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Variability of *Bartonella* Genotypes among Small Mammals in Spain▽

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In order to study which *Bartonella* genotypes are circulating among small mammals in Spain, we analyzed the spleens of 395 animals from three different areas—247 animals from the Basque Country (northern Spain), 121 animals from Catalonia (northeastern Spain), and 27 animals from Madrid (central Spain)—by a triplex PCR combined with a reverse line blot previously described by our group. The prevalence of *Bartonella* was 26.8% (106/395), and in 4.8% (19/395) of the animals more than one *Bartonella* genotype was detected. The study of *gltA* and the intergenic transcribed spacer in the positive samples demonstrated a large diversity, allowing the assignation of them into 22 genotypes. The most prevalent genotypes were 2 and 3, which are closely related to *Bartonella taylorii*. In addition, nine genotypes were associated with specific mammal species. Genotypes close to the zoonotic *Bartonella grahamii*, *Bartonella elizabethae*, and *Bartonella rochalimae* were also detected. Ten genotypes showed a percentage of similarity with known *Bartonella* species lower than 96%, suggesting the presence of potential new species. Further studies of the impact of these pathogens on human health and especially in cases of febrile illness in Spain are strongly recommended. Furthermore, our method has been updated with 21 new probes in a final panel of 36, which represents a robust molecular tool for clinical and environmental *Bartonella* studies.

Bartonella spp. are Gram-negative facultative intracellular alphaproteobacteria that can infect the erythrocytes and endothelial cells of its hosts (10, 45). This zoonotic re-emerging pathogen has a complex cycle in nature, including different reservoir hosts and hematophagous arthropods that play a vector role (24, 45).

More than 30 different *Bartonella* species have been described thus far. In fact, after the implementation of more efficient molecular tools for detection, the number of new species is rapidly increasing. Moreover, four new species pathogenic for humans have been described in the last 3 years: *Bartonella rochalimae* (13), *B. melophagi* (38), *B. tamiae* (31), and "*Candidatus* Bartonella mayotimonensis" (36). The use of more accurate molecular tools will eventually identify additional *Bartonella* species causing human infection, taking into account that any *Bartonella* species can produce disease in humans, as has been hypothesized (36).

Among the different reservoir hosts described for *Bartonella*, small mammals are responsible for maintaining the highest number of species, as well as many others *Bartonella* detected but not yet named (18, 20, 21). Several of these rodent-*Bartonella* species have been linked with human disease. *B. grahamii* has been involved in ocular syndromes (27, 41), *B. elizabethae* has been detected in a case of endocarditis (9), *B. vinsonii* subsp. *arupensis* was identified in a farmer with fever and bacteremia (46) and also in a patient with endocarditis (14), and *B. washoensis* was isolated from a patient with fever and myocarditis (32).

Data on the role of small mammals as *Bartonella* reservoir hosts are scarce in Spain. In the Basque Country (northern Spain), ca. 20% of small-mammal blood smears presented structures compatible with *Bartonella* (16). In Andalusia (southern Spain), *B. tribocorum* was detected in 20% of the analyzed Norwegian rats (*Rattus norvegicus*) and two different *Bartonella* genotypes (GTs) close to *B. elizabethae* were found in 29% of the Algerian mice (*Mus spretus*) studied (39). However, no data are available about other small mammal species or regions in our country.

Environmental studies are essential for identifying which *Bartonella* species are circulating in a specific area and for evaluating their risk for humans. *Bartonella* is a fastidious culture bacterium that hospitals do not include in their routine studies; therefore, molecular detection is recommended. Recently, we have developed a versatile molecular tool that allows not only the detection of a positive sample but the identification the specific *Bartonella* species or GT, as well as potential new variants or new species (15). In the present study, small mammals captured from three different regions in Spain have been analyzed by this method, showing an enormous variability of *Bartonella* circulating among the small mammal populations.

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TABLE 1. Primers and probes used in the study[a]

