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Peripartum dynamics of *Coxiella burnetii* infections in intensively managed dairy goats associated with a Q fever outbreak in Australia

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ABSTRACT

Coxiella burnetii may cause reproduction disorders in pregnant animals but subclinical infection in other animals. Unrecognised disease may delay implementation of control interventions, resulting in transmission of infection to other livestock and to humans. Seroreactivity to *C. burnetii* phase-specific antigens, is routinely used to interpret the course of human Q fever. This approach could be similarly useful in identifying new and existing infections in livestock herds to help describe risk factors or production losses associated with the infections and the implementation of disease-control interventions. This study aimed to elucidate the dynamics of *C. burnetii* infections using seroreactivity to phase-specific antigens and to examine the impact of infection on milk yield in goats in an endemically-infected farm that was associated with a Q fever outbreak in Australia. Seroreactivity pre- and post-partum and milk yield were studied in 164 goats (86 nulliparous and 78 parous). Post-partum, the seroprevalence of antibodies to *C. burnetti* increased from 4.7% to 31.4% throughout goats' first kiddings and from 47.4% to 55.1% in goats kidding for the second or greater time. Of 123 goats that were seronegative pre-partum, 26.8% seroconverted over the three-month peri-partum period, highlighting the importance of controlling infection throughout this time. The risk of seroconversion was comparable in first or later kidders, suggesting constant risk irrespective of parity. No loss in milk production associated with seroconversion to phase 2 was observed within the first nine weeks of lactation. However, seroconversion to only phase 1 was associated with extra 0.276 L of milk per day (95% Confidence Interval: 0.010, 0.543; P = 0.042), which warrants further investigation to ascertain whether or not the association is causal. Further studies on seroreactivity and milk production over longer periods are required, as milk production loss caused by *C. burnetti* may be an additional reason to control the disease in goat herds.

1. Introduction

Coxiella burnetii is the causative agent of Q fever, a zoonotic disease characterized by a flu-like illness in its acute form as well as cardiac malfunction and granulomatous hepatitis in its chronic form in humans (Arricau-Bouvery and Rodolakis, 2005). The bacterium mostly circulates sub-clinically in domestic animals and wildlife although infections in pregnant animals have been reported to result in abortions, still-births, weak offspring and neonatal death (Raoult et al., 2005; Sánchez et al., 2006). Early detection of *C. burnetii* outbreaks in ruminant herds has previously been hindered by the subclinical nature of *C. burnetii* infections in non-pregnant animals and the failure to routinely diagnose causes of abortions on farms (Rodolakis et al., 2007; Schimmer et al., 2009). During the 2007–2010 Q fever epidemic in the Netherlands, the link between infected farms and human cases became apparent after implementation of mandatory reporting of increased abortion rates on farms (Schimmer et al., 2009).

In ruminants, coxiellosis is thought to cause reproductive loss through inflammation of the placenta following massive replica-

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tion of *C. burnetii* in the trophoblast cells (Sánchez et al., 2006); up to 90 fold within three days of infection (Amara et al., 2010). Thus *C. burnetii* infections in ruminants and other domestic animals consequently lead to environmental contamination during parturition or abortion when the placenta and birth fluids are shed. This is considered to be the primary source of human and domestic animal *C. burnetii* infections, with transmission mainly occurring through inhalation of *C. burnetii*-contaminated dust and fluid aerosols (Tigertt et al., 1961; Angelakis and Raoult, 2010).

Intensive ruminant farming, which often involves synchronisation of oestrus and thus parturition, may result in very high rates of both environmental contamination and transmission of infection to susceptible hosts, with bacterial shedding from numerous infected ruminants giving birth within narrow timeframes. Such an effect may explain why a number of previous outbreaks have been associated with intensive ruminant farms: over 4000 human cases of Q fever occurred in the Netherlands between 2007 and 2010, with increased risk of infection associated with living in close proximity to intensively-managed dairy goat herds (Delsing and Kullberg, 2008). Similarly, 147 human cases in the United Kingdom were associated with lambing ewes in the West Midlands in 1992 (Guigno et al., 1992; Smith et al., 1993), and 23% of the residents in a rural German town were considered to have contracted Q fever from a large sheep farm in 1996 (Lyytikäinen et al., 1998).

Testing for antibodies and *C. burnetii* DNA in bulk tank milk (BTM) has been widely used as a surveillance method (Muskens et al., 2011; Van den Brom et al., 2012). However, BTM testing does not describe infection dynamics within herds which is important for identifying infection risk factors (Guatteo et al., 2007). BTM monitoring of *C. burnetii* epidemiology also excludes non-lactating animals and may not detect the initial stages of an outbreak involving fewer animals, leading to falsely negative enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) results (Guatteo et al., 2007). Thus, testing individual animals is required to comprehensively investigate exposure and transmission within herds.

Serological reaction to phase-specific antigens of *C. burnetii* is routinely used to diagnose and interpret the course of Q fever in humans but has not been widely used to study *C. burnetii* infection in domestic animals (Cutler et al., 2007; Böttcher et al., 2011). Recent infections may be identified by the presence of IgM antibodies to phase 2 *C. burnetii* surface glycoproteins, which appear within two weeks of infection, or through detecting seroconversion or a fourfold rise in titre of IgG antibodies to phase 2 *C. burnetii* antigens (Cutler et al., 2007; Rousset et al., 2007; Schimmer et al., 2009; Delsing et al., 2011). Conversely, IgG antibodies to phase 1 *C. burnetii* antigens appear much later in the course of infection—about 114 days after infection in humans—which indicates convalescence, recrudescence or chronic disease, and could also be a marker of constant antigenic stimulation (Powell and Stallman, 1962; Kimbrough et al., 1979; Peacock et al., 1979). The sequential production of antibodies to phase 2 and phase 1 antigens of *C. burnetii* was initially described in guinea pigs (Stoker and Fiset, 1956) and has recently been described in goats (Roest et al., 2013). Phase-specific serology may therefore be a valuable tool for studying the dynamics of infection within herds and for prompt detection of new infections.

