Evaluation of the Presence of *Rickettsia slovaca* Infection in Domestic Ruminants in Catalonia, Northeastern Spain

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Abstract

Rickettsia slovaca is the etiological agent of the human disease tick-borne lymphadenopathy (TIBOLA) transmitted by *Dermacentor* spp. ticks. In our area, *Dermacentor marginatus* is the most important tick vector; adult ticks feed on mammals, especially ungulates such as wild boars and domestic ruminants. The epidemiology of tick-transmitted diseases describes a wild cycle and a domestic cycle and both are connected by ticks. To identify the role of domestic ruminants in the transmission and maintenance of *R. slovaca* infection, blood samples from sheep ($n=95$), goats ($n=91$), and bullfighting cattle ($n=100$) were collected during a herd health program, and livestock grazing was selected to ensure tick contact. Samples were analyzed by serology using an indirect immunofluorescent assay (IFA) and molecular techniques (real-time PCR). Seroprevalence was 15.7% in sheep, 20.8% in goats, and 65.0% in bullfighting cattle. On the basis of molecular methods, *R. slovaca* infection was demonstrated in a goat blood sample with an antibody titer of 1:160. This is the first time that *R. slovaca* has been identified in a goat blood sample. These results suggest that domestic ruminants are exposed to *R. slovaca* infection and, because the domestic cycle is close to the human environment, this could increase the risk of transmitting the pathogen to human beings.

Key Words: *R. slovaca*—Sheep—Goat—Bullfighting cattle.

Introduction

RICKETTSIA SLOVACA, A SPOTTED FEVER group rickettsia (SFG), is considered the etiological agent of an emerging tick-transmitted disease named tick-borne lymphadenopathy (TIBOLA), a relatively mild rickettsiosis. *R. slovaca* was first isolated in *Dermacentor marginatus* ticks in Slovakia, but the first documented case of infection was reported in 1997 in a patient in France (Raoult et al. 1997). Since then, several cases have been reported worldwide. The increase in outdoor activities has resulted in an increased contact with ticks and an increased risk of tick-transmitted diseases (Parola and Raoult 2001).

In Catalonia, northeastern Spain, the seroprevalence of *R. slovaca* in human beings has ranged from 3.7% to 5.5% (Antón et al. 2008), but higher seroprevalence was observed in tick-bitten populations (16.9%) (Lledó et al. 2006). In fact, group age (Antón et al. 2008, Porta et al. 2008) and occupation have had a significant influence on the prevalence recorded (Lledó et al. 2006).

The epidemiology of tick-transmitted diseases describes a wild cycle and a domestic cycle, and both are connected by ticks. The eco-epidemiology of *R. slovaca* is still not completely clarified. In our area, *D. marginatus* constitutes the most important tick vector. Adult *D. marginatus* feeds on mammals, especially ungulates, such as wild boars and domestic ungulates (small ruminants and cattle). Several surveys from different European countries reported prevalence of infection in *D. marginatus* that varied widely and ranged from 0.7% to 13.3% in Germany (Pluta et al. 2009, Silaghi et al. 2011), 32.1% in Italy (Selmi et al. 2009), and 41.5% in Portugal (Milhano et al. 2010). In Spain, prevalence was 17.7% in northeastern Spain (Ortuño et al. 2006) and 24.7% in southeastern Spain (Márquez 2008). However, studies about the eventual role of *D. marginatus* hosts in the epidemiology of *R. slovaca* infection are scarce. Wild boars are exposed to *R. slovaca* infection and may play a role as carriers of rickettsiae-infected ticks (Ortuño et al. 2007), indicating that *R. slovaca* is well established in the wild cycle of *D. marginatus*. But, to our knowledge, there are no reports about the role of domestic ruminants as hosts of the

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tick vector in the maintenance and transmission of *R. slovaca* in the domestic cycle, where contact to human beings is closer.

The aim of this study was to detect *R. slovaca* infection in domestic ruminants, such as goats, sheep, and bullfighting cattle, in our area to clarify which role these hosts could play in the transmission and maintenance of *R. slovaca* in the domestic cycle.

Materials and Methods

Blood samples were collected from sheep ($n=95$), goats ($n=91$), and bullfighting cattle ($n=100$) in the spring of 2009 during a herd health program in Catalonia, northeastern Spain, and were examined for the presence of *R. slovaca* by molecular techniques and serology. Only grazing livestock were selected because this condition ensures a close contact to the environment and increases the risk to tick contact.