Target	Organism	Primer	Probe	Sequence[b]	Concn (μM)[c]
ITS	*Bartonella* spp.	Bart/16-23F		5′-bio-TTG ATA AGC GTG AGG TCG GAG G	0.4
		Bart/16-23R		5′-bio-CAA AGC AGG TGC TCT CCC AG	0.4
	B. taylorii		**S-TAY2**	5′-a-TAT CCA TTT CSC TTA GGC A	3.2
	Bartonella spp.[d]		S-TALP	5′-a-CAG TCC CTT TAG GTC CAT TTA ATC	3.2
	Bartonella GT 8		**S-MUS1**	5′-a-GTC TAT TGG ATT TAA GTG TTG	0.4
	Bartonella GT 9		**S-MUS2**	5′-a-GGT CCG TTT GTT AAG TGT TGG	3.2
	Bartonella GT 21		**S-MUS3b**	5′-a-CTT TGC GAG ACT TTT TCA CTC C	0.4
	Bartonella GT 22		**S-TOPO15b**	5′-a-CTT AAC TTG TTG AAG GCT CCC	3.2
	Bartonella GT 6		**S-CE1MM3b**	5′-a-GAA CTC CAT ATA AAA GGC TTT AAA TAT TG	3.2
	Bartonella GT 14-15		**S-OR3MM1**	5′-a-AAT CAA ATT TAA GCA ATA CAA ATC	3.2
	Bartonella GT 16		**S-R2Eb**	5′-a-AGT GCC TTT GTT AGA GAA TAC C	3.2
	Bartonella GT 17		**S-OR3MM3**	5′-a-AAG AAT AAA AGT CAA AAT AAT ATT G	1.6
	Bartonella GT 18		**S-CAT9b**	5′-a-GTG TAT TAA ACG TAT CAA AGC CTC	3.2
	B. elizabethae		S-ELIZ	5′-a-TAA GTT CCC TTC AAG AGG ATA	3.2
	B. doshiae		S-DOSH	5′-a-TTT GAA CCT TCT CTC TTT AT	3.2
	Bartonella GT 19		**S-APO38**	5′-a- CCT TTT CTC CTT TTT AGG GGC	3.2
	B. grahamii		S-GRAH2	5′-a-ATT CAA GTT GAT GAA TTT GGT TAT	0.4
	Bartonella GT 12		**S-GU1MM1**	5′-a- TCA AAT TGG TGA ATC TGG TTA T	3.2
	Bartonella GT 13		**S-CAT6**	5′-a- TAA AGA GAA GTT TGT CCA AGA G	3.2
	Bartonella GT 20		**S-APS48**	5′-a-ATC ACT GAA AGT TGC TCT GAG T	1.6
	Bartonella GT 10		**S-R24Sh**	5′-a-GCT TTT CTG TTT GCC TGA GGT C	3.2
	B. tribocorum		S-TRIB	5′-a-TTC TAT TAA GTT TGT CAA AGG G	0.4
	B. phoceensis		**S-PHO**	5′-a-GAG AGA CGC TTT TCC CTT TGG	1.6
	B. rattimassiliensis		**S-RAT**	5′-a-CGG TGT TTT GAG GCA AAG TGC	1.6
	B. vinsonii subsp. *arupensis*		S-VIN-A1	5′-a-ACTTGTTGGAATTGCTTAACC	3.2
	B. vinsonii subsp. *vinsonii*		S-VIN-A2	5′-a-ATGAAAATATTGAGAGATTTG	3.2
	B. alsatica		S-ALS	5′-a-GCT GGT GAA ACT TGC TTA TA	6.4
	B. quintana		S-QUIN	5′-a-CGC TTA TCC ATT TGG TTT AA	3.2
	B. bacilliformis		S-BACI	5′-a-CCT ATG ATT GAT TTC TAG GC	0.4
	B. henselae		S-HENS	5′-a-ATC GGT TCA ATC ATA TCG CTT T	3.2
	B. clarridgeiae		**S-CLARR2**	5′-a-ACG ATG CTA AAA GTT GCT ATA TTG	3.2
	B. koehlerae		S-KOE	5′-a-TTA AAT TAT ATC ACT TTG GGT CAT ACG	0.4
	B. rochalimae		**S-ZOR**	5′-a-AAC AGG GAA AAG AGC AGG CCA	3.2
	Bartonella spp. detected in badger[d]		**S-TEJ**	5′-a-GAT GTT TTG TAA AAG TGC GTC G	3.2
	B. vinsonii subsp. *berkhoffii*		S-VIN-B	5′-a-TTT CGG ACA CTA TTG ATA AA	3.2
	B. bovis		S-BOV2	5′-a-CGT TTT GAT AGT CTT TTG TGT TGC	0.4
	B. chomelii, B. schoenbuchensis, B. capreoli, and B. birtlesii		S-CHOSCA	5′-a-TTA TGA TTG CTG ATA AGT TTG CTG	3.2
	O. grignonense		**S-GRIGNO**	5′-a- GCT TTG ATA AAT GTG ATA AGG	1.6
16S rRNA	*Bartonella* spp.	16S-R		5′-bio-GCC YCC TTG CGG TTA GCA CAG CA	1
		P24Emod		5′-bio-CCT TCA GTT MGG CTG GAT C	1
			S-BART16S	5′-a-CTC GCC CTT AGT TGC CAG CAT T	3.2
TCH synthase	*Cannabis sativa*	CI-F		5′-bio-ATG ATG CTG AGG GTA TGT CCT AC	1
		CI-R		5′-bio-GTT TTC TCC TCC ACC ACC ACG	1
			S-CI2	5′-a-GTG GAC ACT TTA GTG GAG GAG G	3.2
gltA	*Bartonella* spp.	**GLTAF2**		5′-GCT TTK CTG TTC CDT GTG AAG	1
		GLTAR2		5′-GCA AAA AGA ACA GTA AAC ATT TC	1
		GLTAF1		5′-AAA ATG CTA CAA GAA ATH GG	1
		GLTAR1		5′-AGC TTT TAA TGT AAT DCC DG	1

[a] The probes and primers in boldface were designed for this study. The rest of the oligonucleotides have been described previously (15).
[b] Oligonucleotide modifications at the 5′ end. bio, biotin; a, amino link.
[c] That is, the concentration of the oligonucleotides used in the assays.
[d] Garcia-Esteban et al. (15).

MATERIALS AND METHODS

Small mammals sampling. Small mammals were captured in three different regions of Spain: the Basque Country (northern Spain), Catalonia (northeastern Spain), and Madrid (central Spain). The animals were anesthetized with ketamine hydrochloride (Imalgene, Merial, France), 10 mg/kg administered intramuscularly, and euthanized in a CO_2 chamber. Spleen samples were obtained from them and kept at −80°C until they were tested. The animals were classified by external morphological data and skull features (4).

***Bartonella* detection in small mammals.** DNA was extracted from spleen samples with the QIAmp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the molecular detection of *Bartonella*, between 100 and 300 ng of DNA was amplified by a multiplex PCR, and the obtained amplicons were identified by reverse line blotting (PCR/RLB), as previously described (15). This method targets simultaneously the 16S rRNA for the generic detection of any *Bartonella*, the intergenic transcribed spacer (ITS) 16S-23S rRNA for species identification, and an internal control for the detection of potential PCR inhibitors.

Since new ITS sequences were identified in the present study, 16 probes were designed for their proper detection in the PCR/RLB. Moreover, three new probes were added to the method for the recently described species *B. rattimassiliensis*, *B. phoceensis*, and *B. rochalimae*, and two probes (S-TAY2 and S-CLAR2) were modified for efficiency purposes. A list of primers and probes for the PCR/RLB is shown in Table 1.

