

Distribution of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* (Acari: Ixodidae) Ticks from the Basque Country, Spain

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ABSTRACT *Borrelia burgdorferi* was found widespread in ixodid ticks from the Basque Country (Spain) during a two-step study. In the first part, a total of 7,835 ixodids of eight different species was collected from vegetation, classified, and processed using polymerase chain reaction (PCR) for detection of *B. burgdorferi* ospA DNA. *B. burgdorferi* DNA was detected in $\leq 12.5\%$ of adults and $\geq 0.6\%$ of *Ixodes ricinus* (L., 1758) nymphs (mean 1.5 and 0.05%, respectively), and in $\leq 14.3\%$ of adult *Hemaphysalis punctata* (Canestrini & Fanzago, 1877) analyzed (mean 1.2%). The second part of the study was undertaken 2 yr later to characterize *B. burgdorferi* distribution by focusing on the areas where *I. ricinus* was the predominant species. Ten areas were selected from which 1,535 nymphs and adults of *I. ricinus* were collected and processed by PCR and culture techniques. Infected ticks were found in all zones. *B. burgdorferi* DNA was detected in a mean of 9.3 and 1.5% of adults and nymphs, respectively. Nine isolates of *B. burgdorferi* were obtained, belonging to four different genospecies (*B. burgdorferi* sensu stricto, *B. garinii*, *B. valaisiana*, and *B. lusitaniae*). The results indicate that some areas of Spain have a potential risk for Lyme disease agent exposure and that *B. burgdorferi* appears to have an increasing occurrence in ticks in the Basque Country.

KEY WORDS *Borrelia burgdorferi* sensu lato, *Ixodes ricinus*, risk index, Spain, Basque Country

LYME BORRELIOSIS, a multisystemic disorder caused by *Borrelia burgdorferi* sensu lato (Burgdorfer et al. 1982, Benach et al. 1983, Johnson et al. 1984), is an arthropod-borne disease distributed worldwide. It has an enzootic cycle of transmission that involves ticks of the *Ixodes ricinus* (L., 1758) complex (Burgdorfer et al. 1982, Barbour et al. 1983), with a focal distribution in areas that fulfill the requirements of the vectors and reservoirs (Kahl 1991). The disease has been reported in the Old World since the beginning of the last century (Garin and Bujadoux 1922), but the identification of such cases as Lyme was only possible after the identification of its etiological agent in the last decade (Benach et al. 1983, Steere et al. 1983, Johnson et al. 1984).

Different species have been assigned to the *B. burgdorferi* sensu lato group: *B. burgdorferi* sensu stricto (s.s.), *B. garinii* and *B. afzelii* (Baranton et al. 1992), consistently associated with human disease; and also *B. japonica* (Postic et al. 1993), *B. lusitaniae* (Le Fleche et al. 1997), *B. bissettii* (Postic et al. 1998), *B. andersonii* (Marconi et al. 1995), and *B. valaisiana* (Wang et al. 1997), isolated from ticks only.

The first reported case of human Lyme disease in Spain was published in 1977; it described two cases of erythema migrans from northern Spain (Uruñuela-Bernedo and Díaz-Sosa 1977), which is the southwestern European limit of distribution of *I. ricinus* (Cordero del Campillo et al. 1994). During the last decade, several series of cases have been published, mainly occurring in the northern half of the country (Anda et al. 1993, Guerrero et al. 1993, Oteo et al. 1993) where the first Spanish tick isolate of *B. burgdorferi* was described in 1992 (García-Moncó et al. 1992). Spirochetes have also been found in the midgut of *I. ricinus* ticks collected in a nearby area (Oteo-Revuelta and Estrada-Peña 1991), and recently, a *B. garinii* strain of human origin (erythema migrans) has been isolated from the same area (Oteo et al. 1998). In the Basque Country (northern Spain), several clinical cases have been reported, and a serological study carried out among a high-risk population (forest guards, beekeepers, farmers, veterinarians, and mushroom pickers among others) showed a 25% prevalence. However, only 15% of serologically positive individuals had prior illness compatible with Lyme borreliosis, and the predominant symptoms were neurological (Arteaga et al. 1998).

