

Exploratory investigation of Q fever in apparently healthy meat sheep flocks in Belgium

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Summary

Q fever is a cosmopolitan disease affecting both humans and many animal species. Although sheep are often implicated in human Q fever outbreaks, the disease remains largely underestimated in meat sheep flocks. In order to fulfil this gap, a preliminary study was performed aiming to investigate the serological and molecular aspects of infection with *Coxiella burnetii* among meat sheep flocks in Belgium. Five Belgian sheep flocks were recruited for this work. Indirect ELISA was used, and in addition, real-time PCR was performed on samples of milk, rectal and vaginal swabs, to understand the dynamics of bacterial shedding. Despite the low overall apparent seroprevalence of 1.39% (95% CI: 0.04–7.5), a high rate of bacterial shedding was found, with 27.7% of tested sheep ($N = 72$) with a positive result to PCR, especially through the rectal and vaginal routes and in seronegative animals. Furthermore, *Coxiella burnetii* DNA was detected in 26.76% of seronegative animals. It can be concluded that an overall good clinical condition of the sheep cannot be used to exclude the presence of *C. burnetii* in a flock. Furthermore in the diagnosis of Q fever in sheep, serology alone was not a sensitive diagnostic tool. On the contrary, molecular biology allowed to detect bacterial shedding, which is an essential element in order to assess the risk due to the contact with shedding animals. At the light of these results, the role of meat sheep flocks in the epidemiology of Q fever in Belgium needs to be better understood.

KEYWORDS

Coxiella burnetii, diagnosis, meat sheep breeds, Q fever, serology, shedding

1 | INTRODUCTION

Q fever is a cosmopolitan disease affecting both humans and animals (Arricau-Bouvery & Rodolakis, 2005), caused by *Coxiella burnetii*, a small pleomorphic, Gram-negative and obligatory intracellular bacterium (Maurin & Raoult, 1999). Small ruminants and in particular sheep are often at the origin of human infections through aerosol and/or contact with animal products (EFSA 2010; Roest et al., 2010). Unlike most of the animals in which the infection is usually asymptomatic, small ruminants often present clinical manifestations

of the disease, resulting in reproductive problems such as abortions, stillbirths and infertility (Van Den Brom, Van Engelen, Roest, Van Der Hoek, & Vellema, 2015). However, subclinical cases in small ruminants remain possible, making the diagnosis more complicated (Van Den Brom et al., 2015). The absence of pathognomonic clinical manifestation makes diagnosis particularly delicate. In addition, there is no reference diagnostic technique, which represents a problem for case detection and surveillance (Porter, Czaplicki, Mainil, Guatteo, & Saegerman, 2011). Q fever laboratory diagnosis in animals can rely on serology. Indeed, the detection of antibodies directed against

C. burnetii is possible using several serological tests, such as immunosorbent enzymatic (ELISA), immunofluorescence (IFA), complement fixation test (CFT) and microagglutination (MAT). ELISA and CFT are the most used in small ruminants (Van Den Brom et al., 2015). They both show great specificity with still greater sensitivity in the ELISA (Rousset et al., 2007). On the other hand, molecular biology allows detection of bacterial DNA and consequently investigates the occurrence of bacterial excretion, which is of crucial importance in Q fever epidemiology (Van Den Brom et al., 2015).

The majority of sheep farms in Belgium are not professional and count a low number of animals, estimated at a mean of 14 and a median of five animals (L. Delooz, personal communication). Indeed, in Wallonia (southern part of Belgium), the majority of farms are hobbyists and only 3% of them are professionals (Association wallonne d'élevage, 2016).

In Belgium, Q fever is endemic in the domestic ruminant population (Saegerman, Speybroeck, Dal Pozzo, & Czaplicki, 2013). Since 2014, a compulsory declaration of the disease after detection of bacterial DNA in milk or aborted products exists (Royal Decree of 3 February 2014). Despite this measure, Q fever remains largely underestimated. Different epidemiological studies have been performed on dairy goats (Boarbi et al., 2014), cattle dairy and meat herds (Czaplicki et al., 2012; Vangeel et al., 2012), but the prevalence in sheep flocks remains unknown in Belgium. Indeed, while for dairy sheep flocks, a national surveillance exists (by serology and PCR on the bulk tank milk), and for meat sheep flocks, which constitute the majority of the sheep population, surveillance occurs on aborted products after declaration of abortion. In Wallonia, the mean abortion declaration rate in sheep farms with more than 20 adult females is $0.086\% \pm 0.01\%$ (L. Delooz, personal communication).

