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Research Article

Cyclical Shedding and Sustained anti-Phase 1 Antibody Responses in a Goat Herd Naturally Infected with *Coxiella burnetii*

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Abstract

Coxiella burnetii is a worldwide zoonotic pathogen that can infect a variety of species and can cause Q fever in humans. Although an infected goat herd is a risk to human health, the duration of shedding and antibody responses in infected goats have not been clearly defined. In 2011, an outbreak of human Q fever in Washington and Montana was associated with infected goats, and one herd offered an opportunity for longitudinal sampling to investigate antibody responses and shedding of *C. burnetii*. Serum, vaginal swab, and milk samples were taken from the herd during the initial outbreak investigation, with follow-up sampling 3, 11, and 14 months later. Serum was tested for anti-*C. burnetii* antibodies by immunofluorescence assay (IFA), and vaginal swabs and milk were tested by polymerase chain reaction (PCR). Phase 2 antibody titers were undetectable in 22/23 goats by the end of the study, but 17/23 goats still had a detectable Phase 1 titer. The goats also shed high amounts of *C. burnetii* in both vaginal secretions and in milk. The shedding was cyclical with high amounts in kidding season, lower amounts 3 months later, and higher amounts returning in the next kidding season. The correlation between shedding and antibody titer was poor. The data show that goats infected with *C. burnetii* pose a risk for human exposure for at least 18 months after infection, and this risk is elevated during the kidding period.

Keywords: Q fever; *Coxiella burnetii*; Goat; Herd; Shedding

Introduction

Coxiella burnetii is an intracellular bacterium found in most parts of the world. The organism can infect mammals, reptiles, fish, and arthropods [1]. Cattle, sheep, and goats are the reservoir species most commonly associated with human *C. burnetii* infections, and the organism can replicate to very high levels in the placenta of these species [2-5]. This can result in abortion, stillbirth, or weak offspring, and the release of large amounts of *C. burnetii* into the environment at parturition [6-8]. Transmission to humans primarily occurs via inhalation of aerosols. Approximately 50% of humans will have symptomatic Q fever after *C. burnetii* infection [9]. Symptoms typically include fever, headache, and myalgia, and in some cases pneumonia and/or hepatitis can be present [9].

Although infection of humans with *C. burnetii* has been linked with exposure to many different species, in recent years goats have taken a prominent role as a reservoir for human exposure. Infected goats have been responsible for multiple human outbreaks in various parts of the world [10-13]. Most notably, dairy goats were implicated in an epidemic of Q fever that took place in the Netherlands from 2007-2010 which resulted in over 4,000 human cases [14, 15]. A nationwide goat culling and vaccination campaign ap-

peared to bring an end to the epidemic and the country has repopulated with goats vaccinated against Q fever [16]. The number of human cases in the Netherlands has declined after the control measures were implemented, with 20 notifications of Q fever in 2013 [17].

The prominent role of goats as a *C. burnetii* reservoir has raised questions regarding the natural history of *C. burnetii* infection in goat herds. The ability of *C. burnetii* to spread through a herd and the duration of shedding once a herd has become infected are important parameters that will determine the overall risk of infection for humans exposed to the herd. These parameters will also influence the reproductive fitness of the herd, which can be compromised by the presence of viable *C. burnetii*. Introduction of *C. burnetii* into a naïve goat herd may result in an "abortion storm" which can be the first indication that the herd is infected [2].

Previous studies have examined *C. burnetii* infection in goats. A 2003 study examined infected goats in Canada and found that antibodies against Phase 1 *C. burnetii* were present at high levels in the herd for at least two kidding seasons after the initial infection, but shedding of *C. burnetii* from placentas was limited in the second kidding season [18]. In a 2006 study from France, most of the goats in a naturally infected dairy goat herd were seropositive 6 weeks after the initial abortion storm and this was maintained for at least 10 months [19]. Shedding in vaginal secretions was documented at least 20 months after the initial episode. These studies indicate that *C. burnetii* may set up a persistent infection in goats and a strong antibody response may not be sufficient to eliminate *C. burnetii*. Experimentally infected goats shed very high amounts of *C. burnetii* at parturition, but shedding declines to low levels by 40 days postpartum [20]. These data suggest that exposure to an infected herd may pose a human health risk for at least 2 years after introduction of *C. burnetii* into the herd, but it is not clear whether or not shedding of *C. burnetii* is a serious concern outside of the periparturient period.

