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PREVALENCE OF *COXIELLA BURNETII* IN WESTERN GREY KANGAROOS (*MACROPUS FULIGINOSUS*) IN WESTERN AUSTRALIA

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ABSTRACT: We investigated the role of the western grey kangaroo (*Macropus fuliginosus)* in the maintenance and transmission of *Coxiella burnetii* in Western Australia. Sera from 1,017 kangaroos were tested using an indirect enzyme-linked immunosorbent assay (ELISA) for the presence of *C. burnetii* antibodies. The overall antibody prevalence across 12 locations throughout mid- to southwestern Western Australia was 24.1% (95% CI: 21.6–26.8). Feces from 990 of the same animals were tested using PCR to identify active shedding of *C. burnetii* in excreta. *Coxiella burnetii* DNA was detected in 4.1% (95% CI: 3.1–5.6) of samples. Our results suggest that kangaroos are reservoirs for *C. burnetii* in Western Australia and may contribute to transmission of the organism to domestic livestock and humans.

Key words: *Coxiella burnetii*, kangaroo, Q fever, wildlife.

INTRODUCTION

Q fever is a re-emerging zoonosis caused by the Gram-negative intracellular bacterium *Coxiella burnetii* (Arricau-Bouvery and Rodolakis, 2005). Livestock have traditionally been associated with transmission of the organism, yet there is increasing evidence that a link exists between infection in humans and livestock and contact with wildlife (Gardon et al., 2001). Before the 1970s, it was proposed that marsupials were a potentially significant reservoir for *C. burnetii* (Derrick et al., 1939; Pope et al., 1960). More recently, Banazis et al. (2010) surveyed the prevalence of *C. burnetii* in domestic and native animals in Western Australia. Their results suggested that kangaroos may be potential reservoirs for *C. burnetii* (Banazis et al., 2010). Further investigation is required to better understand the epidemiology related to infection and transmission dynamics of Q fever. We studied the antibody prevalence and fecal prevalence of *C. burnetii* DNA in western grey kangaroos (WGK; *Macropus fuliginosus*) throughout Western Australia. Analysis incorporated the age and sex of kangaroos, the location and season (quarter) in which samples were collected, and the accumulated rainfall that fell prior to specimen collection to identify any risk factors that predisposed kangaroos to infection. Our results may assist in further defining the wildlife reservoirs of *C. burnetii* and in providing information to assist in reducing the incidence of Q fever in humans and livestock.

MATERIALS AND METHODS

Sample collection

Blood ($n=1{,}017$) and fecal samples ($n=990$) were collected from harvested WGKs at 12 locations throughout mid- to southwestern Western Australia including Badgingarra, Boyup Brook, Bridgetown, Capel, Eneabba, Greenbushes, Manjimup, Nannup, Northcliffe, Preston Beach, Scott River, and Whiteman Park (Table 1). The number of samples collected at each site ranged from 12–281. Blood was collected by cardiac puncture in adult and subadult kangaroos and by free-catch from severed jugular and carotid vessels in pouch young. Fecal samples were collected directly from the intestines of adult and subadult kangaroos during evisceration, approximately 40 min after death. Due to the immaturity of pouch young, no accompanying fecal samples were collected.

For each sample, we recorded the location and date of collection and the sex and approximate age of the animal. Shooters subjectively categorized kangaroos into subadult (<3yr) and adult (≥3yr) based on size and apparent sexual maturity. The sex of each kangaroo was determined and recorded for

TABLE 1. Percentages (+95% confidence interval [CI]) of serum and fecal samples from western grey kangaroos (*Macropus fuliginosus*) with evidence of infection with *Coxiella burnetii* from 12 locations in Western Australia. Serum samples were tested for antibody by enzyme-linked immunosorbent assay (ELISA); fecal samples were tested for bacterial DNA by quantitative polymerase chain reaction (qPCR). Locations are listed in increasing order of antibody prevalence.

