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Short Communication

A Model for *Coxiella burnetii* Monitoring on a Sheep Dairy Farm

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Abstract

The aim of our study was to develop a model for farm monitoring, to be used in the case of the suspected presence of C. *burnetii*. Such a model could also be used to identify possible threats to human health. Thus, a C. *Burnetii* positive sheep farm was selected for this pilot study. Samples were taken from various areas on the farm and tested for the presence of C. *burnetii* using molecular methods. A possible relationship between C. *burnetii* presence and human seroconversion in farm workers was also investigated.

Results showed that air and surface samples taken in areas of the farm used to house pregnant animals and for parturition could represent a useful tool for monitoring C. *burnetii*. Seroprevalence was found in farm workers, but not in veterinarians, who occasionally spent limited time on the farm. This suggests that the threat of infection is restricted to workers who are continuously present on site.

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- Occupational disease
- Monitoring

ABBREVIATIONS

BTM: Bulk Tank Milk; Ct: Threshold Cycle; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

INTRODUCTION

Coxiella burnetii is an obligate intracellular bacterium responsible for Q fever. Cattle, sheep and goats are the main reservoir for the bacterium and considered to be the main source of infection for humans. In ruminants, clinical manifestations of the disease are associated with reproductive disorders: premature birth, abortions and stillbirth [1]. Infected animals can shed the bacterium in milk, faeces, urine, vaginal secretions and birth products (placenta, birth fluids) [2].

In humans, Q fever is mainly asymptomatic or occurs as a nonspecific flu-like illness [3].

In 2014, a total of 777 confirmed cases of Q fever in humans were reported in the EU, resulting in one fatality [4]. *C. burnetii* is remarkably stable in extracellular environments, able to survive in the environment for long periods [5], and extremely infectious for humans: 1-10 viable organisms suffice to induce an infection via the aerogenic route [6], mainly by inhalation of contaminated aerosols originating from parturient animals and their birth products [2]. Organic material contaminants present in stables are particularly dangerous in the dry state, when infectious particles pulverize and rise up in the air. Q fever is predominantly known as an occupational hazard in farmers, veterinarians, abattoir workers, i.e., those in contact with infected animals and dairy products [7]. Thus, since *C. burnetii* may represent a public health threat, preventive and control measures against this emerging problem are needed.

The aim of our study was to develop a model for farm monitoring to be used in the case of the suspected presence of *C. burnetii.* Such a model could also be used to identify possible threats to human health. Hence, a *C. burnetii*-positive sheep dairy farm was selected for this pilot study. Samples were taken from various areas on the farm and tested for the presence of *C. burnetii* using molecular methods. A possible relationship between *C. burnetii* presence and human seroconversion in farm workers was also investigated.

MATERIALS AND METHODS

Pilot farm selection

Our selection of a *C. burnetii*-positive sheep dairy farm was based on the following criteria: reported abortions due to *C.*

burnetii during the last 12 months; bulk tank milk (BTM) samples testing positive using real-time PCR and no vaccination against *C. burnetii*.

The farm was located in the Marches region, Central Italy, an area where sheep farming and artisan cheese making is widespread.

Sampling and environmental matrices

During a 6-month period, corresponding to the lambing season, four samplings were conducted at the farm.

The study was performed on a total of 89 environmental samples of different origins (Table 1). A preliminary filter evaluation used for air sampling was carried out comparing PTFE (FHLP04700), Mixed Cellulose Esters (AAWP04700) and Polycarbonate (GTTP02500) membrane filters (Millipore S.A.S., Molsheim, France). Air samples (n. 20) were obtained by using an active single-stage Surface Air System sampler (SAS, International PBI, Milan, Italy) with an airflow rate of 180 L/min for 3 min (540 L). Filters (0.45 µm, 47 mm of diameter) were held in place by a 65-mm polystyrene contact plate and prewet with a sterile aqueous 1% peptone solution.

Surface dust samples (n. 56) were obtained from horizontal (dust-accumulating) areas in the stables and milking parlour by swabbing a sterile cotton swab (VWR International, Milan, Italy), prewet in 1% peptone solution, in a single motion over a length of approximately 50 cm. Nine deep-litter and 4 BTM samples were also collected.

Samplings were conducted at different sites in the main farm building, namely, in the large open sheep pen and drinking trough area (Area 1), in the pregnant animal and lambing pen (Area 2) (Figure 1), as well as inside the milking parlour. Due to logistical constraints, sampling was performed after milking.

After collection, all obtained samples were cooled with ice packs and processed for analysis within 24 h.

Molecular detection of *C. burnetii*

Samples were examined for the presence of *C. burnetii* DNA by means of a qualitative duplex real-time PCR assay (ADIAVET COX REAL TIME, Adiagene, BioMérieux, Marcy l'Etoile, France).