Since antibodies are produced within a short timeframe of usually 2–3 weeks in newly infected animals (Roest et al., 2013), detecting antibodies is useful for timely diagnosis of new infections. In contrast, detectable *C. burnetii* DNA is usually only present in secretions or excreta during late pregnancy and after parturition following the massive replication of organisms in the placenta (Sánchez et al., 2006; Berri et al., 2007; Rodolakis et al., 2007). There seems to be no evidence of regular shedding of *C. burnetii* by infected ruminants outside the peri-parturient period (Woldehiwet, 2004).

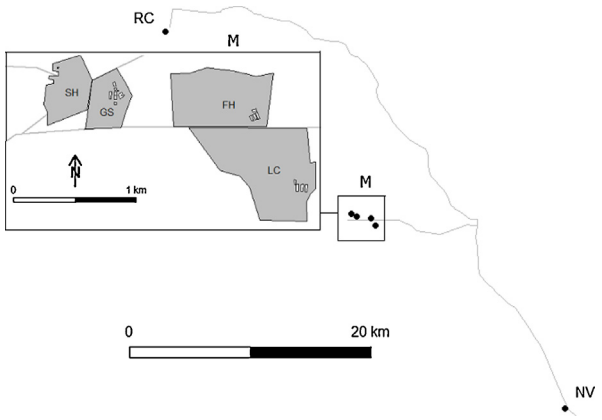

Fig. 1. Map showing the location and distance between the different farms on the dairy enterprise. NV and RC are over 40 km from the main property (M). The main property consists of SH (sheep flock) and goat farms (GS, LC and FH), all within 2 km from each other. The white small polygons within the grey areas (FH, LC and GS) are the shed where the goats are kept.

Early detection and timely implementation of disease control interventions could reduce production losses attributed to *C. burnetii* infections in ruminant herds. These are mainly due to reproductive wastage following *C. burnetii*-induced abortions and perinatal deaths (Masala et al., 2004; López-Gatius et al., 2012). Economic losses due to the cost of interventions such as vaccination and cessation of breeding as well as the cost of treating human infections and weeks of lost work have also been previously described (Garner et al., 1997; Van Asseldonk et al., 2013). For example, in the Netherlands outbreak, the retention payoff index (value of future profitability foregone) was estimated to be €250 per goat for breeding prohibition and €300 per goat culled (Van Asseldonk et al., 2013).

There is limited information on direct production losses due to *C. burnetii* infection in livestock other than those arising from reproductive anomalies. However, one study reported a strong association between shedding of *C. burnetii* in milk and the occurrence of chronic mastitis in dairy cattle (Barlow et al., 2008). In this study, we aimed to describe the dynamics of *C. burnetii* infections and also to examine the impact of infection on milk production in goats on an intensively-managed goat enterprise associated with a Q fever outbreak in Australia, using phase-specific seroreactivity and milk yield.

2. Materials and methods

2.1. Study population

The study was undertaken on an intensive goat and sheep dairy enterprise at the centre of a Q fever outbreak that lasted 2 years (2012–2014) and comprised of 18 confirmed and 6 probable human cases in Victoria, Australia (Bond et al., 2015). The enterprise grazed sheep on pasture, then converted into an intensive goat and sheep dairy in the late 1990s. Stock numbers have steadily risen and now there are 5000 milking goats and 1100 sheep reared in six herds on five different farms, three of which are in close proximity (Fig. 1). Although the sheep dairy herd is still managed extensively on pasture, the dairy goats are reared in large sheds and their reproductive cycles are synchronised to ensure short kidding periods four times a year namely; February-March, June-July, September-October and November-December.

2.2. Bulk milk monitoring of herd-level status

2.2.1. Testing for antibodies to C. burnetii in milk using ELISA

To describe the distribution of infected herds, 50 mL of BTM samples were collected on five different samplings from all the six herds over a four-month period in 2014. Two of the bulk milk samplings were done two weeks prior to a kidding period (August, before the September kidding and October, before the November kidding) and three samplings were done weekly in the three weeks following the November 2014 kidding period (first, second and third week of December). The milk samples were tested for antibodies to *C. burnetii* using ELISA (IDEXX) and indirect immuno-fluorescence assay (IFA), and for *C. burnetii* DNA with a quantitative PCR assay targeting the *com1* gene.

To test for antibodies against *C. burnetii* using the Q fever ELISA (IDEXX), 15 mL from the BTM samples was centrifuged at 5000*g* for 15 min and 50 μL of the resultant milk whey from each sample was diluted 1:5 using kit washing buffer. The diluted milk whey was tested for antibodies to phase 1 and phase 2 *C. burnetii* antigens as per the IDEXX kit instructions. Colour intensity in the ELISA plate wells was measured as optical density (OD) at 450 nm wavelength which is directly proportional to the concentration of antigen-antibody complexes in each well. A corrected OD (COD) of each well was then calculated using the formula:

$$COD = \left(OD_{sample} - OD_{negative\ control}\right) / \left(OD_{positive\ control} - OD_{negative\ control}\right)$$

OD values were interpreted according to the kit manufacturer's protocol, i.e. $0 \leq COD < 0.300$ as negative, $0.30 \leq COD < 0.40$ as suspect and $COD \geq 0.40$ as positive.