Recently, we described the characterization of 15 isolates of *B. burgdorferi* sensu lato, some of them isolated from the same area, and we found a high variability in the pathogenicity of the strains to C3H mice (Escudero et al. 2000). But there is still a lack of

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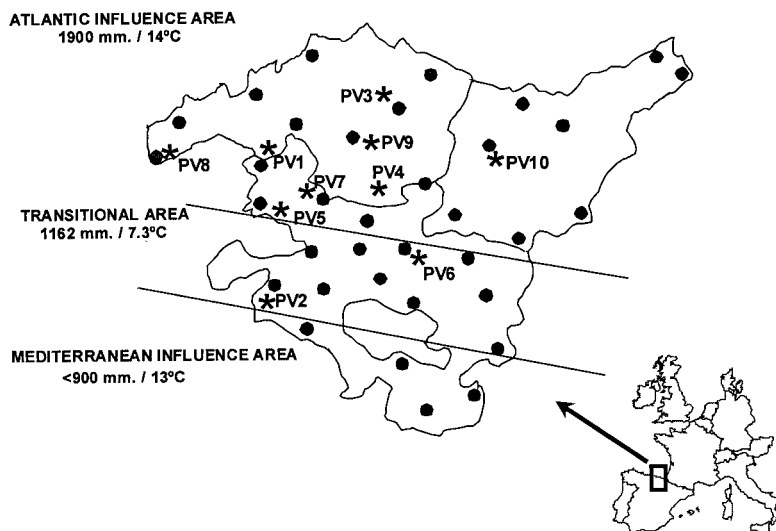


Fig. 1. Basque Country (Spain). Locations of sampling sites in the two phases of the study general survey (●) and in the directed study (*) (PV1-PV10).

prospective studies about the distribution of infected ticks in Spain.

This study was conducted to assess the potential risk of contracting Lyme disease in the Basque Country by describing the pattern of occurrence of *B. burgdorferi*-infected *I. ricinus* ticks.

Materials and Methods

Study Area. The study was carried out in the Basque Country, a mountainous area of northern Spain, located in the Bay of Biscay (7,482 km²). Temperatures were mild throughout the year, with frequent rainfall during the spring and autumn (ranging from 600 to 1,500 mm annually). The average temperature in summer was 20 and 8°C in winter. Landscape consisted of abundant undergrowth and mountain pastures. Pine, beech, or oak woodlands were also common, depending on the area (Table 2). Ticks were frequently present, with *I. ricinus* and *Hemaphysalis punctata* (Canestrini & Fanzago, 1877) as the most frequently found species (Barral et al. 1993).

Tick Collection. In a first phase of this study (general survey), 35 areas that covered most of the ecological biotopes of the Basque Country were studied from March 1992 to April 1993 (Fig. 1). Data on the climate, soil, vegetation, altitude, presence of livestock, farming, and human utilization were compiled. Questing ticks were collected from vegetation by blanket dragging, using a white piled cotton blanket (1.6 by 2 m). Sampling at each site was conducted monthly for 5 min and the blanket was examined twice to collect the ticks. Most of the sampling took place between 0900 and 1500 hours both in sunny and cloudy days. All specimens were transported to the laboratory in humidified plastic tubes, counted, and identified to species (Nosek and Sixl 1972, Gil-Collado

et al. 1979). Adult ticks were individually analyzed by polymerase chain reaction (PCR), whereas nymphs and larvae were grouped in pools of 10 and 100, respectively.

In the second phase of this study (directed survey), 10 areas (PV1-PV10) (Fig. 1) were selected on the basis of a high incidence of *I. ricinus* during the general survey. These sites were sampled between 1995 and 1997. Questing ticks were collected as described above and the same environmental data were also compiled. Most of the sampling took place on sunny days between 0900 and 1500 hours. Each area was sampled for 20 min. If no *Borrelia* isolation was obtained from ticks in a given sample, the area was resampled. In this directed study, ticks were analyzed in pools of up to three adults or 30 nymphs.

DNA Extraction and *ospA*-Based Nested PCR. Ticks were surface disinfected by immersion in decreasing concentrations of ethanol (95, 70, 35%) and washed in sterile phosphate buffered saline (PBS). Samples were then broken up in either a mortar using liquid nitrogen (general survey) or in sterile BSKII using two needles (directed study). These homogenates were digested with proteinase K (200 µg/ml) and SDS (1%), and DNA was extracted with phenol-chloroform-isoamyl alcohol (Sambrook et al. 1989). DNA was resuspended in 50 µl of TE and 2.5 µl were used as a template in a nested PCR for amplification of *ospA* (*ospA*-N1 and C1 primers in the first round, and *ospA*-N2 and C2 primers in the second round), as previously described (Guy and Stanek 1991). PCR reaction mixtures and cycling conditions as well as the detection of the products were conducted as previously described by Oteo et al. (1998).