The specificity of the new set of probes was checked with 10^2 genome equivalents (GE) of different *Bartonella* species or 10^2 plasmid copies of the new ITS (PCITS) found in the study (Table 1). These latter controls were built by cloning the ITS amplicons of interest with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Further characterization of the positive samples. Two PCRs targeting the ITS and *gltA* were performed with the positive samples. In the case of the ITS, the PCR was performed by reamplifying 2 μl of the initial multiplex PCR product in a 50-μl reaction volume with 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 200 μM concentrations of each deoxynucleoside triphosphate (Promega, Madison, WI), 1.5 U of *Taq* Gold DNA polymerase (Applied Biosystems, Branchburg,

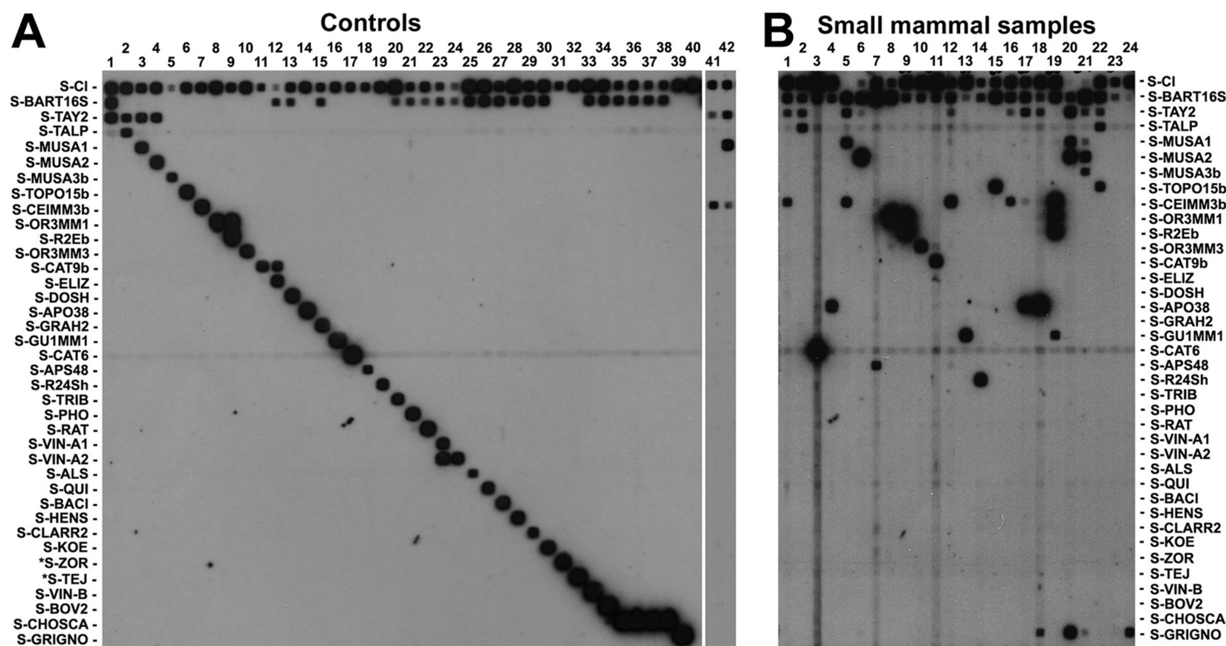

FIG. 1. Reverse line blotting results. (A) Hybridization signal obtained with different controls. Lanes: 1, 10^2 GE *B. taylorii* CIP 107028; 2, 10^2 plasmid copies with the insert ITS (PCITS) *Bartonella* spp. from a Spanish mole (15); 3, 10^2 PCITS from GT 8; 4, 10^2 PCITS from GT 9; 5, 10^2 PCITS from GT 21; 6, 10^2 PCITS from GT 22; 7, 10^2 PCITS from GT 6; 8, 10^2 PCITS from GT 14; 9, 10^2 PCITS from GT 16; 10, 10^2 PCITS from GT 17; 11, 10^2 PCITS from GT 18; 12, 10^2 GE *B. elizabethae* CIP 103761; 13, 10^2 GE *B. doshiae* CIP 107026; 14, 10^2 PCITS from GT 19; 15, 10^2 GE *B. grahamii* CIP 107024;16, 10^2 PCITS from GT 12; 17, 10^2 PCITS from GT 13; 18, 10^2 PCITS from GT 20; 19, 10^2 PCITS from GT 10; 20, 10^2 GE *B. tribocorum* CIP 105476; 21, 10^2 GE *B. phoceensis* CIP 107707; 22, 10^2 GE *B. rattimassiliensis* CIP 107705; 23, 10^2 GE *B. vinsonii* subsp. *arupensis* CIP 106848; 24, 10^2 GE *B. vinsonii* subsp. *vinsonii* CIP 103738; 25, 10^2 GE *B. alsatica* CIP 105477; 26, 10^2 GE *B. quintana* CIP 103739; 27, 10^2 GE *B. bacilliformis* CIP: 77.27; 28, 10^2 GE *B. henselae* CIP 103737; 29, 10^2 GE *B. clarridgeiae* CIP 104772; 30, 10^2 GE *B. koehlerae* CIP107025; 31, 10^2 PCITS *B. rochalimae* from a Spanish fox (15); 32, 10^2 PCITS *Bartonella* spp. from a Spanish badger (15); 33, 10^2 GE *B. vinsonii* subsp. *berkhoffii* CIP 104960; 34, 10^2 GE *B. bovis* CIP 106692; 35, 10^2 GE *B. schoenbuchensis* CIP 107819; 36, 10^2 GE *B. capreoli* CIP 106691; 37, 10^2 GE *B. chomelii* CIP 107869; 38, 10^2 GE *B. birtlesii* CIP 106294; 39, 10^2 PCITS *Ochrobactrum* sample PVR70-01Sh; 40, negative PCR control; 41, 10^2 PCITS from GT 2; 42, 10^2 PCITS from GT 4. Lanes where clones were tested have no hybridization signals with the S-BART16S probe because the control only contains the ITS target. (B) Examples of hybridization reactions with several small mammal samples.