2.2.2. Testing for antibodies to C. burnetii in milk using IFA

The milk whey obtained from the centrifugation above was also tested using IFA. Undiluted milk whey and 1:5 and 1:10 dilutions of the whey in phosphate buffered saline (PBS) were incubated with *C. burnetii* phase 1 (Henzerling strain) and phase 2 (Nine Mile, Clone 4) antigen on 40 well microscopic slides as previously described (Da Silva et al., 2014). Negative controls were 2% casein in PBS, whey obtained from full cream pasteurised goat milk negative for *C. burnetii* antibodies on the ELISA, and a 1:160 dilution of known negative sera from New Zealand goats in whey obtained from full cream pasteurised milk. A positive control was created from known positive serum diluted 1:160 in whey obtained from full cream pasteurised goat milk. The diluted samples were tested for IgG antibodies to *C. burnetii* using a fluorescent-labelled rabbit anti-goat IgG conjugate as described previously (Muleme et al., 2016).

2.2.3. Testing for C. burnetii DNA in bulk tank milk

To identify herds shedding *C. burnetii*, BTM from each of the six herds was also tested for *C. burnetii* DNA. DNA was extracted from two 200 μL aliquots of each BTM sample using a HiYield Genomic DNA Mini Kit (Real Biotech Corporation), with a protocol validated for the extraction of DNA from milk (Lockhart, 2010). This protocol produced greater and more consistent DNA yields than the Chelex-100 (Sigma-Aldrich) extraction method, see supplementary materials Tables S1–S4 (Roest et al., 2011). Pasteurised milk spiked with *C. burnetii* strain Nine Mile Clone 4 was used as a positive control and, pasteurised goat milk and PBS as negative controls. *C. burnetii* DNA was detected using a real-time PCR assay targeting the *com1* gene (Lockhart, 2010). The number of *C. burnetii* genome equivalents in positive samples was determined using a standard curve created from a cloned *com1* amplicon. Positive samples with a *C. burnetii* concentration < 1 copy/μL were retested and considered positive if they contained ≥ 1 copy/μL of the target gene at subsequent assays.

2.2.4. Demonstration of freedom of infection on farms negative for C. burnetii antibodies on bulk tank testing

To confirm whether farms with negative ELISA, IFA and PCR results on BTM were free of infection, and to demonstrate freedom from infection in preparation for a separate vaccination study, 400 blood samples were collected from individual goats on one of the negative farms and tested for IgG and IgM antibodies to *C. burnetii* using a validated IFA (Muleme et al., 2016). This sample size provides 99% confidence of detecting a ≥ 1% prevalence of infection in a 1000-goat herd (Cannon and Roe, 1982), assuming the IFA has 95% diagnostic sensitivity (DSe) and 93% diagnostic specificity (DSp) for antibodies to *C. burnetii* in serum (Muleme et al., 2016).

2.3. Individual animal testing for C. burnetii and milk yield

To study peri-partum infection dynamics of *C. burnetii* in goats, paired blood samples were collected 30 days pre-kidding and 30 days post-kidding from 164 goats, randomly selected from a 252-head milking herd that was positive for *C. burnetii* antibodies and DNA on BTM samples. Sampling was conducted with the approval of the University of Melbourne Animal Ethics Committee (application number 1413118). Blood samples were centrifuged at 10,000*g* for 4 min to obtain serum, which was stored at 4 °C and tested for the presence of IgG and IgM antibodies against phase 1 and phase 2 *C. burnetii* antigens using a validated IFA estimated to have a diagnostic sensitivity of 94.8% (95% CI: 80.3, 99.6) and specificity of 92.5 (95% CI: 77.1, 99.3) for antibodies to *C. burnetii* in goat sera (Muleme et al., 2016).

To assess the relationship between seroreactivity and milk yield, individual daily milk yield data for the first 9 weeks of lactation were obtained from farm records and were used for comparison with individual animal serological testing results for *C. burnetii*. This timeframe of monitoring milk yield was aligned with the period when pre- and post-kidding serum sampling were taken and ended well before commencement of the subsequent kidding period. Birth dates, kidding dates and the number of prior parities for each of the goats was also recorded.

2.4. Statistical analysis

Bulk milk monitoring data were cross-tabulated by farm and sampling point, and comparisons made between ELISA and PCR as well as the IFA and ELISA results using the exact binomial test for correlated proportions in Rstudio. A non-significant binomial exact test indicating limited evidence that the two tests detected different proportions of positive samples, was followed by comparisons using Cohen's kappa test statistic (*K*) (Cohen, 1960; Sim and Wright, 2005), considering neither diagnostic test gold standard for herd-status.

Infection dynamics were described by comparing paired pre- and post-partum phase-specific antibody titres and estimation of the proportion of seroconversions (defined as a four-fold or greater rise in titre), stratified by parity of the goats under investigation. The parity for each of the goats was defined based on its pre-partum parity: i.e. parity 0 (nulliparous) were those goats that had never kidded prior to the pre-partum sampling, parity 1 (primiparous) were those that had previously kidded once and parity ≥ 2 (multiparous) were those that had previously kidded twice or more.

The difference in seroprevalence over the kidding period was also estimated by parity as detected by the various classes of antibodies to *C. burnetii*. The risk of seroconversion, by parity groups, was estimated amongst animals that were seronegative on pre-partum sampling. The McNemar test of paired proportions was performed using MedCalc statistical software to compare the proportions of goats initially seronegative that seroconverted, seropositive initially with increasing titres, seropositive initially

Table 1
Occurrence of antibodies to *C. burnetii* and detection of DNA in bulk tank milk samples from herds on an intensive goat and sheep dairy enterprise in Victoria, Australia, 2014.