DNA extraction

DNA was extracted from 200 μ L of EDTA-blood using the MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's recommendations. The DNA concentration and purity were determined using a NanoDrop spectrophotometer ND1000 (NanoDrop Technologies, Wilmington, DE).

Molecular detection

The detection of *Rickettsia* spp. was performed by real-time PCR. Specific primers for the amplification of the outer membrane protein A gene (*ompA*) were designed in our laboratory using the *ompA* sequences published in GenBank for *R. slovaca*, *R. conorii*, and *R. massiliae*. Conserved regions between species in the *ompA* gene were detected with the multiple sequence alignment algorithm ClustalW (MegAlign, DNASTar v5, Madison, WI). Conserved regions between species were subsequently used to design a primer set with PrimerSelect (DNASTar v5.0). Primers were: 5'-GGG CAT TTA CTT ACG GTG GTGAT (Rr190.314, forward) and 5'-CTT TGA CGG AGC TGC AGA TTG TAT (Rr190.630, reverse). Primers were synthesized by Stab Vida (Setúbal, Portugal). SYBR Green quantitative PCR (qPCR) was performed using the SensiMix dU DNA Kit (Quantace, London, UK). The final reaction mixtures were composed of 1 $\times$ Sensimix, 1 $\times$ SYBR Green, 1 U uracil-DNA glycosylase, 2 mM MgCl₂, 50–100 ng of DNA, and 200 nM of each primer. The amplification conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 50°C for 30 s, 72°C for 30 s, and 78°C for 35 s (acquisition of the SYBR Green fluorescence). After amplification, a melting step was performed. PCRs were performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). This PCR amplified a fragment of 317 bp.

To determine the presence of PCR inhibitors, an additional real-time PCR was performed with primers targeting the β -globin gene (Saiki et al. 1985).

DNA sequencing

Identity of the PCR products was confirmed by direct sequencing using the ABI BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) and electrophoresed in a 3130 Genetic Analyzer (Applied Biosystems). Sequence data were analyzed with SeqMan (DNASTar v5, Madison, WI).

Sequences were compared with available databases using BLAST (www.ncbi.nih.gov/blast/).

Serology

Serum samples were stored at -80°C until use. Antibodies to *R. slovaca* were detected by using the indirect immunofluorescence antibody test (IFA). *R. slovaca* antigen was kindly provided by Dr. Lledó (Pharmacy Faculty, Universidad de Alcalá de Henares, Madrid). To amplify the antigen production, 0.5 mL of *R. slovaca* antigen was mixed with 0.5 mL of minimum essential medium (MEM) and the suspension inoculated into a Vero cell culture. The cells were incubated in MEM with fetal calf serum (FCS) and L-glutamine at 37°C under a 5% CO₂ atmosphere. The antigen was fixed on 10-well microscope slides (BioMerieux, Marcy l'Etoile, France), previously cleaned with an ethanol-acetone mixture (equal volume). A drop of antigen was placed onto each well slide with a pen nib and allowed to dry at room temperature for 30 min. The slides were fixed in acetone for 10 min at room temperature.

Briefly, sera were initially diluted at 1:40 in phosphate-buffered saline with 1% bovine serum albumin (PBS-BSA) and incubated in a humidified chamber at 37°C for 30 min. The slides were removed from the humidified chamber and washed twice in PBS-Tween for 10 min. The slides were air dried and overlaid with fluorescein isothiocyanate (FITC) anti-goat immunoglobulin G (anti-goat IgG; whole-molecule FITC Conjugate[®], Sigma-Aldrich, St. Louis, MO) for goat samples, anti-sheep IgG (anti-sheep IgG, whole-molecule FITC Conjugate[®], Sigma-Aldrich, St. Louis, MO) for sheep samples, and anti-cow IgG (anti-bovine IgG; whole-molecule FITC Conjugate[®], Sigma-Aldrich, St. Louis, MO) for bullfighting cow samples at the dilutions recommended by the manufacturer. The slides were incubated and washed as described above. Once dried, slides were mounted with buffered glycerol and examined with fluorescence microscopy (400 $\times$). Sera giving uniform fluorescence of rickettsiae at antibody titers $\geq 1:40$ were considered positive and were then titrated in a 2-fold dilution until fluorescence was lost.

Statistics

For comparison of prevalences, the chi-squared test was performed by using the Statcalc, Epi-Info v.6 program computer package. Differences were considered statistically significant when $p < 0.05$.

