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Molecular detection of *Coxiella burnetii* in raw meat intended for pet consumption

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Abstract

The discovery of antibodies against *Coxiella burnetii* in cattery-confined breeding cats indicating prior or current exposure (Shapiro *et al.*, 2015) prompted an investigation into possible sources of infection. One hypothesis was that raw meat diets containing reservoir species may provide a source of *C. burnetii* transmission. The aim of this pilot study was to determine whether *C. burnetii* DNA was present in raw meat sold exclusively for companion animal consumption. The sample population consisted of raw meat packages (n = 58) of primarily kangaroo origin, with three to four aliquots (50–120 mg) randomly selected from each package. Genomic DNA was extracted from whole tissue in each of these aliquots using a modified protocol. Three quantitative PCR assays were used for the detection of *C. burnetii* targeting the IS*1111* gene, the heat shock operon *htpAB* and the *C. burnetii* outer membrane protein-coding gene, *com1*. *Coxiella burnetii* DNA was detected in 25/58 samples (43%) using the IS*1111*, *htpAB* and/or *com1* PCR assays and confirmed by DNA sequencing. All samples amplifying a product in the *com1* assay also amplified a product in the *htpAB* and IS*1111* assays. A total of 17/58 (29%) packets were positive with all three genes, 4/58 (7%) were positive with two genes (IS*1111* and *htpAB*) and 4/58 (7%) were positive with the IS*1111* gene only. *Coxiella burnetii* DNA was five times more likely to be found in offal than skeletal muscle meat samples. All meat samples in which *C. burnetii* DNA was found were from kangaroo tissues, while samples labelled as non-kangaroo meat (n = 4) were negative. Multi-locus variable number of tandem repeat analysis (MLVA) identified three different genotypes of *C. burnetii* that have all been identified previously from Australian human clinical Q fever cases. Further investigations are required to determine the potential role of certain raw meats in the transmission of *C. burnetii* to cats and humans.

KEYWORDS

cats and dogs, *Coxiella burnetii*, kangaroo, pet food, Q fever, quantitative Polymerase Chain Reaction (qPCR), raw meat

1 | INTRODUCTION

Coxiella burnetii is the gram negative bacterium responsible for Q fever, a worldwide zoonotic disease with considerable reservoir animal species (Babudieri, 1959). Domestic ruminants are the traditional host animals (Fournier, Marrie, & Raoult, 1998), but there is growing evidence that this emerging zoonosis may be transmitted from a variety of other animal hosts to humans (Kopecny, Bosward, Shapiro, & Norris, 2013;Massey, Irwin, & Durrheim, 2009). A Q fever surveillance report used to inform vaccination policy in New South Wales (NSW), Australia, concluded that the epidemiology of the disease in this state has changed (Massey et al., 2009) and that a new framework for defining those individuals at-risk of acquiring Q fever is required. Furthermore, sources of Q fever infection have recently been highlighted including domestic pets, wildlife, dust and soil (Tozer et al., 2014).

Q fever outbreaks in Australia attributable to an infected dog and two cats occurred in 2007 (Gibbons & White, 2012), 2010 (Kopecny et al., 2013) and 2016 (Malo et al., 2018), respectively. Two of these outbreaks (Gibbons & White, 2012;Kopecny et al., 2013) resulted in the acquisition of feline and canine positive control sera, upon which two Australian *C. burnetii* seroprevalence studies were conducted (Shapiro, Bosward, Heller, & Norris, 2015;Shapiro et al., 2016). These studies found that cattery-confined breeding cats had the highest seroprevalence of *C. burnetii* within cat populations (9.3%) while 6.5% of free-roaming dogs from Aboriginal communities were seropositive. Sources and mechanisms of infection for dogs and cats have not been investigated, yet it is presumed that they are exposed similarly to humans primarily via inhalation and potentially through ingestion and tick bites (Babudieri, 1959;Egberink et al., 2013;Maurin & Raoult, 1999;Tigertt, Benenson, & Gochenour, 1961). Studies into the potential sources of infection for cats and dogs are essential in expanding our understanding of the risk factors involved in the transmission of *C. burnetii* from these animals to humans (resulting in Q fever), as well as the aetiopathogenesis of the disease (coxiellosis) in companion animals.