Farms (Distance in km from farm GL)	Before kidding (August)	Before kidding (October)	After kidding (week 1 December)	After kidding (week 2 December)	After kidding (week 3 December)
ELISA results (corrected OD)					
Sheep (0)	S (0.30)	– (0.23)	– (0.24)	– (0.17)	– (0.26)
GL (0)	+ (0.90)	+ (0.60)	+ (0.79)	+ (0.78)	+ (0.76)
FH (1.6)	+ (1.15)	+ (0.74)	+ (0.81)	+ (0.91)	+ (0.92)
LC (1.9)	+ (0.57)	+ (0.39)	+ (0.52)	+ (0.51)	+ (0.48)
RC (44.1)	– (0.03)	– (0.00)	– (0.00)	– (0.00)	– (0.01)
NV (54.1)	– (0.00)	– (0.01)	– (0.00)	– (0.00)	– (0.00)
IFA results (1:10 dilution of milk whey with PBS)					
Sheep (0)	+	+	–	–	–
GL (0)	+	+	+	+	+
FH (1.6)	+	+	+	+	+
LC (1.9)	+	+	+	+	+
RC (44.1)	–	–	–	–	–
NV (54.1)	–	–	–	–	–
PCR results (mean CT, where positive)					
Sheep (0)	–	–	–	–	–
GL (0)	+ (39.1)	–	+ (36.5)	–	+ (36.0)
FH (1.6)	+ (36.3)	+ (36.8)	–	+ (37.1)	+ (37.2)
LC (1.9)	+ (35.6)	–	–	–	+ (35.5)
RC (44.1)	–	–	–	–	–
NV (54.1)	–	–	–	–	–

For ELISA results OD ≥ 0.40 are positive, 0.30 ≥ OD < 0.40 are suspects and OD < 0.30 are negative. GL, FH, LC, RC and NV are intensive goat herds. The sheep herd is extensively reared on pastures. The estimate of the concentration of *C. burnetii* DNA in each of the PCR positive samples is presented in Table S6.

with decreasing titres and those that remained seronegative (Fagerland et al., 2013).

Mixed effects linear regression models were constructed to assess the relationship between seroreactivity to *C. burnetii* (by IgG antibody phase and by combined IgG antibody profiles) and milk production in the first 9 weeks of lactation, adjusting for parity, day of lactation and accounting for repeated measures and individual animal variability with a random effect based on goat ID. In the mixed effects model to assess the impact of *C. burnetii* infection on milk yield, seroconversion was based on presence or absence of a fourfold rise in antibody titre over the kidding period. The 9 weeks of lactation was the period between the kidding period studied and the start of the next kidding season on the same farm, a high-risk period for further exposure to *C. burnetii*. The mixed effects model used to assess effect of seroconversion on milk volumes was of the form:

$$Milkvol_{ij} = \beta_0 + \beta_1 day_j + \beta_2(parity1)_i + \beta_3(parity \geq 2)_i + \beta_4(Seroconverted(IgGP1\ only)_i$$
$$+\beta_5(Seroconverted(IgGP1\ and\ IgGP2)_i + \beta_6(Seroconverted(IgGP2\ only)_i + u_i + \varepsilon_{ij}$$
$$u_i \sim N\left(0, \sigma^2_{goat}\right), \qquad \varepsilon_{ij} \sim N\left(0, \sigma^2\right)$$

where $Milkvol_{ij}$ is the milk yield of the i^{th} goat on the j^{th} *day* of lactation; β_0 is the mean milk volume for the reference group ; β_1 is the fixed linear regression coefficient of the variable days in milk (day_j); β_2, β_3, β_4, β_5 and β_6 are class effects of indicator variables for the parity and seroconversion for the i^{th} goat; u_i is the Gaussian-distributed random effect for each goat and & ε_{ij} is the residual error (assumed to be independent and to also follow a Normal distribution). The outcomes of multivariable mixed linear regression of daily milk volumes and seroreactivity were compared to those of multivariable linear regression of seroreactivity and average daily milk volume for each day of the 9-week lactation period derived using the area under the lactation curve fitted for each goat using the Wood's model in Rstudios, after adjusting for parity (Thomson et al., 2011; Wood, 1967; Raadsma et al., 2009). All statistical analyses and plots were performed using STATA version 13.0 (StataCorp, College Station, TX) and R statistical package version 3.1.1 (R Core Team, 2014).

3. Results

3.1. Bulk milk monitoring

Antibodies to *C. burnetii* were detected in all BTM samples from goat herds on the three farms in close proximity to each other (GL, LC and FH). BTM samples from the sheep flock at GL were also positive but less frequently than the goats, with one suspect positive ELISA and two positive IFA results (Table 1 and Fig. 1). No antibodies were detected in BTM samples from the other two farms (NV and RC). *C. burnetii* DNA was detected only in BTM samples from farms GL, LC and FH. There was evidence of disagreement between the PCR and ELISA results (exact binomial test p value = 0.031), with the ELISA testing positive for all samples that tested positive by PCR, and for six further samples (Table 1). The IFA detected two more *C. burnetii* positive BTM samples than the ELISA although there was almost perfect agreement between the two tests (K = 0.87, [95% confidence interval (CI): 0.70, 1.00]). Estimates of the DSe and DSp of the ELISA, PCR and IFA on BTM samples are yet unavailable but approximations obtained by the composite reference standard procedure on our limited sample size are shown in supplementary Table S5 (Dohoo et al., 2009).

All 400 blood samples collected from individual adult goats on farm RC (that tested negative on all ELISA and PCR bulk tank milk samples) tested negative for IgG and IgM antibodies to *C. burnetii* using the IFA.

