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Title

A survey of Western Australian sheep, cattle and kangaroos to determine the prevalence of *Coxiella burnetii*

Authors

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Abstract

The objective of this study was to investigate the prevalence of *Coxiella burnetii* in two domestic ruminant species (cattle and sheep) and the western grey kangaroo (*Macropus fuliginosus)* in Western Australia (WA). The IDEXX CHEKiT Q Fever ELISA and CFT were used to test sera from 50 sheep and 329 head of cattle for anti-*C. burnetii* antibodies and 343 kangaroo sera were tested using an indirect ELISA developed specifically for this study. Faecal or urine samples collected from the same animals were tested with two PCR assays to identify active shedding of *C. burnetii* in excreta. Only two of the 379 ruminant sera had detectable levels of anti-*C. burnetii* antibodies according to the ELISA while the CFT did not detect any positive samples. In contrast 115 of the 343 western grey kangaroo serum samples were positive when tested with the antibody-ELISA. The first qPCR assay, targeting the *IS1111*a element, identified 41 of 379 ruminant and 42 of 343 kangaroo DNA samples as positive for *C. burnetii* DNA. The second qPCR, targeting the *JB153-3* gene, identified nine *C. burnetii* DNA-positive ruminant samples and six positive kangaroo samples. Sequence comparisons showed high degrees of identity with *C. burnetii.* Isolation of *C. burnetii* from faeces was also attempted but was not successful. From the results presented here it appears that domestic ruminants may not be the most significant reservoir of *C. burnetii* in WA and that kangaroos may pose a significant threat for zoonotic transfer of this pathogen.

Key words

Coxiella burnetii; cattle; sheep; kangaroo; Q fever; immunosorbent assay; polymerase chain reaction

Introduction

It has been demonstrated that *C. burnetii* has a strong association with domestic ruminants

(Raoult and Marrie, 1995) as well as native Australian marsupials (Derrick, 1939; Pope et al., 1960). Thus, the causative agent of Q fever is recognised as a serious occupational hazard for people who work with or around waste and birth products of livestock or marsupials and may include farmers, veterinarians and zoo and slaughterhouse workers (Garner et al., 1997). However, there have been no published surveys for *C. burnetii* in domestic animals or native marsupials in WA and therefore the zoonotic risk posed by transmission in WA is unknown.

While molecular detection of *C. burnetii* in ruminants is well established (Guatteo et al., 2006), prior to this study no tests had been optimised for use as wildlife surveillance tools. Testing of native Australian marsupials for exposure to, or infection with, *C. burnetii* has predominantly been performed using the CFT (Dane and Beech, 1955; Pope et al., 1960), microscopic agglutination (Derrick, 1939) or animal inoculation (Derrick, 1939; Smith, 1940). However, the limitations of these methods (Field et al., 2000; Peter et al., 1987; Sobsey and Leland, 2001) highlight the need for an improved test to assess the role of native Australian marsupials in the lifecycle of *C. burnetii*.

Each year more than 300,000 Western grey (*Macropus fuliginosus*) and Red (*M. rufus*) kangaroos are harvested commercially in WA (Management, 2002). The introduction of European farming methods and fox baiting programs has allowed kangaroo populations to flourish. Consequently, it is not unusual to find both species mixing with domestic stock as they graze on irrigated pastures and drink from man-made water sources (Management, 2002). This cohabitation, in conjunction with high risk animal husbandry practices may be key factors in the transmission of *C. burnetii* (Soliman et al., 1992) and perhaps provide a basis for cycling between wild and domestic animals and, subsequently, humans. This study aimed to provide preliminary immunological and molecular data on the presence of *C. burnetii* in domestic ruminants and Western grey kangaroos in WA. Kangaroo test results were interpreted in light of sex, age, location and month information to reveal

epidemiological patterns. The patterns observed and detection techniques described here may be useful for more substantive surveys of livestock and native marsupials in Australia.

Materials and methods

Sample collection

Paired samples of blood and faeces were collected from 124 cattle held at a feedlot in the South West of Western Australia (WA) which consisted of approximately 80% *Bos indicus* (all steers except for one heifer) and 20% Angus steers from the South West of WA. All animals were between 18 and 24 months of age although the exact age of each animal was not recorded. Paired blood and urine samples were also collected from 157 mixed age *Bos taurus* heifers from another farm in the South West of WA that had been experiencing an outbreak of leptospiral abortions. A further 48 paired faecal and blood samples were collected from mixed age *Bos taurus* cattle and 50 merino ewes of approximately 5 years of age housed on the Murdoch University farm.

