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Research article
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Seroprevalence of *Coxiella burnetii* among ewes and associated risk factors in Constantine (Northeastern Algeria)

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ABSTRACT. Q fever is a zoonotic disease caused by the rickettsia-like *Coxiella burnetii* and leads to abortions and decreased reproductive performances in domestic ruminants. A serological survey, using ELISA test, was conducted to assess the prevalence of this infection in 226 ewes belonging to 39 flocks localized in Constantine (North-eastern Algeria). A pretested questionnaire has been submitted to farmers/shepherds to collect information related to relevant risk factors.

Results revealed the presence of *C. burnetii* antibodies in 12.4% (95% *CI*: 8.08%−16.72%) of individual animals while 35.9% (95% *CI*: 21.20%−52.82%) of sampled flocks accounted at least one seropositive ewe. Significant causative associations were observed for origin of animals (χ^2=14.29, *P*=0.001), vaccination against enterotoxaemia (χ^2=12.12, *P*=0.002) and pox (χ^2=5.30, *P*=0.025), access to the farm by foreign visitors (χ^2=10.87, *P*=0.004), farmers/shepherds' visits to other farms (χ^2=6.31, *P*=0.021), disinfection frequency (χ^2=7.98, *P*=0.046), pest infestation within farms (χ^2=9.55, *P*=0.049) and abortion history (χ^2=5.54, *P*=0.029). This recorded prevalence of Coxiella infection would indicate a possible responsibility of this agent in causing abortion and reproductive failures in the tested flocks.

Implementing active surveillance programs and further investigations using more accurate analyses and including large samples of more animal species from several provinces are needed to elucidate the real occurrence and dynamics of this infection in the national livestock.

Keywords: Coxiellosis; prevalence; ELISA; risk factors; ewes; Constantine

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INTRODUCTION

Q fever is a widespread zoonosis acknowledged and named for the first time by Edward Derrick in abattoir workers in Australia. It is caused by a small obligate intracellular bacterium *Coxiella burnetii* (de Valk, 2012). A large variety of domestic and wild animal species (mainly cattle, and small ruminants, pets and rodents) are known natural reservoirs for this pathogen (Cutler *et al.*, 2007). Transmission is possible through inhalation of contaminated aerosols generated during birthing or slaughtering of infected asymptomatic animals, which also shed the bacterium in faeces and many physiological secretions (Rodolakis, 2009; Schimmer *et al.*, 2010). In nature, arthropods such as ticks may play a role in the epidemiology of *C. Burnetii* because of their ability to harbour and transmit it trans-stadially and trans-ovarially to their descendants. Nevertheless, in livestock and humans, tick bite is an uncommon route of Coxiellosis (Sprong *et al.*, 2012; Mancini *et al.*, 2014).

In humans, the acute illness is self-limiting and evolves in general as a non-specific flu-like syndrome with inconstant pneumonia and hepatitis; whereas the chronic form (causing meningoencephalitis, myocarditis, endocarditis and other vascular infections) is life-threatening if untreated (Angelakis and Raoult, 2010). Pregnant women generally do not exhibit any clinical signs but their infection leads to obstetrical complications, spontaneous abortions and premature delivery (de Lange *et al.*, 2014).

In domestic ruminants, sheep appear to be more infected than goats and cattle (McQuiston *et al.*, 2002). Q fever is often latent and is responsible for abortion (with placentitis and endometritis) and other reproductive disorders characterized by premature births, weak or unviable progeny, infertility and mastitis (Tissot-Dupont and Raoult, 2008; Georgiev *et al.*, 2013).

Since Q fever has no specific symptoms, the diagnosis confirmation is made through laboratory analyses among which, serology remains the most commonly used because of easy serum samples collection and complicated cultivation/isolation of the bacterium. In veterinary medicine, the ELISA test allows detection of phase I and II antibodies, and due to its sensitivity, it is preferred to CFT and IFA which remains the gold standard for detection of antibodies against *C. burnetii* during acute human Coxiellosis (Horigan *et al.*, 2011; Stephen *et al.*, 2017).

