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Coxiella burnetii Circulation in a Naturally Infected Flock of Sheep: Individual Follow-Up of Antibodies in Serum and Milk

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ABSTRACT The control of Q fever, a zoonotic disease caused by the *Coxiella burnetii* bacterium, remains a scientific challenge. Domestic ruminants are considered the main reservoir, shedding *C. burnetii* essentially through parturition products during abortion or birth. Sheep are particularly frequently associated with human outbreaks, but there are insufficient field data to fully understand disease dynamics and to instigate efficient control measures. A longitudinal follow-up study of a naturally infected sheep flock was performed (i) to investigate relationships between seropositivity and bacterial shedding in the vaginal mucus, (ii) to describe the kinetics of antibodies, including responses to vaccination, (iii) to monitor maternal antibodies in ewe lambs, and (iv) to compare serological results for milk and serum samples. For 8 months, we collected blood samples every 3 weeks from 11 aborting and 26 non-aborting dairy ewes, 20 nonaborting suckler ewes, and 9 ewe lambs. Individual milk samples were also obtained from lactating females. All serum and milk samples were tested by enzyme-linked immunosorbent assay (ELISA), whereas vaginal swabs were tested by quantitative PCR. We found that some dairy females did not seroconvert despite shedding *C. burnetii* in their vaginal mucus. Overall, antibody levels in adult females were found to remain stable over time, with exceptions during the mating and lambing periods. Maternal antibodies decreased during the first month after birth. Interestingly, antibody levels in milk were correlated with those in serum. This study provides valuable field data that will help improve Q fever surveillance and within-flock management measures.

IMPORTANCE Field data are necessary to improve the surveillance, diagnosis, and sanitary management of Q fever in livestock. Here, we provide extensive serological data obtained from serum and milk samples from infected and vaccinated ewes belonging to a naturally infected flock of sheep. We show that antibody levels are stable over time and seropositivity and vaginal shedding are not clearly correlated, whereas antibody levels in milk are strongly correlated with those in serum. Accordingly, we find that antibody levels in bulk tank milk are consistent with the variations observed in the serum of dairy females over time. We report the existence of maternal antibody transmission to ewe lambs and we show that the presence of maternal antibodies at birth does not prevent the development of a serological response to vaccination at the age of 4 months. Finally, we report that adult ewes generally seroconvert after vaccination, including during pregnancy.

KEYWORDS Q fever, ruminant, ELISA, serology, cohort study, zoonosis, maternal antibody

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Q fever is a widespread zoonosis caused by *Coxiella burnetii*, a Gram-negative intracellular bacterium that has been reported in a broad range of host species. Livestock is the main source of human infections, with small ruminants being the most frequently involved (1–4). Both animals and humans become infected mainly through the inhalation of airborne particles contaminated with *C. burnetii* (4–6). In humans, *C. burnetii* infections range from asymptomatic to severe. The clinical signs are polymorphic and nonspecific; acute Q fever most often results in a flu-like illness, hepatitis, or pneumonia, whereas chronic Q fever may develop in patients with predisposing factors, with the most severe manifestation being endocarditis (7). The major clinical manifestations of Q fever in ruminants, in contrast, are abortions, stillbirths, and delivery of weak offspring, leading to significant economic losses (1, 3, 4). However, infected ruminants are most often asymptomatic, even while shedding infectious bacteria. Assessing the Q fever status of livestock farms may thus prove very challenging (3, 4).

PCR assays are commonly used to detect *C. burnetii* directly in biological materials, such as placentas, genital swabs, feces, or milk samples, and they reveal the existence of ongoing infections associated with bacterial shedding (8). In contrast, serological assays are used to detect specific antibodies, and they reveal any past exposure to the bacterium. They are typically used to perform serosurveys, which are designed to provide epidemiological data on Q fever at a relatively low cost, or to complement the results of direct laboratory tests in order to confirm the diagnosis of Q fever abortion at the flock level (3, 4). At an individual level, however, serological responses, bacterial shedding, and clinical signs are not clearly correlated (4). Overall, despite a few experimental infections (9–11), Q fever pathogenesis is still poorly understood. It is necessary to investigate the relationships between bacterial shedding and antibody responses within infected flocks to improve Q fever diagnosis and monitoring at the farm level.

The few longitudinal follow-up studies performed with cattle (12–15), goats (15–18), or other ruminant species (19) have been particularly valuable in providing descriptive data on individual shedding patterns and serological responses. Fewer data exist for sheep (15, 20, 21) than for the aforementioned species, despite the fact that sheep are frequently associated with clusters of human Q fever cases (22–24).

The purpose of this study was to provide extensive serological data from an infected flock of sheep. The aims were (i) to describe individual relationships between shedding and seropositivity, (ii) to assess the stability of antibodies over time and the responses to vaccination, (iii) to monitor maternal antibodies in ewe lambs, and (iv) to compare serological results from milk and serum samples.

