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Identification of a New *Borrelia* Species among Small Mammals in Areas of Northern Spain Where Lyme Disease Is Endemic

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The role of small mammals as reservoir hosts for *Borrelia burgdorferi* was investigated in several areas where Lyme disease is endemic in northern Spain. A low rate of infestation by *Ixodes ricinus* nymphs was found in the small mammal populations studied that correlated with the near-absence of *B. burgdorferi* sensu lato in 184 animals tested and with the lack of transmission of *B. burgdorferi* sensu lato to *I. ricinus* larvae that fed on them. In contrast, questing ticks collected at the same time and in the same areas were found to carry a highly variable *B. burgdorferi* sensu lato repertoire (*B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia valaisiana*, and *Borrelia afzelii*). Interestingly, the only isolate obtained from small mammals (R57, isolated from a bank vole) grouped by phylogenetic analyses with other *Borrelia* species but in a separate clade from the Lyme disease and relapsing fever organisms, suggesting that it is a new species. This new agent was widely distributed among small mammals, with infection rates of 8.5 to 12% by PCR. Moreover, a high seroprevalence to *B. burgdorferi* sensu lato was found in the animal sera, suggesting cross-reactivity between *B. burgdorferi* sensu lato and R57. Although small mammals do not seem to play an important role as reservoirs for *B. burgdorferi* sensu lato in the study area, they seem to be implicated in the maintenance of spirochetes similar to R57.

Lyme disease (LD) is a multisystemic zoonotic disorder caused by *Borrelia burgdorferi* sensu lato and transmitted by hard ticks (family *Ixodidae*) (11, 15, 42). There are currently 11 different genospecies of *B. burgdorferi* sensu lato (55, 81). In Europe, the major vector of *B. burgdorferi* sensu lato is the tick *Ixodes ricinus* and five genospecies, *B. burgdorferi* sensu stricto (42), *Borrelia garinii* (6), *Borrelia afzelii* (16), *Borrelia valaisiana* (80) and *Borrelia lusitaniae* (51), are present in this continent. The first three produce disease in humans (81), and *B. valaisiana* and *B. lusitaniae* have shown to infect laboratory mice (18, 23, 82). Moreover, *B. lusitaniae* has been isolated recently from a skin biopsy of a patient with a chronic skin lesion (18). In different European studies, small mammals (rodents and shrews) are the most important reservoir hosts for the Lyme disease agent (21, 30, 41, 48, 58, 59, 78), but birds can also play this role (39, 46, 61, 68).

The genus *Borrelia* has been classified using 16S rRNA and *flaB* (27, 53, 64) into two major groups: the LD and the relapsing fever (RF) groups. The latter group includes the species responsible for human RF in America (*Borrelia hermsii*, *Borrelia parkeri*, and *Borrelia turicatae*), and in southern Europe and Africa (*Borrelia duttonii*, *Borrelia hispanica*, and *Borrelia crocidurae*). The main vectors of the species of the RF group are the soft ticks (family *Argasidae*), while the human body louse transmits *Borrelia recurrentis* (77). However, new species of *Borrelia* transmitted by hard ticks (family *Ixodidae*) have been classified closer to the RF group: "*Borrelia lonestari*" (proposed name), transmitted in the United States by *Amblyomma americanum* (8) and *Borrelia miyamotoi*, transmitted in Asia by *Ixodes persulcatus* (28). This latter genospecies seems to have a larger distribution area, since related species have been found in Europe in *I. ricinus* (26, 67) and in America in *Ixodes scapularis* (74). The number of species of the RF group is increasing, as new species have been identified in *Hyalomma aegyptium* feeding in tortoises in Turkey (34) and in patients and soft ticks in Tanzania (44).

In southern Spain, a new *Borrelia* species has been isolated from patients and soft ticks (3) in areas where RF is endemic (5, 14, 72). Moreover, in the north of Spain there are areas where LD is endemic that coincide with the distribution of *I. ricinus* (19). In these areas, several series of LD cases have been described (2, 33, 63), and epidemiological studies of *B. burgdorferi* sensu lato in questing ticks (9), in ticks collected from animals (24), and in ticks collected from humans (25) have been performed. Since the first isolation of *B. burgdorferi* sensu lato in Spain (29), only a few isolates have been obtained (9, 62) and their characterization has shown a wide genospecies diversity and virulence in a mouse model (23). In the Basque country, our study region, cases of Lyme disease in humans have been reported; a serological survey showed 25% prevalence in outdoors workers, with antecedents compatible with LD in 15% of those who were seropositive (4). Moreover, our previous data confirmed the wide distribution of the vector *I. ricinus* and *B. burgdorferi* sensu lato in several areas of the Basque country (9).

This study considers the biological cycle of *B. burgdorferi* sensu lato in previously identified areas where of Lyme disease

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endemicity in the Basque country (9), with a special interest in the role of the small mammals as reservoir hosts for *B. burgdorferi* sensu lato, showing that they do not play an important role in our area. However, a new spirochete has been identified and is prevalent in our small mammals. The role of organisms similar to this new spirochete in the ecology of *B. burgdorferi* sensu lato is discussed.

MATERIALS AND METHODS

Small mammal and tick sampling. Small mammals were captured between October 1998 and September 2000 in six different areas of the Basque country, where *B. burgdorferi* sensu lato was previously detected in *I. ricinus* (9). The features and localization of the study areas have been previously described (9). Fifty Sherman traps (Sherman Traps, Tallahasee, Fla.) and 150 INRA traps (BTS Mecanique, Besançon, France) were placed overnight, and trapped animals were brought to the laboratory for tick collection and classification (31, 54, 60). Questing ticks were also collected (by flagging in the same places where the traps were placed) and classified (31, 54, 60).