Western grey kangaroo (*M. fuliginosus*) blood and faecal samples were taken from six locations in the South West and central region of WA, hereafter referred to as 'Capel', 'Manjimup', 'Badgingarra', 'Preston Beach', 'Eneabba' and 'Whiteman Park'. The approximate age of each animal sampled was recorded as either '1' (pouch young; joeys too young to leave the pouch), '2' (juvenile; young at foot who could return to the pouch at will), '3' (sub-adult; kangaroos who had not yet reached mature body weight) or, '4' (adult; fully grown). Information regarding the age classification of Western grey kangaroos has been discussed elsewhere (Dawson 2002, Norbury et al. 1988).

Immunological testing of ruminant sera

Serum from all ruminants and a preliminary selection of kangaroos was tested using the CFT

by the Department of Agriculture and Food Western Australia (DAFWA) according to their in-house procedures which used a seropositivity cut-off of 1/8. All samples that reacted strongly at a 1 in 8 dilution were heat-inactivated at 58°C for 30 minutes and subsequently re-tested.

Samples were also tested using the CHEKiT Q Fever ELISA kit (IDEXX Laboratories Inc., Switzerland) according to the manufacturers' instructions.

Testing serum from kangaroos using an ELISA

Nunc Maxisorp flat bottom microtitre plates (Nalge NUNC International, New York) were coated overnight at 4°C with 100 µl of phase I (1 in 50) and phase II (1 in 50) *C. burnetii* antigens (Institut Virion/Serion GmbH, Germany) diluted in carbonate/bicarbonate buffer (pH 9.6). Diluted antigen was discarded and the plates were inverted and dried at 37°C for 30 minutes. Wells were blocked for 60 minutes with 150 µl of Tris EDTA/0.05% Tween 20 (TEN-T, pH 8) plus 3 % w/v skim milk powder (SMP) after which the blocking solution was discarded. All serum samples were diluted 1 in 400 in TEN-T/1% SMP and allowed to stand at room temperature for 30 minutes. One hundred microliters of diluted control and test sera were added to four wells each and two wells each respectively before incubating at 37°C for 60 minutes in a humid chamber and then washed three times with TEN-T. One hundred microliters of rabbit anti-kangaroo IgG heavy and light chains (Bethyl Laboratories Inc., Montgomery, Texas, USA) diluted 1 in 500 in TEN-T/1% SMP was added before being incubated at 37°C for 60 minutes. Plates were washed, 100 µl of donkey anti-rabbit-HRP (Bethyl, Montgomery, Texas, USA) diluted 1 in 4,000 in TEN-T/1% SMP was added and then the plates were incubated at 37°C for 60 minutes. The microtitre plates were washed a final time and 100 µl of TMB substrate (Pierce, Quantum Scientific, Murrarie, Queensland, Australia) was added, followed by incubation at room temperature for 15 minutes before

addition of 100 µl of 1M H_3PO_4 to stop colour development. The plates were read using a BioRad Microplate Reader 6800 (BioRad, Regents Park, New South Wales, Australia) and the final optical density (OD) of each well was determined by subtracting the OD at a reference wavelength (OD_{570nm}) from the test wavelength (OD_{450nm}) to reduce background interference. The ELISA described above was used to identify proxy 'positive' and 'negative' sera which were used for all unknown sample testing. Three high reacting serum samples were pooled and used as 'positive' controls and three low reacting serum samples were pooled and used as 'negative' controls. The OD values of test samples were converted to a percentage of the mean positive control OD's ('PP') from the same plate and all samples with values equal to or greater than 40% were classified as positive.

Isolation of Coxiella burnetii DNA from faeces and urine

Whole genomic DNA was extracted from faecal samples (marsupials and ruminants) and urine (some cattle). Purification of DNA from faecal samples was done using a modified version of the MoBio PowerSoil™ DNA isolation kit (MO BIO, Carlsbad, California, USA). Briefly, 0.2 g of faeces was added to the supplied bead beating tubes, Solution 'C1' was added and all tubes were vortexed at maximum speed for 30 seconds. Samples were then boiled for five minutes, vortexed again for one minute and then boiled for a further five minutes. The standard DNA isolation procedure was then followed from step five onward. The standard DNA isolation procedure was performed on all kangaroo and ruminant faecal samples on two separate occasions to verify qPCR results. Bovine urine DNA samples were kindly donated by Dr. Peter Wai-in for use in this study. Five millilitres of urine was centrifuged for 30 minutes at 3,000 × *g* and all but 0.5 ml of the supernatant was discarded. The pellet was resuspended in the retained supernatant and centrifuged for 10 minutes at 7,500 × *g* before the supernatant was removed. Whole genomic DNA was then purified

according to one of the two following methods. For clear urine samples the pellet was resuspended in 50 µl of sterile water, incubated at room temperature for two minutes then incubated at 95°C for 10 minutes. This method was obtained from the Animal Research Institute, Queensland Department of Primary Industry and Fisheries, Moorooka Brisbane, Queensland. For urine samples that were contaminated by faecal matter a Qiagen Tissue Minikit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions.

