Estimation of *Coxiella burnetii* prevalence in dairy cattle in intensive systems by serological and molecular analyses of bulk-tank milk samples

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ABSTRACT

A large-scale investigation on *Coxiella burnetii* was carried out in dairy cattle herds from a Q fever-endemic region to evaluate the degree of exposure to *C. burnetii* and to estimate prevalences. This study included all of the dairy cattle herds from the province of Bizkaia, Northern Spain ($n = 178$). Herds were visited between September 2009 and February 2010, and 100 mL of bulk-tank milk (BTM) per farm was collected to be analyzed by ELISA and PCR. Blood samples were also taken from about 15 animals randomly selected from each herd. One hundred nineteen of the 178 studied herds ($66.9 \pm 6.9\%$) were positive for the presence of anti-*C. burnetii* antibodies in BTM. Serum samples from 1,306 cows, 654 heifers, and 502 calves were analyzed by ELISA, and cows showed a statistically significantly higher seroprevalence ($12.3 \pm 1.8\%$) than heifers ($1.1 \pm 0.8\%$) and calves ($0.0 \pm 0.0\%$). Eighty-nine herds ($50.0 \pm 7.3\%$) had at least 1 seropositive animal, but within-herd prevalences higher than 20% were only observed in 24 herds ($13.5 \pm 5.0\%$). A significant correlation was observed between BTM ELISA sample-to-positive control ratios and within-herd seroprevalence, being higher when considering only cows ($R^2 = 0.21$). Animals from herds with negative BTM by ELISA showed a mean seroprevalence of 2.5%, whereas animals from herds with positive BTM samples had a statistically significantly higher seroprevalence (8.9% , $F = 19.7$, degrees of freedom = 1). The proportion of herds *C. burnetii* positive by BTM PCR was $51.7 \pm 7.3\%$ (92/178). The widespread distribution of *C. burnetii* in cattle advocates for the implementation of Q fever control strategies.

Key words: *Coxiella burnetii*, dairy cattle, bulk-tank milk

INTRODUCTION

In northern Spain, Q fever is endemic, and a large series of human Q fever pneumonia cases have been re-

ported (Maurin and Raoult, 1999; Montes et al., 2006). Cattle, sheep, and goats are considered the primary source of transmission to humans (Woldehiwet, 2004; Angelakis and Raoult, 2010). Clinical signs of *Coxiella burnetii* infection are abortion in sheep and goats and reproductive failure in cattle. Uterus and mammary glands are primary sites of infection in the chronic phase of *C. burnetii* infection. Shedding of this pathogen into the environment occurs mainly during parturition via birth products, but shedding through milk and feces has also been documented in sheep, cattle, and goats (Rodolakis et al., 2007). Recent studies in Northern Spain revealed the importance of sheep as a reservoir of this zoonotic pathogen and source of infection for other species, including humans (Oporto et al., 2006; García-Pérez et al., 2009; Ruiz-Fons et al., 2010). However, no data were available on the role of dairy cattle in the domestic cycle of Q fever in this region. The serological analysis of bulk-tank milk (BTM) samples can provide inexpensive, valuable information of herd exposure to *C. burnetii* and prevalence estimations. Furthermore, PCR can be used to identify *C. burnetii*-specific genetic sequences in different samples, including vaginal fluids, milk, and feces (Willems et al., 1994; Berri et al., 2000), and quantitative PCR (qPCR) allows quantification of the bacterial load (Guatteo et al., 2007b).

This study presents a large-scale investigation of the exposure and prevalence of *C. burnetii* in dairy cattle herds from a region where human Q fever is endemic. Correlations between estimations provided by BTM analysis and the real herd seroprevalence, as well as with *C. burnetii* bacterial load, were assessed. Finally, the possible effect of *C. burnetii* on cattle reproduction was also evaluated.