Processing of small mammals. Live animals were maintained in the laboratory for 24 to 72 h to complete the repletion of the ticks that were feeding naturally. The engorged ticks obtained were kept at 18°C, 98% humidity, and a 12-h light cycle until molted.

Animals were anesthetized with ketamine hydrochloride (Imalgene; Merial) at a dose of 10 mg/kg intramuscularly and euthanized in a CO_2 chamber. Samples from different tissues were collected (ear, urinary bladder, spleen, liver, brain, kidney, heart, mesenteric and popliteal ganglia, and blood) for culture and PCR. The animals were classified by external morphological data and skull features (1, 12).

Isolation of *B. burgdorferi* sensu lato Organs were cultured in 4 ml of BSK (Barbour-Stoener-Kelly) II medium prepared as previously described (7), supplemented with 6% of rabbit serum (BSK-RS) (Sigma-Aldrich Quimica S.A., Alcobendas, Madrid, Spain), 50 μg of rifampin/ml, 50 μg of phosphomycin/ml, and 2.5 μg of amphotericin B/ml (52, 76). Before the preparation of the BSK medium, five batches of bovine serum albumin (Sigma) were tested to judge the medium's performance with low-passage autochthonous strains; the best was used to make 12-liter batches of medium. Urinary bladder and ear punch biopsies (EPB) were cultured individually for comparison with previous studies (23, 73, 76). For this, EPB were first disinfected by successive immersion in iodine, 70% ethanol, and phosphate-buffered saline (PBS), and both bladder samples and EPB were ground in BSK. Half of the homogenates were used for culture. The rest of the organs were homogenized individually in 100 μl of BSK-RS, and a 30-μl aliquot was pooled and inoculated in the same medium with antibiotics.

In some experiments, rabbit serum was replaced by sera from different animal species (horse and fetal calf sera from Biological Industries, Beit Haemek, Israel; and mouse serum from Centro Nacional de Microbiología, Majadahonda, Spain) to improve the culture of fastidiously growing isolates.

For the isolation of *B. burgdorferi* sensu lato from ticks, the specimens were disinfected by successive immersion in 95, 70, and 35% ethanol and PBS and homogenized in 100 μl of BSK-RS. A 30-μl aliquot was inoculated in the same medium supplemented with antibiotics. Ticks that molted under the conditions described above were cultured in pools of up to 10 of the same species, stage, and animal in which they were feeding. Ticks collected from the vegetation were cultured in pools of up to 30 nymphs or three adults.

Cultures were maintained at 33°C for 3 months and examined by dark-field microscopy to monitor the presence of spirochetes. After 3 months, the cultures were centrifuged for 5 min at 7,000 × *g*, and the sediments were washed twice with sterile PBS and reserved for PCR.

Animal model. The LD animal model with C3H mice (10) was used for the recovery of isolates that grew poorly in BSK-RS medium, as previously described (23). Briefly, 3- to 4-week-old mice were inoculated intradermally with 10^5 spirochetes. At 2 and 4 weeks postinoculation, an EPB was cultured and subjected to PCR. Thirty days after inoculation, mice were euthanized, and internal organs and blood were processed as described above. A blood sample was taken daily from the tail vein during the first 2 weeks and examined by dark-field microscopy as previously described (3). All animals were processed according to protocols approved by the accredited animal care and use committees at Instituto de Salud Carlos III, following international regulations.

PCR analysis. DNA from the culture sediments and mammal organs was extracted by the guanidine thiocyanate method (17). A negative control for the extraction (distilled water) was included with every 10 samples. DNA concentration was determined for each sample by spectrophotometry; 100 to 300 ng was used in the PCR. An initial screening of the samples was done by a *Borrelia* generic PCR targeting 16S rRNA (16S-PCR) with the oligonucleotides BORF and 16S3B (Table 1). Positive samples were analyzed by a second PCR targeting the 5S (*rrf*)-23S (*rrl*) intergenic spacer (5S-23S-PCR) specific for *B. burgdorferi* sensu lato, with primers previously described (Table 1) (69). PCRs were performed in a 30-μl volume with 10 mM Tris-HCl, 50 mM KCl, 1.67 mM $MgCl_2$, 333 μM each deoxynucleoside triphosphate (Promega, Madison, Wis.), and 2.15 U of *Taq* DNA polymerase (Applied Biosystems, Branchburg, N.J.). Primers were used at a concentration of 30 pmol/reaction mixture for 16S-PCR and at 5 pmol/reaction mixture for 5S-23S-PCR. Cycling conditions for the 16S-PCR were 3 min at 94°C; 50 cycles each of 10 s at 94°C, 1 min at 45°C, and 1.5 min at 72°C; and a final elongation of 7 min at 72°C. For 5S-23S-PCR, the cycles consisted of 1 min at 94°C; 50 cycles each of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C; and a final elongation at 72°C for 5 min. All the amplifications were done with a PTC-200 thermocycler (MJ Research, Waltham, Mass.). DNA from *Borrelia japonica* (strain HO14^T) was used as a positive control, and one negative PCR control (distilled water) was included with every 10 PCRs. Serial dilutions of DNA of *B. garinii* (PBi strain) were tested to check the sensitivity of the 16S-PCR.

For further characterization of unusual isolates, several PCRs with primers based in *ospA* (35), flagellin (*flaB*) (reference 27 and this study), *rpoB* (65), and *p66* and *groEL* (this study; Table 1) were performed. DNA from *B. burgdorferi* sensu stricto (strain B31^T) and *B. hermsii* (strain HS1^T) were used as positive PCR controls.

Reverse line blotting (RLB). Samples amplified by 16S-PCR were subjected to RLB with probes designed in this study (Table 1). The positive samples obtained were analyzed by 5S-23S-PCR/RLB with probes described previously (69). The RLB method was performed as previously described (69), with few modifications. In brief, 56 pmol of each probe was attached to the membrane by a 10-min incubation, and the hybridization of the PCR products was performed at 48°C for 1 h.