NJ), and 1 μM Bart/16-23F and Bart/16-23R primers (Table 1). PCR cycling consisted of an initial denaturing step of 9 min at 94°C, followed by 40 cycles of 30 s at 94°C, 1 min at 64.3°C, and 1 min 30 s at 72°C, with a final elongation step of 7 min at 72°C.

In the case of *gltA*, 100 to 300 ng of DNA was amplified with a nested PCR designed in the present study. First, the primers GLTAF2 and GLTAR2, which amplify a 531-bp fragment, were used. Then, 2 μl of the product was reamplified with the primers GLTAF1 and GLTAR1, yielding a final fragment of 300 bp. Both reactions were performed under the same conditions described above for the ITS, except for the annealing temperature, which was 50°C in both PCR cycles.

For positive samples whose hybridization pattern in the RLB was different from the ones obtained with the controls (Fig. 1A) and suggested the presence of more than one *Bartonella* GT, the ITS amplicons were cloned with the TOPO TA cloning kit (Invitrogen), and 10 clones were analyzed by PCR/RLB. Coinfection was confirmed in samples whose clones showed a hybridization pattern, which explains the hybridization results observed in the original sample.

Sequencing and analysis. PCR products were run in 1% low-melting agarose (Pronadisa, Torrejón de Ardoz, Spain), and the bands of interest were purified by using the QIAquick gel extraction kit (Qiagen) and sequenced with the Big-Dye terminator cycle sequencing kit (Applied Biosystems, Branchburg, NJ) according to the manufacturer's instructions.

ITS and *gltA* sequences were aligned with reference sequences from GenBank (http://www.ncbi.nlm.nih.gov) with the Multi-Alignment Fast Fourier Transform (MAFFT) method (26). Pairwise distance matrices for the aligned sequences were determined by using the Kimura two-parameter method (28) with MEGA4 software (42), and phylogenetic trees were constructed applying the neighbor-joining algorithm (7) with the internal-branch test for evaluation of their topology. Dendrograms were collapsed by using a cutoff bootstrap value of 50. The percent similarities between the *gltA* and ITS sequences identified here and the type strain sequences of each *Bartonella* species were calculated using the MEGA4 software. Sequences were also analyzed by BLAST (1; http://blast.ncbi.nlm.nih.gov/blast.cgi) to identify the closest relative.

Nucleotide sequence accession numbers. The sequences obtained in the present study have been deposited in GenBank under the following accession numbers (ITS, *gltA*): GT 1 (HM596431, HM596455), GT 2 (HM596432, HM596466), GT 3 (only ITS HM596433), GT 4 (HM596434, HM596454), GT 5 (only ITS HM596435), GT 6 (HM596436, HM596462), GT 7 (HM596437, HM596469), GT 8 (HM596438, HM596461), GT 9 (HM596439, HM596457), GT 10 (HM596440, HM596458), GT 11 (only *gltA* HM596453), GT 12 (HM596441, HM596463), GT 13 (HM596442, HM596467), GT 14 (only ITS HM596443), GT 15 (HM596444, HM596465), GT 16 (HM596445, HM596452), GT 17 (HM596446, HM596464), GT 18 (HM596447, HM596468), GT 19 (HM596448, HM596460), GT 20 (HM596449, HM596459), GT 21 (HM596450, HM596456), GT 22 (only ITS HM596451), and sample PVR71-02Sh (only ITS HM596470).

RESULTS

Presence of *Bartonella* spp. in small mammals. We analyzed 395 animals (Table 2) captured in three different regions of Spain: 247 from the Basque Country, 121 from Catalonia, and 27 from Madrid. Small mammals were assigned to nine different species (Table 2), with *Apodemus sylvaticus* (wood mouse) the species with the highest number of studied animals (220/395, 55.7%).