The aim of this study was to investigate the serological and molecular aspects of infection due to *Coxiella burnetii* among meat sheep flocks in Belgium.

2 | MATERIALS AND METHODS

Five hobbyist meat sheep flocks located in Belgium were visited between March and September 2013. Among them, four flocks participated to a previously unpublished epidemiological survey (M. Mori and D. Fretin, unpublished results) in which Q fever seropositive animals were found. The Q fever condition of the fifth flock was unknown at the time of recruitment. The agreement of the Animal Ethics Committee of the University of Liege (No. 1249) for the realization of this study was obtained, as well as the written consent of the owners. In the visited flocks, the most frequent breeds were "Texel," "Ile de France" and "La Romane." The size of the flocks ranged from 20 to 100 animals. Within each flock, ewes from 1 year old were randomly selected ($N = 72$). Individual samples (serum, rectal and vaginal swabs) were taken during the same visit. When lambs were present and the ewes were lactating, individual milk was also collected. At the time of the visit, the owner did not report any clinical signs and the flock was apparently healthy.

2.1 | Serological analysis

The indirect ELISA LSI Vet™ Ruminant Q Fever Serum/Milk (Thermo Fisher Scientific, UK) (ELISA LSIV) has been used, following manufacturer's instructions. This kit uses an inactivated strain of *Coxiella burnetii* (phase I and II) isolated from a naturally infected sheep in France (Porter et al., 2011). Analyses were performed following manufacturer's recommendations. Results were expressed as the percentage of sample/positive (S/P) values, which were obtained using the optical density (OD) values and by applying the following formula:

$$S/P = \left[\frac{(\text{OD sample} - \text{OD-negative control})}{(\text{OD-positive control} - \text{OD-negative control})} \right] \times 100$$

The cut-off value indicated by the manufacturer was used. A sample was considered positive with an S/P value >40.

2.2 | DNA extraction and Real-time PCR analyses

Milk samples, vaginal and rectal swabs have undergone a DNA extraction using the NucleoSpin® Tissue Kit 250® (Macherey-Nagel, Germany). A volume of 200 µl of milk was used for extraction of the DNA. The same volume was used to elute the swabs in sterile PBS. The protocol provided by the kit manufacturer was respected. The DNA extracted from milk and vaginal swabs was amplified using the PCR kit LSI VetMAX™ *Coxiella burnetii*-Absolute Quantification® (Life Technologies, UK). The target of this PCR is the transposon IS 1111. A LSI PCR kit VetMAX™ Feces & Environment® (Life Technologies, UK) was used for amplification of DNA extracted from rectal swabs. Quantitative PCR was performed using a thermocycler CFX96 Touch (Bio-Rad, CA, USA) and CFX Manager 3.0 software (Bio-Rad, CA, USA). According to the manufacturer's instructions, if an amplification was obtained above the threshold cycle of 45, the sample was considered negative. The results were expressed as the logarithm of number of genomic copies (GC)/ml.

2.3 | Statistical analysis

The 95% confidence interval (CI) of the seroprevalence was estimated using an exact binomial distribution. The correlation between the results of the ELISA LSIV and the PCR results for each one of the three routes of excretion was tested using Pearson's and Spearman's correlation tests. A logistic regression was made to find relationships between bacterial excretion and the serological status of the animal (Petrie & Watson, 2013).

3 | RESULTS AND DISCUSSION

To the authors' knowledge, this is the first study reporting serological and molecular investigations of Q fever among meat sheep flocks in Belgium.

TABLE 1 Serological and PCR results in the five tested meat sheep flocks

Flock	Province	Size flock ^a	Sample size ^b	Serology ELISA LSIV Positive/Total tested (%)	Shedding PCR				
					Positive ^c /Total tested N = (%)	Vaginal Positive/Total tested (%)	Faeces Positive/Total tested (%)	Milk Positive/Total tested (%)	Cumulative positive ^d /Total tested (%)
1	Namur	60	16	0/16 (0%)	4/16 (25%)	1/16 (6.25%)	4/16 (25%)	0/9 (0%)	5/41 (12.2%)
2	Luxembourg	70	20	0/20 (0%)	8/20 (40%)	0/17 (0%)	6/20 (30%)	2/17 (11.76%)	8/54 (14.81%)
3	Liege	50	15	1/15 (6.66%)	7/15 (46.66%)	5/14 (35.71%)	3/14 (21.43%)	0/10 (0%)	8/38 (21.05%)
4	Limbourg	100	13	0/13 (0%)	0/13 (0%)	0/13 (0%)	0/13 (0%)	0/0 (0%)	0/26 (0%)
5	Liege	20	8	0/8 (0%)	1/8 (12.5%)	1/8 (12.5%)	0/8 (0%)	0/0 (0%)	1/16 (6.25%)
Total		300	72	1/72 (1.39%)	20/72 (27.7%)	7/68 (10.29%)	13/71 (18.3%)	2/36 (5.55%)	22/175 (12.57%)

^aNumber of animals present in the flock at the time of sampling.