The potential impact to companion animals consuming *C. burnetii*-infected native species and livestock is unknown. Preliminary results of a survey of Australian cat breeders regarding Q fever knowledge and attitudes and general husbandry practices (Shapiro, Norris, Bosward, & Heller, 2017) indicated that 89% of survey participants feed raw meat as a regular constituent of the diet of cattery-confined breeding cats. Of those cat breeders feeding raw meat, nearly half-fed raw kangaroo (*Macropus* spp.) meat. In comparison, feeding practices of the general pet cat-owning community in Australia has indicated that they fed raw meat at a much lower rate (16%) than that of Australian cat breeders (Toribio et al., 2009). Raw food feeding appears more common in Australia than elsewhere in the world (Laflamme et al., 2008), varying from 1.6% to 9% with a maximum of 25% of Australian pet cats fed raw diets (Laflamme et al., 2008;Robertson, 1999;Toribio et al., 2009). This disparity in feeding practices warrants further study regarding the role of raw meat in the transmission of *C. burnetii* to cats. In dogs, the highest cohort of seropositive animals was within free-roaming dogs from Aboriginal communities; again the consumption of raw meat including native species such as kangaroo is possible amongst this group of dogs which often scavenge and hunt for food in environments where kangaroos comprise a significant proportion of the wildlife (Shapiro et al., 2016). The higher percentage of animals observed to be seropositive in dogs from Aboriginal communities and in breeding cats, implies that there may be a likelihood of these subpopulations being exposed to raw meat diets from native species. This led to an extension of the hypothesis that native wildlife species, such as marsupials found exclusively in Australia may be an important source of *C. burnetii* exposure for some groups of companion animals.

Impacts

- *Coxiella burnetii* was isolated from raw kangaroo meat intended for companion animal consumption.
- Isolated genotypes were identified in previous human clinical Q fever cases.
- Findings warrant further investigation into novel sources of *C. burnetii* infection.

Detection of *C. burnetii* DNA has been made possible via PCR assays with many diagnostic laboratories preferentially using a multi-copy gene target, the transposase gene insertion sequence element IS*1111* (Denison, Massung, & Thompson, 2007). Other single-copy targets used include the following: superoxide dismutase gene (Stein & Raoult, 1992), isocitrate dehydrogenase gene (Klee, Ellerbrok, Tyczka, Franz, & Appel, 2006), the outer membrane protein, *com1* gene (Turra, Chang, Whybrow, Higgins, & Qiao, 2005) and the *C. burnetii* heat shock promoter operon, *htpAB*; so called because it encodes the two heat shock proteins: htpA and htpB (GenBank Accession M20482)(Vincent, 2014). Multiple- and single-copy targets are often used concurrently, utilizing their combined sensitivity and quantitative properties respectively.

Research investigating the presence of *C. burnetii* in raw meat for human or pet consumption is minimal. One report from India found no evidence (using a PCR IS*1111* assay) of *C. burnetii* in goat and chicken meat in a study assessing the risk of *C. burnetii* transmission to humans from animal origin food sources (Malik et al., 2013). Australia has highly accessible annual Q fever notification rates (NNDSS-Australian Government, 2015), while in India Q fever is not notifiable, disease is reported infrequently and seroprevalence studies are not current (Joshi, Padbidri, Rodrigues, & Gupta, 1979;Randhawa, Dhillon, & Jolley, 1973). Due to the technical difficulties, expenses and high risks associated with the culturing requirements of *C. burnetii*, the current molecular study focused on determining whether *C. burnetii* DNA could be detected in raw meat of mainly kangaroo origin produced only for pet consumption. The null hypothesis of this study was that *C. burnetii* is not present in raw meat produced for pet consumption. The aims of this study were to (a) develop molecular methods for the detection of *C. burnetii* from

raw meat, and (b) determine whether *C. burnetii* DNA could be detected in raw meat intended for pet consumption.

2 | MATERIALS AND METHODS

2.1 | Sample population

2.1.1 | Sample size determination

Sample size determination was based on prevalence values reported in the Australian *C. burnetii* survey conducted in cattle, sheep and kangaroos, where *C. burnetii* DNA was found in 10.8% of ruminant and 12.2% of kangaroo samples (Banazis, Bestall, Reid, & Fenwick, 2010). The estimation of *C. burnetii* DNA prevalence used in the current study for kangaroo was 12%, margin of error 10% and confidence interval 95%. Using the formula below for estimating a proportion of samples required in a population, the minimum sample size was calculated to be $n = 41$ for kangaroo samples.

$$n = z^2 \frac{p(1-p)}{e^2}$$

Where n = sample size, $Z^2 = (1.96)^2$ for 95% confidence, p = prevalence estimate and e^2 = margin of error (Thrusfield, 2013).