DNA extraction from PTFE filters was carried out using the same binding columns and solutions from a DNA isolation kit specific for filters (*Legionella* DNA Extraction kit, Diatheva, Fano, Italy).

DNA from swabs and deep-litters was extracted with the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) after pretreatment according to Kersh et al [8]. DNA extraction was performed directly from raw milk according to Petruzzelli et al [9].

The template DNA (5 µl) of each extracted sample was used in the real-time PCR assay targeting the specific *C. burnetii* IS1111 sequence. A DNA sequence (GAPDH gene) naturally found in the genome of ruminants was simultaneously amplified as an endogenous control. An exogenous control "EPC-extraction" available in the same amplification kit was added in all lysates from acellular samples.

Table 1: Sampling location, sample type and presence of *C. burnetii* DNA based on qualitative duplex real-time PCR results.

Sampling site	Sample type	Positive samples/tot. samples	Positivity percentage
Stable			
Area 1. Sheep circulation and drinking trough area	Air Deep-litters	0/5 0/2	0% 0%
Area 2. Pregnant animal and lambing pen	Air Surfaces Deep-litters	4/8 8/36 4/7	50% 22% 57%
Milking parlor			
	Air Surface return lane Surface rotary-parlor	0/7 8/12 4/8	0% 67% 50%
	Bulk Tank Milk	0/4	0%
		Total 28/89	

Table 2: Results of the Focus Diagnostics Q Fever Indirect immunofluorescent (IFA) assay for the diagnosis of Q fever.

Professional category	*C. burnetii* IgG	*C. burnetii* IgM
	Cut-off 1:16	**Cut-off 1:16**
1.Farmer	1:64 Positive	Negative
2.Cleaner	1:32 Positive	Negative
3.Shepherd	1:128 Positive	Negative
4.Farmer	1:128 Positive	Negative
5. Farmer	1:128 Positive	Negative
6. Farmer	1:64 Positive	Negative
7. Farmer	1:64 Positive	Negative
8. Farmer	1:64 Positive	Negative
9. Veterinarian	Negative	Negative
10. Veterinarian	Negative	Negative

The PCR assays were run on a Rotor-Gene Q (Qiagen) with the following amplification conditions: 2 min 50°C, 10 min 95°C, 15 sec at 95°C and 1 min at 60°C during 45 cycles.

The PCR detection limit declared by the manufacturer is 1.5 *C. burnetii* /5µl PCR.

Serology

Human blood samples (n. 10) were collected aseptically by qualified personnel using approved venipuncture techniques from farmers and other farm-employees, as well as veterinarians who routinely frequent the farm for official sanitary controls.

Serum samples were tested by means of a commercial Focus Diagnostics Q Fever Indirect immunofluorescent (IFA) assay for the detection and semi-quantitation of human IgM and IgG antibody response to Phase I and Phase II *C. burnetii* and for the diagnosis of Q fever (Focus Diagnostics, Cypress, CA).

Statistical analysis

The results are presented as means (average Ct of all samples in each sample type) ± SD. Data were analyzed using 1-way ANOVA following Bonferroni *post hoc* test. Differences were considered significant at $p<0.05$. The statistical analysis was performed using Prism5 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION

Detection of *C. burnetii* DNA in all samples collected on the sheep dairy farm

A preliminary optimization of the air sampling protocol was performed, including membrane type and DNA extraction from filter. The comparison among membrane types and subsequent DNA extraction allowed us to select PTFE membranes which gave the best results in terms of yield and amplificability (data not shown). Hence, those membranes were used for all subsequent air samplings.

Results of the analyses for all sample type are reported in Table (1). Out of these 89 samples, 28 tested positive. Thus, approximately 32 % of the samples were positive for *C. burnetii* DNA. The percentage of positive samples in each location varied widely. Interestingly, in the stable, in particular in the large open sheep pen and drinking trough area (Area 1), *C. burnetii* DNA was not found. In the pregnant animal and lambing area (Area 2) the highest percentage of *C. burnetii* DNA-positive air (50%) and deep-litter (57%) samples were found, whereas only 22% of the surface samples analyzed tested positive for *C. burnetii*.

These results suggest that the presence of infected animals' birth products, such as the placenta and vaginal secretions at a given site in farm, may increase the possibility of finding *C. burnetii*-positive samples at that particular site and raise the possibility of human exposure. Therefore, such areas should be considered at risk for transmission, especially to farm workers.

Conversely, in the milking parlour, all air samples collected tested negative, while 8 out of 12 (67%) surface return lanes and 4 out of 8 (50%) surface rotary-parlor samples tested positive for *C. burnetii* DNA.