3.2. Individual animal testing

Overall seroprevalence (serological positivity to any of the antibody classes) by parity of the goats, pre- and post-kidding is presented in Table 2. Overall seroprevalence increased from 25.0% to 42.7% over the kidding period (Table 2). Most of this increase was driven by seroconversions in nulliparous animals with a rise

Table 2
Comparison of pre- and post-partum overall seroprevalence of IgG and IgM antibodies against phase 1 and phase 2 *C. burnetii* in adult goats of different parity tested using indirect immunofluourescence assay.

Parity	N	Pre-partum to post-partum change in serological status, n				Number of IFA positive goats, n (%)		Difference in prevalence (%) over the kidding period (95% CI)[a]
		+ +	+ –	– +	– –	Pre-partum	Post-partum	
≥2	42	16	2	5	19	18 (42.9)	21 (50.0)	7.1 (−7.0, 15.4)
1	36	17	2	5	12	19 (52.8)	22 (61.1)	8.3 (−8.2, −18.0)
0	86	4	0	23	59	4 (4.7)	27 (31.4)	26.7 (18.8, 26.7)
Total	164	37	4	33	90	41 (25.0)	70 (42.7)	17.7 (11.1, 21.2)

Parity grouping was based on pre-partum parity status: parity 0 is nulliparous, parity 1 is primiparous and parity ≥2 is multiparous.
[a] Calculated using McNemar's test for paired proportions. [+ +] are goats that remained positive, [+ –] are goats that changed from positive to negative, [– +] are goats that seroconverted, [– –] are goats that remained negative.

Table 3
Seroconversions among goats to *C. burnetii*, by parity.

Parity (n)	Seronegative pre-partum, n	Seroconverted, n (%)	Relative risk (95% CI)
≥1 (78)	41	10 (24.4)	0.87 (0.46, 1.65)
0 (86)	82	23 (28.0)	1.0 (reference)
Total (164)	123	33 (26.8)	

Parity grouping was performed based on pre-partum parity status. Parity 0 is nulliparous, parity ≥ 1 are parous (multiparous and primiparous groups were collapsed due to small numbers).

in seroprevalence of 26.7% (95% CI: 16.0, 37.5) across the kidding period, whereas seroprevalence only rose slightly amongst primiparous and multiparous goats (Table 2).

Majority of the infected animals were detected through testing for IgG antibodies to phase 1 and 2; as IgG to phase 1 was most prevalent (23.2%) on pre-partum testing and IgG to phase 2 was the most detected (32.9%) antibody type on post-partum sampling. Detailed data are provided as supplementary material by antibody class, antigen phase and parity, across the kidding period (Table S7). Furthermore, at pre-partum sampling, the prevalence of all antibody types (IgG and IgM to phase 1 and phase 2) was higher in parous (multiparous and primiparous) compared to nulliparous goats. At post-partum sampling, the prevalence of all antibody classes were comparable across the different parity categories of animals, except for IgG phase 1, which was 35.9% higher in parous compared to nulliparous goats (95% CI: 22.8, 49.1) (Table S7).

Amongst the goats that were seronegative pre-partum, 26.8% seroconverted to *C. burnetii* over the kidding period (Table 3). The risk of seroconversion among goats that were seronegative on pre-partum sampling was similar for nulliparous and parous goats. Phase 1 IgG antibodies were the only antibody class that differed between parity categories among previously negative does that seroconverted during kidding: Parous does had a higher but not statistically significant risk of seroconversion with IgG phase 1 antibodies than nulliparous ones (RR = 2.00; 95% CI: 0.81, 4.92); whilst the risk of seroconversion based on IgG phase 2 antibodies was similar across the different parity categories (RR = 1.0; 95% CI: 0.47, 2.14; P = 0.99), see supplementary Table S8.

Serological status pre- and post-partum, by IgG to each antigen phase and by parity is presented in Table 4, allowing comparison for the proportions of four categories of changes in antibody response: [– –] = goats seronegative pre-partum that remained negative over the kidding period, [– +] = goats seronegative pre-partum that seroconverted or goats that were seropositive and had a fourfold rise in antibody titres, [+ +] = goats with constant titres or a rise in titre that was < four-fold, and [+ –] = goats seropositive pre-partum with decreasing titres. The trend of pre- to post-kidding IgG titres (Table 4) showed that significantly more primiparous and multiparous does were already seropositive prior to kidding than nulliparous does while more nulliparous does remained seronegative than parous ones.

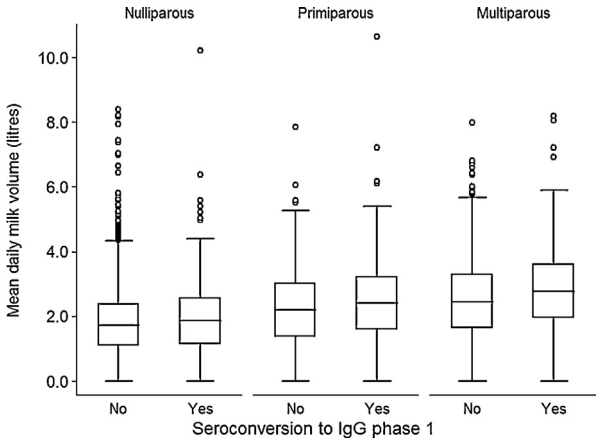

Fig. 2. Daily milk volumes of goats by parity and seroconversion status (Yes = positive based on IgG antibodies to phase 1 *C. burnetii*).