Because of the use of tick pools, the percentage of infection was expressed as the minimum level of in-

fection, assuming that a positive pool would contain no more than one infected tick (Matuschka et al. 1998).

Spirochetes Culture. Ticks collected during the directed study were surface disinfected and broken up in 200 μ l of sterile BSKII as above. Fifty microliters of each tick homogenate were reserved for PCR analysis (*opsA*-based nested PCR), and the 150 μ l remaining were processed for *Borrelia* isolation. Two different procedures were used to culture spirochetes. Some tick homogenate samples were filtered through a syringe filter (μ Star 0.45 μ m Costar, Cambridge, MA) and added to a 5-ml screw cap culture tube with 4 ml of BSK supplemented with 6% rabbit serum (BSK-RS) (Coleman and Benach 1987). Some other tick homogenate samples were added directly without previous filtration to a BSK-RS tube supplemented with 50 μ g/ml of rifampicin (Berger et al. 1992) and 50 μ g/ml of fosfomycin (Pearc-Mursic et al. 1991). Both media were prepared from the same batch of BSK. All the cultures were maintained at 33°C \leq 12 wk with weekly darkfield microscope examination to monitor the presence of viable *Borrelia*. At the end of the 12-wk incubation period, all cultures were centrifuged and the pellet kept for PCR analysis (*opsA*-based nested PCR). All spirochetes isolated were stored at -80°C in the presence of 10% dimethylsulphoxide for further studies.

Restriction Fragment-Length Polymorphism (RFLP) Analysis of *rrf-rrl* PCR Amplicons Obtained from *B. burgdorferi* Isolates. DNA amplification was performed as described by Postic et al. (1994). PCR products were digested with 5 U of *MseI* (MBI Fermentas, Amherst, NY, USA) in 40 μ l, according to the instructions of the manufacturer. Restriction fragments were analyzed by electrophoresis through 5% MS-4 agarose gels (Hispanlab, Madrid, Spain) at 5 V/cm for 4 h in a cold room, stained with ethidium bromide, and photographed. The following strains were included for reference purposes: *B. burgdorferi* B31 (Benach et al. 1983), *B. lusitanae* PotiB2 (Le Fleche et al. 1997), *B. valaisiana* VS116 (Wang et al. 1997), *B. afzelii* VS461, and *B. garinii* PBi (Baranton et al. 1992), and were provided by J. L. Benach (Center for Infectious Diseases, State University of New York, Stony Brook, NY, USA), S. Nuncio (Centro de Estudos de Vectores e Doenças Infeciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Aguas de Moura, Portugal), I. Saint Girons (Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, Paris, France) and B. Wilske (Max von Pettenkofer-Institut, Ludwig-Maximilians-Universität, Munich, Germany), respectively.

Sequencing of the *rrf-rrl* PCR Amplicon. The PCR amplification product was purified using the Qiaquick PCR purification columns (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions, and sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Wellesley, MA, USA) on an ABI 377 DNA sequencer.

Potential Risk of Transmission of *B. burgdorferi* to Humans. An index (Jaenson 1991, Telford et al. 1996) was calculated to assess the potential transmission risk

to humans for each area, and was defined as the product of a measure of tick density (number of ticks captured per minute) and the percentage of infected ticks. The pathogenicity to C3H mice of each strain (Escudero et al. 2000), the month of the year when the infection was detected in ticks in each area and the frequency of human presence, were also taken into account. The score for pathogenicity in the animal model was constructed monitoring signs of inflammation of the tibiotarsal joints, dissemination of spirochete through the skin, and the number of organs colonized (Escudero et al. 2000). Differences in pathogenicity to C3H mice found for each isolate could be used, given the constraints of extrapolation to humans, to hypothesize about the risk for human of contracting Lyme disease in a certain area (Escudero et al. 2000).

Nucleotide Sequence Accession Number Generated in This Study. The sequence of the *rrf-rrl* intergenic spacer of the strain PV8 was deposited in GeneBank (accession number AF320780).