RESULTS

Samples. A total of 564 blood samples and 235 milk samples were obtained from the 66 females studied, as detailed in Table 1. Additionally, 37 vaginal swabs were collected at the beginning of the study, from the 37 dairy females investigated.

Postabortion or postlambing seropositivity and shedding. We found that most of the aborting dairy females (9/11 females) and nonaborting dairy females (17/26 females) were seropositive (or highly seropositive) about 1 month postabortion or postlambing, respectively; however, the remaining aborting dairy females (2/11 females) and nonaborting dairy females (9/26 females) were seronegative or had doubtful results, although most of them (10/11 females) shed *C. burnetii* in their vaginal mucus (Fig. 1). Overall, seropositive females were more frequently PCR positive for *C. burnetii* in their vaginal mucus during the first month postabortion or postlambing than were seronegative females (Fig. 1), although the difference between the two groups was not significant (Fisher's exact test, $P = 0.08$). Indeed, no vaginal shedding was detected for 6 seropositive ewes (including 5 highly seropositive ewes) (Fig. 1). Similarly, we did not observe any significant difference in the proportions of seropositive shedders between aborting and nonaborting ewes or between nonaborting primiparous and multiparous ewes (Fisher's exact test, $P > 0.05$). However, we found that most

TABLE 1 Numbers of serum and milk samples tested by ELISA, according to the pen and aborting status of the investigated females

Animals investigated (no. sampled)	No. of sampling campaigns	No. of blood samples	No. of milk samples
Aborting dairy females (<i>n</i> = 11)	10	105	18 ^a
Nonaborting dairy females (<i>n</i> = 26)	8	193	235 ^b
Nonaborting suckler females (<i>n</i> = 20)	8	169	0
Ewe lambs (<i>n</i> = 9)	10	97	0
Total ^c	10	564	253

^aOnly 2 aborting females were monitored; the other 9 were transferred to the suckler pen.

^bSome females were occasionally sampled between sampling campaigns.

^cSome females could not be sampled at every sampling campaign, for technical reasons.

ewes that shed >10⁴ *C. burnetii* genome equivalents in their vaginal swabs were seropositive (13/17 females), while those that shed >5 × 10⁶ *C. burnetii* genome equivalents per swab were highly seropositive (5/37 females) (Fig. 1).

Variations in S/P values over time. Sample/positive (S/P) values remained stable for both seronegative and seropositive aborting females, including the vaccinated primiparous ewe, throughout the 8 months during which the females were monitored (Fig. 2a); an exception was observed for the multiparous aborting female that was kept in the dairy pen after adopting a lamb, for which the serological profile varied over time. Similarly, nonaborting dairy females had stable S/P values for 4 months after their parturition and then most of the females that were seronegative seroconverted upon vaccination, while the S/P values for those that already had antibodies increased (Fig. 2b). Similarly, nonaborting suckler females (except 1) seroconverted upon vaccination, although the latter took place during their gestation; S/P values remained stable thereafter (Fig. 2c). Interestingly, we observed that the mating period was generally associated with a moderate decrease in S/P values (Fig. 2).

Maternal antibodies in ewe lambs. Although the vaginal mucus of all 9 females whose lambs were monitored was PCR positive during the first month postlambing, only 5 of the females were seropositive 2 to 3 weeks postlambing (Fig. 1 and 2b).

