Measuring antibody levels in bulk-tank milk as an epidemiological tool to search for the status of *Coxiella burnetii* in dairy sheep

F. RUIZ-FONS, I. ASTOBIZA, J. F. BARANDIKA, R. A. JUSTE, A. HURTADO
AND A. L. GARCÍA-PÉREZ*

NEIKER–Instituto Vasco de Investigación y Desarrollo Agrario, Department of Animal Health, Bizkaia, Spain

SUMMARY

Pooling samples may provide a valuable alternative to individual testing for pathogen surveillance purposes. We studied the reliability of measuring the level of antibodies against *Coxiella burnetii* in bulk-tank milk (BTM) to estimate the seroprevalence of *C. burnetii* in dairy sheep in 34 flocks. We then estimated the seroprevalence of *C. burnetii* in 154 dairy sheep flocks according to the level of antibodies in BTM. We tested for the accuracy of our estimation at the population level by comparing predicted mean *C. burnetii* flock seroprevalence with that obtained in another survey performed on the same population. Our findings showed that testing BTM by ELISA is a cost-effective and relatively good index of the seroprevalence of *C. burnetii* in dairy sheep and may be a useful tool for epidemiological surveillance at the population level.

Key words: *Coxiella*, ELISA, Q fever, zoonoses.

INTRODUCTION

One of the main aims of epidemiology is to provide accurate information on the status of pathogens in populations. The economic costs involved, however, are key considerations when designing the sampling methodology. Pooling individual samples may provide a valuable alternative for reducing the associated costs. In dairy livestock, this can be done by using bulk-tank milk (BTM) since this is a good, easy to collect and representative sample of animals under milking [1, 2]. BTM has proven to be a good tool for pathogen surveillance such as border disease virus [3], bovine viral diarrhoea virus [4] or helminths [5].

The determination of specific antibody levels in BTM has proved to be an accurate tool for establishing the status of our target pathogen, *Coxiella burnetii*, at a large geographic scale in dairy cattle [6, 7]. However, no information on the usefulness of BTM for the study of *C. burnetii* in small domestic ruminants has been reported. Q fever, the disease caused by *C. burnetii*, is seen as an emerging disease of humans and livestock in many areas of the world with The Netherlands currently experiencing a major public health problem from this disease [8, 9]. Its emergence has been frequently linked to small ruminant production [10], which is particularly true in the case of The Netherlands where goats and sheep are deemed to be the main reservoirs of *C. burnetii* [11]. However, determining the health status of domestic ruminant populations is expensive and time consuming and, therefore, a reduction in time, effort and economic costs is crucial. In a previous study [1],

* Author for correspondence: Dr A. L. García-Pérez, NEIKER–Instituto Vasco de Investigación y Desarrollo Agrario, Department of Animal Health, Berreaga 1, 48160 Derio, Bizkaia, Spain.
(Email: agarcia@neiker.net)

we investigated the presence of *C. burnetii* DNA in BTM samples from 154 sheep flocks and its relationship with flock seroprevalence in a selected group of flocks. In this study, we retested these same BTM samples to assess the usefulness of measuring the level of antibodies against *C. burnetii* in BTM in order to estimate the seroprevalence in dairy sheep flocks.

METHODS

Survey approach

Flocks included in this study were those previously selected for a survey on border disease in the region (for further details see [3]). Briefly, 154 flocks were selected during March–April 2005 throughout the Basque Country (northern Spain) to obtain a representative sample of dairy sheep flocks in the region that included most of the full-time professional flocks and all the flocks belonging to the Latxa Breed Farmers Association. A BTM sample (90–100 ml) from each flock was collected into sterile containers. When BTM samples were collected it was mostly adult sheep (i.e. those aged >2 years) that were being milked. Later, in autumn 2005, a representative sample of flocks (22%, 34/154) was randomly selected. Any biased results due to sample selection were ruled out (for further details see [1]). Blood samples were collected from 30 animals per flock randomly selected from the following three age groups: 10 replacement ewes (6 months–1 year), 10 yearling ewes (1–2 years) and 10 adult ewes (>2 years).