Quantitative PCR detection of DNA isolated from faeces and urine

Coxiella burnetii DNA was amplified using two separate qPCR assays; one targeting the *IS1111a* element (GenBank accession number M80806) and one targeting the *JB153-3* sequence (GenBank accession number AF387640). The primer and probe sequences and final reaction concentration of the oligonucleotides are shown in Table 1. All reaction mixtures contained primers and probe at the concentrations indicated in Table 1, 12.5 µl UDG SuperMix (Invitrogen, Mount Waverley, Victoria, Australia), 3 mM (*JB153-3* assay) or 4.5 mM (*IS1111a* assay) magnesium chloride and 5 µl of template in a total volume of 25 µl. All samples were tested in duplicate on a Rotorgene 3000 (Corbett Life science, Mortlake, New South Wales, Australia) according to the following cycling parameters: One hold at 50°C for two minutes, a second hold at 95°C for two minutes followed by 40 cycles of 95°C for 20 seconds and 60°C (*JB153-3* assay) or 64°C (*IS1111a* assay) for 40 seconds. Two 'no template' controls (NTC) were included with every run. Each PCR run included a six-point standard curve comprising DNA extracted from Q-Vax™ vaccine (CSL, Parkville, Australia) according to the method of Klee and colleagues (2006). The concentration of DNA from the Q-Vax™ vaccine was determined using a Nanodrop spectrophotometer and the number of *C. burnetii* genomes per microliter of cell suspension was calculated according to the molecular weight of the *C. burnetii* genome (Coleman et al., 2004). The Rotorgene 3000 software was

used to automatically select optimal cycle threshold cut-offs based upon the slope of the standard curve and the R^2 value. The DNA concentrations of the standards were then used by the software to provide estimates of the DNA quantity of unknown samples. Results were expressed as genomes/µl of DNA template.

These conditions were used to evaluate the analytical sensitivity and reproducibility of the *IS1111a* qPCR in buffer and in faecal samples with standard

Conventional PCR and sequencing

To provide template of sufficient length for sequence comparison purposes *Coxiella burnetii* DNA was amplified using a conventional PCR with the OMP1/OMP2 primer set as described previously (Zhang et al., 1998) with the exception that the annealing step was done at 54^{o}C. Cycling was performed on an Applied Biosystems GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, California, USA).

The purified PCR products were sequenced using the Big Dye version 3.1 terminator kit (Applied Biosystems, Foster City, CA, USA) using the dideoxynucleotide chain termination method (Sanger et al., 1977). The sequence was determined using an ABI Prism Applied Biosystems 377 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA) at the State Agriculture and Biotechnology Centre (Perth, Western Australia). Chromatogram sequencing files were edited using Chromas Lite version 2.0 (Technelysium P/L, Helensvale, Queensland, Australia). Sequence information obtained was compared to sequence information previously submitted to GenBank using BLAST software available from http://www.ncbi.nlm.nih.gov.

Sequencing was performed on all samples that were positive when tested with the *JB153-3* qPCR and a random selection of samples that were positive when tested with the *IS1111*a qPCR. In all, amplicons from 13 ruminant and 16 kangaroo samples were sequenced.

Assessment of faecal PCR inhibition

Faecal DNA samples that produced no detectable amplification were randomly selected to assess the amount of PCR inhibition caused by factors that were co-purified during DNA isolation. Forty two bovine samples, 20 ovine samples and 38 kangaroo samples were used. Eighteen microliter aliquots were taken from each sample and 2 µl of DNA extracted from Q-Vax vaccine was added. Two microliters of Q-Vax DNA was also added to 18 µl of high-pure water to serve as an uninhibited control. Amplification of 5 µl of template per reaction, in duplicate, was carried out using the *IS1111a* assay as described previously and data were analysed with the Rotorgene 3000 software.

