Management of a *Coxiella burnetii*-infected sheep flock after an outbreak of Q fever in humans

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Abstract: Following an outbreak of Q fever in a group of students who contracted the infection during a training course on a sheep farm, a detailed investigation of the sheep flock involved was conducted. Of 478 flock animals, 60 *Coxiella burnetii* ELISA-positive and 60 ELISA-negative ewes were selected for the trial and divided into four groups. A month after the initial ELISA screening, all ewes in the flock (except the control group) were vaccinated. Sequentially collected blood samples were tested with ELISA and PCR; feces, milk, manure, bedding, and soil were tested with PCR. The immune response to the vaccination was 92.7%, while the overall *C. burnetii* seroprevalence in the flock after the human outbreak was 64.9%. PCR was positive for 0.2% of milk samples and 34.4% of fecal samples of animals from all four trial groups. *C. burnetii* DNA was not detected in any of the blood samples. Manure was PCR-positive for about 35 months; bedding from the stable was also positive while samples of pasture soil were negative. It appears that extensive cleaning and disinfection combined with vaccination could be regarded as an appropriate approach to control/prevent Q fever in farm settings even in the short term.

Key words: *Coxiella burnetii*, Q fever, sheep, vaccination, environment

1. Introduction

Q fever, a zoonosis caused by *Coxiella burnetii*, has a worldwide distribution. Even though it is present in a wide range of animal species, including arthropods, it affects mostly ruminants and humans. In cows, ewes, and goats, Q fever has been associated mostly with late abortion and reproductive disorders such as premature birth and dead or weak offspring (1,2). Acute infections with *C. burnetii* in humans commonly include a self-limiting febrile episode, pneumonia, or granulomatous hepatitis, while the main clinical manifestation of chronic Q fever is endocarditis (3).

Even though the source of human infection is often unknown, livestock, most frequently small ruminants, are considered as the main source (1,2). The main route of infection for humans is inhalation of contaminated aerosols or dust containing *C. burnetii* shed by infected female animals at parturition, when a great number of organisms can be found in fetal fluids and the placenta. In addition, after delivery, animals shed *C. burnetii* via urine, feces, and milk for several months (4,5). Able to produce highly resistant spore-like forms, *C. burnetii* can survive in the environment as infectious particles for long periods (6).

Awareness of Q fever is usually heightened during human outbreaks (7). This was also the case for a group of veterinary secondary school students who contracted Q fever during a training course on a sheep farm in Slovenia in 2007. The infection was laboratory-confirmed in 68 of 84 exposed individuals and 91% of seropositive individuals manifested clinical signs of acute Q fever (8). This led to a detailed investigation of the sheep flock involved, including microbiological monitoring of the farm environment and a vaccination trial that was used in Slovenia for the first time. The purpose of this work was to assess the effectiveness of vaccination in a study group by sequentially monitoring the immune response and fecal shedding of *C. burnetii* and to investigate the persistence of *C. burnetii* in the farm environment.

2. Materials and methods

2.1. Study flock
The study flock comprised 120 of 478 sheep housed on the farm at that time. The sheep for the study were selected randomly. The farm was a part of a research center for sustainable recultivation located in the Slovenian Karst, a region in which Q fever has been endemic for decades.

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The sheep were of two Slovenian autochthonous breeds, the Istriana sheep (about 90%) and Jezersko-Solčava sheep (about 10%). Animals were kept on pastures from March to September and stabled over autumn and winter. Before and during the lambing season early in 2007, there were no abortions or other reproductive disorders observed in the flock. Later on in 2007, only two abortions were recorded while 385 ewes lambed normally.

2.2. Study design

In June 2007, after the outbreak in humans was confirmed, blood samples from all the animals in the flock (n = 478) were collected and tested for the presence of antibodies against *C. burnetii* (ELISA 1). The presence of antibodies against *C. burnetii* was tested with the CHEKIT Q-fever ELISA Test Kit (Dr. Bommeli AG, Idexx, Switzerland) in accordance with the manufacturer's instructions.

