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Borrelia lusitaniae and Lyme borreliosis in Portugal: A contribution to the study of an emerging bacterial zoonosis with an impact on Public Health.

Isabel Lopes de Carvalho

PhD in Biology

(Microbiology)

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_Resumo

A borreliose de Lyme, também conhecida como doença de Lyme, é uma doença emergente em algumas regiões do mundo, nomeadamente em Portugal. Caracteriza-se por ser ume patologia multifásica e multissistémica, com manifestações dermatológicas, reumatológicas e cardíacas.

O agente etiológico responsável por esta patologia é a bactéria do complexo *Borrelia burgdorferi* sensu lato (s.l.) que, até ao momento, inclui 16 espécies diferentes. O principal vector é ixodídeo do complexo *Ixodes ricinus*.

O principal objectivo desta dissertação é estudar os aspectos biológicos e moleculares de *B. lusitaniae*, focando-se essencialmente em dois aspectos, a infecção nos Humanos e o estudo da eco-epidemiologia de *B. burgdorferi* s.l. em Portugal.

Para corresponder aos objectivos acima citados, inicialmente foi realizado um estudo para avaliar o impacto desta doença em Saúde Pública. Como resultado foi possível constatar que, apesar de ser uma doença de notificação obrigatória, a análise dos dados obtidos pelo laboratório de referência CEVDI/INSA entre 1990-2004, relativamente aos casos diagnosticados *versus* os casos notificados revela que, tal como outras doenças transmitidas por ixodídeos, se trata claramente de uma doença sub-notificada em Portugal. A incidência estimada anual é de 0.4 por 100 000 habitantes, relativamente baixa quando comparada com alguns países europeus. Outro objectivo deste trabalho foi avaliar a potencial acção patogénica de *B. lusitaniae*. Neste sentido, foi feito um grande esforço para a obtenção de amostras biológicas, posteriormente utilizadas nas tentativas de isolamento. Como resultado, neste trabalho é descrito o segundo caso de isolamento de *B. lusitaniae* a partir de uma amostra de sangue total de uma doente que apresentava uma vasculite-*like,* o que confirma que esta genoespécie é patogénica ao Homem. Este caso clínico suporta a hipótese de que as manifestações clínicas associadas à infecção por *B. lusitaniae* incluem alguns sinais clínicos considerados, até ao momento, inespecíficos no diagnóstico de borreliose de Lyme. De forma a contribuir para o esclarecimento do quadro clínico da borreliose de Lyme em Portugal, dada a elevada prevalência de *B. lusitaniae* nos ixodídeos, foi realizado um estudo retrospectivo,onde foram analisados 12 doentes com diagnóstico clínico confirmado. Este trabalho permitiu a detecção, pela primeira vez, de DNA de *B. afzelii* num doente com sintomas neurológicos.

Na vertente eco-epidemiológica, foi dada particular importância à identificação das espécies de vectores e hospedeiros vertebrados responsáveis pela circulação destes agentes na

natureza. No que se refere aos artrópodes vectores, o estudo realizado nos ixodídeos na Ilha da Madeira e na região sul de Portugal revelou que ambas são regiões importantes para a presença de *B. lusitaniae*. No entanto, outras genospécies de *Borrelia* foram detectadas na Ilha da Madeira. Além disso, a espécie *I. ricinus* foi confirmada como principal vector na transmissão desta zoonose em Portugal. No entanto, outras espécies de ixodídeos, como *Dermacentor marginatus,* mostraram poder estar também implicadas na transmissão de borreliose de Lyme, uma vez que foi detectado DNA de *Borrelia* em alguns exemplares. Contudo, a capacidade e competência para transmitir *B. burgdorferi* s.l. continua desconhecida. A análise das sequências nucleotidicas obtidas a partir dos isolados dos ixodídeos da região sul de Portugal sugerem a presença de algumas espécies recombinantes, o que confirma a presença de variabilidade genética intra-especifica de *B. lusitaniae* nestas populações

Alguns estudos publicados anteriormente demonstraram que por vezes a presença de alguns agentes etiológicos condicionam o ciclo natural de outros agentes. Assim, foi também objecto de estudo investigar a existência de co-infecções nos artrópodes vectores. Nesta dissertação é também descrita pela primeira vez a presença de co-infecções nos ixodídeos de *B. lusitaniae* com *Rickettsia helvetica*, *R. monacensis* ou *R. slovaca,* sublinhando o possível aumento de infecção em humanos por múltiplos agentes infecciosos.

Relativamente ao estudo dos hospedeiros reservatórios, inicialmente foi investigado o papel desempenhado pelas aves limícolas migratórias. Como principais resultados salienta-se que foi possível detectar a presença de DNA de *B. garinii* em três diferentes géneros (*Limosa l. limosa, Limosa l. islandica* e *Calidris minuta)* e de *Francisella tularensis* subsp. *holarctica* em um *C. minuta*, realçando a possibilidade de disseminação de novos agentes infecciosos por estas espécies de aves.

Outros estudos direccionados nos animais, provaram o envolvimento dos pequenos mamíferos como potenciais reservatórios de *B. lusitaniae,* uma vez que foi possível isolar pela primeira vez esta genoespécie a partir de uma amostra de *Apodemus sylvaticus.* A análise filogenética agrupou este novo isolado perto de outras estirpes de *B. lusitaniae* isoladas durante o decurso desta dissertação e muito próximas do “cluster” de Norte de África. Foram também obtidas amostras sero-positivas das espécies *M. spretus* e *R. rattus*. Este resultado é extremamente importante em termos ecológicos, pois permite conhecer melhor os mecanismos que permitem a manutenção de *B. lusitaniae* na natureza.

A importância das lagartixas, especialmente as pertencentes à espécie *Teira dugessi* na

manutenção da infecção de *B. burgdorferi* s.l., foi confirmada. O isolamento de *B. valaisiana* a partir de tecidos de lagartixa e a detecção de DNA a partir de ixodideos colectados destes animais, foi descrito pela primeira vez. No entanto, a análise das sequências nucleotidicas dos isolados obtidos sugerem a co-existência de *B. lusitaniae*. Este trabalho permitiu ainda detectar DNA de *B. lusitaniae* nos tecidos e nos ixodídeos das lagartixas, confirmando a possibilidade destes animais servirem como reservatórios desta genoespécie. Os dados obtidos, relativamente a estes estudos, indicam que existe um ciclo silvático específico para *B. lusitaniae* que é mantido por uma variedade de hospedeiros e reservatórios vertebrados.
Muitos aspectos focados neste trabalho necessitam de uma investigação mais detalhada. No entanto, esta dissertação contribuiu para clarificar importantes aspectos epidemiológicos de borreliose de Lyme, essenciais para se estabelecerem medidas de controlo e prevenção, para além de contribuir para o estabelecimento de um diagnóstico clínico e laboratorial mais preciso da doença com impacto na Saúde Pública em Portugal.

Palavras-chave: *Borrelia* spp., *B. lusitaniae*, isolado humano, *Ixodes ricinus*, co-infecção, reservatório.

_Abstract

Lyme borreliosis, is a tick-borne disease caused by *Borrelia burgdorferi* sensu lato, that is considered an emerging disease in some regions of the world, namely in Portugal. The *B. burgdorferi* s. l. complex includes 16 different species that are transmitted to the vertebrate host by the tick vector. The principal vector in Europe is *Ixodes ricinus.* LB is a multisystem disease involving many organs such as the skin, the nervous system, the joints, and the heart.

The main goal of this project is to examine specific biological and molecular aspects of *B. lusitaniae*. The aims of this dissertation are divided into two main topics: Human infection and the eco-epidemiological study of *B. burgdorferi* s.l. in Portugal.

Although LB is a mandatorily notifiable disease in Portugal, the evaluation of CEVDI/INSA data concerning human cases and the number of notified cases shows that this disease is clearly underreported in Portugal. In the period of 1999-2004, the estimated incidence was 0.4 per 100 000 inhabitants, which is relatively low when compared with some endemic countries. In this work it was reported an isolation of *B. lusitaniae* from a human patient presenting with a vasculitis-like syndrome that confirm the patogenicity of this genospecies. This clinical report supports also the hypothesis that the clinical manifestations associated with infection by *B. lusitaniae* include some clinical symptoms until this moment considered unspecific for LB. In a prospective study, twelve patients with clinical diagnostic of LB were analysed and enabled the first detection of *B. afzelii* DNA in a patient with neurological symptoms.

A survey of ixodid ticks collected in Madeira Island and southern region revealed that both are hotspot regions for the presence of *B. lusitaniae*, however other *Borrelia* genospecies were detected in Madeira Island. Moreover, *Ixodes ricinus* species was confirmed as the principal vector of this zoonosis in Portugal. Other species such as *Dermacentor marginatus* were found to contain *Borrelia* DNA, but the ability of this tick to transmit *B. burgdorferi* is unknown. The sequences analysis of the isolates obtained from ticks from the southern region reveal that some recombinants strains are present which could indicate that an intersection in the allelic profiles of the *B. lusitaniae* populations exists. In this dissertation is also described the first report of dual infections of *Rickettsia helvetica, R. monacensis* and *R. slovaca*, each of which infected with *B. lusitaniae*, highlighting the increased likelihood of infection by multiple agents to humans. Concerning the study on migratory shorebirds the detection of *B. garinii* DNA was observed in three different genera (black-tailed godwits *Limosa l. limosa,* Icelandic black-tailed

godwit *Limosa l. islandica* and little stints *Calidris minuta)* and also *Francisella tularensis* subsp. *holarctica* DNA in a little stint.

The studies conducted in animal species prove the involvement of the small mammals, *Apodemus sylvaticus*, as a potential reservoir of *B. lusitaniae* with the first isolate from this animal species. Seropositive samples were obtained from *M. spretus* and *R. rattus species.* Phylogenetic analyses grouped this new isolate near other *B. lusitaniae* strains isolated within this dissertation and most closely aligned to the North African clade. Furthermore the importance of lizards (*Teira dugessi*) in the maintenance of *B. burgdorferi* s.l. was confirmed. Potential *B. valaisiana* isolation from lizard tissues and detection on parasitizing ticks was reported for the first time, but this observation needs conformation. *B. lusitaniae* DNA was also detected in lizard tissues and in ticks that are collected from them. However, the sequence data from lizards' isolation suggest the co-existence of *B. lusitaniae.* This data indicates that there may exist a sylvatic cycle specific to the *B. lusitaniae*, maintained by a variety of hosts and reservoirs.

Many aspects introduced and explored in this work will require more detailed investigations. However, this dissertation contributes to clarify some important epidemiological aspects of the clinical and laboratorial diagnosis of LB, a disease with impact in public health in Portugal.

Keywords: *Borrelia* spp., *B. lusitaniae*, human isolate, *Ixodes ricinus*, co-infection, reservoir.

ACA- Acrodermatitis chronica atrophicans

BSK- Barbour- Stonner- Kelly medium

CDC- Centers for Disease Control and Prevention

CEVDI- Centro de Estudos de Vectores e Doenças Infecciosas

CSF- Cerebrospinal fluid

DNA- Deoxyribonucleic acid

ELISA- Enzyme-linked immunosorbent assay

EM- Erythema migrans

EUCALB- European Union Concerted Action on Lyme borreliosis

IFA-Indirect immunofluorescence assay

LA- Lyme arthritis

LB- Lyme borreliosis

LNB- Lyme neuroborreliosis

MLST- Multilocus sequence analysis scheme

MSF- Mediterranean spotted fever

Osp- Outer surface proteins

USA- United States of America

PCR- Polymerase chain reaction

TBE- Tick-Borne Encephalitis virus

SFG- Spotted fever group

This thesis will be presented in four parts.

- **Part I - Introduction.** Includes the main objectives of the present study and reviews state of the art information presenting a perspective on Lyme borreliosis in Europe in two chapters.
- **Part II - Human infection.** Focuses on clarification of the pathogenic effect of *B. lusitaniae* in humans and is divided into three chapters.
- **Part III - Ecology.** Presents the study of the eco-epidemiology of *B. lusitaniae* and is divided into five chapters.
- **Part IV - General discussion and concluding remarks.** Discussion of the findings and final considerations of the present dissertation.

Part I – Introduction

Chapter I. Objectives

Scope of the thesis

The main goal of this project is to examine specific biological, microbiological and molecular aspects of *B. lusitaniae* in Portugal. The aims of this dissertation are divided into two main topics as follow:

- **Human infection**

i) Develop and/or standardize laboratory diagnostic techniques such as PCR and immunoblotting assays to accurately, diagnose and detect human disease. Special emphasis is given to improve the method of isolation of strains from human samples.

ii) Characterize specific clinical signs and symptomatology associated with Lyme borreliosis caused by *B. lusitaniae*.

- **Ecology**

    i) Evaluate the diversity and prevalence of strains of *Borrelia* isolated from ticks, rodents, lizards and birds.

    ii) Characterize the genotype and phylogenetic analysis of the isolated strains especially those belonging to *B. lusitaniae* species.

    iii) Evaluate the co-infection of *Borrelia* with other tick-borne bacteria in ticks.

Chapter II. State of the Art

1. Introduction

Arthropod borne spirochetes have long caused human suffering and disease (Piesman and Gern, 2004). The genus *Borrelia* is a member of the spirochaete phylum that contains 37 species of spirochetes, many of which cause disease in humans and domestic animals (Schwan and Piesman, 2002). Except the Louse borne relapsing fever that is transmitted by the human body louse (*Pediculus humanus*) and is caused by *Borrelia recurrentis,* all the other species are transmitted by ticks. Two major pathogenic groups are associated to human disease: relapsing fever spirochetes, transmitted by the fast-feeding argasid ticks of the genus *Ornithodoros,* and Lyme borreliosis spirochetes, transmitted by the relatively slow-feeding ixodid ticks of the genus *Ixodes* (Piesman and Schwan, 2010).

Lyme borreliosis (LB) is caused by Gram-negative spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) group. It is an extracellular organism belonging to the order Spirochetales and is the most frequent tick-borne human infection in the world, with an estimated 65,500 patients annually in Europe (Hubálek 2009). The etiological agents are maintained in nature by complex zoonotic transmission cycles involving ticks of the family *Ixodidae* and a vast number of vertebrate species. Humans are accidental hosts and not involved in the life cycle and evolution of the spirochetes (Steere et al. 2004).

LB has a foci in USA and Europe and extending to Asia, including China and Japan. The disease is present throughout Europe except for some southernmost regions (e.g. southern Spain), and the cold northern parts of Scandinavia (Stanek et al. 1993, Hytonen et al. 2008). In Portugal, LB is also an emerging infection where the *B. lusitaniae* is the most prevalent species of the *B. burgdorferi* s.l. complex and was first isolated from ticks collected in southern Portugal (Núncio et al. 1993).

This review will focus mostly on LB in Europe since in North America the disease presents a different pattern.

2. History

In 1977, Allen Steere and colleagues reported an outbreak of arthritis that had been detected mainly in children in eastern Connecticut in the communities of Old Lyme (Steere et al. 1977). The majority of the new patients with joint symptoms were identified during the summer and early autumn, and the arthritis was preceded by an erythematous skin lesion in some patients. The arthritis was characterized by recurrent attacks of swelling and pain in a few large joints, especially the knee. Cultures of joint samples and synovial fluid of the patients were negative for agents known to cause arthritis. Furthermore, some patients noted a skin rash approximately 4 weeks before the onset of arthritis. The skin lesion was found to be compatible with erythema migrans (EM) described in Europe many decades before (Afzelius 1910). However, the disease in Lyme residents was thought to be a previously uncharacterized clinical entity and was named Lyme Arthritis (Steere et al. 1977). During the following years some patients developed arthritis and some also acquired neurological and cardiac abnormalities. Thus, a complex multisystem disorder was recognized and the name was changed from Lyme arthritis to Lyme disease. Furthermore, the vector of the disease was identified as *Ixodes scapularis* (then called *Ixodes dammini*) tick (Steere et al. 1979). The last proof of the association between the spirochete and Lyme disease (now also called Lyme borreliosis) came when the bacteria was isolated from the skin, blood, and cerebrospinal fluid of Lyme patients (Burgdorfer et al. 1982).

In Europe, *Borrelia* was detected for the first time in *I. ricinus* collected in Switzerland in 1983 (Burgdorfer et al. 1983). Since then, several thousands of human cases have been detected in most part of European countries.

