Title: The importance of lizards and small mammals as reservoirs for *Borrelia lusitaniae* in Portugal

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Running title*:* *B. lusitaniae* in lizards and small mammals in Portugal

Summary

Borrelia lusitaniae is a pathogen frequent in the Mediterranean area. Apart from lizards, evidence for birds and small mammals as competent reservoirs for this genospecies has been occasional. We collected questing ticks, skin biopsies and *Ixodes* sp. ticks feeding on lizards, birds and small mammals in a *B. burgdorferi* s.l. enzootic area to assess their importance in the maintenance of *B. lusitaniae*. *B. lusitaniae* was the most prevalent genospecies in questing ticks and was commonly found in larvae feeding on *Psammodromus algirus*. One biopsy infected with *B. lusitaniae* was collected from the tail of one *Podarcis hispanica*, which suggests systemic infection. *I. ricinus* larvae feeding on *Apodemus sylvaticus* were infected with *B. lusitaniae* but with a lower prevalence. Our results reinforce the importance of lizards as reservoirs for *B. lusitaniae*, suggesting that *P. algirus*, in particular, acts as main reservoir for *B. lusitaniae* in Portugal.

Introduction

Borrelia lusitaniae is a genospecies frequent in the Mediterranean area with focal distribution in central and northern Europe. In Portugal, this is the most frequent genospecies infecting questing ticks (Baptista, 2006; Lopes de Carvalho et al., 2008a). This is also the case in Tapada de Mafra, a mixed deciduous forest, where *Ixodes ricinus* ticks are abundant and are frequently infected with *Borrelia burgdorferi* s.l., with a higher prevalence of *B. lusitaniae* when compared to other genospecies (Norte et al., 2013a). *B. lusitaniae* reservoir hosts have not been studied in detail, apart from reptiles, which are confirmed reservoirs through xenodiagnosis (Dsouli et al., 2006). Some observations have suggested that both small mammals (Lopes de Carvalho et al., 2010) and birds (Poupon et al., 2006) can also act as reservoirs for this genospecies but further support is needed. In one previous survey at Tapada de Mafra (see Norte et al. 2012), where *B. lusitaniae* was the most prevalent genospecies in questing ticks, we found no evidence of reservoir competency of avian hosts for *B. lusitaniae* (Norte et al., 2013b). This is a pathogenic genospecies (da Franca et al., 2005; Lopes de Carvalho et al., 2008b), and it is important to better understand which factors contribute to its maintenance in nature, to eventually minimize infection and disease risk. In this study, we collected samples (feeding ticks and tissues) from mammals, birds and lizards, and questing ticks, to assess the potential of each of these vertebrate groups as reservoir for *B. lusitaniae*, and elucidate which species contributes the most to the maintenance of *B. lusitaniae* in Tapada de Mafra enzootic focus..

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Results and discussion

B. burgdorferi *s.l infection in questing ticks*

A total of 749 *Ixodes* spp. were collected from the vegetation, including 748 *I. ricinus* and one *I. frontalis* nymph (Table 1). From these, 373 were tested for *B. burgdorferi* s.l. infection using a nested PCR targeting the 5S-23S rDNA intergenic spacer region (Rijpkema et al., 1995; Supplemental Material 1). *B. burgdorferi* s.l. prevalence in questing *I. ricinus* was 0% in larvae (0/100), 5.3% (14/262) in nymphs and 16.7% (1/6) in adults (Table 1). As expected, *B. burgdorferi* s.l. prevalence increased from larval to adult stage of *I. ricinus* ticks, which is in support of the low transovarial transmission of this agent in this tick species (Bellet-Edimo et al., 2005), and increasing number of hosts on which the ticks fed along their life stages. Therefore, detection of infection in *I. ricinus* larvae feeding on a host strongly suggests acquisition from the host (except in cases of co-feeding; Randolph et al., 1996). The following genospecies were detected in questing ticks: *B. lusitaniae*, *B. valaisiana*, *B. garinii* and *B afzelii* (Table 1). The most prevalent genospecies was *B. lusitaniae*, infecting 3.0% (8/262) of the *I. ricinus* nymphs (Table 1), which is in agreement with previous findings for the same area (Baptista, 2006; Norte et al., 2013b). The finding of one *I. frontalis* nymph questing in the vegetation is worth highlighting because collections of this tick species by flagging are rare (Pérez-Eid, 2007; Bona and Stanko, 2013).