Results

Distribution of *B. burgdorferi* sensu lato in the Study Area. From a total of 49,172 ticks collected in the general survey, 7,835 were analyzed in 749 pools. Adults (371) belonging to eight different species were collected: 131 *I. ricinus*, 161 *H. punctata*, 32 *H. inermis* (Birula, 1985), 20 *Rhipicephalus bursa* (Canestrini & Fanzago, 1877), 16 *Dermacentor reticulatus* (Neumann, 1897), five *D. Marginatus* (Sulzer, 1776), one *R. sanguineus* (Latreille, 1806), and one *H. Sulcata* (Canestrini & Fanzago, 1877). Nymphs and larvae collected belonged to only two species: 2,011 nymphs and 2,257 larvae of *I. ricinus* were sampled as were 959 nymphs and 2,237 larvae of *H. punctata*. No immature stages of *Dermacentor* or *Rhipicephalus* were identified. *I. ricinus* was captured in 27 of the 35 areas analyzed, and *H. punctata* in 33 of them. *B. burgdorferi ospA* gene was amplified in three of the 35 areas studied (from *I. ricinus* in two areas and from *H. punctata* in one area). Thus, *B. burgdorferi* was detected in 1.5% of the adult *I. ricinus* analyzed (2 of 134) and the infection in the different areas was 0% (25 areas), 2.2% (one area with one of 42 adults infected), and 12.5% (one area with one of eight adults infected). Overall, the level of infection detected in nymphs was \geq 0.05% (1 pool positive from a total of 2,011 nymphs). Nymphs were positive for *B. burgdorferi ospA* gene in only one area where the prevalence was \geq 0.6%. All the *I. ricinus* larvae pools were negative. Of the adults of *H. punctata*, 1.2% (2 of 161) were positive and the infection was only detected in one area where the prevalence was 14.3% (2 of 14). No nymphs or larvae of *H. punctata*, or any adults of the other species, were positive. Infection in *I. ricinus* was detected in May and July, whereas *H. punctata* infected ticks were detected in March and October.

Regarding the directed study, 3,070 *I. ricinus* were collected and 64 adults and 1,471 nymphs were analyzed in 83 pools. Taking the PCR results from tick

Table 1. Comparison of *B. burgdorferi* detection by PCR in *I. ricinus* ticks from the 10 areas studied in the general survey and the directed study

	General study				Directed study			
	No. adults ^a	+ (%)	No. nymphs ^a	+ (%)	No. adults ^a	+ (%)	No. nymphs ^a	+ (%)
PV1	8	0	189	0	4	0	53	1 (1.9)
PV2	12	0	198	0	24	2 (8.3)	373	5 (1.3)
PV3	6	0	234	0	13	2 (15.4)	334	5 (1.5)
PV4	0	—	0	—	1	1 (100)	18	1 (5.5)
PV5	0	—	0	—	6	0	197	2 (1.0)
PV6	8	1 (12.5)	155	1 (0.6)	1	0	96	4 (4.2)
PV7	3	0	139	0	0	—	45	1 (2.2)
PV8	42	2.2	261	0	2	1 (50)	80	1 (1.3)
PV9	18	0	291	0	12	0	223	1 (0.4)
PV10	0	—	96	0	1	0	52	1 (1.9)
Total	97	2 (2.1)	1,563	1 (0.06)	64	6 (9.3)	1,471	22 (1.5)

+, Number of positive pools; (%), Minimum percentage of infected specimens, percentage of positives is calculated assuming that any pool would contain more than one infected tick.

^aTotal number of adults or nymphs analyzed from each area.

homogenates and from pellets obtained from cultures, *B. burgdorferi* infected ticks were collected from all the 10 areas (Table 1). The overall level of infection detected was 9.3% of the adults (6 positive pools from a total of 64 adults analyzed) and 1.5% of the nymphs (22 pool positives from a total of 1,471 nymphs analyzed). Infected nymphs were detected in the 10 areas studied and the level of infection in the different areas ranged from 0.5 to 5.6%. Infected adults were found in four areas, but due to the small number of ticks analyzed from some areas it was difficult to assess the level of infection. *B. burgdorferi* infection was detected in January, March, May, June, and October. A comparison of PCR results from the 10 areas where both the general survey and directed studies were carried out is presented in Table 1.

Spirochetes were isolated in 11% of the 83 cultures prepared: 13.3% of the nymph cultures (8 out of 60) and 4.4% of adult cultures (1 out of 23), representing nine of the 10 areas. *B. burgdorferi* was isolated in 13.2% (7 of 53) of the cultures supplemented with antibiotics but only in 6.1% (2 of 30) of the cultures prepared with filtered homogenates without antibiotics. All isolates were identified as *B. burgdorferi* using the *ospA*-based nested PCR.