3. Epidemiology

The surveillance of LB patients in Europe does not allow a direct comparison of disease incidence between countries, due to different case definitions, laboratory methods used and due to the fact that LB is a not a mandatory reportable disease in a number of European and North American countries.
LB occurs between approximately 35° and 60° N in Europe, and between 30° and 55° N in North America. In countries at the southern limits of the LB range, incidence decreases rapidly along a north to south gradient (Hubálek 2009).
In USA, LB is currently the most prevalent vector-borne disease with more than 23,000 reported cases in 2005 (Piesman and Eisen, 2008). Since surveillance began by the Centers for Disease Control and Prevention (CDC), in 1982, the number of reported cases has increased dramatically, and the disease has become an important public health problem in some areas of the country (Bacon et al. 2008). In Europe, the lower annual incidence rates for LB basis on the statutory notifiable disease is found in Portugal (0.04 per 100,000 from 1999 to 2006) and in the UK, where the annual incidence in 2006 was 1.5 per 100,000. In Portugal, this disease is considered a notifiable disease since 1999, but only a few cases are reported each year, which does not allow consistent analysis of risk factors and the impact on public health. In northern and central Europe the highest incidences are found in southern Sweden (464 per 100,000 from 1997 to 2003) and in Slovenia (135 per 100,000 in 2005) respectively (Heyman et al. 2010). However, many experts admit that there is a significant underreporting of LB, and some of them estimate that the real LB incidence rate may be 2-3 times higher than reported (Hubálek 2009).
Both, disease incidence and antibody prevalence are higher in the central and eastern parts of Europe than in the western parts, with a decreasing incidence from south to north in Scandinavia and from north to south in Italy, Spain and Greece (Smith and Takkinen, 2006, EUCALB). This pattern closely reflects the distribution and abundance of ixodid vectors of LB, with the lower incidence at the edges of *I. ricinus* distribution ranges, which are determined by types of climate (mainly temperature and humidity) permissive for this tick species (Talleklint and Jaenson, 1998, Hubalek 2009). Moreover, several countries with increasing incidence of LB have observed changes in tick abundance, as well as changes in latitudinal or altitudinal distribution of ticks during the same time period (Gray et al. 2009). From a public health perspective, the highest risk periods of LB infection occurs when there is an overlap of activities between reservoir hosts, ticks and humans (Heyman et al. 2010). Future climate change in Europe will possibly facilitate a further spread of LB and contribute to increased

disease occurrence in endemic areas. By contrast, in some locations, where climate conditions will become too hot and dry for tick survival, LB may disappear (Talleklint and Jaenson, 1998, Gray et al. 2009).

As already mentioned, Portugal is also included in the list of countries with reported cases of LB and more detailed considerations will be discussed in next chapter.

4. The spirochete

Borrelia is a Gram-negative spirochete and it is an extracellular organism belonging to the order Spirochetales.

Spirochetes are an ancient monophyletic group of bacteria that have a characteristic spiral shape and a unique mode of motility that enables them to swim efficiently through highly viscous media, where the movement of other bacteria is reduced or inhibited (Charon et al. 2002, Tsao 2009). This spirochete has a double envelope membrane and varies in length from 10 to 30 µm and from 0.2-0.5 µm in width (Barbour and Hayes, 1986).The structure of borrelia species is a protoplasmic cylinder that is surrounded first by a cytoplasmic membrane, then by the periplasm, which contains the flagella, and finally by an outer membrane that is only loosely associated with the underlying structures (Bergström and Zückert, 2010- Samuels book). Several periplasmic flagella attach to each end of the cell cylinder and overlap at the cell center, giving the bacterium its characteristic flat wave shape (Figure 1). The asymmetric rotation of the periplasmic flagella bundles generates backward moving waves along the cell body that propel it forward (Moriarty et al. 2008, Tsao 2009). The remarkable aspect of the *B. burgdorferi* s.l. genome is the large number of sequences for predicted and known lipoproteins, including the plasmid-encoded outer surface proteins (Osp) A through F (Fraser 1997). These and other differentially expressed outer surface proteins presumably help the spirochete to adapt and survive in markedly different arthropod and mammalian environments. In addition, during the disseminated phase of the infection, another surface exposed lipoprotein called VlsE, undergoes extensive antigenic variation (Zhang et al. 1997). The organism has few proteins with biosynthetic activity and apparently depends on the host for much of its nutritional requirements.

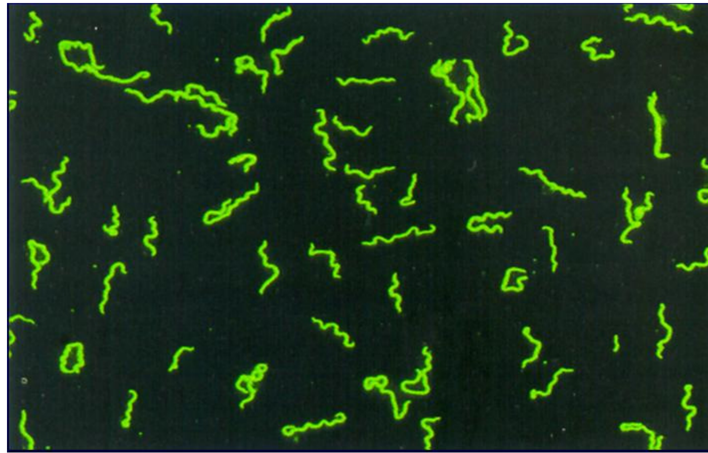

Figure 1. Image of *Borrelia burgdorferi* sensu lato in microscopy of fluorescence. (Amplification 10X40)

LB spirochete genomes (1,5 Mb) are comprised of one linear chromosome and many linear and circular plasmids, more than any other characterized bacteria. A high degree of homology among species exists at chromosome level, but the plasmid portion of the genomes varies greatly even among strains within species (Glockner 2006, Terekhova et al. 2006, Tsao 2009). Plasmids encode 40% of the genome of B31 strain of *B. burgdorferi* s.s., 29% of the PBi strain of *B. garinii*, and 36% of the PKo strain of *B. afzelii* (Glockner 2006, Tsao 2009). The chromosome contains many homologs of known genes, including housekeeping genes (Rosa et al. 2005, Margos et al. 2009). In contrast many plasmid-encoded genes require experiments to infer their functions. Some recent studies suggest that many plasmid genes encode proteins important to reproduction, infection, transmission and persistence of the spirochete in vertebrate hosts that will have implications on the ability to cause disease (Xu et al. 1996, Glockner 2006).

5. Causative agents

The first etiological agent of LB, *B. burgdorferi*, presently *B. burgdorferi* sensu stricto, was discovered in 1982 by Willy Burgdorfer and collaborators (Burgdorfer et al. 1982). Since then several other strains have been identified and molecular analyses of these new spirochetes have revealed that they form a species complex- *B. burgdorferi* s.l.

To date, sixteen species have been named within *B. burgdorferi* s.l. complex. These include *Borrelia burgdorferi* s.s. (Johnson et al. 1984), *B. garinii* (Baranton et al. 1992), *B. afzelii* (Canica et al. 1993), *B. japonica* (Kawabata et al. 1993), *B. andersonii* (Marconi et al. 1995), *B. tanukii* (Fukunaga et al. 1996), *B. turdae* (Fukunaga et al. 1996), *B. valaisiana* (Wang et al. 1997), *B. lusitaniae* (Le Fleche et al. 1997), *B. bissettii* (Postic et al. 1998), *B. sinica* (Masuzawa et al. 2001), *B. spielmani* (Richter et al. 2004), *B. californiensis* (Postic et al. 2007) and, recently recognized, *B. americana* (Rudenko et al. 2009), *B. bavariensis* (Margos et al. 2009) and *B. carolinensis* (Rudenko et al. 2010) (Table 1). However, three of these are novel species that have been recently proposed: *B. californiensis,* up to now restricted to California and is associated with the kangaroo rat *Dipodomys californicus*; *B. americana*, that has a geographic distribution in South Carolina and California and are associated with rodent and bird hosts; and *B. bavariensis* a rodent-associated ecotype of *B. garinii,* are still waiting to have the name validate (Postic et al. 2007, Margos et al. 2009, Rudenko et al. 2009).

All these species are differently distributed throughout the world, differently associated with the vectors and hosts, and have different pathogenicity patterns (Baranton 2001) (Figure 2).

Table 1. *Borrelia* species, geographical distribution, ecological characteristics and references.

***Borrelia* species**	**Reference**	**Geographical distribution**	**Vector**	**Host/Reservoir**
B. burgdorferi s.s.	Johnson et al. 1984	USA; Europe and Asia	*I. scapularis*; *I. pacificus*; *I. ricinus I. persulcatus*	Mammals; birds
B. garinii	Baranton et al. 1992	Europe and Asia	*I. ricinus; I. persulcatus*	Birds and small mammals
B. afzelii	Canica et al. 1993	Europe and Asia	*I. ricinus; I. persulcatus*	Small mammals
B. japónica	Kawabata et al. 1993	Japan	*I. ovatus*	Small mammals
B. andersonii	Marconi et al. 1995	USA	*I. dentatus*	Rabbit
B. tanukii	Fukunaga et al. 1996	Japan	*I. tanuki*	Small mammals
B. turdae	Fukunaga et al. 1996	Japan	*I. turdus*	Small mammals
B. valaisiana	Wang et al. 1997	Europe and Asia	*I. ricinus; I. granulatus*	Birds
B. lusitaniae	Le Fleche et al. 1997	Europe; North Africa	*I. ricinus*	Unknown
B. bissettii	Postic et al. 1998	USA	*I. pacificus; I. neotomae; I. scapularis*	Rodents and Birds
B. sinica	Masuzawa et al. 2001	China	*I. ovatus*	Rodents
B. spielmanii	Richter et al. 2004	Europe	*I. ricinus*	Rodents
B. carolinensis	Rudenko et al. 2010	USA	*I. minor*	Small mammals

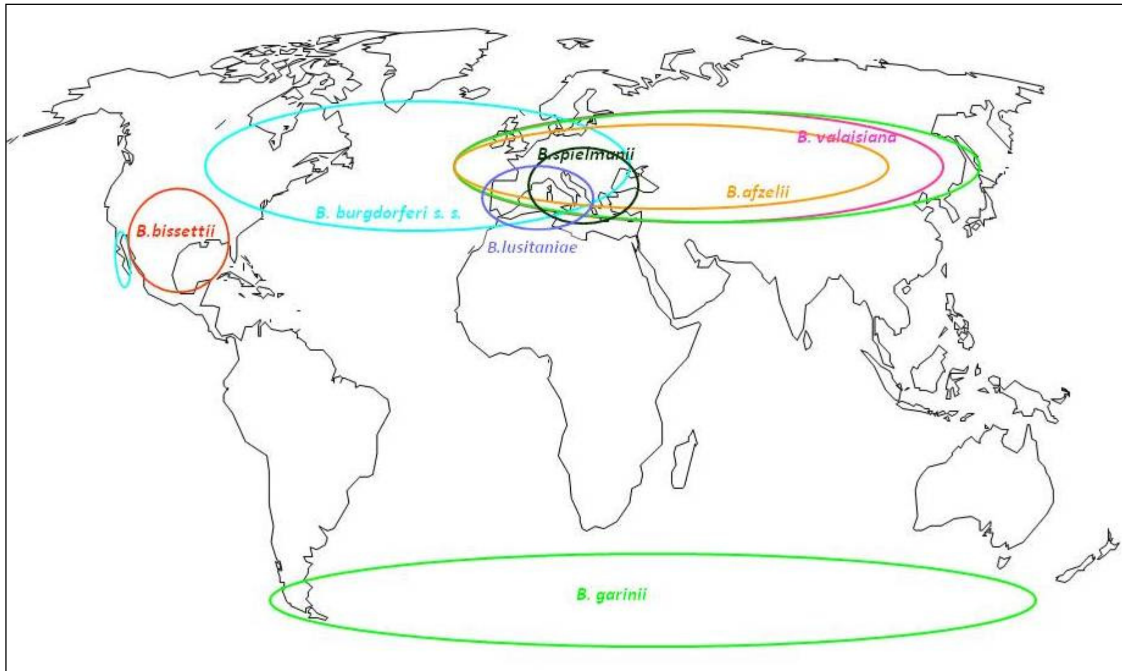

Figure 2. The geographical distribution of pathogenic species of *Borrelia burgdorferi* s.l. complex. □B.burgdorferi s.s. □ B.garinii □ B. bissettii □ B. afzelii □ B. valaisiana □ B. spielmanii □ B.lusitaniae

For many years, in Europe it was of consensus that LB was associated with the infection of one of three species: *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* (Assous et al. 1993, Van Dam et al. 1993, Richter et al. 2004). However, other genospecies have already been implicated in human cases: *B. bissettii, B. valaisiana, B. lusitaniae*, and *B. spielmanii* (Picken et al. 1996, Rijpkema et al. 1997, Collares-Pereira et al. 2004, Fingerle et al. 2008). In contrast, until now, *B. burgdorferi* s.s. is the only human pathogenic species in USA. Each of the pathogenic species has been found preferentially responsible for specific clinical presentations. Bacteria isolate from skin primarily belong to *B. afzelii*, especially those from patients with acrodermatitis chronica atrophicans (ACA), a chronic skin disease not present in USA. Isolates from cerebrospinal fluid (CSF) and ticks are heterogeneous with a predominance of *B. garinii*. Polymerase chain reaction (PCR) from synovial fluid of Lyme arthritis patients revealed higher a prevalence of *B. burgdorferi* s.s. (Canica 1993, Wilske 1993, Wilske 1996).

Concerning *B. lusitaniae*, which was isolated from *I. ricinus* ticks for the first time in Portugal, it was showed firstly that this bacterium could cause disease in the C3H/HeN mouse model (Núncio et al. 1993, Zeidner et al. 2001). In 2004, it was isolated from a human skin biopsy in Portuguese patient (Collares-Pereira et al. 2004). At first, some authors hypothesized that this species has an ecological niche that involves host species restricted to the western

Mediterranean Basin (Núncio et al. 1993, De Michelis et al. 2000, Gern and Humair 2002, Younsi et al. 2005). However, the reports of the presence of *B. lusitaniae* in countries outside the Mediterranean basin, Poland (Wodecka and Skotarczak, 2005), France (Richter et al. 2003) and in Switzerland (Jouda et al. 2003) demonstrate that this species can be found outside of it well previously defined foci in southern Europe (Piesman and Gern, 2004). Nevertheless, the fact that *B. lusitaniae* appears clearly as the dominant species in *I. ricinus* ticks in countries of southern Europe, such as Portugal, Tunisia and Morocco, indicates that the prevalence of other genospecies of *B. burgdorferi* s.l. decrease towards the southern margin of its European distribution (De Michelis et al. 2000, Gern and Humair, 2002, Piesman and Gern, 2004, Younsi et al. 2005).

6. The tick vector

Ticks are obligate hematophagous arthropods that parasitize every class of vertebrates in almost every region of the world (Soneshine 1991). Ticks and mites are grouped as subclass Acari, which is the largest subclass in the class Arachnida of the suborder Ixodida within the order Parasitiformes. There are two major tick families, the *Ixodidae*, also called “hard ticks” because of their sclerotized dorsal plate, and the Argasidae, commonly known as “soft ticks”, because of their flexible cuticle (Soneshine 1991). The Nuttalliellidae is the third family, which as only one species geographically limited to Southern Africa (Olivier, 1989).

The family Ixodidae comprises the highest number of species and is the medically most important tick family (Parola and Raoult, 2001).

The vectors of LB are several closely related ixodid ticks that are part of the *Ixodes ricinus* complex.
The two principal vectors of LB in North America are the blacklegged tick, *I. scapularis*, in the eastern half on the continent and the western blacklegged tick, *I. pacificus*, in the western half of the continent. The vast majority of LB infections in the North America are acquired through the bites of *I. scapularis* both biotic and abiotic factors control the distribution of these tick (Piesman and Gern, 2004). *I. scapularis* is distributed along the eastern seaboard from Florida to Maine and as far west as central Texas, but is not continuous distribution (Ogden et al. 2006). The *I. pacificus* tick is found along the Pacific Coast from British Columbia and Canada (Banerjee et al. 1994).

I. persulcatus is widely distributed over a huge part of the globe in Eurasia, from Japan in the Pacific Ocean trough China and Russia up to Baltic Sea (Korenberg et al. 2002). This tick can be found in broad leaved, mixed or southern Taiga forests (Filippova 1991). In the Russian Federation, *I. persulcatus* appears to be a more efficient vector of LB than does the European vector, *I. ricinus* (Korenberg et al. 2002, Piesman and Schwan, 2010).

The European tick, *I. ricinus,* known commonly as the sheep tick, has a very wide geographical distribution throughout Europe. It is distributed along a northsouth axis extending from Northern Scandinavia to the Kroumiry Mountain in Tunisia, and along an east-west axis extending from Ireland to the European section of the Russian Federation (Piesman and Schwan, 2010). This wide geographical distribution of *I. ricinus* implies that this tick survives under various environmental conditions (Piesman and Gern, 2004). This tick to encounter its hosts quests on the tip of low vegetation. During questing, ticks very often have to face desiccating conditions and have to quit their questing place to move to the litter zone where they regain lost body water (Randolph and Storey, 1999, Gern 2008). Interestingly, *I. ricinus* moves preferentially when desiccation risk is the lowest in nature, at sundown (Gern 2008). If high desiccating conditions are lasting too long, tick mortality is increased resulting in questing tick population decrease (Perret et al. 2000, 2004). Immature *I. ricinus* have been reported to infest an extremely wide range of hosts including rodents, birds, insectivores and lagomorphs, as well as medium sized mammals. Adult *I. ricinus* feed on larger hosts, including roe deer, sheep and wild boar (Humair et al. 2007, Moran-Cadenas et al. 2007). Besides *I. ricinus*, other tick species have been found infected by spirochetes but only *I. hexagonus*, that feeds mainly in carnivores and *I. uriae*, that is associated with seabirds, are able to support natural transmission of *Borrelia* and to significantly contribute to the persistence of spirochetes within endemic areas (Gern et al. 1997, Duneau et al. 2008, Gern 2008).