In Algeria, there are about 26.6 million sheep, mainly located in the steppes and high plateaus, where they are reared under traditional practices and play an important role in the livelihood of many families (MADR, 2014). Nevertheless, the incidence of asymptomatic abortion in late gestation ewes is frequently reported by veterinarians causing substantial economic losses while Q fever continues to be neglected or under-diagnosed as one of the possible aetiologies of these outbreaks.

Furthermore, few studies have been undertaken on this infection because of the deficiency in diagnostic tools; even though it seems to be disseminated all over the country since it has been reported in cattle in Bejaïa (North) and Sétif (North-East), sheep in Médéa (Central North) and camels in Biskra, El-Oued, Ouargla and Ghardaia (South) (Yahiaoui *et al.*, 2013; Agag *et al.*, 2016; Benaissa *et al.*, 2017; Menadi *et al.*, 2019).

The present study determines the prevalence and risk factors of Q fever in ewes' population in Constantine province (North-East Algeria) and brings more insights on this infection in the local livestock, which may result in implementing more efficient preventive and control approaches.

MATERIAL AND METHODS

Study area

All the 12 municipalities of Constantine province were included in this survey. This region is located in the northeast of Algeria, at about 80 km from the Mediterranean Sea coast. It is considered as the third most populated city to which converge all the eastern high plateaus. It is about 2 297.2 km^2 with an altitude ranging from 300 to 1 000 m. Constantine is characterized by a semiarid climate with a typical hot and relatively dry season between June and August, and a wet season from December to April. Rain season corresponds to December, January and February with 350-500 mm of rainfall. The temperature is 25°- 40°C during summer and 0°-12°C during winter.

The ovine population counts about 179 220 heads (111 290 ewe) belonging to 1 938 flocks. Sheep breeding season usually begins in July when the daylight starts to decline. All flocks enrolled in this study grazed along the spring season till the end of August

with no feed supplementation. Along fall and winter, animals are housed and fed straw, barley, and wheat bran. Lambing period habitually lasts from December to March.

Sampling procedure

The study protocol was evaluated and approved by the ethical committee of the scientific board of the institute of veterinary sciences. University Frères Mentouri Constantine 1, Algeria.

From March 2011 to January 2012, livestock owners were contacted and informed about the purpose and the methods of the study to obtain their verbal consent. The animal welfare guidelines were rigorously followed.

The needed sample size estimation was performed in two steps (random selection of a defined number of flocks then the number of sheep to be selected was individually determined by flocks) using formulas for simple random sampling given by Thrusfield (2007).

$$N = \frac{(1.96)^2 \ P(1-P)}{L^2}$$

Where N is the needed sample size, $4 = (1.96)^2$ is the error alpha, P is the disease prevalence and L is the allowed error or required precision (0.06).

At the herd level, total number of flocks to be sampled was calculated by dividing the total individual sample size by the number of animals to sample from each flock. Thus, 39 flocks of 20 to 500 heads were randomly selected. Flocks were stratified according to their size: 6 (≤20 heads), 8 (>20 heads ≤50), 5 (>50 heads ≤100), 10 (>100 heads ≤300) and 10 (>300 heads ≤500).

At the individual level, sample size was determined for each flock so as to detect the existence of the disease. Calculations were made using the formula commonly applied in veterinary epidemiological investigations:

$$n = \left[1 - (1-p)^{1/d}\right] \times \left(N - \frac{d}{2}\right) + 1$$

Where ***n*** is the essential sample size, ***p*** is the probability of detection of at least one seropositive ewe, ***N*** is the herd size, and ***d*** is the number of seropositive ewes in the herd. The probability to detect at least one seropositive ewe in a herd was determined at 95% *(p=0.95)*, while the number of seropositive ewes in each herd (***d***) was estimated assuming that within herd prevalence equals 10% (since there was no previous study in this area).