B. burgdorferi *s.l infection in ticks and tissues from vertebrates*

Lizards

Fifty lizards, including 2 recaptures, belonging to 4 species were sampled (Table 2). From these, 426 *I. ricinus* (379 larvae and 47 nymphs) were collected (Table 2). 346 *I. ricinus* were tested for *B. burgdorferi* s.l. infection and the prevalence was 13.3% (46/346; Table 2), with all the infections belonging to *B. lusitaniae.* Infected ticks were feeding on *Psammodromus algirus*, *Podarcis hispanica* and *Lacerta shreiberi*. However, from these lizard species, only *P. algirus* carried *B. lusitaniae* infected larvae and some of those individuals were infested only by larvae. This suggests reservoir competence of this lizard species for *B. lusitaniae*. *P. algirus* had already been confirmed as reservoir for *B. lusitaniae* in Tunisia by xenodiagnosis (Dsouli et al., 2006), and additional evidence of the role of lizard species as reservoirs for *B. lusitaniae* has been adding up (Richter and Matuschka, 2006; Foldvari et al., 2009; De Sousa et al., 2012).

From the 47 tail biopsies collected (42 in duplicate: one for inoculation into Barbour-Stoenner-Kelly complete (BSK II) medium, and the other for direct DNA extraction – see Supplemental Material 1), in one biopsy from *P. hispanica, B. lusitaniae* DNA was detected, suggesting systemic infection. *B. burgdorferi* s.l. detection in biopsies was rather low (in lizards and other vertebrates groups, see bellow). In the case of lizards, this may be attributed to the fact that the tissue from the lizards' tail is not the best for *Borrelia* detection, when compared to e.g. the collar tissue. Majláthová et al. (2006) collected both tail samples and collar skin biopsies from *Lacerta viridis* but only detected *B. burgdorferi* s.l. in the collar biopsies (18.6%). Foldvari et al. (2009) collected both collar scales and toe clips from lizards, and the percentage of infected tissues was higher for collar scales (8.2%) than for toe clips (2.0%). However, detection from collar scales may reflect local infection, because most of the feeding ticks are attached in this area, rather than a systemic infection. *B.*

burgdorferi s.l. has been detected in lizard tail tissues with success in other studies (Amore et al., 2007; De Sousa et al. 2012). Another potential difficulty in obtaining isolates from field derived inoculations in BSK-II medium arises from the susceptibility of these cultures to contamination. Cultures contaminated soon after inoculation and this may have inhibited *Borrelia* growth and/ or the PCR on the DNA extracted from these cultures.

The high *I. ricinus* larval tick infestation intensity in *P. algirus* (Table 2), than in other sampled vertebrate species (see bellow), suggests the great potential of this species to infect a large number of ticks in this area, acting as the source of *B. lusitaniae* infection to questing nymphs in the area. The other captured lizards in our study (*P. hispanica, L. shreiberi* and *Timon lepidus,* formerly *Lacerta lepida*) do not seem to have such an important role as reservoirs for *B. lusitaniae* as *P. algirus,* because either they were not infested by ticks or the infection prevalence in their infesting larvae was zero. Although the number of *Lacerta* sp. individuals captured was low, precluding a reliable evaluation of their reservoir status, the number of *P. hispanica* sampled individuals was relatively high, and the lack of infection in its large number of infesting larval *I. ricinus* contrasts with the finding of one *B. lusitaniae* positive biopsy collected from this lizard species. The reservoir competence for a *Borrelia* genospecies may vary between related species occurring sympatrically, and which are both important tick hosts (e.g *P. algirus* and *P. hispanica*). Although the reservoir competence of lizards for *B. burgdorferi* s.l. may be species' specific, the range of lizard species for which evidence as reservoirs for *B. lusitaniae* has been compiled is quite large and includes: *L. viridis, Podarcis muralis, Podarcis taurica* and *Lacerta agilis* in central Europe (Richter and Matuschka, 2006; Majláthová et al., 2006; Foldvari et al., 2009), *P. muralis* in Tuscany (Amore et al.,

2007), and *Teira dugesii* in Madeira's island, Portugal (De Sousa et al., 2012). More studies are needed to confirm the reservoir potential of *P. hispanica* for *B. lusitaniae*.