I. ricinus- persulcatus complex ticks have three post embryonic developmental stages- larva, nymph and adult. With the exception of the adult male, each life stage requires a blood meal from a vertebrate hosts. LB spirochetes primarily are maintained in nature through horizontal transmission between their tick and vertebrate hosts (Tsao 2009). Larvae and nymphs feed mostly during spring and summer while adults feed mostly in autumn. The larvae and nymphs feed primarily on small rodents whereas adult ticks feed on variety of larger animals. The feeding period of *Ixodes* species ticks is rather long (several days to over a week) and contributes to their geographic dispersal along with the movement of the host (Wilske 2005).

Ticks transmit the infection by cutaneous inoculation of borrelia contained in saliva. In unfed ticks, borrelia lives in the midgut. During the blood meal, borrelia migrates from the midgut of the tick into its salivary glands and, as the feeding process continues, the spirochetes move along the flow of saliva into the new host (De Silva and Fikrig, 1995, Hÿtonen et al. 2008). Usually, borrelia are transmitted to humans by nymphal or adult ticks and the transmission of the disease requires attachment of the tick for as long as 48-72h, and if the tick has been attached for less than 24h, risk of infection is low (Piesman 1993). The uptake of blood triggers *B. burgdorferi* s.l. to multiply in the midgut of the tick (Piesman et al. 1990). Virtually all spirochetes in the midgut of an unfed nymph express OspA at high levels. This protein is also the predominant antigen expressed by the spirochetes *in vitro*. OspA has been found to bind a tick midgut receptor, TROSPA that is essential for the spirochete colonization. Some authors demonstrated that *I. scapularis*-TROSPA is a specific ligand for *B. burgdorferi* s.l. OspA and that in vitro OspA selectively bound TROSPA but not to other tick proteins (Pal et al. 2004). OspA expression ensures that *B. burgdorferi* s.l. remains in midgut until the tick attaches to the mammalian host. As the tick partakes of a nutritious blood meal, *B. burgdorferi* s.l. begins to shift the expression of its surface proteins. The decrease in surface OspA releases *B. burgdorferi* s.l. from tick epithelial cells in preparation for infection of the mammalian host (Piesman and Schwan, 2010). Tick feeding exposes *B. burgdorferi* s.l. to changes not only in temperature, but also in redox potential, pH and osmolarity. As the nymphal ticks start to feed and spirochetes in the midgut begin to multiply rapidly, most spirochetes cease expressing OspA on their surface (De Silva 1996, Schwan and Piesman, 2000). Simultaneous with the disappearance of OspA, the spirochete population in the midgut begins to express OspC and the organisms begin their migration to the tick's salivary glands, which prepares the organism for host invasion by binding the tick salivary protein, Salp15 (Pal et al. 2004, Pal and Fikrig, 2010).

Figure 3.The four stages of *Ixodes ricinus*. Left to right: female, male, nymph and larvae

7. Co-infection of *Borrelia* with other tick-borne agents

Borrelia spp. may cause nonspecific systemic symptoms and co-infection with other tick-borne agents may lead to more severe and acute illness (Krauser et al 1996). Cases of multiple infections in humans with atypical clinical symptoms have been described (Sexton et al. 1998). Sometimes manifestations caused by different pathogens may overlap sufficiently for one of the infections to be overlooked, resulting in failure to provide appropriate treatment, as in the case of TBE and early neuroborreliosis (Cimperman et al. 1998). Serodiagnostic confusion may also arise where cross reactions occur, to lead an occasional false positive LB serology. It is possible that some of the diagnostic difficulties and treatment problems encountered in LB may be due to co-infections with other pathogens.

Ticks of the *I. ricinus* complex besides *B. burgdorferi* s.l., are also potential or confirmed vectors of other zoonotic pathogens with Public health relevance including *Anaplasma phagocytophilum*, *Babesia microti*, *Bartonella* spp. *Coxiella burnetii*, *Francisella tularensis*, *Rickettsia helvetica and R. monacensis,* and some tick-borne virus (Rehacek et al. 1994, Estrada-Peña and Jongejan, 1999, Sekeyova et al. 2000, Parola and Raoult, 2001, Fernandez-Soto et al. 2004, Holden et al. 2006, Swanson et al. 2006). Frequency of co-infection of ticks with several pathogens varies depending on tick species and countries. The true prevalence of

co-infecting pathogens among *Ixodes* ticks remains largely unknown in the majority of geographic locations.

Ticks that are co-infected can transmit more than one pathogen in a single tick-bite (Leutenegger et al. 1999). In Portugal several of these pathogens have already been detected.

A. phagocytophilum the causative agent of human granulocytic anaplasmosis, primarily recognized as a pathogen of domestic animals, has received increasing attention with confirmation of cases of human infection, especially in the North America but also in Europe (Dumler et al. 2005). It is geographically distributed worldwide in regions infested with suitable ticks' vectors. *A. phagocytophilum* is a prime candidate as a potential influence on the development of atypical clinical manifestations of LB. However, *A. phagocytophilum,* is not known to cause chronic infection, as with untreated *B. burgdorferi* s.l. infection (Steer 2005, EUCALB). Several studies have detected both *B. burgdorferi* s.l. and *A. phagocytophilum* species in the same individual *I. ricinus* tick (Leutenegger et al. 1999, Christova et al. 2001, Stanczack et al. 2004). In Portugal, studies conducted in ixodid ticks prove the involvement of two *Ixodes* species in *A. phagocytophilum* cycles, namely *I. ricinus* on Madeira Island and *I. ventalloi* on the mainland (Santos et al. 2004). Some previous studies based on serological data have also suggested dual infections in LB patients with *A. phagocytophilum* (Santos et al. 2006, Santos et al. 2009).

Babesia spp. is a protozoon that causes the babesiosis in both animal and man although it is quite rare in humans in Europe, transmitted by several ixodid species to the vertebrate host. A few symptomatic cases have been reported, mainly in splenectomised patients (Sambri et al. 2004). Previous studies reported co-infections with *Borrelia* and *Babesia* spp. (Wojcik-Fatla et al. 2009) and in Portugal one case of human babesiosis was reported in a splenectomised patient (Centeno-Lima et al. 2003).

Bartonella spp. is an intracellular bacteria that cause characteristic host-restricted hemotropic infections in mammals and are typically transmitted by arthropod vectors (Chomel et al. 2009). Some reports, from Europe and Russia, described the detection of *Bartonella* DNA in patients exposed to tick bites (Billeter et al. 2008) Furthermore, other studies conducted in *I. ricinus* ticks detected *Bartonella* spp. with *B. burgdorferi* s.l. and *Babesia* spp. co-infections (Halos et al. 2005). However, the role of ticks in transmitting *Bartonella* is still under evaluation. In Portugal, a case of bacillary angiomatosis in HIV infected patient was associated with *B. quintana* (Santos et al. 2000).

Coxiella burnetii, the causative agent of Q fever, has been found throughout the world. The faeces of ticks infected with *C. burnetii* have very high concentrations of viable organisms, which may persist for long periods in the environment, and instead the existence of other infection routes, ticks may play an important role in the dissemination of the organism. This agent can be also be detected in different tick species (Maurin and Raoult, 1999). No cases of co-infection in ticks have been found in literature. In Portugal, Q fever is an endemic disease with mandatory notification. In average 0.08 cases are reported annually per 100.000 inhabitants (2004-2008), however sub notification and subdiagnosis are a problem and official data may not accurately reflect Q fever incidence (Santos et al. 2009, Santos- personal communication).

F. tularensis, the causative agent of tularemia, can infect humans in different ways, including transmission by arthropods such as ticks, tabanids and mosquitoes, as well as by direct contact with infected animals and contaminated environmental materials (Ellis et al. 2002). In Europe, *D. reticulatus* and *I. ricinus* ticks are the main vectors (Petersen et al. 2009). In Portugal, recent studies have confirmed the presence of *F. tularensis* subsp. *holarctica* in *D. reticulatus* and in humans (Lopes de Carvalho et al. 2007).

The spotted fever group (SFG) rickettsiae are distributed worldwide and are one of the oldest known arthropod-borne diseases. Rickettsiae are associated with arthropods, which may act as vectors, reservoirs, and/or amplifiers in the life cycles of the bacteria.
In Europe, the *Rickettsia* species that are pathogenic to humans and that are associated with the *Ixodes* complex are: *R. helvetica* and *R. monacensis* (Sreter-Lancz et al. 2005, Blanco and Oteo 2006). Some descriptions of dual infection of *B. burgdorferi* s.l. with *R. monacensis* and *R. helvetica* in *Ixodes* tick have been made (Fernández-Soto et al. 2004). In Portugal, *R. helvetica* is already detected in *I. ricinus* (Bacellar et al. 1995, Santos-Silva et al. 2006). However, the most important tick-borne agent is *R. conorii* which cause Mediterranean spotted fever (MSF), a tick borne disease associated with *Rhipicephalus sanguineus* tick. In period of 1985-2005, the incidence rate of MSF was $8.4/10^5$ inhabitants, one of highest rates compared with other endemic countries (De Sousa et al. 2008).

One of the most important viruses transmitted by ticks and that have a high relevance in Public Health is the Tick-Borne Encephalitis virus (TBE). TBE is endemic in an area ranging from northern China and Japan, through far-eastern Russia to Europe, and is maintained in cycles involving Ixodid ticks (*I. ricinus* and *I. persulcatus*) and wild vertebrate hosts. The virus causes a potentially fatal neurological infection, with thousands of cases reported annually throughout Europe. TBE has a significant mortality rate depending upon the strain of virus or may cause long-term neurological/neuropsychiatric sequelae in people affected (Mansfield et al. 2009). Some studies demonstrate dual infection with *B. burgdorferi* s.l. and TBE in patients residing in geographic areas where TBE and *B. burgdorferi* s.l. are endemic (Oksi et al.1993, Cimperman et al. 2002). In Portugal, cases of TBE infection have never been described.

8. The vertebrate host

Borrelia can survive and multiply in vertebrate reservoirs, from which they are taken up by ticks (Humair and Gern, 1998). The enzootic cycle involves larval and nymphal ticks becoming infected with *B. burgdorferi* s.l. while feeding on their hosts (Piesman and Gern, 2004). A wide range of vertebrate species is parasitized by *Ixodes* complex ticks. But not all species are competent reservoir. A reservoir host must be able i) to host vector ticks ii) to acquire the agent from infectious ticks iii) to allow the agent to multiply and/or develop and persist in its body and iv) to transmit the agent back to subsequently feeding vectors ticks (Kahl et al. 2002).

Several works have revealed the importance of small mammals for the enzootic transmission cycles of LB spirochetes. Small mammals have been the group most extensively investigated up to now in Europe. In particular, evidence that the mice *Apodemus sylvaticus, A. flavicollis, A. agrarius* and the vole, *Clethrionomys glareolus,* act as reservoirs for *B. burgdorferi* s.l. has been obtained in many European countries (Humair et al. 1993, Gern et al. 1994, Kurtenbach et al. 1998, Hanincová et al. 2003, Piesman and Gern, 2004). Currently, the ability of *Apodemus* mice and *Clethrionomys* voles to infect ticks with borrelia is unanimously recognized (Humair et al. 1999). Colonial seabirds and ground-foraging birds, such as pheasants (*Phasianus colchicus*), blackbirds (*Turdus merula*), European and American robins (*Erithacus rubecula* and *T. migratorius*) were soon recognized as being efficient reservoir hosts as well (Kurtenbach et al. 1998, Richter et al. 2000, Hanincová et al. 2003, Poupon et al. 2006, Duneau et al. 2008).

Studies on hares, squirrels and hedgehogs revealed that they contribute to *Borrelia* infection in ticks (Gern and Humair, 2002).

Among large size mammals, red foxes are also able to infect ticks with *Borrelia* but the infectivity of red foxes appears to be low (Gern 2008). Green lizards (*Lacerta viridis*), sand lizards (*L. agilis*), and common wall lizards (*Podarcis muralis*) play also a role in the enzootic cycles of *Borrelia* in southern Europe (Amore et al. 2007, Grego et al. 2007).

Another important finding is that the different strains of *B. burgdorferi* s.l. are maintained in nature by different spectra of vertebrate host species (Kurtenbach et al. 2002). It is evident that a variety of extrinsic ecological factors such as climate, vegetation or geology, determine the relative role a vertebrate host population plays in the maintenance and geographical distribution of a particular strain of *Borrelia* (Kurtenbach et al. 1995, Randolph and Craine, 1995). However, it is obviously that intrinsic factors also play important roles in the biology of *B. burgdorferi* s.l. (Kurtenbach et al. 2002). First studies strongly suggested that a specific association existed between small mammals and *B. afzelii*; *B. burgdorferi* s.s and a subtype of *B. garinii* were also associated with rodents; that *B. burgdorferi* s.s. and *B. afzelii* were transmitted from squirrels to feeding ticks; that *B. garinii* and *B. valaisiana* were also associated with birds (Kurtenbach et al. 1998). The current knowledge of *B. lusitaniae* situation is especially interesting. In fact, recently, different vertebrate groups such as lizards, birds and hedgehogs were identified as potential reservoirs of *B. lusitaniae* (Dsouli et al. 2006, Poupon et al. 2006, Richter and Matuschka, 2006, Amore et al. 2007).

The explanation of these associations was provided by Kurtenbach et al. (1998); the complement present in the blood of the hosts shown to be the active component in the *Borrelia* host specificity. However, it seems that the situation is not clear and that some exceptions exist, and that different associations are present in nature, even in low frequency (Gern 2008). In fact, results from other studies conflicted with this situation, for example not only *B. afzelli* or *B. burgdorferi* s.s. but also additional *Borrelia* species and subtypes were isolated, or PCR detected, in small mammals (Korenberg et al. 2002, Baptista 2006). Differences in biological and ecological factors such as different *Borrelia* subtypes, different tick vector species and different areas could explain the discrepancy observed (Gern 2008). Recently, analysis of LB spirochetes, using a novel multilocus sequence analysis scheme (MLST), revealed that OspA serotype 4 strains of *B. garinii* (a rodent associated ecotype) were sufficiently genetically distinct from bird-associated *B. garinii* strains to deserve species status, *B. bavariensis* (Margos et al. 2009).

9. Clinical manifestations and diagnosis

The disease covers a wide spectrum of clinical manifestations affecting the skin, nervous and musculosketal systems, the heart and the eyes (Table 2). Due to the diversity of clinical symptoms, LB is often considered in differential diagnosis (Wilske 2003, 2005). Three stages of LB have been described, but the disease itself does not necessarily develop in stages (Stanek et al. 2002).

Early infection (**stage 1**) The most frequent clinical manifestation of LB consists of primary erythema migrans (EM), an annular skin rash that begins days to weeks after a tick bite and is the direct result of the spirochete migrating through the skin (Reed 2002, Heyman et al. 2010, EUCALB). The initial EM (Figure 4) is commonly a homogeneous lesion, which may either be bright red or turn bluish-red, and the homogeneous nature may not change in some patients until treatment or spontaneous healing (Stanek et al. 2002). In European patients, constitutional symptoms accompanying EM (most often fatigue, malaise, arthralgia, myalgia and headache) are present in fewer than 50% and are regularly mild, and local symptoms such as itching, burning and/or mild local pain are reported by about 50% of patients. Constitutional symptoms are more frequent in the USA (Stanek et al. 2002).

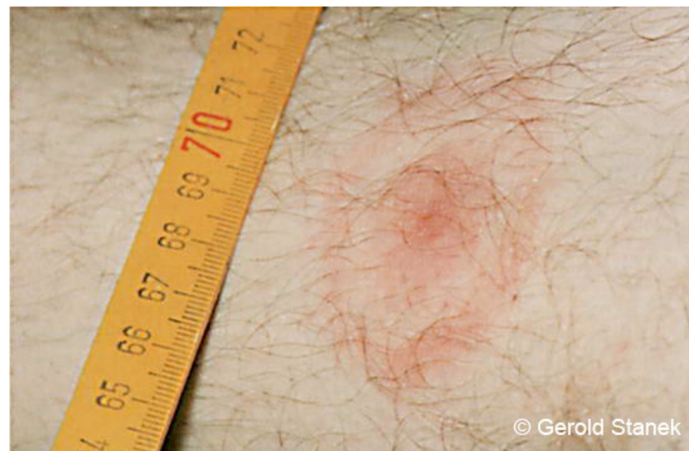

Figure 4. Erythema migrans, the most clinical manifestation of Lyme borreliosis

(EUCALB http://meduni09.edis.at/eucalb/cms/index.php?lang=en)

Early disseminated disease (**stage 2**) shows an hematogenous dissemination of spirochetes over subsequent weeks or months and can result in multiple skin lesions (secondary EM), as well as meningitis, radiculoneuritis, atrioventricular block, myocarditis, and oligoarticular arthritis. Borrelial lymphocytoma presents as a bluish red tumour-like skin infiltrate, up to a few centimeters in diameter, with lymphoreticular proliferation in the dermis and/or subcutis. The targeting of any of these systems can cause a wide variation of clinical features presenting that included Lyme neuroborreliosis (LNB). The full picture of neuroborreliosis is defined as meningoradiculoneuritis (Stanek et al. 2002, Stanek and Strle, 2008). LNB is an infectious disorder of the nervous system caused by the spirochetes and is the most frequent syndrome of disseminated infection in Europe and is also becoming a common symptom in North American LB patients (Garcia-Monco and Benach, 1995, Ciesielski et al. 1989, Mygland et al. 2010).