In the current study, a goat herd in Montana was examined for the presence of *C. burnetii* in vaginal secretions and milk, and for serum antibody against *C. burnetii*. This herd experienced abortions, stillbirths, and weak neonates in the 5 months prior to the first sampling. Q fever cases in people living on the farm during this time period were also documented [21]. Samples were taken from the goats on four occasions extending to 18 months after the suspected introduction of *C. burnetii* into the herd to evaluate transmission dynamics and long-term shedding.

Materials and Methods

Animals

The animals examined in this study were Boer goats. The farm was located in Montana and linked to a multi-state human Q fever outbreak in 2011 [21]. The date that *C. burnetii* was introduced to the farm cannot be stated with certainty, but was likely due to the purchase of goats from a herd in Washington experiencing a documented outbreak of *C. burnetii* infection at the time of purchase in early 2011 . Reproductive problems in this goat herd were noted in the spring of 2011. The goats were sampled in June 2011, September 2011, May 2012, and August 2012. The number of goats on the farm varied, with a low of 55 goats (JUN2011) and a high of 188 goats (MAY2012). Other animals on the farm included 2 cows and 13 sheep as well as multiple dogs, pigs and poultry. Female goats with specimens collected at all 4 sampling occurrences were included in this analysis (N=23).

Serology

Blood was drawn from the goats by venipuncture into serum separator tubes. The tubes were centrifuged and serum removed and stored at 4˚C until analysis. Antibodies against *C. burnetii* were detected using an in-house indirect fluorescent antibody test (IFA). Wells on glass slides were coated with acetone-fixed yolk-sac preparations of either Nine Mile Phase 1 or Phase 2 antigen, and serial two-fold dilutions of the goat serum were incubated on the slides. Goat antibody was detected using a fluorescein conjugated rabbit anti-goat antiserum (KPL, Inc.)

Vaginal swabs

Vaginal samples were taken by insertion of a dry and sterile cotton swab into the vagina of the goats. Swabs were stored at 4˚C until analysis. To extract DNA from the swabs they were incubated in 0.8 ml of phosphate buffered saline (PBS), pH 7.4 at 35˚C for 1 hour with shaking at 200 rpm. DNA was purified from 0.2 ml of the PBS extract using the QIAamp DNA mini kit tissue protocol (Qiagen, Valencia, CA).

Placenta

Two placenta specimens were also collected and analyzed from this farm in April and September as has been previously reported [8]. Placenta tissue DNA was isolated by processing 0.2 grams of tissue using the QIAmp DNA mini kit (Qiagen) according to the manufacturer's tissue protocol.

Milk

DNA was isolated from milk as described previously [22]. Milk from goats was collected in sterile plastic tubes without preservative and stored at 4˚C. The milk was centrifuged for 15 minutes at 1,700 x g, washed with PBS, pH 7.4 and then centrifuged again for 15 minutes at 1,700 x g. The pellets were re-suspended in 1 ml PBS and then centrifuged at 20,000 x g for 5 minutes. The pellets were re-suspended in 200 microliters of ATL buffer from the QIAmp DNA mini kit (Qiagen), and DNA was purified using the tissue protocol from that kit.

PCR

Quantitative PCR to detect the IS1111a insertion sequence was performed as described previously [23]. One microliter of DNA extract was used in each 25 microliter

PCR reaction. Samples were run in duplicate and samples for which both reactions crossed the threshold (C(t)) in less than 40 cycles were considered positive. Samples that had one positive and one negative reaction were run a second time in duplicate and only samples where both reactions had a C(t) of less than 40 were considered positive. Genomic equivalents (GE) of *C. burnetii* were calculated by comparing experimental C(t)'s to a standard curve of *C. burnetii* Nine Mile Phase 1 genomic DNA. It was determined that *C. burnetii* isolated from this farm had approximately 57 copies of IS1111a, whereas Nine Mile Phase 1 has only 19 copies of the PCR target. The GE were then adjusted accordingly, with the assumption that all *C. burnetii* detected in these goats had 57 copies IS1111a.

