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Prevalence of *Coxiella burnetti* infection in wild and farmed ungulates

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Abstract

The aim of this study was to evaluate by serology and PCR analyses the prevalence of *C. burnetti* infection in ungulates in Spain. Sera were collected from red deer (*Cervus elaphus*; n=116), roe deer (*Capreolus capreolus*; n=39), fallow deer (*Dama dama*; n=13) and cattle (n=79). Sera were tested for anti-*C. burnetii* antibody detection by means of an immunofluorescence antibody assay (IFA) and *C. burnetii* DNA was amplified by PCR in samples from ungulates that had antibodies to phase II antigens. Twenty-nine, fifteen and thirty-nine percent of the red deer, roe deer and cattle had antibodies against *C. burnetii*, respectively. None of the fallow deer sera tested positive. Seroprevalence was statistically higher in farmed than in wild red deer and higher in northern than in southern populations, whereas an inverse pattern was observed for the roe deer. Most of the seropositive animals had only anti-*C. burnetii* phase II antibodies, thus showing the acute nature of infections in the sampled ungulates. These results show that *C. burnetii* circulates in wild ungulates in Spain and suggest that they can act as pathogen reservoirs for both domestic animals and humans.

Keywords: *Coxiella*; Q fever; tick; vector-borne diseases; wildlife

43 Introduction

44 Q fever is a worldwide zoonosis produced by the gram-negative bacteria, *Coxiella*
 45 *burnetii*. Multiple hosts can serve as reservoir of infection, including many wild and
 46 domestic mammals, birds and ticks (Willeberg et al., 1980; Marrie et al., 1986; Webster et
 47 al., 1995; Dunbar et al., 1998; Maurin and Raoult, 1999; Komiya et al., 2003). However,
 48 domestic ruminants represent the most frequent source of *C. burnetii* infection in humans
 49 (Maurin and Raoult, 1999).

50 Over 40 tick species are naturally infected with *C. burnetii*, including
 51 *Rhipicephalus*, *Haemaphysalis*, *Amblyomma*, *Dermacentor*, *Ixodes* and *Otobius* species.
 52 However, ticks are not considered essential in the natural cycle of *C. burnetii* in livestock
 53 because other sources of infection are more important in animals that live in close contact
 54 (Maurin and Raoult, 1999). In contrast, ticks may play a significant role in the transmission
 55 of coxiellosis among wild vertebrates (Marrie et al., 1986; Maurin and Raoult, 1999).

56 Despite efforts to eradicate coxiellosis from cattle, goat and sheep herds, the disease
 57 remains a serious risk for human and animal health in Spain (Tellez et al., 1988; Maurin
 58 and Raoult, 1999; Maltezou and Raoult, 2002; Bolaños et al., 2003; Marrie, 2004;
 59 Cardenosa et al., 2006; Oporto et al., 2006; Sanz et al., 2006). The disease seems to be
 60 more prevalent in the Basque and Navarra provinces in northern Spain than in the central
 61 and southern regions of the country (Tellez et al., 1988; Maurin and Raoult, 1999). Recent
 62 research efforts have been focused on the characterization of *C. burnetii* infection in
 63 domestic ruminants and humans (reviewed by Woldehiwet, 2004; Kazar, 2005). However,
 64 little is known about the prevalence of *C. burnetii* in wild ungulates (Enright et al., 1971;
 65 Ejercito et al., 1993; Marrie et al., 1993) and to our knowledge, *C. burnetii* infection has not
 66 been characterized in Spanish wild and farmed cervids. Wild and farmed cervid populations

are growing in Spain and in other countries due to their value as game animals, thus increasing the risk for disease transmission to humans and domestic animals (Gortázar et al., 2006; Vicente et al., 2005).

The objective of the study reported herein was to evaluate by serology and PCR analyses the prevalence of *C. burnetii* infection in wild and farmed cervids from two regions in northern and southern Spain.

Materials and Methods

Study sites

In southern Spain, we collected sera of Iberian red deer (wild and farmed), European roe deer and cattle in a 3,000 hectares fenced hunting estate (LO) located in the Natural Park of “Los Alcornocales” in the province of Cádiz, Andalucía. We also collected European roe deer and cattle sera from a neighbouring hunting estate (OJ). In northern Spain, Iberian red deer, European roe deer and fallow deer serum samples were collected in Asturias region. The number of samples through ruminant species and habitat are summarized in table 1.