MATERIALS AND METHODS

Study Design and Sampling Approach

This study included all of the dairy cattle herds from the province of Bizkaia (Basque Country, northern Spain), all managed under intensive systems. All 178 dairy cattle herds were visited between September 2009 and February 2010. One hundred milliliters of BTM per farm was collected into sterile plastic tubes to be

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processed and analyzed by ELISA and PCR methods. Blood samples (5 mL) were also taken from the jugular vein of a maximum of 15 animals randomly selected among different age categories (5 calves 6–12 mo old, 5 heifers 1–2 yr old, and 5 cows older than 2 yr). However, in several cases this initial distribution of samples had to be modified due to the age structure composition of each herd (e.g., no replacements or heifers moved to breeding centers). A small questionnaire was filled in to compile information regarding the size of the herds and reproductive problems, if present.

ELISA Technique

Bulk-tank milk or individual blood serum samples were used to detect *C. burnetii* antibodies using a commercial indirect ELISA according to manufacturer's instructions (LSIVET Ruminant Milk/Serum Q fever kit; Laboratoire Service International, Lissieu, France). The sample-to-positive control (S/P) ratio in individual serum samples was calculated as follows:

$$S/P = (OD \text{ sample} - OD \text{ NC}) / (OD \text{ PC} - OD \text{ NC}),$$

where OD sample = optical density of the sample, OD NC = optical density of the negative control, and OD PC = optical density of the positive control. The results were expressed as an index: $S/P \times 100$. In the case of serum samples, S/P indices ≤ 40 were considered negative, whereas indices > 40 were indicative of positive serum. On the other hand, BTM samples with S/P indices ≤ 30 were considered negative, whereas samples with indices > 30 were considered positive for *C. burnetii* antibodies. Bulk-tank milk ELISA results were categorized according to the manufacturer's instructions into 4 different ascending categories based on the S/P index: negative ($S/P \leq 30$), slightly positive ($+$; $30 < S/P \leq 100$), positive ($++$; $100 < S/P \leq 200$), and highly positive ($+++$; $S/P > 200$).

Individual data obtained from analyzing blood sera by ELISA were used to calculate population and within-herd seroprevalence values for the population of study, as well as age class-specific (calf, heifer, or adult) seroprevalence values. In addition, BTM ELISA results were used to estimate the percentage of positive herds in the population of study.

Molecular Methods

Bulk-tank milk samples were subjected to DNA extraction using BioSprint 96 DNA Blood Kit (Qiagen Iberia SL, Madrid, Spain) following the manufacturer's instructions. Negative extraction controls were included

every 10 samples to rule out DNA contamination. The PCR amplification was performed using primers targeting a transposon-like repetitive region of *C. burnetii* as described elsewhere (Willems et al., 1994; Berri et al., 2000) adding 100 ng of DNA template to each reaction.

Additionally, to quantify the number of bacteria in PCR-positive BTM, samples from 44 herds with different S/P values in ELISA [4 negative, 21 slightly positive ($+$) and 19 positive ($++$)] were selected and analyzed by real-time qPCR using a commercial kit (LSI Taq-Vet *Coxiella burnetii*; Laboratoire Service International), according to the manufacturer's instructions. This is a duplex qPCR assay that includes a probe targeting the repetitive transposon-like region of *C. burnetii*, and a probe that detects a host-encoded gene (*GAPDH*) used as internal amplification control. The PCR assays were performed using an ABI PRISM 7500 FAST thermocycler (Applied Biosystems Inc., Carlsbad, CA). A sample was considered positive if the cycle threshold (C_t) value of the target gene was below 40 and amplification of the internal amplification control was required to rule out false negatives caused by PCR inhibition. Loads of *C. burnetii* were quantified following the recommendations given by the kit manufacturer. The C_t values of the target gene were converted into estimated quantities of *C. burnetii* using serial dilutions of known concentration of the external positive control. Results were expressed as the logarithmic transformation ($\log_{10}$) of the number of *C. burnetii* per milliliter.