Western blotting (WB). Blood samples from mice were taken by intracardiac puncture. Serum samples were analyzed by WB as previously described (75) with strain Esp1 of *B. burgdorferi* sensu stricto (29) and strain Rio4 of *B. garinii* (23) as antigens, based on previous data about the frequency of these two genospecies in the study areas (9, 23). Sera were tested at a 1:50 dilution. For each strip, the grade of reactivity of p93, p58, p56, p41, p30, OspC, p21, p19, and p17 was recorded (22), and a value was assigned to each band depending on the intensity (strong reactivity, 1; medium reactivity, 0.5; weak reactivity, 0.25; no reactivity, 0). A serum was considered positive when the sum of the values for each of the selected proteins was ≥4, inconclusive when the values were between 4 and 3, and negative when the values were <3.

Sequencing and analysis. PCR products were run in 0.8% low-melt agarose gels (Roche Diagnostics GmbH, Mannheim, Germany), and the bands of interest were purified by using the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany) and sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems) on an ABI 377 DNA sequencer.

16S rRNA sequences were obtained by amplifying overlapping fragments with primers 16S1 to 16S9 (3) and *groEL* was obtained with primers designed in this study (Table 1). Sequences generated in this study were aligned with sequences from databases (Table 2) by using ClustalX (79). Pairwise distance matrices for the aligned sequences were determined by the Kimura two-parameter method (43) with MEGA2 software (45) and phylogenetic trees constructed by neighbor joining (71) with bootstrap analysis with 1,000 replications for evaluation of their topology (13).

Nucleotide sequence accession numbers. The 16S rRNA and *groEL* sequences from R57 have been deposited in GenBank with accession numbers AY626138 and AY707888, respectively.

RESULTS

The ratio of infestation of *I. ricinus* nymphs to larvae was low in small mammals. A total of 184 animals were captured, belonging to seven different species (Table 3). The most frequently found species in all areas was the wood mouse, *Apodemus sylvaticus* (Table 3). A total of 901 *I. ricinus* larvae and only two nymphs were collected from the small mammals. No adults were found feeding on them. Other tick species (*Dermacentor reticulatus*, *Haemaphysalis inermis*, and *Ixodes trianguliceps*)

TABLE 1. Primers and probes used in this study

Primer or probe	Sequence	Gene(s)	Source or reference
Primers			
BORF	5′-CGC TGG CAG TGC GTC TTA A	*rrs*	This study
16S3B	5′-biotin-GCG GCT GCT GGC ACG TAA TTA GC	*rrs*	3
16S1	5′-CGA AGA GTT TGA TCC TGG CTTAG	*rrs*	3
16S2	5′-GCT AAT TAC GTG CCA GCA GCC GC	*rrs*	3
16S5	5′-GTG CGG GCC CCC GTC AAT TCC	*rrs*	3
16S7	5′-CCA TGA TGA TTT GAC GTC ATC	*rrs*	3
16S9	5′-CCT TGT TAC GAC TTC ACC CC	*rrs*	3
23SN2	5′-ACC ATA GAC TCT TAT TAC TTT GAC CA	*rrf-rrl*	69
5SCB	5′-biotin-GAG AGT AGG TTA TTG CCA GGG	*rrf-rrl*	69
N1	5′-GAG CTT AAA GGA ACT TCT GAT AA	*ospA*	35
C1	5′-GTA TTG TTG TAC TGT AAT TGT	*ospA*	35
N2	5′-ATG GAT CTG GAG TAC TTG AA	*ospA*	35
C2	5′-CTT AAA GTA ACA GTT CCT TCT	*ospA*	35
FlaBorF1	5′-GCW TCT GAT GAT GCT GCT GG	*flaB*	This study
FlaBorF2	5′-GCA AMY CAR GAY GAR GCD ATT GC	*flaB*	This study
FlaBorR1	5′-GCY ACA AYH TCA TCK GTC	*flaB*	This study
FlaBorR2	5′-GCA ATH GCY TCR TCY TGR KTT GC	*flaB*	This study
C	5′-GCA GTT CAA TCA GGT AAC GG	*flaB*	27
D	5′-AGG TTT TCA ATA GCA TAC TC	*flaB*	27
66F1	5′-GAA KTA GGC AAR GAY GAY CC	*p66*	This study
66F2	5′-GAR GRA CAA TTC TTG CAA GAG G	*p66*	This study
66F3	5′-CCA ACT TTA TCA AAT KCW KC	*p66*	This study
66F4	5′-GGT GAA AAA GAA TCH TGG	*p66*	This study
66R1	5′-TTT GAR TCC CAK CCA AG	*p66*	This study
66R2	5′-CCA DGA TTC TTT TTC ACC	*p66*	This study
66R3	5′-GMW GMA TTT GAT AAA GTT GG	*p66*	This study
66R4	5′-CCT CTT GCA AGA ATT GTY CYT C	*p66*	This study
1730F	5′-CTT GGI CCI GGI GGA CTT TC	*rpoB*	65
2900R	5′-AGA AAT IAA IAT IGC ATC CTC	*rpoB*	65
GroEL-F	5′-TAC GAT TTC TTA TGT TGA GGG	*groEL*	This study
GroEL-R	5′-CGY CTA TCA CCA AAA CCR GGM G	*groEL*	This study
Probes[a]			
16S	5′-a-GAG GAA TAA GCT TTG TAG GAA ATG ACA A[b]	*rrs*	This study
R57	5′-a-AGT CAT TAA AGA TGT TTA ATG	*rrs*	This study
SL	5′-a-CTT TGA CCA TAT TTT TAT CTT CCA	*rrf-rrl*	69
SS	5′-a-AAC ACC AAT ATT TAA AAA ACA TAA	*rrf-rrl*	69
GA	5′-a-AAC ATG AAC ATC TAA AAA CAT AAA	*rrf-rrl*	69
AF	5′-a-AAC ATT TAA AAA ATA AAT TCA AGG	*rrf-rrl*	69
VA	5′-a-CAT TAA AAA AAT ATA AAA AAT AAA TTT AAG G	*rrf-rrl*	69

