Applied and Environmental Microbiology, Oct. 2007, p. 6166–6171 Vol. 73, No. 19
0099-2240/07/$08.00+0 doi:10.1128/AEM.00590-07

Tick-Borne Zoonotic Bacteria in Wild and Domestic Small Mammals in Northern Spain▽

J. F. Barandika,[1] A. Hurtado,[1] C. García-Esteban,[2]† H. Gil,[2] R. Escudero,[2] M. Barral,[1] I. Jado,[2] R. A. Juste,[1] P. Anda,[2] and A. L. García-Pérez[1]*

NEIKER, Instituto Vasco de Investigación y Desarrollo Agrario, Department of Animal Health and Production, 48160 Derio, Bizkaia, Spain,[1] and Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid, Spain[2]

Received 14 March 2007/Accepted 26 July 2007

The prevalence and diversity of tick-borne zoonotic bacteria (*Borrelia* spp., *Anaplasma phagocytophilum*, *Coxiella burnetii*, and spotted fever group rickettsiae) infecting 253 small mammals captured in the Basque Country (Spain) were assessed using PCR and reverse line blot hybridization. Trapping sites were selected around sheep farms (study 1, 2000 to 2002) and recreational parks (study 2, 2003 to 2005). The majority of the studied mammals (162) were wood mice (*Apodemus sylvaticus*), but six other different species were also analyzed: yellow-necked mice (*Apodemus flavicollis*), shrews (*Crocidura russula* and *Sorex coronatus*), bank voles (*Clethrionomys glareolus*), domestic mice (*Mus domesticus*), and moles (*Talpa europaea*). The results showed an infection rate ranging from 10.7% to 68.8%, depending on the small mammal species. One *C. russula* shrew and one *A. sylvaticus* mouse gave positive reactions for *A. phagocytophilum*, and *C. burnetii* was detected in two domestic mice and one *A. sylvaticus* mouse in a farm. The DNA of *Borrelia* spp. was detected in 67 animals (26.5%), most of them presenting positive hybridization with the probe for *Borrelia* sp. strain R57, the new *Borrelia* species previously detected in small mammals in our region. Furthermore, a second PCR and reverse line blot hybridization specific for *B. burgdorferi* sensu lato revealed the presence of *Borrelia afzelii* in 6.3% of *C. glareolus* voles and 14.3% of *S. coronatus* shrews. All small mammals were negative for spotted fever group rickettsiae. These results highlight the relevance of small mammals as reservoirs of some zoonotic bacteria.

Ticks are important vectors of various pathogenic bacteria, protozoa, and viruses that cause disease in humans and animals worldwide. Some of these agents, such as *Coxiella burnetii*, are now recognized as important emerging vector-borne pathogens as well as agents of bioterrorism (3). *Ehrlichia* species, rickettsiae, and some *Borrelia* species have been reported across the world and have also been associated with disease in animals and humans (33, 35).

A wide range of mammalian reservoir hosts, including rodents, are involved in the natural cycle of various bacterial diseases. Different species of small mammals, mainly mice (*Apodemus* spp.) and voles (*Microtus* spp. and *Clethrionomys glareolus*), are recognized vertebrate reservoirs of tick-borne bacterial zoonoses such as Lyme disease (borreliosis) and human granulocytic ehrlichiosis, among others. In Europe, several rodent species seem to be the natural reservoirs of *Borrelia burgdorferi* sensu lato (35), *Anaplasma phagocytophilum* (7, 25), and *C. burnetii* (44), but there are few reports on the role of such species as possible reservoirs of tick-borne zoonotic bacteria in Spain. The role of small mammals in the biological cycle of *B. burgdorferi* sensu lato was recently investigated in areas of northern Spain where Lyme disease is endemic (14), and a low prevalence of infection was found (0.5%), but a new *Borrelia* sp. (*Borrelia* sp. strain R57), whose clinical and pathogenic importance remains unknown, was widely distributed among small mammals (12.5%). A limited number of small mammals from the same area was also analyzed for the presence of DNA of *A. phagocytophilum*, but all the specimens were negative (31). All these results were explained by the low number of *Ixodes* nymphs parasitizing small mammals in this area, with a 1:450 ratio of infestation of nymphs to that of larvae (14). The study area is particularly relevant due to the endemicity of Q fever pneumonia (29) and its proximity to an area where a new species of *Rickettsia* causing human disease, *Rickettsia monacensis*, has recently been identified in a patient (20a). However, no data are available regarding the presence of *C. burnetii* or *Rickettsia* spp. in small mammals in Spain. All these data create an interest in the search for potential reservoirs of such organisms.

PCR-based methods have become widely used as rapid and effective tools for the detection and identification of tick-borne pathogens in ticks and animal reservoirs. Increased sensitivity and specificity can be achieved by combining PCR with a specific hybridization by means of reverse line blot (RLB) hybridization, a macroarray that is able to identify mixed infections (39, 40). This study was undertaken to investigate the prevalence of tick-borne pathogens in domestic and wild small mammals in northern Spain. The main aim of the study was to determine the risk of disease in areas of potential contact between humans and small mammals carrying pathogens. In recreational areas, large numbers of human beings might come in contact with ticks and tick-borne pathogens from wildlife. Sheep farms, on the other hand, were selected since sheep are the most abundant livestock species in the area. Peridomestic small mammals were selected to investigate the role of these

* Corresponding author. Mailing address: NEIKER, Instituto Vasco de Investigación y Desarrollo Agrario, 48160 Derio (Bizkaia), Spain. Phone: 34 94 403 4312. Fax: 34 94 403 4310. E-mail: agarcia@neiker.net.

† Present address: Hospital Universitario de Getafe, 28905 Getafe (Madrid), Spain.

