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Clinical aspects of C. burnetii infection in dairy cattle

Joan Tutusaus Batlle

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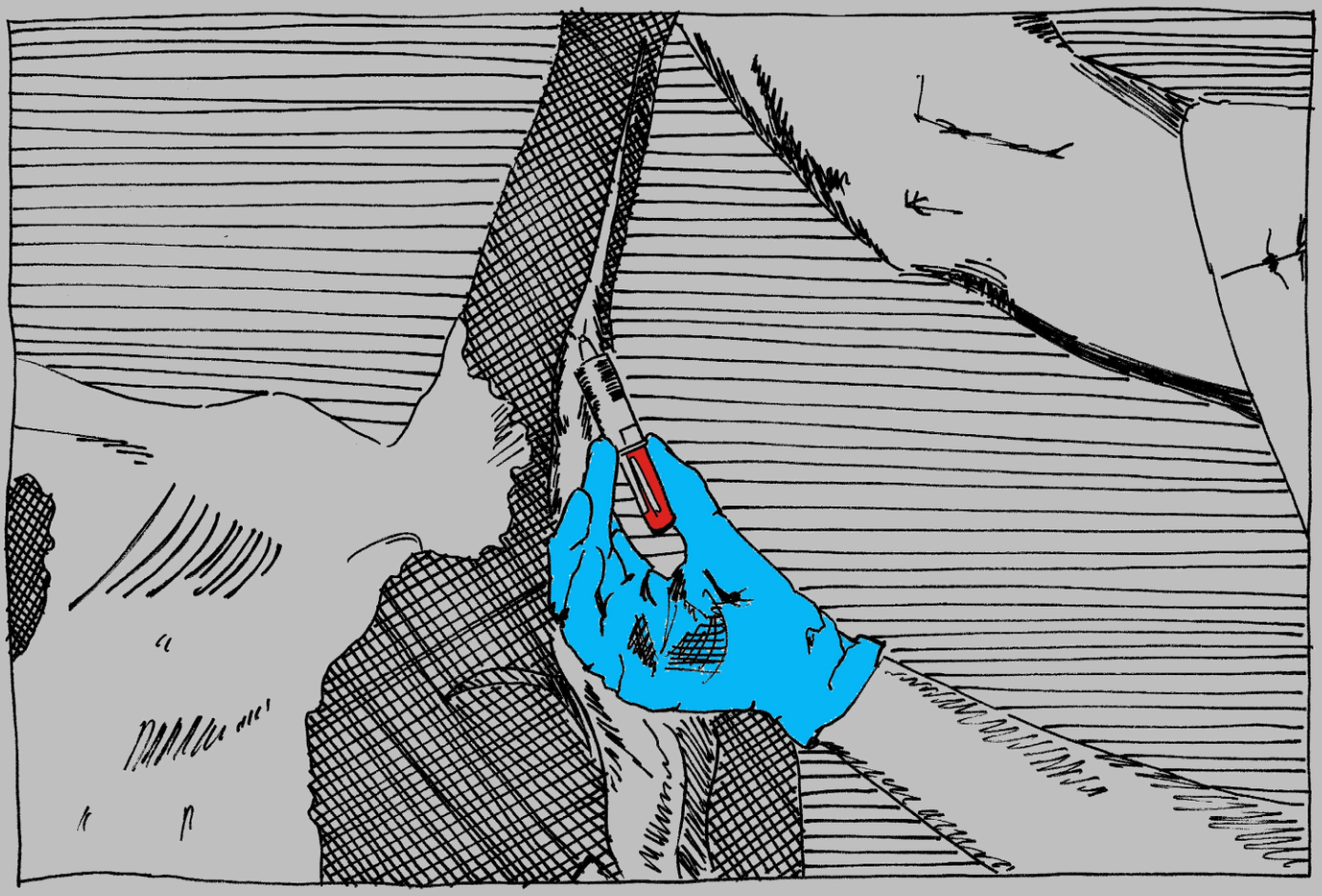

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Clinical aspects of *C. burnetii* infection in dairy cattle

DISSERTATION

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Cover picture by Alba Guiral Herrera, "*Cow blood sampling"*.

Back cover pictures: Hauptner syringe, 7.5 MHz-Ultrasonography of a cross section of a uterine horn, blood plasma samples, Vero cell exposing the contents of a vacuole where *Coxiella burnetii* are growing, dairy cattle farm.

‘Just as the largest library, which is badly arranged, is not as useful as a well arranged moderate one, therefore, the greatest amount of knowledge if not elaborated by your own thoughts, is worth much less than a far smaller volume that has been abundantly and repeatedly thought over.’

Arthur Schopenhauer

“De la mateixa manera que una biblioteca gran i desordenada és menys útil que una de modesta però ben ordenada, una gran quantitat de coneixements, si no han estat elaborats a partir dels teus propis pensaments, tenen menys valor que un volum inferior de coneixents, treballats i pensats de forma abundant i repetida.”

Arthur Schopenhauer

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SUMMARY

Q fever is a worldwide re-emerging zoonosis caused by an intracellular Gram negative bacillus, *Coxiella burnetii*. Domestic ruminants are the main source of infection to human population. Despite the infection is mainly asymptomatic in cattle, it has been linked with several reproductive disorders. Thus, the aim of this thesis was to provide clinical information about *C. burnetii* infection in order to improve its control in dairy herds. To achieve these objectives, four studies, published in peer-reviewed journals, were performed.

In the first study, vertical transmission of *C. burnetii* and links between shedding and seropositivity were examined. The results of this study indicated no detectable precolostral antibody response in calves born from dams with *C. burnetii*-qPCR-positive cotyledons.

In the second study, the effects of *C. burnetii* shedding and seropositivity on postpartum recovery and subsequent fertility in high-producing dairy cows were assessed. *C. burnetii*-shedding seropositive animals showed both, an earlier return to luteal activity and improved conception rate. Moreover, seropositive cows exhibited a lower risk of suffering endometritis on Days 15-21 postpartum than seronegative ones.

The third study sought to assess the effects of an inactivated phase I vaccine against *C. burnetii* at the beginning of the third trimester of gestation on serological profiles and bacterial shedding patterns in dairy cows. A subset of 70 of the 156 analysed cows underwent more intensive monitoring. Results indicated that the inactivated *C. burnetii* phase I vaccine failed to reduce bacterial shedding during the study period.

In the last study, the effect of an inactivated phase I vaccination against *C. burnetii* on 171-177 days of pregnancy on the subsequent reproductive performance of high producing dairy cows was assessed. The results of this study proved that phase I vaccination against *C. burnetii* on advanced pregnant dairy cows improves subsequent fertility, especially in seronegative cows.

RESUM

La Febre Q és una zoonosi re-emergent i endèmica a nivell mundial. Està causada per *Coxiella burnetii*, un bacil Gram negatiu intracel.lular obligat. El reservori principal per a la població humana el constitueixen els remugants domèstics. Tot i que la infecció és principalment asimtomàtica en boví, se l'ha relacionat amb desordres reproductius. L'objectiu de la present tesi doctoral ha estat proporcionar informació sobre la infecció per *C. burnetii* des d'un punt de vista clínic, a fi de millorar el seu control en les explotacions de boví de llet. Per tal d'assolir aquests objectius s'han realitzat quatre estudis que han estat publicats en revistes especialitzades.

En el primer estudi s'ha evaluat la transmissió vertical de *C. burnetii* i la relació entre excreció i seropositivitat. Els resultats d'aquest estudi indiquen que no s'han detectat anticossos precalostrals en els vedells nascuts de vaques amb cotiledons q-PCR positius a *C. burnetii*.

En el segon estudi s'han analitzat els efectes de l'excreció i la seropositivitat de *C. burnetii* sobre la involució postpart i la fertilitat en la següent lactació en vaques lleteres d'alta producció. Els animals excretors i seropositius han presentat un retorn a l'activitat luteal més prompte i una millor taxa de concepció que la resta de vaques. A més, el risc de presentar endometritis en els dies 15-21 postpart ha estat més baix en les vaques seropositives que en les seronegatives.

En el tercer estudi s'han evaluat els efectes d'una vacuna inactivada contra *C. burnetii* en fase I administrada a l'inici del tercer terç de la gestació sobre els perfils serològics i els patrons d'excreció bacteriana en vaques lleteres. Un subgrup de 70 de les 156 vaques estudiades han estat sotmeses a una monitorització més intensiva. Els resultats indiquen que la vacuna inactivada contra *C. burnetii* en fase I no ha estat capaç de reduïr els nivells d'excreció bacteriana durant el periode d'estudi.

En el quart estudi s'ha analitzat l'efecte d'una vacuna inactivada contra *C. burnetii* en fase I administrada en els dies 171-177 de gestació sobre el rendiment reproductiu de la lactació següent en vaques lleteres d'alta producció. Els resultats d'aquest estudi mostren que la vacunació contra *C. burnetii* en fase I de vaques en un estat de gestació avançat millora el rendiment reproductiu de la lactació següent, especialment en les vaques seronegatives.

RESUMEN

La Fiebre Q es una zoonosis re-emergente y endémica mundialmente. Está provocada por un bacilo Gram negativo e intracelular obligado, *Coxiella burnetii*. El reservorio principal para la especie humana lo constituyen los rumiantes domésticos. Aunque en general la infección es asintomática en bovino, se la ha relacionado con desórdenes reproductivos. El objetivo de la presente tesis doctoral ha sido proporcionar información sobre la infección por *C. burnetii* bajo un punto de vista clínico con el fin de mejorar el control de la infección en las explotaciones de vacas lecheras. Para cumplir estos objetivos se han realizado cuatro estudios que han sido publicados en revistas especializadas.

En el primer estudio se ha evaluado la transmisión vertical de *C. burnetii* y la relación entre excreción y seropositividad. Los resultados indican que no se han detectado anticuerpos precalostrales en los terneros nacidos de vacas con los cotiledones q-PCR positivos a *C. burnetii*.

En el segundo estudio se han analizado los efectos de la excreción y la seropositividad de *C. burnetii* sobre la involución postparto y la fertilidad en la lactación siguiente en vacas lecheras de alta producción. Los animales excretores y seropositivos han mostrado un retorno a la actividad luteal más temprano y una mejor tasa de concepción que el resto de las vacas. Además, las vacas seropositivas han presentado menor riesgo de endometritis en los días 15-21 postparto que las seronegativas.

En el tercer estudio se han evaluado los efectos de una vacuna inactivada contra *C. burnetii* en fase I administrada en el inicio del tercer tercio de gestación sobre los perfiles serológicos y los patrones de excreción bacteriana en vacas lecheras. Un subgrupo de 70 de las 156 vacas estudiadas fue sometido a una monitorización más intensiva. Los resultados indican que la vacuna inactivada contra *C. burnetii* en fase I no ha sido capaz de reducir los niveles de excreción bacteriana durante el periodo de estudio.

En el cuarto estudio se ha analizado el efecto de una vacuna inactivada contra *C. burnetii* en fase I administrada en los días 171-177 de gestación sobre el rendimiento reproductivo en la lactación siguiente en vacas lecheras de alta producción. Los resultados de este estudio muestran que la vacunación contra *C. burnetii* en fase I de las vacas en un estado avanzado de gestación mejora el rendimiento reproductivo de la lactación siguiente, especialmente en las vacas seronegativas.

INTRODUCTION

INTRODUCTION

The prevalence of zoonoses and emerging diseases is increasing worldwide. Despite efforts to control and eradication, these diseases cause great economic losses for the involved countries and creats health crises in both, animal and human populations. In 1951 regulations in order to prevent the international spread of diseases were created (WHO 2007). Before that, disease situation was relatively stable. Nowadays, the world has dramatically changed. The population growth, international travelling, misuses of antimicrobials and intensification of animal production facilitates the appearance of health crises that can rapidly spread worldwide. The most remarkables processes had been foot and mouth disease, bovine spongiform encephalopathy, and, more recently, highly pathogenic avian influenza and Q fever (Arricau-Bouvery and Rodolakis 2005; Ducrot *et al.* 2008; Rodriguez *et al.* 2009; Capua and Cattoli 2013).

Change in the dynamics of markets, increasing the price of power and raw materials for animal feed have been the most remarkable effects of globalization. Dairy industry is one of the most important agroalimentary industries in Spain: From April 2012 to March 2013 both milk and dairy products consumption was 112.14 kg per capita, higher than consumption of meat (MAGRAMA 2013). However, milk price received by the producer is subjected to a downward trend (EUROSTAT 2013). Moreover, despite the improvement in genetic selection and nutrition, a declining of reproductive performance since 80's has been detected. This trend has been frequently associated with an increment in milk production, but its origin is multifactorial and not always related to milk yield (Lucy 2001; López-Gatius 2003; López-Gatius *et al.* 2006). Identifying causes of reproductive impairment becomes necessary to apply corrective measures and improve the productivity of dairy herds.

The multifactorial factors that impaired the reproductive parameters of high producing dairy cows include non-infectious causes, such cow, environment, management, and infectious agents such as *Infectious Bovine Rhinotracheitis virus* (IBR) (Nandi *et al.* 2009), *Bovine Viral Diarrhoea virus* (BVD) (Rüfenacht *et al.* 2001), *Brucella spp*. (Seleem *et al.* 2010), and *Neospora caninum* (Bartels *et al.* 2006) among others. All these infectious and parasitic agents can be diagnosed and monitored relatively easy in commercial dairy herds due to the existence of an efficient and viable serological and molecular biology techniques. Moreover, vaccines against these infections are available at clinical level (Moriyón *et al.* 2004; Livingstone and Longbottom 2006; Alvarez *et al.* 2007; Mughini-Gras *et al.* 2013). Despite the progress in molecular biology and biotecnology, there are still infections that remain undiagnosed and consequently, untreated in dairy herds. This is the case of Q fever.

Coxiella burnetii is an obligate intracellular bacillus, the etiological agent of Q fever, a re-emerging zoonosis worldwide (Maurin and Raoult 1999; Arricau-Bouvery and Rodolakis 2005) described in the thirties by Derrick (1983). In the last thirty years *C.*

burnetii has caused outbreaks in human population of different countries of the European Union (Georgiev *et al.* 2013). Although Q fever is often subclinical in humans, it can cause a flu-like syndrome and abortion in pregnant women. Moreover, in immunocompromised patients the process becomes chronic and cause hepatitis, valvular heart diseases and pneumonia (Maurin and Raoult 1999). In small ruminants, Q fever causes epidemic waves of abortion. The most recent case took place between 2007 and 2010 in The Netherlands, with affectation of the human population (Dijkstra *et al.* 2012). Although Q fever symptomatology is well known in humans and small ruminants, pathogenesis of the disease in cattle is not yet fully understood (Porter *et al.* 2011; Agerholm 2013). Thus, clinical signs in cattle, one of the most important reservoirs of the bacteria, are not well characterized and sometimes controversial (To *et al.* 1998; Bildfell *et al.* 2000; Hansen *et al.* 2011; Muskens *et al.* 2011; Garcia-Ispierto *et al.* 2012; López-Gatius *et al.* 2012). Despite the presence of bacterium in fetal membranes and fetal abortions has been demonstrated (Bildfell *et al.* 2000; Muskens *et al.* 2012), vertical transmission has not been described. In addition, the existence of seronegative shedders and seropositive non-shedders demands the combination of serological and molecular biology techniques for an adequate laboratorial diagnosis (Guatteo *et al.* 2007).

In order to control the disease at herd level, *Coxiella burnetii* vaccines have been developed. Phase I vaccines seems to be the most protective, reducing bacterial shedding in ruminants, while phase II vaccines are far less effective (Zhang *et al.* 2007). These results were obtained only for seronegative and non-pregnant females or in early inseminated animals (Guatteo *et al.* 2008). Due to the management of animals in dairy herds, vaccination of cows during the dry-off period could be easy applied due to the management policy. However, vaccination effects against *C. burnetii* in pregnant cows in the third trimester of pregnancy are not yet defined.

The need to understand the dynamics of serological profiles, shedding patterns and its relationship of reproductive performance in commercial high producing dairy herds has motivated the development of this thesis. The inactivated phase I vaccine effects on third trimester pregnant females on serological profiles, shedding patterns and reproductive parameters has also been evaluated.

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MAIN OBJECTIVES

MAIN OBJECTIVES

- To examinate serology of *Coxiella burnetii* of newborn calves from both, seropositive and seronegative dams.

- To determine possible links between dam shedding during the peripartum period and seropositivity of the dams.