Our findings showed that *C. burnetii* mainly accumulates on surface areas. Although a qualitative real-time PCR assay was employed in this study, Ct values could give a rough estimation about contamination levels in sampled matrices. Average Ct values obtained in the different environmental matrices (Figure 2) indicate a low *C. burnetii* DNA concentration, and thus a low pathogen level. Statistical analysis showed no significant differences among average Ct of environmental matrices analysed (1-way ANOVA following by Bonferroni *post hoc* test, $p > 0.05$).

However, this result was in part expected since environmental samples usually contain lower levels of contamination than veterinary samples such as vaginal swabs or placenta samples, which can contain over 10^9 bacteria per gram of tissue [10], as previously reported [11,12].

Moreover, since the most likely route for *C. burnetii* to enter the environment (air and surface dust) is by shedding through placenta materials and amnion fluids during the lambing season,

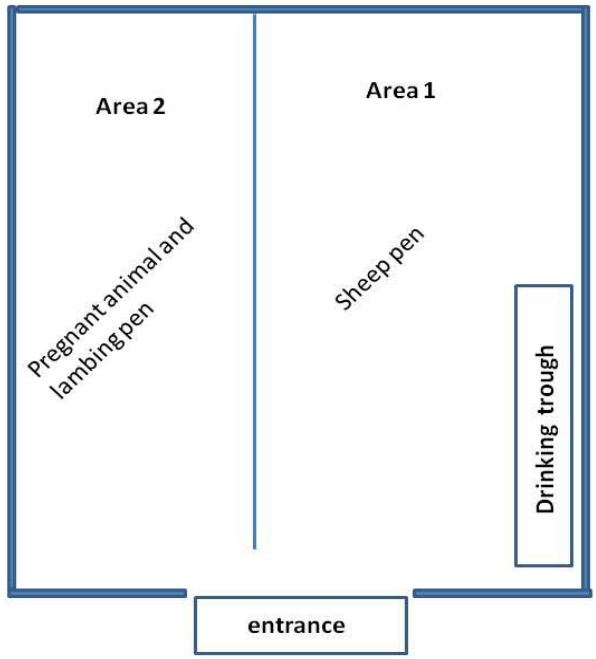

Figure 1 Map of the farm indicating the areas where the samples used in this study were collected.

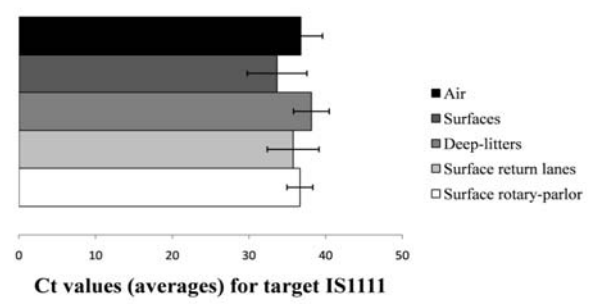

Figure 2 Ct values (averages) ± Standard Deviation obtained from the real-time PCR assay carried out on DNA from different matrices at the sheep dairy farm. High Ct values for target IS1111 indicate low *C. burnetii* DNA content.

this would explain the higher number of positive samples found in Area 2 than in Area 1. Finally, BTM always tested negative, indicating that *C. burnetii* DNA environmental presence is not necessarily linked to its shedding in milk, as described by de Rooij et al. [13], according to whom *C. burnetii* positive animals could be present on BTM negative farms.

Since only DNA was analyzed in the environmental samples, the viability of the bacteria is unknown. *C. burnetii* cultivation is impracticable due to the associated risks and difficulties. Nevertheless, it would be of interest to know the ratio of dead versus viable *C. burnetii* bacteria, which would aid the interpretation of the observed DNA levels.

Serology

Serological analysis carried out on farmers and veterinarians showed seroconversion in 8/10 operators; only the veterinarians

who sporadically visited the farm tested negative (Table 2). The interpretation of the data, made according to the manufacturer's datasheet, indicated that positive samples derived from past infections, since the analysis of antibody classes showed the absence of specific IgM, while anti-phase I IgG, ranging from 1:32 and 1:128, were always higher than anti-phase II IgG, which would be highly concentrated only in patients with acute Q fever.

CONCLUSION

This pilot study allowed us to determine the most appropriate sample types and sampling locations for *C. burnetii* monitoring on a sheep farm.

Environmental matrices play a leading role as reservoirs of microorganisms. Due to the accumulation of dust over long periods of time, surface area swabs may give insight into the presence of *C. burnetii* DNA on a farm over a long period. Moreover, dairy farm sheep environmental control procedures (air, surfaces and deep-litters) could be a more effective support in *C. burnetii* monitoring than BTM analysis.

Seroprevalence found in farm workers, but not in veterinarians, who only visited the farm occasionally, suggests that the health threat is restricted to workers who are continuously present on site.

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Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

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