2.1.2 | Sample sources

An opportunistic sampling strategy was used, whereby 58 unique packages of meat for pet consumption were purchased from retail outlets in close proximity to the two veterinary campuses of the University of Sydney (Camperdown and Camden) in NSW, Australia which are 65 km apart. Samples were purchased over a three-month period and sourced from seven independent suppliers: two were the largest local chain supermarkets ($n = 15$), four were large chain pet stores ($n = 42$) and one an online retailer ($n = 1$). Each sample purchased had a unique batch number, barcode, and expiry date. Samples chosen were of two varieties: 'minced meat' which primarily consisted of skeletal muscle tissue ($n = 25$) or samples that were primarily composed of offal tissues ($n = 33$) which included any of the following tissues: heart, liver, kidney, stomach, intestines and bone. Thirteen different pet meat brands were selected in total, but due to store availabilities these were not evenly distributed across the sample population (one sample was obtained from three brands, two samples were obtained from two brands, three samples were obtained from four brands, five samples were obtained from one brand, six samples were obtained from two brands and 25 samples were obtained from one brand). Fresh and frozen vacuum sealed packages sold exclusively for dog and cat consumption were preferentially selected if they contained kangaroo as the primary ingredient. Selection of a single brand 25 times was based on packaging labelling and frequency of availability in the stores, with specific kangaroo tissues listed in the first four to five ingredients. Samples were classified as containing: only kangaroo ($n = 47$) or kangaroo mixed with beef, lamb and/or chicken ($n = 7$). A small selection of samples containing no kangaroo (beef, lamb and/or chicken only; $n = 4$) were also obtained for comparison. While this sampling strategy is not optimal, it was intended as a preliminary investigation to determine if *C. burnetii* DNA could be amplified from these commercially available products.

2.2 | Molecular detection

2.2.1 | DNA extraction from pet meat samples

DNA was extracted from the meat samples using the QIAamp® DNA Mini Kit (QIAGEN) according to the manufacturer's instructions utilizing the tissue protocol with adjustments as noted below. Meat packages ($n = 58$) were independently purchased and weighed between 100 g to 3 kg. Tissue sub-samples weighed between 50 and 120 mg, with three to four aliquots excised from each meat package at random. DNA extraction and quantitative PCR was performed on a total of 195 aliquots. Meat samples were manually excised using individual sterile disposable surgical blades, (No.24; Swann-Morton® Ltd) and sterile Petri dishes (Sarstedt). Meat aliquots were then placed in sterile 1.5 ml microcentrifuge tubes (Eppendorf South Pacific) to which 180 µl of Buffer ATL (Qiagen) preheated to 56°C was added, followed by 20 µl of proteinase K (>600 mAU/ml) (Qiagen). The samples were mixed by pulse-vortexing for 15 s and incubated overnight (16–20 hr) at 56°C on an orbital shaking incubator (Ratek) set at 200 RPM, to optimize tissue lysis. Further adjustments in extraction method were the removal of supernatant only following the addition of Buffer AL and incubation at 70°C for 10 min, to prevent clogging of the column membrane with cellular debris. The amount of elution buffer (Buffer AE) added to the column was adjusted to 100 µl to concentrate the DNA. DNA yield was quantified using the Nanodrop 1,000 (Thermo Fisher Scientific) with yields ranging from less than 1 to greater than 900 ng/µl of DNA. Extracted DNA samples were stored at −20°C until required.

2.2.2 | Quantitative PCR detection of *C. burnetii* and host species DNA

Quantitative PCR (qPCR) reactions were performed in three separate PCR assays using primers targeting *C. burnetii*. These included primers targeting the multicopy insertion sequence IS*1111* gene, and two primer sets targeting single copy genes: *htpAB* (heat shock operon) and the *C. burnetii* outer membrane protein-coding gene, *com1* (Table 1). An additional assay targeting the Eastern Grey kangaroo (*Macropus giganteus*) mitochondrial DNA (mtDNA) gene, cytochrome b (*cyt b*), was also used as an endogenous control to confirm the presence and integrity of DNA. The assays targeting IS*1111* and Eastern Grey *Cyt b* were performed at the University of Sydney using a Bio-Rad-CFX

TABLE 1 Sequence, product lengths and concentrations of *Coxiella burnetii* and endogenous control gene primers