Statistical Analyses

Linear regression was performed with the following purposes: 1) to analyze if general and age class-specific herd *C. burnetii* seroprevalence and the level of antibodies in BTM were related, 2) to determine if herd size had any effect on the observed general and age class-specific herd seroprevalence in the level of antibodies in BTM or in BTM *C. burnetii* bacterial load, 3) to study if any relationship existed between general and age-specific herd seroprevalence and the BTM *C. burnetii* bacterial load, and 4) to search for any association between the level of antibodies and BTM *C. burnetii* bacterial load. Later, we tested for differences in average seroprevalence values and *C. burnetii* levels in BTM for herds with different levels of antibodies in BTM [categorical; negative, slightly positive ($+$) and positive ($++$)] by means of ANOVA. We finally tested for the effect of different herd factors, such as *C. burnetii* seroprevalence level (continuous; in percentage), herd size (continuous), herd management (categorical; presence/absence of outdoor premises), and coexisting domestic ruminant species within the herd (categorical;

presence/absence), on the risk of cattle herds suffering from reproductive disorders (categorical; reported/non-reported) by means of logistic regression models. Univariate models were initially performed with each of the explicative variables and the dependent variables. Later, explicative variables were introduced into bivariate models according to their Akaike Information Criterion (AIC) values from univariate models (in an ascendant order) by a forward stepwise procedure. The final model was selected as the one with the lowest AIC value (Akaike, 1974) from all of the models performed. The level of significance was set at $P < 0.05$. Statistical uncertainty associated with the calculation of proportions was assessed by estimating the 95% confidence interval according to the expression $CI_{95\%} = 1.96[p(1 - p)/n]^{1/2}$, where p is the seroprevalence (as a fraction of unity) and n is the sample size.

RESULTS

BTM Serology

A total of 178 BTM samples were tested, with 119 being positive to the presence of anti-*C. burnetii* antibodies ($66.9 \pm 6.9\%$; $\pm$ CI). Fifty-six out of the 178 ($31.5 \pm 6.8\%$) studied herds showed slightly positive (+) results in ELISA, 63 were positive (++); $35.4 \pm 7.0\%$), and none of the herds was classified as highly positive (+++).

Within-Herd Seroprevalence and Antibody Levels in BTM

A total of 1,306 cows, 654 heifers, and 502 calves were sampled and analyzed. Age could not be recorded for 83 of the surveyed animals. Antibodies against *C. burnetii* were observed in $6.7 \pm 1.0\%$ of the animals tested. Cows showed a statistically significant higher seroprevalence ($12.3 \pm 1.8\%$) compared with heifers ($1.1 \pm 0.8\%$) and calves ($0.0 \pm 0.0\%$; $\chi^2 = 69.3$, $P < 0.001$).

Eighty-nine herds ($50.0 \pm 7.3\%$) had at least 1 seropositive animal, but high prevalences ($>20\%$) were only observed in 24 of 178 ($13.5 \pm 5.0\%$) herds. Within-herd seroprevalences ranged between 0 and 66.7%. A significant correlation was observed between BTM ELISA S/P values and within-herd seroprevalence ($R^2 = 0.18$, $P < 0.001$). However, this relationship improved when considering age categories separately, especially when herd seroprevalence was calculated for cows only ($R^2 = 0.21$, $P < 0.001$; Figure 1). Animals from herds with negative BTM by ELISA showed a mean seroprevalence of $2.5 \pm 0.8\%$ (range 0–25%), whereas animals from herds with positive BTM samples had a statisti-

cally significantly higher seroprevalence of $8.9 \pm 0.9\%$ (range 0–66.7%; $F = 19.7$, $df = 1$, $P < 0.001$). An increasing seroprevalence was observed also in herds with negative, slightly positive, and positive BTM samples. When only cows were considered, mean seroprevalence increased to 3.5 ± 1.2 , 11.7 ± 1.8 , and $22.9 \pm 2.9\%$ in herds with negative, slightly positive, and positive BTM samples, respectively (Figure 2).