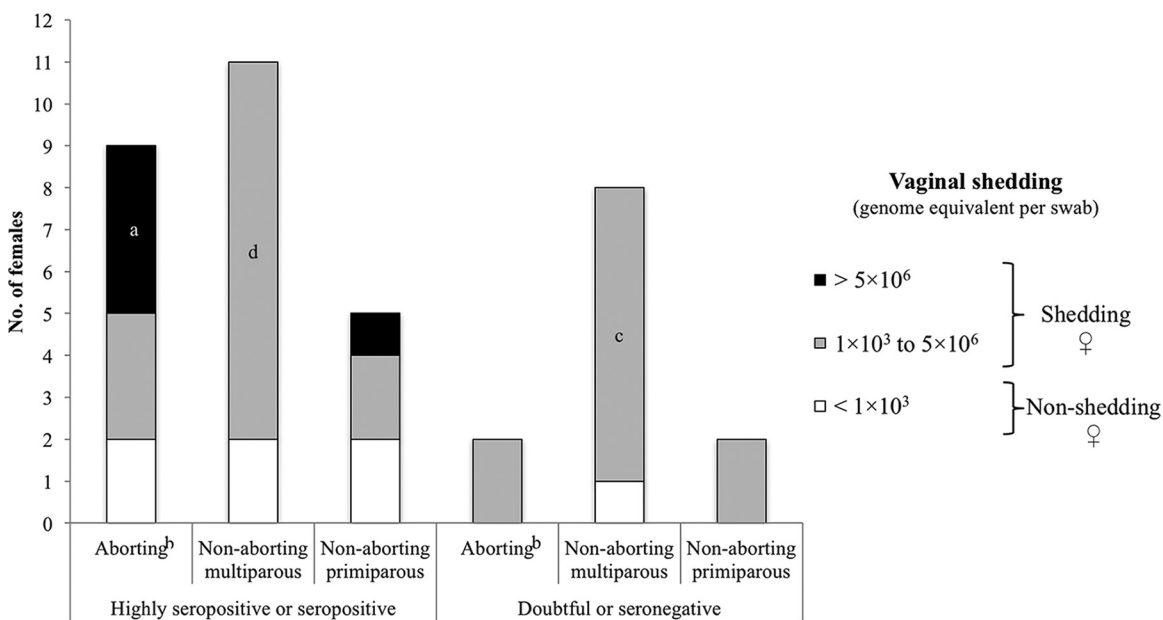


FIG 1 Histogram showing the numbers of females shedding *C. burnetii* in their vaginal mucus during the first month postlambing or postabortion, according to their serological status during this month and their aborting status and parity. ^aIncluding the only aborting primiparous ewe. ^bIncluding 1 primiparous and 10 multiparous ewes. ^cIncluding the dams of ewe lambs 1 to 5. ^dIncluding the dams of ewe lambs 6 to 9.