▽ Published ahead of print on 10 August 2007.

TABLE 1. Oligonucleotide sequences of primers and probes used in PCR and RLB hybridization for detection and identification of different pathogens

Target species	Target gene	Primer/probe name	Primer/probe sequence (5′ to 3′)	Primer/probe concn (μM)	Annealing temp (°C)	Reference
Primers						
C. burnetii	*htpAB*	Trans 1	TAT GTA TCC ACC GTA GCC AGT C	0.2	63	45
		Trans 2	biotin-CCC AAC AAC ACC TCC TTA TTC			
A. phagocytophilum	*msp2*	Msp2–3F	CCA GCG TTT AGC AAG ATA AGA G	0.4		46
		Msp2–3R	biotin-GCC CAG TAA CAA CAT CAT AAG C			
SFG rickettsiae	*ompA*	Rr190.70p	ATG GCG AAT ATT TCT CCA AAA	0.4	46	36
		Rr190.602n	biotin-AGT GCA GCA TTC GCT CCC CCT			
Borrelia spp.	16S rRNA	BORF	CGC TGG CAG TGC GTC TTA A	0.4		14
		16S	biotin-GCG GCT GCT GGC ACG TAA TTA GC			
B. burgdorferi sensu lato	5S-23S rRNA	23SN2	ACC ATA GAC TCT TAT TAC TTT GAC CA	0.2	57	39
		5SCB	biotin-GAG AGT AGG TTA TTG CCA GGG			
Probes						
C. burnetii	*htpAB*	*C. burnetii*	amino-GCA AGA ATA CGG ACT CAC GA	2		This study
A. phagocytophilum	*msp2*	*A. phagocytophilum*	amino-GGT TAC GAG CGC TTC AAG ACC	2		This study
Rickettsia spp.	*ompA*	OmpA-All	amino-GGC AAA AGC TTA ACT TTA AA	2		This study
Borrelia spp.	16S rRNA	*Borrelia* sp.	amino-GAG GAA TAA GCT TTG TAG GAA ATG ACA	2		14
Borrelia sp. strain R57	16S rRNA	*Borrelia R 57*	amino-AGT CAT TAA AGA TGT TTA ATG	16		14
B. burgdorferi sensu lato	5S-23S rRNA	*Bb* SL	amino-CTT TGA CCA TAT TTT TAT CTT CCA	0.3		39
B. burgdorferi sensu stricto	5S-23S rRNA	*Bb* SS	amino-AAC ACC AAT ATT TAA AAA ACA TAA	0.3		39
B. afzelii	5S-23S rRNA	*Bb* AF	amino-AAC ATT TAA AAA ATA AAT TCA AGG	0.3		39
B. garinii	5S-23S rRNA	*Bb* GA	amino-AAC ATG AAC ATC TAA AAA CAT AAA	0.3		39
B. valaisiana	5S-23S rRNA	*Bb* VA	amino-CAT TAA AAA AAT ATA AAA AAT AAA TTT AAG G	0.3		39
B. lusitaniae	16S rRNA	*Bb* LU	amino-TCT ATT TTA TTT TTT ATA TTT TTT T	0.9		13

species as reservoirs of some tick-borne bacteria affecting sheep (*C. burnetii* and *A. phagocytophilum*) that might put at risk professionals living and working around farms. Rapid screening of a selection of bacteria infecting small-mammal tissues was carried out using multiplex PCR, followed by RLB hybridization using genus-specific or species-specific probes. Using this approach, we report herein the infection rates in small mammals for *A. phagocytophilum*, *C. burnetii*, spotted fever group (SFG) rickettsiae, *B. burgdorferi* sensu lato, and *Borrelia* sp. strain R57, with the purpose of providing an assessment of the role of small mammals as reservoir hosts for tick-borne bacteria.

MATERIALS AND METHODS

Study area and small-mammal sampling. The Basque Country is a 7,200-km^2 region located in Atlantic northern Spain where ticks are abundant (4), there is a maritime climate with mild winters, and annual rainfall ranges from 600 to 1,500 mm. Livestock, raised at pasture several months a year, is abundant as well as wildlife, which consists mainly of rodents, foxes, badgers, wild boar, roe deer, and red deer.

Small mammals were captured between years 2000 and 2005 in two consecutive studies carried out at 15 different sites in the Basque Country. In the first study (study 1), carried out between April 2000 and November 2002, domestic and wild rodents were sampled in areas surrounding five sheep farms and in forested areas nearby. Study 2 started in September 2003 and finished in May 2005, and small mammals were captured in 10 recreational areas where, as previously described (4), the tick population was abundant.

After the authority's permission was obtained, Sherman traps (7.6 cm by 8.9 cm by 22.9 cm; Tallahassee, FL) and INRA traps (5 cm by 5 cm by 15 cm; BTS Mechanique, Besançon, France) were used for the live capture of rodents. Captures were carried out throughout the year, but mainly in the spring and autumn months. In study 1, 120 traps (20 Sherman traps placed inside sheep farms and 100 INRA traps placed outdoors) were set for two consecutive nights, and 100 INRA traps were set overnight in study 2. In study 1, moles were also captured using 25 pincer traps (Michel Touchard et Fils, Grainville Langannerie, France) per sampling. To compare the abundances of these animals between studies, the small-mammal abundance index (SAI) was calculated as follows: $SAI = (SC \times 100)/(T \times N)$, where SC is the number of small mammals captured, T is the number of traps, and N is the number of nights.