On the basis of the ELISA 1 results, 60 seropositive and 60 seronegative ewes were selected for the trial. The vaccination group included 30 seropositive and 30 seronegative ewes. The control group consisted of 30 seropositive and 30 seronegative unvaccinated ewes. All groups were housed together. At the end of July 2007, all ewes in the flock were vaccinated, with the exception of 60 animals from the control group. The only one available at that time, an OIE manual-recommended (9) inactivated phase I vaccine was used, which was prepared with the *C. burnetii* Nine Mile strain in the Laboratory for Diagnosis and Prevention of Rickettsial and Chlamydial Infections, Institute of Virology, Bratislava, Slovakia. This is a formalin-killed corpuscles vaccine (concentration: 100 μg/mL) intended for protective and emergency vaccinations against coxiellosis in cattle and sheep. The recommended dose is 1 mL s.c. At the same time, blood samples (n = 120) for ELISA 2 and milk samples (n = 119; milk 1) were taken for molecular tests. Additionally, 20 samples of feces (feces 1) from seropositive ewes (10 from vaccinated and 10 from unvaccinated ewes) were screened for the presence of *C. burnetii* DNA by PCR.

Two months after the vaccination, blood samples were collected and tested from all ewes (n = 115) left in the trial (ELISA 3). In January 2008, before the parturition period, ewes (n = 115) were tested again with ELISA (ELISA 4), and blood (n = 97), fecal (n = 97; feces 2), and milk samples (n = 97; milk 2) were collected for molecular analyses by PCR. Additionally, other sheep in the flock (n = 301) were also tested with ELISA to determine the overall seroprevalence of *C. burnetii* in the flock before the parturition period. The final ELISA testing was performed in November 2008 in the 93 ewes remaining in the trial (ELISA 5). Fisher's exact test was used for the comparison of the groups in trial.

In order to gain insight into environmental contamination by *C. burnetii*, a total of 70 samples from the environment were investigated. Manure, bedding, and soil from the pastures were sampled eleven times between 2007 and 2011. Manure was collected at different locations and at different depths (from the surface to 70 cm deep).

2.3. PCR tests

DNA from blood and milk samples was extracted using the BioSprint 15 DNA Blood Kit (QIAGEN, Germany) using a KingFisher magnetic particle processor (Thermo Scientific, Germany). Before DNA extraction, milk samples were processed as described previously (10). DNA from fecal and environmental samples was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN). Briefly, approximately 1 g of feces was thoroughly homogenized in 10 mL of ASL buffer (QIAGEN). After the particles were allowed to settle, 1.8 mL of supernatant was incubated at 95 °C for 5 min and then processed according to the manufacturer's instructions. Suspensions of manure/bedding/soil were first shaken for 20 min; after the particles settled, 2 mL of supernatant was mixed with 6 mL of ASL buffer (QIAGEN) and vortexed. Two milliliters of the mixture was incubated at 95 °C for 5 min and then processed in accordance with the manufacturer's instructions.

PCR amplification was performed using primers targeting a transposon-like repetitive region IS*1111a* of *C. burnetii* as described previously (10). Samples with unclear PCR results (e.g., unspecific bands) and all environmental samples were additionally tested with the commercial PCR kit ADIAVET COX REALTIME (Adiagene, France) targeting IS*1111* in accordance with the manufacturer's instructions, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). The kit included internal control for the detection of PCR inhibitors.

3. Results

The overall seroprevalence of *C. burnetii* in the sheep flock after the human outbreak was 64.9% (310 positive samples out of 478). Furthermore, 29 samples were equivocal in ELISA 1. The seroprevalence before the parturition period in 2008 increased to 92.7% (332 positives out of 358 samples and 7 equivocal ELISA 4 results). Results for unvaccinated animals were excluded from the calculation of the overall prevalence based on ELISA 4 results. The number of animals decreased over the course of the trial in all groups due to different reasons (e.g., death, depredation by wolves).

After vaccination, an increase in seronegative animals was observed among negative vaccinated ewes (from 3.6% to 17.4%). In the group of positive vaccinated ewes, 23.3% of animals became seronegative before vaccination; after vaccination, these animals were found to be seropositive in ELISA 3. In the following two tests (ELISA 4 and 5), the

proportion of seronegative animals remained below 7%. Among seropositive unvaccinated ewes, the proportion of seronegative animals increased from 23.3% in ELISA 2 to 30.8% in ELISA 5. The results of testing the ewes in the trial for the presence of antibodies against *C. burnetii* are summarized in Table 1.