- To assess the effects of *Coxiella burnetii* shedding or seropositivity on postpartum recovery and subsequent fertility in high producing dairy cows.

- To determine the effect of an inactivated phase I vaccine against *Coxiella burnetii* at the starting of the third trimester of gestation (on Day 171-177) on serological profiles and shedding patterns of high producing dairy cows.

- To assess the effect of phase I vaccination against *Coxiella burnetii* at the starting of the third trimester of gestation (on Day 171-177) on the subsequent reproductive performance of lactating high producing dairy cows.

CHAPTER 1

No detectable precolostral antibody response in calves born from cows with cotyledons positive for *Coxiella burnetii* by quantitative PCR

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NO DETECTABLE PRECOLOSTRAL ANTIBODY RESPONSE IN CALVES BORN FROM COWS WITH COTYLEDONS POSITIVE FOR *COXIELLA BURNETII* BY QUANTITATIVE PCR

Abstract

Samples from 45 dams (milk/colostrum, faeces, vaginal fluid and blood on days 171-177 of gestation and at parturition, and cotyledons at parturition) and their calves (blood collected before colostrum intake and weekly until days 29-35) were analyzed to examine the vertical transmission of *C. burnetii* and links between shedding and seropositivity. All calves were born *C. burnetii* seronegative. Only those born to seropositive dams seroconverted following colostrum intake. Logistic regression analyses indicated that the likelihood of dam seropositivity was 21 and 4.85 times higher for multiparous than for primiparous (65.6% vs. 8.3%, P=0.006), and for prepartum shedding cows (75% vs. 38.2%, P=0.03) compared to the remaining animals, respectively. In conclusion, the results of this study indicate no detectable precolostral antibody response in calves born from dams with cotyledons positive for *C. burnetii* by qPCR. In order to analyze the possibility of persistent infection due to immunotolerance to an early *in utero* infection, further studies will need to test for *C. burnetii* DNA. In addition, in the present study multiparous cows showed a significantly higher seroprevalence than primiparous cows and heifers, colostral antibodies were efficiently transferred to newborn calves, and there was a link between bacterial shedding on days 171-177 of gestation and *Coxiella* seropositivity of the dam.

Key words: Bovine, reproduction, Q fever, *Coxiella burnetii*, colostrum, antibody response

1. Introduction

Q fever is a re-emerging zoonosis that is endemic worldwide (Maurin and Raoult 1999). Its etiological agent, *Coxiella burnetii*, is an obligate intracellular Gram-negative bacterium able to produce endospore-like forms (McCaul and Williams 1981). Symptoms caused by *C. burnetii* are well known in humans (Maurin and Raoult 1999) and small ruminants (Sánchez *et al.* 2006), but there is still a lack of knowledge of the clinical course of disease in cattle, one of the main reservoirs for human infection (Maurin and Raoult 1999; Arricau-Bouvery and Rodolakis 2005). There is some controversy regarding the symptoms and lesions associated with seropositivity to the *C. burnetii* species (Guatteo *et al.* 2006; 2007).

Airborne bacteria constitute the main route of transmission in the form of contaminated aerosols generated from the placenta and fluids expelled during parturition and abortion. However, *C. burnetii* can be shed into the environment through milk, vaginal fluid and faeces and this can occur outside the calving or abortion period (Guatteo *et al.* 2007).

Thus, Q fever is difficult to diagnose in field conditions, since both seropositive non-shedding and seronegative shedding animals exist (Guatteo *et al.* 2007; Rodolakis *et al.* 2007; Hansen *et al.* 2011).

In general, there is little epidemiological information on the serologically *C. burnetii*-infected dairy cattle. In natural infections in cattle, sheep and goats, a non-immune (often neo-natal) animal is supposed to be infected from the environment or by ticks and undergo a primary subclinical infection (Woldehiwet 2004). In high-producing dairy herds, seroprevalence of infection has been demonstrated to be highly stable throughout gestation (Garcia-Ispierto *et al.* 2011). However, a small rate of seroconversion has also been observed, determining the existence of an active infection (Nogareda *et al.* 2012). Transplacental infection of the foetus *in utero* by *C. burnetii* is possible, but its consequences are still unknown (Angelakis and Raoult 2010). If infection occurs in early gestation, immunotolerance of the fetus is possible, such as in calves persistently infected with bovine viral diarrhoea virus (McClurkin *et al.* 1984). For all these reasons, the aim of the present study was to examine the serology of *Coxiella burnetii* in newborn calves from seropositive or seronegative dams. Possible links between dam shedding during the peripartum period and seropositivity of the dams were also investigated.

2. Materials and methods

2.1. Cattle and herd management

The study was performed on two commercial Holstein-Friesian dairy herds in northeastern Spain, each comprising 625 and 125 lactating animals, from October 2010 to October 2011. In Herd 1, cows were milked three times daily and in Herd 2 twice daily. For Herds 1 and 2, respectively, mean annual milk production was 11.343 Kg and 8.846 Kg, and the culling rate was 29% and 23%. The cows calved all year round and were fed complete rations. Rations were in line with the National Research Council recommendations (2001).

All cows were bred by artificial insemination (AI) using semen from bulls of proven fertility. Dry cows were kept as a separate group and transferred, depending on their body condition score and age, 7-25 days before parturition to a ‘parturition group’.

All animals were tuberculosis and brucellosis free, as shown in yearly tests from 1985 to 2011. Based on serology and polymerase chain reaction (PCR) analysis of bulk tank milk samples, the herds were known to be chronically infected with *C. burnetii*. Vaccination programs for the prevention of bovine viral diarrhoea (BVD) and infectious bovine rhinotracheitis (IBR) included modified live vaccines for animals 6-8 months old. Pregnant animals were given killed vaccines during the 7th month of each gestation

period. Parous cows that were not pregnant on Day 150 postpartum received a further killed vaccine.

Only calves born to healthy cows free of clinical disease during the study period were included in the study. Exclusion criteria were: mastitis, lameness and digestive disorders. Efforts were made to reduce variation in the general health status of the animals so that serological changes could be attributed to factors other than the clinical condition of the cows during the study. The final data analyzed corresponded to 46 calves and their corresponding mothers.

2.2. Experimental design

Dams were serologically tested from day 171 to 177 of gestation. Forty-five dams were then followed into parturition based on their serological patterns (21 seropositive and 24 seronegative). On gestation days 171-177 and at parturition, milk, faeces, vaginal fluid and blood, plus colostrum and cotyledons (only at parturition) were collected. Blood samples from their corresponding calves were collected at birth before colostrum intake and at the ages of 1-7, 8-14, 15-21, 22-28 and 29-35 days. First sampling was performed on days 171-177 due to the management policy of the herd and because at that time period there were no dried-off animals.

2.2.1. Blood

Blood samples were collected from the coccygeal vein in dams and the jugular vein in calves into heparinized vacuum tubes (BD Vacutainer^{TM}, Becton-Dickenson and Company, Plymouth, UK). Tubes were centrifuged (10 min, 1600×*g*) within 30 min after collection and the plasma stored at -20ºC until analysis.

2.2.2. Milk

Milk and colostrum samples were collected from Day 171-177 of gestation and on the day of parturition, respectively, in a plastic sterile container for PCR. To minimize the risk of contamination during the collection process, teats were washed in clean water and then each teat end was scrubbed with teat wipes impregnated with an antiseptic solution. Finally, milk and colostrum were collected from the four teats after elimination of the first streams. Samples were frozen at -20°C prior to analysis.

2.2.3. Vaginal fluid

After disinfection of the vulva with iodine solution, a vaginal swab was obtained and stored at -20º C.

2.2.4. Faeces

Faecal samples were collected using a glove for rectal examination into sterile containers.

2.2.5. Placenta

Placenta specimens were obtained immediately after parturition. After washing the perineum with iodine solution, three cotyledons were excised using rectal palpation gloves. All specimens were stored after their collection at -20ºC until PCR analysis.

2.3. Laboratory analyses

Plasma samples were screened for antibodies against *C. burnetii* by ELISA. In the remaining samples, the possible presence of the bacterium was detected by PCR.

2.3.1. *Coxiella burnetii* antibodies

Antibodies to *C. burnetii* were detected in plasma samples by indirect enzyme-linked immunosorbent assay (ELISA) using the CoxLS kit (LSIVET RUMINANT Milk/Serum Q FEVER from Laboratoire Service International, Lissieu, France). This validated test (García-Pérez *et al.* 2009) was performed according to the manufacturer's instructions. The sensitivity and specificity values for the ELISA test are 85% and 95%, respectively (Courcoul *et al.* 2010). A cocktail of both antigen phases (I and II) was used in this assay to detect total anti-*C. burnetii* immunoglobulin G antibodies (IgG) (Guatteo *et al.* 2008).

2.3.2. Polymerase chain reactions on individual samples

Coxiella burnetii was detected in the milk, faeces and vaginal fluid samples obtained between 171 and 177 days of gestation and at parturition, and in the colostrum and cotyledons obtained only at parturition using a commercial kit targeting the repetitive transposon-like region of *C. burnetii* (LSI Taqvet *Coxiella burnetii*®; Laboratoire Service International, Lissieu, France) according to the manufacturer's instructions. The positive control used was a solution containing 10^5 *C. burnetii*/ mL (provided by UR INRA IASP, 37380 Nouzilly, France). The negative control sample used was DNase Rnase-free water. DNA was extracted from the different samples using the QIAmp DNA minikit® (Qiagen S.A., France) according to the manufacturer's instructions. For the milk or vaginal mucus samples, DNA was extracted directly from 200 µL of raw milk or 200 µL of the obtained vaginal mucus dilution. For the faecal samples, 1 g of the original sample was weighed and mixed by vortexing for 30 seconds with 4 mL of DNase Rnase-free water and 400 µL then collected. Finally, samples were centrifuged at 6000 xg for 1 min and 200 µL of supernatants were used for DNA extraction. For the

cotyledon samples, DNA was extracted from 25 mg of tissue cut into small pieces and placed in a 1.5 mL microcentrifuge tube. Only the samples presenting a typical amplification curve with a cycle threshold below 40 were considered to be positive.

2.4. Data collection and analyses

The following data were recorded for each animal: herd, calf sex, calving date, milk production by the cow on day 50 postpartum of the previous lactation (<40 Kg versus ≥40 Kg), parity (heifers and primiparous versus multiparous cows), *C. burnetii* seropositivity and antibody titres for the dam and calf, and *C. burnetii* shedding by the cow from day 171 to day 177 of gestation and at parturition. When one or more PCR-positive samples (milk, faeces, vaginal fluid, colostrum or placenta) were recorded between gestation days 171 and 177 or at parturition, the animal was recorded as shedding-positive for the corresponding sampling day.

All statistical procedures were performed using the SPSS package version 18.0 (SPSS Inc., Chicago, IL, USA) with the level of significance set at $P<0.05$. Fisher exact tests were used to compare differences in the percentages of calf *C. burnetii* seropositivity between *C. burnetii* seropositive and seronegative cows. Two binary logistic regression analyses were performed, considering dam *C. burnetii* seropositivity as the dependent variable. The first analysis assessed the effects of herd, *C. burnetii* shedding from day 171 to day 177 of gestation and at parturition, and parity, on dam *C. burnetii* seropositivity. Since parity was the most important factor affecting *C. burnetii* seropositivity, in the second logistic regression this factor was excluded. Regression analyses were conducted according to the method of Hosmer and Lemeshow (1989).

3. Results

Of the 45 dams and their calves included in the study, 21 dams were *C. burnetii* seropositive and 24 were seronegative. One of the seropositive cows delivered twins. Table 1 shows the frequency distributions of the dams based on serology and shedding patterns.

Table 1. Classification of the calves based on the serological profiles and shedding patterns of their mothers

Cow serology	Cow shedding pattern	Number of calves
Seropositive (n = 21)	Shedding gestation days 171-177	5
	Shedding parturition	2
	Shedding gestation days 171-177 and parturition	5
	Non-shedding	10
Seronegative (n = 24)	Shedding gestation days 171-177	2
	Shedding parturition	4
	Shedding gestation days 171-177 and parturition	1
	Non-shedding	17

All calves were seronegative to *C. burnetii* at birth, and only those born from seropositive dams underwent seroconversion following colostrum intake (Fisher test, P< 0.001) and remained seropositive until the end of the study. On the contrary, seroconversion was not observed during the study period in seronegative dams or calves born to seronegative mothers.

In total, 286 samples were submitted for PCR. Among these, positive results for the identification of *C. burnetii* were recorded in 32 samples: 13 of 122 (10.6%) and 19 of 164 (11.6%) collected between Day 171 and 177 of gestation, and at parturition, respectively (Table 2). Milk was the sample returning most positive results from gestation days 171-177 (12 positive samples, 92.31%), and vaginal fluid was the sample returning most positive results at parturition (9 positive samples, 47.4%). At parturition, 4 faecal samples and 6 cotyledon samples were positive for *C. burnetii* DNA.

Table 2. Samples scoring positive (+) or negative (–) for the PCR detection of *Coxiella burnetii*

Time of collection	Vaginal fluid		Faeces		Milk		Cotyledons		Total	
	+	-	+	-	+	-	+	-	+	-
Gestation days 171–177	1	42	0	43	12	24			13	109
Parturition	9	35	4	39	0	41	6	30	19	145
Total	10	77	4	82	12	65	6	30	32	254

The first binary logistic regression revealed no significant effects of herd, milk production and *C. burnetii* shedding from gestation days 171-177 and at parturition on dam *C. burnetii* seropositivity (table 3). Based on the odds ratio, the likelihood of dam seropositivity was 21 times higher (P=0.006) for multiparous than for the remaining cows (65.6% vs 8.3%, respectively).

Table 3. Odds ratios of the variables included in the first logistic regression model for factors affecting *Coxiella burnetii* seropositivity

Factor	Class	n (%)	Odds ratio	95% CI[b]	*P* - value
Parity	Non-multiparous[a]	1/12 (8.33%)	Reference		
	Multiparous	21/32 (65.63%)	21.0	2.3- 84.5	0.006

[a] heifers plus primiparous cows; [b] Confidence interval; R^2 Nagelkerke = 0.339. P value for the model *P*< 0.01

After eliminating the age effect, the second binary logistic regression revealed no significant effects of herd, milk production and *C. burnetii* shedding at parturition on dam *C. burnetii* seropositivity (table 4). Based on the odds ratios, the likelihood of dam seropositivity was 4.8 times higher (P=0.036) for cows shedding the pathogen from days 171-177 of gestation compared to non-shedding cows (75% vs 38.2%, respectively).

Table 4. Odds ratios of the variables included in the second logistic regression model for factors affecting *Coxiella burnetii* seropositivity

Factor	Class	n (%)	Odds ratio	95% CI[a]	*P* - value
C. burnetii shedding	Non-shedding	13/34 (38.24%)	Reference		
in prepartum period	Shedding	9/12 (75%)	4.846	1.105-21.255	0.036

[a] Confidence interval; R^2 Nagelkerke= 0.136. *P* value for the model 0.026

4. Discussion

To the best of our knowledge, this is the first report on the absence of precolostral antibody response in calves of *C. burnetii*-seropositive dams. All 46 calves analyzed delivered by both *C. burnetii*-seronegative and seropositive dams, were born seronegative, although *C. burnetii* DNA was detected in the placental cotyledons of 6 seropositive dams. In addition, seroconversion was only observed in calves born to seropositive dams after colostrum intake, and these calves remained seropositive during the entire study period. Multiparous cows and cows shedding the bacterium between day 171 and day 177 of gestation were more likely to show antibodies against *C. burnetii* than the remaining cows.

It is well known that the epitheliochorial placenta of ruminants acts as a barrier to the passage of antibodies against any pathogen from the dam to its fetus. Thus, a newborn calf testing seronegative before colostrum intake would indicate no contact with the pathogen at the intrauterine level or immunotolerance to the given pathogen of the infected calf, as has been observed in the case of calves persistently infected with bovine viral diarrhoea virus (McClurkin *et al.* 1984). Conversely, antibodies found in newborn animals before colostrum intake are indicative of fetal infection. In the present study, seroconversion was only observed after colostrum intake in calves delivered by seropositive dams. The results of earlier work in mice (Baumgartner and Bachmann 1992) had already suggested that fetoplacental union prevents the vertical transmission of *C. burnetii.* These authors also proposed that the infection of newborns was determined by aerosol inhalation at the moment of parturition. However, in the present study, seronegative calves remained seronegative for at least one month after parturition. Further studies are needed to establish the exact time of infection in cattle.