Serological and molecular analyses

BTM ($n=154$) and blood samples ($n=1011$ from 34 sheep flocks) were analysed for *C. burnetii* antibodies. Milk serum was obtained by centrifugation and frozen at -20 °C until serological analyses were performed. Whole blood samples were allowed to coagulate and, after centrifugation, serum was obtained and frozen at -20 °C until required. The detection of antibodies against *C. burnetii* in both milk and blood sera was performed using a commercial indirect ELISA [LSIVET Ruminant milk/serum Q fever, Laboratoire Service International (LSI), France]. Sample optical density/optical density of the positive control ratio (S/P) was calculated according to the manufacturer's instructions. The level of antibodies present in BTM was classified into four different categories: negative ($S/P < 0.3$), slightly positive

($+$, $0.3 < S/P < 1$), positive ($++$, $1 < S/P < 2$) and highly positive ($+++$, $S/P > 2$). General flock seroprevalence was calculated for the 34 individually surveyed flocks. Additionally, seroprevalence was calculated at the flock level for each of the age groups of sheep under milking (yearling + adult and adult ewes).

Although results of the presence of *C. burnetii* in BTM by PCR have already been published by our group elsewhere (for details see [1]), they are briefly presented here to allow the analysis of serological data presented herein in the context of those results. In brief, BTM aliquots were treated for DNA extraction as previously described [12, 13] and PCR amplification was performed using primers targeting a transposon-like repetitive region of *C. burnetii* as described elsewhere [14, 15]. Twenty-two percent of the 154 BTM samples were *C. burnetii*-positive by PCR.

Statistical assessment

The relationship between BTM antibody levels (continuous; measured as the S/P ratio percentage) and flock seroprevalence (continuous; percentage) in the 34 selected sheep flocks was assessed by linear regression. The relationship between the level of antibodies against *C. burnetii* in BTM and the seroprevalence value in the flock for yearling + adult ewes and adult ewes only was also assessed by linear regression. Additionally, we aimed to determine the relationship between the categorized level of antibodies in BTM (negative, $+$, $++$, $+++$) and the calculated seroprevalences (at the flock level and for each considered age group separately), for which ANOVA tests were performed. Finally, difference in the level of antibodies in BTM depending on the presence/absence of *C. burnetii* DNA in milk was analysed by ANOVA.

The regression model performed between the level of antibodies against *C. burnetii* in BTM and mean flock seroprevalence allowed us to extrapolate the level of seroprevalence to the 154 sheep flocks in which only BTM was analysed by ELISA. We aimed to obtain a regional seroprevalence value calculated from the levels of antibodies in BTM from a representative sample of the regional sheep population. Since the total variance of flock seroprevalence was not explained completely by the level of antibodies in BTM, we estimated the 95% confidence intervals for each of the predicted flock seroprevalences by
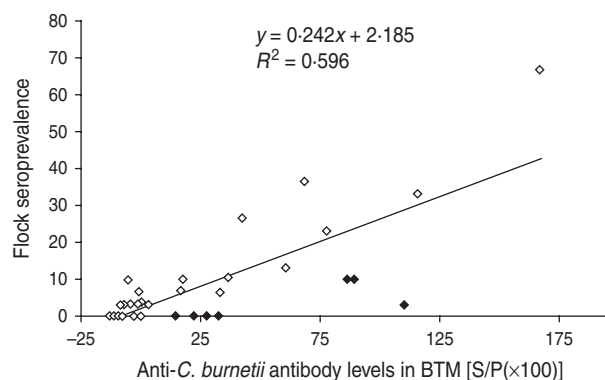
Fig. 1. Relationship between continuous bulk-tank milk (BTM) ELISA level of antibodies against *Coxiella burnetii* and seroprevalence values for the 34 studied flocks. Seven flocks that apparently show a different relationship of BTM antibody level and flock seroprevalence are differentiated in the graph (◆). The equation of the linear regression and the correlation coefficient are shown.