Accordingly, the minimum required sample size was calculated to be about 100 ewes. However, and in order to increase the precision of the study, a total of 226 ewes were enrolled belonging to 39 flocks localized all over Constantine. Number of ewes and flocks to be sampled per municipality was proportional to its total number of animals and flocks respectively. Herds' distribution is shown in Figure 1.

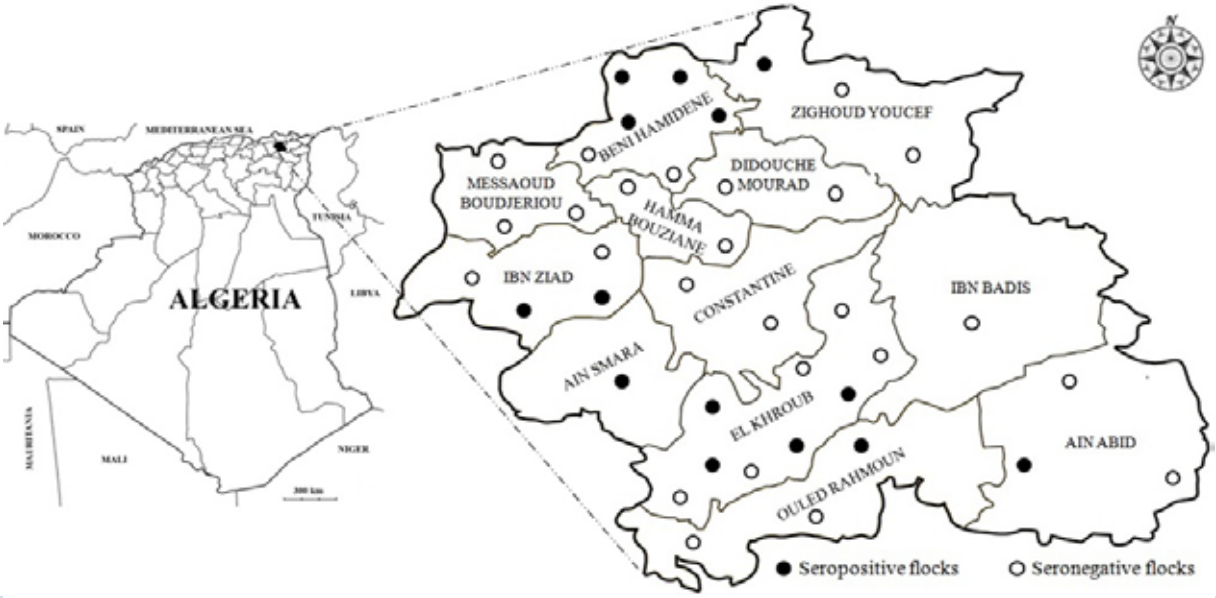

Figure 1. Distribution of seropositive and seronegative flocks among the 12 municipalities of Constantine

Blood was collected from jugular vein with disposable needle in plain Vacutainer® tubes labeled and conveyed quickly on ice to the laboratory. Sera were then separated from the clot by centrifugation and stored at −20°C until analyses. Each flock was assigned an identification number (ID n°) and its characteristics were recorded.

Serological tests

Detection of *C. burnetii* specific antibodies was carried out by using a commercial Enzyme-Linked ImmunoSorbent Assay (ELISA) according to the manufacturer's recommendations and protocols (LSIVET Ruminant Milk/Serum Q Fever ELISA COXLS LSI, Lissieu, France). The antigen is a sheep strain (phase I-II). Sensitivity of this ELISA test reaches 87% and specificity 100%. Absorbance values were measured at 450 nm (OD_{450}) using an ELx800™ absorbance microplate reader (Bio-Tek Instruments®, INC, Vermont, USA). Antibody reactivity was estimated using the sample to positive ratio (S/P) calculated as (Sample OD − Negative OD) / (Positive OD − Negative OD) × 100. The S/P values were classified as negative (S/P ratio≤40) or positive (S/P ratio>40). A flock was defined as infected if it included at least one seropositive ewe.