A total of 26.8% (106/395) of the small mammals were

TABLE 2. Presence of *Bartonella* GTs in small mammals

Probe	GT[a]	No. of positive small mammal species (% positive)[b]									
		As (n = 220)	Af (n = 3)	Mg (n = 16)	Mdo (n = 34)	Ms (n = 30)	Sc (n = 14)	Mde (n = 1)	Cr (n = 56)	Te (n = 21)	Total (n = 395)
16S rRNA	Generic *Bartonella*	69 (31.4)	2 (66.7)	3 (18.8)	3 (8.8)	1 (3.3)	5 (35.7)		8 (14.3)	15 (71.4)	106 (26.8)
S-TAY	GT 1	2 (0.9)									2 (0.5)
S-TAY + S-CE1MM3b	GT 2/3	27 (12.3)	1 (33.3)	1 (6.3)							29 (7.3)
S-TAY + S-MUS1 + S-CEIMM3b	GT 4/5	6 (2.7)		1 (6.3)	1 (2.9)						8 (2.0)
S-TAY + S-MUS1	GT 8	7 (3.2)									7 (1.8)
S-TAY + S-TALP	GT 7				2 (5.9)					13 (61.9)	15 (3.8)
S-CE1MM3b	GT 9	1 (0.5)									1 (0.3)
S-CAT6	GT 13								7 (12.5)		7 (1.8)
S-APO38	GT 19	4 (1.8)									4 (1.0)
S-TAY + S-MUS2	GT 9						1 (7.1)				1 (0.3)
S-APS48	GT 20	2 (0.9)									2 (0.5)
S-OR3MM1	GT 14/15	2 (0.9)									2 (0.5)
S-OR3MM1+S-R2EB	GT 16	2 (0.9)									2 (0.5)
S-OR3MM3	GT 17	1 (0.5)									1 (0.3)
S-CAT9+S-ELIZ	GT 18					1 (3.3)					1 (0.3)
S-GU1MM1	GT 12	1 (0.5)									1 (0.3)
S-R24SH	GT 10								1 (1.8)		1 (0.3)
S-TOPO15b	GT 22									1 (4.8)	1 (0.3)
S-TAY + S-CE1MM3b + S-APO38	GT 2/3 and GT 19*	7 (3.2)	1 (33.3)	1 (6.3)							9 (2.3)
S-TAY + S-MUS1 + S-CEIMM3b + S-APO38	GT 4/5 and GT 19*	2 (0.9)									2 (0.5)
S-TAY + S-CE1MME3b + S-APS48	GT 2/3 and GT 20*	1 (0.5)									1 (0.3)
S-TAY + S-MUS1 + S-CE1MM3b + S-APS48	GT 4/5 and GT 20*	1 (0.5)									1 (0.3)
S-OR3MM1 + S-RE2B + GU1MM1	GT 12 and GT 16*	1 (0.5)									1 (0.3)
S-TAY + S-MUS1+ S-MUS2	GT 8 and GT 9 *						3 (21.4)				3 (0.8)
S-TAY + S-MUS1+ S-MUS2+ S-MUS3	GT8, GT 9, and GT2*						1 (7.1)				1 (0.3)
S-TAY + S-TALP + S-TOPO15b	GT 7 and GT 22*									1 (4.8)	1 (0.3)
No signal with ITS probes	*Bartonella* spp.	2 (0.9)									2 (0.5)
S-GRIGNO	*O. grignonense*	12 (5.5)			5 (14.7)		3 (21.4)				20 (5.1)

[a] That is, the genotype (GT) associated with each combination of hybridization signals obtained in the RLB. GTs 2/3, 4/5, and 14/15 cannot be distinguished by RLB. *, the presence of coinfections was checked by clones (see Materials and Methods).

[b] Small mammal species: As, *Apodemus sylvaticus* (wood mouse); Af, *A. flavicollis* (yellow-neck mouse); Mg, *Myodes glareolus* (bank vole); Mdo, *Mus domesticus* (domestic mouse); Ms, *M. spretus* (Algerian mouse); Sc, *Sorex coronatus* (Millet shrew); Mde, *Microtus duodecimcostatus* (Mediterranean pinevole); Cr, *Crocidura russula* (common shrew); Te, *Talpa europaea* (mole). The number of animals studied (n) is indicated in parentheses with each abbreviation.

TABLE 3. GTs identified in small mammals

GT	*Bartonella* species (% similarity)[a]		Reactivity with the ITS probes	
	gltA	ITS	Pattern	Lane[b]
1	*B. vinsonii* subsp. *arupensis* (93.5)	*B. taylorii* (98.9)	S-TAY	1
2	*B. taylorii* (94.2)	*B. taylorii* (97.8)	S-TAY + S-CE1MM3b	41
3	ND	*B. taylorii* (93.5)	S-TAY + S-CE1MM3b	NS
4	*B. taylorii* (94.6)	*B. taylorii* (97.8)	S-TAY+ S-MUS1+ S-CE1MM3b	42
5	ND	*B. taylorii* (98.4)	S-TAY+ S-MUS1+ S-CE1MM3b	NS
6	*B. vinsonii* subsp. *arupensis* (94.5)	*B. taylorii* (99.0)	S-CE1MM3b	7
7	*B. vinsonii* subsp. *vinsonii* (95.0)	*B. taylorii* (73.9)	S-TAY + S-TALP	2
8	*B. vinsonii* subsp. *vinsonii* (93.8)	*B. taylorii* (91.0)	S-TAY + S-MUS1	3
9	*B. vinsonii* subsp. *arupensis* (91.9)	*B. taylorii* (90.2)	S-TAY + S-MUS2	4
10	*B. birtlesii* (91.9)	*B. vinsonii* subsp. *berkhoffii* (67.9), *B. cooperplainsense* (67.0)	S-R24Sh	19
11	*B. birtlesii* (100.0)	ND	NA[c]	NA
12	*B. grahamii* (100.0)	*B. grahamii* (98.8)	S-GUIMM1	16
13	*B. tribocorum* (95.8)	*B. elizabethae* (77.4), *B. tribocorum* (77.1)	S-CAT6	17
14	ND	*B. elizabethae* (89.3), *B. grahamii* (89.3)	S-OR3MM1	8
15	*B. elizabethae* (97.3)	*B. elizabethae* (89.6), *B. grahamii* (89.6)	S-OR3MM1	NS
16	*B. elizabethae* (96.9)	*B. elizabethae* (85.3)	S-OR3MM1+S-R2Eb	9
17	*B. elizabethae* (96.9)	*B. grahamii* (91.9)	S-OR3MM3	10
18	*B. elizabethae* (97.3)	*B. elizabethae* (95.0)	S-ELIZ+S-CAT9	11
19	*B. doshiae* (96.2)	*B. doshiae* (89.6)	S-APO38	14
20	*B. rochalimae* (95.8)	*B. rochalimae* (65.4)	S-APS48	18
21	*B. rochalimae* (96.2)	*B. rochalimae* (71.5)	S-MUS3b	5
22	ND	*B. tribocorum* (73.5), *B. rochalimae* (73.0)	S-TOPO15b	6

[a] The highest percent similarity found with the indicated *Bartonella* species is indicated in parentheses. ND, not determined (a sequence could not be obtained).
[b] The corresponding lane in Fig. 1A with an example of this hybridization pattern. NS, not shown.
[c] NA, not applicable.

infected with *Bartonella* according to the PCR/RLB results (Table 2). All of the animal species were found to be infected except for the one specimen of *Microtus duodecimcostatus* (Mediterranean pine vole). By animal species, the percentage of infection ranged from 3.3% (1/30) in *Mus spretus* (Algerian mouse) to 71.4% (15/21) in *Talpa europaea* (mole).

