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Research paper

Molecular identification of *Borrelia* genus in questing hard ticks from Portugal: Phylogenetic characterization of two novel Relapsing Fever-like *Borrelia* sp.

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ABSTRACT

In the last decades, several studies have reported pathogenic species of *Borrelia* related to those that cause Tick-borne Relapsing Fever (RF), but unexpectedly suggesting their transmission by hard ticks, known vectors of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) species, rather than by soft ticks. This study was designed to update the presence of *B. burgdorferi* s.l. species in ticks from several districts of mainland Portugal, where *Ixodes ricinus* had been previously described.

Ticks (a total of 2915 specimens) were collected in seven districts throughout the country, and analyzed using molecular methods. Three nested-PCR protocols, targeting the flagellin gene (*flaB*), the intergenic spacer region (IGS) located between 5S and 23S rRNA, and the *glpQ* gene, and a conventional PCR targeting the 16S rRNA, were used for *Borrelia* DNA detection.

Borrelia DNA was detected in 3% of the ticks from Braga, Vila Real, Lisboa, Setúbal, Évora and Faro districts. The obtained amplicons were sequenced and analyzed by BLASTn, and 15/63 (24%) matched with homologous sequences from *Borrelia lusitaniae* and 15/63 (24%) with *B. garinii*, being these the most prevalent species. DNA from *B. burgdorferi* sensu stricto (*s.s.*), *B. valaisiana* and *B. afzelii* were detected in 7/63 (11%), 6/63 (10%), and 2/63 (3%) of the specimens, respectively. Unexpectedly, DNA sequence (*flaB*) analysis from eight (13%) samples, two from *Rhipicephalus sanguineus* and six from *Haemaphysalis punctata* tick species, revealed high homology with RF-like *Borrelia*. Phylogenetic analyses obtained from three genetic markers (16S rRNA, *flaB*, and *glpQ*) confirmed their congruent inclusion in a strongly supported RF cluster, where they segregated in two subgroups which differ from the other Relapsing Fever species.

Therefore, the results confirm the circulation of multiple species of *B. burgdorferi* s.l. over a wide geographic range, covering most of the Portuguese mainland territory. Surprisingly, the obtained data also revealed two putative Relapsing Fever-like *Borrelia* species in different species of hard ticks, possibly disclosing the circulation of novel RF-like *Borrelia* species with different associated tick vectors.

1. Introduction

The genus *Borrelia* is a group of helical-shaped, motile bacteria that form a monophyletic lineage within the phylum Spirochetes, and comprises two major clades. In some cases, associations of specific groups of bacteria with certain species of tick vectors have been postulated, but this is still open to debate.

Traditionally, spirochetes classified in the so-called *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) complex are transmitted by hard ticks of *Ixodes* genus. On the other hand, the Tick-borne Relapsing Fever *Borrelia* (RFB) are usually described as being transmitted by soft *Argasidae* ticks (Cutler, 2015), with the exception for *B. theileri* that, despite being classified as a RFB, is usually associated with hard ticks (*Rhipicephalus* spp.) (McCoy et al., 2014).

Phylogenetic studies carried out in the last decades, whether based on 16S ribosomal RNA (rrs) or on flagellin gene (*flaB*) sequences have come to challenge the simplistic vector/host division, suggesting that a proposal of somewhat strict associations between certain species of *Borrelia* and their vectors might be more difficult to defend than initially

Abbreviations: RF, Relapsing Fever; *B. burgdorferi* s.l., *Borrelia burgdorferi* sensu lato; *flaB*, flagellin gene; IGS, intergenic spacer region; *B.*, *Borrelia*; *B. burgdorferi* s.s., *Borrelia burgdorferi* sensu stricto; RFB, Relapsing Fever *Borrelia*; STARI, Southern Tick-Associated Rash Illness; LD, Lyme Disease; ML, Maximum Likelihood; NJ, Neighbor-Joining; TOT, transovarial transmission.