Mammals

Seventy-eight small mammals belonging to 5 species were captured, including 30 recaptures. The most common species captured was the wood mouse *Apodemus sylvaticus*. Forty-one small mammals were infested by ticks, and 239 *Ixodes* spp. ticks were collected from them, including 234 *I. ricinus* in larval stage (Table 3). 226 *Ixodes* spp. ticks were screened for *Borrelia* infection, whose prevalence was 0.9% (2/226). All infections corresponded to *B. lusitaniae* and they were detected in *I. ricinus* larvae feeding on an *A. sylvaticus*. Those infected ticks were derived from the same animal, infested only by larvae. This finding supports previous results that *A. sylvaticus* acts as competent reservoir for *B. lusitaniae* (Lopes de Carvalho et al. 2010). The percentage of *B. lusitaniae* infected larvae feeding on *A. sylvaticus* was much lower than those feeding on *P. algirus*, suggesting a less prominent role of this small mammal as amplification agent of this genospecies in the study area. However, the number of small mammal recaptures was quite high (30/78; 38.5%). Because they were not individually marked, *Borrelia* prevalence in ticks derived from small mammals could have been underestimated if spirochete–free individuals were captured significantly more often than others.

Forty-six ear biopsies were collected (38 in duplicate) from 36 *A. sylvaticus*, 7 *Mus* spp., 2 *Microtus lusitanicus* and 1 *Crossidura russula*. This tissue has been used previously with success in direct detections and to obtain *B. burgdorferi* s.l. isolated cultures (Sinsky and Piesman, 1989; Hanincová et al., 2003a), but none of the collected

biopsies in this study were found infected (either through culture on BSK II medium or direct DNA extraction). One out of the two cultures from the heart of *A. sylvaticus* was positive, but the genospecies was impossible to determine. None of the two bladder derived cultures from the same individuals were positive.

Additionally 1 (larva) and 11 (9 nymphs and 2 adult females) *I. ricinus* were collected from a rabbit and a wild boar, respectively, but were negative for *B. burgdorferi* sl. Two biopsies were also taken from the ear of the rabbit but were negative for *B. burgdorferi* s.l., both through culture and direct DNA extraction.

We did not detect *B. afzelli* either in ticks feeding on any of the sampled hosts or their tissues. Although the prevalence of this genospecies was relatively low in questing nymphs, it was unexpected that ticks collected feeding on potential *B. afzelii* reservoirs hosts, such as small mammal species (Hanincová et al., 2003a; Cadenas et al., 2007) and wild boars, were negative. In North Eastern Europe, *I. ricinus* larvae feeding on *Lacerta* sp. were infected with *B. afzelii* (Majláthová et al., 2006; Foldvari et al., 2009), but our sampled lizard species did not harbour *B. afzelii* positive ticks. Possibly other mammal species which were not sampled, such as hedgehogs (Skuballa et al., 2012), foxes or some Artyodactil species (Cadenas et al. 2007) act as *B. afzelii* reservoirs in this area.

Birds

One-hundred and eight birds were captured, including 5 recaptures, belonging to 22 species, and 31 were infested with ticks: 243 *Ixodes* spp. ticks were collected, including 236 *I. ricinus* (204 larvae and 32 nymphs; Supplemental Material 2). We screened 220 *Ixodes* sp. for *B. burgdorferi* s.l. infection. Only *Turdus merula* (3 out of 5