Serological analyses were performed at the Department of Reproductive Pathology, École Nationale Vétérinaire de Nantes ONIRIS. France.

Epidemiological data collection

A questionnaire has been pre-established and pre-tested on farmers/shepherds non-included in this study to verify its accuracy and clarity. The final version included 65 questions of which 75% were close ended. Data related to farm characteristics, flock composition and characteristics, feed and water origin and quality, origin of animals (home bred or purchased), reproductive performances and problems (mainly abortion), health status of animals, treatments and vaccinations, contact with other animals or humans, biosecurity measures, disinfection and pest management were collected. Ages (in years) of sampled ewes were divided into three classes: ≤2yrs, 2<yrs≤3 and 3<yrs≤4.

Data analysis

Data collected through the questionnaire and the results of serological analysis were coded, stored, and analysed using SPSS 20 software (2011). Determination of risk factors associated with *C. burnetii* seroprevalence was realized in two stages. A univariate analysis (using *chi-square test*) was performed at first to check for significant associations between tested variables and the seroprevalence of Q fever coded as 0 (negative) or 1 (positive). In a second step, factors that show moderate statistical significance *($p\leq0.25$)* with counts ≥5 in each cell were introduced to a multivariable logistic regression model. The variable flock was included in the model as a fixed effect variable. The logistic model was developed using the stepwise forward approach using a likelihood ratio test at each step with 0.1 as significance level for removal or entry. In the final model, any variable with a *$p<5\%$* was considered statistically significant and was retained in the model. The fit of the model was assessed using the *Hosmer and Lemeshow goodness-of-fit test* (Abu-dalbouh *et al.* 2012).

RESULTS

In the study area, the seroprevalence of Coxiellosis was 12.4% (95% CI: 8.08% − 16.72%) in individual animals, while 35.9% (95% CI: 21.20% − 52.82%) of sampled flocks had one or more positive ewes. Most infected animals and flocks were from Beni-Hamidene and El Khroub municipalities (Table 1; Figure 1).

There was no tendency of Coxiella infection with ewes' age *($X^2=0.31$, $p=0.85$)* since ewes at different ages had recorded approximately the same seroprevalence (ys≤2: 13.72%; 2<ys≤3: 11.21%; 3<ys≤4: 11.76%).

As shown in Table 2, 71.42% of positive ewes had aborted, mainly those aged over 2 years. Abortion at first gestation was exclusive to seropositive females aged of ≤3 years old. Abortion at 2nd stage of gestation was more frequent in age class 2<ys≤3 and specific to ewes of 3<ys≤4 old. It is worth noting that in females with Coxiella antibodies, 21.42% had known repeated abortion. We could also find an association between history of abortion and seropositivity *($X^2=5.54$, $p=0.02$)*.

The risk factors that were significantly associated to Coxiella seroprevalence are shown in Table 3. They are represented by origin of animals, vaccination against enterotoxaemia and pox, access to the farm by foreign visitors, farmers/shepherds' visits to other farms, disinfection frequency and pest infestation within the farm. It was not possible to verify with certainty a causal relationship between *C. bumetii* seropositivity and any other farming activities, other flocks' characteristics or therapeutic practices.

Conversely, the multivariate logistic regression model did not establish a significant association between the potential risk factors defined by univariate analysis and seropositivity to *C. burnetii*.