Initial identification of *Bartonella* GTs in small mammals. The reactivity observed in the PCR/RLB with the ITS-specific probes of our initial panel (15) identified 64 (60.4%) animals that presented hybridization with S-TAY (probe for *B. taylorii*), 16 (15.1%) animals with both S-TAY and S-TALP, and 26 (25.4%) small mammals that presented hybridization only with the generic probe for 16S rRNA. This latter hybridization pattern suggested the presence of potential new species or variants in these 26 samples, different from those included in our initial panel of probes.

Sequence analysis of the positive samples. From the 106 positive samples, 63 (59.4%) were further characterized by sequencing the ITS and the *gltA*. These samples were selected according to the hybridization pattern and the small mammal species from which they were obtained. Analyzing the sequences, 22 *Bartonella* GTs were assigned in the present study with correlative numbers (Table 3). The percentage of similarity of the *gltA* sequence (260 bp, excluding the primers) with known *Bartonella* type strains ranged from 91.9 to 100%. GT 9 (91.9% with *B. vinsonii* subsp. *arupensis*) and GT 10 (91.9% with *B. birtlesii*) showed the lowest values (Table 3). Moreover, some remarkable hits identified by BLAST were as follows. GTs 15 to 17 showed 99 to 100% similarity to *Bartonella* from fleas in Portugal (AY877422 and AY877423), GTs 20 and 21 showed 97 to 98% similarity to voles from western Siberia (EF682090), GT 10 showed 97% similarity to a *Bartonella* strain from a small mammal captured in equatorial Africa (FJ851111), GT 8 showed 98% similarity to a *Bartonella* strain from a *Sorex araneus* in the United Kingdom (EF031548) and, finally, GT 13 showed a similarity of 98% to a *Bartonella* strain from a *Suncus murinus* in China (FJ464239). In the phylogenetic analysis, these *gltA* sequences were placed along the dendrogram in seven different clades that we designated 1 to 7: *B. taylorii*/"*Candidatus* Bartonella mayotimonensis", *B. birtlesii*, *B. doshiae*, *B. grahamii*, *B. tribocorum*, *B. elizabethae*/*B. rattimassiliensis*, and *B. clarridgeiae*/*B. rochalimae*, respectively (Fig. 2).

Overall, the ITS showed a lower percentage of similarity among type strains, compared to *gltA* sequences, ranging from 67 to 99% (Table 3). Apart from the more similar *Bartonella* described species, some high-similarity hits were found by BLAST. GTs 13 to 17 showed 97 to 99% similarity to *Bartonella* from an *M. spretus* captured in southern Spain (EU218552), GTs 20 to 22 showed 75 to 89% similarity to a *Bartonella* from a vole captured in western Siberia (EF682087), GT 6 showed 100% similarity to a *Bartonella* from an *A. flavicollis* from Slovenia (DQ155391), GT 19 had 99% similarity to a *Bartonella* from an *A. sylvaticus* from the United Kingdom (AJ269792) and, finally, GT 9 showed 98% with a *Bartonella* from an *S. araneus* captured in the United Kingdom (EF031550). In the ITS dendrogram the sequences were placed in six clades (A to F). Although the distribution of some of the GTs was similar compared to the *gltA* dendrogram (Fig. 2), like GTs located in the *B. taylorii*, *B. doshiae*, and *B. rochalimae* clades, the other GT had a different position in the dendrogram.