^bNumber of animals sampled randomly per flock.

^cNumber of animals with a PCR-positive result to at least one shedding route.

^dCumulative number of PCR-positive samples.

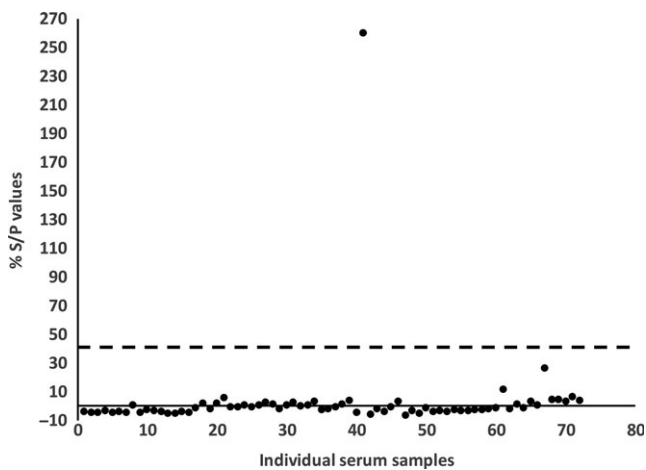


FIGURE 1 The results of the individual ELISA expressed as % S/P ratio as black points. Legend: the horizontal line represents the cut-off value of 40% S/P indicated by the kit manufacturer

The overall apparent seroprevalence was 1.39% (95% CI: 0.04–7.5). The apparent seroprevalence for each flock is reported in Table 1. The S/P values obtained by testing each individual samples are presented in Figure 1. These results were obtained using a commercialized ELISA in which the antigen is a bacterial strain isolated from ruminants (Porter et al., 2011). The sensitivity and specificity of the ELISA LSIV test were 87% and 99.1%, respectively, when applied to a set of sera derived from sheep, goats and bovines and using test accuracy in the absence of a gold standard (TAGS) (Horigan, Bell, Pollard, Sayers, & Pritchard, 2011). In this study, the low apparent seroprevalence measured in sheep is comparable to the one described in the Netherlands in two studies on dairy and non-dairy sheep and goats (Schimmer, De Lange, Hautvast, Vellema, & Van Duynhoven, 2014; Van Den Brom, Moll, Van Schaik, & Vellema, 2013). In particular, these Dutch studies showed that dairy sheep and goats had a significantly higher chance of being seropositive than non-dairy sheep and goats. In 2004, serum samples of 1714 sheep from 95 flocks located in Lower Saxony (Germany) were

investigated by ELISA and 2.7% of these samples were seropositive (Runge, Binde, Schotte, & Ganter, 2012).

In four of the five flocks recruited in this study, Q fever seroprevalence was previously measured using the same commercial indirect ELISA in 2010 (data not shown, M. Mori and D. Fretin unpublished data). Comparing the results of the two studies, a decrease in the apparent seroprevalence within the same flock could be observed. Unfortunately, in the 2010 study, *Coxiella burnetii* DNA detection was not performed. However, flocks 1–3 (Table 1) with a high apparent herd seroprevalence in 2010 (between 14% and 70%) had in the current study a high percentage of shedders (between 25% and 46.66%, Table 1), which could be explained by a long-time infection or reinfection of the animals present in the flock. Within flock 4, with an apparent herd seroprevalence of 5.26% in 2010, no shedders were detected in the present study. Knowing that the Q fever serological status of an animal is not stable over time, it could be speculated that some sheep were transiently seronegative, as already noted in other works (Garcia-Perez et al., 2009). Furthermore, it is highly possible that 3 years apart, different animals were sampled in the course of the two studies.

In the current study, *Coxiella burnetii* DNA was detected in 27.7% of the tested sheep, subsequently defined as shedders. Among them, 90% were shedding through a single route while none were shedding by the three routes simultaneously (Table 1). These results match those previously described (Joulié et al., 2015; Rodolakis et al., 2007). Sheep were more likely to shed the bacteria through the faecal (18.3%) and the vaginal route (10.29%) rather than via the milk (5.55%). This has already been described previously (Joulié et al., 2015; Rodolakis et al., 2007; Sting et al., 2013). This shedding pattern may explain that sheep are most often involved in human outbreaks of Q fever, with the shedding of the bacteria and consequent environmental contamination (Berri et al., 2005; Tissot-Dupont & Raoult, 1993). The lowest rate of excretion through the milk may be due to the discontinuous character of this shedding route among the sheep flocks (Berri et al., 2005). However, no domination of one route of shedding was noted compared to the others.