Isolation of Coxiella burnetii from bovine and kangaroo faeces

Isolation of viable *C. burnetii* cells was attempted with four ruminant and six kangaroo samples which had tested positive with the *IS1111a* qPCR assay and had estimated genomes/g of faeces greater than 1,800 for ruminant samples and greater than 1,200 for kangaroo samples. All in vitro culture work was conducted by Michelle Lockhart in the PC-3 biocontainment laboratory [AQIS approved] of the Australian Rickettsial Reference Laboratory (ARRL), in the Hunter Area Pathology Service, John Hunter Hospital, Newcastle, NSW. In the procedure developed by the ARRL 0.5 g of faeces was resuspended in 10 ml of PBS and mixed thoroughly. Solid matter was removed by centrifugation at 100 × *g* for five minutes and the resulting supernatant was centrifuged at 5,000 × *g* for 15 minutes to pellet bacterial cells. Cells were resuspended in 5 ml of PBS and subjected to two further slow speed/high speed centrifugation steps (100 × *g*/5,000 × *g*) before passing the suspension through a 0.45 µm filter. Half of the filtrate was divided into two confluent cultures of vero cells and two microfuge tubes for DNA extraction according to the procedure described by

Klee and company (2006). The remaining filtrate was passed through a 0.22 µm filter and the resulting liquid was again divided equally between two flasks of vero cells and two microfuge tubes for DNA extraction (Klee et al., 2006) and subsequent qPCR using the *IS1111a* assay. Faecal samples had been frozen after collection and subjected to at least three freeze-thaw cycles prior to attempts at isolation.

Statistical analysis of sample test results

All statistical analyses were performed with the SPSS statistical package (version 15.0, SPSS Inc., Chicago, USA) unless indicated otherwise.

For the PCR inhibition results the concentration of genome equivalents estimated to be present in control and test samples were compared using a one-way ANOVA and post-hoc (LSD) analysis to identify individual samples that deviated from the population variance by a significant degree (P=0.05).

Due to the low number of immunologically-positive ruminant samples no statistical analyses were performed using these data. The apparent prevalence and binomial confidence intervals were calculated using results from testing marsupial serum and marsupial, bovine and ovine faeces with ELISA and qPCR respectively.

Where more than two groups were tested simultaneously for having a significant impact on sample positivity a one-way ANOVA with post-hoc analysis (Tukey's) was used. For kangaroo samples one-way ANOVA was used to test the significance of the effect collection location, month and age of the animals had on the *IS1111a* qPCR and ELISA results. All individual factors of each group were included in the analyses. The same test was also used to determine significant differences within the Capel kangaroo results with comparisons made between collection month.

Where only two groups were tested for significance in relation to assay result an independent

sample t-test was used. This test was used to discern differences in the mean genome equivalent copy number estimated using the *IS1111a* qPCR for ovine faecal samples and bovine urine and faecal samples. For kangaroo samples the t-test was used to determine if there was a statistically significant difference between the number of qPCR positive results and ELISA positive results and to determine if sex had a significant impact on *IS1111a* qPCR and ELISA results and to determine significant differences within the Capel kangaroo results with comparisons made between animal sexes.

Results

Immunological testing of ruminant and kangaroo serum

All bovine and ovine serum samples were negative when tested with the CFT. None of the sera reacted strongly at a dilution greater than 1 in 8. No strong reactions were seen in any of the samples that were heat inactivated.

A random selection of 20 serum samples from kangaroos was tested using the CFT to determine if the test was applicable to this species. Strong non-specific reactions, which could not be reduced through heat inactivation, were observed in all samples and this made interpretation of results impossible.

Two of the 329 bovine sera (0.61%, ± 0.84% for 95% CI) and no ovine sera were positive using the CHEKiT Q fever ELISA. Table 2 shows the number of ELISA and qPCR positive samples, according to sample type, and the mean estimated genome equivalents per gram of faeces or millilitre of urine.

A total of 115 of 343 (33.53%, ± 5% for 95% CI) sera from kangaroos were positive when tested using the ELISA developed for this study. Table 3 shows the percent of samples that were ELISA-positive for each collection location. The mean PP for samples collected in Capel was significantly lower than the PP for samples collected in all other locations with the

exception of Badgingarra ($P<0.001$). When tested with the ELISA, samples collected from Manjimup and Badgingarra had PP values that were significantly lower than the PP values of samples collected at Preston Beach ($P<0.05$).

Table 4 shows the percent of samples that were ELISA-positive for each sex and age group of kangaroo. There was no significant difference in the mean PP values of male and female kangaroos and there was no significant association between the calculated PP values and the age group of the kangaroos. No statistically significant association was found between the mean PP values and the sex of the animals sampled in Capel. No statistically significant association was found between the mean PP values and the sex of the animals sampled in Manjimup.

Table 5 shows the percent of samples that were ELISA-positive for each collection month. The mean PP values of samples collected in June were significantly higher than those of samples collected in March, April and May ($P <0.05$). The mean PP values of samples collected in July were significantly higher than those of samples collected in March and April ($P <0.01$). Of the samples collected in Capel, the mean PP values for samples collected in March and May were found to be significantly lower than those for samples collected in July ($P <0.05$). Of the samples collected in Manjimup, the mean PP values for samples collected in April were found to be significantly lower than those of samples collected in all other months ($P <0.001$).