Persistent infection (**stage 3**) occurs months to years after the initial exposure and can be associated with ACA, varying degrees of encephalopathy and encephalomyelitis, and persistent arthritis. ACA usually begins on the extensor sites of the extremities, most commonly on the lower leg with initial involvement of one foot. ACA does not heal spontaneously (Stanek et al. 2002). Of note, clinical manifestations of LB among patients in North America seem to differ somewhat from those residing in Europe and Asia. For example, acrodermatitis and severe encephalomyelitis due to LB are more common in Europe and Asia but are infrequent among patients in North America. These discrepancies can be explained, at least in part, by the different genospecies of *Borreli*a responsible for LB in various geographic areas and possibly by genetic differences among the affected populations (Reed 2002).

The distinct clinical manifestations of LB are predominantly associated with infection by different *B. burgdorferi* s.l. strains: Lyme arthritis is associated with *B. burgdorferi* s.s., neuroborreliosis is associated with *B. garinii* and ACA is associated with *B. afzelii*. Therefore, the clinical manifestations are somewhat distinct in North America and in Europe, reflecting the global distribution of the different spirochetes genotypes (Wang et al. 1999).

In Europe, LB occurs with similar frequencies in women and men with the exception of ACA which is more frequent in women. Early neuroborreliosis cases showed a bimodal age distribution with a lower frequency in the age range of 20 to 29 years old whereas ACA occurs primarily in older patients (Wilske 2005).

Table 2. Main clinical manifestations of Lyme borreliosis (adapted from Stanek et al. 2002)

Organ system	**Clinical feature**
Skin	Erythema migrans
	Erythema migrans multiple lesions
	Borrelial lymphocytoma
	Acrodermatitis chronica atrophicans
Nervous system	Meningitis
	Meningoencephalitis
	Meningo-radiculoneuritis
	Encephalomyelitis
	Cerebral vasculitis
	Pheripheral neuropathy
Musculoskeletal	Arthritis
	Myositis
Heart	Carditis
Eye	Conjunctivitis, endophtalmitis, panophthalmitis

10. Treatment

A tick bite, which leads to infection with *B. burgdorferi* s.l., can result in an infection which remains subclinical (asymptomatic) and is self-limiting. All cases with clinical symptoms should, however, receive antibiotic treatment to prevent progression to later stages. However, when the infection remains untreated, the spirochete can disseminate and cause severe disease months or years after infection.

The type of antibiotic treatment given will depend on the clinical manifestations but also on antibiotic strategies in different parts of Europe. Various antibiotics are used for the therapy of LB, with a usual duration of treatment of 14 days. Patients who have solitary EM or borrelial lymphocytoma are treated orally with doxycycline, penicillin V, amoxicillin, cefuroxime-axetil or azithromycin, and in exceptional cases with erythromycin (Wormser et al. 2006, Stanek and Strle, 2008). To date, there is no evidence for acquired antimicrobial resistance against the antibiotics that are commonly used for the treatment of LB. Nervous system involvement and Lyme carditis are treated with intravenous ceftraxione or penicillin G for 2-3 weeks and only in exceptional cases with oral doxycycline or amoxicillin (Brouqui et al. 2004, Steer 2005, Stanek and Strle, 2008, Heyman et al. 2010).

11. Laboratory Diagnosis

The diagnosis of LB usually requires laboratory confirmation by means of a microbiological diagnostic assay, except in cases with the clinical manifestation EM (Steere 2005, Wilske 2005).

The European Union Concerted Action on Lyme borreliosis (EUCALB) and the CDC have developed a case definition of LB for surveillance purposes that includes either physician-diagnosed EM along with solitary lesions with diameters of at least 5 cm or at least one late joint, neurologic, or cardiac manifestation along with laboratory confirmation (EUCALB, CDC). This definition is not intended to be total sensitive or specific for clinical diagnosis but is useful as a starting point for the development of a differential diagnosis and highlights the central role of laboratory testing, especially for extracutaneous LB.

Laboratory tests have improved considerably over the last years and clinicians now have available a lot of options for the direct detection of organism in tissues, serological detection of organisms in tissues, serological detection of immune responses, and molecular detection of specific nucleic acid sequences and antigens. All of the various methodologies have their inherent advantages and limitations (Reed 2002).

Culture isolation of *B. burgdorferi* s.l. from clinical specimens remains the *gold standard* for diagnosis and is most commonly attempted with skin biopsy or cutaneous lavage specimens from EM lesions and blood from patients with early-disseminated disease (Wilske 2005). Positive culture rates of nearly 90% for secondary EM lesions, 50% for primary EM lesions, and 48% for large volume of blood or plasma specimens from patients with early LB have been reported (Reed 2002). Isolation of *B. burgdorferi* s.l. from other sites, such as CSF and synovial fluid is uncommon and the low recovery rate observed probably reflects the small number of viable organisms present in those anatomic locations. Culture of *B. burgdorferi* s.l. involves incubating a specimen in Barbour-Stoenner-Kelly medium (BSK) and detecting the presence of characteristic spirochetes by dark-field microscopy or by fluorescent microscopy with a specific fluorescent antibody. Time to detection of a positive specimen can be within a clinically relevant time frame, provided cultures are examined for the presence of spirochetes at frequent intervals, especially during the first two weeks of incubation (Reed 2002). Culturing may be of help in individual cases if the clinical picture suggests LB despite a negative antibody assay and /or for untreated patients having lesions suspected to be primary EM, particularly those that appear atypical (Reed 2002, Wilske 2005). However is a very time consuming method.

Many laboratories have turned to molecular assays in an attempt to increase sensitivity and specificity and decrease the turnaround time for laboratory testing of LB. The majority of the assays utilize PCR to amplify specific *B. burgdorferi* s.l. nucleic acid sequences from tissue biopsy specimens or samples of blood, CSF, joint fluid. Both conventional and nested PCR assays have been developed, and detections methods vary from gel electrophoresis and Southern hybridization to real-time PCR. Both plasmid and chromosomal targets have been used, and each has its advantages. Targets carried on plasmids, such as *ospA*, *ospC*, and *vlsE*, are present in multiple copies within each bacterium, and assays with these targets have greater sensitivity than those employing single-copy chromosomal targets such as *fla*, *recA*, *rpoB*, 16S and 23S ribosomal DNA, and the rDNA intergenic spacers. Several specific sequences are available in databases, so nowadays is easy to select the optimal DNA sequences for amplification (Wang et al. 2010).
PCR can be used to confirm EM lesions before the appearance of serum antibodies and without the delay associated with culture isolation (Reed 2002, Wilske 2005). The highest detection rate of *Borrelia* with PCR is achieved in skin biopsy samples from patients with EM and ACA, where the median sensitivity is around 70%, and in joint samples of Lyme arthritis (LA) patients with sensitivity up to 80% (Aguero-Rosenfeld 2005, Wang et al. 2010). However, a negative result in a PCR test cannot be held as an exclusion of LB. Given that the number of spirochetes in infected tissues or body fluids of patients is generally very low, appropriate procedures for sample collection, transport and DNA preparation from clinical samples are critical for reliable and consistent PCR results (Wang et al. 2010). One of the limitations of nucleic acid amplification methods is the generation of false positive results due to contamination. Amplicon contamination is extremely troublesome in assays tuned for maximum sensitivity, a necessity for the diagnosis of *B. burgdorferi* infections (Schmidt 1997).

Borrelia serology is the diagnostic test of choice in most cases where LB is suspected. Currently, the EUCALB and CDC recommends, for serologic serodiagnosis, a two-test approach in which samples are first detected by enzyme-linked immunosorbent assay (ELISA) and those with a borderline or positive results are tested by immunoblotting (EUCALB, CDC).
According to EUCALB an IgM and IgG immunoblot are considered positive if three bands are present (20, 30, 40 kDa), however the interpretation criteria depend on the strain or species used as source of antigen and it is necessary to characterize diagnostic antigens with monoclonal antibodies for proper subsequent identification of immunoblot bands (Hauser et al. 1999).

According to the CDC criteria, an IgM immunoblot is considered positive if two of the following three bands are present: 23, 29, and 41 kDa; however the combination of the 23 – and 41 kDa bands may still be a false positive result. An IgG immunoblot is considered positive if 5 of the following 10 bands are present: 18, 23, 28, 30, 39, 41, 45, 58, 66, and 93 kDa. Approximately half of the normal population has IgG reactivity with the 41 kDa flagellar antigen of the spirochete, and this response by it self, has no diagnostic significance.
In Europe, no single set of criteria for immunoblot interpretation give high levels of sensitivity and specificity in all countries (Robertson 2000). However, the development of immunoblots using defined recombinant or synthetic antigens provide a high sensitivity and specificity, and the interpretation criteria varies according to the test in use.

Serodiagnosis has low sensitivity during the first or second weeks of infection. During this period, approximately of 20% to 30% of the patients have positive responses in acute phase samples, usually of the IgM isotype, but by the convalescence 2 to 4 weeks later about 70% to 80% have seroreactivity, even after antibiotic treatment (Steer 2005). After 1 month, almost all patients with active infection have positive IgG antibody responses. In persons with illness longer than a one month, a positive IgM test alone is likely to be a false positive result, and thus a positive IgM response should not be used to support the diagnosis after the first month of infection. In patients with acute neuroborreliosis, especially those with meningitis, intrathecal production of IgM, IgG, or IgA antibody to *B. burgdorferi* s.l. may often be demonstrated by the antibody capture, but this test is less often positive in those with chronic neuroborreliosis (Steere 1991). After an antibiotic treatment, antibody titers decline slowly, but IgG and even IgM responses may persist for many years after treatment (Philipp et al. 2005).

12. Prevention of Lyme borreliosis

Control measures can be divided into measures applied on an individual basis (personal protection and vaccination) and measures applied to the environment (vector control) (Stafford and Kitron 2002).

The first line of defense against tick bites is to avoid high risk habitats during peak tick activity periods. If exposure to tick habitats can not be avoided, there are simple measures that can be taken to minimize the risk of tick bites and pathogen exposure (Piesman and Gern, 2004, Piesman and Eisen, 2008). Wearing appropriate clothing mechanically decreases the risk that a tick finds a feeding site. Repellents have been demonstrated to effectively decrease the risk of bites by a variety of tick species, DEET (N,N-diethyl-meta-toluamide) and permethrin based products are reasonably safe to use, although they are perceived as a potential source of toxicity. Other repellents based on natural products are available (Gardulf et al. 2004).

Vaccination remains a viable approach to be pursued in the prevention of LB.
A recombinant vaccine for use in humans FDA approved in USA in 1999, directed against the OspA of *B. burgdorferi* s.l. was tested and shown to be effective (Steere et al. 1998). However, the tenure of this vaccine proved relatively short, as it was voluntarily removed from the market by its manufacturer in 2002. Today, considerable research efforts continue towards the development of a broadly protective LB vaccine. Several proteins have been assessed as potential vaccine candidates to date (Marconi and Earnhart, 2010).

The environmental strategies include: (i) area-wide or host targeted tick control and oral vaccination of reservoir hosts; (ii) landscape modifications to render the environment less suitable for tick survival and for tick hosts; (iii) future anti-tick vaccines developed specially to reservoir hosts (Stafford 2004, Piesman and Eisen 2008).

13. State of art of *B. lusitaniae* in Portugal

The first clinical case of LB in Portugal was described in 1989 by David de Morais et al. in Alentejo region (David de Morais et al. 1989). Subsequent studies in the same region confirmed the presence of seropositives, some of them with confirmed clinical signs of LB (Filipe et al. 1990, Núncio et al. 1992).

In 1993, the first strains of *Borrelia* were isolated from *I. ricinus* complex ticks (Núncio et al. 1993) that were identified as a new species, named *B. lusitaniae* (Le Fleche et al. 1997). The type strain –PoTiB2- was defined based on complete sequences of rrs genes.

Recognizing the Public Health importance of this zoonosis, Direcção Geral de Saúde in 1999, has included it in the list of compulsory notifiable diseases (www.dgs.pt). However, the low number reported shows that LB is an underreported disease.

PCR-based study found *B. afzelli, B. valaisiana, B. garinii* and *B. burgdorferi* s.s. in *I. ricinus* ticks from Madeira island (Matuschka et al. 1998). The genetic diversity of *B. burgdorferi* s.l. in tick populations from a sylvatic habitat in mainland Portugal was analyzed and revealed a diverse population of *B. lusitaniae, B. afzelli, B. valaisiana, B. garinii* and *B. burgdorferi s.s.* in questing adult *I. ricinus* ticks (De Michelis et al. 2000, Kurtenbach et al. 2001). The prevalence infection in ticks could vary the region from region: some studies show prevalences of 11.9% (n= 234, collected in several regions), 11.8% (n=2806, Mafra region), 34.7% (n=206, Grandola region); and 31.2% (n= 285, Madeira Island) (Núncio et al. 2001, Núncio 2002, Baptista et al. 2004).

In 2001, the first animal model was developed, and demonstrated that in C3H/HeN mice *B. lusitaniae* induced pathology. The lesions were characterized as a severe necrotizing endarteritis of the aorta, with a minimal mixed inflammatory infiltrate extending into the adjacent myocardium. The analysis of the immunoblot demonstrated that mice with lesions resulting from the infection with *B. lusitaniae* reacted only to flagellin or both flagellin and OspC proteins (Zeidner et al. 2001).

In 2003, the presence of *B. garinii* and *B. lusitaniae* were detected in the cerebrospinal fluid of patients with neuroborreliosis (Santos et al. 2003).

A study conducted in two different sites in *I. ricinus* revealed some isolates of *B. garinii*, *B. valaisiana* and *B. lusitaniae* strains (Baptista et al. 2004).

A study on the dermatological aspects of LB in Portugal was performed which resulted in the isolation of *B. lusitaniae* from a human skin biopsy in a patient with chronic skin lesions and a weak serological response (Collares-Pereira et al. 2004, Da Franca et al. 2005); and also a DNA detection of a co-infection of *B. garinii* and *B. burgdorferi* s.s. in another patient (Da Franca 2004).

The host geographic structure shapes evolution and epidemiology of *B. lusitaniae* were analyzed with MLST using *I. ricinus* ticks from two different regions in Portugal. The results show that the regional *B. lusitaniae* populations constitute genetically distinct populations (Vitorino et al. 2008).

This data indicates the importance of researching this bacterial zoonosis in Portugal. This study was conducted in order to investigate the microbiological and molecular aspects of this strain.

Part II – Human Infection

Chapter III. Laboratory Diagnosis of Lyme Borreliosis at the Portuguese National Institute of Health (1990-2004)

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Surveillance report

LABORATORY DIAGNOSIS OF LYME BORRELIOSIS AT THE PORTUGUESE NATIONAL INSTITUTE OF HEALTH (1990-2004)

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Lyme borreliosis is considered to be an emerging infection in some regions of the world, including Portugal. The first Portuguese human case of Lyme borreliosis was identified in 1989. Since 1999, this disease is considered a notifiable disease (DDO) in Portugal, but only a few cases are reported each year, which does not allow consistent analysis of risk factors and the impact on public health. In this study the authors analyse the data available at the Centre for Vectors and Infectious Diseases Research (CEVDI) laboratory, at the Instituto Nacional de Saúde Dr. Ricardo Jorge (National Institute of Health, INSA) during the past 15 years (1990-2004) and evaluate them against the registry of national reported cases (1999-2004). Serological tests were the basis for laboratory diagnosis. Data on year of diagnosis, sex, age, geographical origin and clinical signs are available for 628 well documented Portuguese positive cases. The number of cases per year varied between 2 and 78, with the highest number of cases reported in 1997. Of the positive cases, 53.5% were female and the age group most affected was 35-44 years old. Neuroborreliosis was the most common clinical manifestation (37.3%). Human cases were detected in 17 of the 20 regions of Portugal, and the highest number of laboratory confirmed cases were from the Lisbon district. The comparison of the number of notified cases and the number of positive cases confirmed by our laboratory show that Lyme borreliosis is clearly an underreported disease. Due to the scattered distribution of the positive cases and the low prevalence of the tick species *Ixodes ricinus*, the most effective prevention measure for Lyme borreliosis in Portugal is education of the risk groups on how to prevent tick bites.

Introduction

Lyme borreliosis has been reported throughout Europe where it is the most common tickborne infection, as it is in the United States [1]. Clinically, it shows up as a multisystemic disease, presenting dermatological, rheumatic, neurological and cardiac manifestations. The first reported human case of Lyme disease in Portugal was identified in 1989 [2]. Diagnosis is preformed by the Centre for Vectors and Infectious Diseases Research (CEVDI) at the Instituto Nacional de Saúde Dr. Ricardo Jorge (National Institute of Health, INSA), using several techniques including culture, PCR, and antibody detection. The first strains of *Borrelia burgdorferi* sensu lato were isolated from ticks captured in the south of Portugal [3] and the study showed that they belong to a new species, *B. lusitaniae* [4]. Subsequent studies confirm the presence of several *B. burgdorferi* s.l. species (*B. lusitaniae, B. afzelii, B. garinii* and *B. valaisiana*) in ticks and the infection prevalence could vary between: studies have found prevalences of 11.9% (n= 234, collected in several regions), 11.8% (n= 2806, Mafra region), 34.7% (n=206, Grandola region); and 31.2% (n=285, the island of Madeira) [5, 6, 7, 8]. In all the studies made so far, B. lusitaniae is the most prevalent borrelia species. Recently, a strain of this species was isolated from a human sample, indicating that it could cause disease in humans [9]. Other species of borrelia*, B. garinii,*
B. afzelii B. burgdorferi sensu stricto and *B. valaisiana* have already been detected in mainland Portugal and/or the island of Madeira [5, 10]. Since 1999, Lyme borreliosis has been a mandatorily notifiable disease in Portugal, but only a few cases are reported each year, which does not allow consistent analysis of risk factors and the impact on public health. The aim of this study was to contribute to a more precise evaluation of the epidemiological situation of Lyme borreliosis in Portugal, analysing the data available at the CEVDI's laboratory concerning the serological diagnosis of this disease and data available on the statutory notifiable disease register.