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anticipated. Indeed, and as an example, many of these studies reported that several different species of *Borrelia* classified as RFB were, in fact, transmitted by hard ticks. These included *B. miyamotoi*, firstly found in *Ixodes persulcatus* in Asia (Fukunaga et al., 1995) but also present in other *Ixodes* species, *B. lonestari* detected in *Amblyomma americanum* in North America (Barbour et al., 1996), *Borrelia* sp. found in *Amblyomma geomydae* and in *Haemaphysalis* spp. in Japan (Takano et al., 2012), and *B. turcica* which was found to be transmitted among reptiles by *Hyalomma aegyptium* ticks (Güner et al., 2004; Kalmár et al., 2015). These findings also showed that *B. theileri*, *B. lonestari* and *B. miyamotoi* branch together as a single monophyletic group in phylogenetic trees, located deep within the Relapsing Fever spirochetes clade (Barbour, 2014), and clearly outside from *B. burgdorferi* s.l. complex. However, and despite their allocation to the RF-cluster of spirochetes, the metastriate-transmitted *Borrelia* spp. should not be assumed to be biologically equivalent to the RFB (maintained by argasid ticks), nor do they cause typical Relapsing Fever (Telford et al., 2015).

Up to the present day, the analysis of the different RFB has not been regularly updated, and with a few exceptions, they have not been as thoroughly studied as *B. burgdorferi* s.l. bacteria. As an exception, the biology of *B. theileri* has been relatively well examined, particularly the clinical aspects associated with bovine borreliosis and vector–pathogen interactions (Callow, 1967; Smith et al., 1985). *B. lonestari* has also been associated with a disease manifesting as what came to be known as Southern Tick-Associated Rash Illness (STARI) or Master's disease, although incrimination of *B. lonestari* as the etiologic agent of these diseases has not yet been demonstrated (Feder et al., 2011). Finally, *B. miyamotoi*, which has been found in different species of *Ixodes* ticks in different regions of North America, Europe and Asia (Geller et al., 2012; Cochez et al., 2015; Cosson et al., 2014; Crowder et al., 2014; Dibernardo et al., 2014; Hansford et al., 2014; Kiewra et al., 2014; Mukhacheva and Kovalev, 2014; Takano et al., 2014; Nunes et al., 2015; Venczel et al., 2015), has also been associated with human disease cases in Europe (Hovius et al., 2013; Jahfari et al., 2014), USA (Gugliotta et al., 2013; Krause et al., 2013), Russia (Platonov et al., 2011), and Japan (Sato et al., 2014).

Relapsing Fever has sporadically been reported in the Iberian Peninsula, mainly in Spain during the twentieth century (Sanchez-Yebra et al., 1997), but with an incidence that is most probably underestimated. In Portugal, reduction of human tick-borne RFB cases may have been an indirect consequence of African swine fever outbreaks from 1960 until 1993, resulting in decreasing numbers of Alentejano pig herds and traditional pigpens in this region (Boinas, 1994; Morais et al., 2007). Consequently, pig production housing was modified, with modern shelters being constructed with glass fiber or metal, unsuitable for tick survival. It became evident that traditional shelters constructed with stone and clay, often with cracks and crevices, were essential for *Ornithodoros erraticus* infestation, with no infestation being found in pigpens with smooth walls and floor (Palma et al., 2012). Nevertheless, *B. hispanica* was recently detected in *O. erraticus* (detection rate of 2.2%), from a swinery in the Alentejo region (in the south of Portugal), which proves that RF-causing bacteria still circulate naturally, and suggest that they may be responsible for the cases of fever-illness with an indeterminate etiology (Palma et al., 2012). In addition, very little is known about which RFB agents are potentially transmitted by hard ticks in Portugal. In fact there are only two recent studies reporting the molecular identification of *B. miyamotoi* at Tapada Nacional de Mafra in Lisboa region (≈35 km North of the capital). The first one in an *I. ricinus* nymph that had fed on a *Turdus merula* (Norte et al., 2012), and the second one in an *I. ricinus* nymph collected from the vegetation (Nunes et al., 2015).