Location	Latitude, longitude	n	ELISA positive % (95% CI)[a]	qPCR positive % (95% CI)
Capel	33°33′8′S, 115°33′14′E	299	10.7 (7.5, 14.9) A	3.9 (2.1, 7.2)
Bridgetown	33°57′27′S, 116°8′13′E	54	13.0 (6.1, 24.7) AB	3.7 (0.3, 13.3)
Northcliffe	34°40′56′S, 116 9′42′E	40	17.5 (8.4, 32.3) ABC	7.5 (1.9, 20.6)
Nannup	33°58′52′S, 115°45′53′E	34	20.6 (10.1, 37.1) ABCD	0.0 (0.0, 8.8)
Manjimup	34°14′24′S, 116°8′42′E	113	23.0 (16.2, 31.6) BC	3.5 (1.1, 9.0)
Scott River	34°15′2′S, 115°16′24′E	12	25.0 (8.3, 53.9) ABCDE	0.0 (0.0, 21.6)
Badgingarra	30°20′17′S, 115°32′22′E	141	27.0 (20.3, 34.8) BCE	4.3 (1.8, 9.2)
Eneabba	29°48′57′S, 115°15′53′E	105	27.6 (19.9, 36.9) BCE	2.9 (0.6, 8.4)
Boyup Brook	33°50′4′S, 116°23′18′E	86	34.9 (25.6, 45.4) CDE	9.3 (4.6, 17.5)
Greenbushes	33°50′50′S, 116°3′26′E	24	45.8 (27.9, 64.9) DE	0.0 (0.0, 12.1)
Preston Beach	32°51′52′S, 115°40′23′E	75	48.0 (27.9, 64.9) E	1.3 (0.0, 7.9)
Whiteman Park	31°50′3′S, 115°57′5 E	34	56.7 (39.2, 72.6) E	3.3 (0.0, 18.1)
Total		1,017	24.1 (21.6, 26.8)	4.1 (3.1, 5.6)

[a] Values that share the same letter were not significantly different between locations ($P<0.05$).

997 individuals. The number of males and females were well distributed across all sample collection sites except for Scott River, which had only 12 samples in total. Out of the 760 samples for which age was recorded, 27 were pouch young, 60 were subadults, and 673 were adults. Age was not consistently recorded for 227 animals harvested in Badgingarra, Preston Beach, and Eneabba. Kangaroos were sampled in all accumulated rainfall and quarter categories in Capel only.

Serum and fecal testing

Antibodies to *C. burnetii* in kangaroo sera were detected using an enzyme-linked immunosorbent assay (ELISA) as described by Banazis et al. (2010). Whole genomic DNA was extracted from each fecal sample using the MoBio PowerSoil™ DNA isolation kit (MO BIO, Carlsbad, California, USA) with an optimized method (Banazis et al., 2010). Fecal samples were tested using two quantitative polymerase chain reaction (qPCR) assays (one targeting the *IS1111a* element and one targeting the *JB153-3* sequence; Banazis et al., 2010).

Environmental data

Daily rainfall data were obtained from the Bureau of Meteorology for weather stations closest to the sampling site. Accumulated rainfall was calculated for the preceding 30 days (RainCat30) and 60 days (RainCat60) at each site for collection date. Data were grouped into four categories: <25 mm, 25–49 mm, 50–99 mm, and ≥100 mm for RainCat30; and <50 mm, 50–99 mm, 100–199 mm, and ≥200 mm for RainCat60. Data were also aggregated based on the quarter of the year in which they were collected: Q1=January–March; Q2=April–June; Q3=July–September; Q4=October–December.

Data analysis

A generalized linear model, which assumed a binomial distribution for the presence of *C.*

burnetii, was fitted to the ELISA and qPCR data to determine whether there was an association with sex, age, quarter, or rainfall category (McCullagh and Nelder, 1989). The agreement between the two tests was assessed by calculating the Kappa statistic using the Statistical Package for the Social Sciences (SPSS v.17, SPSS Corporation, Chicago, Illinois, USA). Chi-square or Fisher's exact tests were used at the 95% confidence limit to determine whether any two proportions were significantly different. Data from the Capel location were included in this initial analysis but were also analyzed separately because it was the only location where a large number of samples were collected across all rainfall and quarter categories and across sex and age groups.