On the other hand, the infection of fetuses in the absence of antibodies could be indication of persistent infection due to immunotolerance to an early *in utero* infection, as suggested in other infections such as bovine viral diarrhoea virus (Houe 1995). However, in the present study, DNA was not analyzed in fetuses and calves. Further studies will need to test for *C. burnetii* DNA as well as antibodies for the detection of exposed and/or infected animals.

Colostrum could be a possible source of infection for newborn calves, but all of the colostrum samples analyzed, including those from seropositive dams, were PCR negative for *C. burnetii*. The ingestion of raw milk from infected farms has been

described to cause seroconversion in humans (Benson *et al.* 1963). Seroconversion of the present calves was attributed only to dam immunoglobulins transferred to the calves via the colostrum, as an efficient vehicle of passive immunity from mother to calf. Since the bacterium was observed in 12 raw milk samples obtained between day 171 and day 177 of gestation, it was surprising that most of the colostrum samples examined lacked the pathogen. It has been suggested that some samples (such as faeces) contain large numbers of Taq polymerase inhibitors and that detecting the pathogen in this type of sample may be more complicated than in other samples (Guatteo *et al.* 2006). However, in the present study, the internal control used indicated no interference in the PCR, and four faecal samples were PCR positive. In effect, the presence of this organism in the colostrum has been previously detected in cattle (Huijsmans *et al.* 2011).

The detection of *C. burnetti* in 6 of the 36 cotyledon samples examined is consistent with the findings of several studies (Hansen *et al.* 2011) in which *C. burnetii* was able to colonize the placenta and multiply inside the trophoblasts. In a recent study (Ben-Amara *et al.* 2010), it was concluded that the trophoblast allows *C. burnetii* replication, without interfering with the normal course of pregnancy and that this is rarely accompanied by inflammation in cattle (Hansen *et al.* 2011). However, it seems that the bacterium could cause some placental damage (López-Gatius *et al.* 2011). Reduced Pregnancy-Associated Glycoprotein (PAG) levels in the second half of gestation and increased cortisol levels around day 180 of pregnancy (Garcia-Ispierto *et al.* 2010) have been observed in *C. burnetii*-seropositive cows, and this could reinforce the idea of placental damage.

Multiparous cows showed a significantly higher seroprevalence than primiparous cows and heifers, probably due to the increased possibility of contact with *C. burnetii* with age. McCaughey *et al.* (2010) have also recently reported increasing odds with age of cattle being infected with Q fever. This constitutes further proof that horizontal transmission is the main route of *C. burnetii* infection in cattle. In addition, between day 171 and day 177 of gestation, the prevalence of seropositivity was higher among shedding cows than non-shedders. This is likely to indicate reactivation of the bacterium during this period, when cortisol levels have been described to peak in seropositive cows (Garcia-Ispierto *et al.* 2010). However, shedding during parturition was not significantly related to seropositivity of the dam. The fact that shedding cows were detected indicates active infection in the herds analyzed.

Seropositive animals are more likely to be shedders than their seronegative counterparts, in agreement with others (Arricau-Bouvery *et al.* 2005; Guatteo *et al.* 2007). Moreover, none of the 24 seronegative animals detected in our study seroconverted during the study period, albeit short, as observed in previous studies (Guatteo *et al.* 2007; Garcia-Ispierto *et al.* 2011). Longer-term studies are needed to determine when seroconversion takes place in chronically *C. burnetii*-infected dairy herds.

In conclusion, the results of this study indicate no detectable precolostral antibody response in calves born from dams with cotyledons positive for *C. burnetii* by qPCR. In order to analyze the possibility of persistent infection due to immunotolerance to an early *in utero* infection, further studies will need to test for *C. burnetii* DNA. In addition, in the present study multiparous cows showed a significantly higher seroprevalence than primiparous cows and heifers, colostral antibodies were efficiently transferred to newborn calves, and there was a link between bacterial shedding on days 171-177 of gestation and *Coxiella* seropositivity of the dam.

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CHAPTER 2

Coxiella burnetii shedding during the peripartum period and subsequent fertility in dairy cattle

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COXIELLA BURNETII SHEDDING DURING THE PERIPARTUM PERIOD AND SUBSEQUENT FERTILITY IN DAIRY CATTLE

Abstract

The objective of this study was to assess the effects of *Coxiella burnetii* shedding or seropositivity on postpartum recovery and subsequent fertility in high-producing dairy cows. Given the difficulty in diagnosing *C. burnetii* infection at the farm level, an exhaustive series of tests in 43 pregnant animals that delivered at least one live calf were conducted, including blood serology and PCR of milk or colostrum, cotyledons (only at parturition), faeces, vaginal fluid against C. *burnetii* on gestation Day 171-177, at parturition and on Days 1-7, 8-14, 15-21, 22-28, 29-35 and 91-97 postpartum. During scheduled herd visits, ultrasonography (US) of the genital tract and examination of vaginal fluid were performed on Days 15-21 (V1), 22-28 (V2), 29-35 (V3) and 51-57 (V4) postpartum by the same veterinarian. Logistic regression analysis revealed that the likelihood of suffering endometritis (the presence of echogenic intrauterine fluid (IUF), cervical diameter of ≥4cm or endometrial thickness ≥0.75cm) was lower in *C. burnetii*-seropositive animals (OR= 0.10), compared with *C. burnetii*-seronegative animals. According to Kaplain-Meier survival analysis, *C. burnetii*-seronegative and non-shedding cows showed a delayed return to luteal activity and conception was delayed in non-shedding animals, compared with the remaining animals. Overall, the results of our study provide useful insight into the effects of *C. burnetii* infection on postpartum recovery and subsequent fertility. In particular, animals not infected with *Coxiella* seem to be susceptible to infection and not protected against the bacterium in dairy herds. The elevated costs of determining infection at the farm level, make monitoring of cows virtually impossible from a clinical point of view.

Keywords: Q fever, bovine, reproductive performance, pregnancy rate, infection

1. Introduction

Coxiella burnetii is an intracellular bacterium distributed worldwide that causes Q fever in animals and also in humans. Domestic ruminants, such as cattle, goats and sheep, are the primary reservoir species for exposure to humans (Maurin and Raoult 1999; Arricau-Bouvery and Rodolakis 2005). In the cow, the pathogenesis of the bacterium is not completely understood and most infections escape diagnosis at the farm level.

Prevalences of *C. burnetii* in Europe has been described to rage from 38% to 79% in cattle according bulk tank milk (BTM) antibodies (Agger *et al.* 2010; Muskens *et al.* 2011; Ryan *et al.* 2011). Reproductive disorders related to coxelliosis are frequently described in cattle, but the data available are inconsistent. In the cow, bacteria have been found in the placenta or aborted foetuses using polymerase chain reaction (PCR)

procedures (Parisi *et al.* 2006), and the main clinical manifestations are late abortion (Woldehiwet 2004), infertility (To *et al.* 1995; To *et al.* 1998), metritis and placenta retention (Bildfell *et al.* 2000; López-Gatius *et al.* 2011). The bacterium is shed by ruminants through birth products, milk, faeces and vaginal mucus (Guatteo *et al.* 2006). Serology is not always indicative of shedding or illness (Guatteo *et al.* 2007). Hence, blood monitoring is not sufficiently precise and PCR testing of samples representing all possible shedding routes is necessary to understand *Coxiella* infection. Thus, at the clinical level, detecting infected animals is sometimes difficult if not impossible.

The postpartum period has been recognized as a critical time for the reproductive performance of dairy cattle (LeBlanc 2008). In effect, calving is a considerable risk to high-producing dairy cows and infection of the uterus is a physiological process (Sheldon and Dobson 2004). After calving, the cow has to reach both adequate uterine and cervix involution and return to ovarian cyclicity (Morrow *et al.* 1969; Shrestha *et al.* 2004; LeBlanc 2008). Thus, it could be that *C. burnetii* affects one of these important processes of the reproductive cycle of a cow. The objective of this study was to assess the effects of *C. burnetii* shedding or seropositivity on postpartum recovery and subsequent fertility in high-producing dairy cows. Given the difficulty in diagnosing *C. burnetii* infection at the farm level, we conducted an exhaustive series of tests in 43 pregnant-delivering animals.

2. Materials and methods

2.1. Cattle and herd management

The data examined were generated during a reproductive control program conducted on two well-managed, high-producing, Holstein-Friesian commercials dairy herds in north-eastern Spain, each comprising 625 and 125 lactating animals, from October 2010 to November 2011. Cows were milked three and two times daily, with a mean annual milk production of 11343 and 8846 Kg, respectively. The culling rate was 29% for Herd 1 and 23% for Herd 2. The cows calved all year round and were fed mixed rations. Feeds consisted of cotton-seed hulls, barley, corn, soybean and bran; and primarily corn, barley, or alfalfa silages and alfalfa hay were provided as roughage. Rations were in line with National Research Council recommendations (2001). All animals were tuberculosis and brucellosis free, as shown in yearly tests from 1985 to 2011. All cows were bred by artificial insemination (AI) using semen from bulls of proven fertility and oestrus was confirmed at insemination (López-Gatius 2000) by a previous palpation of the genital tract. Mean (%) calf mortality, conception rate and endometritis at 15-21 days postpartum during the period of study were 9, 33 and 37, respectively.

The farms were kept dog-free. Vaccination programmes for the prevention of bovine viral diarrhoea and infectious bovine rhinotracheitis included modified live vaccines (Cattlemaster; Pfizer, New York, NY, USA) for animals 6-8 months old. Pregnant

animals were given killed vaccines (Triangle 4; Boehringer Ingelheim, Barcelona, Spain) during the 7th month of each gestation period. Parous cows that were not pregnant on Day 150 postpartum received a further killed vaccine. The presence of *C. burnetii* DNA in the BTM was detected by polymerase chain reaction (PCR) in both herds (Garcia-Ispierto *et al.* 2010, 2011; López-Gatius *et al.* 2011). Based on previous ELISA and PCR analyses of BTM samples, herds were known to be chronically infected with *C. burnetii*.

Only animals free of clinical disease during the study period were included in the study. Exclusion criteria were the following: mastitis, lameness and digestive disorders. Those exclusions were made to reduce variation in the general health status of the animals so that serological changes could be attributed to factors other than the clinical condition of the cows during the study. The final data analyzed corresponded to 43 cows.

2. 2. Experimental design

Dams were serologically tested on gestation Day 171-177. Forty-three dams were then followed into parturition based on their serological patterns (23 seropositive and 20 seronegative). On gestation Day 171-177, at parturition and on Days 1-7, 8-14, 15-21, 22-28, 29-35 and 91-97 postpartum, milk, faeces, vaginal fluid and blood and colostrum and cotyledons (only at parturition) were collected.

2.2.1. Blood

Blood samples were collected from the coccygeal vein into heparinized vacuum tubes (BD VacutainerTM, Becton-Dickenson and Company, Plymouth, UK). Tubes were centrifuged (10 min, 1600×*g*) within 30 min after collection and the plasma stored at -20°C until analysis.

2.2.2. Milk

Milk and colostrum samples were collected in a plastic sterile container for PCR. To minimize the risk of contamination during the collection process, teats were washed in clean water and then each teat end was scrubbed with teat wipes impregnated with an antiseptic solution. Finally, 10 ml of milk and colostrum were collected from the four teats in one sterile container after elimination of the first streams. Samples were frozen at -20°C prior to analysis.

2.2.3. Vaginal fluid

After vulva disinfection with iodine solution, a vaginal swab was obtained and stored at -20ºC. Faeces samples were collected using a glove for rectal examination into sterile containers.

2.2.4. Placenta

Placenta specimens were obtained immediately after parturition, before membranes were expelled. After washing the perineum with iodine solution, one hand was introduced in the vagina with a rectal palpation glove and three cotyledons were excised. All specimens were stored after their collection at -20ºC until PCR analysis.

2.3. Clinical examination

During scheduled herd visits, exams were performed on Days 15-21 (V1), 22-28 (V2), 29-35 (V3) and 51-57 (V4) postpartum by the same veterinarian. The clinical examination during the first three visits included ultrasonography (US) of the genital tract and ovaries and examination of vaginal fluid (aspect and odour). At V4 only ultrasonography of ovarian structures was performed.

The entire reproductive tract was examined by ultrasound using a portable B-mode ultrasound scanner (Easi-scan with a 7.5 MHz transducer-BCF Technology Ltd., Livingston, UK). Scanning was performed carefully and slowly along the dorsal/lateral surface of the cervix and each horn and then the ovaries. Cranial cervical size and endometrial thickness were measured using the internal callipers of the ultrasonographers, and IUF was scored as absent, anechogenic or echogenic fluctuant or compact contents (Figure 1). The presence of a corpus luteum (CL) in one or both ovaries was also recorded. Although there are previous studies that describe threshold values for a cow with endometritis (LeBlanc *et al.* 2002; Kasimanickam *et al.* 2004), based on prior experimental findings (López-Helguera *et al.* 2012), cows on V1 were classed as suffering endometritis according to the following criteria: presence of echogenic IUF, cervical diameter of ≥4cm or endometrial thickness ≥0.75cm. It was considered these threshold values as reference.

3. Laboratory analyses

Plasma samples were screened for antibodies against *C. burnetii* by ELISA. In the remaining samples, the presence of the bacterium was detected by PCR.

3.1. *Coxiella burnetii* antibodies

Antibodies to *C. burnetii* were detected in plasma samples by indirect enzyme-linked immunosorbent assay (ELISA) using the CoxLS kit (LSIVET RUMINANT Milk/Serum Q FEVER; Laboratoire Service International, Lissieu, France), validated for bovine, sheep and goat. This validated test (García-Pérez *et al.* 2009) was performed according to the manufacturer's instructions. The sensitivity and specificity values for the ELISA test are 100% and 95%, respectively. The antigen for the ELISA CoxLS kit was isolated from domestic ruminants by INRA in Nouzilly (France). A cocktail of both antigen phases (I and II) was used in this assay to detect total anti-*C. burnetii* immunoglobulin G antibodies (IgG) (Guatteo *et al.* 2008). Results are expressed as optical densities (OD). For each sample, the sample-to-positive (S/P) ratio was calculated as follows: sample OD minus negative control OD/positive control OD minus negative control OD and expressed as an antibody titre (titre = S/P x 100).

3.2 Polymerase chain reactions on individual samples

C. burnetii was PCR-detected in the milk, colostrum, faeces, vaginal fluid and cotyledons samples using a commercial kit targeting the repetitive transposon-like region of *C. burnetii* (LSI Taqvet *Coxiella burnetii*®; Laboratoire Service International, Lissieu, France) according to the manufacturer's instructions. The positive control used was a solution containing 10^5 *C. burnetii*/ mL (provided by UR INRA IASP, Nouzilly, France). The negative control sample used was DNase Rnase-free water. DNA was extracted from the different samples using the QIAmp DNA minikit® (Qiagen S.A., Courtaboeuf Cedex, France) according to the manufacturer's instructions. For the milk or vaginal mucus samples, DNA was extracted directly from 200 µL of raw milk or 200 µL of the obtained vaginal mucus dilution. For the faeces samples, 1 g of the original sample was weighed and mixed by vortexing for 30 s with 4 mL of DNase Rnase-free water and 400 µL then collected. Finally, samples were centrifuged at 6000 x *g* for 1 min and 200 µL of the supernatants used for DNA extraction. For the cotyledon samples, DNA was extracted from 25 mg of tissue cut into small pieces and placed in a 1.5-mL microcentrifuge tube.

4. Data collection and analyses

The following data were recorded for each animal: herd, calving date, milk production on Day 50 postpartum, lactation number, retention of the placenta (retention of the foetal membranes >24 hours), ultrasonography findings such as IUF, measurements of the cervix and endometrium (cm), vaginal content and odour and presence of a CL at visits V1, V2, V3; The animals that had a CL on the clinical examinations were recorded as returned to luteal activity. Fertile AI date, semen providing bull and AI technician, *C. burnetii* seropositivity and *C. burnetii* shedding were also recorded.

When one or more PCR-positive samples (milk, faeces, vaginal fluid, colostrum or placenta) were recorded on gestation Day 171-177, at parturition, or on Days 1-7, 8-14, 15-21, 22-28, 29-35 or 91-97, the cow was scored as shedding-positive in the pre-partum, parturition and postpartum, respectively. Table 1 shows the PCR-positive samples recorded at each time point.