Of the 343 kangaroo serum samples tested by ELISA six came from three mother/pouch young pairs. In one instance both mother and young were negative and in the other two instances the mothers were both immunologically positive but the pouch young were negative.

Quantitative PCR testing of ruminant faeces and urine and kangaroo faeces

All no template controls were negative in all runs of the PCR. Out of the 26 bovine samples that were PCR-positive according to the *IS1111a* assay (7.90% ± 2.92 for 95% CI), 14 were from animals sampled in Pinjarra (urine samples), 11 were from cattle that were sampled in Vasse (faecal samples) and one positive sample was from an animal on the Murdoch University farm (faecal samples). Six of the 50 ovine faecal samples collected were qPCR-positive according to the *IS1111a* test (12.00% ± 8.9 for 95% CI). The results from testing of re-isolated faecal DNA samples with the *IS1111a* qPCR were found to be qualitatively the same as from the primary isolation as described above. There were three primary DNA isolation bovine samples that were positive when tested with the *JB153-3* qPCR. No significant associations were found between ruminant qPCR results and species.

The 42 kangaroo faecal samples that were positive according to the *IS1111a* qPCR had a mean bacterial load of 1,131.58 genome equivalents per gram of faeces (± 457.01 for 95% CI) while the testing the same DNA samples with the *JB153-3* test gave results with median, 1st and 3rd quartiles of 175, 151.25 and 1,115.63 copies/g of faeces respectively. Table 3 shows the number of *IS1111a* qPCR-positive kangaroo samples that were detected in each collection location, Table 4 shows the number of *IS1111a* qPCR-positive samples that were detected for each sex and age group of kangaroo and Table 5 shows the number of *IS1111a* qPCR-positive samples that were detected for each collection month. The results from testing of re-isolated faecal DNA samples with the *IS1111a* qPCR were found to be qualitatively the same as from the primary isolation shown in Tables 3-5. As there were only six primary DNA isolation kangaroo samples that were positive when tested with the *JB153-3* qPCR these results have been excluded from the tables. According to both the *JB153-3* and *IS1111*a qPCR tests there were no significant differences observed for the data when any of the factors described previously were examined. However, restricting the data to results from samples collected in

Capel revealed that the mean result in March was significantly greater than the mean observed for both May and July. No significant difference was found according sex.

Sequencing

Sequencing of the *com1* amplicon was successful in all but one instance with all matches showing greater than 99% identity with *C. burnetii* Dugway strain (GenBank accession number CP000733.1).

Inhibition of PCR

Five of the 42 bovine samples of faeces tested using the qPCR had significantly lower estimates of *C. burnetii* DNA concentration than the mean estimated concentration of all samples ($P < 0.05$). None of the 20 ovine extracts tested using the qPCR had significantly lower estimates of *C. burnetii* DNA concentration than the mean estimated concentration of all samples ($P < 0.05$). Five of the 32 kangaroo faecal extracts had significantly lower estimates of *C. burnetii* DNA concentration than the mean estimated concentration of all samples ($P < 0.05$).

Agreement between immunological tests and qPCR

It was found that the *IS1111a* qPCR detected less positives than ELISA for kangaroo samples ($P < 0.05$).

Isolation of Coxiella burnetii from faeces

Attempts to isolate *C. burnetii* from four ruminant and six kangaroo faecal samples were unsuccessful. Testing of the DNA extracts made during the *C. burnetii* isolation process at ARRL confirmed the presence of *C. burnetii* DNA.

Discussion

CFT is generally considered to be less sensitive than ELISA but was included here to provide additional serological evidence and because it was hoped it could be used for testing kangaroo sera. Results from testing ruminant samples with the CFT and CHEKiT Q fever ELISA showed low to negligible seroprevalence. Seropositivity and actual infection by *C. burnetii* are not always well correlated in ruminants (Berri et al., 2001) so the low number of serologically positive animals observed here may not truly represent the transmission potential present for WA sheep and cattle. It has been proposed that using antigen made with the Nine Mile strain of *C. burnetii* may not be appropriate for use in Australian studies and may lead to an underestimation of the serological prevalence of this pathogen (Rodolakis et al., 2007b). However, a serological survey of abattoir workers in Queensland found that only 1% of individuals had detectable antibodies against *C. burnetii* (McKelvie, 1980) perhaps indicating that transmission from domestic livestock is not as common in Australia as it is in other countries.