195 birds infested) harboured infected ticks, and the genospecies detected were *B. garinii*,
196 the most prevalent genospecies (16/97; 16.5%), followed by *B. valaisiana* (10/97;
197 10.3%) and *B. turdi* (3/97; 3.1%; Table 4). A mixed infection by these three
198 genospecies was detected in one *I. ricinus* larva through a Reverse Line Blot (Gil 2005)
199 using specific probes described previously (Ripkema 1995, Gil 2005, Gern 2010; see
200 Supplemental Material 1). The relatively high prevalence of *B. garinii* and *B. valaisiana*
201 in ticks derived from *T. merula* is consistent with previous findings from the same area
202 and, with other studies which found that birds from the genus *Turdus* are competent
203 reservoirs for these genospecies (Taragel'ová et al., 2008; Norte et al., 2013a; Norte et
204 al., 2013b). *B. turdi*, detected in a lower prevalence, is a genospecies associated with
205 bird-reservoirs (Norte et al., 2013a,b).
206 Twenty-four biopsies were collected in duplicate from 24 birds which
207 corresponded to 77% (24/31) of the birds which carried ticks, belonging to 10 species
208 (Supplemental Material 2), but all were negative for *B. burgdorferi* s.l.
209
210 *Genetic variability of* B. lusitaniae *strains in circulation at Tapada de Mafra*
211
212 In this study, we detected 3 genetic variants of the analysed partial *5S-23S* intergenic
213 spacer region (from the positions 254 to 453). The sequences were aligned using
214 Multalin software (Corpet, 1988) and compared with other published sequences. Two
215 variants were reported before in questing ticks from the same area (Norte et al., 2013b)
216 but a new one was found in *I. ricinus* larvae and nymphs feeding on *P. algirus* and in *I.*
217 *ricinus* larvae feeding on *A. sylvaticus* (PoTiBlus4). This variant differed between 3 and
218 9 base pairs from the other genetic variants reported from the same area (PoTiBlus1,
219 PoTiBlus2, PoTiBlus3, PoTiBmf364 and PoTiBl37; Norte et al. 2013b).

The results of this study are in partial agreement with those of Amore et al., (2007) in Tuscany, where *B. lusitaniae* was the most prevalent *Borrelia* genospecies in questing ticks and the hosts harbouring *B. lusitaniae* infected ticks were lizards (in their case *Podarcis* sp.). Amore et al. (2007) also found that 19-25% of the blood and biopsy samples from *Podarcis* sp. were positive for this genospecies, but, neither mice nor passerine birds were infected or harboured *B. lusitaniae* infected ticks. This shows that host specificity for *Borrelia* genospecies may vary with *Borrelia* subtypes, different tick vector species/races, and host composition in different geographic areas differing in habitat structure and biocenosis (Gern, 2008). Therefore, specific and localised studies are needed in each enzootic area to properly understand the ecology of this zoonosis.

Nucleotide sequence accession numbers

B. lusitaniae rrf (5S)-rrl (23S) intergenic spacer region nucleotide sequences obtained in this study, and in Norte et al. (2013b) study from the same area, have been deposited in GenBank with the accession numbers: KJ857486 (PoTiBlus2) and KJ857487 (PoTiBlus4).

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Table 1. *Borrelia burgdorferi* s.l. infection prevalence in *Ixodes* sp. ticks collected questing in the vegetation at Tapada de Mafra from February to June 2013.

Species	stage	sex	no. collected	no. tested	no. *Bb*sl positive ticks (%)	No. of ticks infected with *Bb*sl genospecies (%)			
						LU	VA	GA	AF
Ixodes ricinus	L		371	100	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	N		367	262	14 (5.3)	8 (3.0)	3 (1.1)	2 (0.8)	1 (0.4)
	A	M	6	6	1 (16.7)	0 (0)	1 (16.7)	0 (0)	0 (0)
		F	4	4	0/4 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ixodes frontalis	N		1	1	0/1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

L- larva; N- nymph; A- adult; M – male; F - female

*Bb*sl – *Borrelia burgdorferi* s.l.

LU = *B. lusitaniae;* VA = *B. valaisiana*; GA = *B. garinii*; AF = *B. afzelii*.

B. burgdorferi s.l infection was assessed through a nested PCR targeting the 5S-23S rDNA intergenic spacer region (Rijpkema et al., 1995) and sequence analysis, followed by a Reverse Line Blot (Gil 2005) on the positive samples, using specific probes described previously (Ripkema 1995, Gil 2005, Gern 2010) to detect mixed infections. For further details on methods see Supplemental Material 1.