Processing of small mammals. Trapped animals were immediately transported to the laboratory and examined for attached ticks and other ectoparasites. Live captured rodents were anesthetized with ether and with ketamine hydrochloride (Imalgene 500; Merial, France) at a dose of 10 mg/kg of body weight intramuscularly and euthanized in a CO_2 chamber. At necropsy, samples from different tissues were collected (ear, urinary bladder, spleen, liver, kidney, lung, and brain) and stored at −80°C. Small mammals were classified by external morphological data and skull features (1, 6, 32). Collected ticks were identified using taxonomic keys (15, 26).

DNA extraction and multiplex PCR. DNA was extracted from pools of tissues and ear samples by using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany), with a previous treatment with proteinase K for 3 h. A negative control was included for every 10 samples. The DNA concentration was determined for each sample with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE).

DNA was subjected to two multiplex PCR amplifications, one for the detection of *C. burnetii* and *A. phagocytophilum* and a second one for the detection of SFG rickettsiae and *Borrelia* spp. The oligonucleotide sequences of the primers, the gene targets, the concentration of each primer, and the annealing temperature of the PCR are shown in Table 1. Extracted DNA (100 to 200 ng) was used in each PCR.

One amplicon from each of the genes targeted in this study was routinely cloned, sequenced, and used as a positive control. Briefly, the amplified products were purified with a GFX PCR kit (Amersham Biosciences, Uppsala, Sweden), cloned into a pCR4-TOPO vector, and introduced into *Escherichia coli* according to the manufacturer's instructions (TOPO TA cloning kit for sequencing; Invitrogen, CA). Recombinant plasmid DNA was purified using a FlexiPrep kit (Amersham Biosciences) and subjected to automatic dye terminator cycle sequencing. The different plasmids were used to calculate the absolute detection limit of the technique. The concentration of each plasmid was calculated spectrophotometrically, and the plasmids were serially diluted in Tris-EDTA buffer to reach concentrations ranging from 10^8 to 1 copies per microliter.

The prevention of cross-contamination and false-positive results was managed by using plugged tips, setting PCRs in a room separate from that used for DNA extraction, and including a negative (water) control in each run.

RLB hybridization. To increase the detection limit of the PCR assay, PCR amplicons were hybridized to DNA probes specific for *C. burnetii*, *A. phagocytophilum*, *Borrelia* sp. strain R57, SFG rickettsiae, and *Borrelia* spp. by RLB hybridization. The probes, synthesized by MWG Biotech AG (Germany) with a C_6 amino linker, were as listed in Table 1. The preparation of RLB membranes and hybridization were carried out as previously described by Gubbels et al. (17) with the following adaptations: the complete amplification reaction mixture (25

μl) was loaded onto the blotter after dilution with 2× SSPE-0.1% sodium dodecyl sulfate (1× SSPE is 0.18 NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) to a total volume of 160 μl, incubation was carried out at 48°C for 60 min, and washing steps were performed at 40°C. After hybridization, PCR products were stripped from the membrane as previously described by Gubbels et al. (17) and the membrane was rinsed and stored in 20 mM EDTA (pH 8.0) at 4°C until the next hybridization and reused a maximum of eight times.

To exclude false-positive results, negative controls included during DNA extraction and PCR amplification were also subjected to RLB hybridization. The specificities of the probes were tested against those of the previously constructed plasmids, which were also used as positive controls in each assay.

Identification of species of *Borrelia*. Samples positive for *Borrelia* spp. by multiplex PCR (16S rRNA gene) and RLB hybridization were analyzed using a PCR specific for *B. burgdorferi* sensu lato, targeting the 5S-23S rRNA intergenic spacer, followed by RLB hybridization with specific probes described previously (13, 14, 39, 40) for *B. burgdorferi* sensu lato, *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. valaisiana*, and *B. lusitaniae* (Table 1). RLB hybridization was performed as previously described (14).

Statistical analysis. The prevalence of each bacterial species was analyzed according to independent variables, such as host species, study (study 1 and study 2), and date of sampling (season and year), by chi-square or Fisher's exact test by using the SAS statistical package (version 8.0; SAS Institute Inc., Cary, NC). Significance was set at a P value of <0.05. Different logistic regression models including the same variables were performed on the data for the only agent (*Borrelia* sp. strain R57) that had a large enough set of positive results, and the model that better fitted the rates of infections was used.

RESULTS

Small mammals captured and tick infestation. A total of 6,580 trap nights were used over the two studies (3,680 in study 1 and 2,900 in study 2), and 334 small mammals belonging to seven species were trapped (186 in study 1 and 148 in study 2), with a global SAI of 5.1 (5.05 for study 1 and 5.10 for study 2). During study 1, 450 pincer traps were also placed and 30 moles (*Talpa* spp.) were captured. *A. sylvaticus* was the most frequently found species (69.8%) and the most ubiquitous in both studies.

One hundred twenty-four of the 334 animals captured harbored at least one tick, with a maximum of 202 ticks observed in one *A. sylvaticus* mouse. The total amount of collected ticks was 1,961, accounting for 1,934 larvae (98.6%), 26 nymphs (1.3%), and one adult (an *Ixodes acuminatus* female). The percentage of small mammals parasitized and the number of ticks collected varied between studies, and the individual tick infestation followed a Poisson distribution. Whereas in study 1 the percentage of animals infested with ticks was low (9.1%), in study 2, 1,883 larvae were collected from 72.3% of the captured animals. Very few nymphs were found over the study periods, and all of them were collected in study 2 and consisted of 26 nymphs that were detached from 10 *A. sylvaticus* mice and one *C. glareolus* vole, giving a 1:72 average ratio of infestation of nymphs to that of larvae.