calculating the standard error (S.E.) of the residuals in the regression according to a Student's *t* distribution. We were hence able to calculate minimum, mean and maximum predicted seroprevalence values for the subsample of 154 sheep dairy flocks. Later, in order to test for the predictive power of the estimated mean flock seroprevalence in the region, we compared predicted with observed mean flock seroprevalence values obtained from a recent survey performed in dairy sheep flocks for *C. burnetii* serological status in the Basque Country (for further details see [16]). Predicted (the present study) and observed [16] flock seroprevalence values in the same sheep population were grouped into three categories (0%, 0–10%, ≥10%). Later, relative frequencies of predicted and observed flocks in each seroprevalence category were calculated. Predicted and observed mean population seroprevalences were compared by means of non-parametric Mann–Whitney *U* tests. Statistical uncertainty was assessed by calculating the 95% confidence interval (CI) for each of the proportions according to the expression: S.E. 95% CI $= 1 \cdot 96[P(1-P)/n]^{1/2}$. The *P* value was set at 0·05.

RESULTS

The level of antibodies in BTM and flock sero-prevalence in the 34 selected sheep flocks was highly correlated ($R^2 = 0 \cdot 596$, $P < 0 \cdot 001$; Fig. 1). Additionally, BTM antibody level and flock seroprevalence for >1-year-old ewes were also highly correlated ($R^2 = 0 \cdot 581$, $P < 0 \cdot 001$). A slightly better relationship

was observed between BTM antibody level and flock seroprevalence for adult ewes ($R^2 = 0 \cdot 615$, $P < 0 \cdot 001$). Seven of the 34 sheep flocks displayed an apparently different relationship between antibody levels in BTM and *C. burnetii* seroprevalence in the flock (Fig. 1). We could not identify any difference in management, size and geographical location between these seven flocks and the rest of the sheep flocks. Thus, they were not excluded for the calculation of the predictive value of BTM analysis by ELISA. Additionally, a positive statistically significant relationship was also evidenced between the categorized antibody level in BTM and flock ($F = 18 \cdot 9$, D.F. $= 2$, $P < 0 \cdot 001$), yearling + adult ewe ($F = 1 \cdot 7$, D.F. $= 2$, $P < 0 \cdot 001$) and adult ewe only ($F = 21 \cdot 6$, D.F. $= 2$, $P < 0 \cdot 001$) seroprevalence values. Analyses of BTM detected the presence of antibodies against *C. burnetii* (values are $\pm$ S.E.) in 62 ($40 \cdot 3 \pm 7 \cdot 8\%$) of the 154 surveyed flocks. Out of the positive flocks, 49 ($79 \cdot 0 \pm 10 \cdot 1\%$) were found slightly positive, 13 ($21 \cdot 0 \pm 10 \cdot 1\%$) positive and none highly positive, indicating that most of the BTM-positive flocks presented low to medium antibody levels in BTM at sampling. Mean flock seroprevalence was $2 \cdot 8 \pm 0 \cdot 7\%$, $15 \cdot 2 \pm 3 \cdot 8\%$ and $34 \cdot 4 \pm 18 \cdot 3\%$ for BTM-negative, slightly positive, and positive flocks, respectively (Table 1).

Despite the observed positive relationship between flock seroprevalence and antibody level in BTM, flock seroprevalences overlapped between slightly positive and positive flocks (ranges 0–37% and 3–67%, respectively; see Table 1). However, 75% (9/12) of the flocks testing slightly positive and positive in BTM ELISA had seroprevalence levels ≥10%. Of sero-negative flocks only 9·1% (1/11) presented antibodies in BTM. On the other hand, more than 50% (12/22) of BTM-seronegative flocks had seropositive animals but flock seroprevalence was not higher than 10%.

Twenty-two percent of BTM samples tested positive by PCR (previously reported in [1]). Interestingly, the presence of *C. burnetii* DNA and antibody levels detectable by ELISA in BTM were statistically related ($F = 9 \cdot 9$, D.F. $= 1$, $P < 0 \cdot 01$), with mean S/P($\times 100$) values of 24·9 in PCR-negative, compared to 50·0 in PCR-positive, samples.