Results

C. burnetii was introduced into the herd in early 2011 and serum was drawn in JUN2011, SEP2011, MAY2012, and AUG2012. The serum drawn from the goats in JUN2011 was tested as part of a multi-state Q fever outbreak investigation [21, 24]. The testing was done at the U.S. Department of Agriculture National Veterinary Services Laboratories (NVSL) by ELISA and included goats from multiple farms. Twenty-three goats met sampling criteria and were included in this study. Of the 23 goats, 71% were found to be positive in JUN2011. Follow-up testing was done on the Montana farm in SEP2011, MAY2012, and AUG2012. Using a cut-off of 1:64 for a positive result, the IFA test demonstrated that 91%, 87%, and 74% of the 23 goats were positive for anti-Phase 1 antibodies at the three follow-up dates, whereas 22%, 57%, and 4.3% of the goats were positive for anti-Phase 2 antibodies on those testing dates, respectively (Table 1). In addition to a predominantly Phase I antibody response, the titers against Phase 1 *C. burnetii* were much higher than Phase 2 titers in the herd. Titers against Phase 1 ranged up to 1:32768 with geometric means of 1:1024, 1:1709, and 1:264 at the three testing dates. Phase 2 titers did not exceed 1:2048 and geometric means were 1:23, 1:71, and 1:9.3 at the three sampling dates (Table 1). Negative samples were assigned a value of 1:8 for the calculation of the geometric means.

Table 1. Serological responses in goats at successive sampling dates.

Sampling date	positive % Phase 1	positive % Phase 2	Geometric mean Phase 1	Geometric mean Phase 2
SEP2011	91	22	1024	23
MAY2012	87	57	1709	71
AUG2012	74	4.3	264	9.3

The presence of *C. burnetii* in vaginal secretions was tested by PCR analysis of swab samples taken at all four sampling dates. The percentage of positive swabs was high at the first three sampling dates (91, 87, and 100 percent positive) but declined to 32% positive in AUG2012 (Table 2). The mean number of genome equivalents in the swabs was elevated in JUN2011 (33,368 GE/swab), declined in SEP2011 (54.4 GE/swab), highest in MAY2012 (244,209 GE/swab), but decreased again in AUG2012 (66.6 GE/swab). The sampling dates with high vaginal shedding (JUN2011 and MAY2012) were during the kidding season on this farm. Although breeding and kidding were sporadic in this herd, the majority of pregnant does were kidding between February and June. The high mean of genome equivalents shed in JUN2011 and MAY2012 was to some extent influenced by a small number of goats with very high shedding. In particular, one goat sampled in MAY2012 had nearly 5 million genomes on its swab sample. However, median values for GE/swab (Table 2) show that higher shedding was widespread among goats tested in JUN2011 and MAY2012 (787 and 1312 GE/swab respectively) compared to the SEP2011and AUG 2012 sampling dates (35 and 0 GE/swab respectively).

Table 2. Shedding of *C. burnetii* in goat vaginal mucus at successive sampling dates.

Sampling date	of PCR positive % vaginal swabs	Mean GE*/ swab	Median GE/ swab
JUN2011	91	33,368	787
SEP2011	87	54.4	35
MAY2012	100	244,209	1312
AUG2012	32	66.6	0

*GE=genome equivalent

The placenta samples collected in April and September had a mean number of genome equivalents of 1.5 X 10^8/gram and 2.5 X 10^8/gram, respectively [8]. *C. burnetii* shed in milk followed a similar pattern to the shedding in the vaginal secretions. Although only 5/23 goats were tested in JUN2011, 100% of those were positive with a mean GE/ml of 13,418. In SEP2011, 15 samples were tested and 73% were positive with the mean GE/ml dropping to 495. In MAY2012, 22 samples were tested and 100% were positive with a mean GE/ml of 3,115. In AUG2012 81% of the 21 tested samples were positive with a mean GE/ml of 1,330. Median values did not fluctuate as much for the milk samples but did follow the pattern of increasing after each of the two kidding seasons (Table 3).

Table 3. Shedding of *C. burnetii* in goat milk at successive sampling dates.

Sampling date	PCR positive milk %	Mean GE*/ml	Median GE/ml
JUN2011	100	13,418	455
SEP2011	73	495	130
MAY2012	100	3,116	668
AUG2012	81	1,330	120

*GE=genome equivalent

Whether or not antibody titers against *C. burnetii* can be used to predict shedding is not known. Some goats in this study had very low titers but very high shedding on the same sampling date. Figure 1A shows the Phase 1 antibody titers of the 23 goats in MAY2012 plotted against the shedding in

vaginal secretions. The animal shedding the highest amounts in vaginal secretions has a negative titer, so clearly positive serum antibody was not a requirement for high shedding. Although there may be a trend toward higher titers in some of the higher shedding goats, overall the correlation between shedding and titer was not statistically significant (Pearson correlation, p=0.76). Likewise, shedding in milk did not correlate with the Phase 1 IgG titer (p=0.73; Figure 1B) or shedding in vaginal secretions (p=0.2; Figure 1C).