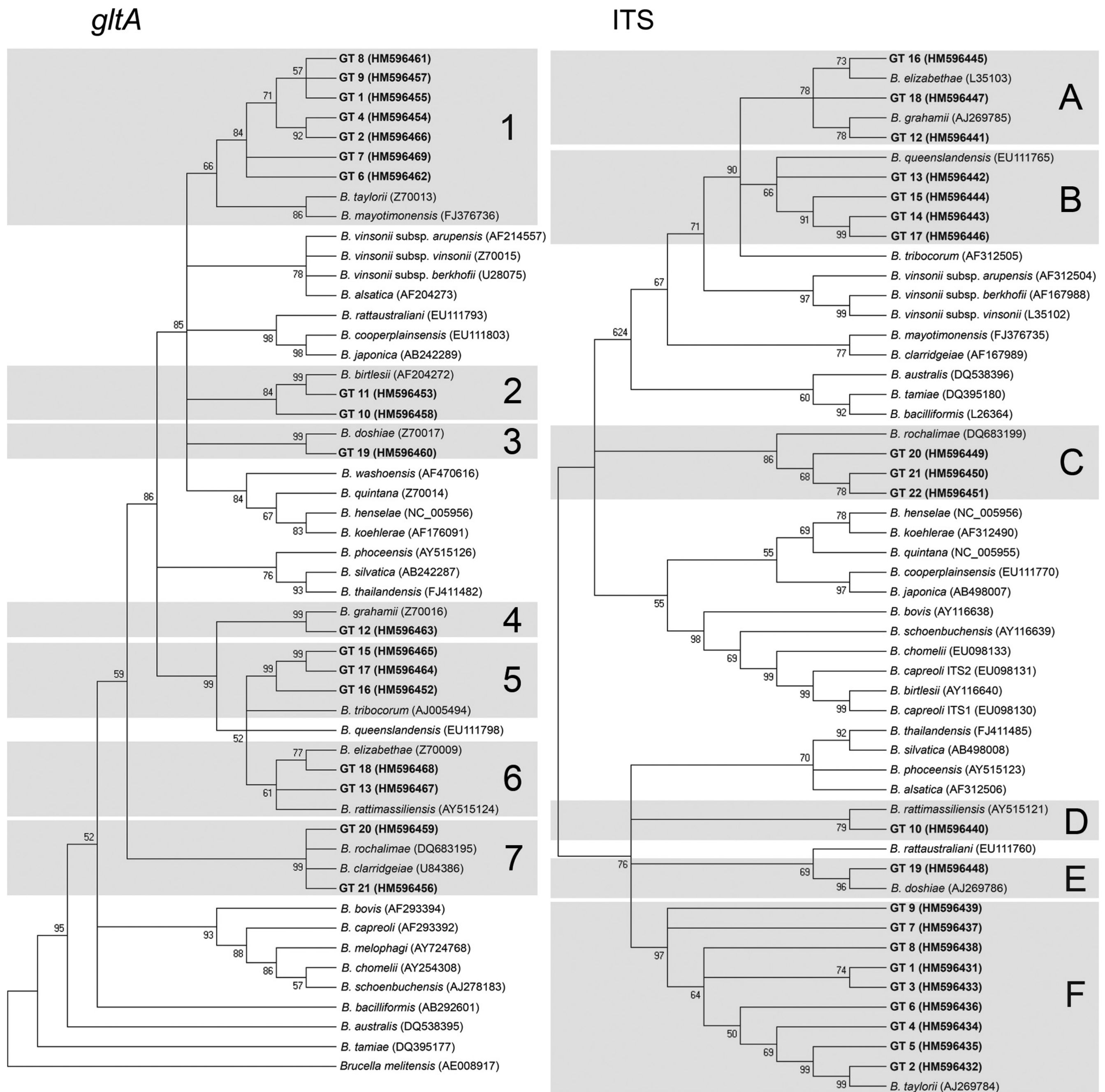

FIG. 2. Phylogenetic relationships of *Bartonella* spp. detected in this study. Dendrograms of *gltA* and ITS were built with reference sequences obtained from GenBank. The accession numbers are indicated in parentheses. The clades found in the study are shaded.

Identification of *Bartonella* GTs in small mammals with the new probes. Based on these preliminary results we designed 21 ITS probes, indicated in Materials and Methods, for improving the RLB (Table 1, probes in boldface) and distinguishing the GTs identified above. To check the specificity of the updated method, the 36 ITS probes were tested against genomic DNA from different *Bartonella* GTs, as well as with the cloned controls, with the purpose of determining the accuracy of their differentiation by hybridization (Fig. 1A). We were able to differentiate all of the GTs detected in the study except GTs 2 and 3, GTs 4 and 5, and GTs 14 and 15 that shared the same hybridization pattern (Table 3); therefore, these will be referred from now on as GTs 2/3, 4/5, and 14/15, respectively, since they cannot be differentiated by PCR/RLB.

Afterward, all of the samples (n = 395) were tested again with the updated method. An example of these results is shown in Fig. 1B. The most frequently found *Bartonella* were GTs 2/3, detected in 10.1% (40/395) of the animals, followed by GT 7 in 4.1% (16/395) and GT 19 in 3.8% (15/395) of the studied animals (Table 2). On the other hand, some *Bartonella* GTs

were quite infrequent, such as GT 10, GT 17, or GT 18, which were detected in only one specimen each. Moreover, 4.8% (19/395) of the animals showed coinfections with at least two different *Bartonella* GTs, which were assessed by analysis of the cloned PCR products and PCR/RLB. The most common combinations were GTs 2/3 and 19, detected in 2.3% (9/395) of the animals (Table 2).

The small mammal species that presented a larger variability of *Bartonella* was *A. sylvaticus*. Interestingly, some GTs were found to be associated with some small mammal species such as GTs 9 and 21 with *Sorex coronatus*, GT 18 with *M. spretus*, GTs 10 and 13 with *Crocidura russula*, GTs 7 and 22 with *T. europaea*, or GTs 2 to 5 and 19 with *Apodemus* spp. and *Myodes glareolus* (Table 2).

The updated method with the new probes designed in the present study allowed us to perfectly identify all of the samples, including the 26 positive samples that initially reacted only with the generic probe S-BART16S, except for two animals. A further characterization of these specimens was performed, although only in one of them (GT 11) was it possible to sequence *gltA*, showing a perfect match with *B. birtlesii* (Table 3 and Fig. 1B, lane 23).

Finally, five small mammals presented only a faint hybridization signal with the S-BART16S probe (Fig. 1B, lane 24). However, they were not considered *Bartonella*-infected animals because the ITS sequence, identical in all of them (GenBank accession no. HM596470) showed a 99% similarity with the ITS of *Ochrobactrum grignonense* (AJ242581). Therefore, a specific probe for this ITS was designed (Table 1) and used in the updated method. This microorganism was detected in 5.1% (20/395) of the animals and specifically in 14.7% (5/34) of *Mus domesticus* (Table 3).

DISCUSSION

The use of molecular tools in epidemiological studies is essential for a fastidious culture bacterium such as *Bartonella*. The method designed previously by us (15) has been updated with 21 ITS new probes in a final panel of 36, allowing the detection of new variants and potentially new species among the small mammals that could not be identified by a traditional approach. Only two samples could not be properly identified by this method, probably due to the low number of the microorganisms present in the samples. Using this method, we determined which *Bartonella* GTs are circulating in the small mammal populations in three areas of Spain.

Bartonella spp. had infected 26.8% of the animals studied. This percentage is similar to the values found in other studies: 26% in Japan (22), 28% in Denmark (12) and Spain (39), 30% in Greece (43) and France (17), and 31% in Poland (44).