RESULTS

Serologic testing of kangaroo sera

The overall prevalence of antibody to *C. burnetti* in 1,017 kangaroos across all 12 locations was 24.1% (95% CI: 21.6–26.8). Results varied significantly among locations ($P<0.001$) with the estimated prevalence at Capel being significantly lower than all sampling locations (10.7%, 95% CI: 7.5–14.9: $P<0.005$) except for Bridgetown, Northcliffe, Nannup, and Scott River (Table 1). The estimated antibody prevalences at Whiteman Park (56.7%, 95% CI: 39.2–72.6) and Preston Beach (48%, 95% CI: 37.1–59.1) were significantly higher ($P<0.01$) than all other locations except for Boyup Brook, Scott River, and Greenbushes (Table 1).

RainCat60 was significantly associated with antibody prevalence ($P=0.034$); RainCat30 was not ($P=0.427$). There was a significant interaction between RainCat60 and location ($P<0.001$), indicating that differences in the number of antibody-positive animals between rainfall categories varied by location. At Eneabba and Badgingarra, prevalence decreased with increasing rainfall ($P<0.05$) while at Capel, prevalence was significantly higher following increased rainfall ($P<0.05$).

When considering all collection locations, antibody prevalence was significantly lowest in the 4th quarter ($P<0.05$). At Capel alone, quarter did not have a significant effect on prevalence ($P<0.05$). The proportion of antibody-positive kangaroos was not influenced by the age or sex of the animal across the 12 collection sites, including Capel ($P>0.05$).

Testing of kangaroo feces

Coxiella burnetii DNA was detected in 4.1% (95% CI: 3.1–5.6; $n=990$) of fecal samples. There were no significant differences in the proportions of fecal samples that were qPCR-positive among collection locations (Table 1) and no association with rainfall, quarter, age, or sex. At Capel, where the estimated prevalence of fecal DNA was 3.9% (95% CI: 2.1–7.2), the association with rainfall, age, and sex was similarly not significant.

Agreement between ELISA and qPCR

There was poor agreement between the qPCR and ELISA results (Kappa=0.120). However, the probability of a fecal sample being positive for *C. burnetii* DNA using qPCR was more likely if the ELISA result was positive (OR=7.1; $P<0.001$).

DISCUSSION

Q fever has traditionally been considered an occupational hazard for workers involved with the farming or slaughter of ruminants (Garner et al., 1997). More recent studies have failed to demonstrate a convincing link between these classic reservoir sources and infection in humans (Gardon et al., 2001; Massey et al., 2009). Contact with wildlife and feral animals, in addition to livestock, is now considered an important risk factor for Q fever (Massey et al., 2009). The high antibody prevalence in kangaroos found in our study supports the work of Derrick et al. (1939), who proposed that marsupials were a potentially significant reservoir host of *C. burnetii*. More significantly, these results provide evidence to support Pope et al. (1960) and Banazis et al. (2010), who suggested that kangaroos are likely to play

an important role in the maintenance and transmission of *C. burnetii*.

The statistically significant differences in estimated antibody prevalence among collection locations are of interest. Because fecal shedding is likely to be seasonal and intermittent, antibody prevalence may give a better indication of the risk of infection in local kangaroo populations. The high prevalence at Preston Beach is notable because the property from which the kangaroos were harvested was destocked approximately 3 yr earlier. The property borders on residential and bush land and there are no livestock within approximately 2 km. Persistence of the organism in the environment following shedding from infected livestock many years earlier may account for the high level of exposure to *C. burnetii*. Alternatively, it may be possible that a wildlife–tick transmission cycle can maintain *C. burnetii* without the presence of livestock. Given that the daily home range of WGKs may be less than 2 km (Arnold et al., 1991), it is unlikely that the kangaroos sampled in this study would have regularly travelled far enough to come into contact with livestock (Arnold et al., 1991).