[a] 16S, generic probe to 16S rRNA of *Borrelia* spp. R57, specific probe for 16S rRNA of spirochetes similar to R57; SL, generic probe for 5S-23S rRNA of *B. burgdorferi* sensu lato, SS, specific probe for *B. burgdorferi* sensu stricto; GA, specific probe for *B. garinii*; AF, specific probe for *B. afzelii*; VA, specific probe for *B. valaisiana*.
[b] a, aminolink modification of the oligonucleotides in 5′.

were collected infrequently. The ratio of infestation of *I. ricinus* nymphs to larvae in the small mammals was 1:450.

***Borrelia* spp. were widespread in tissues of small mammals.** Animal samples were analyzed by a generic 16S-PCR/RLB designed in this study for the detection of *Borrelia* spp. (Table 1; Fig. 1A). This method was able to detect 8.5 fg (eight spirochetes) and was used for initial screening. Cultures from 108 animals, as well as tissue samples from 76 specimens found dead, were analyzed. Twenty-three (12.5%) of the 184 animals were positive by this technique (Table 3; Fig. 1B). EPB was the positive tissue in 20 animals; the other three positive tissues were two brain samples and one urinary bladder sample. Five of the seven species of small mammals captured yielded positive results (Table 3).

The *B. burgdorferi* sensu lato genospecies composition of the PCR-positive samples was determined by 5S-23S-PCR/RLB. From the 23 samples of small mammals that were positive by the generic 16S-PCR/RLB, only one organ sample, a brain from a wood mouse (*A. sylvaticus*) (Fig. 1B, line 11), was positive by this second method, and was identified as *B. burgdorferi* sensu stricto (Table 3). Consequently, the prevalence of the infection for *B. burgdorferi* sensu lato in small mammals was 0.5% (1 of 184 mammals) and 0.8% (1 of 130 mammals), when only the *A. sylvaticus* specimens studied were considered (Table 3).

***B. burgdorferi* sensu lato was isolated and detected from questing ticks but not from ticks feeding on small mammals.** *I. ricinus* was the most abundant questing species found in all the areas studied. A total of 233 adults and 2,540 nymphs of this species were collected from vegetation; approximately half (98 adults in 36 pools and 1,298 nymphs in 49 pools) were cultured in BSK-RS with antibiotics. Twenty isolates from four different genospecies were obtained (Fig. 2). In addition, all the cultures were analyzed by 16S-PCR/RLB; eight additional positive samples were found (Table 4). Afterward, all positive samples from questing ticks cultures were analyzed by 5S-23S-PCR/RLB to determine the genospecies presented. *B. burgdorferi* sensu stricto was the most frequent genospecies identified (14 pools),

TABLE 2. Different bacteria from the order *Spirochaetales* used in phylogenetic analyses

Species	Strain	Source	Country of origin	GenBank accession no.	
				16S rRNA	*groEL*
B. afzelii	DK4	Erythema migrans; skin	Denmark	X85194	
	VS461[T]	*I. ricinus*	Switzerland		AF517954
	Pko-85	Erythema migrans; skin	Germany		AF517956
B. andersonii	21038[T]	*Ixodes dentatus*	United States	L46701	
	21123	*I. dentatus*	United States		AF517975
B. anserina	ES-1	*Argas persicus*	Unknown	U42284	
B. bissettii	DN127[T]	*I. pacificus*	United States	AJ224141	AF517974
B. burgdorferi sensu stricto	B31[T]	*I. dammini*	United States	U03396	
	Sh-2-82	*I. dammini*	United States		AF517948
	20004	*I. ricinus*	France		AF517951
B. coriaceae	Co53[T]	*O. coriaceus*	United States	U42286	
B. crocidurae	UESV/523SIS	Blood of patient	Mali	U42301	
B. duttonii	UESV/117DUTT	*Ornithodoros moubata*	Zaire	U42288	
B. garinii	PBi	CSF[a] of patient	Germany	X85199	
	G25	*I. ricinus*	Sweden		AF517962
	Sika2	*I. persulcatus*	Japan		AF517964
B. hermsii	HS1[T]	*O. hermsi*	United States	U42292	AF518000
B. hispanica	UESV/246	*O. erraticus*	Morocco	U42294	
B. japonica	HO14[T]	*I. ovatus*	Japan	L46696	AF517970
B. lonestari	Texas 20	*A. americanum*	United States	U23211	
	010298	Unknown	United States		AY552786
B. lusitaniae	PotiB2[T]	*I. ricinus*	Portugal	X98228	AF517971
B. miyamotoi	HT31[T]	*I. persulcatus*	Japan	D45192	
B. parkeri	M3001	*O. parkeri*	United States	U42296	
B. persica	UESV/340	*O. tholozani*	Iran	U42297	
B. recurrentis	A1	Blood of patient	Ethiopia	U42300	
B. sinica	CNM3[T]	*Niviventer confucianus*	China	AB022101	
B. tanukii	Hk501[T]	*I. tanuki*	Japan	D67023	AF517973
B. turdi	Ac502	*I. turdus*	Japan	D67024	
	Ya501[T]	*I. turdus*	Japan		AF517972
B. turicatae	M2007	*O. turicata*	United States	U42299	
B. valaisiana	CKA4a	*Apodemus agrarius*	China	AB022143	
	VS116[T]	*I. ricinus*	Switzerland		AF517976
Borrelia sp.	HN7	*I. granulatus*	Korea		AF517987
Borrelia sp.	Antequera	Blood of patient and *O. erraticus*	Spain	U28502	
Borrelia sp.	R57	*C. glareolus*	Spain	AY626138	AY707888
Borrelia sp.	TXW-1	*Dermacentor variabilis*	United States	AF467976	
Brachyspira hyodysenteriae	B204	Pig feces	Unknown	M57741	
Brevinema andersonii	L31544	*Blarina brevicauda*	United States	MV116	
Cristispira sp.	CP1	Oyster	United States	U42638	
Leptonema illini	3055	Unknown	Unknown	M88719	
Leptospira interrogans serovar canicola	Moulton	Blood of dog	Unknown	X17547	
L. interrogans serovar Lai	56601	Unknown	Unknown		NC_004342
Spirochaeta aurantia	J1	Pond mud	Unknown	M57740	
Treponema denticola	ATCC 35405	Human periodontal pocket	Canada		NC_002967
Treponema pallidum	Nichols	Patient	Unknown	M88726	NC_000919