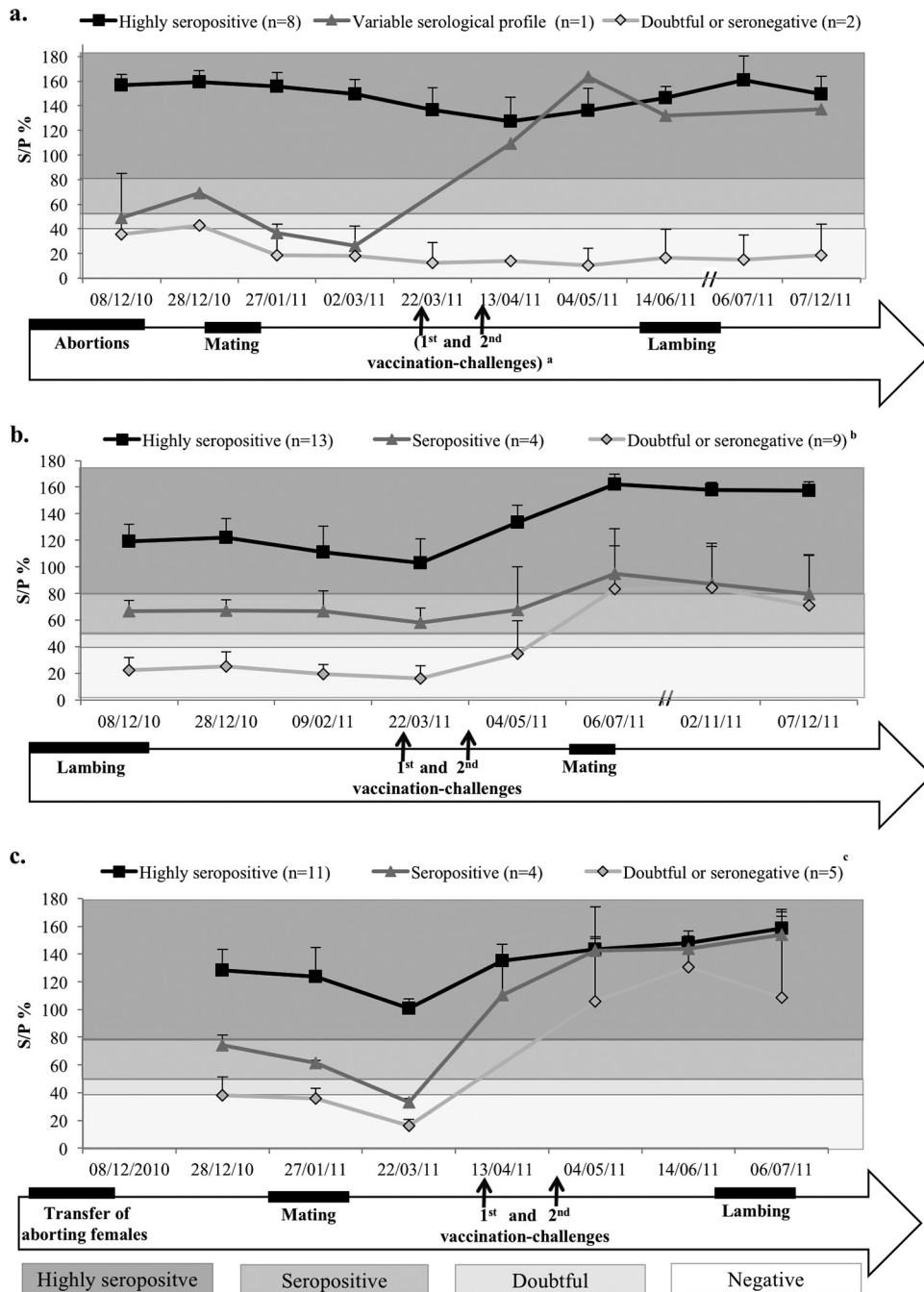


FIG 2 S/P values obtained over time for serum samples from aborting dairy ewes ($n = 11$) (a), nonaborting dairy ewes ($n = 26$) (b), and suckler ewes ($n = 20$) (c). For simplicity, only positive confidence intervals are represented. ^aOnly the aborting primiparous ewe (represented by the black curve among the highly seropositive females) and 1 of the multiparous ewes (represented by the dark gray curve), to which the farmer gave a lamb for adoption, were vaccinated. ^bThree of the seronegative nonaborting dairy ewes remained seronegative. ^cOne of the suckler ewes remained seronegative.

Accordingly, we detected antibodies in 4 of the 5 ewe lambs born to these seropositive dams, whereas lambs born to seronegative females had doubtful or negative enzyme-linked immunosorbent assay (ELISA) results (Fig. 3). Subsequently, S/P values decreased as the ewe lambs grew older, with all lambs becoming seronegative by 2 months after birth.

By the age of 8 months, i.e., 4 months after vaccination, 4 lambs had seroconverted. The S/P values for the others had increased but had not reached the threshold for