Table 1. PCR samples recorded for each period of days on 43 dairy cows

Period[a]	Vaginal fluid		Faeces		Milk/colostrum		Cotyledons		Total nº. of samples	
	+	-	+	-	+	-	+	-	+	-
Day 171-177 of gestation	0	43	1	42	10	33	_	_	11	118
Parturition day	5	38	1	42	1	42	5	38	12	160
1-7 days postpartum	4	39	2	41	1	42	_	_	7	122
8-14 days postpartum	3	40	2	41	0	43	_	_	5	124
15-21 days postpartum	3	40	1	42	2	41	_	_	6	123
22-28 days postpartum	3	40	1	42	3	40	_	_	7	122
29-35 days postpartum	2	41	0	43	0	43	_	_	2	127
91-97 days postpartum	0	43	1	42	1	42	_	_	2	127
Total nº. of samples	20	324	9	335	18	326	5	38	52	1023

[a]Samples taken on day of the period

All statistics procedures were performed using the SPSS package version 18.0 (SPSS Inc., Chicago, IL, USA) with the level of significance set at $P<0.05$. Three binary logistic regression analyses were performed, considering retention of placenta, endometritis and repeat breeding syndrome (cows receiving 4 or more AI) as the dependent variable and the above-mentioned variables as independent factors.

Kaplan-Meier survival analysis was used to compare the mean time to return to luteal activity and pregnant AI for *C. burnetii*-seropositive/shedding cows in each time period. Cows that were culled before 150 days postpartum were considered as censored cases. Cox proportional-hazards regressions were used to confirm the Kaplan-Meier analyses.

3. Results

The herds returned a positive polymerase chain reaction test for *C. burnetii* in the BTM indicating an excretion rate higher than 10^4 *Coxiella* /ml. The study included 43 pregnant non-aborting cows with a mean lactation number of 3.2 ranging from 1 to 6. At the beginning of the study, 23 were seropositive for *C. burnetii* (53.4%) and 20 seronegative, and 11 (25.6%), 12 (27.9%), and 17 (39.5%) shed the bacterium in the prepartum, at parturition or in the postpartum (at least on one postpartum sample), respectively. Cows testing negative for *C. burnetii* did not undergo seroconversion during the period of study, and all seropositive cows remained seropositive for *C. burnetii*.

After parturition, eight cows had retention of the placenta (23.5%). Three of the 43 animals included in the study were never inseminated due to economic decisions (culling). Nine of the inseminated cows presented the repeat breeding syndrome (22.5%).

3.1. Logistic regression analysis

No effects were found for the dependent variables retention of placenta and repeat breeding syndrome for any factor included in the analyses. Based on the odds ratios, the likelihood of suffering endometritis was lower in *C. burnetii*-seropositive animals (OR= 0.10, 10 times higher for seronegative animals), compared with *C. burnetii*-seronegative animals (Table 2).

Table 2. Odds ratios of the variables included in the final logistic regression model for factors affecting endometritis on Day 15-21 postpartum

Factor	Class	n	% endometritis	Odds Ratio	95% Confidence Interval	p
Coxiella burnetii seropositivity	Seronegative	6/20	30.0	Reference		
	Seropositive	1/23	4.3	0.10	0.01-0.90	0.04

R^2 Nagelkerke: 0.21

3.2. Kaplan Meier analyses

Figures 1 and 2 show Kaplan-Meier survival curves until Day 57 postpartum for seropositive and seronegative animals, and for shedding and non-shedding animals during the study period (*C. burnetii* shedding in the prepartum, at parturition or postpartum), respectively. *Coxiella burnetii*-seronegative and non-shedding cows showed a delayed return to luteal activity compared to the remaining animals.

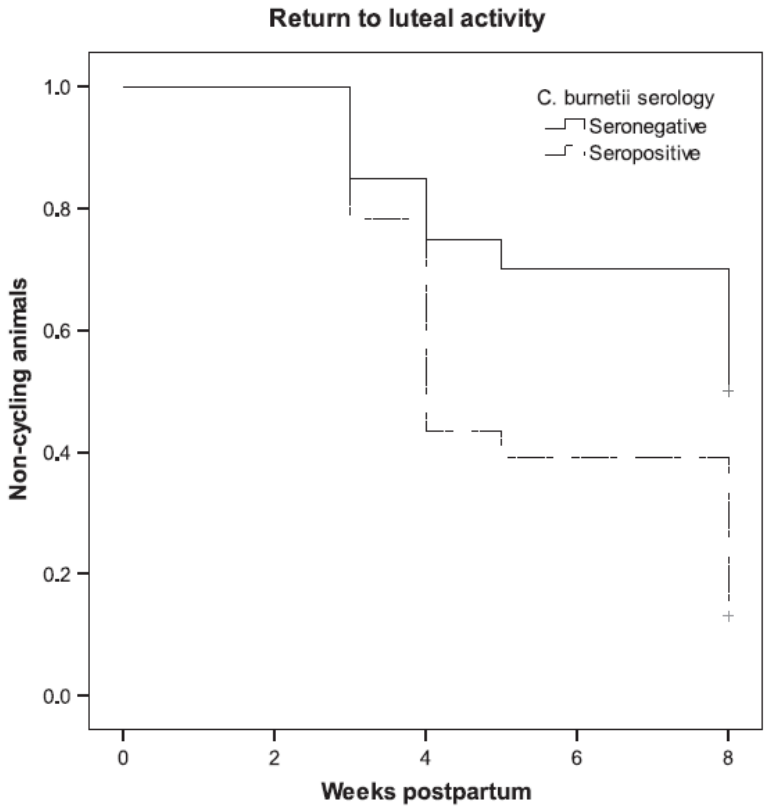

Figure 1. Kaplan-Meier survival curves for analysis of time to return to luteal activity until Day 57 postpartum for *Coxiella burnetii*-seronegative (n=20) and *C. burnetii*-seropositive (n=23) cows (Log Rank p=0.01)

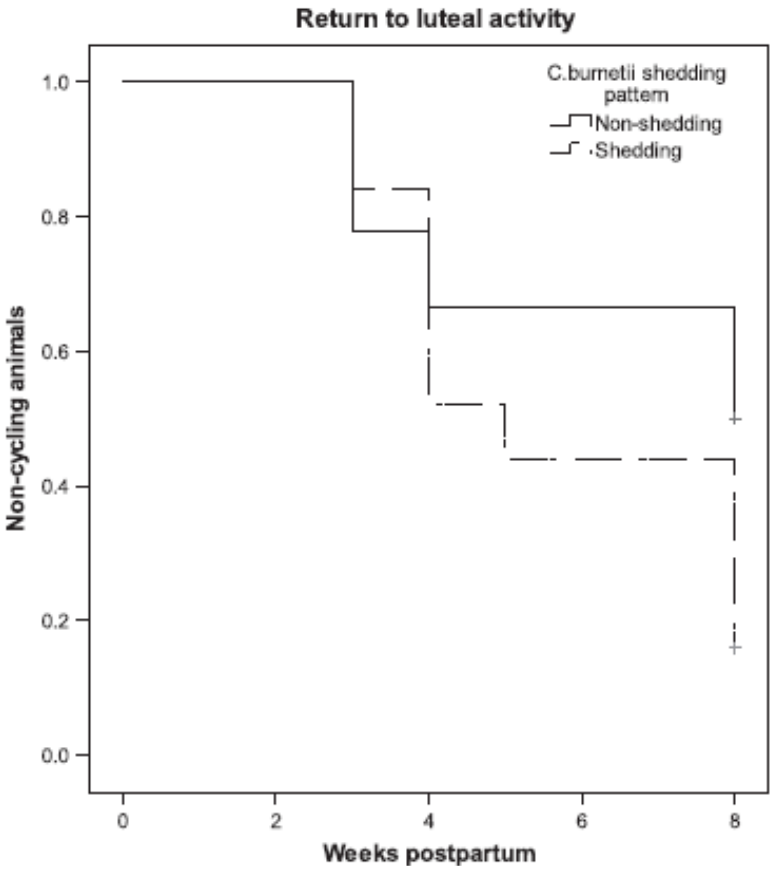

Figure 2. Kaplan-Meier survival curves for the analysis of time to return to luteal activity until Day 57 postpartum for non-shedding (n=18) and shedding (n=25) animals (Log Rank p=0.04)

Figure 3 shows Kaplan-Meier survival curves until Day 150 postpartum for shedding patterns at parturition. Conception was delayed in non-shedding animals compared with the remaining animals.

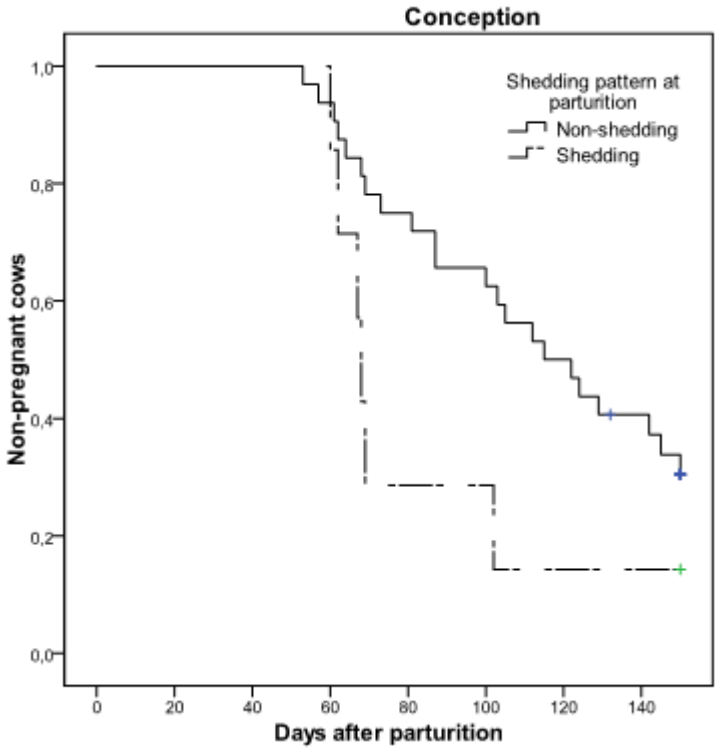

Figure 3. Kaplan-Meier survival curves for the analysis of time to conception until Day 150 postpartum for non-shedding (n=31) and shedding (n=12) animals (Log Rank p=0.001)

3.3. Cox proportional-hazards regressions

Cox proportional-hazards regression was performed to determine factors affecting the return to luteal activity. Based on the hazard ratio, the likelihood of an early return to luteal activity was higher in *C. burnetii* seropositive compared with seronegative cows (by a factor of 2.55) (Table 3).

Table 3. Final Cox's proportional-hazards regression models of factors found to be related to time to return to luteal activity

Factor	Class	n	Hazards ratio	95% Confidence Interval	P
C. burnetii	Seronegative	20	Reference		
Seropositivity	Seropositive	23	2.55	1.4-3.4	0.019

P value for the model 0.001

Finally, a last Cox proportional-hazards regression was performed to determine factors affecting time to conception. Based on the hazard ratio, *C. burnetii* shedders at parturition were 2.3 times more likely to conceive during the established period than non-shedders (Table 4).

Table 4. Final Cox proportional-hazards regression models of factors found to be related to time to fertile insemination

Factor	Class	n	Hazards ratio	95% Confidence Interval	*P*
Coxiella burnetii shedding	Non shedding	31	Reference		
at parturition	Shedding	12	2.3	1.01-3.6	0.05

P value for the model 0.001

4. Discussion

In this study, we examined the relationship between *C. burnetii* seropositivity and shedding and subsequent reproductive performance in high-producing dairy cows with a history of a positive PCR test for the presence of the bacterium in the BTM. An exhaustive analysis of cows was performed to detect infection. Cows were followed each week postpartum and PCR analyzed for each excretion route (three samples were tested before parturition and 21 samples postpartum for each cow).

One of the most surprising results of this study was that *C. burnetii*-seropositive shedding animals showed both an earlier return to luteal activity and conception. Moreover, seropositive cows exhibited a lower risk of suffering endometritis than seronegative ones. No effects on placenta retention or repeat breeding syndrome were detected. Our findings are similar to those obtained in recent studies (Hansen *et al.* 2011; López-Gatius *et al.* 2011) yet differ from those reported by others (To *et al.* 1998; Vaidya *et al.* 2010). Such discrepancies suggest we are still far from understanding the precise mechanisms of this and other infections. For example, toxoplasmosis was discovered in 1908 (Nicolle and Manceaux 1908), but it was not until recently, a century later, that scientists discovered that latent toxoplasmosis in women is beneficial, because it protects pregnant women against the acute form of the infection and their children from the consequences of congenital toxoplasmosis (Bojar and Szymańska 2010). Thus, our results could suggest that in *C. burnetii*-infected cows, something similar could be happening. Probably, infected animals are protected against the detrimental effects of a new infection or even from recrudescence of the bacterium during fertile days. However, testing such theories is difficult because numerous PCR procedures are required to identify *C. burnetii*-infected animals. According to Guatteo *et al.* (2007), cows can sporadically or persistently shed the bacterium and shedding routes are rarely concomitant. Moreover, a seronegative result does not provide assurance that the animal is not infected (EFSA 2010). Of course, this is not sustainable from a clinical perspective. Hence, if a cow is suffering a new infection, shedding may not be the correct tool to predict this. Perhaps an IgM or IgGII ELISA test would be needed to confirm this situation. Unfortunately, no commercial kits are available for this bacterium.

Another possible explanation is the elevated culling rate of high-producing dairy herds (López-Gatius *et al.* 2006). Althought in this study there were no possibility to

determine the infection in culled cows, it is possible that only infected fertile cows survive in the farm and others were rapidly eliminated. Thus, studies that follow lifespan of animals are needed.

In the present work, the samples that more often proved PCR-positive for *C. burnetii* were vaginal fluid and milk. According to Guatteo *et al.* (2007), this could be because faeces samples are heterogeneous and contain a large number of Taq polymerase inhibitors (Guatteo *et al.* 2006). The time when most positive samples were obtained was the day of parturition, followed by the pregnancy period (171-177 days). Interestingly, Day 90 postpartum was the time point returning the fewest positive samples. In human, Q fever has been linked to immunocompromised hosts (Raoult 1990). Similarly, cows in the last third of gestation and on the day of parturition could immunosuppressed due to effects of progesterone and cortisol (Lewis 2004) and this allows for recrudescence of the bacterium. In contrast, at 90 days postpartum, the cow has recovered from delivery and the immune system is again competitive.

Overall, the results of our study provide useful insight into the effects of *C. burnetii* infection on postpartum recovery and subsequent fertility. In particular, animals not infected with *Coxiella* seem to be susceptible to infection and not protected against the bacterium in dairy herds. The elevated costs of determining infection at the farm level, make monitoring of cows virtually impossible from a clinical standpoint.

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CHAPTER 3

Serological and shedding patterns after *Coxiella burnetii* vaccination in the third gestation trimester in dairy cows

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CHAPTER 4

Reproductive performance of high producing lactating cows in *Coxiella*-infected herds following vaccination with phase-1 *Coxiella burnetii* vaccine during advanced pregnancy

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GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of this thesis was to provide information about epidemiology and control of a re-emerging zoonosis worldwide: Q fever. Although this disease is not new, there is a lack of studies clarifying the effects of the bacterium on reproductive performance of dairy cattle. Herein, the results found can be useful from a clinical point of view, not only for increasing knowledge on shedding patterns or serological profiles, but also to prevent the possible negative effects of this infection in dairy cattle. No antibodies against *C. burnetii* were detected in the newborn calf, even in calves born from seropositive animals. Moreover, no negative effects of *C. burnetii* shedding or seropositivity were found on the fertility of cows. In effect, a positive relationship was demonstrated between shedding patterns and return to postpartum ovarian cyclicity and conception rate at first AI. Finally, a positive effect of vaccination against *C. burnetii* in the third trimester of gestation on subsequent reproductive performance was determined, especially in seronegative cows.