Material and methods

The results of previous testing of all the sera and/or cerebrospinal fluid (CSF) of patients with clinical suspicion of Lyme borreliosis received at CEVDI's laboratory between 1990 and 2004 were analysed retrospectively. The antibodies were detected by indirect immunofluorescence in-house assay using a strain of B. garinii and a cut-off of 1:256 for IgG in sera and 1:4 in CSF were adopted. All borderline and positive samples were confirmed by immunoblot assay also an in-house test, using a strain of B.garinii. The interpretation was done according to the European group recommendations [11]. All the positive sera were tested to Treponema spp. and rheumatoid factor and all sera with a positive result were considered to be false positives for Lyme borreliosis. The laboratory definition of a positive case is when we detected a seroconversion (significant change in levels of the specific antibodies IgG and/or IgM in two samples), or when we detected a positive titres of specific antibodies in one sample, in patiens with clinical suspicion of Lyme borreliosis [12]. The data from the laboratory confirmed positive cases were compared with the available data from the cases of Lyme borreliosis notified during the period of 1999-2004. The notification of human cases of Lyme borreliosis was done directly by the clinician to the competent health authority, the Direcção Geral de Saúde (DGS), at the health Ministry. The case definition establish to the clinicians by the health

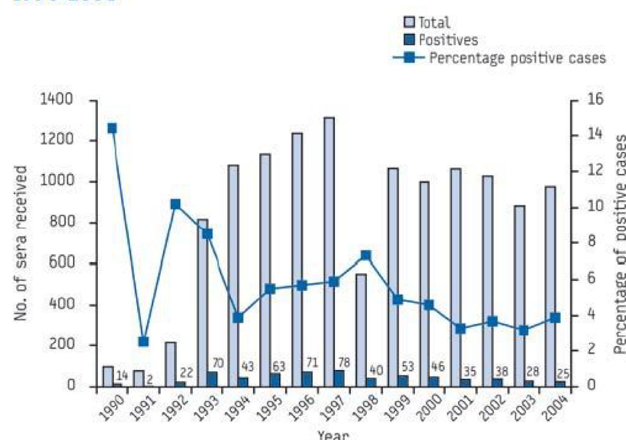
authority must fit the following criteria. Confirmed case: Erythema migrans confirmed by laboratory findings or at least one of the late manifestations of Lyme borreliosis with laboratory confirmation

Results

Among 12 535 biological samples taken for analysis from patients with clinical suspicion of Lyme borreliosis, 628 (5%) tested positive using the EUCALB diagnostic criteria. In patients with neurological symptoms, CSF was sometimes sent for analysis (21%). Data is available describing the 628 Portuguese patients, 129 of whom tested positive for both CSF and sera. The remaining 499 patients were diagnosed based in the result of sera analysis, with the observation of seroconversion. The number of cases per year varied between 2 and 78, with the highest number of cases in 1997 [FIGURE 1].

FIGURE 1

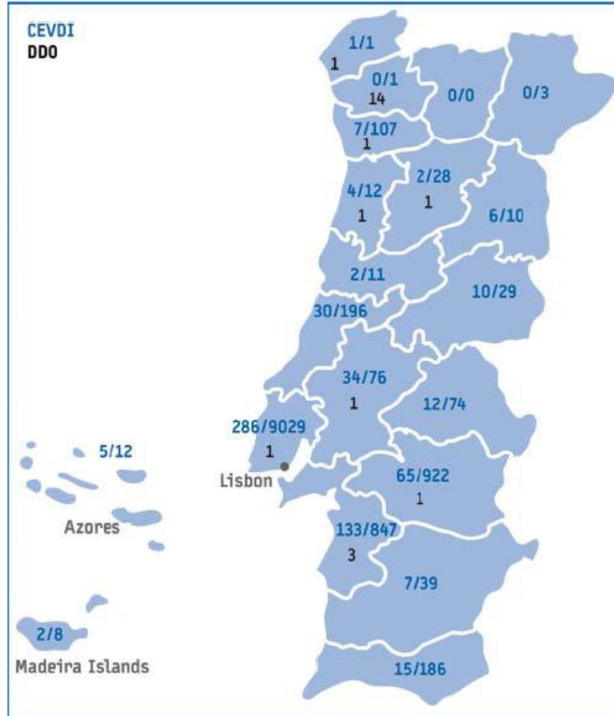
Number of samples received at CEVDI/INSA, and percentage of Lyme borreliosis cases found to be positive, Portugal, 1990-2004



The geographical distribution of the positive cases, based in the patients' home addresses, shows that Lyme borreliosis infection has been seen in 17 of the 20 districts of Portugal [FIGURE 2].

FIGURE 2

Geographical distribution of the positive cases by cases studied at CEVDI (1990-2004) and number of notified cases, Portugal, 1999-2004



There were slightly more female patients (53.5%) than male patients (46.5%). The notification forms were frequently not filled in completely, which may have caused some distortion in the data analysis of age and clinical manifestations. Information on patient age was available on only 62.3% of the forms. Analysing the available data, the mean age was 44 years old (range: 2 months to85 years) and the age group most affected was 35-44 years old (21.3%) [FIGURE 3].

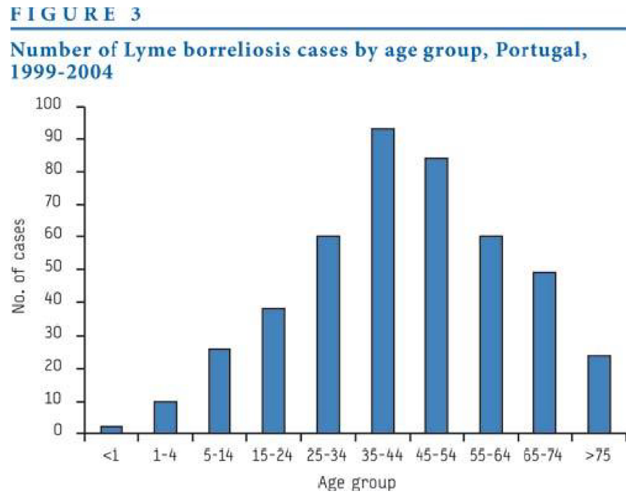
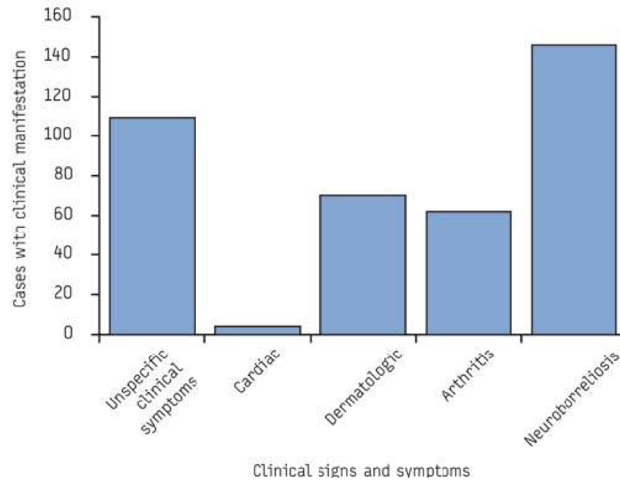


FIGURE 3
Number of Lyme borreliosis cases by age group, Portugal, 1999-2004

No clinical symptoms were reported in 237 (37.7%) of the 628 positive cases ([FIGURE 4]. Analysis of the information provided by the physician in the remaining 391 cases showed that the most frequently reported manifestations were neurological, reported in 146 patients (37.3%), followed by nonspecific symptoms in 109 cases (27.8%). Five of the cases with nonspecific symptoms had hepatic symptoms (4.5%), nine had myalgia (8.3%), 19 had optical symptoms (17.4 %) and 76 reported only fever (69.7%).

FIGURE 4

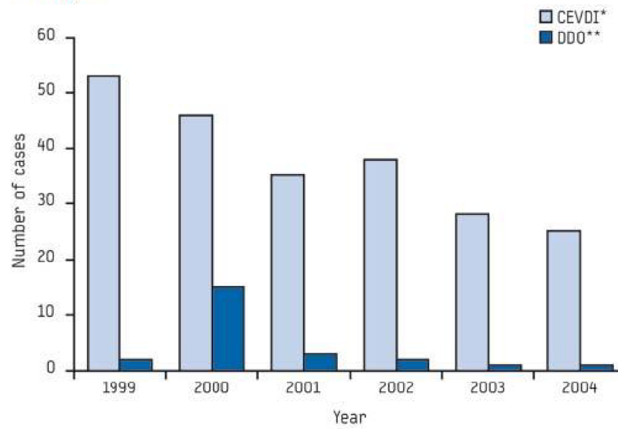
Distribution of positive Lyme borreliosis cases, by clinical signs and symptoms, Portugal, 1999-2004



The evaluation of the number of cases reported nationally between 1999 and 2004 (n=24) [13] and the number of positive cases confirmed by our laboratory (n=225) during the same period, show that is clearly an underreported disease. The annual incidence, estimated on the basis of the statutory notifiable disease is 0.04 per 100 000 inhabitants. However, when laboratory data are taken into account, we assume that this rate could be on average 10 times higher, 0.4 per 100 000 inhabitants [FIGURE 5].

FIGURE 5

Number of notified Lyme borreliosis cases and number of cases diagnosed at CEVDI during the period 1999-2004, Portugal



* CEVDI : Centro de Estudos de vectores e doenças infecciosas
** DDO : Doença de Declaração Obrigatória (Mandatory notification of disease)

Discussion/Conclusion

Although Lyme borreliosis is a mandatorily notifiable disease in Portugal, the evaluation of CEVDI data concerning human cases of Lyme borreliosis and the number of notified cases during the same period (1999-2004) shows that this disease, like other vector borne diseases, such as boutonneuse fever (the most prevalent tick borne disease in Portugal), is clearly underreported in our country [14]. According to our data, between 1999-2004 we detected an average of 35 new cases of Lyme borreliosis each year. Other diseases such as AIDS and tuberculosis have a bigger impact on public health and the general impression gained is that Lyme borreliosis cases are not considered important enough to notify and to publish. The major problem of underreporting is the impossibility of realise an epidemiological analysis of Lyme borreliosis in Portugal. For example, according to the notification data, Lyme borreliosis is more common in the Braga district (n=14) in northern Portugal, but when the results are analysed, the only sample from this district to be sent for analysis was negative and the districts showing higher number of confirmed cases are Lisbon (n=286), Setubal (n=133) and Evora (n=65) districts located in central and southern Portugal. It is also possible that the results have been influenced by the proximity of the CEVDI's facilities to these regions, and the hospitals and physicians located at Northern regions of Portugal may usually send their samples to other regional laboratories that also perform these tests. For example, if sufficient samples from Braga district and other northern regions were sent to our laboratory, perhaps the proportion of positive cases in these regions would increase. Also, if we analyse not only the number of positive cases but also the proportion of it, the district of Lisbon is simultaneously the district with a higher

number of positive cases and one of the districts with a lower proportion of positive cases.
As the laboratory data are not cross-checked with the official data, it is impossible to know which cases detected at CEVDI were reported to the health authorities, which laboratories performed the laboratory testing and why the clinicians did not notify the positive cases that they diagnose. Also, the fact that some of the positive cases may have been in patients who acquired their infections in districts or countries other than their area of residence should be considered, although patients in Portugal usually use the health facilities in their area of residence. In our experience, fewer than 10 patients during the time period considered (1999-2004) mentioned the possibility that they may have acquired their infection outside of their area of residence. However, the number of positive cases of Lyme borreliosis detected is undoubtedly higher than the number of cases reported. The reported incidence of Lyme borreliosis in Portugal is among the lowest reported in Europe. However, if we analyse the proportion of positive cases detected during this study (5%), we can see that this value is similar to the detected in other studies of seroprevalence in risk populations performed in several European countries [15]. After 15 years performing laboratory diagnosis, even knowing the limitations of laboratory results and being aware that the diagnosis of Lyme borreliosis should be always established by the clinician, these data, could contribute to the better understanding of the epidemiology of Lyme borreliosis in our country. To improve the notification of this disease, a network should be established to link all laboratories performing Lyme borreliosis diagnosis, aggregating all laboratory detected cases. This would allow the competent health authority to compare this information with the cases notified by clinicians and to make a more accurate analysis. The distribution of positive cases is influenced by clinicians' awareness of vector borne diseases, but the size of the I. ricinus population and the prevalence of infected ticks are also contributory factors to the incidence of the disease. The estimated annual incidence for Lyme borreliosis in Portugal is 0.04 per 100 000 inhabitants. A higher estimated can be obtained if we take laboratory data into consideration (0.4 per 100 000 inhabitants). However, as other laboratories also perform this test, it seems likely that underreporting is even higher, and consequently the true incidence of Lyme borreliosis in Portugal should be similar to the published values detected in other countries such as Scotland (0.6 per 100 000 inhabitants), United Kingdom (0. 3 per 100 000 inhabitants) and much lower than that detected in countries such as France (16 per 100 000 inhabitants), Germany (17.8-25 per 100 000 inhabitants), Bulgaria (55 per 100 000 inhabitants), Slovenia (120 per 100 000 inhabitants) and Austria (130 per 100 000 inhabitants) [1, 16, 17]. It would be interesting to compare the incidence detected in Portugal with geographical areas such as southern Spain, Morocco, Tunisia and Algeria but, to our knowledge, there are no available data published concerning the incidence of the disease in the these regions. All these areas share with Portugal some eco-epidemiological aspects such as vector population abundance and prevalence of infection, lack of information about the vertebrate reservoirs and the presence of the different Borrelia burgdorferi s.l. strains with particular relevance to B. lusitaniae., During the past five years, the number of human cases detected each year at CEVDI seems to have stabilised at approximately thirty five cases per year. This reduction may perhaps be explained by the increased number of other laboratories performing this diagnosis. Also, due to the diversity of the possible clinical presentations of Lyme borreliosis that may be confused with other aetiologies, the benign course of the majority of clinical cases, and the usually very positive response to the timely application of antibiotics, a large percentage of cases are never sent to the laboratory to confirm a clinical diagnosis. In this study, the positive cases which mention erythema migrans are very rare, probably because many clinicians are aware that this stage frequently does not evoke an antibody response and that laboratory confirmation cannot be expected, and

therefore do not request a laboratory confirmation of their clinical diagnosis. Considering that the incidence of Lyme borreliosis is directly linked to the density of the tick vector I. ricinus, and knowing that this species is not found in high tick population densities, we would expect the incidence of Lyme borreliosis to also be low. However, we should also consider the I. ricinus has been found to exist all over the country, but due to differing environmental characteristics, especially climate, distribution is not uniform throughout Portugal but focused in some regions where conditions are more suited to the survival of this tick species, and where this species predominates, achieving high population density.

In the absence of publications describing clinical cases, the information available in the clinical forms is very useful because the analysis allows us to clarify some epidemiological aspects such as risk factors concerning age, sex and geographic localisation. Other information that would help laboratory diagnosis, such as symptom onset date, information about occurrence of recent tick bites, and recent trips, are frequently unavailable. This is why collaboration and exchange of information between clinicians and laboratories are so important. Research concerning the eco-epidemiology of Lyme borreliosis in Portugal has so far been slow to advance, and it is difficult to study the impact and risk factors. However this knowledge is essential if we are to implement adequate prevention programmes, which are currently considered the best approach to solving the problem of vectorborne diseases. Sixteen years after the report of the first human case of Lyme borreliosis in Portugal this is still a poorly understood disease in Portugal. Due to the scattered distribution of the positive human cases and the scattered nature of the tick vector distribution throughout Portugal, the most effective prevention measure for Lyme borreliosis in Portugal is probably educating risk groups about how to avoid tick bites.

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Chapter IV. Vasculitis like syndrome associated with *Borrelia lusitaniae* infection

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CASE REPORT

Vasculitis-like syndrome associated with *Borrelia lusitaniae* infection

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Abstract We report the isolation of *Borrelia lusitaniae* from a 13-year-old female child presenting with a vasculitis syndrome. The patient was treated with doxycycline, 100 mg bid for 20 days, and is in remission after a follow-up of 2 years. These results should alert clinicians to the fact that *B. lusitaniae* may be pathogenic in humans, highlighting that patients may be seronegative or present with minimal positive antibody titres and clinical signs that are not specific for Lyme borreliosis. In order to prevent the occurrence of more serious disease manifestations via timely treatment, the analysis by molecular methods may be a useful approach when antibody titres are uninformative.