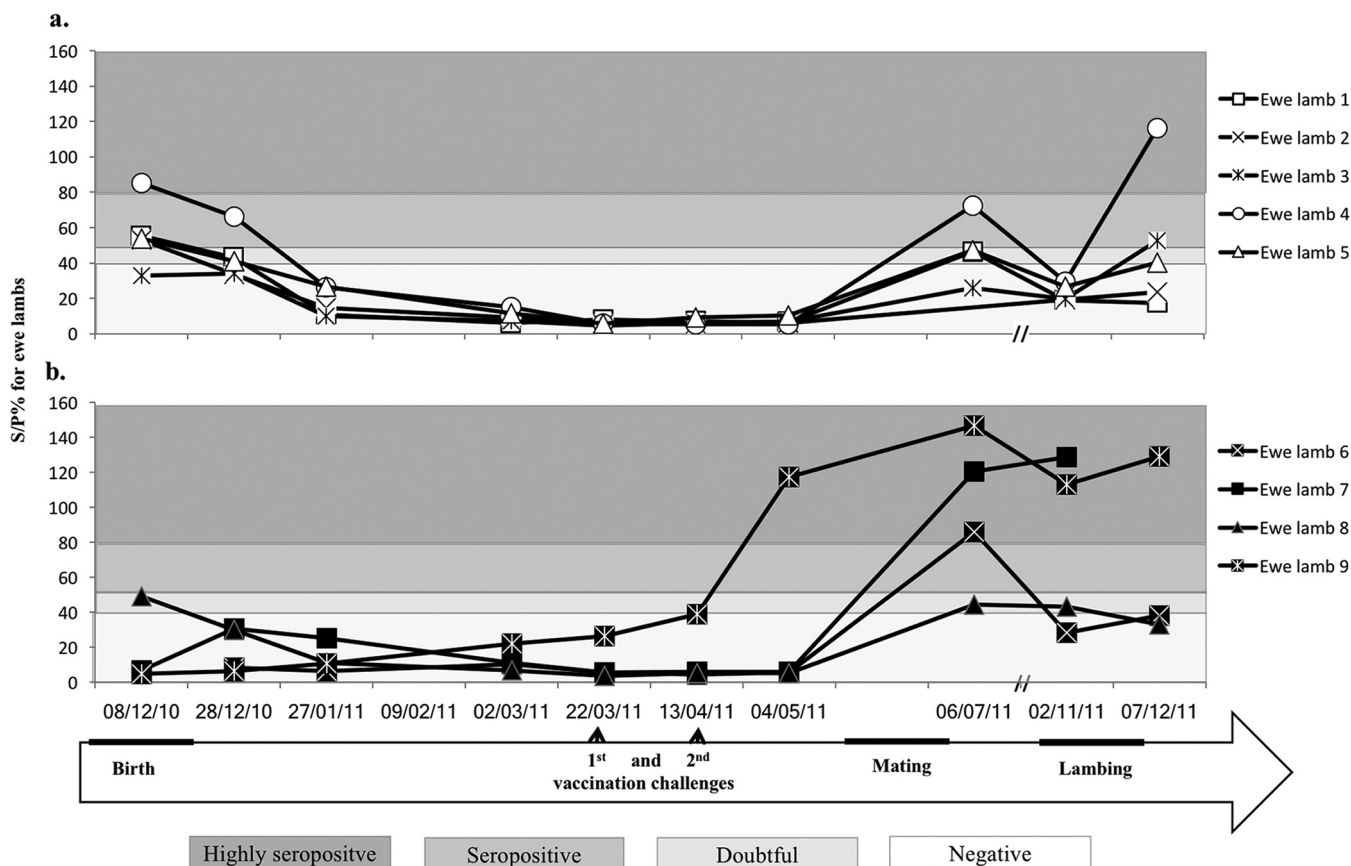


FIG 3 S/P values obtained from serum samples collected from ewe lambs between 1 month and 1 year of age, according to the presence ($n = 5$) (a) or absence ($n = 4$) (b) of detected antibodies in the mother’s serum on the date the lambs were first sampled (i.e., 8 December 2010).

seropositivity. Interestingly, ewe lamb 3, which was born to a highly seropositive dam, remained seronegative despite vaccination (Fig. 3). We then observed a decrease in S/P values during the lambing period. Finally, at the end of the study, 7 months after the booster vaccination, the S/P values for only 4 of the 9 monitored ewe lambs were above the threshold for seropositivity.

Antibody levels in milk. All negative serum samples were associated with negative milk samples. Despite a strong correlation ($r = 0.89$) between the S/P values for individual seropositive serum and milk samples, some milk samples were considered negative while the corresponding serum samples were positive or even highly positive ($\text{kappa} = 0.48$) (Fig. 4). Accordingly, the S/P values obtained for the bulk tank milk were consistent with the variations observed for the serum samples from dairy females over time (Fig. 2b); values were high at the beginning of the study and decreased slightly thereafter but remained above or very close to the positivity threshold before increasing after vaccination (Fig. 5).

DISCUSSION

Longitudinal studies are essential for providing relevant health risk indicators. Although they are complex and costly to set up, longitudinal studies such as the one described here are particularly useful because they provide valuable data that will improve the understanding of *C. burnetii* circulation in infected flocks. Indeed, it is essential to repeat the sampling process with the same animals so as to understand how serological responses and shedding dynamics vary over time and between individuals; such data are critical for contextualizing transversal serosurveys and improving the interpretation of results at different levels.

Here, we took up the challenge of carrying out a longitudinal study within a naturally infected flock of sheep. The originality of our study resides in the fact that we

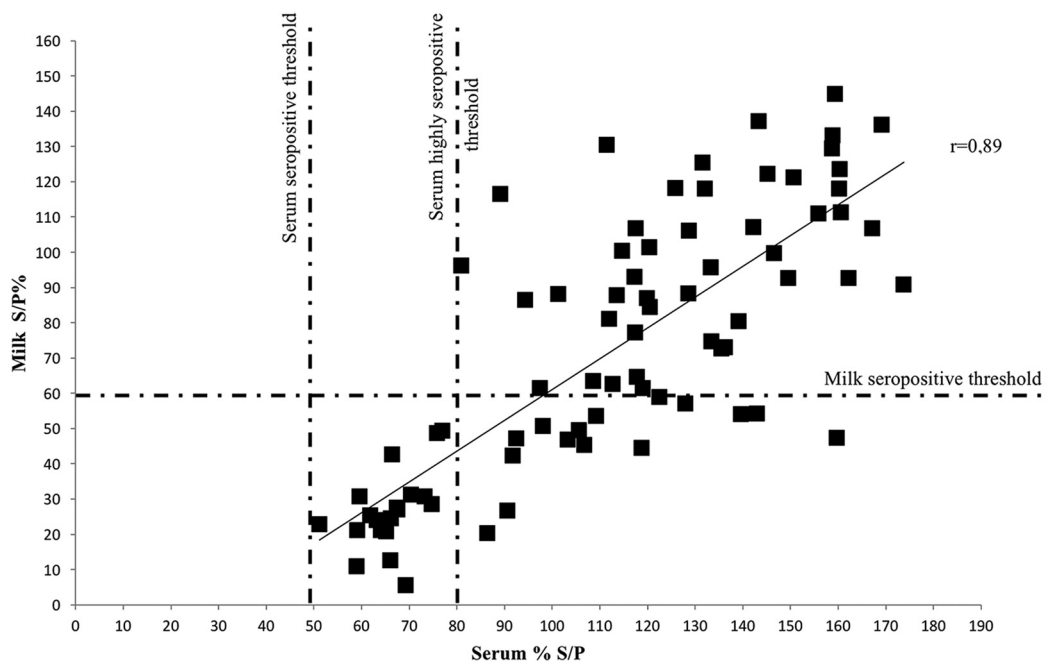


FIG 4 Correlation between S/P values obtained for individual serum and milk samples from dairy females for which S/P values were above the positivity threshold (i.e., >50%) ($n = 82$ samples from 26 nonaborting dairy females and 2 aborting females that had adopted a lamb).

concomitantly assessed the relationship between seropositivity and shedding, described antibody kinetics in both milk and serum samples, and investigated passive immunity in ewe lambs.