1. Clinical relevance of *C. burnetii* infection in dairy herds

1.1. Calves

Calves play an important epidemiological role as reservoirs of infection in diseases with vertical transmission (Houe 1995; Whittington and Windsor 2009; Williams *et al.* 2009; Santman-Berends *et al.* 2010), especially in those farms with self-production reposition of heifers. Thus, monitor and cull calves born from infected animals may be an important tool to control an infection with a demonstrated vertical transmission. Scarce information on *C. burnetii* antibody levels of the neonate is available in literature. Vertical transmission of *C. burnetii* has not been demonstrated. In this thesis we found that precolostral antibody response was not detectable in calves born from dams with *C. burnetii*-qPCR-positive cotyledons (Chapter 1). After the colostrum intake of seropositive animals, all calves seroconverted. What is necessary now is to determine whether those seronegative calves born from infected dams have the presence of the bacteria, determined by PCR. If not, what is the mechanism that does not allow bacteria the cross-over of the placenta to the foetus in the case of live newborns? Maybe, failure of that mechanism determines abortion or stillbirth. Studies performed in mice (Baumgartner and Bachmann 1992) suggested that foetus-placental union resists *C. burnetii* vertical infection and that the infection of the newborn is established by aerosol inhalation at the moment of parturition. On the contrary, if the vertical transmission is possible, this could be indication of persistent infection due to immunotolerance to an early *in utero* infection, as suggested in other infections such as Bovine Viral Diarrhea Virus (Houe 1995) or *Mycobacterium avium paratuberculosis* (Whittington and Windsor 2009). Probably, a combination of both, serology and molecular techniques are necessary to understand better effects of in the newborn calf.

1.2. Postpartum diseases

Parturition is a crucial period in all mammals, but especially in dairy cows (Sheldon and Dobson 2004; Sheldon 2008). In addition, postpartum immunity is depressed during 2-3 weeks after calving (Hammon *et al.* 2006; LeBlanc 2008). In this critical period, the cow has to overcome the natural contamination of the genital tract after parturition and be able to get pregnant again (LeBlanc 2008; Sheldon *et al.* 2008). Moreover, postpartum recovery is even more complex in high producing systems due to postpartum immunosupression of high milk production (Dobson *et al.* 2007; Walsh *et al.* 2011). This fact can facilitate recrudescence or *the novo* infections. Thus, any calving and postpartum complications, such as *C. burnetii* infection, could delay both, return to cyclicity and uterine involution. Endometritis, inflammation of the endometrium without affecting the remaining uterine layers (LeBlanc *et al.* 2002; Sheldon *et al.* 2006), is usually underestimated when the cervix closes rapidly after parturition (Kasimanickam *et al.* 2004). Moreover, definition and diagnosis of subclinical endometritis is still under discussion (Lewis 1997; Barlund *et al.* 2008; Senosy *et al.* 2009; López-Helguera *et al.* 2012). Thus, determine at clinical level whether *C. burnetii* increases this uterine pathology is a really difficult task. There is controversy regarding the relationship *C. burnetii*-endometritis. While some authors revealed significant differences between seropositive or *C. burnetii* shedding animals (Woernle and Müller 1986), other authors have not (Sting *et al.* 2000). In this thesis we could demonstrate that seropositive cows exhibited lower risk of suffering endometritis diagnosed by ultrasonography than the remaining animals (Chapter 2). To first determine a clear relationship between this infection and endometritis, scientific community has to clarify what subclinical endometritis is, and to put some light on what a *C. burnetii* infected animal is. For example, the differences among studies could be simply because definitions of endometritis are different, or because seropositive animals are protected against coxiellosis because they just suffered the acute infection weeks ago.

1.3. Conception rate

Fertility declining besides a milk production increase is being described in dairy cattle since 1980's (López Gatius 2003; López Gatius *et al.* 2006). This is a multifactorial process where genetics, nutrition, production and management play an important role. Therefore, studies of the effects of an infectious disease on conception rate should be done carefully. Moreover, postpartum pathologies such as retention of placenta, metritis and endometritis lead to decreased conception rate (Grön *et al.* 1990; Fourichon *et al.* 2000; López-Gatius *et al.* 2006; Bell and Roberts 2007; Mee 2008).

C. burnetii infection in dairy cattle is often associated with infertility (Krauss *et al.* 1987; Aitken 1989; To *et al.* 1998). However, it has been demonstrate that early fertile cows (cows becoming pregnant before Day 90 of lactation) were more likely to be

seropositive than the remaining animals (López-Gatius *et al.* 2012), according to our results (Chapter 2). Furthermore, according to Chapter 3, shedding cows on days 91-97 postpartum showed a higher conception rate at first AI than the remaining cows. Again, these statements should be taken into account carefully. There are several factors which can modify the results of a study trying to associate *C. burnetii* infection with the conception rate:

(1) Definition of infection. How could we define a *Coxiella*-infected animal? Depending of its seropositivity? Or its shedding status?

(2) Circulation of different *C. burnetii* genotypes. What is the importance of the type of *C. burnetii* strain in the cow?

(3) Monitorization or not of other factors traditionally affecting the conception rate such as previous postpartum diseases, days in milk, milk production, the bull inseminating semen, inseminator or season among others.

We should assume that there are no consistent studies that determine the real clinical implication of *C. burnetii* infection on the conception rate of the dairy herds. Furthermore, the enormous cost of the analyses for example, PCR for all routes of shedding such as milk, feces and vaginal fluid during the postpartum and insemination periods question the real necessity of performing these kinds of studies. Finally, the study population should be elevated to try to demonstrate clearly the consequences of a factor on the conception rate.

1.4. Pregnancy losses

Pregnancy loss is the main symptom attributed to Q fever in sheep and goats, especially in late pregnancy (Masala *et al.* 2004; Woldehiwet 2004; Arricau-Bouvery and Rodolakis 2005; Sánchez *et al.* 2006; Wouda and Dercksen 2007; Jones *et al.* 2010). In cattle, this bacterium has been linked to abortion during the third trimester of gestation (van Moll et al, 1993; Bildfell *et al.* 2000; Cabassi *et al.* 2006; Parisi *et al.* 2006; Jensen *et al.* 2007; Jones *et al.* 2010; Pritchard *et al.* 2011; Muskens *et al.* 2012) but there is controversy. In any of our studies, relationships between abortion and *C. burnetii* infection has been found according to other authors (Lange *et al.* 1992; Tramuta *et al.* 2011; López-Gatius *et al.* 2012; Yang *et al.* 2012). In addition, bacterium could not be associated with abortion in cattle under experimental conditions (Agerholm 2013). Probably, the strain has an important role in these differences (Rusell-Lodrigue *et al.* 2009). Thus, as has already been suggested (Agerholm 2013) the importance of *C. burnetii* as an abortifacient agent in dairy cattle is overestimated. As it has been noted above on the conception rate, the main procedure to solve the question whether *C. burnetii* infection is a risk factor of abortion is to monitor all other factors than can be

related to pregnancy loss, such as management, environmental and cow factors besides concurrent infectious diseases. None of the existing published studies controlled all that.

2. Aspects of laboratorial diagnoses

2.1. Serology

Serology is the cheapest laboratorial method currently available to diagnose *C. burnetii* infection in a herd. Seroprevalence of dairy cows range from 37 to 100 in herds in Europe (EFSA 2010). ELISAs are the most commonly used assays for screening herds, but its interpretation at the individual level is difficult. We do not known, the exact time of seroconversion or the duration of antibody titers in the cow (Kennerman *et al.* 2010). Moreover, although there is a positive correlation of serology and shedding (Guatteo *et al.* 2007; Courcoul *et al.* 2010), the presence of seronegative animals that shed *C. burnetii* and seropositive that do not (Guatteo *et al.* 2007; Rousset *et al.* 2009; Hansen *et al.* 2011), questions the significance of serology in dairy cows. Despite of this, studies on dynamics of the antibody titration during the productive life of the cow will help to interpret serological data. Numerous questions regarding serology interpretation and its relationship with clinical symptomatology remain unsolved.

According with previous data (McCaughey *et al.* 2010; Böttcher *et al.* 2011; Paul *et al.* 2012), our findings demonstrated that there is a positive correlation between *C. burnetii* seropositivity and parity. The likelihood of seropositivity is 21 times higher for multiparous than for primiparous cows and heifers (Chapter 1). Therefore, multiparous cows tend to be already immunized, while young animals are susceptible to infection. In addition, pregnant cows can show a very stable pattern of *C. burnetii* antibodies throughout gestation with a postpartum decrease (Garcia-Ispierto *et al.* 2011), probably due to a shunting of serum antibodies to colostrum prior to calving. The postpartum antibody drop was more clearly observed in primiparous cows, which showed higher antibody levels compared to multiparous cows throughout gestation (Garcia-Ispierto *et al.* 2011). In fact, although there is a higher probability of having come into contact with the bacterium in multiparous cows, in a recent study multiparous cows showed a lower risk of being seropositive than primiparous cows (Garcia-Ispierto *et al.* 2011). This apparent contradiction may not be difficult to interpret in the case of dairy cows. In dairy herds, cows with reproductive disorders are culled. Maybe *C. burnetii*-seropositive cows were culled in a higher proportion than their seronegative partners. None of the existing reports studied seroprevalence of culling cows.

In Chapter 1 and 3 it has been described that the likelihood of seropositivity is higher in shedding cows compared with non-shedders, in agreement with previous studies (Guatteo *et al.* 2007; Courcoul *et al.* 2010). The previous authors suggested that this relation between shedding patterns and serological profiles is due to strong stimulation of the immune system of the infected cow. However, previous works have reported a

large number of exceptions to all these findings, describing multiparous and/or shedding cows with a seronegative status (Guatteo *et al.* 2007; Rousset *et al.* 2009; Garcia Ispierto *et al.* 2011; Hansen *et al.* 2011; Nogareda *et al.* 2012). Accordingly, seronegative shedders have also been detected in our studies (Chapter 1, 3). The reason why several cows do not develop a humoral response is still unknown. A genetic resistance to infection or an immunotolerance phenomenon could explain the seronegativity of theses cows. Thus, due to several limitations, serology may not be sufficient to diagnose *C. burnetii* infection, and a combination of serology and molecular biology is necessary to determine the level of infection in cattle.

2.2. Shedding patterns

Shedding of bacteria occur throughout milk, faeces and vaginal mucus. A recent study showed that dairy cows shedders with antibodies shed for a longer period of time than shedders without antibodies (Courcoul *et al.* 2010). Despite of that, serology is not a completely reliable screening test for the detection of shedders within a herd (Natale *et al.* 2012) and PCR performed in all possible routes is necessary to understand *C. burnetii* infection. Moreover, animals do not shed the bacteria continuously so that intermittent shedding is a common status of the infected cow (Guatteo *et al.* 2007). Thus, at clinical level, this can be practically and economically impossible to afford.

There are few publications relating *C. burnetii* shedding with reproductive disorders. The presence of *C. burnetii* DNA in placental cotyledons and in fetal tissues has been associated with abortions (Pritchard *et al.* 2011; Muskens *et al.* 2012). In addition, one study linked *C. burnetii* shedding in milk with chronic subclinical mastitis (Barlow *et al.* 2008). However, a study from Guatteo *et al.* (2006) described that the proportion of shedding cows not differ significantly between aborted and non-aborted cows. In our studies, shedding cows have shown an earlier return to cyclicity and a higher conception rate than non-shedders and all PCR positive cotyledons were collected from full term parturitions (Chapter 2, 3). Thus, the presence of the bacterium does not necessarily imply a clinical infection. The viability of the detected bacterium, strain and immune status of the shedding cow are factors to consider.

Shedding of *C. burnetii* in any of the three routes during parturition and third trimester of gestation is maximized due to a recrudescence of the bacteria (Harris *et al.* 2000; Chapter 1, 2, 3). The immunosuppression status of the cow during parturition due to high plasma concentrations of progesterone and cortisol may explain this increase in the number of shedding cows in these periods (Lewis *et al.* 2004).

During postpartum, shedding animals decrease until 90 days in milk (DIM), probably due to the immune status recovery of animals (Harris *et al.* 2000; Lewis *et al.* 2004). Regarding shedding routes, persistent shedding pattern have only been determined in milk (Guatteo *et al.* 2007), but not in vaginal mucus and feces (Guatteo *et al.* 2007).

These findings indicate that the digestive tract, uterus and vaginal environment may be less comfortable to the bacterium than the environment from the mammary gland. Clinical repercussions of this are not yet determined.

3. Does vaccination against *C. burnetii* improve reproductive performance?

Nowadays, measures to control *C. burnetii* infection in a farm consist in two main routes: (1) Antibiotherapy or (2) vaccination. The first one, although it has been demonstrated to reduce shedding in cattle, does not reduce abortion or prevent shedding (Durand 1993; Rodolakis 2009; Angelakis and Raoult 2010; Taurel *et al.* 2012b) and it is not economically viable in dairy herds. In effect, milk following antibiotic administrations has to be removed from the food chain (European Commission 1996). Thus, vaccination against *C. burnetii* is the only possible solution to prevent shedding of the bacteria in a herd.

Several vaccines against *C. burnetii* have been developed: the phase I and phase II vaccine. Phase I seems the most protective, inducing seroconversion and reducing bacterial shedding and abortion rates in seronegative and/or PCR-negative goats (de Cremoux *et al.* 2012). In dairy cattle, a Th2 immune response and reduced shedding has been observed only in non-pregnant animals that are seronegative and/or PCR-negative (Guatteo *et al.* 2008). When applied in infected animals during peri-insemination period, vaccination does not prevent *C. burnetii* shedding (Guatteo *et al.* 2008; Rousset *et al.* 2009) leading the question the use of vaccination in adults. However, nulliparous heifers, considered most of them to be non-infected animals (Taurel *et al.* 2011, 2012a), are a common target population for vaccination.

Due to the farm management policy, vaccination post-AI is sometimes difficult and requires additional management efforts. Recently, two studies applied this vaccine during the dry period. Although this also did not reduce shedding during postpartum period (Chapter 3), it has been demonstrated to be safe, not increasing abortion rate, and improved subsequent fertility of herd, especially when applied to *C. burnetii* seronegative animals (Chapter 4). The question that arises is how vaccination against *C. burnetii* increases reproductive performance. Two hypotheses emerge: (1) vaccination protects seronegative animals to be infected with *C. burnetii* or (2) there is a non-specific immunostimulation after vaccination that is beneficial for the animal. Thus, probably it is better to vaccinate heifers (usually seronegative animals) and pregnant cows to increase subsequent reproductive performance in an already infected herd.

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CONCLUSIONS

CONCLUSIONS

The main conclusions of this thesis are:

- There was no detectable precolostral antibody response in calves born from dams with *C. burnetii*-qPCR-positive cotyledons.
- Multiparous cows showed a significantly higher seroprevalence than primiparous cows and heifers.
- Colostral antibodies were efficiently transferred to newborn calves
- There was a link between bacterial shedding on Day 171-177 of gestation and *Coxiella*-seropositivity of the dam.
- Animals not infected with *Coxiella* seem to be susceptible to infection and not protected against the bacterium in dairy herds.
- The elevated costs of determining infection at the farm level, make monitoring of cows virtually impossible from a clinical standpoint.
- Vaccination of pregnant dairy cows with an inactivated *Coxiella burnetii* phase I vaccine at the start of the third trimester of pregnancy did not reduce shedding of the bacterium.
- Irrespective of vaccination, shedding levels during the postpartum period were positively related to fertility in response to first AI.
- Phase I vaccination against *C. burnetii* on advanced pregnant dairy cows improved subsequent fertility.
- Vaccination could be implemented in *Coxiella*-infected dairy herds, especially on seronegative animals.

EPILOGUE

CHAPTER 5

Q fever: real threat or false alarm?

Winner paper of “Premi de Comunicació Científica Joan Lluís Vives 2013, XIV edició. Modalitat de ciències bàsiques, ciències de la salut, enginyeries i arquitectures”.

Q FEVER: REAL THREAT OR FALSE ALARM?

1. Introduction

Anton van Leeuwenhoek was a Dutch naturalist who first time observed spermatozoa in 1677 by an own-made microscope. He named them "animalcules" (Karamanou *et al.* 2010). Science revealed that those diminutive animalcules were responsible of the human existence. Later, the German physician Robert Koch discovered the etiologic agent of anthrax, *Bacillus anthracis*, in blood of infected cows (Schmitt 1982). After a long time of skepticism, bacteria were recognized as causative agents of disease. Great efforts have been made by scientific community to classify and characterize microorganisms. Moreover, scientists have found the way to use them in their own benefit. Actually, microorganisms have been used since antiquity. The first evidence of the fermented milk products elaboration appeared around the year 10000 BC (Pederson 1979). Nowadays food industry (wine, beer, yogurt, bread), and human and animal health (mainly antibiotics and vaccines), take advantage of the properties and benefits associated with bacteria. In fact, knowledge of bacteria has improved both, human health and food quality, and allowed the eradication of high morbidity viruses such as *Smallpox* virus (Henderson 2009) and keep under control bacterial diseases such as leprosy (WHO 2012).