Nulliparous animals produced less milk in the first nine weeks after kidding (mean ± standard deviation [SD] = 1.804 ± 1.005 L per day) compared to primiparous (mean ± SD = 2.264 ± 1.221 L per day) and multiparous goats (mean ± SD = 2. 486 ± 1. 295 L per day). Lactation curves were approximately linear, decreasing from an early peak by 2 mL per day of lactation (95% CI: 1, 3 mL per day). Goats that seroconverted to only IgG phase 1 produced an extra 0.276 L of milk per day (95% CI: 0.010, 0.543 L; P = 0.042) compared to those that did not seroconvert; while milk volumes of goats that seroconverted to both IgG phase 1 and phase 2 and that of goats that seroconverted to only IgG phase 2 were comparable to milk volumes of goats that did not seroconvert (Table 5). These results were also comparable to those obtained by linear regression of seroreactivity and average daily milk volumes obtained from lactation curves fitted using the Wood's model (see Table S9).

Similar results were obtained on comparison of milk yield among goats with different combined seroconversion profiles; with goats that seroconverted based on IgG antibodies to phase 1 *C. burnetii* produced 0.206 L more milk per day (95% CI: 0.045, 0.367 L per day; P = 0.012) in the first 9 weeks of lactation compared to those that did not seroconvert, after adjusting for parity and day of lactation (Fig. 2; Supplementary Table S10). Further comparison of seroconversion based on IgG phase 1 to other changes in antibody titres i.e. [+ –] (seroreversion), [+ +] (remaining positive) and [– –] (remaining negative), did not affect the statistical significance of the association between mean daily milk volume and seroconversion to IgG phase 1 (Supplementary Table S11). There was no statistically significant interaction between the effects of parity and serological status on milk volume.

Table 4
Comparison of the proportion of goats with different patterns of pre-partum to post-partum changes in IgG antibody titres to *C. burnetti* over the kidding period.

Antibody type	Parity	N	Pre-partum to post-partum changes in antibody titres, n (%)				Difference in proportions (McNemar)
			− −	+ −[a]	+ +[b]	− +[c]	
IgG phase 2	≥2	42	20 (47.6)	7 (16.7)	2 (4.8)	13 (30.9)	14.3 (−8.8, 33.0)
	1	36	17 (47.2)	4 (11.1)	6 (16.7)	9 (25.0)	13.9 (−8.3, 29.5)
	0	86	66 (76.7)	0 (0)	0 (0)	20 (23.3)	23.3 (15.4, 23.3)
IgG phase 1	≥2	42	22 (52.3)	7 (16.7)	2 (4.8)	11 (26.2)	9.5 (−12.2, 28.0)
	1	36	12 (33.3)	7 (19.5)	4 (11.1)	13 (36.1)	16.7 (−10.2, 38.5)
	0	86	74 (86.0)	1 (1.2)	0 (0)	11 (12.8)	11.6 (3.2, 13.9)

[+] = positive, [−] = Negative. [− −] is the proportion of goats that remained negative; [+ −] is seroreversion but also includes.
[a] (goats with decreasing antibody titres). [+ +] are goats that stayed with constant titres or.
[b] (goats with increasing titres that were less than fourfold). [− +] are goats that seroconverted and those [c](those that had a fourfold rise in antibody titres).

Table 5
Mixed effects linear regression models for differences in milk volume (litres) in presence of different IgG antibody titres to either phase 1 or phase 2 *C. burnetii*.

Model: Outcome variable	Explanatory variable	Categories	Coef.	SE(Coef.)	(95% CI)	P-value[a]
Seroconversion based on IgG	Day of lactation (l)		0.002	0.001	(0.001, 0.003)	0.001
	Parity[b]	≥2	0.728	0.086	(0.280, 0.617)	<0.001
		1	0.448	0.079	(0.574, 0.883)	<0.001
		0	0	-	(reference)	-
	Seroconverted[c]	P1 only	0.276	0.136	(0.010, 0.543)	0.042
		P2 and P1	0.173	0.095	(−0.013, 0.360)	0.069
		P2 only	−0.010	0.105	(−0.215, 0.195)	0.922
		Negative	0	-	(reference)	-
	Constant		1.690	0.053	(1.587, 1.794)	<0.001
			Est.	SE (Est.)		
	Random effect	per goat	0.149	0.019	(0.116, 0.191)	<0.001
	−Log likelihood		12807.057			

[a] P-values estimated using Wald test statistic for explanatory variables and the likelihood ratio test for random effect terms.
[b] Parity grouping was done based on pre-partum parity status. Parity 0 is nulliparous, parity 1 is primiparous, parity ≥2 is multiparous.
[c] Seroconversion defined as a ≥ fourfold increase in antibody titre between samplings. P1 = phase 1, P2 = Phase 2, Coef = coefficient, SE = standard error, CI = confidence interval, Est = Estimate.

4. Discussion

The use of paired samples and detection of a four-fold rise in antibody titre is routinely used in humans and occasionally in some domestic animals to detect *C. burnetii* infections using IFA (Wisniewski and Krumbiegel, 1970; Moore et al., 1991; Marrie, 1995; Fiorillo et al., 2013). Using paired sera to detect increasing antibody titres and to diagnose active *C. burnetii* infection may not have been widely adopted for livestock herds given the cost considerations (Böttcher et al., 2011). However, when properly implemented, paired testing of individual animals provides important information on infection dynamics that can be crucial for identifying risk factors as well as for developing control protocols.

Our results showing a more marked increase in seroprevalence among nulliparous goats after exposure to kidding (Table 2) points to higher susceptibility among these animals compared to those that have previously kidded. The occurrence of a similar risk of seroconversion amongst those seronegative prior to kidding, irrespective of parity, implies relatively constant risk of infection among all goats. This also suggests a common source and intensity of exposure such as from a heavily contaminated environment. Therefore, there is a need to implement control protocols among all goats in infected herds, regardless of parity.