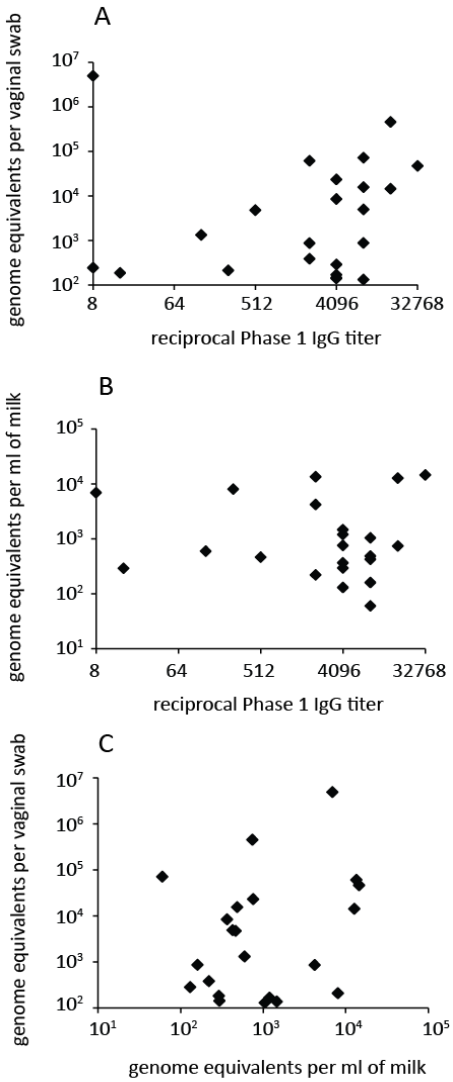

Figure 1. Comparison between shedding in vaginal mucus (A) or milk (B) and serum IgG antibody titers against Phase 1 *C. burnetii*. Panel C shows a comparison between shedding in vaginal mucus and milk

Discussion

The data from this study indicates that goats can shed *C. burnetii* for at least 16-18 months after documented infection, and shedding from goats can be cyclical with increased shedding during the kidding season followed by a reduction in shedding 3-4 months later. Although the highest amounts of *C. burnetii* are shed from goats via the placenta, this study also found high levels of *C. burnetii* in vaginal secretions and milk. When compared to the first sampling date (JUN2011) the shedding in vaginal secretions and milk had declined to low levels at the final time point tested for this study (AUG2012). However, the shedding was cyclical and the amount of *C. burnetii* shed from positive goats did not dip as low in AUG2012 as it did in SEP2011. This suggests that the low shedding in AUG2012 did not signify the end of shedding from this herd. The shedding rebounded to high levels between SEP2011 and MAY2012, and shedding could increase again in subsequent peripartum periods. However, the herd has been sold and this cannot be measured.

Higher amounts of *C. burnetii* were shed from these goats in May and June compared to August and September. The reason for the cyclical nature of the shedding is not known. Most of the kidding on this farm occurred between February and June. The periods of higher shedding therefore corresponded to the kidding season. It is possible that pregnancies in these goats resulted in growth of more *C. burnetii* in the placenta which was followed by increased shedding for a period after parturition which would then decline until a subsequent pregnancy. Complete information on pregnancies and kidding for these goats is not available, so it is not possible to say with certainty that all of the increased shedders were pregnant each year. Another possible contributing factor to enhanced shedding in May and June is re-infection of the goats each year. Even goats that were not pregnant could be exposed to large amounts of *C. burnetii* from the birth products of infected goats that gave birth in February-June. Large numbers of *C. burnetii* could come directly from the released placentas, or could come from *C. burnetii* deposited in the environment. Analysis of environmental samples on this farm found very high amounts of *C. burnetii* in environmental samples taken from goat birthing areas in JUN2011 and MAY2012 [8]. Another possible route of re-infection is sexual transmission from male goats [25].