Animals and sample preparation

Wild cervids from Asturias region were necropsied after being hunter harvested. Blood from shot wild cervids was collected directly from the heart or thoracic cavity into sterile tubes without anticoagulant. Wild cervids from southern Spain were life-captured by gamekeepers with fixed capture boxes and bled by cervical puncture. Cattle were bled during the tuberculosis control campaign. All samples were collected in 2004 and 2005. Blood from captured/farmed wild cervids and cattle from southern Spain was collected into sterile tubes with and without anticoagulant (EDTA) and maintained at 4°C until arrival at the laboratory. Plasma and serum were then separated after centrifugation and stored at -

20°C. DNA was extracted from sediments of serum samples using TriReagent (Sigma, St. Louis, MO, USA). Ticks were collected from cervids and cattle and classified as previously described (Ruiz-Fons et al., 2006).

C. burnetii serologic tests

Serum antibodies were determined for *C. burnetii* using an indirect immunofluorescence antibody (IFA) test (Fuller Laboratories, Fullerton, CA, USA) and following manufacturer's recommendations. The test for *C. burnetii* uses phase I and II antigens with a cut-off dilution of the test serum of 1:16 (Torina et al., 2007). The IFA test has been previously used to test deer sera for anti-*Coxiella burnetii* antibodies (Yasumoto et al., 1997).

C. burnetii *htpB* polymerase chain reaction (PCR) and sequence analysis

The *C. burnetii* *htpB* gene, coding for a 62 kDa antigen (Vodkin and Williams, 1988) was amplified by nested PCR as previously reported (To et al., 1996). To check the specificity of the PCR, amplified fragments were resin purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both strands by double-stranded dye-termination cycle sequencing (Secugen SL, Madrid, Spain). At least two independent clones were sequenced for each PCR analyzed.

Statistical analysis

A chi-square test was used to test for the distribution patterns of *C. burnetii* seroprevalence between sampling area (northern vs. southern), ungulate species and habitat (wild vs. farmed). The same statistical test was used to evaluate the differences in the anti-phase I and II antibodies seroprevalence in cattle from LO farm. Statistical analyses were performed using the SPSS v14.0 (SPSS Inc., Chicago, USA) package. The *p* value was set at 0.05.

Results

Evidence of *C. burnetii* infection was found in red deer in northern and southern Spain and in roe deer and cattle in southern Spain. Seroprevalence of *C. burnetii* in the sampled ruminants was 15.4% in roe deer, 5.6% in wild red deer, 40% in farmed red deer and 39% in cattle. None of the fallow deer analyzed showed evidence of contact with *C. burnetii*. Seroprevalence level through species, habitat and location is shown in table 1.

Hyalomma marginatum marginatum and *Hy. lusitanicum* were found parasitizing on red deer and cattle. Roe deer had infestations with *Hy. m. marginatum*. *Rhipicephalus bursa* and *R. pusillus* were collected from roe deer and cattle and red deer and roe deer, respectively. *R. (Boophilus) annulatus* was found on cattle only. *Ixodes ricinus* and *Haemaphysalis concinna* were found infesting red deer and roe deer in Asturias.

The observed seroprevalence to *C. burnetii* antigens was higher in the southern red deer population ($\chi^2=4.85$, $df=1$, $p<0.05$; Table 1) and it was higher in farmed than in wild animals ($\chi^2=9.05$, $df=1$, $p<0.01$; Table 1). Furthermore, the observed seroprevalence of *C. burnetii* infection in wild red deer was higher in northern than in southern populations (Table 1). However, the opposite result was obtained for wild roe deer with higher observed seroprevalence in the southern population ($\chi^2=8.27$, $df=1$, $p<0.01$; Table 1).

In the study reported herein, anti-phase I antibodies were found in three cattle only in the farm LO in southern Spain (Table 1), which statistically differed from the number of anti-phase II antibodies positive cattle from the same farm ($\chi^2=22.6$, $df=1$, $p<0.001$). Interestingly, the observed seroprevalence of *C. burnetii* in cattle was higher in farm LO than in farm OJ in Andalucía, southern Spain ($\chi^2=21.5$, $df=1$, $p<0.001$; Table 1).

C. burnetii DNA was amplified by PCR in samples from wild and farmed red deer and cattle that had antibodies to phase II antigens (Table 1). As expected, the sequence of

the amplicons corresponded to the *C. burnetii* *htpB* gene. Sequence analysis evidenced that all sequences were identical to the sequence reported for the Nine Mile phase I isolate (Genbank accession number M20482; Vodkin and Williams, 1988).

Discussion

This is to our knowledge the first report on *C. burnetii* infection in Spanish wild ungulates. As abovementioned, there is a need of research on pathogens circulating in wild ungulate populations due to its increasing demographic trend and especially due to their possible role as pathogen reservoirs for domestic animals and humans (see for example Ruiz-Fons et al., 2007).