Ixodes ricinus was the most abundant tick species found, corresponding to 89.3% of larvae (100% in study 1 and 89.1% in study 2) and 96.2% of nymphs (all from study 2) collected. Three other species (*Ixodes trianguliceps*, *Rhipicephalus turanicus*, and *Haemaphysalis concinna*) were sporadically collected. *I. ricinus* larval infestation varied strongly among captured small mammal species. *A. sylvaticus* was the most parasitized species with 7.3 larvae/animal, followed by *C. glareolus* with 1.1 larvae/animal. Other small mammal species harbored very few larvae, i.e., *Apodemus flavicollis* with 0.7, *Sorex coronatus* with 0.5, and *Crocidura russula* with 0.2, whereas *Mus domesticus* and *Talpa europaea* had none.

Tick-borne bacterial infection in small mammals. All the nucleotide probes designed for hybridization assays gave positive results with their corresponding positive controls and did not show any cross-reaction. The sensitivity of the hybridization assay was assessed by RLB hybridization processing serially diluted PCR products and was found to be 1 or 2 orders of magnitude higher than that calculated for the multiplex PCR only. The absolute limit of detection for the combined PCR and subsequent RLB hybridization procedures using gene clones as templates was between 6 and 60 gene molecules for the different multiplex PCR amplifications.

Pools of tissues from 253 animals (127 from study 1 and 126 from study 2), belonging to *A. sylvaticus* (162; 64%) and six other species, were subjected to the two multiplex PCRs described above and RLB hybridization. The prevalences of infection were different between animal species (Table 2). The most frequently infected species was *C. glareolus*, with 68.8% of the studied specimens (11/16) harboring DNA from some of the bacteria investigated, followed by *A. sylvaticus* (33.3%), *C. russula* (16.7%), *S. coronatus* (14.3%), and *M. domesticus* (10.7%). All the moles and *A. flavicollis* were negative, though only three specimens of the latter were examined. The prevalences were similar between studies (Table 2), with 26.0% of the analyzed animals being positive in study 1 and 30.1% in study 2.

Borrelia sp. DNA was detected in 67 (26.5%) animals, and most of these positive animals also hybridized with the specific probe for *Borrelia* sp. strain R57 (62/67). A total of 37.5% of small mammals trapped in spring were positive, whereas the prevalences among summer, autumn, and winter captures were 20.0, 15.0, and 21.1%, respectively. Statistical differences were observed only between spring and autumn prevalences ($P < 0.05$). Infection rates varied significantly among species ($P < 0.0001$), from 3.6% in *M. domesticus* to 68.7% in *C. glareolus*, while none of the *T. europaea*, *A. flavicollis*, and *C. russula* strains analyzed harbored DNA of *Borrelia* spp. The best logistic regression model included study, host species, season, and *Ixodes* larval number and showed that *C. glareolus* had an odds ratio to be infected that was 5.62 (95% confidence interval [CI], 1.63 to 19.33) for *A. sylvaticus*, 29.22 (95% CI, 5.43 to 157.10) for *M. domesticus*, and 16.95 (95% CI, 2.54 to 113.13) for *S. coronatus*. Also, the odds ratio was 3.37 (95% CI, 1.62 to 6.99) higher in the spring than in the fall. Five animals hybridized with the generic probe for *Borrelia* spp. but not with that for *Borrelia* sp. strain R57 after RLB analysis targeting the 16S rRNA gene. To check whether *B. burgdorferi* sensu lato could be found in these five animals and in mixed infections with *Borrelia* sp. strain R57, all *Borrelia*-positive samples were subjected to 5S-23S rRNA RLB hybridization. *B. burgdorferi* sensu lato was detected in two *S. coronatus* shrews (14.3%) and one *C. glareolus* vole (6.3%), and *B. afzelii* was the genospecies identified. In one animal (*C. glareolus*), *B. afzelii* was detected in a mixed infection with *Borrelia* sp. strain R57. Other genospecies such as *B. garinii*, *B. valaisiana*, *B. lusitaniae*, and *B. burgdorferi* sensu stricto were not detected. Three animals positive for *Borrelia* spp. did not hybridize with any of the specific probes.

Two domestic mice (*M. domesticus*) captured in the spring

TABLE 2. Results of RLB analysis for the two studies and the different species of small mammals

Infecting pathogen(s)	No. (%) of infected animals									
	Study 1 (186/127)[a]	Study 2 (148/126)	Total (334/253)	*A. sylvaticus* (233/162)	*A. flavicollis* (3/3)	*C. glareolus* (17/16)	*S. coronatus* (17/14)	*C. russula* (6/6)	*M. domesticus* (28/28)	*T. europaea* (30/24)
RLB hybridization results										
C. burnetii	2 (1.6)	0	2 (0.8)	0	0	0	0	0	2 (7.1)	0
A. phagocytophilum	1 (0.8)	1 (0.8)	2 (0.8)	1 (0.6)	0	0	0	1 (16.7)	0	0
Borrelia spp.	1 (0.8)	2 (1.6)	3 (1.2)	3 (1.9)	0	0	0	0	0	0
Borrelia sp. strain R57	27 (21.3)	33 (26.2)	60 (23.7)	49 (30.2)	0	10 (62.5)	0	0	1 (3.6)	0
B. afzelii	1 (0.8)	1 (0.8)	2 (0.8)	0	0	0	2 (14.3)	0	0	0
B. afzelii/*Borrelia* sp. strain R57	0	1 (0.8)	1 (0.4)	0	0	1 (6.3)	0	0	0	0
C. burnetii/*Borrelia* sp. strain R57	1 (0.8)	0	1 (0.4)	1 (0.6)	0	0	0	0	0	0
Total single and mixed infections	33 (26.0)	38 (30.2)	71 (28.1)	54 (33.3)	0	11 (68.8)	2 (14.3)	1 (16.7)	3 (10.7)	0
Results by genus										
C. burnetii	3 (2.4)	0	3 (1.2)	1 (0.6)	0	0	0	0	2 (7.1)	0
A. phagocytophilum	1 (0.8)	1 (0.8)	2 (0.8)	1 (0.6)	0	0	0	1 (16.7)	0	0
Borrelia spp.	30 (23.6)	37 (29.4)	67 (26.5)	53 (32.7)	0	11 (68.8)	2 (14.3)	0	1 (3.6)	0