Contrary to one would think, humans are far from bacteria control. Due to the increasing occurrence of antibiotic resistant strains, there are groups of bacteria responsible for pandemic diseases whose treatment is no longer effective. Examples are the multiple drug-resistant *Mycobacterium tuberculosis*, penicillin-resistant *Streptococcus pneumonia*, vancomycin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*, a major cause of hospital-acquired infections (Domin 1998; Cole 2012).

Zoonoses are a specific group of infectious or parasitic diseases able to be transmitted from animals to human population (Pastoret 2009; WHO 2013). Currently, 60% of human pathogens are zoonotic (Cleaveland *et al.* 2001) and so 75% of human emerging diseases (Slingenbergh *et al.* 2004). Emerging diseases are defined as those have appeared recently in humans or that existed previously but nowadays are rapidly increasing in incidence (Cleaveland *et al.* 2001; WHO 2013).

2. Q fever, a XXI century zoonosis

Q fever is a good example of an emerging zoonosis. The disease was first described by Derrick (1935), a doctor from Queensland (Australia). He described an outbreak of an unspecific disease in a group of workers from a cattle slaughterhouse (Derrik 1937). A year later, and after several failed attempts to associate the outbreak with a previous known microorganism, Derrick sent samples from his patients to his colleague Burnet, a

doctor from Melbourne. Burnet was successful in isolating this unknown bacterium. Meanwhile, in 1937, Herald Cox isolated the same microorganism from ticks in United States of America. The "Q fever" name was given by its first discoverer, Derrick. The name's etymology includes: (1) "Fever", the main symptom and (2) "Q" from the word "Query" (question), referred to the mystery of what microorganism caused those episodes of fever. Etiologic bacterial agent was named as "*Coxiella burnetii*" in honor of its discoverers Cox and Burnet (Maurin and Raoult 1999).

C. burnetii main characteristics are the high resistance in the environment and high infectious capacity by aerosols (Azad 2007). Probably for that reason, this bacterium was object of many experiments to use it as a biological weapon during Second World War by the ancient URRS, USA and Japan (Alibek 1999; Garrett 2000). However, Q fever was not really considered as a disease of a public health importance until the first decade of XXI century. At that moment, an epidemic wave of Q fever appeared in several countries of the European Union (Georgiev *et al.* 2013). The most significant one was an outbreak in the Netherlands between years 2007 and 2010. In 2009, 2.357 human cases were reported, with six mortal victims (van der Hoek *et al.* 2010; Georgiev *et al.* 2013). In addition, livestock sector, especially goat herds, reported large economic losses due to miscarriages (Roest *et al.* 2011). Furthermore, the stamping out applied to the infected herds involved the sacrifice of a large number of goats (van der Hoek *et al.* 2010; Roest *et al.* 2011). Until that moment, Q fever has been considered as a reemerging zoonosis and started being the subject of several scientific studies worldwide (Arricau-Bouvery and Rodolakis 2005).

3. What is known today about Q fever?

As previously said, *Coxiella burnetii* is the etiologic agent of Q fever, an endemic worldwide disease. It is an obligated intracellular bacillus that can be isolated from a large number of hosts such as ticks, wild and domestic mammals and humans. However, domestic ruminants (sheep, goat and cow) are the main reservoir and source of infection for humans (Maurin and Raoult, 1999; Arricau-Bouvery *et al.* 2005). Infected animals can shed the bacterium to the environment through milk, feces, vaginal fluid, urine, semen and abortion and parturition products (fluids and placenta) (Kruszewska and Tylewska-Wierzbanowska 1997; Heinzen *et al.* 1999; Guatteo *et al.* 2006). Despite shedding is possible at any time, abortion or parturition are the periods when bacterial shedding is maximized (Guatteo *et al.* 2007; Harris *et al.* 2010). Contaminated aerosols can travel long distances transported by wind. Thus, it is possible that *C. burnetii* infect not only to risk personnel such as veterinarians or farmers, but also general population (Figure 1). Once inhaled, bacteria reach lungs of the host and are phagocyted, spread throughout the host organism and finally excreted out of the body to the environment (Maurin and Raoult 1999).

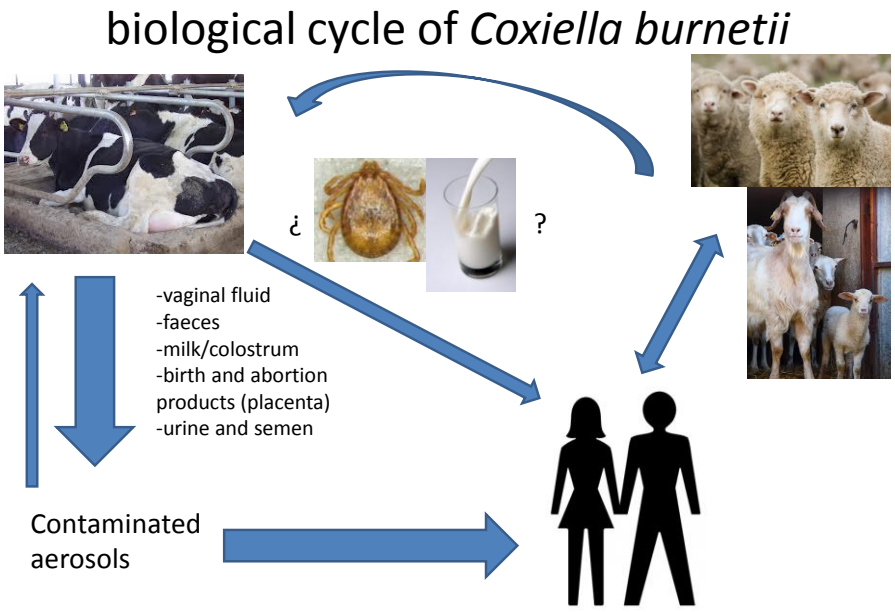

Figure 1. Schematic representation of *Coxiella burnetii* biological cycle. In this diagram, the epidemiological role of the transmission by oral ingestion of contaminated raw milk or the tick as a possible vector is questioned.

Clinical signs produced by *Coxiella burnetii* in humans are well known. It has been calculated that in 60% of cases the infection are subclinical. However, in some cases it can cause a flu-like syndrome with high fever and respiratory problems. Moreover, in the chronic form, hepatitis or endocarditis may appear. These symptoms mainly occur in immunocompromised or advanced age patients. In addition, abortions in pregnant women have also been reported (Porter *et al.* 2011). In fact, abortions caused by *Coxiella burnetii* are underdiagnosed in women (Marrie 1993; Carcopino *et al.* 2009). Furthermore, seroconversion occurs in infected patients 2-3 weeks after the onset of symptoms (Tissot *et al.* 1994; Fournier *et al.* 1998; Fournier and Raoult 2003).

4. What does still remained unknown about Q fever?

In domestic mammals this disease has not been extensively studied as in humans. Moreover, different studies have shown contradictory results (To et al, 1998; Woldehiwet *et al.* 2004; Ruiz-Fons *et al.* 2010; Agerholm 2013). Reproductive problems such as abortions, infertility, premature births with weak or dead calves, endometritis, placentitis, placental retention at parturition and even mastitis have been linked to *C. burnetii* infection (Krauss *et al.* 1987; Tainturier 1987; Aitken 1989; van Moll *et al.* 1993, Sanford *et al.* 1994; To *et al.* 1998; Bildfell *et al.* 2000; Arricau-Bouvery and Rodolakis 2005; Jensen *et al.* 2007; Barlow *et al.* 2008; Jones *et al.* 2010; López-Gatius *et al.* 2012, Muskens *et al.* 2012). In cattle, this infection is still more contradictory, maybe because in this specie *C. burnetii* is usually subclinical and difficult to detect at farm level (Porter *et al.* 2011). Thus, this bacterium can not only be

a public health problem, but also can cause an economical stroke in herds. To control this disease in human population, scientific community should start controlling the bacterium in farms, especially in ruminant herds. This could only be possible whether the knowledge of the pathogenesis of *C. burnetii* improves.

To control the disease it is essential to detect infected animals. However, the diagnosis is now not accurate at clinical level. There are two laboratorial techniques to detect the bacteria: Serology such as Enzyme-Linked ImmunoSorbent Assay (ELISA), based on plasma detection of anti-*C. burnetii* antibodies, and molecular biology such as Polymerase Chain Reaction (PCR), based on the detection and amplification of DNA fragments of the bacterium. A combination of both is frequently required to detect the infection, because there are seropositive cows that do not shed the bacterium and seronegative animals that shed it (Guatteo *et al.* 2007; Rousset *et al.* 2009; Hansen *et al.* 2011). The question that still remains to be solved in cattle is whether the pattern of shedding and serology has a meaning as in humans or is just an individual variance.

5. Effects of Q fever in bovine, the controversy

Nowadays, as it is discussed above, the implication of *C. burnetii* in reproductive problems of dairy cows is still not well clarified. In one hand, several reproductive problems are described, such as placenta retention, abortions and infertility in seropositive animals (Krauss *et al.* 1987; To *et al.* 1995; Hässig and Lubsen 1998; To *et al.* 1998, López-Gatius *et al.* 2012). On the other hand, several scientists demonstrated no effect of this bacterium on reproduction (Lange *et al.* 1992; Nielsen *et al.* 2011; Muskens *et al.* 2012; Paul *et al.* 2012; Agerholm 2013) or even found a positive correlation with fertility (Garcia-Ispierto *et al.* 2012). Maybe the confounding results are due to the diagnosis of this disease. While in some papers consider that a cow is suffering Q fever using serology, others consider shedding patterns or even a combination of both, serology and molecular techniques. Moreover, culled animals should be considered in the analyses because less productive and infertile animals are culled for economical reasons. It is possible that *C. burnetii* is present in those cows.

The controversy found in literature, could be also explained by the bacteria strain. It has been demonstrated that *C. burnetii* virulence is variable according to the strain. Thus, *Nine Mile* has a higher virulence than *Priscilla* and *Dugway* strains (Rusell-Lodrigue *et al.* 2009). Recently it has been demonstrated that strains of our region, Spain, have low virulence (Jado *et al.* 2012). Thus, it is necessary that scientists determine the *C. burnetii* strain before performing analyses regarding effects of this bacterium on animals.

6. Can Q fever be treated in animals?

In human medicine, treatment for coxiellosis with antibiotics is well established (Porter *et al.* 2011). In contrast, in animals, antibiotherapy has a restricted use for three obvious reasons. First, there must be a period of suppression for meat or milk consumption. Second, for animal use, the treatment is expensive and third, the effectiveness of antibiotherapy in animals remains unclear (Muskens *et al.* 2007). Moreover, results of existing studies are contradictory (Behymer *et al.* 1977; Astobiza *et al.* 2010). Generally, antibiotherapy is associated with the decrease of the bacterial load shed. However, the treatment is not able to prevent shedding nor limit the duration of bacterial excretion (Durand 1993; Rodolakis 2009; Angelakis and Raoult 2010; Taurel *et al.* 2012). For these reasons, control methods acquire relevance.

7. How to Control Q fever in dairy cattle farms

Control Q fever involves the application of measures to reduce the possibility of cow to cow or cow-another specie transmission, including humans, especially staff. The most relevant control methods are the following:

-To keep housings an especially calving parlour in good hygienic conditions, and maintain well-ventilated environments (Arricau-Bouvery and Rodolakis 2005; EFSA 2010).

-To remove the placenta after an abortion or parturition as fast as possible, always manipulating it with gloves (Woldehiwet 2004; Arricau-Bouvery and Rodolakis 2005; EFSA 2010).

-To avoid remove manure on windy days for not to spread the bacteria (Berri *et al.* 2004).

- To prevent the entry of wild animals, dogs and cats on the farm (Madariaga 2005).

- To pasteurize or sterilize milk for human consumption (Enright *et al.* 1957; Anon 2004; Woldehiwet 2004; Cerf and Condron 2006).

- Vaccination to immunize animals. Nowadays, inactivated phase I vaccines are the main immunological products used to be applied in domestic ruminants (Arricau-Bouvery and Rodolakis 2005). This vaccine reduces shedding levels in infected females (Sadecky *et al.* 1975; Sadecky and Brezina 1977; Brooks *et al.* 1986; Guatteo *et al.* 2008; de Cremoux *et al.* 2012; Taurel *et al.* 2012). In addition, in goats, it reduces the incidence of abortions (Arricau-Bouvery *et al.* 2005). However, these positive effects are only described in both, non-infected and non-pregnant females (Guatteo *et al.* 2008). Recently, a study has described an improved reproductive performance in vaccinated

cows during the third trimester of pregnancy, compared to the remaining animals (López-Helguera *et al.* 2013).

8. The reality on Q fever

In the last years, Q fever has been a very controversial issue among the scientific community. On one hand, there are defenders of the public health importance of this zoonosis and on the other hand, others believed that Q fever is just a subclinical infectious process, appearing sporadic clinical manifestations in subjects with predisposing factors.

After the last outbreak produced in the Netherlands from 2007 until 2010, no new epidemic waves have been reported in Europe. However, *C. burnetii* infection is detected in the all 5 continents (Guatteo *et al.* 2011). The question that arises is whether Q fever is now under control. It is probable that the continuous mutation of all bacteria, including *C. burnetii*, has created a low virulence strain. As a conclusion, more studies are needed to determine the consequences of this infection in dairy herds. To achieve this objective, laboratorial methods should improve, allowing an economic and practical diagnosis of Q fever infection in herds. Furthermore, to avoid the spread of virulent strains from one area to another, a strict control of livestock movements is necessary. In fact, probably with the current biosecurity methods, this infection should be under control. Thus, management practices seem to be the key to solve Q fever.

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APPENDIX

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Com es sol dir, no sé per on començar. Pel principi per exemple.

De baixa laboral, a causa d'una injecció accidental de tuberculina realitzant tasques de sanejament, vaig decidir que necessitava una feina amb reptes, incentius, i sentir-me veterinari. Per una sèrie d'esdeveniments combinats amb atzar vaig acabar concertant una cita amb la Doctora Irina Garcia Ispierto i el Doctor Fernando López Gatius a l'Escola Tècnica Superior d'Enginyeria Agrària de la Universitat de Lleida. Recordo perfectament el dia que vaig entrar per primera vegada al despatx dels meus directors de tesi. Ells em van explicar el què feien, a què es dedicaven i em van proposar si volia formar part d'un projecte que estaven a punt d'enjegar. -"Saps que és la febre Q?"- em preguntà la Irina mentres el Fernando escoltava atent des de la seua taula. De seguida em van contagiar els seus ànims i la passió amb què parlaven de la seua feina. Vaig dir el SI VULL aquella mateixa tarda.

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SHORT COMMUNICATIONS AND POSTERS SUBMITTED TO NATIONAL AND INTERNATIONAL CONFERENCES

NATIONAL CONFERENCES

Tittle: Estudio de la evolución de anticuerpos de *Coxiella burnetii* en un rebaño de vacas lecheras de alta producción en el noreste de España durante un año
Authors: Tutusaus J, Garcia-Ispierto I, López-Gatius F, Nogareda C
Presentation form: Oral communication
Date: 19/11/2010
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Organizing entity: Asociación de Veterinarios Especialistas en Diagnóstico de Laboratorio (AVEDILA)
Publication: Book of Congress reports

ESTUDIO DE LA EVOLUCIÓN DE ANTICUERPOS DE *COXIELLA BURNETII* EN UN REBAÑO DE VACAS LECHERAS DE ALTA PRODUCCIÓN EN EL NORESTE DE ESPAÑA DURANTE UN AÑO

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Coxiella burnetii, agente causante de Fiebre Q, es una zoonosis distribuida mundialmente. Esta bacteria puede producir desórdenes reproductivos tales como abortos, muerte fetal, crías débiles, mastitis, metritis e infertilidad en el ganado vacuno lechero. El diagnóstico serológico por ELISA se considera una de las pruebas de elección para el estudio seroepidemiológico. El objetivo de este trabajo fue evaluar los anticuerpos de *C. burnetii* en 478 vacas lecheras de alta producción. El análisis serológico se realizó mediante un kit comercial de ELISA indirecto LSIVET. Una muestra se consideró negativa, positiva 1, 2, 3 o 4 según si el título era ≤ 40, de 40 a ≤ 100, de 100 a ≤ 200, de 200 a ≤ 300 y > 300, respectivamente. La prevalencia de anticuerpos fue del 51% en 2009 y 49,4% en 2010. El 65% de los animales se mantuvieron estables en el mismo grupo, el 10,5% disminuyeron y el 24,5% aumentaron durante el periodo de estudio. El 4,8% de los animales seropositivizaron, mientras que un 6,5% seronegativizaron. El análisis de la leche de tanque por RT-PCR en los dos años estudiados confirmó una fuerte excreción bacteriana (>10.000 bacterias/ml). Los resultados obtenidos son similares a los publicados por Guatteo y col. (2007), sugiriendo la amplia distribución de la infección y la estabilidad de los anticuerpos.

