15-1 | R. felis | Suspect | Negative | 24.92 | Suspect |
| HH-16-1 | R. felis | Suspect | Negative | 26.05 | Suspect |

All C. felis were typed using COX1 gene sequencing, percent identity against reference genome of R. felis (CP000053).

**Table 5**

| Sample | Flea species | qPCR Bartonella | Sanger gltA/SSR | C. value | Result |
|--------|--------------|-----------------|-----------------|---------|--------|
| HH-2-1 | R. felis | Negative | Positive | 30.62 | Suspect |
| HH-5-1 | R. felis | Negative | Positive | 28.98 | Suspect |
| HH-6-1 | R. felis | Negative | Positive | 33.68 | Suspect |
| HH-13-1 | R. felis | Suspect | Negative | 24.21 | Suspect |
| HH-15-1 | R. felis | Suspect | Negative | 24.92 | Suspect |
| HH-16-1 | R. felis | Suspect | Negative | 26.05 | Suspect |

All C. felis were typed using COX1 gene sequencing, percent identity against reference genome of R. felis (CP000053).
lated areas in Australia have the highest incidence of human infection cases recorded (Teoh et al., 2016; Hii et al., 2017). Therefore, (Wedincamp and Foil, 2002) which might explain why densely populated areas. The focus in Australia has been on the transmission of R. felis (Macaluso, 2017). The cat flea (C. felis) was confirmed as the most common flea on dogs and cats in New South Wales, Australia, and there was an absence of cox1 clades other than clade ‘Sydney’. While DNA of the zoonotic pathogens R. felis and Bartonella spp. was demonstrated in fleas, C. burnetii DNA was not detected in this investigation, consistent with previous studies. A combination of molecular tools to characterise both the arthropods and the potential zoonotic pathogens enabled us to detect co-infection of Bartonella spp. and R. felis. Illumina next-generation amplicon sequencing was also applied to demonstrate co-infection of B. henselae and B. clarridgeiae.

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CRediT author statement

Holly Hai Huai Huang: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Roles/Writing - original draft; Writing - review & editing. Rosemonde Isabella Power: Formal analysis; Methodology; Validation; Writing - review & editing. Karen Olivia Mathews: Investigation; Data curation; Methodology; Resources; Writing - review & editing. Katrina L. Bosward: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing - review & editing. Jan Slapeta: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing - review &
The nucleotide sequence data generated in this study were deposited in GenBank (NCBI) under the accession numbers MZ381608-MZ381642 and MZ420158-MZ420169. Raw fastq sequence data was deposited at SRA NCBI BioProject: PRJN737164. Sequence data, associated supplementary and additional data are available at LabArchives (https://doi.org/10.25833/2bct-2276).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crviwd.2021.100045.

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