A description of the principal characteristics of the areas where *B. burgdorferi* was found in *I. ricinus* is shown in Table 2.

Genomic Characterization of *B. burgdorferi* Isolated Strains. As described by Postic et al. (1994), PCR amplification of the spacer region located between the *rrf* and *rrl* genes generated a fragment of ≈ 230 bp (Fig. 2). According to the RFLP patterns, the isolates from PV4, PV5, and PV6 belonged to the *B. garinii* genospecies. They consisted of three bands of 108, 95, and 50 bp (Fig. 2) that correspond to the pattern B previously described (Postic et al. 1994). Strains PV1, PV2, and PV3 comprised four fragments (108, 51, 38, and 29 bp) that corresponded to pattern A of *B. burgdorferi* sensu stricto. Isolate PV7 had two bands of 175 and 50 bp corresponding to *B. valaisiana* (pattern F). Isolate PV8 could not be analyzed by RFLP because of its extremely slow growth rate, but the amplified product was sequenced and found to be identical to that of PotiB2 strain of *B. lusitanae*.

Potential Risk of Transmission of *B. burgdorferi* to Humans. The different variables used to assess the risk index were studied in the 10 areas that presented the infection. This index was calculated only for the nymphs, because they were more frequently captured

Table 2. Description of the areas where *B. burgdorferi* infection was found in *I. ricinus*

Area	Altitude, m	Use	Dominant trees (woodland presence)	Predominant type of ground cover	Livestock frequency	Livestock species present	Climate
PV1	450	L	Pine, oak (yes)	Heather	Low	Sheep (goat)	Atlantic
PV2	830	L, F	Pine (yes)	Heather	High	Cattle (horse)	Transition
PV3	760	M, L, R	Pine (yes)	Heather	High	Sheep (cattle, horse)	Atlantic
PV4	600	R	Cypress (yes)	Grass	NP	NP	Atlantic
PV5	700	M, L	Pine, beech (no)	Heather	High	Sheep (cattle, horse)	Atlantic
PV6	575	R, L	Pine (yes)	Furze, Meadow	High	Sheep (horse)	Transition
PV7	600	R	NP	Heather, Furze	Low	Sheep (horse)	Atlantic
PV8	640	L	NP	Furze, Bracken	High	Cattle (horse)	Atlantic
PV9	500	F, L	Pine (yes)	Bracken	Low	Sheep (cattle)	Atlantic
PV10	620	R, F	Pine (yes)	Bracken, Heather	Low	Sheep (horse)	Atlantic

R, recreational; L, livestock; M, mountain route; F, forestry; NP, not present.

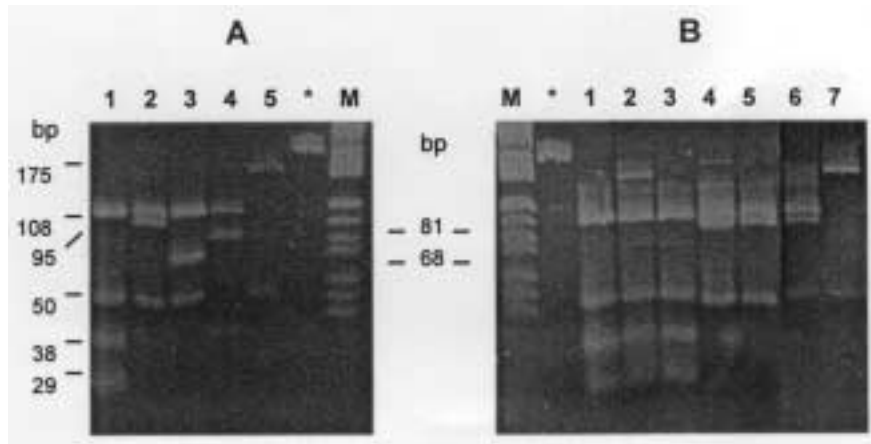


Fig. 2. *MseI* restriction patterns of *B. burgdorferi* sensu lato isolates. DNA was electrophoresed on a 5% MS-4 agarose gel, stained with ethidium bromide, and UV illuminated. On every panel the lane M corresponds to the DNA size marker V and the lane * corresponds to the not *MseI*-digested DNA fragment. (A) *B. burgdorferi* sensu lato reference strains. Lane 1: *B. burgdorferi* sensu stricto (B31^T); lane 2: *B. garinii* (PBi); lane 3: *B. afzelii* (VS461^T); lane 4: *B. lusitaniae* (POTIB2^T); lane 5: *B. valaisiana* (VS116^T). (B) *B. burgdorferi* sensu lato isolated during the directed study. Lane 1: PV1; lane 2: PV2; lane 3: PV3; lane 4: PV4; lane 5: PV5; lane 6: PV6; and lane 7: PV7.