Results

Seropositivity was detected in 15 sheep (15.7%), 19 goats (20.8%), and 65 bullfighting cattle (65.0%). Two sheep, 3 goats, and 4 bullfighting cattle showed antibody titers $\geq 1:320$. Table 1 shows the distribution of antibody titers in each host. Statistical differences were observed when comparing seroprevalence detected in bullfighting cattle and seroprevalence detected in goats and in sheep ($p < 0.05$).

Rickettsia was detected by real-time PCR in 1 goat blood sample with an antibody titer of 1:160. The cycle threshold (Ct) for this sample was 29.9 and the melting temperature (T_m) was 79.5°C. Sanger cycle sequencing and BLAST analysis identified it as *R. slovaca*. The GenBank accession number for this DNA fragment is JX128267. All PCR reactions performed

TABLE 1. DISTRIBUTION OF ANTIBODY TITERS IN EACH HOST

	1:40	1:80	1:160	1:320	1:640
Sheep	7	4	1	1	1
Goat	5	5	6	1	2
Bullfighting cattle	38	8	15	4	—

on the DNA extracted from EDTA-blood samples were positive for β -globin, indicating that no PCR inhibitors were present.

Discussion

Seropositivity detected in our study showed circulation of *R. slovaca* infection in the domestic cycle, suggesting that domestic ruminants are exposed to *R. slovaca* infection. It is interesting to note that the seroprevalence of *R. slovaca* in the bullfighting cattle was higher than in sheep and goats. In fact, seroprevalence in bullfighting cattle was similar to that obtained by our group in a previous study in wild boars (52.1%) (Ortuño et al. 2007). It could be suggested that habitat may be an important factor to take into account, as sheep and goats live on grazing land that is not a proper habitat for *D. marginatus*. In that sense, in a study reporting the abundance and distribution of the ticks of grazing sheep, *D. marginatus* was observed in only 15.0% of flocks, although it was widely distributed in the surveyed region (Estrada-Peña et al. 2004). Nevertheless, bullfighting cattle and wild boar share the same areas in the forest. Wild boars are considered a tick-trophic support for *D. marginatus* and an infected-tick carrier.

An earlier report showed the prevalence of *R. slovaca* infection in *D. marginatus* ticks captured from wild boars to be 30.5% (Ortuño et al. 2007). Although serology is considered the easiest method for the detection of SFG rickettsiae, the interpretation of serological data is difficult because of the cross-reactivities among SFG when using IFA assays (Brouqui et al. 2007). Moreover, the possibility that seropositivity may be due to a coinfection should not be ruled out. Ruminants constitute important hosts of *Rhipicephalus* species ticks such as *R. turanicus*, *R. bursa*, or *R. pusillus*, and all of them could act as vectors of SFG rickettsiae (Márquez 2008).

To date, nothing is known about the serological response to SFG rickettsiae infection in ruminants. In suspected cases of Mediterranean spotted fever in human beings, IgG titers of 1:128 or above are considered to be indicative of infection by *R. conorii*, whereas IgG titers of 1:64 or more are considered to be indicative of infection by other rickettsia species in other rickettsioses (Brouqui et al. 2007). Applying these standards suggested for humans, seroprevalence to *R. slovaca* infection in ruminants could be lower.

Furthermore, serological responses in patients infected with *R. slovaca* appear to be slower and also delayed (Porta et al. 2008). This fact could explain the antibody titer (1:160) detected in the goat sample where *R. slovaca* could be isolated. This is the first time that *R. slovaca* has been demonstrated from an animal blood sample. This finding could suggest that goats may develop rickettsiemia and, hypothetically, could be capable of transmitting and amplifying the infection to other tick vectors.

On the other hand, no animal sampled showed clinical symptoms. Although *R. slovaca* is recognized worldwide as one of the most important tick-borne rickettsioses in humans, the relevance of infection in animals seems to be minimal or insignificant.

In conclusion, this is the first time that *R. slovaca* has been demonstrated in a goat blood sample. Our results suggest the circulation of *R. slovaca* in the domestic cycle. This is important because the domestic cycle is closer to human environments than the wild cycle, thus increasing the risk of transmitting the infection to humans. Further studies will be necessary to define the role of domestic ruminants in the maintenance *R. slovaca* infection.

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Author Disclosure Statement

No competing financial interests exist.

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