Seropositivity and vaginal shedding are not clearly correlated. To control Q fever transmission among animals and from animals to humans, it is vital to have tools that reliably detect *C. burnetii* shedders. In this study, we found that seropositivity was not indicative of whether ewes had recently been shedding *C. burnetii*, an observation that has already been reported for sheep (21, 25, 26), cows (22), and goats (27). Indeed, we observed that some ewes remained seronegative although they were shedding *C. burnetii* in their vaginal mucus, while others were seropositive although no vaginal shedding was detected. Interestingly, however, most of the seropositive females displayed PCR results above 10^4 genome equivalents per swab, which is the

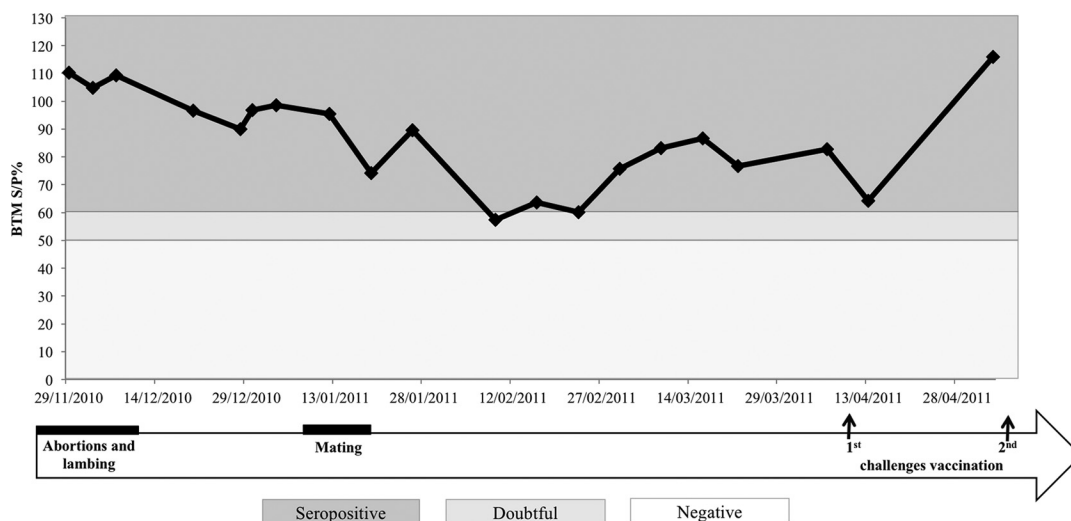


FIG 5 S/P values obtained over time for bulk tank milk (BTM) samples ($n = 26$).

threshold suggested by a group of French experts for considering that abortion in ruminants is caused by *C. burnetii* (28). Additionally, we found that all ewes whose burdens were greater than 5×10^6 genome equivalents per vaginal swab were systematically strongly seropositive 1 month postabortion or postlambing. Overall, these results suggest that, while serology is a useful tool for assessing exposure to *C. burnetii* at the flock level, it cannot be used alone to assess the infection status of an individual sheep.

Antibody levels in milk samples are correlated with those in serum samples.

Bulk tank milk serological analyses are occasionally used for cattle (12, 29, 30), goat (31, 32), and sheep (33, 34) herds to reveal exposure to *C. burnetii*. Here, we found that antibody levels in bulk tank milk were generally indicative of the serological results obtained from individual serum samples from the dairy ewes included in the individual follow-up study, as reported previously for other sheep and goat farms (35, 36). Therefore, our findings suggest that serological analysis of bulk tank milk is a relevant tool for assessing within-flock seroprevalence. Accordingly, we found a strong correlation between antibody levels detected in the serum and milk samples collected on the same day from individual ewes, as investigated previously in cattle (12, 37). With the positivity threshold currently recommended by the kit manufacturer, however, the ELISA used in this study appeared to detect positive serum samples more frequently than positive milk samples. Because no reference test is available for the serological diagnosis of Q fever (3), the sensitivity and specificity of all Q fever serological tests have been insufficiently characterized; in this context, it might be useful to reanalyze the serum and milk samples from this study using other commercial ELISA kits, in order to confirm the observed status and to identify potential discordant test results.

Furthermore, as part of a previous study (38), we tested the same milk samples for the presence of *C. burnetii* DNA; shedding was detected only for certain females with small bacterial burdens, over short periods just after abortion or lambing. These results confirm that milk may be used to reveal past exposure to *C. burnetii* through serological analyses (15, 39, 40) but it is not a reliable matrix for detecting ongoing shedding in sheep, contrary to findings for goats (32, 41).

Breeding conditions may explain variations in antibody levels over time.