Nonetheless, control measures are likely to achieve the greatest benefit when applied to cohorts containing many seronegative animals, such as young does kidding for the first time, whose seroprevalence increased from 4.7% to 31.4% in our study. Presumably widespread infection occurs in this cohort when they are exposed to high loads of *C. burnetii* shed in the placenta and birth fluids of their older infected pen-mates giving birth at the same time. This not only underscores the need to protect these animals before they are exposed to kidding but also suggests the separation of nulliparous goats from older does at the time of their first kidding could be an additional strategy to reduce transmission of infection (Berri et al., 2001; Guatteo et al., 2008; Astobiza et al., 2011; Böttcher et al., 2011).

The low pre-partum seroprevalence in nulliparous goats also seems to suggest that these goats may possibly have had limited exposure to *C. burnetii* at the time they were born, a presumably high risk period for exposure and transmission of *C. burnetii*. Additionally, given that this study was performed on a goat farm where *C. burnetii* infection was highly endemic, it still remains unclear why so many of the nulliparous goats had remained seronegative before their first kidding, given their close proximity to sheds of adult goats with very high seroprevalence. However, low pre-partum prevalence among nulliparous goats has also been previously reported in a study undertaken during a Q fever outbreak on a goat farm in France; where 94.7% of seronegative goats were nulliparous (de Cremoux et al., 2012). Perhaps, close proximity or contact with birth fluids and tissue, accentuated by the sniffing and consumption of birth materials by goats (Yilmaz et al., 2012), is a more important mode of *C. burnetii* transmission in goats than airborne transmission through contaminated dust which is believed to occur at distances of as far as 5 km in humans (Leski et al., 2011; De Bruin et al., 2012).

An alternative explanation may be that exposure to lower doses of *C. burnetii* only triggers low and short-lived antibody titres that had diminished by the point of sampling (in the last month of gestation). However, it is highly unlikely that goats infected with *C. burnetii* would fail to develop detectable antibody titres by the last month of gestation given the affinity that the organism has to placental tissue and the enormous replication of the organism within the placenta reported to occur in pregnant goats particularly late in gestation (Baumgärtner and Bachmann, 1992; Amara et al., 2010). This also explains our sampling of goats one month before kidding and highlights the robustness of our serological findings,

as pre-partum samples provide a pre-exposure baseline while the post-partum samples illuminate changes arising from exposure to high loads of *C. burnetii* released in the placenta and birth fluids at the time of kidding (Berri et al., 2001; Guatteo et al., 2008; Astobiza et al., 2011; Böttcher et al., 2011).

The finding that the seroprevalence was significantly higher in parous (parity ≥1) compared to nulliparous goats suggests a build-up in the overall herd seroprevalence as more animals join the kidding herd. This may also point to the development of higher and longer-lived antibody titres after repeated exposure to high doses of *C. burnetii* shed at kidding as suggested by our finding that parous goats had, and maintained, higher titres of IgG to phase 2 and phase 1 antigens, both before and after kidding, than nulliparous goats (Table 4). It is, however, not clear whether a build-up of herd humoral immunity is reflective of protection versus re-infection and subsequent contamination of the environment.

The protective ability of antibodies to *C. burnetii* has been demonstrated in a study in which administration of immune serum in experimental mice before challenge with *C. burnetii* led to increased resistance to infection and clearance of *C. burnetii* organisms from the spleens of the mice (Humphres and Hinrichs, 1981). Another study performed in mice showed that B-cells producing IgG and IgM antibodies against phase 1 *C. burnetii* play a major role in protection against infection, providing further evidence for a role of humoral immunity in protection against *Coxiella* infections (Zhang et al., 2007; Zhang et al., 2013). However, even if humoral immunity is protective against subsequent challenge with *C. burnetii*, only 42.7% of the herd was seropositive to *C. burnetii* after exposure to kidding.

From the BTM testing results, the occurrence of *C. burnetii* infection on the enterprise seemed to be centred on the three goat farms (LC, FH and GL) within a distance of 2 km from each other and not on the other two (RC and NV) goat farms located >40 km away. We also serologically demonstrated disease freedom in one of the BTM-negative herds (farm RC), even though it shares a milk truck and other vehicles, plus some staff, with the infected farms GL, FH and LC. This suggests that the risk of transmission of *C. burnetii* on fomites is very low.

Fewer samples from the milking sheep flock on farm GL were positive for antibodies, despite the flock's proximity to the goat herds on the three infected farms. This may have been because excretion of *C. burnetii* in ovine milk is reportedly short-lived (Woldehiwet, 2004). Alternatively, the difference may be because the sheep are raised extensively on pastures at much lower stocking densities than the goat enterprise, reducing the rate of intra-flock transmission and intensity of environmental contamination.

The DSe of the tests used on BTM samples could have been affected by the dilution of milk from positive goats by milk from negative goats, the intermittent shedding of *C. burnetii,* or even the proportion of positive goats at the time of sampling (Guatteo et al., 2007; Woldehiwet, 2004). Cross-reactivity with other agents like *Chlamydia* spp. (Lukáčová et al., 1999) could also have occurred resulting in reduced DSp. However, all the three tests have previously been reported to be highly specific for *C. burnetii* antibodies in goat serum (Muleme et al., 2016) and to have a high analytical specificity for *C. burnetii* DNA (Lockhart et al., 2011). Lockhart et al., 2011; demonstrated that the PCR targeting the com1 gene of *C. burnetii*, which was used in this study, was negative on DNA extracted from a wide range of bacteria (Lockhart et al., 2011).