As has been shown in the studies described above, the variability of *Bartonella* GTs in small mammals is large, and the detection of potentially new species is frequent. Likewise, we have identified 22 different GTs in our study, which were distributed along the *gltA* dendrogram in seven different clades, which represents a high variability. Moreover, it has been proposed that a similarity percentage of less than 96% of a *gltA* fragment of 321 bp suggests the presence of a new species (34). In our study, although the *gltA* fragment analyzed was slightly smaller, 10 GTs had a percent similarity of less than 96%. The isolation and further characterization of these GTs will allow us to determine their exact taxonomic positions.

In the case of the ITS, the percent similarity to the described *Bartonella* species was lower compared to the *gltA*, as was previously found (34). It is known that the ITS hypervariability prevents the accurate alignment of the sequences (3), and the ITS phylogenetic information should be interpreted with caution. However, this hypervariability allowed us to design highly specific probes to differentiate the *Bartonella* GTs.

B. grahamii and *B. taylorii* are well distributed in Eurasia. However, in some countries, such as Sweden (19), China (37), or Japan (22), *B. grahamii* is the predominant species, whereas *B. taylorii* is predominant in Poland (44) or Greece (43). In the present study, GTs 2/3, which are closely related to *B. taylorii*, were the most frequently detected *Bartonella* GTs.

The percentage of infection in moles (*T. europaea*) was the highest among all of the small mammals studied, with GT 7 being the most prevalent GT in this species. This is the first study on the prevalence of *Bartonella* in moles. *B. talpae* is the only species that has been associated with this animal species until now (45). However, no type strain or molecular data are available from this species, and GT 7 cannot yet be assigned to this *Bartonella* species until a type strain is isolated and well characterized.

GTs 15 to 18, which were closely related to *B. elizabethae*, were found less frequently infecting small mammals in the present study. Interestingly, they presented a high similarity with *Bartonella* identified in rodent fleas from Portugal (11) and *M. spretus* in Andalusia, southern Spain (39), indicating that these GTs are well distributed in the Iberian Peninsula. However, the percentage of infection in *M. spretus* was quite low (3%) compared to the percentage found for this species in Andalusia (29%). This was probably due to a more efficient cycle of transmission in southern Spain than in the rest of the country, although more studies are needed to confirm this point.

Whereas some GTs (2/3, 4/5, or 19) were detected in several animal species, other GTs (10, 12, 13, 18, 19, or 22) were only found in one species. Some of these associations have already been proposed in other studies. Indeed, some of our GTs demonstrated a high similarity to GTs detected in the United Kingdom in shrews (6, 19) or in Algerian mice in Spain (39). This host association could be related to the higher copy number of genes for putative host-adaptability factors that have been identified in the genome of *B. grahamii* (2, 40) and are likely to be present in other rodent *Bartonella* strains. Another explanation could be the existence of restricted cycles between some vectors and animals, such as a GT transmitted by a specific arthropod species. A previous study in the Basque Country (16) found an association between the *Palaelopsylla soricis* flea and the *Echinonyssus soricis* mite with *S. coronatus* and the *Polyplax reclinata* louse with *C. russula* (16). These associations could be responsible for the transmission of the specific GTs found in shrews. In contrast, also in the Basque Country, other vectors, such as the *Ctenophtalmus baeticus avernus* flea, were less host specific (16) and could be responsible of the transmission of *Bartonella* GTs present in different small mammals such as GTs 2/3. In fact, *Ctenophtalmus nobilis* is a competent vector for *B. taylorii* (closely related to GTs 2/3) and *B. grahamii* (5). Additional studies are needed to confirm

the role of these arthropods as vectors in the transmission of these GTs.

In the present study, 4.8% of the studied animals were infected with more than one *Bartonella* GT. We have been able to detect these coinfections by using the PCR/RLB we have designed. The presence of coinfection has been observed in different studies performed in southern Spain (39), the United Kingdom (3, 5), the United States (30, 33), and Japan (22), and it seems to be a common event in small-mammal populations. Also, subsequent infection with different species has been observed in nature (3, 30). Although it has been shown that there is cross serological reactivity between *Bartonella* species, there is a lack of heterologous protection between *B. henselae* and *B clarridgeiae* or even between different *B. henselae* serotypes in cats (47), explaining subsequent *Bartonella* infections or the possibility of coinfections, such as those we observed in small mammals.

A surprising finding was the high prevalence of a bacterium closely related to *O. grignonense* in the studied animals. This species is an environmental microorganism (35) which is phylogenetically close to the recently described species *O. pseudogrignonense* and *O. haematophilum* that can infect humans (25). The potential zoonotic role of the agent detected in the present study is unknown, although it can infect small mammals. Our design of a specific probe for this microorganism could be useful for future clinical and environmental surveys. In addition, since the *Ochrobactrum* genus is closely related to *Bartonella*, the use of 16S rRNA as a target in *Bartonella* studies can produce false positives and overestimate the real prevalence of this bacterium and should be carefully surveyed in environmental studies using only this target.

Bartonella is an emerging zoonotic pathogen; the number of *Bartonella* species implicated in human disease is increasing rapidly. Apart from the rodent-*Bartonella* zoonotic species *B. grahamii*, *B. elizabethae*, *B. washoensis*, and *B. vinsonii* subsp. *arupensis*, there is serological evidence of rodent-associated *Bartonella* involved in febrile illness in the southwestern United States (23), among intravenous drug users (8) and, more recently, in patients with febrile illness in Thailand, where *Bartonella* spp. closely related to *B. elizabethae*, *B. rattimassilensis*, and *B. tribocorum* (29) were detected. In our study, GTs close to the zoonotic *B. grahamii*, *B. elizabethae*, and *B. rochalimae* have been detected. These data call for performing additional studies to determine the impact of these pathogens on human health and especially in the case of febrile illness for which no etiological agent has been identified.

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