C. burnetii DNA Detection

The proportion of herds in which DNA of *C. burnetii* in BTM was detected was $51.7 \pm 7.3\%$ (92/178). A high percentage of the PCR-negative herds ($37.2 \pm 10.2\%$, 32/86) had detectable antibodies by ELISA in BTM, whereas only $5.4 \pm 4.6\%$ of PCR-positive herds (5/92) tested negative in BTM ELISA. Herds with positive results by both PCR and ELISA showed a higher within-herd seroprevalence ($9.1 \pm 1.2\%$) than did herds with negative results ($2.5 \pm 0.8\%$) and these difference were statistically significant ($F = 16.7$, $df = 1$, $P < 0.001$). Table 1 shows a comparison of results obtained with both techniques considering categories describing the level of antibodies by ELISA.

A positive and statistically significant relationship ($R^2 = 0.31$, $P < 0.001$) was observed between estimated bacterial load from qPCR and S/P ELISA results in BTM samples (Figure 3). Mean bacterial load was 1.7, 2.6, and 3.3 *C. burnetii*/mL ($\log_{10}$ basis) in negative, slightly positive (+), and positive (++) ELISA BTM samples, respectively, which differed significantly, especially between negative and positive (++) BTM samples ($F = 3.9$, $df = 2$, $P < 0.05$).

Effect of Herd Factors on *C. burnetii* and Reproductive Disorders

The surveyed cattle herds had an average size of 63.8 ± 3.9 animals, ranging from 5 to 290 animals, with 59.2% of the herds under this mean herd size. Interestingly, 25.6% of the herds were under the twenty-fifth percentile (28 animals) and 75.6% were under the seventy-fifth percentile (82 animals). In spite of the observed differences in herd size, no relationship was found between the size of the herd and the seroprevalence level; in addition, no effect of herd size on BTM antibody levels and the number of copies of *C. burnetii* DNA in BTM was evidenced.

Only 34 (46.6%) of the 73 farmers who filled out our questionnaire reported reproductive disorders consisting on infertility and abortion. Surprisingly, animals from herds with reported reproductive disorders showed a slightly lower seroprevalence against *C. burnetii* ($7.0 \pm 1.5\%$) than those from herds not reporting reproduc-

Figure 1. Correlation between sample-to-positive control (S/P) ratios in bulk-tank milk (BTM) ELISA with within-herd seroprevalence in cows.

tive problems ($8.4 \pm 1.3\%$). Nevertheless, none of the herd variables included in the logistic regression models significantly affected the risk of cattle herds suffering reproductive disorders.

DISCUSSION

Q fever in humans is endemic in the Basque Country (Northern Spain), with pneumonia as the main symptom (Montes et al., 2006). Sheep have been involved in human outbreaks in the area and several studies have been carried out to investigate the relevance of sheep in the domestic cycle of Q fever (Oporto et al., 2006; García-Pérez et al., 2009; Ruiz-Fons et al., 2010). The present study is the first to evaluate the distribution of *C. burnetii* in dairy cattle in the region. The prevalence of *C. burnetii* determined by BTM ELISA was higher than that reported in England or Ireland (21.0 and 37.9%, respectively; Paiba et al., 1999; Ryan et al., 2011), but similar to that found in Denmark (59%; Agger et al., 2010). A recent study published in the Netherlands on 341 cattle herds (Muskens et al., 2011a) revealed an even higher seroprevalence of 78.6%, accompanied by a high prevalence of *C. burnetii* DNA detection in BTM samples (56.6%; Muskens et al., 2011a). Interestingly, the sampling period coincided

Figure 2. Correlation between bulk-tank milk (BTM) ELISA categories with mean seroprevalence in cows. NEG = sample-to-positive control (S/P) ratio was ≤ 30 ; POS+ = slightly positive ($30 < \text{S/P} \leq 100$); POS++ = positive ($100 < \text{S/P} \leq 200$).