The seroprevalence of *C. burnetii* found in wild roe (15.4%) and red deer (5.6%) populations in Spain is within the range reported for other wild cervids. Recent seroepidemiological studies in wild cervids have shown anti-*C. burnetii* antibody prevalence of 16.5% and 1.5% in moose (*Alces alces*) and white-tailed deer (*Odocoileus virginianus*) in Nova Scotia, USA, respectively (Marrie et al., 1993) and 69% and 56% in Hokkaido deer (*Cervus nippon yesoensis*) and Japanese deer (*Cervus nippon centralis*) in Hokkaido, Japan, respectively (Ejercito et al., 1993). We found no fallow deer to show evidence of contact with *C. burnetii*, but the number of samples tested was smaller than in other host species included in the study. Alternatively, this species may have innate resistance to infection.

Our results suggest that in farmed red deer, animal-to-animal contact may increase the risk of *C. burnetii* transmission. Additionally, differences in the observed prevalence of *C. burnetii* infection in wild cervid populations may depend upon living environments (Komiya et al., 2003), which are different between northern and southern Spain. Management widely differs between the areas sampled in northern and southern Spain.

While sampled wild cervids in southern Spain live in fenced estates, northern wild cervids live in natural conditions. Feeders for both red and roe deer are present in the sampled southern estates and cervids use to concentrate on them, thus increasing the risk of *C. burnetii* and other pathogens transmission between individuals (Acevedo et al., 2007).

Anti-*C. burnetii* phase II antibodies are generally associated with acute infections whereas in chronic infections, the antibody level to phase I is usually higher than that to phase II (Maurin and Raoult, 1999). Our results suggest that most of the animals analyzed had acute *C. burnetii* infections, which may correlate with heavy tick infestations in these animals. Tick species found are common on wild ungulates in Spain (de la Fuente et al., 2004; Ruiz-Fons et al., 2006) and have been suspected to play an important role as vectors of *C. burnetii* among wild vertebrates (Marrie et al., 1986; Maurin and Raoult, 1999). Nevertheless, in order to definitively determine the real role of wild ungulates as *C. burnetii* reservoirs for domestic animals and humans, an experimental approach is needed.

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Table 1. Results of IFA test to phase I and II *C. burnetii* antigens and *htpB* gene nested PCR in wild and domesticated cervids and cattle.

Host	Habitat	Location	N° of samples	Seropositive (% $\pm$ SE at 95% CI)		Nested PCR positive/total tested (%) ^a
				Phase I	Phase II	
Red deer	Wild	Northern Spain	21	0 (0 $\pm$ 0)	2 (9.5 $\pm$ 12.9)	1/2 (50)
	Wild	Southern Spain	15	0 (0 $\pm$ 0)	0 (0 $\pm$ 0)	None tested
	Farm LO	Southern Spain	80	0 (0 $\pm$ 0)	32 (40 $\pm$ 10.8)	4/32 (12)
<i>Subtotal red deer</i>			<i>116</i>	<i>0 (0 $\pm$ 0)</i>	<i>34 (29.3 $\pm$ 8.2)</i>	<i>5/34 (14.7)</i>
Roe deer	Wild	Northern Spain	21	0 (0 $\pm$ 0)	0 (0 $\pm$ 0)	None tested
	Wild	Southern Spain	18	0 (0 $\pm$ 0)	6 (33 $\pm$ 22.4)	0/6 (0)
<i>Subtotal roe deer</i>			<i>39</i>	<i>0 (0 $\pm$ 0)</i>	<i>6 (15.4 $\pm$ 11.6)</i>	<i>0/6 (0)</i>
Fallow deer	Wild	Northern Spain	13	0 (0 $\pm$ 0)	0 (0 $\pm$ 0)	None tested
Cattle	Farm LO	Southern Spain	49	3 (6 $\pm$ 4.9)	26 (53 $\pm$ 10.6)	3/26 (12) ^b
	Farm OJ	Southern Spain	30	0 (0 $\pm$ 0)	2 (7 $\pm$ 5.1)	0/2 (0)
<i>Subtotal cattle</i>			<i>79</i>	<i>3 (3.8 $\pm$ 4.3)</i>	<i>28 (35.4 $\pm$ 10.6)</i>	<i>3/28 (10.7)</i>

^a Only samples from seropositive animals were tested by *C. burnetii htpB* gene nested PCR.

^b Samples from animals seropositive for phase I *C. burnetii* antibodies were negative for the *htpB* gene nested PCR assay.