More ruminant samples were found to be PCR-positive than were detected using the Idexx CHEKiT ELISA. This observation may be attributed to early stage infections in the animals sampled where the host may not have generated an immune response to *Coxiella* but may be shedding organisms in bodily secretions and faeces (Berri, et al. 2002). Alternatively, *C. burnetii* DNA detected in the faeces could have been ingested by the animal through contaminated feed but may have passed through the digestive system without establishing an infection although this possibility seems unlikely given the bacterial load in the faecal samples. Approximately half of the bovine DNA samples were purified from urine, which had much lower estimated bacterial load than the remainder that were from faecal samples and therefore the overall results must be treated with caution due to the affect different sample

type can have on PCR results (Berri et al., 2001). In contrast, a study by Vaidya et al. (2008) detected much higher bacterial loads in urine than in faeces. The study by Vaidya and colleagues (2008) may have used less faecal material per extraction than was used in this work, and did not add facilitators of PCR to reaction mixtures which can have a significant effect on sensitivity (Jiang et al., 2005) and these two factors may account for this disparity. Overall, Vaidya and colleagues (2005) found qPCR to have higher sensitivity than ELISA and the data presented here for ruminant samples appear to support those results. However, the issue of contamination of test samples must also be considered as it is possible that this could have occurred before or after DNA extraction with either PCR product, or with *C. burnetii* cells themselves (Kwok and Higuchi, 1989). The likelihood of contamination of new reaction mixtures with PCR product was reduced through the use of a commercial qPCR master mix which prevents amplification of carried over PCR products (Kwok and Higuchi, 1989). Performing re-isolation of DNA from all ruminant and kangaroo faecal samples, and subsequently testing these samples with the *IS1111a* qPCR returned the same results as for the primary DNA isolation. Thus, confirmatory testing with a qPCR assay targeting the *JB153-3* region, found only in phase I cells, was also undertaken. Cultured phase II *C. burnetii* cells were manipulated for other experiments in the same laboratory as the DNA purification took place and this may have provided an opportunity for contamination of test samples. Assays targeting the *IS1111a* repetitive element can be highly sensitive (Hoover et al., 1992) but because this element is found in both phase I and phase II strains they cannot distinguish between a wild type positive and contamination by phase II *C. burnetii* DNA. Thus, the quantitative PCR targeting the *JB153-3* genetic element was developed to clarify PCR test results. While a study found that the *JB153-3* element was present in the genomes of all four phase I strains tested and absent from the four phase II strains of *C. burnetii* that were analysed (Hoover et al., 2002), this gene lies in a redundant genomic region and

therefore it may not be present in all wild-type strains thus its use as a screening assay is limited.

Culture of *C. burnetii* was attempted on a small selection of qPCR-positive faecal samples to support the PCR results presented here and to provide isolates which could be used for genetic comparisons with other well characterised strains. Unfortunately none of the attempts resulted in the isolation of viable *C. burnetii* cells although given the low success rate of this procedure (Enright et al., 1971) this was not unexpected. It is also possible that several cycles of freeze-thawing may have impaired the viability of any cells present.

The finding that the prevalence of antibodies to, and shedding of, *C. burnetii* was negligible in the ruminants sampled is at odds with epidemiological studies from other countries. Despite the low prevalence of anti-*C. burnetii* antibodies found in abattoir workers by McKelvie (1980), domestic livestock are involved in transmission of *C. burnetii* to humans in Australia as human cases are reported every year in red meat industry workers and Q fever is a recognised occupational hazard in this group (Worksafe, 2001). But, as was implicated by Dane and Beech (1955), domestic ruminants may not be the most important reservoir of *C. burnetii* in Australia and the interaction between domestic and wildlife cycles of *C. burnetii* in Australia remains unknown.

The results from testing serum from kangaroos from WA suggest that Australian marsupials may play a significant role in the maintenance of *C. burnetii* in the environment. The ELISA results indicate an exposure rate of nearly 34% in all collection locations spanning approximately 500 kilometres. A very high prevalence of anti-*C. burnetii* antibodies was observed in some areas, although the strong relationship between seropositivity and collection month may have skewed the results for locations that were only sampled once or twice. The majority of samples were collected in two of the six locations and thus the overall results may not truly represent the state-wide situation. However, where enough samples were collected,

inferences could be made within results for one location. Samples collected in Capel did not yield test results that were significantly influenced by month whereas those collected in Manjimup did, indicating that seropositivity of kangaroos may also be linked to the home range of a particular population. The lower overall seropositivity of kangaroos in Capel may indicate that *C. burnetii* exposure is not endemic in all kangaroo populations and perhaps hints at other factors than those considered here being involved in the seroprevalence differences observed. An earlier study in Australia (Pope et al., 1960) that suggested that the kangaroo tick, *Amblyomma triguttatum*, may be responsible for the transfer of *C. burnetii* between host species and future work testing ticks for *C. burnetii* DNA may be warranted.