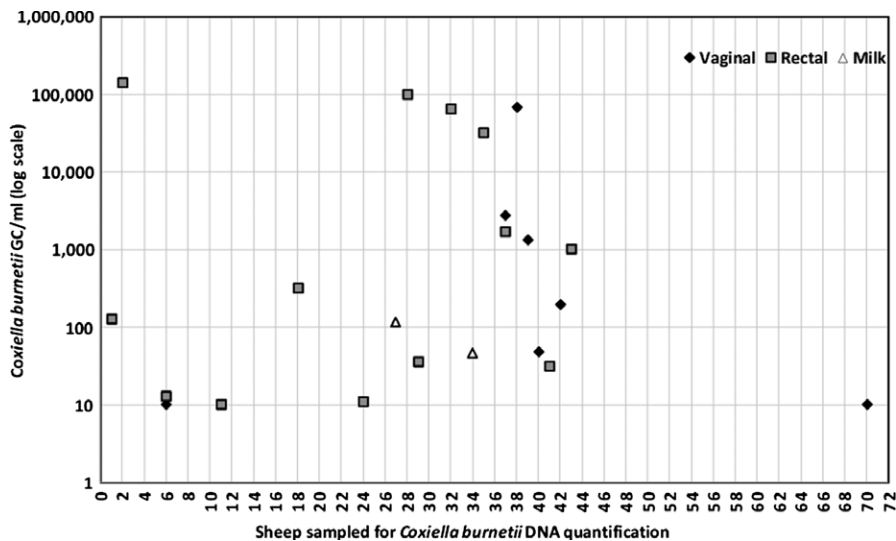


FIGURE 2 The results of the quantification of the genomic copies GC/ml of *Coxiella burnetii* (expressed in a logarithmic scale) from 72 sheep and three shedding routes (vaginal, rectal and milk)

Consequently, the three routes should be investigated simultaneously and repeatedly in epidemiological surveys (Joulié et al., 2015). Next to its frequency, shedding of *Coxiella burnetii* DNA was quantified and expressed in GC/ml. These results are presented in Figure 2. In tested sheep, the magnitude of GC/ml shedding was ranging between Log 1 and 5 for the faecal and vaginal routes and was about Log 2 in the milk.

No correlation was found between the serology status and the shedding (bacterial DNA detection in one of the three investigated routes) (p value $>.05$). S/P values obtained at the ELISA were tested using a Pearson's and a Spearman's test for a correlation with the PCR result obtained with each of the three shedding routes (all p values $>.05$). Indeed, 26.76% of the seronegative animals were shedders. These results confirm those obtained in a previous study performed by Rodolakis et al. (2007), while it contradicts the one described by Berri et al. (2005), where a higher concordance between the results of serology and shedding of bacteria through the milk was noted. It could not be excluded that the absence of correlation was related to an insufficient number of animals tested in the present study. It could be envisaged to include in a second study a larger number of sheep. Faecal shedding in seronegative sheep could also be explained by bacterial transient along the gastrointestinal tract as a consequence of the environmental contamination and the oral ingestion of the *Coxiella*. Furthermore, the absence of correlation between serology and shedding may be influenced by the sensitivity and specificity of tests as well as by their performances according to the epidemic or endemic condition of the disease.

Based on the results obtained in the flocks included in this study, *Coxiella burnetii* shedders were found in apparently healthy flocks. Furthermore, shedders were found among seronegative and seropositive sheep. These preliminary results suggest that in the diagnosis of Q fever in sheep, serology should be coupled to PCR in order to increase sensitivity. More investigations will be necessary to better understand the epidemiology of Q fever in a representative sample of the Belgian sheep population and using a larger number of

animals. The apparently healthy condition of the sheep flocks associated with the contemporarily bacterial shedding questions the role of sheep in the transmission of *Coxiella burnetii* to humans.

At present, the detection of Q fever in meat sheep flocks is based on passive surveillance and mostly relies on the abortion declaration programme. At the light of the results obtained in this study and based on the low abortion declaration rate in sheep, the authors believe that Q fever in Belgium remains an underestimated disease in meat sheep flocks. Furthermore, Q fever diagnosis (by serology and PCR) should be systematically implemented and required during animal trade.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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