Table 1. Distribution of sampled ewes and flocks over the 12 municipalities of Constantine province

Municipality	Number of sampled ewes (%)		Number of sampled flocks (%)		Flock identification number (Number of positive ewes)
	Positive	Negative	Positive	Negative	
Ain Abid	1 (0.4%)	3 (1.3%)	1 (2. 6%)	2 (5.1%)	33(1), 15(0), 37(0)
Ain Smara	4 (1.8%)	5 (2.2%)	1 (2. 6%)	0 (0%)	13(4)
Beni-Hamidane	8 (3.5%)	25 (11.0%)	4 (10.3%)	2 (5.1%)	1(1), 2(4), 3(1), 26(2), 4(0), 5(0)
Constantine	0 (0%)	8 (3.5%)	0 (0%)	2 (5.1%)	12(0), 48(0)
Didouche Mourad	0 (0%)	4 (1.7%)	0 (0%)	2 (5.1%)	22(0), 24(0)
El Khroub	9 (4%)	37 (16.4%)	4 (10.3%)	5 (12.9%)	45(3), 46(2), 47(3), 54(1), 31(0), 38(0), 44(0), 52(0), 53(0)
Hamma Bouziane	0 (0%)	6 (2.7%)	0 (0%)	2 (5.1%)	11(0),14(0)
Ibn Badis	0 (0%)	6 (2.7%)	0 (0%)	1 (2. 6%)	32(0)
Ibn Ziad	3 (1.3%)	33 (14.6%)	2 (5.1%)	2 (5.1%)	41(2), 49(1), 27(0), 40(0)
Messaoud Boudjeriou	0 (0%)	11 (4.9%)	0 (0%)	3 (7.7%)	10(0), 29(0), 30(0)
Ouled Rahmoune	2 (0.9%)	37 (16.4%)	1 (2. 6%)	2 (5.1%)	42(2), 43(0), 50(0)
ZighoudYoucef	1 (0.4%)	25 (11.0%)	1 (2. 6%)	2 (5.1%)	9(1), 23(0), 28(0)
Total	28 (12.4%)	198 (87.6%)	14 (35.9%)	25 (64.1%)	/

Table 2. Prevalence of seropositive ewes to *C. burnetii* according to their age and abortion characteristics

Age category (years)	Number (%) Positive ewes	Abortion history	Abortion in first pregnancy	Abortion at (Stage of gestation)		Repeated abortions	Number (%) Negative ewes
				2nd	3rd		
ys≤2	14 (50%)	7 (25%)	5 (17.85)	2 (7.14%)	5 (17.85)	3 (10.71%)	88 (44.44%)
2<ys≤3	12 (42.85%)	11 (39.28%)	4 (14.28)	6 (21.42%)	5 (17.85)	3 (10.71%)	95 (47.97%)
3<ys≤4	2 (7.14%)	2 (7.14%)	0 (0%)	2 (7.14%)	0 (0%)	0 (0%)	15 (7.57%)
Overall	28 (100%)	20 (71.42%)	9 (32.14%)	10 (35.71%)	10 (35.71%)	6 (21.42%)	198 (100%)

Table 3. Potential risk factors associated with *C. burnetii* seropositivity in ewes at individual level

Risk factors	χ^2	*p*-value χ^2	Adjusted OR (95% CI)
Origin of animals	14.29	0.001	/
Vaccination against enterotoxaemia	12.12	0.002	0.32 (0.16-0.61)
Foreigners get access to the farm	10.87	0.004	2.02 (1.01-4.01)
History of abortion	5.54	0.029	0.34 (0.15-0.79)
Shepherds visit other farms	6.31	0.021	0.34 (0.15-0.79)
Vaccination against pox	5.30	0.025	0.41 (0.18-0.92)
Disinfection frequency	7.98	0.046	/
Pest presence within the farm	9.55	0.049	/

DISCUSSION

In Algerian livestock, *C. burnetii* infection is still very poorly investigated and often goes unsuspected by veterinarians during abortion outbreaks. The current survey attempts to bring more insights on the dissemination of this disease in ewes and the associated risk factors at different municipalities of Constantine province. Thus, the seroprevalence recorded at individual level (12.4%) is approximately the same as the one reported by Khaled *et al.* (2016) (12.2%) in eight Algerian provinces (including Constantine). However, it is less than the ones described by Yahiaoui *et al.* (2013) and Rahal *et al.* (2012) in Ksar El Boukhari (Médéa province) (26.08% and 19% respectively). On the other hand, the surveyed herds of our study seem to be less infected (35.9%) than the ones analyzed in those previous studies (71.4%, 80% and 60% in that order).