We observed slight variations in both serum and milk antibody levels over time, which could be explained by several physiological and environmental factors. First, we observed slight decreases in antibody levels just after mating for all of the monitored females and just before lambing for females that were included in the study as ewe lambs. Our observations are novel, to our knowledge, for small ruminants but are consistent with those already described for gestating dairy cows (42–45). These variations during the birthing and mating periods might be related either to an increase in stress due to pen movements or to hormonal variations.

Second, we also observed variations in antibody levels after vaccination. For most of the monitored females, a serological response was clearly noticed following the first and second vaccination challenges. Although the vaccine manufacturer does not recommend vaccination during pregnancy, we did not observe any adverse clinical signs among the suckler females that were vaccinated at 2 months of gestation. This finding is in agreement with the absence of adverse vaccination effects reported previously for goats (10, 15) and cows (46). Interestingly, the antibody levels detected for these females were similar to those detected for nonvaccinated aborting females. This result highlights the need to develop serological methods that differentiate between infected and vaccinated animals.

Finally, as described previously for calves (47), colostrum intake from seropositive mothers is essential for transferring maternal antibodies to offspring, possibly having major consequences for the health of juveniles (48, 49). Here we report maternal antibody transmission in a naturally infected flock of sheep. We observed that one of the ewe lambs born to a seropositive dam did not harbor maternal antibodies, which suggests that its colostrum intake was insufficient. The levels of antibodies in all of the other lambs born to seropositive dams decreased during the first 2 months after birth,

TABLE 2 Numbers of aborting and nonaborting females sampled, according to the pen, breed, parity, and sampling period

Sampling period (every 3 wk)	Pen	Breed	Parity	No. of aborting females	No. of nonaborting females
From November 2010 to July 2011	Dairy	Lacaune	Multiparous	1 ^a	19
			Primiparous	1 ^a	7
From December 2010 to July 2011	Dairy and then suckler ^b	Lacaune	Multiparous	9 ^b	0
			Multiparous	0	14
	Suckler	Lacaune-Rouge de l'Ouest	Primiparous	0	6
			Nulliparous	0	9 ^d
Dairy renewal and then dairy ^c	Lacaune	Nulliparous	0	9 ^d	
Total				11	55

^aThe farmer made these females adopt a lamb, to keep them in the dairy pen.

^bDairy females aborted in the dairy pen and were progressively transferred to the suckler pen after abortion.

^cEwe lambs were transferred to the dairy pen before the mating period (i.e., 7 months after the start of the study).

^dThese ewe lambs were born to nonaborting multiparous dairy females monitored in this study.

which is consistent with the expected kinetics of maternal antibodies. Unexpectedly, the responses to vaccination varied between ewe lambs regardless of the serological status of their mothers. Interestingly, one of the ewe lambs born to a seronegative dam displayed an early increase in S/P values, perhaps in response to natural exposure to *C. burnetii*.

Conclusion and perspectives. This study provides serological data that complement previously published data on shedding dynamics and environmental contamination in a sheep flock (38). Such field data are particularly valuable for optimizing the understanding of *C. burnetii* circulation dynamics in naturally infected flocks. However, complementary pathophysiological research is needed to explain the regulating mechanisms of seroconversion in both shedding and nonshedding females and to understand the respective roles of humoral and cell-mediated immune responses (10, 39).

Overall, our results highlight the complexity of deciphering *C. burnetii* epidemiology and confirm the need to use a combination of indicators to assess the Q fever status of livestock farms. In particular, this longitudinal study provides new knowledge that can be exploited in future studies regarding (i) the appropriate females to monitor, (ii) the appropriate samples to collect, (iii) the appropriate analyses to perform, and (iv) the optimal sampling periods. These data will help improve field protocols tailored to specific objectives, e.g., the surveillance and diagnosis of Q fever abortive episodes, the identification of shedding farms, and follow-up monitoring of sanitary measures. The optimization of such protocols is crucial for the implementation of efficient animal and public health management measures in the framework of the One Health global approach.

MATERIALS AND METHODS

Field sampling. (i) Flock selection. The study was carried out on a flock of 610 sheep divided into two pens of 360 purebred Lacaune dairy sheep and 250 crossbred Lacaune-Rouge de l'Ouest sheep. The lambing season took place in late autumn and late spring for the dairy and suckler pens, respectively. The flock was selected for study following abortions by 10 multiparous dairy ewes (an additional primiparous dairy ewe aborted about 1 month after the start of the study). Four of the aborting dairy ewes were screened for diverse infectious abortive microorganisms. *C. burnetii* was identified as the etiological agent; indeed, all of the laboratory results were negative for toxoplasmosis, chlamydiosis, listeriosis, salmonellosis, campylobacteriosis, and border disease, whereas quantitative PCR (qPCR) tests were positive for *C. burnetii*. With the exception of 2 ewes (1 primiparous and 1 multiparous) to which the farmer had given a lamb for adoption, the aborting females were transferred from the dairy pen to the suckler pen (Table 2). None of the sheep had been vaccinated against Q fever before the start of the study. However, the farmer administered an inactivated vaccine (Coxevac; CEVA-Santé Animale, Libourne, France) to all nonaborting females approximately 5 months after the start of the study, i.e., 2 months before mating (dairy pen) or lambing (suckler pen). The 2 aborting females that had adopted a lamb were vaccinated along with all of the other dairy females in the dairy pen. The farmer made all decisions regarding vaccination independently of our cohort study.