In other animals *C. burnetii* is shed in milk, urine and faeces (Arricau Bouvery et al., 2003; Berri et al., 2001). While a relatively low proportion of PCR-positive results were observed for kangaroo samples it is apparent that shedding of *C. burnetii* by kangaroos does occur via excreta as well although, as mentioned for ruminant PCR results, it is possible that *C. burnetii* cells could have passed through the digestive tract without establishing infection. The relatively low bacterial load in kangaroo faeces, and indeed in ruminant faeces, indicates that large-scale proliferation of bacteria probably doesn't occur in the gastrointestinal tract. The disparity observed between qPCR and ELISA results for kangaroos may be attributed to the situation where even after the host has cleared the *C. burnetii* infection detectable levels of antibodies may remain for several months (Enright et al., 1971). It is also possible that other sample types, such uterine swabs, could provide a more sensitive PCR assay and this should be investigated in future studies. Testing DNA-spiked buffer has shown that the *IS1111a* qPCR was able to detect 0.16 *C. burnetii* genomes per reaction in 1/3 of tests, was successful in half of reactions containing 1.59 genomes and in all reactions containing 15.90 genomes (data not shown) which equate to theoretical limits of detection of 19.90, 198.75 and 1,987.50 copies/g of faeces respectively. As such, it is possible that a proportion of samples with low

bacterial loads were not detected. On the three occasions that dams and their pouch young were sampled and tested immunologically in this study there was no evidence that the young had been exposed to *C. burnetii* despite not being weaned from mother's milk . This might indicate that kangaroos do not shed coxiellae in milk but given the small samples size it is not possible to draw any conclusions with confidence. Conducting histopathology experiments in kangaroos could help to define such unknowns and would allow the natural transmission cycle to be defined, thus helping to determine the role of Australian marsupials as reservoirs of *C. burnetii*. Parturition in Western grey kangaroos occurs in approximately February (Dawson, 2002) but because products of parturition are minimal in quantity, spread of infection from kangaroo birth products is unlikely. However, animals may have depressed immune systems around this time, increasing their susceptibility to infection via the faecal-oral route and possibly enabling increased pathogen proliferation and, subsequently, increased shedding. Infection by this mechanism may be facilitated by increased rainfall in April/May, which leads to a sudden proliferation of new green feed, perhaps disrupting the kangaroos natural gut flora and subsequently reducing their intrinsic resistance to enteric pathogens. This may be compounded by concentration of animals close to food and water sources at this time. This theory is supported by other work where incidence of Q fever was found to have a strong correlation with rainfall (Gardon et al., 2001). However, finding consistent seasonal trends for outbreaks of *C. burnetii* infections both overseas (Hellenbrand et al., 2001; Raoult et al., 2000) and in Australia (Garner et al., 1997; McKelvie, 1980) has been difficult and further data are needed to support the conclusions proposed here.

Conclusions

The high seropositivity observed in the western grey kangaroos tested indicates that these marsupials may be a significant reservoir for *C. burnetii* in Western Australia and may pose a

threat for zoonotic transfer of this pathogen. The risk of direct transmission to humans could be particularly relevant for individuals involved in the commercial harvest and processing of kangaroos. However, a more extensive risk could be posed by transmission to domestic ruminants and subsequently to a wider human population. Therefore, further work is required to fully elucidate the role that kangaroos, as a putative wildlife reservoir, play in transmission to both domestic animals and humans. This study has also provided methods that can be used to detect *C. burnetii* DNA in faecal and urine samples and Australian marsupial serum samples. These tools could enable future surveillance studies for *C. burnetii* in native marsupials and livestock in Australia.

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Table 1. Primer and TaqMan probe sequences for a quantitative PCR targeting *C. burnetii* genomic DNA

Name	5` to 3` sequence	5` label	3` label	Reaction Concn
*IS1111a*F	GTTTCATCCGCGGTGTTAAT	none	none	25 pmol
*IS1111a*R	TGCAAGAATACGGACTCACG	none	none	20 pmol
*IS1111a*P	CCCACCGCTTCGCTCGCTAA	6-FAM	BHQ-1	1.25 pmol
*JB153-3*F	TATTCGGCATCCCTTGGATA	none	none	15 pmol
*JB153-3*R	TTGTAACGCGCCACTATCTG	none	none	20 pmol
*JB153-3*P	TCACGCGCAATATTTGCAGCATG	6-FAM	BHQ-1	3.75 pmol

Table 2. qPCR results, including bacterial load, of ruminant urine and faecal samples