Table 2. *Ixodes ricinus* ticks, skin biopsies and *B. lusitaniae* prevalence in ticks from lizards captured at Tapada de Mafra between March and June 2013.

Species	No. of individuals	No. of recaptures	No. of skin biopsies collected[a]	No. of infested	*Ixodes ricinus* collected from lizards			
					Larvae		Nymphs	
					No. collected	*B. lusitaniae* prevalence % (No. positives/ No. tested)	No. collected	*B. lusitaniae* prevalence % (No. positives/ No. tested)
Lacerta shreiberi	1	1	1	1	1	0 (0/1)	16	43.8 (7/16)
Podarcis hispanica	27	1	27	25	100	0 (0/99)	12	16.7 (2/12)
Psammodromus algirus	18	0	17	17	278	16.6 (33/199)	19	21.0 (4/19)
Timon lepidus	2	0	2	0	0	-	0	-

[a] Biopsies were collected in duplicate except in 4 cases (2 collected only for direct DNA extraction, 2 only for culture in BSK II medium); Refer to text for results on *Borrelia* prevalence in biopsies.

B. burgdorferi s.l prevalence was assessed through a nested PCR targeting the 5S-23S rDNA intergenic spacer region (Rijpkema et al., 1995) and sequence analysis, followed by a Reverse Line Blot (Gil 2005) on the positive samples, using specific probes described previously (Ripkema 1995, Gil 2005, Gern 2010) to detect mixed infections. For further details on methods see Supplemental Material 1.

Table 3. Ixodidae ticks and skin biopsies collected from mammals at Tapada de Mafra sampled between March and June 2013.

Species	No. of individuals	No. of recaptures	No. of skin biopsies [a]	No. of infested	Ticks collected from mammals[b]				
					I. ricinus			*I. acuminatus*	
					Larvae	Nymphs	Adult females	Larvae	Nymphs
Apodemus sylvaticus	38	26	36	36	226	0	0	3	2
Crossidura russula	1	0	1	1	3	0	0	0	0
Microtus lusitanicus	2	3	2	2	2	0	0	0	0
Mus domesticus	5	1	5	0	0	0	0	0	0
Mus spretus	2	0	2	2	3	0	0	0	0
Oryctolagus cuniculus	1	0	1	1	1	0	0	0	0
Sus scrofa	1	0	0	1	0	9	2	0	0

[a] Biopsies were collected in duplicate except in 4 cases (3 collected only for direct DNA extraction, 1 only for culture in BSK II medium); Refer to text for results on *Borrelia* prevalence in biopsies.

[b] Refer to text for results on *Borrelia* prevalence in ticks.

365 **Table 4.** *Borrelia burgdorferi* s.l. infection prevalence in *Ixodes* sp. ticks collected

366 feeding on five *Turdus merula* captured from March to June 2013 at Tapada de Mafra.

Tick family/ species	**Stage**	**No. infected/ no. tested (%)**	**No. of ticks infected with *Bb*sl genospecies (%)**			
			GA	**VA**	**TU**	**GA, VA, TU**
Ixodidae	N	1/1 (100)	0 (0)	1 (100)	0 (0)	0 (0)
Ixodes ricinus	L	22/67 (32.8)	13 (19.4)	8 (11.9)	0 (0)	1 (1.5)
	N	2/27 (7.4)	1 (3.7)	0 (0)	1 (3.7)	0 (0)
Ixodes frontalis	L	1/1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
	N	1/1 (100)	0 (0)	0 (0)	1 (3.7)	0 (0)

367 Ticks classified as Ixodidae lacked body structures needed for identification.

368 L- larva; N- nymph.

369 *Bb*sl – *Borrelia burgdorferi* s.l.

370 GA = *B. garinii*; VA = *B. valaisiana*; TU = *B. turdi*.

371 *B. burgdorferi* s.l prevalence was assessed through a nested PCR targeting the 5S-23S

372 rDNA intergenic spacer region (Rijpkema et al., 1995) and sequence analysis, followed

373 by a Reverse Line Blot (Gil 2005) on the positive samples, using specific probes

374 described previously (Ripkema 1995, Gil 2005, Gern 2010) to detect mixed infections.

375 For further details on methods see Supplemental Material 1.