(ii) Animal sampling. Overall, 66 sheep were monitored (Table 2), i.e., 37 dairy ewes (including 11 that had aborted [10 multiparous and 1 primiparous] and 26 that had not [19 multiparous and 7 primiparous]), 20 nonaborting suckler ewes (14 multiparous and 6 primiparous), and 9 ewe lambs (born

TABLE 3 IDvet ELISA interpretation thresholds defined by the manufacturer

Sample type	S/P value (%)	Status
Serum	<40	Negative
	40 to <50	Doubtful
	50 to ≤80	Positive
	>80	Highly positive
Milk	<50	Negative
	50 to ≤60	Doubtful
	>60	Positive

to 9 of the 19 nonaborting multiparous dairy ewes). The ewe lambs were monitored from their first month of life (i.e., between 16 and 20 days of age) until their first lambing. Descriptive data about the *C. burnetii* shedding dynamics of the 37 dairy ewes have been reported elsewhere (38); here we considered only the vaginal samples collected from those females during the first month postabortion or postlambing. When two vaginal samples were available, only the most loaded (i.e., the one that displayed the greatest bacterial burden by qPCR) was considered. Blood samples were collected from each monitored female every 3 weeks for 8 months. Samples of 3 ml of whole blood were collected in dry tubes from the jugular vein; serum was recovered after centrifugation (10 min at $3,000 \times g$, room temperature) and frozen at -20°C . Additional blood samples were collected from all of the monitored females 1 year after the beginning of the study, i.e., after their next lambing (dairy pen) or 1 month before mating (suckler pen). Milk samples were collected from dairy ewes every 3 weeks for 8 months. Individual milk samples (5 ml) were collected in a sterile flask after the teats were disinfected with an alcohol wipe and the first spurts were eliminated. Finally, 5 ml of bulk tank milk was collected in a sterile flask once a week for 5 months.

Laboratory analyses. Serum and milk samples were analyzed using the IDvet ID Screen Q fever indirect multispecies ELISA kit (IDvet, Grabels, France). This kit is based on a mix of phase I and II antigens obtained after the purification and inactivation of a *C. burnetii* strain isolated from the placenta of an aborting cow. The results were read at 450 nm using a μ Quant spectrophotometer (BioTek Instruments, Winooski, VT). Positive and negative internal controls were included in each plate. The optical density (OD) values obtained were expressed as the mean percentage of sample/positive (S/P) values, as recommended by the kit producer, as follows: $S/P \text{ value} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{neg.control}}) / (\text{OD}_{\text{pos.control}} - \text{OD}_{\text{neg.control}})$, where $\text{OD}_{\text{pos.control}}$ and $\text{OD}_{\text{neg.control}}$ are the OD values for the positive and negative controls, respectively.

Serum and milk samples were classified, based on S/P values, as highly seropositive, seropositive, doubtful, or seronegative, in accordance with the manufacturer's recommendations (Table 3). We considered a female to have seroconverted when its S/P values increased over time from below the doubtful threshold to above the positivity threshold.

Bacterial burdens in vaginal mucus samples were estimated by a real-time qPCR method targeting the multicopy *IS1111* gene, as described previously (38). Serial dilutions of genomic DNA prepared from the Nine Mile phase II RSA 493 isolate (ANSES, Sophia Antipolis, France) were used as quantitative standards. The minimum limit of quantification of the method was assessed as 5×10^2 genome equivalents per swab, which corresponds to the lowest concentration of the Nine Mile isolate standard curve; similarly, the maximum limit of quantification was assessed as 5×10^6 genome equivalents per swab, which corresponds to the highest concentration of the Nine Mile isolate standard curve. A female was considered a shedder when *C. burnetii* DNA was detected at levels above twice the minimum limit of quantification (1×10^3 genome equivalents per swab) for at least one of the vaginal samples collected during the first month postabortion or postlambing.

Statistical tests. We used Fisher's exact test to investigate the relationships between seropositivity and shedding. We calculated a correlation coefficient and carried out a kappa test to assess the quantitative and qualitative correlations, respectively, of the serological results obtained from serum and milk samples. Statistical analyses were performed using R version 3.1.0 software. Our α level for statistical significance was set at 0.05.

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