[a] CSF, cerebrospinal fluid.

followed by *B. valaisiana* (10 pools) and *B. garinii* (9 pools). *B. afzelii* was detected in only one pool (Table 4). In six of the nymph pools, mixed infections were detected: one pool with *B. afzelii* and *B. garinii*, one pool with *B. burgdorferi* sensu stricto and *B. valaisiana*, two pools with *B. garinii* and *B. valaisiana*, and two pools with *B. garinii* and *B. burgdorferi* sensu stricto.

Engorged ticks, which were feeding naturally on the captured mammals, were allowed to molt and cultured to test for the potential of the small mammals in the transmission of *B. burgdorferi* sensu lato. A total of 284 molted ticks (275 *I. ricinus* larvae, seven *H. inermis* larvae, one *I. ricinus* nymph, and one *D. reticulatus* nymph) belonging to 48 small mammals were processed in 96 cultures; no spirochetes were isolated.

In addition, all these cultures were analyzed by 16S-PCR/RLB to detect the presence of *Borrelia* spp.; all of them were negative.

A new organism was isolated from a bank vole. Necropsy material from 108 small mammals was cultured, and one isolate (R57) was obtained from an EPB of a bank vole (*Clethrionomys glareolus*) that showed the typical *Borrelia* morphology and movement by dark-field microscopy. The organism grew well during the first passage, but subsequent attempts to grow it in culture were unsuccessful. Attempts to recover viable organisms from infected C3H mice were unsuccessful; no spirochetemia was detected by dark-field visualization of blood smears.

The whole 16S rRNA gene from R57 (1,440 bp) was sequenced by using overlapping primers (Table 1). Also, a frag-

TABLE 3. Small mammals captured and PCR/RLB results

Species	No. (%) of animals captured[a]	No. (%) positive by PCR/RLB[b]		
		16S generic	16S specific R57	5S-23S rRNA
A. sylvaticus (wood mouse)	130 (70.7)	16 (12.3)	10 (7.7)	1 (0.8)
C. glareolus (bank vole)	18 (9.8)	4 (22.2)	4 (22.2)	
Sorex coronatus (millet shrew)	15 (8.2)	1 (6.7)		
Apodemus flavicollis (yellow-necked mouse)	9 (4.9)	1 (11.1)		
Crocidura russula (common shrew)	9 (4.9)	1 (11.1)	1 (11.1)	
Microtus lusitanicus (Lusitanian pine vole)	2 (1.1)			
Sorex minutus (pygmy shrew)	1 (0.5)	0 (0.00)		
Total	184	23 (12.5)	15 (8.2)	1 (0.5)

[a] Values are percentages of captured animals from the total number of captured animals.
[b] Values are percentages of positive animals from the total number of each animal species.

ment of *groEL* (243 bp) was amplified and sequenced with degenerate primers (Table 1), based on aligned sequences of other members of the order *Spirochaetales* (Table 2). However, attempts to amplify 5S-23S-PCR/RLB (Fig. 2) and *ospA*, *flaB*, *rpoB*, and *p66* from R57 were unsuccessful, while *B. burgdorferi* sensu stricto and *B. hermsii* yielded amplicons of the expected size (data not shown).

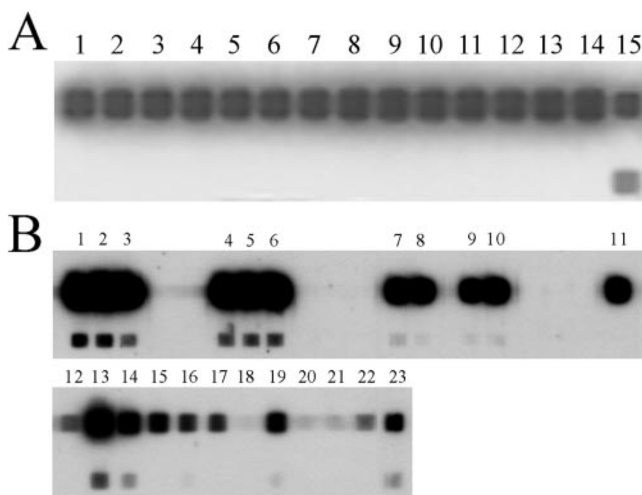

FIG. 1. (A) RLB based on the 16S rRNA of different species of the genus *Borrelia* and R57. Specific probes for the genus *Borrelia* and R57 were used in this assay. Lanes: 1, *B. japonica*; 2, *B. burgdorferi* sensu stricto; 3, *B. garinii*; 4, *B. afzelii*; 5, *B. valaisiana*; 6, *B. lusitaniae*; 7, Spanish relapsing fever spirochete; 8, *B. turicatae*; 9, *B. duttonii*; 10, *Borrelia anserina*; 11, *B. hermsii*; 12, *B. crocidurae*; 13, *B. recurrentis*; 14, *B. hispanica*; 15, R57. (B) Hybridization with samples from small mammal tissues. The upper spots in each panel correspond to the 16S genetic probe for *Borrelia* spp.; the lower spots correspond to the 16S-specific probe for R57 spirochetes.