It is difficult to interpret the association between rainfall and antibody prevalence, as samples were not collected in all rainfall categories at all locations, and disease incidence cannot be evaluated by antibody prevalence alone. In the individual analysis at Capel, antibody prevalence was significantly higher following increased rainfall in the 2 mo prior to sample collection. However, at Badgingarra and Eneabba, the highest prevalence was found among animals collected during the driest conditions. Given that no significant association was found with fecal shedding, it is not possible to evaluate the effects of rainfall on the rate of infection with *C. burnetii*. This relationship requires further investigation, as Gardon et al. (2001) reported a strong correlation between accumulated rainfall and disease incidence in humans, with a lag period peaking at the second month. Although wetter conditions do not generally support aerosolization of infectious particles, these conditions do favor the presence of wildlife and arthropod vectors, whose activity is often dependent upon rainfall (Gardon et al., 2001). While the role of the tick in transmission of *C. burnetii* is unknown, a positive correlation between disease and rainfall is frequently seen in arthropod-borne disease (Gardon et al., 2001). Following isolation of *C. burnetii* from 13 kangaroo ticks (*Amblyomma triguttatum*), four of which were found on goats and sheep, Pope et al. (1960) suggested that the 3-host tick may act as a vector between the different host species. Although engorged kangaroo ticks were noted on the animals sampled, no formal study was undertaken. The influence of rainfall on Q fever incidence may also be related to increased reproduction in fast-breeding potential reservoir species, such as rodents and lagomorphs, following increased availability of food (Fiedler, 1994; Webster et al., 1994). With increased shedding and aerosolization of *C. burnetii* associated with a greater presence of reservoir hosts, it is plausible that disease incidence rises following increased rainfall.

The link between quarter and antibody prevalence may have been a chance association, despite the statistical significance, given that antibody presence is not a marker of acute infection. Despite the absence of an association between quarter and fecal shedding of *C. burnetii* in this study, others have shown that infection in animals, including ruminants, tends to be seasonal (Enright, Franti, Longhurst et al., 1971; Yanase et al., 1997). This relationship in kangaroos requires further investigation, as it is possible that the small number of positive samples in this study were insufficient to demonstrate a seasonal trend in shedding of *C. burnetii*. Future research is needed for a genetic comparison of *C. burnetii* in kangaroos and domestic stock in order to characterize the relationship between the two cycles.

When interpreting the antibody results from this study, it is important to consider the reliability of the serologic assay. Banazis et al. (2010) reported that the reproducibility of the ELISA could be further improved. In its development, selection of an appropriate cut-off point was difficult due to an absence of known negative samples and of a validated test by which to confirm the positive controls. Samples tested by the complement fixation test did not provide meaningful results due to strong, nonspecific reactions that could not be reduced through heat inactivation (Banazis et al., 2010). Although the immunofluorescence assay (IFA) is the current reference method for detection of Q fever in humans (Rousset et al., 2010), this test has not been validated for use in kangaroos either. The IFA is subjective in nature and difficult to standardize between operators, resulting in an unreliable means of confirming positive status in kangaroo samples. In the development of the ELISA, three of the highest-reacting serum samples were pooled to form the positive control. Each of the three samples was subsequently positive on fecal qPCR. PCR products showed a high level of homology to *C. burnetii.* Antibody-positive blood samples were not tested by PCR. This may have been useful in confirming positive samples, but was not attempted in this study due to the lack of a validated means to do so and to the likelihood that the blood-borne stage of infection is transient. Bacteremia has only been detected for up to 7 days following infection in mice (Kruszewska and Tylewska-Wierzbanowska, 1993) and 10 days following infection in monkeys (Waag et al., 1999). As it takes 2–3 wk following infection for antibody development (Kishimoto and Burger, 1977), it is likely that an antibody-positive sample would be negative on blood PCR. Finally, the strain of antigen used may not have been representative of the antigenic profile of *C. burnetii* in Australian marsupials, as regional differences may occur in antigenic strains (Banazis et al., 2010). Isolation and characterization of *C. burnetii* from Australian marsupials would further improve antigen selection. To minimize the effect that the variability of the antigen may have had on results, absorbance values were normalized by expressing the test sample absorbance values as a percentage of the positive control absorbance mean from the same plate (Banazis et al., 2010). The possibility of cross-reactivity must also be considered (Banazis, 2009). Although no evidence of its occurrence has been found in kangaroos, cross-reactivity between *C. burnetii* and other organisms has been documented in humans, mice, and rabbits (La Scola and Raoult, 1996; Lukacova et al., 1996).