The effect of pooling milk from infected and non-infected goats in BTM on the DSe and DSp of the tests could not be satisfactorily assessed using the samples available for this study. This would require Bayesian latent class modelling using reasonably high numbers of samples from farms of unknown *C. burnetii* status as well as BTM samples from known *C. burnetii* positive and negative farms (Branscum et al., 2005). However, the ELISA and the IFA detected exposure to *C. burnetii* in more BTM samples than the PCR, as has been previously reported (Muskens et al., 2011) and as reflected in the composite reference standard estimates of DSe (PCR 56%; ELISA 89%) (Supplementary Table S5). Given that the intention of BTM testing here was to identify herds exposed to *C. burnetii,* we consider limitations due to decreased DSe of the tests on BTM samples to have been reduced by the regime of multiple testing (5 times) of each farm and the interpretation of three tests in parallel.

Similarly, the Dse and Dsp of the IFA on serum would imply that between 0.4 and 19.7% of truly positive goats could be undetected by the IFA and between 0.7–22.9% of goats classified as test positive could be false positives due to cross-reaction of the conjugate with components of the goat sera or other microorganisms. This could possibly lower the estimated prevalence and may have introduced misclassification of individuals. We consider this would not have major implications on the key findings of this study given that it affects all comparison groups equally (i.e. a non-differential misclassification).

Our finding of comparable milk yields amongst goats that seroconverted to IgG phase 2 titres and those that did not seroconvert suggests that acute *C. burnetii* infections has little if any effect on milk yields in the first 9 weeks of lactation. We did not use data for the full 330-day lactation period, because there were only 9 weeks between the kidding period studied and the start of the next kidding season on the same farm, a high-risk period for further exposure to *C. burnetii*. Prior information on the infection status of the goats outside the study period was also not available, but serological findings were not affected by vaccination since vaccination of livestock against *C. burnetii* is not available in Australia. Additionally, there could have been confounding factors unknown to us that could have influenced seroconversion and milk yield. Future research could longitudinally sample and test does from each of the four kidding seasons in the year to describe the effect of acute infections on milk production for the full lactation period.

An unexpected finding was the increased milk yield in goats that had seroconverted to IgG antibodies against phase 1 *C. burnetii* antigens, compared to those that had not. IgG antibodies against phase 1 antigens appear later than antibodies targeting phase 2 antigens, thus seroconversion based on IgG phase 1 antibodies may be indicative of a longer or repeated exposure to *C. burnetii* as well as a fully developed humoral immune response when the goats may possibly have acquired the ability to cope with the effects of acute infections on milk yields (Powell and Stallman, 1962; Kimbrough et al., 1979; Peacock et al., 1979; Zhang et al., 2012). Greater milk yields in seropositive animals have also been reported in cattle infected with *Mycobacterium avium paratuberculosis* and were attributed to the ability of seropositive animals to overcome the effects of infection (Nielsen et al., 2009).

Further investigations into this relationship are indicated to ascertain whether or not it is a causal association; as this study found comparable milk yields among goats that seroconverted to phase 2 and those that did not seroconvert. A limitation of this study that may provide an explanation for this finding is that numerous statistical comparisons were conducted and this may have introduced multiple testing error. However, it is also possible that the acute infections take longer than 9 weeks to cause reductions in milk yields in affected goats. We therefore recommend further research to assess the effect of seroreactivity on milk yields over the entire lactation period as evidence of *C. burnetii*-associated milk production losses could be an additional motivation to implement *C. burnetii* control programs on intensive dairy goat farms.

This study involved Saneen goats on five farms of a large intensive goat dairy enterprise; and all the farms had similar husbandry and management protocols. Thus, a random selection of 65% (164/252) of the goats due to kid is unlikely to have posed considerable selection bias. Also, given that only the one breed of goats

is used across the enterprise and the same animal husbandry protocols are implemented, the results are highly likely to be valid for other farms on the property. Additionally, the findings of this study are most likely to be generalisable to other large intensive farms with synchronised kidding seasons. No other study has estimated the risk of seroconversion to *C. burnetii* in adult goats in intensive farms across the kidding season, but results from a study undertaken during a Q fever outbreak in England reported high (78%) seroprevalence of *C. burnetii* in goats kidding for the first time, 24 days after the last abortion, which points to the susceptibility of this cohort of goats to *C. burnetii* shed by their older infected pen-mates kidding at the same time (Reichel et al., 2012).

5. Conclusions

In this herd, there was a greater increase in seroprevalence over the kidding period in goats kidding for the first time (26.7%) compared to older goats (8.3%) which points to higher susceptibility to *C. burnetii* infection among goats kidding for the first time and the possibility of controlling *C. burnetii* transmission through the separation of first-time kidders from older goats. Many nulliparous goats had remained seronegative until kidding whilst in close proximity to sheds of adult goats with very high seroprevalence, suggesting that contact with birth fluids and tissue at kidding is a more important mode of *C. burnetii* transmission in goats than airborne transmission through contaminated dust.

Importantly, the risk of seroconversion over the kidding period was similar in all goats that were seronegative pre-partum irrespective of parity, which underscores the need to implement control strategies involving all goats on affected farms and to time these interventions to ensure all goats are protected from infection before each kidding period.

We did not detect a milk production loss associated with acute *C. burnetii* infections based on seroconversion to IgG phase 2 antigens within the first nine weeks of lactation. However, goats that seroconverted to IgG against phase 1 antigens produced more milk than those that did not seroconvert; a result we did not expect which should be investigated to ascertain whether or not, the association is causal. We therefore recommend further research to assess the effect of seroreactivity on milk yields over the entire lactation period.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2017.02.006.

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