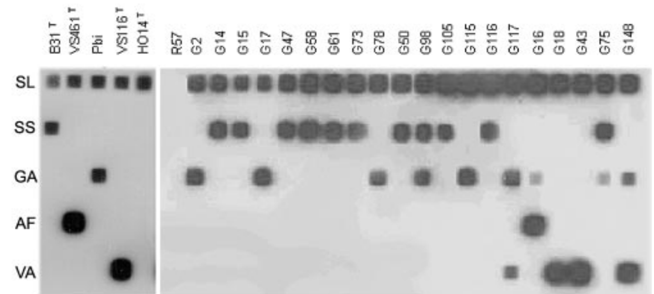

FIG. 2. 5S-23S-PCR/RLB of the different isolates obtained in this study. The left panel shows the positive controls belonging to the different genospecies; the right panel shows the results with different samples. The name of the probes used is shown on the left. SL, generic probe for *B. burgdorferi* sensu lato; SS, specific probe for *B. burgdorferi* sensu stricto; GA, specific probe for *B. garinii*; AF, specific probe for *B. afzelii*; VA, specific probe for *B. valaisiana*. The names of the strains used as controls and the names of the isolates obtained in this study are on the top of the figure.

16S rRNA and *groEL* sequences were used to determine the taxonomic position of this isolate. Sequences from representative species of the order *Spirochaetales* (Table 2), including members of the *B. burgdorferi* sensu lato and RF groups, were used to build trees (Fig. 3 and 4). The relative position of R57 was stable in both trees and grouped this organism closer to the genus *Borrelia* but in a clade different from that of the other *Borrelia* species tested.

Spirochetes similar to R57 are widely distributed among small mammals. A 16S probe specific for R57 was designed to investigate the presence of this organism in the samples (Fig. 1A). When the 22 positive samples from small mammals that were negative by 5S-23S-PCR/RLB were tested, 15 samples (all of them EPB) were positive (Fig. 1B). In six of them, we were able to sequence the 16S-PCR product (491 bp), which was identical to the R57 isolate. The seven remaining samples showed a weak hybridization signal with the generic *Borrelia* probe and a negative result for the R57 probe (Fig. 1B). The hybridization efficiency of the R57 probe was lower than that of the generic probe, as seen by the weaker signal (Fig. 1A). Considering this, all samples positive by 16S-PCR/RLB and negative by 5S-23S-PCR, from which we were unable to amplify a 16S fragment visible in agarose gels, were considered probable R57 homologs (7 of 184 samples). In summary, the prevalence of the infection by spirochetes similar to R57 among the small mammals ranged from 8.5% (15 of 184 samples) to 12% (22 of 184 samples).

In contrast, spirochetes similar to R57 were not detected in ticks. All of the 96 cultures of the molted ticks that fed on the captured mammals were negative by 16S-PCR/RLB, even the ones that fed on positive animals. Moreover, samples from questing tick cultures were also negative for the R57 probe (Table 4).

A high seroreactivity to *B. burgdorferi* sensu lato was found among the small mammals. Sera samples from 80 animals were analyzed by WB. The antibody response detected in the animals was highly variable; in 8.75% (seven animals), reactivity against OspA was observed. In summary, the seroprevalence found was 13.75% (11 of 80 animals), and with 25% of the animals studied (20 of 80 animals), the results were inconclusive.

TABLE 4. PCR/RLB analysis of the cultures of questing ticks[a]

Stage	No.		16S PCR/RLB[b]		5S-23S PCR/RLB[b]				
	Ticks	Pools	16S	R57	SL	SS	GA	AF	VA
Nymph	1,298	49	1.85% (24)	0.00%	1.85% (24)	0.93% (12)	0.61% (8)	0.08% (1)	0.61% (8)
Adult	98	36	4.08% (4)	0.00%	4.08% (4)	2.04% (2)			2.04% (2)

[a] 16S, generic probe for 16S rRNA of *Borrelia* sp.; R57, specific probe for 16S rRNA of spirochetes similar to R57; SL, generic probe for 5S-23S rRNA of *B. burgdorferi* sensu lato; SS, specific probe for *B. burgdorferi* sensu stricto; GA, specific probe for *B. garinii*; AF, specific probe for *B. afzelii*; VA, specific probe for *B. valaisiana*.

[b] Percentages indicate the minimum infection, considering one positive tick per pool. Values in parentheses are the number of positive pools per number of ticks processed (1,298 nymphs or 98 adults).

DISCUSSION

The aim of this study was to identify potential reservoir hosts for *B. burgdorferi* sensu lato in areas previously described as being endemic for LD in northern Spain (9). Small mammals have been described as important reservoir hosts for *B. burgdorferi* sensu lato (21, 30, 41, 48, 58, 59, 78). However, in this study only one isolate (R57) was obtained from a *C. glareolus* EPB among the 108 animals processed by culture. This isolate had the same morphology by dark-field microscopy as other *B. burgdorferi* sensu lato species, and phylogenetic analyses based