PCR tests generated negative results for all blood samples while two (0.2%) milk samples were found to be positive. Among fecal samples, 34.4% were PCR-positive for *C. burnetii*. Results of PCR tests of blood, milk, and feces are shown in Table 2. The highest proportion of PCR-positive environmental samples was detected in manure, followed by bedding from the stable, while soil samples collected from the pasture were negative. An overview of PCR results for environmental samples is given in Table 3.

4. Discussion
In the present study, the focus was on vaccination and its impact on the reduction of *C. burnetii* in individual groups of animals in the trial. In addition, the aim was to determine the reduction of *C. burnetii* presence in the environment. Effects of preventive vaccination against *C. burnetii* usually appear after a prolonged period (11,12). Eibach et al. (13) reported a significant reduction of *C. burnetii* secretion after vaccination performed during an outbreak of the disease in sheep.

An immune response to the vaccination in the present study was recorded in 96.4% of animals in the vaccination trial group (92.7% of the flock), which is similar to the findings of Eibach et al. (13), although a different vaccine was used. To the best of our knowledge, this is the first vaccination study in which the aforementioned vaccine was used; other authors studied the impact of the Coxevac vaccine (Ceva, France) (11–14). To monitor the immune response, a commercial ELISA kit with microplates coated with the *C. burnetii* Nine Mile strain was used. Rodolakis et al. (15) reported a higher number of *C. burnetii* shedders (as confirmed with PCR) among seropositive animals if ELISA with the antigen prepared from ruminant *C. burnetii* isolates was used instead of ELISA with the Nine Mile strain antigen. However, Ohlson et al. (16) reported that ELISA based on ruminant and tick antigens performed in a similar manner.

In the present study, the seroprevalence in the sheep flock in connection with vaccination was followed for 18 months. A statistically significant difference was observed between seronegative unvaccinated and vaccinated ewes during the whole trial (P < 0.05). Berri et al. (17) reported that in a 22-month study, most seropositive sheep remained positive, while none of the negative sheep seroconverted. In the present study, 23.3% of ewes seroconverted before vaccination in both seropositive groups. In the seropositive vaccinated group, all ewes became seropositive after the vaccination (100%) and the proportion of seronegative animals remained below 7% until the end of the study. However, in the seropositive unvaccinated group, further decline in the number of seropositive animals was observed; after 18 months (at the end of the study, ELISA 5), 30.8% of animals were seronegative. A significantly higher number of seropositive animals between seropositive vaccinated and seropositive unvaccinated groups was observed only in ELISA 3 (P < 0.05), while from October 2007 until

Table 1. Results of testing the ewes in a trial for the presence of antibodies against *Coxiella burnetii* (CHEKIT Q-fever ELISA Test Kit, Dr. Bommeli AG, Idexx, Switzerland).

	NV ewes[a]			NU ewes[b]			PV ewes[c]			PU ewes[d]		
	Tested	Pos	Equ	Tested	Pos	Equ	Tested	Pos	Equ	Tested	Pos	Equ
ELISA 1 (outbreak, June 2007)	30	0	0	30	0	0	30	30	0	30	30	0
ELISA 2 (vaccination, July 2007)	30	0	2 (6.7%)	30	1 (3.3%)	1 (3.3%)	30	18 (60.0%)	5 (16.7%)	30	20 (66.7%)	3 (10.0%)
ELISA 3 (October 2007)	28	27 (96.4%)	0	27	1 (3.7%)	2 (7.4%)	30	30 (100.0)	0	30	19 (63.3%)	3 (10.0%)
ELISA 4 (January 2008)	28	23 (82.1%)	2 (10.7%)	29	3 (10.3%)	1 (3.4%)	29	27 (93.1%)	0	29	21 (72.4%)	0
ELISA 5 (November 2008)	23	17 (73.9%)	2 (8.7%)	24	4 (16.7%)	1 (4.2%)	20	17 (85.0%)	2 (10.0%)	26	14 (53.8%)	4 (15.4%)

[a] 30 ELISA 1-negative vaccinated (NV) ewes, [b] control group of 30 ELISA 1-negative unvaccinated (NU) ewes, [c] 30 ELISA 1-positive vaccinated (PV) ewes, [d] control group of 30 ELISA 1-positive unvaccinated (PU) ewes; pos - positive, equ - equivocal.