[a] The first number in parentheses represents the number of animals captured, and the second number represents the number of animals analyzed.

and winter in 2000 in the premises of a sheep farm (study 1) had positive hybridization with the *C. burnetii* probe. The remaining captures of that period were negative for *C. burnetii* (seven *A. sylvaticus* mice, two *C. glareolus* voles, one *A. flavicollis* mouse, and one *S. coronatus* shrew). In the spring of 2001, one of the seven *A. sylvaticus* mice captured in a forest area near the same farm was also positive, whereas other small mammals trapped in this season (five *M. domesticus* mice and two *C. glareolus* voles) were negative. These results indicate that 28.6% of domestic mice (*M. domesticus*) and 7.1% of wood mice (*A. sylvaticus*) were infected in this farm. *A. phagocytophilum* was detected in one *C. russula* shrew captured in study 1 and one *A. sylvaticus* mouse from study 2, which represent 16.7% and 0.6% infection rates for these species, respectively, and an overall infection rate of 0.8%. *Rickettsia* was the only pathogen included in this study that was never detected in any of the small mammals tested. Coinfection with more than one pathogen was found in two small mammal species and accounted for 0.8% (2/253) of the animals analyzed (Table 2). The combinations were those formed by *B. afzelii* and *Borrelia* sp. strain R57 in one *C. glareolus* vole and by *C. burnetii* and *Borrelia* sp. strain R57 in one *A. sylvaticus* mouse.

DISCUSSION

To investigate the role of small mammals as potential reservoir hosts for tick-borne bacterial zoonoses in northern Spain, small mammals were captured between years 2000 and 2005 in two consecutive studies. Almost half of the captured mammals harbored at least one attached tick, but the number of parasitized animals and ticks collected was higher in study 2 than in study 1, probably because sampling sites in study 2 were selected on the basis of tick abundance, whereas in study 1, sampling sites were nearby lowland sheep farms where the presence of ticks was normally scarce. *I. ricinus* was the predominant tick species (91.6% of larvae and 79.4% of nymphs) feeding on small mammals. This observation is consistent with the tick collection rates obtained by blanket dragging the vegetation in this region (4). *A. sylvaticus* was clearly the most abundant species trapped over the two studies, indicating that this species is predominant in woodland areas of northern Spain, which is in agreement with reports from La Rioja, a neighboring region in north-central Spain (11). *A. sylvaticus* was also the rodent species most heavily infested with *I. ricinus* larvae and nymphs, in agreement with other reports from Europe (14, 16, 23). The different tick infection levels observed for different animal species (*C. glareolus* versus *A. sylvaticus*) were explained by acquired immunity to *I. ricinus* larvae after repeated infestations in *C. glareolus* (9).

The mean number of *I. ricinus* larvae in *A. sylvaticus* in both studies (7.3) is clearly lower than in studies from Sweden (49.1) (42) but higher than in several European studies, e.g., 1.7 in Switzerland (25), 2.0 in La Rioja, Spain (11), and 2.1 in Ireland (16). In addition, few nymphs (26 nymphs) were collected, providing a 72:1 overall ratio of larvae to nymphs. This low nymphal infestation may limit the capacity of transmission of tick-borne bacteria to small mammals. This could be especially true when transovarial transmission of pathogens in ticks does not exist or occurs at a very low level and infection must be transmitted in the course of feeding by infected nymphs, as is the case for *B. burgdorferi*. However, the observed 72:1 ratio of larvae to nymphs was markedly lower than the one reported by Gil et al. (14) in the same region (450:1), but the prevalence of *B. burgdorferi* sensu lato in small mammals was higher (0.8% in study 1 and 1.6% in study 2) than in the former study (0.5%), suggesting an increase in nymphal infestation in recent years. Interestingly, this is the first report of *B. afzelii* infection of small mammals in Spain and the first time that *S. coronatus* has been involved as a reservoir of Lyme borreliosis. The prevalence values of *B. afzelii* infection found in *S. coronatus* (14.3%) and *C. glareolus* (6.3%) were markedly lower than those found in shrews and rodents in Central Europe (20, 22, 47). In any case, our results confirm reports by other groups that propose small mammals as reservoirs of *B. afzelii* (18, 19). Despite being the most abundant species captured and ana-

lyzed, none of the *A. sylvaticus* specimens was positive for *B. burgdorferi* sensu lato, suggesting its low contribution in the transmission of these spirochetes to subadult tick stages. These findings are in accordance with previous studies that found lower numbers of ticks and higher infection prevalences in bank voles than in *Apodemus* mice (23, 28). The absence of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. valaisiana* in the analyzed small mammals contrasts with the results reported for questing ticks from the vegetation in our region, where *B. burgdorferi* sensu stricto, *B. garinii*, and *B. valaisiana* and not *B. afzelii* are the most prevalent genospecies (5, 10, 14). Hence, the main reservoir hosts for *B. burgdorferi* sensu lato in northern Spain remain unknown, and other mammal species, lizards, or birds may play a major role in the maintenance of this spirochete in the natural environment.