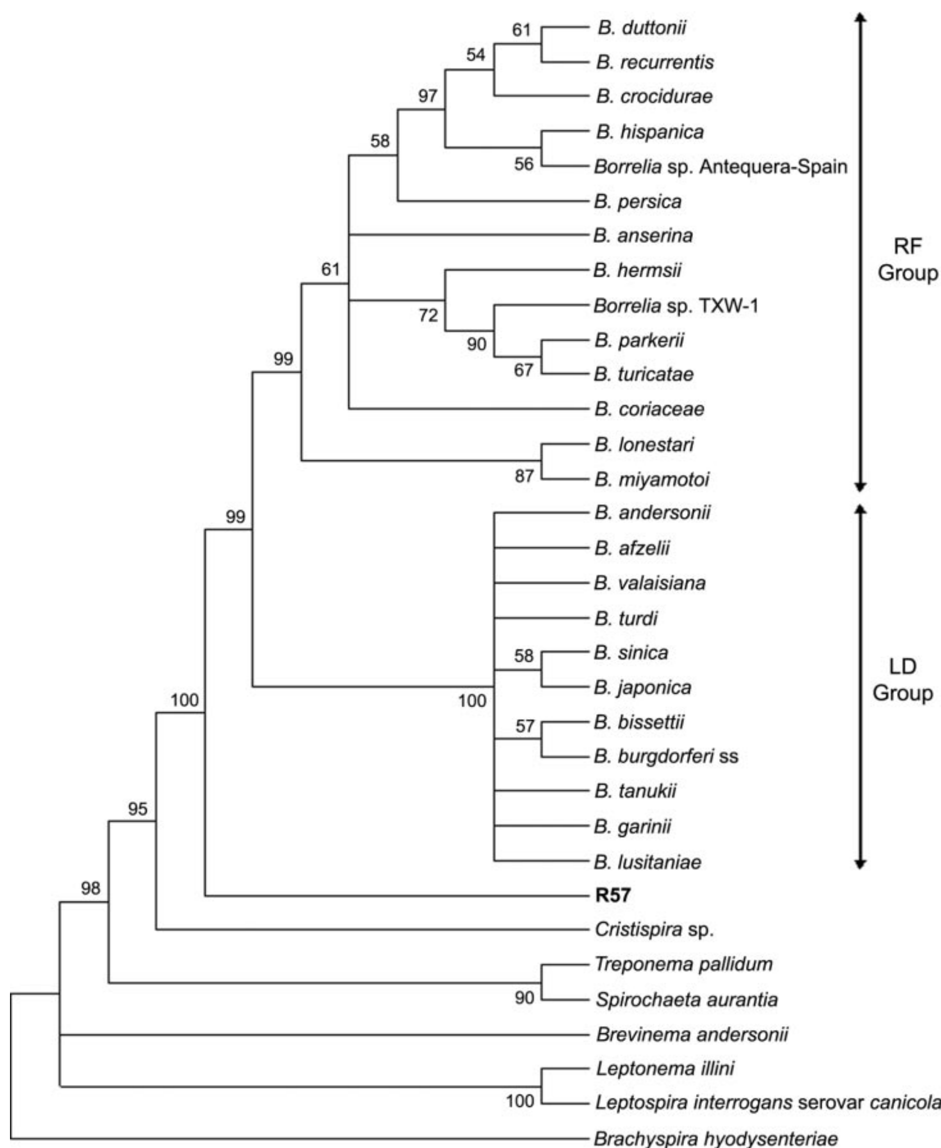

FIG. 3. Neighbor-joining phylogenetic tree based in 16S rRNA sequences of the different species of the order *Spirochetales* and R57. ss, sensu stricto.

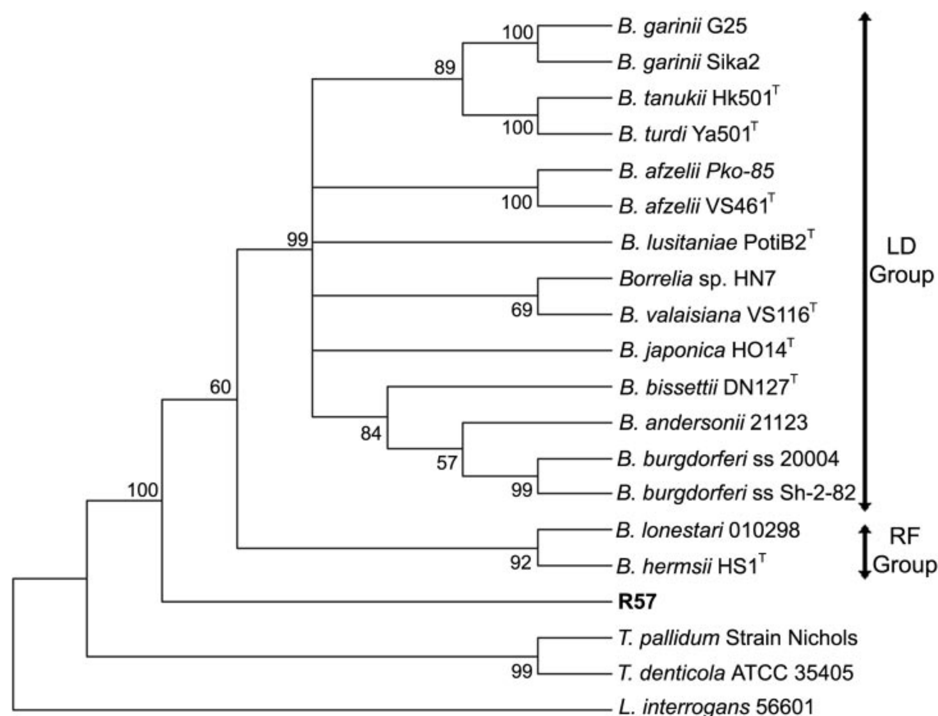

FIG. 4. Neighbor-joining phylogenetic tree based in *groEL* sequences of the different species of the order *Spirochetales* and R57. ss, sensu stricto.

on 16S rRNA and *groEL* placed it in a clade separate from that of LD and RF spirochetes. The position of R57 in both trees was identical, as an ancestor of both LD and RF *Borrelia*. Moreover, PCRs for 5S-23S rRNA, *ospA*, *flaB*, *rpoB*, and *p66* yielded negative results, suggesting abundant sequence differences between this agent and other *Borrelia* species.