Predicted mean flock seroprevalence for the 154 sheep flocks in our study was $9 \cdot 6 \pm 0 \cdot 8\%$, which was slightly under the $11 \cdot 8 \pm 2 \cdot 4\%$ observed when analysing 30 animals from each of 46 sheep flocks in our region [16]. However, these values did not differ statistically. Confidence intervals of predicted flock sero-prevalence showed a minimum flock seroprevalence

Table 1. *Average of the mean flock seroprevalence (sero.) of the 34 studied sheep flocks and the number of flocks in different blood seroprevalence categories (0%, 0–10%, ⩾10%) across BTM ELISA results classified according to the manufacturer's instructions. Standard errors (s.e.) associated with mean seroprevalence as well as seroprevalence ranges are also shown*

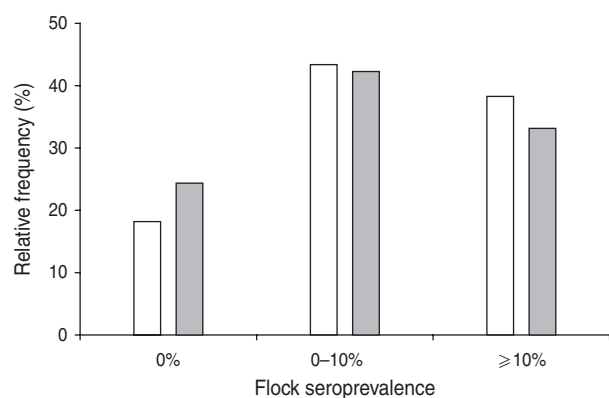| BTM ELISA	No. flocks	Average sero. (s.e.)	Sero. range	No. seronegative flocks	No. flocks sero. 0–10%	No. flocks sero. ⩾10%
Neg.	22	2·8 (0·7)	0–10%	10	10	2
Pos. +	9	15·2 (3·8)	0–37%	1	1	7
Pos. ++	3	34·4 (18·3)	3–67%	0	1	2

BTM, Bulk-tank milk.

Fig. 2. Predicted (this study, □) and observed ([16], ▩) relative frequencies of flock occurrence in different categorical flock seroprevalences (0%, 0–10%, ⩾10%) in the study area.

of $1·6 \pm 0·4\%$ and a maximum seroprevalence of $27·4 \pm 0·8\%$. Interestingly, predicted and observed relative frequencies of flocks in each seroprevalence category did not differ statistically (see Fig. 2).

DISCUSSION

Accuracy of epidemiological surveys is highly dependent on the tools epidemiologists employ for pathogen or disease diagnosis. The higher the number of individuals sampled within the population under study, the higher the accuracy of the population status estimation [17]. Nonetheless, in order to reduce time and cost, a representative sample is usually selected and pooling is a common practice.

We herein demonstrated a good agreement between the level of antibodies against *C. burnetii* in BTM and seroprevalence in dairy sheep flocks. To the best of our knowledge, this is the first report on the reliability of testing BTM by ELISA and its predictive power to establish the serological status at the flock level in sheep. BTM has been extensively used to measure pathogen exposure in livestock subpopulations both by determining the level of circulating specific antibodies [3–5] and by determining the presence of the pathogen or its genomic material [1, 18]. BTM antibody level has been also used for *C. burnetii* epidemiological surveillance in dairy cattle [6, 7]. However, none of these studies corroborated the relationship between antibody levels in BTM and the contact rate of animals on the farm with the pathogen. Although antibodies circulating in blood are expected to easily pass into milk during lactation, no information is available on the extent to which the level of *C. burnetii* antibodies in animals relates to that in BTM. Guatteo et al. [12] found good agreement between the level of antibodies against *C. burnetii* in individual paired blood and milk samples in dairy cows, thus showing that at the individual level milk and blood antibody levels are correlated. Unfortunately, these authors did not provide any evidence on the feasibility of using pooled individual milk samples to estimate the general status of *C. burnetii* in dairy cattle herds. In our study we were unable to assess the agreement between antibody levels in milk and blood sera of individual sheep. Nevertheless, according to the findings of Guatteo et al. [12] and the evidence that higher BTM antibody levels correlate with the percentage of antibody-positive sheep within the flock, a close link between antibody levels in blood and individual milk in sheep would be expected.