and were believed to be responsible for the majority of *B. burgdorferi* infections in man (Junttila et al. 1994). The percentages of infected ticks in each area were those calculated in Table 1.

The density of *I. ricinus* nymphs calculated from sampling times and ticks captured in each area (data not shown), ranged from 1.3 to 12.4 nymphs per minute and the risk index varied from 2.6 to 17.4 (Table 3). In general, the risk index was higher in areas PV2, PV3, PV4, and PV6. A high risk of transmission was calculated for areas PV2 and PV3, where the percentage of infection was low but the density of ticks was high. Conversely, nymphs in PV4 and PV6 presented with the highest level of infection although their density was lower.

The pathogenic strains (Escudero et al. 2000) were found in areas having a high risk index (PV4 and PV6), which were recreational and frequently visited in spring and summer. One of the low pathogenic strains isolated from PV3 occurred in an area that had a high risk index. However, it was also a recreational area with good access and, therefore, frequently visited.

The low pathogenic strains from PV1 and PV5 were collected from an area where the risk index seemed to be lower.

Discussion

We used a combination of PCR and culture to conduct the first prospective study on the distribution of the aetiologic agent of Lyme disease and its tick vector in northern Spain (Basque Country). Our data indicated that *B. burgdorferi* was quite widespread in the region. *I. ricinus* was the most common tick species, being active throughout the year (Barral et al. 1993). The general prevalence of *B. burgdorferi* among the 27 areas where *I. ricinus* was found was low in the general survey (phase I). However, when more intensive sampling was undertaken in selected areas of directed study, all were positive for *B. burgdorferi*, confirming a wide distribution of this pathogen in the region. In this study, the rates of infection observed were similar to those reported for other endemic European areas (Hubalek et al. 1991, Gray et al. 1992). No infection

Table 3. Risk of acquiring infection from host seeking *I. ricinus* nymphs

Area	<i>I. ricinus</i> density	% infection	Month of infection	Risk index	<i>B. burgdorferi</i> strain	Pathogenicity (Escudero et al. 2000)
PV1	1.77	1.90	June	3.4	<i>B. burgdorferi</i> s.s.	LP
PV2	12.43	1.40	January, March, May	17.4	<i>B. burgdorferi</i> s.s.	NP
PV3	6.69	1.50	May, October	10.0	<i>B. burgdorferi</i> s.s.	LP
PV4	1.80	5.60	June	10.1	<i>B. garinii</i>	P
PV5	4.93	1.00	March, May	4.9	<i>B. garinii</i>	LP
PV6	3.20	4.20	January, March, May	13.4	<i>B. garinii</i>	P
PV7	2.25	2.20	March	5.0	<i>B. valaisiana</i>	NP
PV8	4.00	1.30	May	5.2	<i>B. lusitaniae</i>	NP
PV9	7.40	0.50	October	3.7	—	—
PV10	1.35	1.90	January	2.6	—	—

P, pathogenic; NP, no pathogenic; LP, low pathogenicity.

was detected in 2,257 *I. ricinus* larvae analyzed. Some authors have reported different levels of larval infection (Miserez et al. 1990, Matuschka et al. 1992, Zhioua et al. 1994), but because of its rarity, it is considered of little or no significance in the Lyme disease cycle (Burgdorfer 1989, Gray et al. 1998).

A higher percentage of tick infection was observed in the directed study as compared with the general survey. This could be due to a higher distribution and prevalence of the infection because of environmental and epidemiological factors, which could account for the higher availability of reservoirs and consequently cause an increment in the number of *B. burgdorferi* infected ticks. In addition, the use of a combination of techniques in the directed study (PCR from tick homogenates and from sediment of cultures) could have improved the detection of *B. burgdorferi* in the samples studied (Schwartz et al. 1993) or a more intensive and persistent sampling in the directed study could have accounted for the increasing pattern observed.