Primer	Primer sequences (5'–3')	Product length (bp)	Final concentration (nM)	Reference
IS*1111**	CGCAGCACGTCAAACCG	146	300	Adapted from (de Bruin et al., 2011)
Forward primer	TATCTTTAACAGCGCTTGAACGTC		300	
Reverse primer				
Probe	FAM[a]-ATGTCAAAAGTAACAAGAATGATCGTAAC-BHQ1[b]		200	
*htpAB***	GTGGCTTCGCGTACATCAGA	114	400	Designed in house by Sullivan Nicolaides Pathology (Brisbane Queensland) using accession number: M20482 (Vincent, 2014)
Forward primer	CATGGGGTTCATTCCAGCA		400	
Reverse primer				
Probe	FAM[a]-AGCCAGTACGGTCGCTGTTGTGGT-BHQ1[b]		200	
*com1****	AAAACCTCCGCGTTGTCTTCA	76	400	Adapted from (Lockhart, Graves, Banazis, Fenwick, & Stenos, 2011)
Forward primer	GCTAATGATACTTTGGCAGCGTATTG		400	
Reverse primer				
Probe	FAM[a]-AGAACTGCCCATTTTTGGCGGCCA-BHQ2[b]		200	
Eastern grey kangaroo cyt b[†]	CAGACAACTTCTCTCCTGCCAA	174	300	Designed in house using accession number: U87137.1
Forward primer	TGGATGTATGGAGGAGTGGGAT		300	
Reverse primer				
Probe	CFO560[c]-TGATACTTCCTATTTGCCTACGCCATCC-BHQ1 [b]		150	

Note: All primers were sourced from Biosearch Technologies™, CA, USA.

[a]6-Carboxyfluorescein.

[b]Black Hole Quencher-1.

[c]CAL Fluor Orange 560 Amidite.

*Insertion sequence 1111 (IS*1111*).

**Heat shock operon (*htpAB*).

***Outer membrane protein (*com1*).

[†]Mitochondrial cytochrome b gene (*cyt b*).

Real-Time PCR Thermocycler (Bio-Rad Laboratories Pty Ltd). Each reaction contained 1X SensiFAST No-Rox (Bioline), primers and probe (concentrations listed in Table 1), 2 µl of sample DNA (samples varied in concentration from <1 to >100 ng/µl) and nuclease-free water in a total volume of 10 µl. The cycling parameters were as follows: initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 40 s. Each PCR run included two no template controls (NTC) with DNA-free water used in place of sample DNA. DNA extracted from the Q fever vaccine Q-VAX® (Seqirus, Australia) at a dilution of 1/10 and *C. burnetii* DNA of known copy number (Amplirun® Vircell) at 1,100, 550 and 27.5 copies of the *C. burnetii* genome per reaction were used as positive controls in each assay. Samples were considered positive for IS*1111* if they contained more than 27.5 copies of *C. burnetii* genome which corresponded to a quantification threshold (Cq) less than 34. The PCR assays targeting the *htpAB* and *com1* genes were performed by our collaborators at the Australian Rickettsial Reference Laboratory (ARRL). The *htpAB* and *com1* reactions both contained 1X Platinum Quantitative PCR Supermix-UDG (Invitrogen), primers and probe (concentrations listed in Table 1) and 5 µl of template DNA in a final reaction volume of 25 µl. Quantitative PCR was performed on a RotorGene 3,000 (Qiagen) using the following cycling parameters: 50°C for 3 min; 95°C for 5 min; 40 cycles of 95°C for 20s for denaturation and 60°C for 40s for annealing and extension. Positive (*C. burnetii* genomic DNA) and negative controls (water in place of DNA) were included in each assay. Packets were determined to be positive for *C. burnetii* DNA if at least two aliquots of each packet were positive for at least one *C. burnetii* gene target.

2.2.3 | DNA sequencing and analyses

The IS*1111* PCR product of a subset of positive samples were sent to the Australian Genome Research Facility Ltd (AGRF) whereby Sanger sequencing was conducted with the forward and reverse primer using Applied Biosystems 3,730 and 3730xl capillary sequencers. A GenBank nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to determine sequence homology.