In contrast to what is currently known for RFB, in the last decades, the incidence of Lyme Disease (LD) has been increasing in some countries of Europe (Hubálek, 2009). Consequently, LD is likely to become an increasingly relevant health risk in the near future due to complex interactions between diverse environmental and socio-economic factors, which will likely affect various aspects of disease ecology and epidemiology. Currently there are already 20 species of *B. burgdorferi* s.l. described (Margos et al., 2011), and six of them have already been reported in Portugal. The most prevalent species is *B. lusitaniae*, isolated for the first time from the vector in 1993 (Núncio et al., 1993), and lately from a patient skin biopsy in 2003 (Collares-Pereira et al., 2004). Despite the detection of *Borrelia* in several species of Ixodids in Portugal, the only tick species with proven vector competence is *I. ricinus*.

For two years, between 2012 and 2014, an extensive survey of ticks was carried out in several districts of Portugal, where *I. ricinus* ticks are present, with the aim of determining the prevalence of *B. burgdorferi* s.l., species. Thus, the results presented in this study not only confirmed the wide distribution of multiple species of *B. burgdorferi* s.l. throughout Portugal, but they also revealed the presence of two different species of RFB. One RFB DNA was found in questing *Haemaphysalis punctata*, while the other was detected in questing *Rhipicephalus sanguineus* hard ticks.

2. Material and methods

2.1. Study area and tick collection

Portugal, the westernmost country in continental Europe, has climatic conditions influenced by the Atlantic Ocean and the Mediterranean Sea (Information available at http://www.florestar.net. Access in 20-11-2015). Out of its 92,090 km^2 of land surface, 3.4 million ha correspond to forested areas, mainly localized north of the Tagus river, with agroforestry and forest grazing areas localized in the south of the country.

Between May 2012 and May 2014, questing ticks were collected from spring to fall on a monthly basis in Lisboa district, and one to three times during each season for the remaining districts, by flagging with a 1 × 1m cloth over low and high vegetation, with a similar time flag (30 min of sampling at each site). The 23 collecting sites were scattered throughout the country, and located in seven districts: Braga, Vila Real, Aveiro, Lisboa, Setúbal, Évora and Faro (Fig. 1). Collected ticks were identified at the species level using taxonomic keys (Estrada-Peña et al., 2004; Pérez-Eid, 2006) (Table 1), and then stored in vials with 70% ethanol until further use.

2.2. DNA extraction

Ticks were firstly washed in 70% ethanol and secondly in sterile distilled water, then dried on sterile paper and finally subjected to mechanical maceration. Genomic DNA was extracted by alkaline hydrolysis, with NH_4OH (0.7 M) as described by Wodecka et al. (2010), using a volume of 500 μl added to each adult ticks, or 100 μl added to immature ticks (larvae and nymphs). Adult and nymphal specimens were processed individually while larvae were pooled together by species and day of capture (ten specimens per pool). The obtained lysates were stored at −20 °C for further use.

2.3. PCR amplifications

2.3.1. DNA amplification from B. burgdorferi s.l. species

Detection of *B. burgdorferi* s.l. DNA was carried out using two different nested-PCR protocols. One of them targeted the intergenic spacer region (IGS), located between the 5S and 23S rRNA, using the 23SN1 and 23SC1 external primers (which amplify a 320 bp DNA fragment), and the 23SN2 and 5SC inner primers (which amplify a 280 bp DNA fragment), as described by Rijpkema et al. (1995). The nested-PCR protocol used included a denaturation step at 94.5 °C for 1 min, 25 cycles of amplification at 94 °C for 30 s, 52 °C for 30 s (outer primers), or 55 °C for 30 s (inner primers), and 72 °C for 1 min, followed by a 5 min extension phase at 72 °C. The second nested-PCR protocol used, targeted the