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

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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],
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
The level of antibodies in BTM and flock seroprevalence in the 34 selected sheep flocks was highly correlated \( (R^2=0.596, \ p<0.001) \); Fig. 1. Additionally, BTM antibody level and flock seroprevalence for >1-year-old ewes were also highly correlated \( (R^2=0.581, \ p<0.001) \). A slightly better relationship was observed between BTM antibody level and flock seroprevalence for adult ewes \( (R^2=0.615, \ P<0.001) \).

Seven of the 34 sheep flocks displayed an apparently different relationship between antibody levels in BTM and \textit{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 \((F=18.9, \ D.F. =2, \ P<0.001)\), yearling + adult ewe \((F=1.7, \ D.F. =2, \ P<0.001)\) and adult ewe only \((F=21.6, \ D.F. =2, \ P<0.001)\) seroprevalence values. Analyses of BTM detected the presence of antibodies against \textit{C. burnetii} (values are ± s.e.) in 62 (40-3±7-8%) of the 154 surveyed flocks. Out of the positive flocks, 49 (79-0±10-1%) were found slightly positive, 13 (21-0±10-1%) 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 2.8±0.7%, 15.2±3.8% and 34.4±18.3% 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 seronegative 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 \textit{C. burnetii} DNA and antibody levels detectable by ELISA in BTM were statistically related \((F=9.9, \ D.F. =1, \ P<0.01)\), with mean S/P(×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 9.6±0.8%, which was slightly under the 11.8±2.4% 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 seroprevalence showed a minimum flock seroprevalence...
of 1.6 ± 0.4% and a maximum seroprevalence of 27.4 ± 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

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**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.**

<table>
<thead>
<tr>
<th>BTM ELISA</th>
<th>No. flocks</th>
<th>Average sero. (s.e.)</th>
<th>Sero. range</th>
<th>No. seronegative flocks</th>
<th>No. flocks sero. 0–10%</th>
<th>No. flocks sero. ≥10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg.</td>
<td>22</td>
<td>2.8 (0.7)</td>
<td>0–10%</td>
<td>10</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Pos. +</td>
<td>9</td>
<td>15.2 (3.8)</td>
<td>0–37%</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Pos. + +</td>
<td>3</td>
<td>34.4 (18.3)</td>
<td>3–67%</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

BTM, Bulk-tank milk.
[S/P(\times 100) \geq 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 \textit{C. burnetii} in the flock: (i) the high proportion of flocks with seroprevalence levels \geq 10% that tested positive in BTM ELISA in our survey; and, (ii) the high proportion of animals that make contact with \textit{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 \textit{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 \textit{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. \textit{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 \textit{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 \textit{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 \textit{C. burnetii} seroprevalences are higher than 10%.

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**DECLARATION OF INTEREST**

None.

**REFERENCES**