2.2.4 | Multi-locus variable number of tandem repeat analysis

Multi-locus variable number of tandem repeat analysis (MLVA genotyping) was carried out at the ARRL on PCR positive samples, using DNA from *C. burnetii* Nine Mile as a control. Amplification of three MLVA loci (ms24, ms28 and ms33) was attempted using published primers (Arricau-Bouvery et al., 2006;Tilburg et al., 2012). These three loci were shown previously to be the most suitable for discriminating between Australian isolates of *C. burnetii* (Vincent, Stenos, Latham,

Fenwick, & Graves, 2016). The 5' end of each primer was labelled with a fluorescent 6-FAM dye and successfully amplified products were sent to the AGRF for accurate sizing by capillary electrophoresis. A negative control was used in all genotyping reactions (water in place of DNA) and no products were amplified in the negative control reactions. Data analysis was performed using the PeakScanner v1.0 software (Applied Biosystems). The number of repeats present was determined from the product size based on comparison with the *C. burnetii* Nine Mile strain, the repeat profile of which is known to be 27–6–9 for the three loci ms24, ms28 and ms33 respectively (http://mlva.u-psud.fr/MLVAnet/spip.php?rubrique50).

3 | RESULTS

3.1 | Quantitative PCR detection of *C. burnetii* and mitochondrial cytochrome b gene (endogenous control) DNA

The endogenous control *cyt b* PCR confirmed the presence of host DNA of suitable quality and the success of the DNA extraction process. The presence of *C. burnetii* DNA was demonstrated in 25/58 (43%) meat packages, with between two to four aliquots per package identified as exhibiting amplification of IS*1111*, *htpAB* and/or *com1* DNA. Of the 47 samples listed on the packaging as containing only kangaroo, 23 (49%) exhibited *C. burnetii* PCR product amplification. Two (29%) of seven packets containing kangaroo plus beef/lamb/chicken exhibited PCR product amplification for *C. burnetii* DNA, and none of the non-kangaroo samples (beef/lamb/chicken) exhibited amplified product by PCR. Samples (n = 25) exhibiting amplified *C. burnetii* PCR product based on tissue type were comprised of 4/25 (16%) skeletal muscle and 21/33 (64%) offal tissues. Detailed results of all meat samples, species of origin, tissue type, PCR findings and number of *C. burnetii* amplified PCR products in aliquot/s per sample are presented in Table 2.

3.2 | DNA sequencing and analyses

All products exhibited 100% homology to the top matching *C. burnetii* sequence (accession number: KT391017) following BLAST analysis of the sequenced product.

TABLE 2 Quantitative PCR-positive *Coxiella burnetii* raw meat samples indicating meat species of origin, tissue type and qPCR gene result for insertion sequence 1111 (IS*1111*), heat shock operon (*htpAB*) and *C. burnetii* outer membrane protein (*Com1*)

Sample No.	Meat species	Tissue type	IS*1111*	*htpAB*	*Com1*
1	Kangaroo	Offal	+	+	+
2	Kangaroo	Offal	+	+	+
3	Kangaroo	Offal	+	+	+
4	Kangaroo	Offal	+	+	+
5	Kangaroo	Offal	+	+	+
6	Kangaroo and beef	Skeletal muscle	+	−	−
7	Kangaroo, beef and lamb	Skeletal muscle	+	+	−
8	Kangaroo	Offal	+	−	−
9	Kangaroo	Offal	+	−	−
10	Kangaroo	Offal	+	−	−
11	Kangaroo	Skeletal muscle	+	+	−
12	Kangaroo	Offal	+	+	−
13	Kangaroo	Offal	+	+	+
14	Kangaroo	Skeletal muscle	+	+	+
15	Kangaroo	Offal	+	+	+
16	Kangaroo	Offal	+	+	+
17	Kangaroo	Offal	+	+	+
18	Kangaroo	Offal	+	+	+
19	Kangaroo	Offal	+	+	+
20	Kangaroo	Offal	+	+	+
21	Kangaroo	Offal	+	+	+
22	Kangaroo	Offal	+	+	+
23	Kangaroo	Offal	+	+	+
24	Kangaroo	Offal	+	+	+
25	Kangaroo	Offal	+	+	−

Note: (+ = positive; − = negative).

3.3 | MLVA Genotyping

Full MLVA profiles (in which all three of the loci were amplified and the number of repeat units determined) were obtained from six samples, with partial profiles obtained from a further ten. The three different genotypes identified (CbAU05, CbAU10 and CbAU11) differed at only one of the three loci and have been observed previously in *C. burnetii* isolates from Australian human patients with clinical Q fever residing in three Australian states: NSW, Queensland (QLD) and Victoria (VIC). All three genotypes are unique to Australia (Vincent et al., 2016). The results were added to the Coxiella Australia MLVA database (http://mlva.u-psud.fr/mlvav4/genotyping/).