Compared to surrounding countries, the animal level prevalence is very close to the one (11.8%) reported by Barkallah *et al.* (2018) in sheep of central-eastern Tunisia, but less than the 15.3% observed in Morocco by Benkirane *et al.* (2015). These authors have found respectively 20.21% and 54% of their flocks to be positive. In sub-Saharan neighbouring countries, the animal seroprevalence was found to be 21.5% in Mali and 2.55% in Niger (Sidibe *et al.*, 2013; Zecchini *et al.*, 2008).

In southern European countries where climate, terrain and traditional farming of sheep resemble to those in North Africa (including Algeria), the reported animal and flock seroprevalences were respectively 8.6-11.4% and 37.5% in Portugal (Anastácio *et al.* 2013; Cruz *et al.* 2018), 11.8% and 74% in Spain (Ruiz-Fons *et al.*, 2010), 18% and 73.6% in Italy (Villari *et al.*, 2018) and 8% in ewes in Greece (Filioussis *et al.*, 2017). In view of that, the seroprevalence at individual level is approximately comparable to the one recorded in our study (except for Greece and Italy); but in contrast, at flocks' level it appears to be much higher than ours.

Disparities in Q fever seroprevalence (between regions of close proximity and between adjacent countries) have already been described in Europe (Georgiev *et al.*, 2013) and within Mali (Sidibe *et al.*, 2013) for instance, and are related to differences in studies design, sampling methods and samples' size, analysing procedures and strongly to variations in risk factors associated to this infection which are still poorly understood (Vanderburg *et al.*, 2014).

Many studies had established a causative link of Coxiellosis and the geographical location (Asadi *et al.*, 2014; Keyvani Rad *et al.*, 2014). According to Barkallah *et al.* (2018), the likelihood to have a positive animal is higher in rural than in urban areas in relation to the undeveloped livestock farming practices and deficient hygiene. This is consistent with our findings and may explain in part the disparities we have recorded between the different municipalities.

In agreement with our findings, a potential association has been observed between Coxiellosis prevalence and abortions in ovine flocks in many parts of the world (Vanderburg *et al.*, 2014; Barkallah *et al.*, 2018; Ullah *et al.*, 2019), however, the fact that some of our Coxiella seronegative ewes had aborted may be led to other potential infectious or non-infectious abortifacient causes that affect sheep in our region and had not been investigated in this study. According to Palmer *et al.* (1983), abortion caused by *C. burnetii* occurs during late pregnancy and repeated abortions in the same goat and sheep are possible, which is confirmed throughout our results. Usually, sheep under a year old are less likely to experience gestation and parturition than older ones. This is why Coxiella-associated abortions in our study were more frequent in ewes of ≥2 years old. In this same context, and in contrast with our records, age of animals represents a predisposing factor to this infection, since ewes of 2 to 3 years old (Kennerman *et al.*, 2010) and those of ≥5 years old (Mahdavi-Roshan *et al.*, 2018) were described to be more infected in relation to a cumulative contact with the bacterium from their young age (Knobel *et al*., 2013).

Even though we couldn't establish any relation of infection to mixed bred animals (sheep with cattle and/or goats), other studies concur with the fact that in mixed flocks especially with goats, the risk of getting the infection is higher because these animals are able of aborting twice following the infection, and shedding *C. burnetii* for up to 2 gestations, while ewes abort habitually once and do not shed the organism in vaginal mucus at subsequent lambing (Hatchette *et al.*, 2003; Berri *et al.*, 2007).