Although the isolate grew well during the first passage, attempts for subculturing the organism with the same batch of medium were unsuccessful, as the spirochetes remained viable for about 5 days but without signs of multiplication. Changing the source for the serum in the BSK formulation did not improve the culture of R57. This is not surprising, considering that several *Borrelia* species are noncultivable, such as the relapsing fever *Borrelia* isolated from patients and *Ornithodoros erraticus* in southern Spain (3). Also, attempts to recover the agent by passages through C3H mice were done. For this, internal organs, EPB, and blood were cultured at different times, always with negative results by both culture and PCR. Also, spirochetemia was not detected in blood smears.

A probe for the 16S-PCR/RLB specific for this new spirochete (R57) was designed in this study and confirmed the presence of spirochetes similar to R57 in all the study areas and in three of the seven species of small mammals studied. Interestingly, this new agent was highly prevalent among the small mammals (8.5 to 12%), in the absence of *B. burgdorferi* sensu lato.

All the 16S rRNA sequences obtained from samples positive to the R57 probe were identical. Although the samples belong to different areas, all of them are geographically close to each other. The 16S rRNA sequence identity observed in each R57-positive animal from every region would indicate that organisms similar to R57 have a highly conserved 16S rRNA sequence in the 491-bp fragments analyzed.

All the species of *Borrelia* are transmitted by different species of ticks, with the exception of *B. recurrentis*, which is transmitted by the human body louse (77). Organisms similar to R57 do not seem to be transmitted by hard ticks (family *Ixodidae*) or at least not by *I. ricinus*. Spirochetes similar to R57 were not detected in questing ticks from the same areas where R57 was prevalent in small mammals. Moreover, the 284 molted ticks analyzed, which previously fed on the small mammals, were negative by culture and PCR, even those that fed on positive animals. Also, R57 is not species specific for small mammals, since different animal species were found infected. Taking into account that a wide repertoire of ectoparasites other than ticks were found infesting the small mammals studied, including fleas, chiggers, mites, and lice (data not shown), their possible role in the transmission of this new agent should be investigated. The method for detection of R57 developed in this study will enable us to complete the study of the ecology of this agent.

Small mammals have been described as important *B. burgdorferi* sensu lato reservoir hosts in Europe (30), but in contrast to the situation described for other European studies (21, 41, 48, 58, 59, 78), small mammals of different species were found to be infected just occasionally by *B. burgdorferi* sensu lato in this study. The prevalence of the infection by *B. burgdorferi* sensu lato was very low (0.5%; 1 of 184), even considering prevalence only in *A. sylvaticus* (0.8%; 1 of 130). Prevalence was also lower than the data obtained in other studies: 30 to 40% in Germany (47, 58), 25% in the United Kingdom (49), 17.5% in Switzerland (40), 47 to 0% in The Netherlands (21),

or 60% of mice and 23% of shrews in Sweden (78). Moreover, no transmission of *B. burgdorferi* sensu lato from small mammals to ticks that were feeding on them was demonstrated, which confirmed that *A. sylvaticus* at least is not acting as an important reservoir for LD *Borrelia* species in our area. This could be explained by the fact that *I. ricinus* nymphs scarcely infested them. A similar situation has been described in Ireland, where small mammals do not play an important role as reservoir hosts of *B. burgdorferi* sensu lato (32). The major reservoir hosts for *B. burgdorferi* sensu lato in the Basque country are still to be identified. The suitable candidates, with demonstrated roles as reservoirs elsewhere, are birds (39, 46, 68), dormice (56, 57), and squirrels (20, 38).

Four different genospecies have been found infecting questing ticks, the most abundant being *B. burgdorferi* sensu stricto, followed by *B. valaisiana* and *B. garinii*. *B. afzelii* was infrequently founding infecting questing ticks. This is different from other European studies (37, 70), especially those from Central Europe, where *B. afzelii* is highly prevalent. *B. afzelii* seems to be maintained in nature mainly by a tick-small mammal cycle that does not include the involvement of birds (36, 40, 66). The fact that small mammals are not playing a relevant role as reservoir hosts for *B. burgdorferi* sensu lato in the study areas may explain the low prevalence of *B. afzelii* found, similar to that in Ireland (32), where *B. valaisiana* was highly prevalent and birds seemed to play a relevant role as reservoir hosts for this genospecies (39, 50). *B. valaisiana* was also prevalent in our region, suggesting that birds may also play a role in maintaining this genospecies in our area.

Given the high prevalence of antibodies to *B. burgdorferi* sensu lato detected by WB in animals and the infrequent contact between these animals and *B. burgdorferi* sensu lato, we suggest that this seroprevalence is related to a cross-reactivity of R57 with *B. burgdorferi* sensu lato. An organism close to *Borrelia*, like R57, could induce cross-reacting antibodies, which would explain this high level of seroreactivity against *B. burgdorferi* sensu lato. Whether these antibodies play an immunoprophylactic role that could additionally account for the lack of circulation of LD *Borrelia* in small mammals from the Basque country deserves further study. A previous study in the same region showed a high level of seroprevalence against *B. burgdorferi* sensu lato in humans with a low prevalence of LD among the individuals investigated (4). This situation matches our data from the small mammals; spirochetes similar to R57 could be responsible for part of the high seroreactivity found among the population of the Basque country.

In conclusion, we have shown that although small mammals are not important reservoir hosts for *B. burgdorferi* sensu lato in areas of LD endemicity in the Basque country, they are maintaining a new spirochete species. This new species is widely distributed among small mammals within the study area; produces a disseminated infection that involves the skin, brain, and urinary bladder; and is thought to induce antibodies that cross-react with *B. burgdorferi* sensu lato. Signature nucleotides in the 16S rRNA sequence enabled us to design a diagnostic method that will be useful in determining the actual distribution of this agent in the area.

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