4 | DISCUSSION

This pilot study demonstrated the presence of *C. burnetii* DNA in raw meat packages for pet consumption using qPCR. *Coxiella burnetii* DNA was only detected in kangaroo-containing meat packages and was not detected in the small number (n = 4) of packages containing other raw meat sources. PCR results were confirmed by DNA sequencing (100% sequence homology to the gene target IS*1111*, accession number: KT391017), and results were further assessed using MLVA genotyping which identified three different genotypes of *C. burnetii* in the meat samples. These three genotypes have been observed in *C. burnetii* isolates from human patients with clinical Q fever located in three Australian states: NSW, QLD and VIC (Vincent et al., 2016). The two aims of this study were successfully achieved, which were to: (a) develop molecular methods for the detection of *C. burnetii* DNA from raw meat and (b) determine whether *C. burnetii* DNA could be detected in raw meat for pet consumption. An exploration of the potential for *C. burnetii* transmission to humans and animals from raw meat intended for pet consumption was beyond the scope of this study and requires further investigation.

The detection and significance of the presence of *C. burnetii* DNA in meat samples that were primarily of kangaroo origin in this study is supported by the findings of others investigating the association between *C. burnetii* and macropods. Previous exposure of macropods to this pathogen has been detected serologically or by molecular methods aimed at amplifying *C. burnetii* DNA in faeces of kangaroos. Macropods, with emphasis on kangaroos, represent the marsupial family most commonly tested serologically for *C. burnetii*, with exposure to this bacterium a longstanding observation. *Coxiella burnetii* seroprevalence in kangaroos ranges from 23% to 33.5% (Banazis et al., 2010;Pope, Scott, & Dwyer, 1960;Potter, Banazis, Yang, Reid, & Fenwick, 2011), and these findings have led to postulations of a kangaroo-tick cycle (Pope et al., 1960). It has been further suggested that kangaroos potentially present a greater zoonotic threat for acquisition of Q fever than cattle and sheep in Western Australia (WA) (Banazis et al., 2010) and that kangaroos may transmit *C. burnetii* to both humans and domestic livestock (Flint et al., 2016;Potter et al., 2011). Two recent serological studies examining *C. burnetii* seroprevalence in Australian native wildlife highlighted the potential public health implications associated with wildlife species in the transmission of Q fever to humans in residential areas. The first study investigated macropods as atypical reservoirs of *C. burnetii* infection in QLD and WA and found an overall seroprevalence of 20.8% (Cooper, Barnes, Potter, Ketheesan, & Govan, 2012a). The second multi-species seroprevalence study conducted in response to a recent increase in Q fever cases in the absence of contact with traditional reservoir species in QLD, examined *C. burnetii* seroprevalence in six native marsupials and introduced wildlife species and reported seroprevalence in these species ranging from 10.7% to 48.3% (Cooper, Goullet, Mitchell, Ketheesan, & Govan, 2012b). These two studies utilized the same in-house ELISA methodology; however, positive and negative controls for wildlife species were not included. The potential for native marsupials to act as reservoirs of *C. burnetii* transmission for humans and animals was again corroborated through a *C. burnetii* molecular study screening ticks and wildlife in northern QLD (Cooper, Stephens, Ketheesan, & Govan, 2013). Our results add a further dimension to the previous kangaroo and *C. burnetii* observations, with the consequences for human and companion animal health from the handling and ingestion of kangaroo meat produced as pet food still to be determined.

Australian manufacturers of pet meat, defined as 'meat in a raw state that is intended as food for pets' (CSIRO, 2009), are bound by the minimum requirements in the Australian Standard for Manufacturing and Marketing of Pet Food (Australian-Standards, 2017) which focuses on the safety of multi-ingredient, manufactured food for feeding to pet cats and dogs and on ensuring products are accurately labelled and free from misleading statements. An Australian parliamentary enquiry into the regulatory approaches to ensure the safety of pet food (Parliament-of-Australia, 2018) has recommended amongst other things that amendments to the Food Standards Australia and New Zealand (FSANZ) include pet food standards and labelling requirements. This recommendation acknowledges the importance of safety and regulation of pet food for both the benefit of pets and the human households in which the animals reside and the food is stored. No reasonable expectation currently exists for testing any meat (for humans or pets) for the presence of *C. burnetii* DNA.