The detection of *B. burgdorferi* DNA in *H. punctata* was an interesting finding. Other studies have reported *B. burgdorferi* infection in this species (Márquez and Constan 1990, Talleklint 1996), but it remains unknown if this tick could transmit the infection. Further studies are needed to examine this issue.

The season when positive ticks were collected varied between the two studies. In the general survey, infected ticks were collected mainly during May and July, whereas in the directed study, infection was detected during most of the months when enough ticks were collected to analyze. Therefore, it could be concluded that in this area *B. burgdorferi* could be found at any time of the year when ticks were active.

Ixodes ricinus infected with *B. burgdorferi* were mainly collected in pine woodlands, located below 800 m above sea level with Atlantic climate and a predominant undergrowth mainly composed of heather, as well as bracken, furze, and grass. This agrees with other studies that described low levels of infection in open fields and high prevalence in forested areas, therefore, considering woodland the main biotope for Lyme disease (Gray et al. 1992, 1995, Dister et al. 1997). Several studies have indicated that tick survival and, therefore, the number of infected ticks, is higher among forests than in open areas, due to better environmental and climatic conditions and the wider variety of hosts found in forests (Mejlon and Jaenson 1993, Wegner et al. 1997). In the Basque Country, higher tick populations have been observed in forests as compared with open areas (Barral et al. 1993).

Highly infected areas contained grazing fields with abundant undergrowth, both in open areas and forests. Genchi (1992) affirmed that the presence of rodents acting as reservoirs and large mammals that maintain high vector populations are needed for the establishment and maintenance of the endemic infection. In our case, grazing livestock was constantly abundant in most of the areas where infection was detected. Therefore, the number of ticks collected was high and the percentage of infection lower, which

is in agreement with other studies (Gray et al. 1992, Dorn and Sünder 1997). However, it is noteworthy that the PV4 area had a high level of infection despite a low tick population. This could be explained in part by the observations of Gray et al. (1992), who reported a high percentage of infection even with low numbers of ticks in areas without large mammals but with rodents acting as reservoirs. This indicates that further studies are needed to explain the differences in prevalence found, as well as the role of the different wild animals present in the area as possible reservoirs of the pathogen. In summary, we believe that in the Basque Country *B. burgdorferi* is present throughout the distributed area of *I. ricinus*.

Differences in the risk index calculated for the 10 areas studied were found even in nearby areas with similar ecological characteristics. However, it is noteworthy that the risk index of an area is not only conditional on the prevalence of *I. ricinus* infection with *B. burgdorferi*, but also, and probably principally, on the density of *I. ricinus*. Remarkably, half of the areas where the infection was detected were recreational areas that were densely visited in spring and summer, and in PV4 and PV6 the most pathogenic genospecies were found (Escudero et al. 2000). Other studies also suggest that the highest risk for acquiring Lyme disease is linked to outdoor activities (Gray et al. 1998). Thus, in Lyme disease endemic regions like the Basque Country, those areas densely populated by *I. ricinus* and frequently visited by people such as recreational areas, public footpaths, and hiking routes could be considered potentially risky areas (Falco and Fish 1989).

Four of the five European *B. burgdorferi* sensu lato genospecies were identified in this study. The absence of *B. afzelii* in the sample studied correlates with the lack of chronic Borrelia skin manifestations of Lyme disease in Spain (Alonso-Llamazares et al. 1997), whereas in other European areas where *B. afzelii* tick isolates have been reported this disease manifestation is common. *B. valaisiana* has been rarely related to human disease (Rijpkema et al. 1997) and never isolated from a case of Lyme borreliosis.

The lack of properly based surveillance methods, together with a low level of health education, could contribute to the low clinical awareness of this disease in our area. Alternatively, a low human incidence of Lyme borreliosis could account for this low awareness and be explained by several hypotheses. One would be the lack of human exposure to infected ticks, but this would not be expected in an area with a large percentage of rural population. Another explanation could be a low percentage of infected ticks. A third one might be the circulation of nonpathogenic variants of *B. burgdorferi* that could lead to a major percentage of asymptomatic infections in vertebrate hosts (Arteaga et al. 1998).

Finally, a more intensive educational campaign directed to the affected population, together with an effort to define molecular markers that allow us to identify pathogenic Borrelia-carrying ticks are needed.

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