According to our observations, testing BTM by ELISA for predicting flock seroprevalence in sheep flocks where *C. burnetii* circulates at low rates, i.e. <10% (see Table 1) would not provide a high sensitivity. In this case, reducing the threshold of antibody level by which BTM is considered positive

[S/P($\times 100$) $\geqslant 30$] would not improve sensitivity because it ranges between -9.3 to 17.6 in flocks with blood seroprevalences between 0% and 10% (Table 1). On the other hand, two circumstances indicate that BTM ELISA may be a good indicator of epidemic infection by *C. burnetii* in the flock: (i) the high proportion of flocks with seroprevalence levels $\geqslant 10\%$ that tested positive in BTM ELISA in our survey; and, (ii) the high proportion of animals that make contact with *C. burnetii* when it first enters a flock [19], which can be evidenced by a high flock seroprevalence level despite the significant percentage of infected sheep that do not seroconvert [20, 21].

BTM samples were collected in the middle of the lactation season, which started in November–December and finished in June–July. Excretion of *C. burnetii* in dairy sheep flocks takes place mainly around parturition [10, 13, 19], determining the period when risk of infection for non-infected animals is higher. Seroconversion in sheep occurs 3–4 weeks after infection [22, 23]. Therefore, at the time BTM samples were collected, infected animals would have seroconverted and BTM antibody levels would be indicative of the exposure of the flock to *C. burnetii*. In addition, circulating antibody levels detectable by ELISA are long-lasting in sheep [23] and when blood was collected (6 months after BTM sampling), circulating antibodies resulting from infections in the previous reproductive season were expected to be detected [12]. Thus, the time gap between BTM and blood sample collection should not have altered our findings. However, replacement animals introduced during the time period that elapsed between BTM and blood collection may be one of the causes for the imperfect fit between antibody levels in BTM and flock seroprevalence. Future research on the relationship between antibody levels in paired BTM and individual samples would help to properly determine any effect of time, season or management.

Predictions of mean seroprevalence at the population level based on BTM ELISA results differed slightly, although not statistically, from mean values obtained by a cross-sectional survey performed 2 years later [16]. Additionally, the percentages of predicted and observed frequencies of flocks in different seroprevalence categories were very similar (Fig. 2). Moreover, temporal differences as well as differences in the flocks included in both studies might be partly responsible for the observed differences between predicted and observed flock seroprevalences. *C. burnetii* infection is endemic in our study area [16, 24, 25], and

in the absence of any specific control measure at the sheep population level (e.g. vaccination), the global regional seroprevalence is expected to remain virtually unaltered. Altogether, our observations and previously published information on cattle [6, 7, 12], give us confidence to conclude that predictions based on BTM antibody levels are accurate enough to determine the rates at which *C. burnetii* circulates in dairy sheep flocks at the population level.

In conclusion, our findings show that testing BTM by ELISA is a cost-effective and relatively good index of the seroprevalence of *C. burnetii* in dairy sheep flocks and may be a useful tool for epidemiological surveillance purposes at the population level. However, using the threshold values of the ELISA as established by the manufacturer may not properly reflect the status of the flock when seroprevalence is low. Meanwhile, testing BTM by ELISA may prove more accurate when within-flock *C. burnetii* seroprevalences are higher than 10%.

DECLARATION OF INTEREST

None.

REFERENCES

1. **García-Pérez AL,** *et al.* Investigation of *Coxiella burnetii* occurrence in dairy sheep flocks by bulk-tank milk analysis and antibody level determination. *Journal of Dairy Science* 2009; **92**: 1581–1584.
2. **García-Pérez AL,** *et al.* Border disease virus seroprevalence associates to antibodies in bulk tank milk and reproductive performance of dairy sheep flocks. *Journal of Dairy Science* 2010; **93**: 2444–2449.
3. **Berriatua E,** *et al.* Flock-prevalence of border disease virus infection in Basque dairy-sheep estimated by bulk-tank milk analysis. *Veterinary Microbiology* 2006; **118**: 37–46.
4. **Beaudeau F,** *et al.* Assessing the within-herd prevalence of cows antibody-positive to bovine viral diarrhoea virus with a blocking ELISA on bulk tank milk. *Veterinary Record* 2001; **149**: 236–240.