In consistence with our findings, the introduction of new animals of unknown health status without quarantine would consequently facilitate the introduction and the dissemination of *C. burnetii*, especially in the case of shedding females (Porten *et al.*, 2006). Ovine flocks are known to be most deeply infected than other livestock, and discard the bacterium in feces, vaginal mucus, and milk, with ewes able to eliminate *C. burnetii* for up to 2 months (Rodolakis *et al.*, 2007). The excretion of the bacterium reaches its maximum during birth or abortion (Roest *et al.*, 2012), thus birth and abortion products, mainly placentas have a very high bacterial load.

Many studies as in ours' have shown that farm managing practices (Pests, foreign visitors, hygiene and disinfection) can influence the seroprevalence of on farm Coxiellosis.

Cantas *et al.* (2011) had identified poor hygiene as one of the most important on farm risk factors associated with *C. burnetii* abortions. It is well known that *C. burnetii* (Small Cell Variants which are highly infectious) are extremely stable under adverse environmental conditions and can remain infectious for many months (Scott and Williams, 1990). As spore stage, they can survive on wool of sheep at ambient temperature for 7-10 months and large quantities of *C. burnetii* can persist in soil one year after an outbreak (Kersh *et al.*, 2013). Thus, visiting other farms by the shepherds or free access to the farm for foreign visitors (as found in our survey) may represent a potential risk factor for the introduction and the dissemination of the bacterium, especially from foot traffic (Kersh *et al.*, 2013). Cardinale *et al.* (2014), suggest that farm personnel and visitors often act as mechanical carri-

ers/transmitters of the pathogen from infected flocks to uninfected ones. This is why the access of visitors to the farm as well the farmers/shepherds to other farms during parturition period must be avoided.

Pests presence on farm interfere positively with the dissemination of the disease on farms, since the potential role of these animals (rodents, birds, ticks) had been described as a risk factor of Coxiellosis occurrence (Gardon *et al.*, 2001; Angelakis and Raoult, 2010).

The link we have recorded between Coxiellosis prevalence and vaccinations against pox and enterotoxaemia in sheep could be explained by the lack of preventive measures during vaccination campaigns. In fact, veterinarians may participate to the dissemination of the bacterium from farm to farm, flock to flock and animal to animal via their contaminated equipment, clothes and mainly boots. Another possible route of transmission may be represented by sharing previously used and contaminated needles (Scott *et al.*, 2011) between animals during vaccination.

Many other risk factors (such as sheep breed, season, parity, ticks, raising system, flock size…) are potentially associated with seropositivity, but they were not significant in our study.

Finally, it is important to mention that some animals may remain seropositive for several years following an acute infection, while; some others may excrete the bacteria before the appearance of antibodies (Berri *et al.*, 2000). At the time of abortion, small ruminants that abort are frequently seropositive, although antibodies' titers can decrease over time (Berri *et al.*, 2001). Thus, the serological testing is of a subtle interpretation in the case of the Q fever, and the ELISA test should be coupled with a method of direct detection of the bacterium using molecular tools (PCR) which are more sensitive (Mori *et al.*, 2013).

CONCLUSION

In conclusion, the present study reveals that Coxiella infection is more frequent than previously considered. Many factors (mostly related to biosecurity, hygiene and disinfection) play an important role in the occurrence and dissemination of this infection among ovine flocks of our region, which prove that preventive veterinary measures are a key point in the control of Q fever. It appears also that sheep may be potential reservoirs of *C. burnetii* for other animals and humans infection. The dissemination risk of this bacterium is amplified through animals' movements. Sheep can move from the high plateaus to the Northern provinces and sometimes till the seaside provinces. In addition to that, there is an increased animal-human contact in animal operation sectors, in pastures and watering points, in live animal markets, but also through the food chain.

Nevertheless, there still many knowledge deficiencies in determining the true incidence of this economically heavy disease which motivates the need for future studies (using more accurate analyses and including large samples of several animal species from more provinces) to understand its epidemiology among the national livestock and the broader community especially in persons with special risk factors (contact with animals).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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