The decision not to disclose brand names of samples tested in this study was made for a number of reasons. Raw pet meat products cannot be viewed as products with stable and consistent ingredients since the constituents of these products are highly influenced by abattoir by-products and the availability of tissues condemned as not suitable for human consumption; the occurrence of which could be highly variable from batch to batch and geographic location. Thus, the PCR results in this study reflect a snapshot in time and are potentially not consistently representative of the particular brand's product over time. Animal host species contained within packages often exceeded 3–4 different species, and it is envisaged that animal supply and species sources might be expected to change over time and thereby influence the packages' constituents. The alignment of animal sources, abattoirs and meat packaging facilities with specific brands is unknown. In addition, the point at which *C. burnetii*

contamination occurred (in situ while the animal was alive or during any of the carcass processing steps) was also not able to be determined within the scope of the current research project. Similarly, viability and thereby infectivity of bacteria in samples that exhibited positive PCR amplification was not assessed and therefore, the significance of these findings for companion animal and human health are currently unclear. As no assessment of the link between amplification of DNA and any effect on health was undertaken, identification of the brands is of little consequence to the objectives of the study. Furthermore, the number of samples selected to represent each brand was not evenly distributed across the sample population. Further larger-scale molecular studies using stratified sampling framework and extending the investigation to bovine, ovine and caprine as well as kangaroo host tissue samples is required. Sampling would also require expansion of the geographic area for purchasing of samples and investigation of the geographic sources of meat comprising these packages. Until studies utilizing random sampling with even representation of market brands are conducted, release of brand names would be unhelpful.

The development of molecular methods for DNA extraction from raw meat samples followed by the detection of *C. burnetii* DNA using qPCR was successfully achieved in this study. The advent of PCR-based assays allowed for the rapid detection of *C. burnetii* in clinical and research samples (Klee, Tyczka, et al., 2006). Quantitative PCR assays for *C. burnetii* have sensitivities that vary according to the target sequences. The current study targeted three genes: IS*1111* (Hoover, Vodkin, & Williams, 1992), *htpAB* (heat shock operon) and *com1* (Tables 1 and 2). The IS*1111* target has been found to be present in 20 copies in the *C. burnetii* Nine Mile RSA493 strain (Seshadri et al., 2003), and 55 copies are present the Australian isolate AuQ01 (Walter, Vincent, Stenos, Graves, & Frangoulidis, 2014). The sensitivity of the real-time IS*1111* PCR is high due to its multi-copy number within the bacterial genome; however, it is unable to be used for quantification purposes (Klee, Tyczka, et al., 2006), whereas *htpAB* and *com1* PCRs can be used for quantifying organism numbers. In the present study, the detection of *C. burnetii* DNA using the IS*1111* PCR in more samples compared with its presence when using the *htpAB* and *com1* PCR assays clearly indicates the sensitivity difference between single- and multiple-copy gene targets in general as well as variation in assay sensitivity due to different analytical properties of the assays under comparison. The latter includes differences in the following: target regions with different G/C contents; PCR machinery varying in ramp rates and the sensitivity of detectors; reagents, amounts of primers, Taq and DNA used; and cycling parameters.

The sequencing of the PCR amplicons, with resulting 100% homology to the IS*1111* target sequences, as well as genotyping of *C. burnetii* isolates from positive samples (to demonstrate alignment with strains known to have caused disease in humans), alleviates the immediate potential of false-positive amplification from non-target species in the context of the samples analysed. Recently, *Coxiella*-like bacteria have been identified in ticks (Duron, 2015) and horses (Seo et al., 2016). These species are closely related to *C. burnetii* but genetically and, to current knowledge, behaviourally distinct.