The cyclical shedding in goats could explain the differences between previous studies involving *C. burnetii* infection of goats. A study of experimentally infected goats found that shedding declined fairly rapidly after parturition, reaching low levels by 40 days postpartum [20]. This is in contrast to studies of naturally infected goats that found high shedding up to 20 months after the initial infection [18, 19]. However, in the studies of naturally infected goats, the animals were primarily sampled close to the time of kidding. Taken together, the results suggest that goats shed large amounts of *C. burnetii* in placenta, vaginal secretions, milk, and feces when they give birth. Shedding is likely to decline in the weeks postpartum but increase again during at least the subsequent pregnancy.

This study also demonstrates differences in the routes of shedding from goats infected with *C. burnetii*. The highest amounts of *C. burnetii* observed on this farm were in goat placentas. Although only two placentas were analyzed from the farm, both had greater than 10^8 GE per gram in the placental tissue, and viable *C. burnetii* isolates were obtained from both placentas [8]. Examination of long-term shedding

found *C. burnetii* present in a high percentage of both vaginal secretions and milk, with the percentages increasing during the kidding season. The number of GE being shed was more stable in milk, but the numbers of GE went to much higher levels in vaginal secretions in JUN2011 and MAY2012. It is not clear if either the vaginal or the milk route is effective at releasing *C. burnetii* into the local environment. Examination of environmental samples on this farm found that high levels of *C. burnetii* were found primarily in birthing areas and the house[8].Because the animal caretakers on this farm had nursed newborn kids in the house, it is likely that kidding was a primary way for *C. burnetii* to contaminate this environment.

Serological analysis of the goats in this study revealed that responses to Nine Mile Phase 1 *C. burnetii* were more robust and durable than responses to Nine Mile Phase 2. At the final sampling date in AUG2012, all but one of the goats was negative for antibodies against Phase 2, whereas 74% were still positive against Phase 1. The geometric mean titers shown in Table 1 show that on average, titers were much higher against Phase 1 compared to Phase 2. These titers went as high as 1:32768, and 8 goats had titers ≥1:8192. Only one goat had a Phase 2 titer greater than 1:1024 and this goat's titer declined to undetectable levels by the end of the study. All goats except 1 had a peak phase 1 titer in MAY2012 followed by a decline.

The reason for higher titers against Phase 1 *C. burnetii* compared to Phase 2 in this herd is not known. Early in human infections, Phase 2 responses are typically higher than Phase 1 responses. Usually, the bacteria are cleared and the Phase 2 antibody titers will decline very slowly as Phase I titers begin to rise [26]. Occasionally, the bacteria are not cleared, chronic Q fever develops, and Phase 1 titers will gradually increase and stay elevated as long as viable *C. burnetii* are present [9]. There could be a similar pattern of antibody responses in goats. Although there may be some role for re-infection, *C. burnetii* is essentially establishing a persistent infection in the goats, with continued shedding of *C. burnetii* for at least 18 months. This long-term infection may be required for development of Phase 1 antibodies. In this study it was not possible to measure antibody titers immediately after infection because the exact time of infection is not known for any of the goats and some of the goats were likely infected 3-4 months prior to the first sampling. The reason for the slow development of Phase 1 antibodies in humans is not known, so it is not possible to determine if a similar mechanism is operating in goats.

The presence of high Phase 1 titers in the goats does not prevent the shedding of *C. burnetii*. There is a trend toward higher shedding of *C. burnetii* from goats that have high Phase 1 titers; but the correlation is not statistically significant, and both high and low titers can be found in high-shedding goats. Thus, similar to humans with chronic infection, high levels of Phase 1 antibody are not sufficient to clear *C. burnetii* from the animal. The increase in shedding between SEP2011 and MAY2012 corresponds to an increase in Phase 1 titers over the same time frame. This is consistent with a higher bacterial burden driving the increase in Phase 1 titers, but does not exclude other reasons for the increase in titers.

In summary, this study reveals several important features of natural *C. burnetii* infection in goats. First, goats develop weak Phase 2 antibody responses that decline fairly rapidly. Phase 1 titers are elevated and persistent in most goats for at least 18 months. Second, goats with high antibody titers will continue to shed high amounts of *C. burnetii* in vaginal secretions and milk. The shedding is cyclical with higher amounts shed during the time of kidding. The shedding continues for at least 2 kidding seasons after the initial infection. Third, there is a poor correlation between shedding and antibody responses, with both high and low titers being found in goats that shed large numbers of bacteria. These findings demonstrate the need for education of the public and livestock owners regarding the zoonotic risk for Q fever transmission particularly during high-risk periods of shedding.

Note:

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the CDC or other represented agencies.

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