Tittle: Relationship between *Coxiella burnetii* shedding in faeces, milk and vaginal fluid at parturition and the presence of bacterirum in placental cotyledons
Authors: Tutusaus Batlle J, López-Gatius F, Garcia-Ispierto I
Presentation form: Oral communication

Date: 15/05/2013
Place: Zaragoza (Spain)
Name of the congress: XV Jornadas sobre Producción Animal de la Asociación Interprofesional para el Desarrollo Agrario
Organizing entity: Asociación Interprofesional para el Desarrollo Agrario (AIDA)
Publication: Book of Congress reports, Vol. 2, Page 819

RELACIÓN ENTRE LA EXCRECIÓN DE *COXIELLA BURNETII* EN HECES, LECHE Y FLUIDO VAGINAL AL PARTO Y LA PRESENCIA DE LA BACTERIA EN LOS COTILEDONES PLACENTARIOS

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INTRODUCCIÓN

La fiebre Q es una zoonosis endémica a nivel mundial producida por un bacilo Gram negativo intracelular obligado, *Coxiella burnetii* (Maurin y Raoult, 1999). A pesar de que existe un amplio rango de hospedadores, los rumiantes domésticos constituyen los principales reservorios y la fuente de infección más importante para los humanos (Maurin y Raoult, 1999). La sintomatología clínica ha sido extensamente estudiada en humanos y en pequeños rumiantes, pero existe controversia en la vaca. Frecuentemente se asocia con problemas subclínicos, de los cuales destacan la subfertilidad, placentitis, metritis, mastitis (Porter *et al*., 2011) o problemas endocrinos, siendo los abortos esporádicos en esta especie (To *et al*., 1998; Garcia-Ispierto *et al*., 2010). Además, la bacteria se excreta al medio ambiente a través de uno o varios canales, como son el fluido vaginal, los productos del parto y los abortos, la placenta, las heces y la leche y pueden existir animales seropositivos no excretores, y excretores seronegativos. Por todo esto la detección de animales infectados es complicada a nivel de granja (Guatteo *et al*., 2007). El objetivo del presente trabajo fue estudiar la posible relación entre la presencia de *Coxiella burnetii* en los cotiledones placentarios el día del parto con la retención de placenta, los terneros nacidos muertos, la serología y la excreción bacteriana por otras vías como son el fluido vaginal, la leche y las heces durante los días 171-177 de gestación y el día del parto.

MATERIAL Y MÉTODOS

El estudio se realizó en dos explotaciones comerciales de vacuno lechero Frisón de alta producción en el noreste de España con 625 y 125 vacas en lactación de octubre de 2010 a octubre de 2011, en rebaños infectados por Fiebre Q. Un análisis previo al estudio mediante PCR cuantitativa de leche de tanque reveló la existencia de una infección natural en los dos rebaños por *Coxiella burnetii*. Los datos de este estudio se

obtuvieron de 78 vacas lecheras de alta producción procedentes de las dos explotaciones.

Diseño experimental

Las vacas se muestrearon los días 171-177 de gestación y el día del parto. En los dos muestreos se extrajo sangre para la determinación de los niveles plasmáticos de anticuerpos específicos frente a *Coxiella burnetii* y se tomaron muestras de heces, fluido vaginal, leche y calostro y cotiledones el día del parto, para detectar *C. burnetii* mediante PCR cuantitativa (QIAmp DNA minikit®, Qiagen S.A. and LSI Taqvet *Coxiella burnetii*®; Laboratoire Service International, Francia). Para la detección de anticuerpos anti *C. burnetii* se utilizó un kit de ELISA indirecto (LSIVET RUMINANT, Laboratoire Service International, Francia).

Datos registrados y análisis

Se realizaron seis regresiones logísticas binarias mediante el programa estadístico SPSS (versión 18). Para las regresiones se utilizaron la retención de placenta, los nacidos muertos, la serología en los días 171-177 de gestación, la excreción en el preparto, la excreción al parto, y la presencia de *C. burnetii* en los cotiledones como variables dependientes.

RESULTADOS Y DISCUSIÓN

La población de estudio estaba comprendida por 15 primíparas y 63 multíparas. En los días 171-177 días de gestación se diagnosticaron 41 vacas seropositivas (52,6%) frente a *C. burnetii*. Se analizaron un total de 546 muestras mediante PCR, de las cuales el 8,6% resultaron positivas (n=47) (Tabla 1). En concordancia con otros estudios (Harris *et al*., 2000), las muestras analizadas indican un aumento de excreción vía vaginal durante el parto, comparado con otros periodos.

Mediante regresión logística no se encontraron relaciones significativas entre la retención de placenta, los terneros nacidos muertos, la seropositividad y la excreción de *C. burnetii* en los días 171-177 de gestación con la presencia de *C. burnetii* en los cotiledones placentarios. Estos hallazgos refuerzan la idea de que en ganado vacuno las infecciones por *C. burnetii* se caracterizan por su forma subclínica (Guatteo *et al*., 2007). En un estudio reciente (Tutusaus *et al*., 2013, aceptado para publicación) se ha observado que todos los terneros nacen seronegativos, con independencia del perfil serológico y el patrón de excreción maternos. Análisis histológicos son necesarios para determinar las consecuencias de la presencia de la bacteria en la placenta, así como determinar el momento concreto de infección placentaria y/o fetal.

En el presente trabajo la totalidad de las vacas incluidas en el estudio (n=78) tuvieron un parto a término, independientemente de la presencia o no de la bacteria en los cotiledones (17%). Este hecho refuerza la idea de que *C. burnetii* raramente produce abortos en la especie bovina a pesar de que las células trofoblásticas sean una localización habitual de la bacteria (Ben-Amara *et al*., 2010; Hansen *et al*., 2011). Sin embargo la cepa y el estado inmunológico son factores que se deberían tener en cuenta debido a que podrían condicionar la aparición de la sintomatología clínica.

Según la Odds ratio, la probabilidad de presentar cotiledones PCR positivos a *C. burnetii* al parto es 10,3 veces mayor en las vacas excretoras al parto por otras vías (heces, fluido vaginal y calostro) que para las no excretoras en el mismo periodo (P=0.005). Es probable que en el momento del parto la bacteria se reactive y sea excretada al medio con más eficacia, seguramente debido a la inmunosupresión materna. La detección de estos animales excretores de forma rutinaria, eficiente y a un coste razonable, así como conocer las repercusiones sanitarias y económicas asociadas a la excreción debería ser objeto de futuras investigaciones.

Las conclusiones son que las vacas excretoras de la bacteria a través de heces, leche o fluidos vaginales al parto tienen más posibilidades de expulsar la bacteria a través de la placenta que las vacas no excretoras al parto. No se ha detectado en las 2 granjas del estudio ninguna relación entre la excreción de *C. burnetii* durante el preparto o al parto con la retención de placenta o terneros nacidos muertos.

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Tabla1. Relación de muestras PCR positivas en función del momento de muestreo y la naturaleza de las mismas.

Tipo de muestra	171-177 días preparto	Día del parto	En los dos periodos
Fluido vaginal	2 2,6%	14 18%	0
Heces	3 3,8%	5 6,4%	0
Leche/calostro	8 10,3%	2 2,6%	3 1,9%
Cotiledones		13 17%	

RELATIONSHIP BETWEEN *COXIELLA BURNETII* SHEDDING IN FAECES, MILK AND VAGINAL FLUID AT PARTURITION AND THE PRESENCE OF BACTERIUM IN PLACENTAL COTYLEDONS

ABSTRACT

The aim of this study was to analyze the relationship between *Coxiella burnetii* in the cotyledons at parturition and placenta retention, stillborns, *C. burnetii* seropositivity and shedding by other routes during days 171-177 of pregnancy and at parturition in dairy cows. Two herds in northeastern Spain provided 78 cows that were sampled at 171-177 days of pregnancy and at parturition. Samples of blood, faeces, milk, vaginal fluid, and cotyledons were collected for the specific antibodies detection in blood by indirect ELISA and *C. burnetii* DNA detection by quantitative PCR in the remaining samples. The seroprevalence during pregnancy and % of PCR positive samples was 52.6% and 8.6%, respectively. All cows delivered at term. No significant links were found in the study by logistic regression between retained placenta, stillborn calves, *C. burnetii* seropositivity and shedding on days 171-177 of gestation with the presence of *C. burnetii* in cotyledons. However, according to the odds ratio, the probability to present positive PCR cotyledons at parturition is 10.3 times higher in shedding cows at parturition by other routes than non-shedders in the same period (P = 0.005). In conclusion, *C. burnetii* shedding cows at parturition are more likely to have the bacterium in the cotyledons than non-shedders.

Keywords: *Coxiella burnetii*, cattle, cotyledons

INTERNATIONAL CONFERENCES

Tittle: Coxiella burnetii seropositivity is related to placenta retention in high producing dairy cows
Authors: López-Gatius F, Almería, S, Tutusaus J, Garcia-Ispierto I
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Organizing entity: European Society for Domestic Animal Reproduction (ESDAR)
Publication: Reprod Anim 46:124. Poster number 161 (special issue)

COXIELLA BURNETII SEROPOSITIVITY IS RELATED TO PLACENTA RETENTION IN HIGH PRODUCING DAIRY COWS

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ABSTRACT

The possible relationship between *Coxiella*-seropositivity and the reproductive performance of cows during the previous year to the serological screening was examined in three high producing dairy herds, with particular emphasis on placenta retention. The 3 herds had positive polymerase chain reaction test for *C. burnetii* in the bulk tank milk with an excretion higher than 10^4 *Coxiella* /ml. Antibodies against *C. burnetii* were detected in 50.2% of 781 parous cows analyzed, ranging from 46 to 53% for the different herds. From 440 pregnancies recorded, 16.8% (74/440) suffered pregnancy loss: 15% during the early fetal period and 2.1% after Day 90 of gestation. Logistic regression analysis indicated no significant effects of herd, lactation number and *Neospora caninum* seropositivity on retained placenta. Based on the odds ratio, the likelihood of a retained placenta increased by factors of 1.75 or 8.1 in cows showing *C. burnetii* seropositivity or twin pregnancies, respectively. No significant interactions were found. Relationships between *C. burnetii* infection and reproductive disorders such as abortion, stillbirth, weak offspring, postpartum metritis and infertility have been suggested, but to our knowledge, *C. burnetii* seropositivity has not described before as a predisposing factor for placenta retention.

Tittle: Dynamics of *Coxiella burnetii* antibodies in high producing dairy cows in northeastern Spain
Authors: Tutusaus J, Garcia-Ispierto I, Nogareda C, López-Gatius F
Presentation form: poster
Date: 15-17/09/2011
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Name of the congress: 15Th Annual Conference of the European Society for Domestic Animal Reproduction
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DYNAMICS OF *COXIELLA BURNETII* ANTIBODIES IN HIGH PRODUCING DAIRY COWS IN NORTHEASTERN SPAIN

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Q fever is a zoonosis caused by *Coxiella burnetii*, an obligate intracellular gram negative bacterium endemic worldwide. There are many mammal reservoirs of the bacterium, but the most commonly identified sources of human infection are domestic ruminants. Infection in these species is mainly asymptomatic but if clinical signs are present, reproductive disorders are the most frequently reported. In cattle, late abortion and infertility are the main clinical manifestations. The aim of this study was to assess *C. burnetii* antibodies in 478 high-producing dairy cows. Serological analyses were performed using a commercial indirect ELISA kit LSIVET. *C. burnetii* prevalence in the herd was 51% in 2009 and 49.4% in 2010. The results showed that in 65%, 10.5% and 24.5% of animals the titer remained stable in the same group, decreased and increased during the study period, respectively. Twenty four animals (5%) seroconverted, while 31 (6.5%) became seronegative. Bulk tank milk analyses by RT-PCR in the two years of study confirmed a high bacterial excretion (>10000 bacteria/ml). A group of ten cows were selected from the herd: Five seropositive and five seronegative animals. These cows were sampled individually and provided a total of eight milk and ten vaginal fluid samples. All of the fluid and 50% of milk samples were PCR negative. No excretion of *C. burnetii* into vaginal fluid was found. The results suggest a high stability of antibodies and the bacterium shedding by milk, and a broad distribution of the infection.

Tittle: La edad es un factor de riesgo para la seropositividad frente a *Coxiella burnetii* en vacas lecheras de alta producción
Authors: Tutusaus Batlle J, López-Gatius F, Garcia-Ispierto I
Presentation form: Oral communication
Date: 19/04/2012
Place: Santander (Spain)
Name of the congress: XVII Congreso internacional ANEMBE de medicina bovina
Organizing entity: Asociación Nacional de Especialistas en Medicina Bovina de España
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LA EDAD ES UN FACTOR DE RIESGO PARA LA SEROPOSITIVIDAD FRENTE A *COXIELLA BURNETII* EN VACAS LECHERAS DE ALTA PRODUCCIÓN

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Resumen

El objetivo del presente trabajo fue estudiar factores que afectan a la serología frente a *C. burnetii* en vacas lecheras de alta producción, incluyendo la posible relación con los patrones de excreción de *C. burnetii* durante el parto. Se utilizaron 45 vacas lecheras de alta producción de raza Frisona procedentes de dos explotaciones comerciales situadas en el noreste de España. Los animales se muestrearon el día 171-177 de gestación, el del parto y el 29-35 postparto. Se determinaron los niveles plasmáticos de anticuerpos frente a *C. burnetii* y la excreción bacteriana el día del parto en heces, calostro, fluido vaginal y cotiledones placentarios, mediante ELISA indirecto y PCR cuantitativa, respectivamente. Se realizó una regresión logística binaria que reveló que las vacas multíparas tenían 23 veces más riesgo de ser seropositivas frente a *C.burnetii* que las primíparas. La conclusión de este estudio fue que la edad es un factor de riesgo para la seropositividad frente a *C. burnetii* y que no existe relación entre el perfil serológico y el patrón de excreción al parto.

Introducción

La fiebre Q es una zoonosis endémica a nivel mundial producida por un bacilo Gram – intracelular obligado, llamado *Coxiella burnetii* (1). Esta bacteria posee un amplio rango de hospedadores, sin embargo, los rumiantes domésticos constituyen los principales reservorios y la fuente de infección más importante para los humanos (1). La sintomatología clínica ha sido extensamente estudiada en humanos y en pequeños rumiantes, pero existe controversia en la vaca. Frecuentemente se asocia con problemas subclínicos y subfertilidad, placentitis o problemas endocrinos (2, 3). Además, la bacteria se excreta al medio ambiente a través de uno o varios canales, como son el fluido vaginal, los productos del parto y los abortos, la placenta, las heces y la leche y pueden existir animales seropositivos no excretores, y excretores seronegativos. Por todo esto la detección de animales infectados es complicada a nivel de granja (4). El objetivo del presente trabajo fue estudiar factores que afectan a la serología frente a *C. burnetii* en vacas lecheras de alta producción, incluyendo la posible relación con los patrones de excreción de *C. burnetii* durante el parto.

Material y métodos

El estudio se realizó en dos explotaciones comerciales de vacuno lechero Frisón de alta producción en el noreste de España con 625 y 125 vacas en lactación, respectivamente, de octubre del 2010 a octubre de 2011. La media de producción de las granjas fue de 11343 y 8846 Kg por vaca y año, respectivamente. Un análisis previo al estudio mediante PCR cuantitativa de leche de tanque reveló la existencia de una infección natural en los dos rebaños por *Coxiella burnetii*. En las dos explotaciones se aplicaban programas vacunales frente a IBR y BVD. Únicamente se incluyeron en el estudio vacas clínicamente sanas. Los datos se obtuvieron de 45 vacas lecheras de alta producción.