Table 2. Positive results of *Coxiella burnetii* PCR tests of milk, blood, and feces collected from the ewes in trial.

Date	Matrix	NV ewes[a]		NU ewes[b]		PV ewes[c]		PU ewes[d]	
July 2007	Milk 1	0/29	0%	0/30	0%	0/30	0%	0/30	0%
January 2008	Milk 2	1/26	3.8%	0/24	0%	0/24	0%	1/23	4.3%
January 2008	Blood	0/25	0%	0/24	0%	0/24	0%	0/23	0%
July 2007	Feces 1	nt	-	nt	-	0/10	0%	0/10	0%
January 2008	Feces 2	8/25	32%	8/24	33.3%	9/24	37.5%	8/23	34.8%

nt - not tested; [a] 30 ELISA 1-negative vaccinated (NV) ewes, [b] control group of 30 ELISA 1-negative unvaccinated (NU) ewes, [c] 30 ELISA 1-positive vaccinated (PV) ewes, [d] control group of 30 ELISA 1-positive unvaccinated (PU) ewes.

Table 3. Sampling data and *Coxiella burnetii* real-time PCR results for the environmental samples collected at the sheep farm.

Date	Matrix	Number of samples	PCR positive
29 June 2007	Bedding (stable)	5	4
22 August 2007	Soil (pasture)	4	0
7 November 2007	Manure	3	2 (1[a])
21 December 2007	Manure	9	9
20 February 2008	Manure	10	10
	Bedding (stable)	4	3
27 May 2008	Manure	6	4 (2[a])
	Bedding (stable)	4	0
3 September 2008	Manure	10	8 (1[a])
5 November 2008	Manure	4	3 (1[a])
13 April 2010	Manure	3	2
	Bedding (stable)	3	0
29 July 2010	Bedding (stable)	1	0
14 February 2011	Manure	4	0
Total number of samples	Manure	49	38 (5[a])
	Bedding (stable)	17	7
	Soil (pasture)	4	0

[a] Number of samples with PCR inhibition.

the end of the study, approximately 7% of ewes became seronegative in both seropositive groups. Furthermore, among vaccinated animals, the number of seronegative animals increased more in the seronegative group than in the seropositive group. However, the increase was statistically insignificant (P = 0.3508). The comparison of seronegative vaccinated and seropositive unvaccinated groups showed a significant difference in the number of seropositive ewes in ELISA 3 (P < 0.05), but not in ELISA 4 (P = 0.0787) and ELISA 5 (P = 0.0572). According to these findings it could be suggested that the immune protection

of animals provoked by natural infection was comparable to the immune response induced by vaccination. Only the short-term vaccine effect was statistically significant. Nevertheless, it could be hypothesized that the vaccine also had a long-term effect as there were no more *C. burnetii*-related health issues in sheep or outbreaks in humans recorded until the time of writing the manuscript.

During the trial, the proportion of ELISA-positive/equivocal animals in the seronegative unvaccinated group steadily increased (to 20% in ELISA 5). The reason for this could lie in the persistence of *C. burnetii* in the

environment (in manure, ticks, dust, etc.), which might have led to new infections. Dissemination via aerosolized, contaminated dust particles from the positive stable could also be a pathway of *C. burnetii* transmission (18,19). De Bruin et al. (20) showed with quantitative PCR that surfaces (dust samples) contained higher levels of *C. burnetii* DNA than vaginal swabs from goats and sheep. Moreover, it is known that the serological status of the animal and shedding of *C. burnetii* are not related, as shedding is more likely to occur in seronegative than in seropositive animals (21), which especially holds true for sheep (15).

Vaccination reduces (14,22) but does not prevent *C. burnetii* shedding. There are some reports of vaccination preventing or reducing the release of *C. burnetii* in goats (23), but this has not been observed in sheep (24). In the present study, *C. burnetii* was not detected in any animals at the first milk sampling (Table 2), which is consistent with previous findings (15). Six months later, in the second milk sampling, *C. burnetii* was found in one animal (3.8%) in the seronegative vaccinated group and in one (4.3%) in the seropositive unvaccinated group. Vaccination seems a highly unlikely reason for the first finding, as this was a single case in the negative vaccinated group; in addition, there was no such case in the group of seropositive vaccinated animals. Previously present immune response could have prevented the shedding of *C. burnetii* in this group of animals. Furthermore, there was a time lag—the animals were vaccinated 6 months before the sampling— and, finally, an inactivated vaccine was used. Therefore, long-term shedding of *C. burnetii* seems highly unlikely. Positive results in both groups may have occurred because of milk sample contamination from a highly contaminated environment. It should also be taken into account that *C. burnetii* secretion in milk is intermittent (5) and that sheep shed a smaller quantity of *C. burnetii* in milk than cows and goats (2).