This study detected the presence of *C. burnetii* DNA (IS*1111*, *com1* and *htpAB*) in 43% of primarily kangaroo origin meat packages intended for pet consumption. *Coxiella burnetii* DNA was detected five times more frequently in offal than skeletal muscle samples. This finding is not surprising, as *C. burnetii* experimental infections of guinea pigs (Parker & Steinhaus, 1943) and sheep (Abinanti, Welsh, Lennette, & Brunetti, 1953), revealed persistent bacteria localizing in the liver, spleen, kidney, seminal vesicles, lymph nodes, bone marrow and intestines (Harris, Storm, Lloyd, Arens, & Marmion, 2000). *Coxiella burnetii* presence or persistence in skeletal muscle tissues of naturally or experimentally infected animals has not been recorded; however, *C. burnetii* was detected in multiple adipose tissue sites in a mouse model of Q fever (Bechah et al., 2014). The overall findings need to be seen in perspective of the limitations in the current study. The small sample size (n = 58) was appropriate for a pilot study and met the aims of the investigation; however, the low sample number and disproportionate brand distribution dictates that further investigations are necessary to explore the potential public and animal health implications of these findings. The source of *C. burnetii* DNA detected may represent intracellular infection within the tissues or surface contamination of the meat occurring at any stage along the processing pathways. However, given that non-kangaroo meat did not contain *C. burnetii* DNA but would have been subject to the same potential for surface contamination, this decreases the likelihood of surface contamination. Further investigations exploring the source of *C. burnetii* within raw meat are warranted utilizing fresh tissue samples of host animal species. The presence of *C. burnetii* DNA via PCR gives no indication of the viability of bacteria present. To fully understand the risk pet meat represents for the community at large, further studies including viability testing will be required. Future assessment of *C. burnetii* viability methodologies would require physical containment level 3 facilities, laboratory-specific pathogen-free animal models, a reduction in the overall microbial contamination and complexity of pet meat samples to enable growth in cell or egg-based culture systems, and an increase in *C. burnetii* DNA yields from these samples.

The role of ingestion as a route of transmission of *C. burnetii* and its potential to result in Q fever disease is poorly understood. Despite this, the international standard of time-temperature conditions for pasteurization of bovine milk is predominantly based upon the requisite heat treatment against *C. burnetii* (Enright, Sadler, & Thomas, 1957a, 1957b). Almost all outbreaks of Q fever in the past 50 years in Australia, Europe and the United States were attributed to inhalation and in a few cases arthropod bites (Cerf & Condron, 2006). The ingestion of *C. burnetii* causing clinical Q fever disease in humans is reported infrequently, and the evidence is circumstantial (Durand & Limouzin, 1983) with no published reports associated with the consumption of meat and only a single outbreak of Q fever attributed to the consumption of *C. burnetii*-infected raw milk reported in the literature (Signs, Stobierski, & Gandhi, 2012). However an association between ingestion of unpasteurized *C. burnetii*-infected milk and seroconversion in humans is known (Benson, Brock, & Mather, 1963). The infectivity potential of the various

forms of *C. burnetii* potentially present in contaminated raw meat products is unknown. In one study dating back more than 60 years, cats (*n* = 12) were experimentally infected with *C. burnetii* via different routes. None of the cats were pregnant during the study, *C. burnetii* was detected in blood and urine by intraperitoneal inoculation into guinea pigs, while complement fixation testing (CFT) was used as the serological assay and the clinical parameters monitored included body temperature, appetite and mental alertness (Gillespie & Baker, 1952). Four cats infected orally remained asymptomatic, *C. burnetii* was detected in the blood and urine of three and two cats, respectively, while only one cat developed a low complement-fixing titre (Gillespie & Baker, 1952). It is possible that, as was demonstrated with ingestion of raw infected milk in humans, ingestion of both viable and inactivated *C. burnetii* organisms from pet meat may result in seroconversion in cats and dogs consuming these products. It also follows that it is feasible that inhalation of aerosolized, viable bacteria during consumption of the meat by pets may result in *C. burnetii* infection in exposed companion animals. By the same token, inadvertent ingestion or inhalation of organisms as a result of poor food hygiene practices during handling of raw meat may result in seroconversion possibly in the absence of clinical disease. This study reinforces fundamental household hygiene practices for humans handling all raw meat products which incorporate washing hands with soap and water following contact, not mixing of raw and cooked meat chopping boards and storage of raw meat at the bottom of the refrigerator. The potential for seroconversion to be protective against Q fever disease is another area requiring further study but beyond the scope of this project.

5 | CONCLUSION

The detection of *C. burnetii* DNA (IS*1111*, *htpAB* and *com1)* in 43% of the raw meat packages screened (all samples of kangaroo origin) using qPCR was confirmed through DNA sequencing and further assessed using MLVA genotyping. The three genotypes found have been observed in *C. burnetii* isolates from human patients with clinical Q fever located in three Australian states, NSW, QLD and VIC. The potential for *C. burnetii* transmission to occur via ingestion of, or aerosolization from, raw meat intended for pet consumption to humans and animals requires further investigation.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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