The novel *Borrelia* sp. strain R57, a spirochete closely related to the genus *Borrelia* but forming a clade separate from that of the Lyme disease agent and relapsing fever organisms (14), was widely distributed in rodents and shrews, both in sheep farms boundaries (22.1%) and in recreational parks (27.8%). Especially noteworthy were the detection of *Borrelia* sp. strain R57 in a domestic mouse and its high prevalence in bank voles (68.8%). These two species of small mammals and most of the animals captured in study 1 had low tick infestation levels, suggesting that *Borrelia* sp. strain R57 might be transmitted by ectoparasites other than ticks. This was already proposed by Gil et al. (14), who found that all the ticks collected from *Borrelia* sp. strain R57-positive small mammals were negative. The overall prevalence of *Borrelia* sp. strain R57 obtained in this study was two times higher than that previously reported by Gil et al. for regions in northern Spain where Lyme disease is endemic (14). The only methodological difference among both studies was probe concentration, which was much higher in the present study (16 versus 0.4 μM), thus increasing the sensitivity of the assay. Interestingly, *Borrelia* sp. strain R57 and *B. afzelii*, the only genospecies of *B. burgdorferi* found in northern Spain, were found as a mixed infection in a *C. glareolus* specimen, which suggests that an exclusion phenomenon does not exist between these two species, although the influence of such a possibility on the lack of distribution of other *Borrelia* genospecies should be further investigated.

In the United States, rodents are implicated as natural reservoirs for *A. phagocytophilum* (43). In Europe, this pathogen has been detected in several species of rodents such as *A. flavicollis* (25, 41), *Apodemus agrarius*, *Rattus rattus* (8), *C. glareolus*, *A. sylvaticus* (25), and shrews (*Sorex araneus*) (25), but their role as reservoirs is not clear. In our area, this agent has been associated with ovine and bovine abortions in several mountainous areas (12, 21) and in roe deer (31), but it was not detected among a small number of small mammals tested, probably due to the relatively low number of nymphs found to be infesting them (31). In the present study, we detected *A. phagocytophilum* in *A. sylvaticus* (0.6%) and *C. russula* (16.7%). Here, the relatively higher proportion of nymphs observed would explain the higher prevalence in a manner similar to that described above for *B. burgdorferi*. Also interesting was the detection of *A. phagocytophilum* in *C. russula*, since this is the first time that this shrew species has been involved as a possible reservoir. Nevertheless, the overall prevalence (0.8%) found in this study is clearly lower than in studies from Bulgaria (7.7%) (8) and Switzerland (8 to 10%) (25), where rodents seem to represent important reservoirs for this agent.

C. burnetii has a worldwide distribution, and many wild and domestic mammals (mainly sheep, cattle, and goats), birds, and arthropods, such as ticks, are considered their reservoirs. However, domestic ruminants represent the most frequent source of human infection. The disease occurs throughout Spain, and the incidence of its respiratory manifestations is especially high in our area (Basque Country) (29), where the largest series of Q fever pneumonia in Europe have been reported (27). Furthermore, this agent has a high importance as an abortifacient agent in sheep flocks in northern Spain (30). There are several serological studies that implicate small mammals in the wild and domestic cycles of Q fever (24, 38, 44), but very few studies demonstrate the presence of *C. burnetii* DNA in small mammals by molecular techniques (41). Therefore, the molecular detection of *C. burnetii* DNA in *A. sylvaticus* (0.6%) and *M. domesticus* (7.1%) captured in a sheep farm with previous reports of abortion (P. Gabiria, personal communication) is an interesting finding. These results suggest that mice would have acquired the infection by direct contact with infected sheep or with sheep fetuses or placentae inside the farm or in the pasture and that in the study region, *C. burnetii* developed in a peridomestic cycle rather than in a wild cycle and associated with infected flocks. Consequently, the risk of transmission to humans is associated mainly with domestic ruminants, and control and surveillance of *C. burnetii* in the animal reservoir environment are therefore needed to avoid human infection.

Ixodidae family ticks may act as vectors, reservoirs, and amplifiers of SFG rickettsiae (34), and small rodents have been shown to be susceptible to infection by several *Rickettsia* species (37). However, the DNA of the SFG rickettsiae was not detected in any of the animals analyzed in this study, suggesting that these animals are not involved as reservoirs of these pathogens in this area. This is in accordance with available data on the scarce number of *Rickettsia*-positive tick specimens in the same areas (J. F. Barandika, unpublished data) and with the low incidence of human rickettsiosis in the studied area (2).

In summary, a better knowledge of the wild and peridomestic cycles of tick-borne bacteria has been achieved and questions have been raised concerning the ecology of these zoonotic organisms. The complex cycle of these agents and the variations that could suffer over time suggest the need for continuous environmental surveillance to detect variations and prevent risks for transmission to humans.

ACKNOWLEDGMENTS

This work was conducted under financial support from FEDER, FIS G03/057 and PI051873, INIA RTA02-001, and the Department of Agriculture and Fisheries and Food of the Basque Government.

We thank the EDEN Sixth Framework EU project (contract reference no. 010284-2) for providing resources for ongoing work and an expert tick and tick-borne disease forum for discussions.

REFERENCES

1. **Alvarez, J., A. Bea, J. M. Faus, E. Castién, and I. Mendiola.** 1986. Atlas de los vertebrados continentales de Alava, Vizcaya y Guipúzcoa. Gobierno Vasco, Viceconsejería de Medio Ambiente, Vitoria-Gasteiz, Spain.
2. **Anonymous.** 2005. Informe de Salud Pública 1999–2005. Departamento de Sanidad, Gobierno Vasco, Vitoria-Gasteiz, Spain. http://www.osasun.ejgv.euskadi.net/r52-478/es/contenidos/informacion/informe_saludpublica/es_4041/informe_salud_publica_c.html.
3. **Azad, A. F., and S. Radulovic.** 2003. Pathogenic rickettsiae as bioterrorism agents. Ann. N. Y. Acad. Sci. **990:**734–738.