			ELISA	*IS1111a* qPCR	
	Sample	No. of samples	Positives	Positives	Median, 1st, 3rd quartiles (copies/ml, g)
Bovine	Urine	157	1	14	19.29, 15.06, 24.58
	Faeces	172	1	12	1812, 1593, 1952
Ovine	Faeces	50	0	6	2726, 2244, 3356
	TOTAL	379	2	32	-

Table 3. The percentage of kangaroo samples that were positive for each collection location and significant differences observed between locations

		ELISA positives		*IS1111a* positives	
	Total samples	Percent (95% CI's)	PP values (median, 1st, 3rd quartiles)	Percent (95% CI's)	Positive in both
Capel	123	8.1 (3.3, 13.0)	24.6, 20.9, 29.2*	8.13 (3.3, 13.0)	1
Manjimup	113	38.1 (29.1, 47.0)	31.9, 21.2, 56**	15.04 (8.5, 21.6)	8
Badgingarra	30	40 (22.5, 57.5)	31.2, 18.2, 65.8**	10 (-0.7, 20.7)	2
Preston Bch	28	82.1 (68, 96.3)	61.9, 46.2, 75.6	3.57 (-3.3, 10.5)	1
Eneabba	17	58.8 (35.4, 82.2)	62.1, 26.4, 82.5	11.76 (-3.6, 27.1)	2
Whiteman Pk	32	53.1 (35.8, 70.4)	56.9, 19.6, 77.3	25 (10.0, 40.0)	6
TOTAL	343	33.5 (28.5, 38.5)	27.7, 21.0, 54.5	12.3 (8.5, 15.4)	20

For the ELISA results the OD values of test samples were converted to a percentage of the mean positive control OD's ('PP') from the same plate and all samples with values equal to or greater than 40% were classified as positive. The columns labelled 'percent' describe the percent of samples that were test positive with ELISA or PCR.

* Differences significant at P<0.001.

** Differences significant at P<0.05.

Table 4. The percentage of kangaroo samples that were positive for each sex and age group

			ELISA positives		*IS1111a* positives	
		Total samples	Percent (95% CI's)	PP values (median, 1st, 3rd quartiles	Percent (95% CI's)	Positive in both
Sex	Male	177	36.2 (29.1, 43.2)	29.3, 21.2, 53.9	14.69 (9.5, 19.9)	12
	Female	166	30.7 (23.7, 37.7)	26.4, 21.0, 54.5	9.04 (4.7, 13.4)	8
TOTAL		343	33.5 (28.5, 38.5)	27.7, 21.0, 54.5	12.25 (8.5, 15.4)	20
Age	1	3	0	15.0, 14.0, 22.4	0	0
	2	4	50.0 (1.0, 99.0)	45.9, 25.8, 67.1	0	0
	3	40	30.0 (15.8, 44.2)	27.0, 21.2, 48.4	15.00 (3.9, 26.1)	3
	4	296	34.1 (28.7, 39.5)	27.9, 21, 56.1	11.82 (8.2, 15.5)	17
TOTAL		343	33.5 (28.5, 38.5)	27.7, 21.0, 54.5	12.25 (8.5, 15.4)	20

For the ELISA results the OD values of test samples were converted to a percentage of the mean positive control OD's ('PP') from the same plate and all samples with values equal to or greater than 40% were classified as positive. The columns labelled 'percent' describe the percent of samples that were test positive with ELISA or PCR.

Table 5. The percentage of kangaroo samples that were positive for each collection month

		ELISA positives		*IS1111a* positives	
	Total samples	Percent (95% CI's)	PP values (median, 1st, 3rd quartiles	Percent (95% CI's)	Positive in both
March	36	0	22.5, 20.3, 25.4	22.2 (8.6, 35.8)	0
April	20	0	20.8, 19.1, 22.9	25 (6.0, 44.0)	0
May	65	33.9 (22.3, 45.4)	22.1, 19.7, 52.5	12.3 (4.3, 20.3)	5
June	138	44.2 (35.9, 52.5)	37.6, 26.0, 65.3*	8 (3.5, 12.5)	8
July	84	38.1 (27.7, 48.5)	31.1, 21.5, 68.2 **	10.7 (4.1, 17.3)	7
TOTAL	343	33.5 (28.5, 38.5)	27.7, 21.0, 54.5	12.3 (8.5, 15.4)	20

For the ELISA results the OD values of test samples were converted to a percentage of the mean positive control OD's ('PP') from the same plate and all samples with values equal to or greater than 40% were classified as positive. The columns labelled 'percent' describe the percent of samples that were test positive with ELISA or PCR.

* Differences significant at P<0.05.

** Differences significant at P<0.01.