Diseño experimental

Las vacas se muestrearon tres veces a lo largo del periodo de estudio: día 171-177 de gestación, día del parto y día 29-35 postparto. En los tres muestreos se extrajo sangre para la determinación de niveles plasmáticos de anticuerpos específicos frente a *Coxiella burnetii.* Adicionalmente, el día del parto se tomaron muestras de heces, fluido vaginal, calostro y cotiledones placentarios para detectar la posible excreción bacteriana por esas vías mediante PCR cuantitativa. Para la detección de anticuerpos anti *C. burnetii* se utilizó un kit de ELISA indirecto (LSIVET RUMINANT Milk/Serum Q FEVER de Laboratoire Service International, Lissieu, Francia)

Datos registrados y análisis

Los siguientes datos fueron registrados para cada vaca: explotación, fecha de parto, producción de leche en el día 50 de lactación, nº de partos (primíparas versus multíparas), seropositividad frente a *Coxiella burnetii* (seronegativa: título < 40, seropositiva: título ≥ 40) en los días 171-177, día del parto y día 29-35 postparto y excreción de *C. burnetii* al parto (una o más muestras positiva a la PCR). Se realizó una regresión logística binaria mediante el programa estadístico SPSS, con la variable dependiente seropositividad frente a *C. burnetii* como variable dependiente.

Resultados y discusión

Los perfiles serológicos y los patrones de excreción de las 45 vacas estudiadas se detallan en la tabla 1. No se observó seroconversión en ninguno de los animales durante el periodo de estudio. Se analizaron un total de 164 muestras mediante la técnica PCR, el 13,41% resultaron positivas.

La tabla 2 muestra las variables incluidas en el modelo final. Según la Odds ratio, la probabilidad de una vaca a ser seropositiva a *C. burnetii* era 23 veces mayor en las multíparas que en las primíparas. Estos animales han estado mayor tiempo expuestos a la bacteria, pero la pregunta sigue siendo porqué si la bacteria es tan ubicua y está presente activamente en las granjas estudiadas, no hay más animales seropositivos. Quizás, la clave está en estudiar si la infección es aguda o crónica en estos animales.

En el presente trabajo no se puede establecer ninguna relación entre el perfil serológico y el patrón de excreción al parto. Sin embargo, está descrito que las vacas excretoras tienen más probabilidades de ser seropositivas que seronegativas (4), debido a que la excreción implica la existencia de una infección activa que estimula el sistema inmune, produciéndose seroconversión. No obstante, esta estimulación no se observa en todos los casos, existiendo en menor proporción, animales excretores seronegativos. Se necesitan más estudios para comprender por qué el sistema inmune de algunos animales

infectados no se estimula o, por el contrario, si existen animales resistentes a la infección.

El 17% de los cotiledones analizados fueron positivos a la PCR, demostrando que *C. burnetii* coloniza la placenta. A pesar de ello y de acuerdo con trabajos anteriores (5), no se detectaron problemas reproductivos clínicos en estos animales. Sin embargo se necesita investigar en este sentido con mayor profundidad y con más datos para confirmar o descartar esta hipótesis.

Conclusiones

La edad es un factor de riesgo para la seropositividad frente a *C. burnetii* y no existe relación entre el perfil serológico y el patrón de excreción al parto.

Tabla1. Perfiles serológicos y patrones de excreción de las 45 vacas estudiadas

Perfil serológico	Excreción al parto	
Seropositivas n= 21	n=8	38,1%
Seronegativas n= 24	n=5	21%

Tabla 2. Odds ratio de las variables incluidas en el modelo de regresión logística para los factores que afectan a la seropositividad de *C. burnetii*.

Factor	Clase	N	% seropositividad frente a *C. burnetii*	Odds Ratio	95% IC	*P*
Número de Partos	Primíparas	1/13	7.7	Referencia		
	Multíparas	21/32	65.6	23.0	2.4-84.5	0.005

Agradecimientos

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Tittle: Los niveles de excreción y resultados serológicos individuales no son indicadores individuales de signos de enfermedad producidos por *Coxiella burnetii* en rebaños infectados
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LOS NIVELES DE EXCRECIÓN Y RESULTADOS SEROLÓGICOS INDIVIDUALES NO SON INDICADORES INDIVIDUALES DE SIGNOS DE ENFERMEDAD PRODUCIDOS POR *COXIELLA BURNETII* EN REBAÑOS INFECTADOS

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Resumen

El objetivo del presente trabajo fue estudiar la posible relación entre la serología y la excreción de *C. burnetii* en el periodo preparto y postparto, y los principales parámetros reproductivos en vacas lecheras de alta producción. Para ello se utilizaron 78 vacas de raza Frisona, escogidas al azar, procedentes de dos explotaciones comerciales situadas en el noreste de España, infectadas por Fiebre Q. Los animales se muestrearon los días 171-177 de gestación y 91-97 postparto. Se determinaron los niveles plasmáticos de anticuerpos frente a *C. burnetii* y la excreción bacteriana en heces, leche y fluido vaginal, mediante ELISA indirecto y PCR cuantitativa, respectivamente. Se realizaron siete regresiones logísticas binarias y tres análisis de supervivencia Kaplan-Meier mediante los cuales no se observó ninguna relación con la retención de placenta, la fertilidad en la primera IA, a día 90 y 150 postparto, el síndrome de la vaca repetidora, el retorno a la ciclicidad en los primeros 35 días postparto y la pérdida de gestación en los primeros 90 días post-IA. Los resultados del presente estudio sugieren que la cepa estudiada de *C. burnetii* no afecta al proceso reproductivo de vacas lecheras de alta producción en nuestra zona de estudio.

Introducción

La fiebre Q es una zoonosis endémica a nivel mundial producida por un bacilo Gram negativo intracelular obligado, *Coxiella burnetii* (1). A pesar de que existe un amplio rango de hospedadores, los rumiantes domésticos constituyen los principales reservorios y la fuente de infección más importante para los humanos (1). La sintomatología clínica ha sido extensamente estudiada en humanos y en pequeños rumiantes, pero existe controversia en la vaca. Frecuentemente se asocia con problemas subclínicos, de los cuales destacan la subfertilidad, placentitis o problemas endocrinos (2, 3) también están descritos metritis, mastitis y abortos (11). Además, la bacteria se excreta al medio ambiente a través de uno o varios canales, como son el fluido vaginal, los productos del parto y los abortos, la placenta, las heces y la leche y pueden existir animales seropositivos no excretores, y excretores seronegativos. Por todo esto la detección de animales infectados es complicada a nivel de granja (4). El objetivo del presente trabajo fue estudiar la posible relación entre la serología y la excreción de *C. burnetii* en el periodo preparto y postparto sobre los principales parámetros reproductivos en vacas lecheras de alta producción, como la retención de placenta, la fertilidad, el síndrome de la vaca repetidora, el retorno a la ciclicidad en los primeros 35 días postparto y la pérdida de gestación en los primeros 90 días.

Material y métodos

El estudio se realizó en dos explotaciones comerciales de vacuno lechero Frisón de alta producción en el noreste de España con 625 y 125 vacas en lactación de octubre de 2010 a octubre de 2011. Un análisis previo al estudio mediante PCR cuantitativa de leche de tanque reveló la existencia de una infección natural en los dos rebaños por *Coxiella burnetii*. Únicamente se incluyeron en el estudio vacas clínicamente sanas. Los datos se obtuvieron de 78 vacas lecheras de alta producción.

Diseño experimental

Las vacas se muestrearon dos veces a lo largo del periodo de estudio: días 171-177 de gestación y días 91-97 postparto. En los dos muestreos se extrajo sangre para la determinación de los niveles plasmáticos de anticuerpos específicos frente a *Coxiella burnetii* y se tomaron muestras de heces, fluido vaginal y leche para detectar la posible excreción bacteriana por esas vías mediante PCR cuantitativa. Para la detección de anticuerpos anti *C. burnetii* se utilizó un kit de ELISA indirecto (LSIVET RUMINANT, Laboratoire Service International, Francia). Para el análisis del fluido vaginal, heces y leche se utilizó el kit QIAmp DNA minikit® (Qiagen S.A., Francia) para la extracción del ADN de las muestras y el kit LSI Taqvet *Coxiella burnetii*® (Laboratoire Service International, Lissieu, Francia) para la realización de la PCR cuantitativa.

Datos registrados y análisis

Se realizaron siete regresiones logísticas binarias y tres análisis de supervivencia Kaplan-Meier mediante el programa estadístico SPSS (versión 18). Para las regresiones se utilizaron la retención de placenta, presencia de endometritis (definida por la presencia de fluido intrauterino ecogénico, un diámetro cervical de ≥ 4 cm o un grosor endometrial de ≥ 0,75 cm en los días 15-21 postparto (5)), la fertilidad a la primera IA, la fertilidad en los días 90 y 150 postparto, el síndrome de la vaca repetidora, las pérdidas de gestación, y la serología en los días 171-177 de gestación como variables dependientes. En los análisis de supervivencia Kaplan-Meier se analizaron las fertilidades a día 90 y 150 postparto y la ciclicidad durante las tres primeras semanas postparto (presencia de un cuerpo luteo determinada por ecografía los días 15-21, 22-28 y 29-35 postparto) estratificado para vacas seropositivas (n=30) y seronegativas (n=48) y para excretoras y no excretoras a 171-177 días de gestación y 91-97 días postparto.

Resultados y discusión

De las vacas estudiadas, 19 eran primíparas y 59 multíparas. Se analizaron mediante PCR un total de 468 muestras, de las cuales el 4,5% resultaron positivas a la PCR (n=21). Cuatro primíparas (5,13%) y dos multíparas (2,6%) seropositivizaron, mientras que dos primíparas (2,6%) y una multípara (1,3%) seronegativizaron, indicando que la mayoría de los animales mantienen constantes sus niveles de anticuerpos, tal y como observaron otros autores en trabajos anteriores (6,7). La tabla 1 indica el número de muestras con resultado positivo a la PCR en función de su naturaleza y el momento del muestreo. La ruta fecal y la láctea son las vías más utilizadas por la bacteria y son las que muestran una excreción más prolongada en el tiempo. Estos datos concuerdan parcialmente con trabajos anteriores (4).

Mediante las regresiones logísticas binarias y los análisis de supervivencia Kaplan-Meier no se encontró ninguna relación significativa entre el perfil serológico y/o los patrones de excreción sobre los parámetros reproductivos estudiados. En un trabajo realizado por Garcia-Ispierto y col. (8), se observó que los animales seropositivos y/o excretores tenían menos probabilidades de sufrir endometritis, recuperaban la ciclicidad postparto antes y presentaban mejores tasas de fertilidad comparado con el resto de animales. Esto indica que los animales no infectados (vacas seronegativas y no excretoras) eran susceptibles a la bacteria. Por todo ello, cabe pensar en dos posibles hipótesis: que la cepa estudiada de *C. burnetti* en las dos granjas no afecte a la reproducción de las vacas de nuestra zona de estudio, o que no hayamos sido capaces de relacionar la serología y la excreción con los signos de la infección a nivel clínico posiblemente por falta de potencia estadística (número limitado de vacas en el estudio).

Por lo que respecta a la serología frente a *C. burnetti*, según la Odds ratio, la probabilidad de una vaca a ser seropositiva fue 9,8 y 4,5 veces mayor en las multíparas

y en las excretoras en los días 171-177 de gestación que en el resto de animales, respectivamente (P= 0.04). Estos resultados están en concordancia con otros estudios anteriores (4, 9, 10).

Conclusiones

No se ha podido determinar relación entre el perfil serológico y los patrones de excreción de la cepa estudiada de *C. burnetii* con los principales índices y parámetros reproductivos en vacas lecheras de alta producción en nuestra zona de estudio.

Tablas

Tabla1. Relación de muestras PCR positivas en función del momento de muestreo y la naturaleza de las mismas.

Tipo de muestra	171-177 días preparto	91-97 días postparto	En los dos periodos
Fluido vaginal	2 2,6%	1 1,3%	0
Heces	4 5,1%	2 2,7%	1 1,3%
Leche	8 10,26%	1 1,3%	2 2,7%

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CURRICULUM VITAE

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Clinique Vétérinaire Universitaire, Pôle ruminants. Faculté de Médecine Vétérinaire. Université de Liège. Belgique. 08/01/2014 - 08/04/2014. Estada a l'hospital clínic (Unitat de remugants) realitzant una immersió en els camps de la cirurgia i la medicina interna en els bovins. Visites a explotacions de vaquí de llet i de carn (Blanc-Blau-Belga) de la zona per a realizar el control reproductiu, auditories de sanitat mamària i nutrició. Tutor responsable: Christian Hanzen. Cap del servei de Clínica i Obstetrícia dels animals de producció.

6. Producció Científica. Articles publicats.

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Curs acadèmic 2012-2013:
-8 hores de docència pràctica a Anatomia I. Grau de Ciència i Salut Animal (ETSEA, UdL).
-5 hores de docència pràctica a Reproducció Animal. (Aprenentatge cooperatiu). Grau de Ciència i Salut Animal (ETSEA, UdL).

Curs acadèmic 2013-2014:
-6 hores de docència pràctica a Anatomia I. Grau de Ciència i Salut Animal (ETSEA, UdL).
-6 hores de docència pràctica a Histologia. Grau de Ciència i Salut Animal (ETSEA, UdL).

-2 hores de docència teòrica i 8 h de docència pràctica a Histologia (Complements a la formació). Grau de Ciència i Salut Animal (ETSEA, UdL).
-5 hores de docència pràctica a Reproducció Animal. (Aprenentatge cooperatiu). Grau de Ciència i Salut Animal (ETSEA, UdL).

8. Cursos de postgrau rebuts

Curs teòric-pràctic d'experimentació animal per a l'acreditació com a Personal Investigador Usuari d'Animals d'Experimentació. Personal de la categoria C. Otorgat per la Unitat de formació del professorat universitari. Dins del programa formatiu del Màster en Biotecnologia en Ciències de la Salut. Curs 2010-2011. Universitat de Lleida.

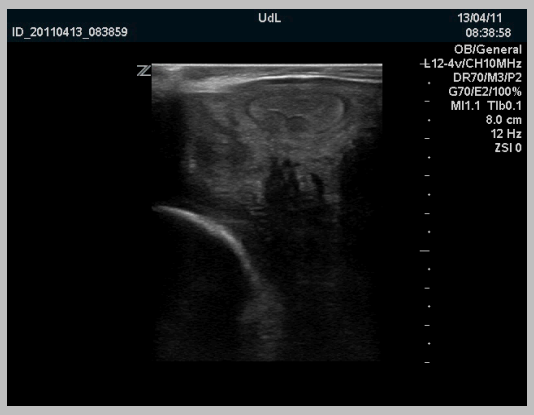
9. Experiència laboral

Contracte laboral en règim general (10/07/06 - 31/08/06). Cooperativa d'Ivars S.C.C.L. secció de crèdit. Ivars d'Urgell (Lleida). Treball en el servei de diagnòstic de gestació per ecografia a granges de porcí i en el Centre d'inseminació porcina realitzant extraccions de semen i tasques diverses al laboratori (processament i envasat de les dosis i anàl.lisi seminal).

Contracte laboral en règim general (14/07/08 - 14/08/08) en una explotació de vaquí de llet a l'empresa "Solà Comas SL". Realització de tasques pròpies de ramader i de veterinari en aquest àmbit.

Contracte laboral en règim general (01/09/2008 - 30/10/2008) en una explotació porcina de cicle tancat i 1000 mares a l'empresa "Cercle Tancat SL". Realització de tasques pròpies de ramader i de veterinari en aquest àmbit.

Jornada laboral completa (03/11/2008- 15/04/2010) en l'empresa Laboratorios Karizoo S.A., com a veterinari clínic en explotacions de vedells mamons i d'engreix. Seguiment d'explotacions, tramitació d'ajudes, sanejament, assessorament clínic i nutrició.



“Q fever is a worldwide re-emerging zoonosis caused by an intracellular Gram negative bacillus, *Coxiella burnetii*. Domestic ruminants are the main source of infection to human population. Despite the infection is mainly asymptomatic in cattle, it has been linked with several reproductive disorders. Thus, the aim of this thesis was to provide clinical information about *C. burnetii* infection in order to improve its control in dairy herds.”