4. **Barandika, J. F., E. Berriatua, M. Barral, R. A. Juste, P. Anda, and A. L. Garcia-Perez.** 2006. Risk factors associated with ixodid tick species distributions in the Basque region in Spain. Med. Vet. Entomol. **20:**177–188.
5. **Barral, M., A. L. Garcia-Perez, R. A. Juste, A. Hurtado, R. Escudero, R. E. Sellek, and P. Anda.** 2002. Distribution of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* (Acari: Ixodidae) ticks from the Basque Country, Spain. J. Med. Entomol. **39:**177–184.
6. **Blanco, J. C.** 1998. Mamíferos de España. Planeta, Barcelona, Spain.
7. **Bown, K. J., M. Begon, M. Bennett, Z. Woldehiwet, and N. H. Ogden.** 2003. Seasonal dynamics of *Anaplasma phagocytophila* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. Emerg. Infect. Dis. **9:**63–70.
8. **Christova, I., and T. Gladnishka.** 2005. Prevalence of infection with *Francisella tularensis*, *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in rodents from an endemic focus of tularemia in Bulgaria. Ann. Agric. Environ. Med. **12:**149–152.
9. **Dizij, A., and K. Kurtenbach.** 1995. *Clethrionomys glareolus*, but not *Apodemus flavicollis*, acquires resistance to *Ixodes ricinus* L., the main European vector of *Borrelia burgdorferi*. Parasite Immunol. **17:**177–183.
10. **Escudero, R., M. Barral, A. Perez, M. M. Vitutia, A. L. Garcia-Perez, S. Jimenez, R. E. Sellek, and P. Anda.** 2000. Molecular and pathogenic characterization of *Borrelia burgdorferi* sensu lato isolates from Spain. J. Clin. Microbiol. **38:**4026–4033.
11. **Estrada-Peña, A., J. J. Osacar, B. Pichon, and J. S. Gray.** 2005. Hosts and pathogen detection for immature stages of *Ixodes ricinus* (Acari: Ixodidae) in North-Central Spain. Exp. Appl. Acarol. **37:**257–268.
12. **García-Pérez, A. L., J. Barandika, B. Oporto, I. Povedano, and R. A. Juste.** 2003. *Anaplasma phagocytophila* as an abortifacient agent in sheep farms from northern Spain. Ann. N. Y. Acad. Sci. **990:**429–432.
13. **Gil, H.** 2002. Zonas endémicas de enfermedad de Lyme en la Comunidad Autónoma del País Vasco: estudio del papel de los micromamíferos en el mantenimiento de *Borrelia burgdorferi* sensu lato en el medio rural. Ph.D. thesis. Universidad de Zaragoza, Zaragoza, Spain.
14. **Gil, H., M. Barral, R. Escudero, A. L. Garcia-Perez, and P. Anda.** 2005. Identification of a new *Borrelia* species among small mammals in areas of northern Spain where Lyme disease is endemic. Appl. Environ. Microbiol. **71:**1336–1345.
15. **Gil-Collado, J., J. L. Guillén-Llera, and L. M. Zapatero-Ramos.** 1979. Claves para la identificación de los Ixodoidea españoles (adultos). Rev. Iber. Parasitol. **39:**107–111.
16. **Gray, J. S., F. Kirstein, J. N. Robertson, J. Stein, and O. Kahl.** 1999. *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks and rodents in a recreational park in south-western Ireland. Exp. Appl. Acarol. **23:**717–729.
17. **Gubbels, J. M., A. P. de Vos, M. van der Weide, J. Viseras, L. M. Schouls, E. de Vries, and F. Jongejan.** 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. **37:**1782–1789.
18. **Hanincová, K., V. Taragelova, J. Koci, S. M. Schafer, R. Hails, A. J. Ullmann, J. Piesman, M. Labuda, and K. Kurtenbach.** 2003. Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. Appl. Environ. Microbiol. **69:**2825–2830.
19. **Humair, P. F., O. Péter, R. Wallich, and L. Gern.** 1995. Strain variation of Lyme disease spirochetes isolated from *Ixodes ricinus* ticks and rodents collected in two endemic areas in Switzerland. J. Med. Entomol. **32:**433–438.
20. **Humair, P. F., N. Turrian, and L. Gern.** 1993. *Borrelia burgdorferi* in a focus of Lyme borreliosis: epizootiologic contribution of small mammals. Folia Parasitol. (Prague) **40:**65–70.

20a. **Jado, I., J. A. Oteo, M. Aldámiz, H. Gil, R. Escudero, V. Ibarra, J. Portu, A. Portillo, M. J. Lezaun, C. García-Amil, I. Rodríguez-Moreno, and P. Anda.** *Rickettsia monacensis* and human disease, Spain. Emerg. Infect. Dis., in press.