Table 1. Distribution of positive and negative herds with respect to results obtained in serological and molecular analyses of bulk-tank milk (BTM) samples and associated mean within-herd seroprevalence of *Coxiella burnetii* (%), standard error of seroprevalence (SE), and seroprevalence range

BTM ELISA ¹	BTM PCR	No. of herds (%)	Within-herd seroprevalence	SE	Range (%)
Negative	Negative	54 (30.3)	2.5	0.8	0–25
	Positive	5 (2.8)	2.7	2.7	0–13.3
Positive+	Negative	23 (12.9)	7.3	1.3	0–20
	Positive	33 (18.6)	5.0	1.1	0–20
Positive++	Negative	9 (5.1)	12.2	4.5	0–42.9
	Positive	54 (30.3)	11.6	1.7	0–66.7

¹Negative indicates that the sample-to-positive control (S/P) ratio was ≤ 30 ; + indicates slightly positive ($30 < \text{S/P} \leq 100$); ++ indicates positive ($100 < \text{S/P} \leq 200$).

with the Q fever outbreak in humans associated with goat and sheep abortions (van den Brom and Vellem, 2009). Similarly, in the present study, 51.4% of the herds shed DNA of *C. burnetii* through milk. In a previous study in the United States, 94.3% of the farms analyzed were PCR positive in BTM, showing that milk was an important *C. burnetii* excretion route in cattle (Kim et al., 2005). These studies suggest that *C. burnetii* shedding through milk is widespread in dairy cattle herds in different countries. The high *C. burnetii* DNA prevalence in dairy cattle could be explained by the long-time excretion of *Coxiella* through

milk, which, in cattle, can extend for several months compared with the shorter excretion periods reported for sheep and goats (Rodolakis et al., 2007; Astobiza et al., 2010). The widespread distribution of *C. burnetii* in cattle milk poses questions about the role of milk as a vehicle for the transmission of this zoonotic bacterium to humans, particularly in those regions of the world where raw unpasteurized milk is consumed (Breurec et al., 2010; Loftis et al., 2010).

It is well known that *C. burnetii* can cause metritis and infertility in cattle, and rarely abortion (Woldehewet, 2004; Parisi et al., 2006). Despite the high preva-

Figure 3. Correlation between sample-to-positive control (S/P) ratios in bulk-tank milk (BTM) ELISA with quantitative results of bacterial load ($\log_{10}$ *Coxiella burnetii*/mL) at the herd level. Bact = *C. burnetii*.

lences found in the present study, no association was found between *C. burnetii* within-herd seroprevalence and reproductive disorders. Interestingly, in the Netherlands, where *C. burnetii* DNA has been detected with high incidence in cattle herds (Muskens et al., 2011a), the implication of this pathogen in cases of metritis was very rare (Muskens et al., 2011b). These data suggest that *C. burnetii* could persist for long time in a herd without causing significant clinical signs.

This wide distribution of antibodies against *C. burnetii* in BTM indicates that this pathogen is endemic throughout our region. The seroprevalence observed here for dairy cattle was similar to individual (6.6%) and herd (42.9%) seroprevalences reported for beef cattle in the region, which is managed in semi-extensive conditions (Ruiz-Fons et al., 2010). However, the percentage of herds with within-herd seroprevalences higher than 20% was higher for dairy cattle herds (13.5%) than beef cattle herds (7.1%), suggesting that intensive and semi-intensive management conditions can favor the expansion of *C. burnetii* within herds, as reported by Capuano et al. (2001). Still, seroprevalence in dairy cattle was much lower than in sheep (Ruiz-Fons et al., 2010), which is in accordance with observations compiled in other countries where Q fever human outbreaks are related to small ruminants more frequently than to cattle (EFSA, 2010). The analysis of seroprevalence in the 3 age categories revealed that prevalence of antibodies increased with age, with negative results in calves and very low prevalence in heifers. This was in agreement with other studies and indicated horizontal transmission and maintenance of infection within adult populations (McCaughey et al., 2010; Ruiz-Fons et al., 2010; Taurel et al., 2011). In the current study, no relationship was found between the size of the herd and the seroprevalence level. Nevertheless, other studies showed controversial results. Thus, whereas Taurel et al. (2011) found a significantly higher seroprevalence in herds with less than 46 animals, others (McCaughey et al., 2010; Ryan et al., 2011) observed higher seroprevalence in larger dairy herds.