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Keywords *B. lusitaniae* · Clinical manifestations · Human isolate · Lyme borreliosis · Portugal

Introduction

Currently, Lyme borreliosis is the most prevalent tick-borne disease in Europe and in USA. The first report of Lyme borreliosis in Portugal dates from 1989 [1]. Since then, several human cases of this disease are annually detected at the Center for Vector and Infectious Diseases Research, Instituto Nacional de Saúde Dr. Ricardo Jorge (CEVDI/INSA) laboratory, confirming the endemicity of this disease in our country [2]. In Portugal, according to the data published since 1997, the following species of *Borrelia burgdorferi* sensu lato have been detected in *Ixodes* ticks: *Borrelia lusitaniae*, *B. afzelli, B. valaisiana, B. garinii* and *B. burgdorferi s.s*. [2]. The pathogenic role of *B. lusitaniae* in humans, the most prevalent borrelia species in Portugal, is still controversial. However, studies performed in animal models [3, 4] and a report of strain isolation from a human patient [5, 6] indicate that infection caused by this strain leads to disease in both mouse and man. Here, we report a vasculitis-like syndrome associated with *B. lusitaniae* infection, confirmed by the isolation of the etiologic agent.

Case report

In February 2006, a 13-year-old female child was observed at the Paediatric Rheumatology outpatient clinic of Santa Maria Hospital in Lisbon, Portugal. She presented with tumefaction, pain, cyanosis and paraesthesia of the 3rd, 4th and 5th fingers of the right hand, together with vasculitis-like punctiform erythematous

skin lesions, occurring with an intermittent pattern over a 2-month period. The erythrocyte sedimentation rate was 22 mm/1st hour, C reactive protein was 1.8 mg/dl, the complement was low (CH_{50} 41, C3 0.89) and IgM rheumatoid factor was negative. The remaining workup for diffuse connective tissue diseases and vasculitis was unremarkable. These included a normal chest X-ray, echocardiogram, echo Doppler of the upper limbs, electromyogram and capillaroscopy. In addition, the serology for Anti Neutrophil Cytoplasm Antibodies (ANCA), anti cardiolipin, anti beta2 glycoprotein 1, lupus anticoagulant, Anti Nuclear Antibodies (ANA), B and C hepatitis virus and B19 parvovirus were negative. Although there were no epidemiologic risk factors noted for Lyme borreliosis, this hypothesis was investigated. Acute and convalescent serology was performed as described previously [2], collected within 1 month of each other. The presence of specific antibodies was confirmed by indirect immunofluorescence assay (IFA) using two different strains, *B. garinii* (VS 102, kindly provided by Dr. Péter) and *B. lusitaniae* (PoTiB2; CEVDI/INSA), ELISA (Serion) and by immunoblot (IB; Euroimmun). Concerning the acute sample, the titres were negative (ELISA) or borderline (IFA and IB) for IgG, and borderline (IFA and ELISA) or positive (IB) for IgM. The results for the convalescent sample showed an increase of the IgM titres by IFA (Table 1).

Whole blood (500 μl) from the convalescent sample was then inoculated in complete Barbour–Stoenner–Kelly II medium (Sigma) and incubated at 34°C. The culture was examined weekly by dark-field microscopy and after 8 weeks motile spirochetes were detected. The spirochetes were centrifuged at 7.000×*g* for 30 min, washed in phosphate-buffered saline, re-suspended in water and stored at −20°C. DNA was then extracted using the Qiagen blood and tissue kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions, and used as template for PCR. A nested PCR reaction, targeting the 5S (rrf)–23S (rrl) intergenic spacer [7], and/or the *flaB* gene encoding the flagella of *B. burgdorferi* s.l. [8] were run. Using multiple alignments of published OspA sequences of *B. lusitaniae* within GenBank, primers were designed (OspAP1-5′ TGA TAA AAA CGA TGG GTG TGG 3′/OspAP2-5′GGT GCC GTT TGC TGT A 3′), and the outer surface protein A (OspA) gene was also amplified. Amplicons of PCR reactions were then visualized on 1.2% agarose gels (Roche Diagnostics GmbH, Mannheim, Germany), purified using the JETquick system (Genomed, Inc.) and sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 377 DNA sequencer. Sequences of the new isolate were aligned with other sequences from databases (Table 2) using ClustalW [9]. Pair-wise distance matrices for the aligned sequences were determined by the Kimura two-parameter method with PAUP* 4.0b10 [10]. A phylogenetic tree was then constructed by Parsimony, with bootstrap analysis using 500 replications for evaluation (Fig. 1). The results of

Table 1 Evaluation criteria and serological results

	Evaluation criteria		1st sample		2nd sample	
	IgM	IgG	IgM	IgG	IgM	IgG
IFA	<16—negative	<128—negative	16	128	64	128
B. garinii	16—borderline	128—borderline	Borderline	Borderline	Positive	Borderline
B. lusitaniae	≥32—positive	≥256—positive				
ELISA[a]	<5 U/ml—negative	<5 U/ml—negative	3,72	0,15	3,85	0,12
	3–5 U/ml—borderline	3–5 U/ml—borderline	Borderline	Negative	Borderline	Negative
	>5 U/ml—positive	>5 U/ml—positive				
IB[a]	no band—negative	no band—negative	Osp C and 3 bands	8 bands	OspC and 3 bands	9 bands
	OspC band weak only—borderline	VlsE weak only—borderline	Positive	Borderline	Positive	Borderline
	OspC band negative with at least one of the specific antigens bands[b]—positive	VlsE band negative with one of the specific antigens bands[c]—borderline				
		VlsE band positive—positive				
	OspC band positive—positive	2 or more specific bands positives, VlsE negative—positive				

[a] According to the manufacturer's recommendation
[b] p83, p39, p31, p21, p19, p17
[c] p83, p39, p31, Osp C (p25), p21, p19

Table 2 Strain and GenBank *OspA* sequences used in this study

Species	Strain or isolate	Origin	Accession no.
B. lusitaniae	MT7	Tick, Morocco	AJ634230
B. lusitaniae	MT4	Tick, Morocco	AJ634229
B. lusitaniae	MT26	Tick, Morocco	AJ634237
B. lusitaniae	TT928	Tick, Tunisia	AJ634243
B. lusitaniae	TT925	Tick, Tunisia	AJ634242
B. lusitaniae	TT916	Tick, Tunisia	AJ634241
B. lusitaniae	TT840	Tick, Tunisia	AJ634236
B. lusitaniae	MT23	Tick, Morocco	AJ634236
B. lusitaniae	MT12	Tick, Morocco	AJ634234
B. lusitaniae	MT10	Tick, Morocco	AJ634233
B. lusitaniae	MT8	Tick, Morocco	AJ634231
B. lusitaniae	TT908	Tick, Tunisia	AJ634240
B. lusitaniae	MT2	Tick, Morocco	AJ634228
B. lusitaniae	MT16	Tick, Morocco	AJ634235
B. lusitaniae	PotiB1	Tick, Portugal	Y10837
B. lusitaniae	PotiB2	Tick, Portugal	Y10838
B. lusitaniae	PotiB3	Tick, Portugal	Y10839
B. lusitaniae	PoHuB1	Human, Portugal	EU863625
B. lusitaniae	ITAh02	Tick /Lizard, Italy	EF457554
B. lusitaniae	PoHL1	Human, Portugal	AY576875
B. lusitaniae	RB-Pm2N6	Tick, Germany	DQ379494
B. lusitaniae	RB-LA1N1	Tick, Germany	DQ379495
B. lusitaniae	ITAh03	Tick, Italy	EF457555
B. lusitaniae	ITAh04	Tick/Lizard, Italy	EF457556
B. lusitaniae	ITAh05	Lizard, Italy	EF457557
B. lusitaniae	ITAh06	Tick, Italy	EF457558
B. valaisiana	NA	NA	AJ249467
B. afzelii	NA	NA	CP000396
B. garinii	NA	NA	NC006129
B. burgdorferi sensu stricto	B31	USA	NC_001857

NA Not applicable

these analyses confirmed the identity of this isolate as *B. lusitaniae.* The patient was then treated with doxycycline 100 mg bid for 20 days. Both symptoms and clinical laboratory data normalized and the patient remained in remission after a follow-up of 2 years.

Discussion

In European endemic areas for Lyme borreliosis, neuroborreliosis and joint manifestations are the most frequent extra-cutaneous features observed. Among the pathogenic species of Borrelia, *B. burgdorferi* s.s. is the most frequently identified species, but other species also seem to be involved [11]. In Portugal, the incidence of this disease is estimated to be 0.4 per 100,000 inhabitants which is relatively low when compared to endemic areas (16–120 per 100,000 inhabitants) [2]. Another distinctive feature is that a large proportion of patients (27.8%) present with non-specific clinical symptoms, against a background of eco-epidemiological parameters characterized by the presence of different *B. burgdorferi* s. l. strains as well as *B. lusitaniae* [2].

We have described a vasculitis-like syndrome associated with the isolation of *B. lusitaniae.* Although the clinical presentation is not typical of Lyme borreliosis, this case had features suggestive of vasculitis, which has been described as one of the characteristic physiopathological aspects of this disease [12]. In addition, acute ischemia of the digits had previously been demonstrated in the context of Borrelia infection [13]. On the other hand, this child had intermittent symptoms, which have also been pointed out as one of the possible characteristics of rheumatic symptoms associated with this condition [11]. Finally, the overall peculiarities of this clinical report supports the hypothesis that the clinical manifestations associated with infection by *B. lusitaniae* are quite non-specific and may overlap with multiple clinical syndromes [6].

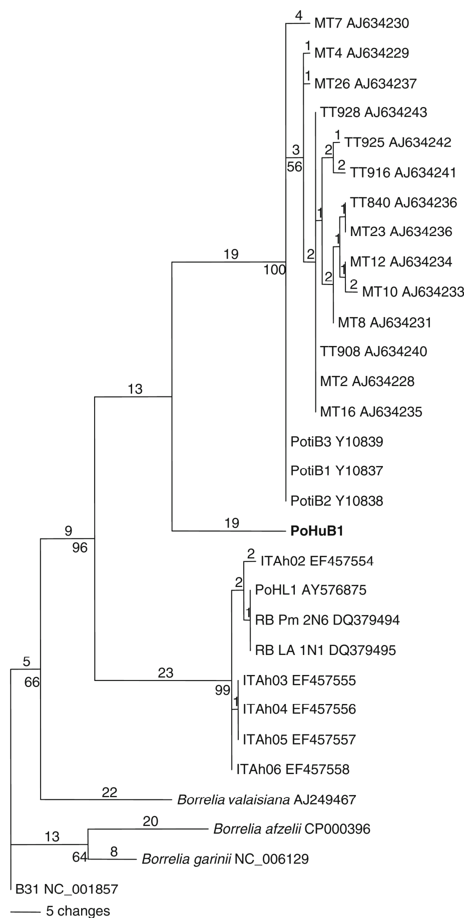

Fig. 1 Phylogenetic tree constructed by parsimony analysis of partial *OspA* sequences from the isolate obtained in this study (*boldface*) compared to Genbank sequence data. The Bootstrap values (500 replicates) are given adjacent to the nodes

The fact that specific antibodies are present in low titre may provide false negative results. In the first human case of Lyme borreliosis with *B. lusitaniae* infection, serology was negative [4, 5], while in the case presented here antibody titres were borderline positive, as seroconversion was detected by a fourfold IgM increase in the convalescent sample. Both the IFA and IB tests demonstrated more sensitivity than the ELISA. This may be due to different antigens used by each test, underscoring the importance of using multiple analyses when performing Lyme disease serology. Although IgG is expected to rise slowly in *Borrelia* infections, it remained borderline months after the beginning of clinical symptoms.

The use of autochthonous strains as antigens in serological diagnosis, recommended by some authors, has not been considered as an advantage in comparison with routine methods in use at our laboratory. Culture of spirochetes from patient material is still the gold standard for laboratory diagnosis of LB [14] and was accomplished in this case. However, it took 8 weeks to identify the viable spirochetes, and when a diagnosis is pending on the result of this test this time gap is clearly a drawback.

Finally, it should be emphasized that the analysis of the phylogenetic tree demonstrated that this new isolate is grouped with the North African clade [15] and with the Portuguese tick isolates, and not with the first human isolate previously described [6].

These results should alert clinicians, not only in Portugal but in many other European countries where *B. lusitaniae* predominates, such as Spain, Slovakia, Switzerland and Italy, and especially other countries from the western Mediterranean basin, such as Tunisia and Morocco, that this *Borrelia* species may be pathogenic in humans. Our experience demonstrates that these patients will probably present as seronegative or with minimal positive antibody titres, and with clinical signs that may be non-specific for Lyme borreliosis. Little is known regarding the progression of the disease associated with *B. lusitaniae*, but our clinical experience suggests that the majority of Portuguese patients with Lyme borreliosis present with neurological symptoms [2], which can give a clue to the probable disease progression pattern of *B. lusitaniae* infection taking into consideration the endemic characteristics of this agent in Portugal. In order to prevent the occurrence of more serious disease manifestations, the culture and/or the analysis by molecular methods may be useful approaches when antibody titres are uninformative, so that timely treatment can be initiated.

Nucleotide sequence accession number The *flaB* and *OspA* gene from PoHuB1 have been deposited in Genbank with accession numbers EU863626, EU863625, respectively.

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Disclosures None

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Chapter V. Clinical and laboratory data of Portuguese patients with diagnostic of Lyme borreliosis

In submission

Clinical and laboratory data of Portuguese patients with a diagnosis of Lyme borreliosis.

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Abstract

Lyme borreliosis caused by the *Borrelia burgdorferi* s.l. complex comprises a diverse group of zoonotic bacteria. Twelve Portuguese patients with indicative serology and clinical diagnosis of Lyme borreliosis were analyzed. Fifty five percent of the patients presented dermatological lesions, 36% presented musculoskeletal (arthritis) and neurological symptoms, and 9% had cardiac signs. The laboratory diagnosis was performed using an IFA in house assay and/or commercial ELISA. All borderline and positive samples were confirmed by immunoblot assay; PCR and culture were performed whenever suitable samples were sent to the lab. *Borrelia* DNA was detected in three patients, one of them referred a trip to Canada, and was probably infected there, consisting on the first imported case of Lyme borreliosis. The sequencing of the three samples allowed the identification of *B. afzelii*, *B. lusitaniae* and *B. burgdorferi* s.l. To our knowledge this is the first detection of *B. afzelii* in Portuguese patients. This study also indicates that an efficient diagnosis of Lyme borreliosis has to be based on clinical and laboratory findings and that the use of molecular methods, in some cases, improves the laboratory diagnosis.

Key words: Lyme borreliosis, *B. afzelii*, *B. lusitaniae*, diagnosis, patients, Portugal

Introduction

Borrelia burgdorferi sensu lato (s.l.) is the causative agent of Lyme borreliosis (LB), one of the most common tick-transmitted diseases in Portugal. LB, a multi-systemic disease, is considered an emerging disease in some regions of the world, particularly in Portugal. The most common agents of human LB are *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. lusitaniae* and *B. spielmanii* (Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008, Margos et al. 2009). In Portugal, *B. lusitaniae* is the most prevalent species found in ticks (Baptista et al. 2004, Lopes de Carvalho et al. 2008, Milhano et al. 2010 (unpublished). However, other species have also already been detected such as *B. afzelii*, *B. valaisiana*, *B. garinii* and *B. burgdorferi* sensu stricto (s.s) (Baptista et al. 2004, Matuschka et al. 1998). Two human strains of *B. lusitaniae* were isolated from Portuguese patients confirming its pathogenicity (Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008). The analysis of laboratory data has confirmed LB as being underreported, with an estimated incidence rate of 0.04 per 100 000 inhabitants (Lopes de Carvalho and Núncio, 2006). Like other spirochete infections, borrelial infection may present with a wide range of clinical manifestations (Stanek et al. 2010). Signs and symptoms of LB occur in stages and involve a variety of tissues and organs, including the skin, joints, heart, and nervous system. Early infection (stage 1) consists of primary erythema migrans (EM), an annular skin rash that begins days to weeks after a tick bite. Hematogenous dissemination of spirochetes over subsequent days to weeks (stage 2) can result in multiple skin lesions (secondary EM), as well as meningitis, radiculoneuritis, atrioventricular block, myocarditis, and oligoarticular arthritis. Persistent infection (stage 3) occurs months to years after the initial exposure and can be associated with acrodermatitis chronica atrophicans, varying degrees of encephalopathy and encephalomyelitis, and persistent arthritis (Stanek et al. 2002, Stanek et al. 2010).

Serological tests for detection of borrelia antibodies are frequently used in laboratory diagnosis of LB (Aguero-Rosenfeld et al. 1996, Aguero-Rosenfeld, 2004). The production of immunoglobulin (Ig)M antibodies starts at around 2 weeks of infection and peaks at 2 months, after which the antibody levels, in most cases, start to decline slowly (Hÿtonen et al. 2008). Subsequently, the antibody production switches to IgG. It is crucial to keep in mind that in some patients IgG and also IgM responses may persist for months or years after successful antimicrobial treatment (Kalish et al. 2001). Data on borderline or negative results associated with LB are scarce (Stanek et al. 2010) but have already been reported (Berger et al. 2002, Holl-Wieden et al. 2007) and may be relevant when the infectious agent is *B. lusitaniae*. A generally accepted principle in borrelia serologyis the so-called two-step (two-tier) approach,

where the first step is a sensitive screening ELISA followed, in cases where the screening is positive or borderline positive, by a specific confirmatory immunoblot assay (Aguero-Rosenfeld et al. 2005, Wilske et al. 2007). Antibodies of both IgG and IgM classes are usually validated, although the significance of IgM-positive results later in the disease, or in areas with high LB endemicity, is controversial (Wilske et al. 2007).