21. **Juste, R. A., G. R. Scott, E. Paxton, J. L. Gelabert, and S. Jimenez.** 1989. Presence of *Cytoecetes phagocytophila* in an atypical disease of cattle in Spain. Vet. Rec. **124:**636.
22. **Khanakah, G., E. Kocianova, V. Vyrostekova, J. Rehacek, M. Kundi, and G. Stanek.** 2006. Seasonal variations in detecting *Borrelia burgdorferi* sensu lato in rodents from north eastern Austria. Wien. Klin. Wochenschr. **118:**754–758.
23. **Kurtenbach, K., H. Kampen, A. Dizij, S. Arndt, H. M. Seitz, U. E. Schaible, and M. M. Simon.** 1995. Infestation of rodents with larval *Ixodes ricinus* (Acari: Ixodidae) is an important factor in the transmission cycle of *Borrelia burgdorferi* s.l. in German woodlands. J. Med. Entomol. **32:**807–817.
24. **Literak, I.** 1995. Prevalence of *Coxiella burnetii* infection in military training areas in the Czech Republic and Slovakia. Acta Vet. Brno **64:**179–186.
25. **Liz, J. S., L. Anderes, J. W. Sumner, R. F. Massung, L. Gern, B. Rutti, and M. Brossard.** 2000. PCR detection of granulocytic Ehrlichiae in *Ixodes ricinus* ticks and wild small mammals in western Switzerland. J. Clin. Microbiol. **38:**1002–1007.
26. **Manilla, G.** 1998. Acari Ixodida. Fauna d′Italia. Edizioni Calderini, Bologna, Italy.
27. **Maurin, M., and D. Raoult.** 1999. Q fever. Clin. Microbiol. Rev. **12:**518–553.
28. **Michalik, J., T. Hofman, A. Buczek, M. Skoracki, and B. Sikora.** 2003. *Borrelia burgdorferi* s.l. in *Ixodes ricinus* (Acari: Ixodidae) ticks collected from vegetation and small rodents in recreational areas of the city of Poznan. J. Med. Entomol. **40:**690–697.
29. **Montes, M., G. Cilla, D. Vicente, V. Nieto, M. Ercibengoa, and E. Perez-Trallero.** 2006. Gipuzkoa, Basque Country, Spain (1984–2004): a hyperendemic area of Q fever. Ann. N. Y. Acad. Sci. **1078:**129–132.
30. **Oporto, B., J. F. Barandika, A. Hurtado, G. Aduriz, B. Moreno, and A. L. Garcia-Perez.** 2006. Incidence of ovine abortion by *Coxiella burnetii* in northern Spain. Ann. N. Y. Acad. Sci. **1078:**498–501.
31. **Oporto, B., H. Gil, M. Barral, A. Hurtado, R. A. Juste, and A. L. Garcia-Perez.** 2003. A survey on *Anaplasma phagocytophila* in wild small mammals and roe deer (*Capreolus capreolus*) in northern Spain. Ann. N. Y. Acad. Sci. **990:**98–102.
32. **Panzironi, C., G. Cerone, M. Cristaldi, and G. Amori.** 1993. A method for the morphometric identification of southern Italian populations of *Apodemus* (*sylvaemus*). Hystrix **5:**1–16.
33. **Parola, P., B. Davoust, and D. Raoult.** 2005. Tick- and flea-borne rickettsial emerging zoonoses. Vet. Res. **36:**469–492.
34. **Parola, P., C. D. Paddock, and D. Raoult.** 2005. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin. Microbiol. Rev. **18:**719–756.
35. **Piesman, J., and L. Gern.** 2004. Lyme borreliosis in Europe and North America. Parasitology **129**(Suppl.)**:**S191–S220.
36. **Regnery, R. L., C. L. Spruill, and B. D. Plikaytis.** 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J. Bacteriol. **173:**1576–1589.
37. **Rehacek, J., J. Urvolgyi, E. Kocianova, and L. Jedlicka.** 1992. Susceptibility of some species of rodents to rickettsiae. Folia Parasitol. (Prague) **39:**265–284.
38. **Riemann, H. P., D. E. Behymer, C. E. Franti, C. Crabb, and R. G. Schwab.** 1979. Survey of Q-fever agglutinins in birds and small rodents in northern California, 1975–76. J. Wildl. Dis. **15:**515–523.
39. **Rijpkema, S. G., M. J. Molkenboer, L. M. Schouls, F. Jongejan, and J. F. Schellekens.** 1995. Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. J. Clin. Microbiol. **33:**3091–3095.
40. **Schouls, L. M., I. van de Pol, S. G. T. Rijpkema, and C. S. Schot.** 1999. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J. Clin. Microbiol. **37:**2215–2222.
41. **Smetanova, K., K. Schwarzova, and E. Kocianova.** 2006. Detection of *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Rickettsia* spp., and *Borrelia burgdorferi* s. l. in ticks, and wild-living animals in western and middle Slovakia. Ann. N. Y. Acad. Sci. **1078:**312–315.
42. **Tälleklint, L., and T. G. T. Jaenson.** 1993. Maintenance by hares of European *Borrelia burgdorferi* in ecosystems without rodents. J. Med. Entomol. **30:**273–276.
43. **Walls, J. J., B. Greig, D. F. Neitzel, and J. S. Dumler.** 1997. Natural infection of small mammal species in Minnesota with the agent of human granulocytic ehrlichiosis. J. Clin. Microbiol. **35:**853–855.
44. **Webster, J. P., G. Lloyd, and D. W. Macdonald.** 1995. Q. fever (*Coxiella burnetii*) reservoir in wild brown rat (*Rattus norvegicus*) populations in the UK. Parasitology **110:**31–35.
45. **Willems, H., D. Thiele, R. Frolich-Ritter, and H. Krauss.** 1994. Detection of *Coxiella burnetii* in cow's milk using the polymerase chain reaction (PCR). Zentbl. Vetmed. **41:**580–587.
46. **Zeidner, N. S., T. R. Burkot, R. Massung, W. L. Nicholson, M. C. Dolan, J. S. Rutherford, B. J. Biggerstaff, and G. O. Maupin.** 2000. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in northern Colorado. J. Infect. Dis. **182:**616–619.
47. **Zore, A., M. Petrovec, K. Prosenc, T. Trilar, E. Ruzic-Sabljic, and T. Avsic-Zupanc.** 1999. Infection of small mammals with *Borrelia burgdorferi* sensu lato in Slovenia as determined by polymerase chain reaction (PCR). Wien. Klin. Wochenschr. **111:**997–999.