5. **Bennema S, et al.** The use of bulk-tank milk ELISAs to assess the spatial distribution of *Fasciola hepatica*, *Ostertagia ostertagi* and *Dyctiocaulus viviparus* in dairy cattle in Flanders (Belgium). *Veterinary Parasitology* 2009; **165**: 51–57.

6. **Paiba GA, et al.** Prevalence of antibodies to *Coxiella burnetii* (Q fever) in bulk tank milk in England and Wales. *Veterinary Record* 1999; **144**: 519–522.

7. **Agger JF, et al.** Prevalence of *Coxiella burnetii* antibodies in Danish dairy herds. *Acta Veterinaria Scandinavica* 2010; **52**: 5.

8. **Schimmer B, et al.** Large ongoing Q fever outbreak in the south of The Netherlands, 2008. *Eurosurveillance* 2008; **13**: 18939.

9. **Schimmer B, et al.** Sustained intensive transmission of Q fever in the south of The Netherlands, 2009. *Eurosurveillance* 2009; **14**: 19210.

10. **Maurin M, Raoult D.** Q fever. *Clinical Microbiology Reviews* 1999; **12**: 518–553.

11. **van der Hoek W, et al.** Q fever in The Netherlands: an update on the epidemiology and control measures. *Eurosurveillance* 2010; **15**: 19520.

12. **Guatteo R, et al.** Performances of an ELISA applied to serum and milk for the detection of antibodies to *Coxiella burnetii* in dairy cattle. *Revue de Médecine Vétérinaire* 2007; **158**: 250–252.

13. **Rodolakis A, et al.** Comparison of *Coxiella burnetii* shedding in milk of dairy bovine, caprine, and ovine herds. *Journal of Dairy Science* 2007; **90**: 5352–5360.

14. **Willems H, et al.** Detection of *Coxiella burnetii* in cow's milk using the polymerase chain reaction (PCR). *Zentralblatt für Veterinärmedizin B* 1994; **41**: 580–587.

15. **Berri M, Laroucau K, Rodolakis A.** The detection of *Coxiella burnetii* from ovine genital swabs, milk and fecal samples by the use of a single touchdown polymerase chain reaction. *Veterinary Microbiology* 2000; **72**: 285–293.

16. **Ruiz-Fons F, et al.** Seroepidemiological study of Q fever in domestic ruminants in semi-extensive grazing systems. *BMC Veterinary Research* 2010; **6**: 3.

17. **Bhopal R.** *Concepts of Epidemiology: An Integrated Introduction to the Ideas, Theories, Principles and Methods of Epidemiology*. Oxford: Oxford University Press, 2002, pp. 346.

18. **Arricau-Bouvery N, Rodolakis A.** Is Q fever an emerging or re-emerging zoonosis? *Veterinary Research* 2005; **36**: 327–349.

19. **Astobiza I, et al.** Kinetics of *Coxiella burnetii* excretion in a commercial dairy sheep flock after treatment with oxytetracycline. *Veterinary Journal* 2010; **184**: 172–175.

20. **Berri M, et al.** Relationships between the shedding of *Coxiella burnetii*, clinical signs and serological responses of 34 sheep. *Veterinary Record* 2001; **148**: 502–505.

21. **Berri M, et al.** Shedding of *Coxiella burnetii* in ewes in two pregnancies following an episode of *Coxiella* abortion in a sheep flock. *Veterinary Microbiology* 2002; **85**: 55–60.

22. **Brooks DL, et al.** Q fever vaccination of sheep: challenge of immunity in ewes. *American Journal of Veterinary Research* 1986; **47**: 1235–1238.

23. **Martinov SP, Pandurov S.** Serological study of experimental and natural Q fever in cattle and sheep. *Biotechnology and Biotechnological Equipment* 2005; **19**: 165–169.

24. **Sanzo JM, et al.** Q fever: prevalence of antibodies to *Coxiella burnetii* in the Basque Country. *International Journal of Epidemiology* 1993; **22**: 1183–1188.

25. **Montes M, et al.** Gipuzkoa, Basque Country, Spain (1984–2004): a hyperendemic area of Q fever. *Annals of the New York Academy of Sciences* 2006; **1078**: 129–132.