The absence of a significant difference in the proportion of fecal samples positive for *C. burnetii* DNA among collection locations was likely due to small sample sizes from some study sites. As the assistance offered by professional shooters in this project was largely voluntary, and caused some disruption to their normal routine, we did not request larger numbers of samples. The low prevalence of *C. burnetii* DNA in feces of kangaroos may reflect the intermittent periods of shedding of *C. burnetii* observed in several studies (Guatteo et al., 2007; Rodolakis et al., 2007). Intermittent shedding significantly reduces the sensitivity of any technique designed to detect fecal organisms, including PCR. Commonly, the prevalence of antibodies against an organism will be much greater than the isolation–detection rate (Yabsley and Pittman Noblet, 2002; Rodriguez-Vivas et al., 2004), which appears to be the case in this study. It may also be possible that fecal shedding is not the primary route of transmission in kangaroos. Shedding varies with the species in question and, for many reservoir hosts, it is possible that the nature of shedding differs from that of ruminants. Kangaroos are unlikely to contribute significantly to environmental

contamination through excretion of birth products due to physiologic differences in reproduction between eutherian and metatherian animals (Dawson, 2002).

The poor agreement between the PCR and ELISA was not unexpected, particularly given the intermittent and seasonal nature of *C. burnetii* shedding demonstrated in previous studies (Yanase et al., 1997; Gardon et al., 2001; Guatteo et al., 2007; Rodolakis et al., 2007) and the persistent nature of antibodies following many infections in animals. The presence of antibody-negative kangaroos apparently shedding the organism in feces has been reported in other species (Berri et al., 2001). In livestock, this could be because the bacteria are localized in the placenta or uterus without inducing systemic antibodies (Berri et al., 2001). In the kangaroos in this study, it may be possible that animals were shedding but had not yet developed detectable IgG. Alternatively, *C. burnetii* antibodies produced during the early stages of infection may have waned over time, yet shedding persisted. Similar observations have been reported in cows, where detectable antibodies against *C. burnetii* disappeared in several months (Grist, 1959; Yanase et al., 1997). Experimental infection studies are needed to determine the extent and duration of the immune response in kangaroos to further explain these findings.

The sex of the kangaroos had no effect on the prevalence of *C. burnetii* antibody or on the proportion of fecal samples positive by qPCR. This finding is in agreement with the work of Willeberg et al. (1980), who noted that there was no sex-associated difference in the prevalence of *C. burnetii* antibodies among cattle, horses, or cats. Despite female ruminants shedding higher amounts of the organism at parturition (Berri et al., 2001), evidence suggests that susceptibility to infection in kangaroos is the same for both sexes. In humans, sex hormones may play a role in the pathogenesis of *C. burnetii*, with men being more symptomatic than women, despite equal antibody prevalence (Raoult et al., 2000; Raoult et al., 2005). A similar finding was noted in C57/BL6 mice clinically infected with *C. burnetii* (Leone et al., 2007). As no investigation was undertaken to determine the pathologic changes associated within infection in kangaroos, it is unknown whether a similar response could be expected in the WGK.

Age had no effect on the prevalence of *C. burnetii* antibodies or the prevalence of fecal shedding in kangaroos from the 12 sample collection sites, a finding noted in other species (Willeberg et al., 1980). Despite this observation, age appears to be a risk factor for Q fever in humans, with the incidence of symptomatic disease increasing with age (Raoult et al., 2000). Leone et al. (2007) also noted that, following clinical infection with *C. burnetii*, mature mice (14 mo) had increased tissue bacterial burden and granuloma formation and defective responses to bacterial stimulation when compared to younger mice (1 mo; Leone et al., 2007). Although kangaroos do not appear to exhibit clinical signs of disease, it is expected that antibody prevalence should increase with age due to increased exposure over time (Ruiz-Beltrán et al., 2004). The relationship between age and infection in kangaroos requires further investigation, as the inevitable age-based selection bias introduced through the kangaroo harvesting industry may have influenced our results.

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