Contrary to expectations, feces of seropositive animals were negative in the first sampling (Table 2); however, a relatively small number of samples was tested. The second sampling revealed the presence of *C. burnetii* DNA in about one-third of the fecal samples tested. The results were also surprising, as a similar and relatively high proportion of animals that shed *C. burnetii* in feces was detected in all trial groups. This was clearly not related to vaccination since the results were very similar in vaccinated and unvaccinated groups. It could perhaps be explained by the emergence of new infections, as the proportion of seropositive animals in the negative unvaccinated group increased steadily during the trial (Table 1). However, it is also possible that the presence of *C. burnetii* in feces is linked to ingestion from the environment and passage through the gastrointestinal tract and not to shedding from the intestines. Nevertheless, additional sampling could give better insight into the shedding patterns, as fecal shedding may be discontinuous (18).

To the best of our knowledge, data on the presence of *C. burnetii* in blood are scarce. Negative results for the presence of *C. burnetii* in the blood of all animals in the trial were expected as bacteremia due to *C. burnetii* usually lasts only 5–7 days (25).

Since *C. burnetii* DNA was confirmed in feces, further focus was put on the presence of *C. burnetii* in manure and the environment (in the stable and on the pasture). Results summarized in Table 3 showed that manure was the most contaminated matrix, as 85.7% samples were positive 18 months after the outbreak. Moreover, *C. burnetii* DNA was found to persist in manure for about 35 months, while in the stable, DNA could be detected up to 8 months after the sampling commenced. Manure was negative in the last sampling, 4 years after the outbreak, while Astobiza et al. (11) reported positive environmental samples (aerosol) even after 4 years. In contrast to our results, de Bruin et al. (26) reported only 57.9% positive manure samples during Q fever outbreaks, while all of the other tested environmental samples (aerosols in stables, milk unit filters, surface area swabs) were positive. Even though it is likely that *C. burnetii* in the environment also derives from birth fluids or other secretions, the results of this study support previous findings that feces may represent a significant source of *C. burnetii* for the farm environment (15,18). Hermans et al. (27) reported a higher incidence of human Q fever cases around contaminated farms and suggested the zoonotic potential of the manure. Therefore, special caution is needed during handling, storage, and application of the manure, as the wind-borne spread of *C. burnetii* has also been reported (28,29). As a preventive measure on the farm studied, manure was stored in a covered dunghill approximately 200 m away from the stable and was allowed to be used when the samples were confirmed as PCR-negative for *C. burnetii*.

With regard to environmental samples taken from the stable, it was found that *C. burnetii* was present in a smaller number of samples (41.2%). Even though the bedding was changed regularly, it remained positive for almost 1 year, probably due to fecal shedding of *C. burnetii*. Dust and/ or aerosols in the stable may represent another source of bedding contamination, although this is unlikely as thorough cleansing and disinfection of the stable with quicklime, sodium hypochlorite, and formalin was performed. Soil samples from the pastures were negative. This result could be linked to sampling, as it was difficult to obtain a representative sample from a fairly large area (10 ha); in addition, a very small number of samples was investigated. Kersh et al. (30) suggested that farm workers played an important role in environmental contamination and transmission of *C. burnetii* to different parts of the farms. Therefore, careful decontamination is of high importance on the farms.

In conclusion, a One Health approach is necessary to successfully control outbreaks of Q fever, as veterinarians

often become alert only after human cases are reported. The results of this study suggest the following conclusions:

· Vaccination combined with extensive stable cleaning and disinfection is a suitable approach to control/prevent the disease, even in the short term;

· Sheep milk used for human consumption is not a main source of *C. burnetii* infection for humans;

· Feces and consequently manure are the most common source of environmental contamination;

· biosafety measures contribute to prevention of *C. burnetii* transmission by manure, which may represent a source of infection for a long time.

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