The fact that only two human cases were reported with unquestionable infection due to *B. lusitaniae* may be related to the lack of specificity of the clinical signs referred, making it extremely difficult to analyse and validate the clinical and laboratory diagnosis. The aim of the present study was to describe 12 human cases of LB studied between 2008 and 2010, using an integrated approach, of analysis of laboratory results, clinical manifestations and epidemiological data.

Material and Methods

Patient samples: The study included all the patients with a clinically suspicious presentation and a positive laboratory result (serological and/or microbiological and/or molecular result) for Lyme borreliosis detected at our laboratory during 2008-2010. Of the initial 15 patients identified, the clinicians were contacted and the final diagnosis of Lyme borreliosis were validated or excluded according to the overall analysis of the data available. Three patients were excluded due to the existence of other co-morbilities such as heart condition, syphilis, and Q fever infection. The remaining 12 patients were diagnosed with Lyme borreliosis. An epidemiological and clinical inquiry was developed and data about patients concerning gender, age, clinical manifestations were obtained and analyzed along with the laboratorial findings.

All cases were analyzed by serology and in four cases whole blood were sent allowing the culture and PCR detection.

Serology: Samples were tested by an indirect immunofluorescence in-house assay using a strain of *B. garinii;* a cut-off of 1:256 for IgG and 1:32 for IgM in sera was adopted for the IFA test. Alternatively, a commercial ELISA kit (Serion) was employed observing the manufacter's recomendations. All borderline and positive samples were confirmed by immunoblot assay (Euroimmun, Germany). The interpretation was done according to the manufacturer. All the positive sera were tested for *Treponema* spp. and rheumatoid factor and all sera with a positive result were considered to be doubtful positives for LB and were eliminated from this study in order to prevent misdiagnosis.

The human cases were established when both laboratory and clinical diagnosis were compatible with an active infection by *B. burgsdorferi* s.l.

Culture: Whole blood samples from three patients was inoculated in complete Barbour-Stoenner-Kelly II medium (BSK-II) (Sigma) and incubated at 34ºC. The culture was examined weekly by dark-field microscopy. After 8 weeks 1 mL of the cultures were centrifuged at 13.000x g for 20 min, washed in phosphate-buffered saline and boiled for 10 min.

DNA extraction and PCR: DNA was extracted using the Blood & Tissue kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The *rrf (5S)-rrl* and *(23S)* intergenic spacer region of *B. burgdorferi* s.l. were used for *Borrelia* DNA detection (Rijpkema et al. 1995). Amplified products of nested-PCR reactions were visualized on 1.2% agarose gels (Roche Diagnostics GmbH, Mannheim, Germany), purified using the JETquick system (Genomed, Inc.) and sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems) on an ABI 377 DNA sequencer.

Results

A total of 12 clinical samples were used in this study (Table 1). Of the total patients analyzed, the majority were from the central and southern regions, namely, two from Setúbal, six from Santarém and two from Lisbon districts and one from the Azores Islands. Most of the patients were from rural areas where agropecuary is the main activity and the presence of *B. burgdorferi* s.l. has already been detected in all the districts mentioned.

The distribution of the patients by district may reflect the proximity to the CEVDI/INSA lab and not significant differences in Lyme borreliosis incidence. Seventy-five percent of the cases were women and 25% were men, but due to the small number of patients statistical analysis could not be performed. Five of the patients were 5-15 years old, and the remaining seven patients were adults with ages between 21 and 57 years old. According to the immunoblot technique, recognized as a confirmatory test, 67% and 8% of the patients had positive or borderline IgM antibodies, 50% and 33% had positive or borderline IgG antibodies.

Borrelia DNA was detected in three patients with positive or borderline serology. In one case (patient nº 8) we detected *B. afzelii* DNA (Genbank accession number HQ176500) from a patient that presented with fever (38º), prostration and neurological injuries (mental confusion). *Borrelia* DNA was also detected in a patient (patient nº 9) that suffered of myalgia and a neuroretinitis of the left eye and had high temperature (39.6º). The epidemiological

inquiries showed that this patient was bitten by a tick during a trip to Canada one month before the first clinical symptoms appears. Due to the small amount of blood sample available it was impossible to identify the genospecies present in this patient. In another case (patient nº12) it was possible to isolate *B. lusitaniae*. This patient presented tumefaction, pain, cyanosis and paraesthesia of the 3rd, 4th and 5th fingers of the right hand, together with vasculitis-like punctiform erythematous skin lesions, occurring with an intermittent pattern over a two month period. In the other nine patients with positive serology, most of them presented with fever (ranging between 37-39º), prostration, myalgia, skin lesions (three of them with EM), arthralgia, cardiac symptoms and three with neurological damage. Considering the major clinical manifestations in this study, fever prevailed among clinical symptoms (82%), followed by dermatological lesions (55%), neurological symptoms (36%), musculoskeletal (including arthritis) (36%) and cardiac signs (9%) (Table 2).

Discussion

The presence of *B. burgdorferi* s.l. (*B. afzelii*, *B. lusitaniae*) was demonstrated by PCR in three patients with positive or borderline serology. One of these patients probably acquired *Borrelia* infection in Canada, since the symptoms became after one month the patient returns, however due to the small amount of sample sent to the laboratory, the genetic identification of the bacteria allowed confirming the presence of *B. burgdorferi* s.l. but hindered the genospecies identification. In one case, besides the PCR positive result, it was possible the isolation of the etiologic agent, *B. lusitaniae*.

Concerning the detection of *B. afzelii* in Portuguese patient, to our knowledge this is the first time that this genospecies is associated with a human infection in Portugal. The occurrence of different species within the *B. burgdorferi* s.l. complex, having a differential tissue tropism, could decide about the character of the illness (Niscigorska-Olsen J et al. 2008). Moreover, infection with *B. burgdorferi* s.l. can result in dermatological, neurological, cardiac, and musculoskeletal disorders (Aguero-Rosenfeld et al. 2005, Stanek et al. 2010). The diagnosis of Lyme borreliosis may be difficult because of variable clinical symptoms and frequently inconsistent laboratory findings. The circulation of more than one borrelia species may increase the complexity of the clinical picture and in many cases laboratory findings are very useful in order to confirm the clinical suspicion of Lyme borreliosis. In our country, previous studies have demonstrated that *B. lusitaniae* isolates derived by culture were achieved in a seronegative and in a borderline seropositive patient (Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008). This fact obligates the laboratory to use a multiple approach, including

not only the serology but also microbiological and molecular methods, to improve the accuracy of the laboratory diagnosis.

Analyzing only the ecological data it would seem that the North region of Portugal, with more humidity and lower temperatures, would be more suitable for the vector tick, *I. ricinus*. Although in this study all the patients were from central/southern region. In fact, several studies performed in the past years allowed the isolation and/or molecular detection of *B. burgdorferi* s.l. in ticks all over the country, in a scattered pattern and in low prevalence. The fact that our laboratory is located in the southern region may also influence these results, especially when the use of molecular methods are needed to detect infection, since sending suitable samples in due time to the laboratory is very expensive. The experience of the Portuguese reference laboratory is that when the infectious strain is *B. lusitaniae* immunological methods may be insufficient due to the low immunoreactions induced by this genospecies (Zeidner et al. 2001).

To achieve the complete characterization of the clinical picture of the disease caused by *B. lusitaniae* it would be necessary to confirm by molecular or microbiological methods the borrelia genospecies present in the human sample. At our laboratory, during the last two years, the number of requests of diagnosis by PCR is increasing. However, most part of the samples are whole blood, even in those cases were the onset of the disease occurred several months before and therefore is not the most adequate. Besides this fact, given that the low number of spirochetes in infected tissues or body fluids of patients in general is very low, appropriate conditions for sample collection, transport and DNA preparation from clinical samples are critical for reliable and consistent PCR results (Wang et al. 2010).

The analysis of the clinical history allowed the assessment of the most frequent symptoms. In previous studies, neurological symptoms were usually the most frequent in Portuguese patients (Lopes de Carvalho and Núncio, 2006). However, in this study, dermatological symptoms were the most frequent after the unspecific reference to fever. Lyme neuroborreliosis appears to occur in a higher proportion of patients in Europe, and Lyme arthritis seems to be a more frequent manifestation in USA (Strle and Stanek, 2009). In countries where more than one *Borrelia* genospecies circulate and cause disease, as in the case of Portugal, gathering information about laboratory data and clinical presentations of human cases, is essential for reliable epidemiological studies. This study indicates that an efficient diagnosis of Lyme borreliosis has to be based on clinical and laboratory findings. Furthermore, the laboratory diagnosis should include whenever it is possible, a combination of various techniques such as serology, PCR and culture, not solely on serology.

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Table 1- Review of patients with Lyme borreliosis screened by five distinct detection methods

No.	Patients		IFA		ELISA		Immunoblot		PCR	Culture
	Age	Gender	IgM	IgG	IgM	IgG	IgM	IgG		
1	38	F			+	-	+	-	-	-
2	8	F					-	+	na	na
3	51	F			+	-	+	+-	na	na
4	32	F			+	-	+-	+	na	na
5	37	F			+	-	+	+	na	na
6	18	F			+	-	+	+-	na	na
7	57	M			-	+	-	+	na	na
8	54	M	+	+-			+	-	B.a	-
9	14	F	+	+-	+	-	+	-	B.b.s.l.	na
10	14	M	+	+			-	+	na	na
11	15	F					-	+	na	na
12	13	F	+-	+-	+-	-	+	+-	B.l.	+

B.a-*B. afzelii*; B.b s.l.-*B.burgdorferi* sensu lato; B.l- *B. lusitaniae*; +- - borderline; + - positive; - - negative; na-not applicable

Table 2 – Principal manifestations of the patients studied.

Principal Clinical Signs	**Percentage (%) of the patients**
Fever	82%
Dermatological	55%
Neurological	36%
Musculoskeletal	36%
Cardiac	9%

Part III – Ecology

Chapter VI. Detection of *Borrelia lusitaniae*, *Rickettsia* sp. IRS3, *Rickettsia monacensis* and *Anaplasma phagocytophilum* in *Ixodes ricinus* collected in Madeira Island, Portugal

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Detection of *Borrelia lusitaniae*, *Rickettsia* sp. IRS3, *Rickettsia monacensis*, and *Anaplasma phagocytophilum* in *Ixodes ricinus* Collected in Madeira Island, Portugal

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ABSTRACT

A total of 300 *Ixodes ricinus* ticks were tested by polymerase chain reaction (PCR) for the presence of *Borrelia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum*. Sequence analysis demonstrated 8 (2.7%) ticks infected with *B. lusitaniae*, 60 (20%) with *Rickettsia* spp., and 1 (0.3%) with *A. phagocytophilum*. Seven (2.3%) ticks were coinfected with *B. lusitaniae* and *Rickettsia* spp., 2 (0.6%) with *R. monacensis*, and 5 (1.7%) with *Rickettsia* sp. IRS3. The results of this study suggest simultaneous transmission of multiple tick-borne agents on Madeira Island, Portugal.
Key Words: Madeira—*Ixodes ricinus*—*B. lusitaniae*—*Rickettsia*—*Anaplasma phagocytophilum*.

INTRODUCTION

Madeira, the main island of the Madeira archipelago, Portugal, is located in the north Atlantic Ocean about 1000 km from the European Coast and 800 km west of Africa. Climatic conditions make this island an ideal setting for *Ixodes ricinus* ticks, the most widely distributed tick species, colonizing various habitats and parasitizing several vertebrate hosts. Human parasitism by this species is a common occurrence in Portugal. In other parts of Europe, *I. ricinus* ticks play an important role in the transmission of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, and *Rickettsia* spp. to domestic animals and humans. In Portugal, *B. lusitaniae* is the most prevalent *Borrelia* spp. and was first isolated from ticks in southern Portugal (Núncio et al. 1993). In 2004, *B. lusitaniae* was isolated from a human patient presenting with erythemic skin lesions, indicating its pathogenicity in humans (Collares-Pereira et al. 2004). Subsequent reports from mainland Portugal indicate an incidence rate for Lyme borreliosis of 0.04/100,000 inhabitants. To date, only 2 clinical cases have been confirmed by laboratory testing on Madeira Island (Lopes de Carvalho and Núncio 2006).

Multiple *Borrelia* spp., including *B. afzelii*, *B. valaisiana*, and *B. burgdorferi* sensu stricto, have been detected in *I. ricinus* ticks collected on Madeira Island (Matuschka et al. 1998, Núncio et al. 2001). *A. phagocytophilum* has also been detected in 4% of actively questing *I. ricinus* collected from vegetation on this island (Santos et al. 2004). To our knowledge, no studies of *Rick-*

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was based on statistical analysis of the prevalence of tick infection with *Borrelia* spp. found previously (Núncio et al. 2001). DNA was extracted from ticks as described (Schouls et al. 1999). Polymerase chain reaction (PCR) assays were performed targeting 2 *B. burgdorferi* sensu lato genes: *fla* (Johnson et al. 1992), using outer 1 and 2 primers for the first reaction and inner 1 and 2 for the nested reaction, and the intergenic spacer region (*rrf-rrl*) (Rijpkema et al. 1995), using primer pairs 23SN1/23SC1 and 5SCB/23SN2 for the nested reaction. *Rickettsia* DNA was detected by amplification of citrate synthase gene *gltA* using RpCs415/RpCs1220 primers (Sousa et al. 2006), and the outer membrane protein A gene *ompA* with the primer pair Rr190.70p/Rr190.602n (Regnery et al. 1991). A single target gene of *A. phagocytophilum* was amplified, the heat shock operon (*groESL*) (Sumner et al. 1997), in a nested PCR, using primer pairs HS1/HS6 and HS43/HS45, respectively (Santos et al. unpublished data).

The resulting amplicons were sequenced and compared with published sequences of representative *A. phagocytophilum*, *Borrelia*, and *Rickettsia* species. Multiple alignments of the nucleotide sequences were generated by the ClustalW program, version 1.6 (Thompson et al. 1994), and phylogenetic analysis was carried out by maximum likelihood analysis in the TREE-PUZZLE program, version 5.1 (Strimmer and von Haeseler 1997), using a quartet puzzling algorithm to generate the tree. The analysis was run with the Hasegawa-Kishino-Yano (HKY-85) model of substitution (Hasegawa et al. 1985), and quartet puzzling support values based on 1000 puzzling steps were calculated.

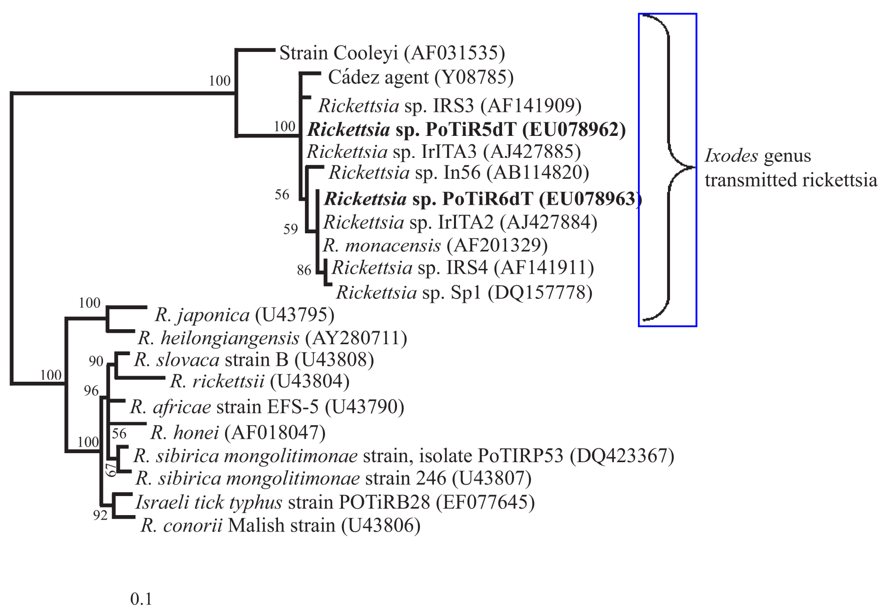

FIG. 2. Phylogenetic analysis based on the *ompA* gene nucleotide sequences. Maximum likelihood tree was performed by the PUZZLE program, with the HKY-85 model generated using a transition/transversion of 1.75, a nucleotide frequency of A = 0.271; C = 0.182; G = 0.228; T = 0.32. Log likelihood = −17914.85. Branch lengths represent genetic distances between sequences. The branch values represent the support based on quartet puzzling steps of 1000 replicates. Only a single representative of 100% identical isolates obtained in this study was included in the tree (boldface).

RESULTS AND DISCUSSION

In this study, *B. lusitaniae*, *Rickettsia* sp. IRS3, *R. monacensis*, and *A. phagocytophilum* were detected in *I. ricinus* collected from Madeira Island. The percentage of positives for each agent is shown in Table 1. Strains PoTiB6, PoTiR5dt, and PoTiR6dt are designations for *B. lusitaniae*, *Rickettsia* sp. IRS3, and *R. monacensis*, respectively.