Bulk-tank milk samples are easy to collect and can provide valuable epidemiological data to assess the health status of dairy herds (Ruiz-Fons et al., 2011). However, the overall correlation obtained between BTM ELISA and within-herd seroprevalence was low. This could have been partially due to variation in the proportion of age classes surveyed within herd, which, in turn, was caused by differences in the age structure of cattle herds in Bizkaia. Thus, the presence of few highly seropositive cows in the herd would cause an increase in BTM ELISA values. In this sense, we have observed BTM samples categorized as highly positive (+++) in herds with only 10% of seropositive animals

(A. Piñero, unpublished data). These animals can also persistently excrete high bacterial loads through milk and have been identified as heavy shedders (Guatteo et al., 2007a). This indicates that serological results from BTM samples should be analyzed with caution, and that other complementary analyses need to be done in the herd. Interestingly, the European Food Safety Authority (EFSA, 2010) report considered that Q fever is active in a herd when the following 3 conditions occur at the same time: 1) herd seroprevalence is around 50%, 2) *C. burnetii* DNA is detected in vaginal swabs or placentas, and 3) abortions account for over 4% in herds with more than 100 animals. Thus, BTM analysis is a useful first approach to evaluate the presence of *C. burnetii* in a herd and, if positive, it needs to be followed by a more complete study to confirm the existence of active Q fever in the herd. Polymerase chain reaction is then a valuable tool that provides additional information. For example, 23 and 9 BTM ELISA that were found to be slightly positive (+) and positive (++), respectively, were negative by PCR, suggesting past infections and, consequently, absence of shedders among milking cows. Conversely, several BTM samples (n = 5) were negative by ELISA and positive by conventional PCR, indicating the presence in the herd of a small number of *C. burnetii* milk-shedding cows that did not seroconvert. Conventional PCR is less informative than qPCR, which not only identifies the presence or absence of the pathogen in the herd, but also measures the bacterial burden in the sample. However, this qPCR targets a repetitive sequence region (IS1111) present in different copy numbers in the genome of different *C. burnetii* strains (Klee et al., 2006). However, considering that genetic diversity among *Coxiella* strains infecting the European dairy cattle population is very low (Horrevorts, et al., 2011), the quantitative values presented here assumed that the copy number of IS1111 was the same in *C. burnetii* strains in all herds. In a similar way, this same technique has been used in individual cattle milk samples to investigate the correlation between within-herd prevalence of milk shedders and estimated titers in positive BTM (Guatteo et al., 2007b). In the present work, a positive and statistically significant relationship was observed between estimated bacterial load from qPCR and S/P BTM ELISA results. A mean bacterial load of 3.3/mL in positive ELISA BTM samples (++) was observed, which, taking into account the results obtained by Guatteo et al. (2007b) using the same qPCR kit, would correspond to approximately 50% of heavy shedders among milk shedder cows.

CONCLUSIONS

The data presented in this study revealed that *C. burnetii* is widespread in dairy cattle herds from the

Basque Country, showing a similar situation to that described in other countries. This situation indicates the need for further studies on the epidemiological consequences of *C. burnetii* shedding in milk of cattle.

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