Seven (2.3%) ticks infected with *B. lusitaniae* were also infected with *Rickettsia* spp., 2 (0.6%) with *R. monacensis*, and 5 (1.7%) with *Rickettsia* sp. IRS3. One (0.3%) tick was infected with *A. phagocytophilum*. Sequence analysis indicated 100% similarity to the *groESL* partial sequence, previously described in ticks from Madeira. The phylogenetic analysis of the intergenic spacer region (*rrf-rrl*) gene of *Borrelia* shows PoTiB6 clustered with the other *B. lusitaniae* strains isolated in Portugal from ticks, such as PoTiB2, and humans, PoHL1, with a nucleotide sequence identity of 99% (Fig. 1).

Concerning the phylogenetic analysis of the *Rickettsia ompA* gene, PoTiR5dt clustered with *Rickettsia* sp. IRS3 and *Rickettsia* sp. IrITA3, and PoTiR6dt clustered with *R. monacensis* and *Rickettsia* sp. IrITA2, both with a nucleotide sequence identity of 100% (Fig. 2).

The overall tree topology for *Borrelia* and *Rickettsia* was identical when phylogenetic analyses were performed for the *fla* and *gltA* genes, respectively (data not shown).

The prevalence of *Borrelia* spp. in *I. ricinus* found in this report, 2.7%, was lower compared with the 31.2% rate demonstrated in a previous study in Madeira (Núncio et al. 2001). This might be due to a difference in the sensitivity of assays used in these studies. However, the rate is similar to the prevalence found in a previous study (Matuschka et al. 1998), even though the authors detected *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto, but not *B. lusitaniae*, as was the case in the study by Núncio et al. (2001).

A. phagocytophilum was also detected in fewer ticks (0.3%) compared with an earlier study performed on Madeira Island (4%) (Santos et al. 2004). This may be attributed to seasonal and geographical changes in this agent's prevalence or to differences in the detection methods used. Furthermore, some archival samples may have been degraded, resulting in false negative amplification.

An outbreak of murine typhus, caused by *R. typhi*, in Porto Santo in 1996 alerted clinicians to the occurrence of this disease in the Madeira archipelago. This points to the possibility of other pathogenic Rickettsiae circulating in Madeira Island. The detection of *Rickettsia* sp. IRS3 and *R. monacensis* in the current study, the latter species having recently been shown to be pathogenic in Spain (Jado et al. 2007), should therefore make competent authorities vigilant toward these potentially pathogenic agents.

Our report is the first documentation of *Rickettsia* sp. IRS3 and *R. monacensis* in *I. ricinus* ticks and their coinfection with *B. lusitaniae*. These findings raise a few questions concerning whether these agents' life cycle is altered by their coexistence in the same vector, for which further studies would be needed, to determine if this would result in coinfection with less typical Lyme borreliosis clinical presentation or disease severity.

As tourism becomes a larger portion of the economy of this island, other studies should be undertaken, looking at a broad range of tick species and agents, as well as specimens from multiple municipalities. In this way, clinical awareness will be enhanced and prevention methods can be readily established.

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Chapter VII. Co-infections of *Rickettsia slovaca and R. helvetica* with *B. lusitaniae* in ticks collected in a Safari Park, Portugal

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Co-infections of *Rickettsia slovaca* and *Rickettsia helvetica* with *Borrelia lusitaniae* in ticks collected in a Safari Park, Portugal

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Abstract

Borrelia and *Rickettsia* bacteria are the most important tick-borne agents causing disease in Portugal. The identification and characterization of these circulating agents, mainly in recreational areas, is crucial for the development of preventive measures in response to the gradually increasing exposure of humans to tick vectors. A total of 677 questing ticks including *Dermacentor marginatus*, *Rhipicephalus sanguineus*, *Ixodes ricinus*, *Hyalomma lusitanicum*, *H. marginatum* and *Haemaphysalis punctata* were collected in a Safari Park in Alentejo, Portugal, to investigate the prevalence rates of infection and characterize *Borrelia* and *Rickettsia* species. From a total of 371 ticks tested by PCR for Borrelia burgdorferi sensu lato (s.l.), of which 247 were tested for *Rickettsia*, a prevalence infection of 18.3% was found for *B. lusitaniae* and 55% for *Rickettsia* spp. Sequence analysis of positive amplicons has identified the presence of *B. lusitaniae* (18.3%), *R. monacensis* strain IRS3 (51.7%) and *R. helvetica* (48.3%) in *I. ricinus*. *R. slovaca* (41.5 %), *R. raoultii* (58.5 %) and also *B. lusitaniae* (21%) were identified in *D. marginatus* ticks. One (5.9%) *H. lusitanicum* was infected with *B. lusitaniae* and *R. massiliae* was also found in one *R. sanguineus*. Co-infection was found in 7 (20%) *I .ricinus* and 34 (23.3%) *D.marginatus* ticks. We report, for the first time, simultaneous infection of *R. helvetica* and *B. lusitaniae* and also *R. slovaca*, the agent of TIBOLA/DEBONEL, with *B. lusitaniae*. Additionally, six isolates of *B. lusitaniae* were established, and successful isolates of *Rickettsia* were also obtained for the detected species using tick macerates cultured in mammalian and mosquito cell lines. This report describes the detection and isolation of tick-borne agents in a Portuguese Safari Park, highlighting the increased likelihood of infection by multiple agents to potential visitors or staff.

Keywords: Ticks, *Rickettsia*, *Borrelia*, Safari Park, Portugal

Introduction

Mediterranean spotted fever (MSF) and Lyme borreliosis (LB) are the two main tick-borne diseases included in the nationally notifiable diseases of the Portuguese Ministry of Health in Portugal that pose major concern to public health. However, *R. conorii*, which causes MSF, is not the only pathogenic *Rickettsia* species circulating in Portugal. There are other members of spotted fever group capable of causing disease that have been detected in Portuguese patients, such as *R. sibirica mongolotimonae* strain, and in ticks, such as *R. slovaca*, *R. aeschlimannii*, *R. helvetica*, *R. massiliae*, *R. monacensis* and *R. raoultii* (De Sousa et al. 2006, Bacellar et al. 1995, Lopes de Carvalho et al. 2008). Some of these spotted fever group rickettsia seem to have low pathogenicity, such as *R. massiliae* (Beati and Raoult, 1993, Vitale et al. 2006), *R. monacensis* (Simser et al. 2002, Jado et al. 2007) and *R. raoultii* (Parola et al. 2009). Nevertheless, they were found to be involved in human disease.

The first human case of LB caused by *Borrelia burgdorferi* sensu lato (s.l.) was detected in Portugal in 1989, however it was only recognised as a notifiable disease in 1999 (David de Morais et al. 1989). Analysis of laboratory data has confirmed LB as being underreported, with an estimated incidence of 0.04 per 100 000 inhabitants (Lopes de Carvalho e tal. 2008). Using molecular biological assays, *B. afzelii*, *B. valaisiana*, *B. garinii* and *B. burgdorferi* sensu stricto (s.s.) were detected in *I. ricinus* collected in Madeira island (Matuschka et al. 1998, Lopes de Carvalho et al. 2008). In other studies conducted in Portugal, *I. ricinus* and *H. marginatum* ticks were found to be infected with *B. lusitaniae*, *B. afzelii*, *B. valaisiana*, *B. garinii* and *B. burgdorferi* s.s. (De Michelis et al. 2000). *B. lusitaniae*, the most prevalent *Borrelia* species in Portugal, was isolated from a human patient in 2004 and 2008, confirming its role in the pathogenesis of LB (Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008a).

Safari parks are popular tourist attractions with thousands of visitors each year, and the possibility of tick bites is considerable in these rich ecological niches. In this study the main objective was to identify which *Rickettsia* and *Borrelia* species are present in questing ticks from one of the largest Safari Parks (approximately 90-ha) in Portugal, harbouring more than 40 species of exotic animals. Furthermore, as this park has imported exotic animals, this study may be also an important control of circulating tick species.

Initially a screening survey on ticks based on the molecular detection was performed to evaluate the prevalence of infection and to assess the potential agents that may be tick-transmitted to humans in this park. After, the main aim was to isolate *Rickettsia* and *Borrelia* species for agent characterization and future use for diagnostic purposes.

Materials and methods

Tick samples

Ticks were collected by flagging in a Safari Park in Alentejo, Portugal (38º 03’ 03.45’’ N), in areas where animals circulated. The identification was performed using morphological characters with standard taxonomic keys. The sampling was performed non-systematically from December 2006 to April 2009.

DNA extraction and amplification

The ticks were washed in 70% ethanol and sterile distilled water, dried on sterile paper and subsequently boiled in 25% ammonium hydroxide solution, as described previously by Schouls et al. (1999). A negative control was included every ten DNA extractions to monitor the occurrence of false positives. The tick lysate was stored at -20ºC until further use. Amplification of rickettsial DNA was performed targeting the gene for

citrate synthase (*glt*A), outer membrane protein A (*omp*A) and B (*omp*B) as described previously (De Sousa et al. 2006). PCR amplification of the 5S (rrf)-23S (rrl) intergenic spacer (Rijpkema et al. 1995) was amplified for the detection and isolation of *Borrelia*. To characterize the isolates the outer surface protein A (*Osp*A) (Lopes de Carvalho et al. 2008) and *Osp*C genes were also performed. The *osp*C primers were designed (OspC1F5'ACGGATCATTGTTAGCAGGAG3'/OspC1R5'GCATCAGTAGCAGCAC CAG), using multiple alignments of published ospC sequences of *B. lusitaniae* within GenBank (EF179585; EF179575) (Vitorino et al. 2008). All amplification reactions included positive and negative controls.

DNA sequencing

Amplicons were purified using Jetquick Purification PCR Product Spin Kit (Genomed, Inc) and sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 377 DNA sequencer, according to the manufacturer's recommendations. The sequencing reactions were performed with the forward and reverse primers used for the PCR amplifications. The sequences were assembled by combining the sequences generated by each primer, using the Lasergene software (DNASTAR). For identity determination, primer sequences were removed and the analysis was conducted with PAUP 4.0b10 software.

Cultivation of *Rickettsiae*

Adult *I. ricinus*, *R. sanguineus* and *D. marginatus* ticks collected from vegetation were washed for 5 min in 10% bleach, 70% ethanol and dried with sterile paper. All these tick species were inoculated in Vero cell line (African green monkey fibroblast cells). *D. marginatus* and *I. ricinus* were also inoculated in mosquito C6/36 cell line (derived

from Aedes albopictus), where half the number of ticks per isolation attempt was randomly inoculated in Vero cells, and the other half in C6/36 cells. When inoculating in Vero cell-coated shell-vials, ticks were firstly triturated in Eagle's minimal essential medium (MEM, Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum and 1% glutamine. For inoculation in C6/36 cell-coated shell-vials, L-15 medium (Leibovitz, Gibco, Invitrogen, Paisley, UK) supplemented with 3% fetal bovine serum was used. Both Vero and C6/36 cell-coated shell-vials were incubated at two temperatures: half the number of shell-vials coated with each cell type was incubated at 28ºC, and the other half at 32ºC. After 6-7 days the cells were scraped with glass beads, transferred onto a confluent monolayer of the same cell type in 25 cm2 flasks and incubated at the respective temperatures for a further 6 days. The monolayers were scraped every 6-8 days for *Rickettsia* detection by Gimenez staining. Positive isolates were established after three successful passages and inoculums were stored at -80º C. Isolate characterization was performed by PCR and sequencing.

Cultivation of *Borrelia* spirochetes

Adult *I. ricinus* ticks were disinfected by successive immersion in iodine, 70% ethanol, and distilled water and inoculated in 8 mL of complete Barbour-Stoenner-Kelly (BSK) II medium (Sigma).

Cultures were maintained at 34ºC for 3 months and examined weekly by dark-field microscopy to monitor the presence of spirochetes. After 6 weeks, 1 mL of each culture was centrifuged for 15 min at 7,000 X g, and DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen Hilden, Germany) according to the manufacturer's recommendations.

Confirmation of *Borrelia* isolates identity was performed by PCR amplification of cultured spirochetes and subsequent sequencing. Positive isolates were collected and stored at -80ºC, after two successful passages.

Results

A total of 677 ticks, 291 *D. marginatus*, 168 *R. sanguineus*, 163 *I. ricinus*, 30 *H. lusitanicum*, 17 *H. punctata* and 8 *H. marginatum*, were collected from vegetation in a Safari Park in Alentejo, Portugal (Table 1). No new tick species that could have been introduced by the exotic animals were detected.

Of the total collected ticks, 371 (54.8%) were tested by PCR for *B. burgdorferi* s.l., of which 247 (36.4%) were then tested for *Rickettsia* (Table 1). Rickettsial DNA was detected in 136 (55%) ticks. Sequence analyses of *Rickettsia* positive amplicons from I. ricinus showed that 14 (48.3%) samples have 100% sequence identity with *glt*A and *omp*B sequences of *R. helvetica* (U59723 and AF123725) and 15 (51.7%) samples have 100% identity with *glt*A and *omp*A of *R. monacensis* strain IRS3 (EF501755 and EU078962).

Forty-four (41.5%) *D. marginatus* tick samples showed> 99% and 100% identity with *glt*A and *omp*A of *R. slovaca* (U59725 and U43808) and 62 (58.5%) tick samples (named PoTiR7dt) showed 100% identity with *glt*A and *omp*A of *R. raoultii* (DQ365804 and GQ404429), previously detected in *Dermacentor* ticks collected in Russia and Spain respectively. In I. ricinus there was no difference in tick's infection by gender for both *R. helvetica* and *R.monacensis*. However, in *Dermacentor* we found a significantly higher percentage of infection in female ticks (65%) for both *Rickettsia* species, when compared with males. One *R. sanguineus* was infected with *R. massiliae*

showing 100% of identity with *glt*A and *omp*A of previous strain of *R. massiliae* (DQ459393 and DQ459388).

B. lusitaniae was detected in 68 (18.3%) ticks using 5S (rrf)-23S (rrl) intergenic spacer, with 100% homology with PoTiB1 (DQ111065). Infection percentages of 21% were found in *D. marginatus*, 22.9% in *I. ricinus* and 5.9 % in *H. lusitanicum*. Co-infection of *Borrelia* and *Rickettsia* was observed in 41 (16.6%) ticks, 7 (20%) *I. ricinus* and 34 (23.3%) *D.marginatus*. Co-infection was found in *I. ricinus* and *D. marginatus* with *R. helvetica* and *R. slovaca*, respectively, each of which infected with *B. lusitaniae.* Concerning isolation attempts performed in this study, Rickettsia isolation attempts were undertaken using two cell lines, Vero and C6/36 cells, incubated at both 28º and 32ºC. *R. monacensis* strain IRS3 and *R. helvetica* were each isolated from five *I. ricinus*, *R. massiliae* from three *R. sanguineus* and *R. slovaca* from 13 *D. marginatus* ticks (Table 2). All five *R. monacensis* strain IRS3 isolations were achieved at 28ºC; four proliferated in Vero cells and one in C6/36 cells after a mean of 14 days. Three out of five successful isolates of *R. helvetica* were detected in C6/36 cells at 28ºC, with the other two isolates were obtained at 32ºC, one in C6/36 cells and the other in Vero cells, after a mean of 14 days. *R. massiliae* was detected in Vero cells after 35 days, two at 28ºC and one at 32ºC. One isolate of *R. slovaca* was detected in C6/36 cells incubated at 32ºC, and the other 12 isolates in Vero cells, four of which were incubated at 28ºC and the remaining eight at 32ºC. *Rickettsiae* were visualised by Gimenez staining after a mean of 15 days incubation at either temperature. All *Rickettsia* isolates were characterized by PCR and sequencing as described for the prevalence of infection. The sequences of the fragments *glt*A, *omp*A and *omp*B genes amplified from rickettsial isolates were submitted to the Genbank and their accession numbers were obtained (Table 2).

Isolation attempts for *Borrelia* spp. were performed on 39 *I. ricinus* ticks, resulting in the successful isolation of *B. lusitaniae* from six (15.4%) ticks, five of which (named PoTiB7) are 100% identical with *osp*A gene of *B. lusitaniae* PoHUB1 (EU863625), PoTiBGr409 (EF179560) and PoTimfP147 (EF179560). Identity of 100% was obtained for *osp*C gene of *B. lusitaniae* PoTiBmfP220 (EF179581) and 100% with IGS sequences TT928 (AY575767). One isolate (named PoTiB8) had the same identity for *osp*A gene, for *osp*C sequences has 100% with PoTiBGr41 (EF179568) and for IGS sequences has 100% with PoTiB1 (DQ111065) and M3-22 (AY575742). The 5S (rrf)-23S (rrl) intergenic spacer, *osp*A and *osp*C genes from PoTiB7 and PoTiB8 have been deposited in Genbank with accession numbers HM193536, HM193537, HM193538, HM193539, HM193540, respectively.

Discussion

This report describes the detection of five *Rickettsia* species and *B. lusitaniae*, capable causing disease in humans, from ticks collected in a Portuguese Safari Park. With the exception of *R. raoultii* detected by PCR, in this study we were able also to isolate four *Rickettsia* species, namely *R. monacensis* strain IRS3 and *R. helvetica* from *I. ricinus* ticks, *R. massiliae* from *R. sanguineus* and *R. slovaca* from *D. marginatus*. *B. lusitaniae* was only isolated from *I. ricinus* because it was the only tick species used for culture attempts. Among the SFG rickettsiae present in *I. ricinus* our study showed high prevalence of *R. helvetica* (48.3%) and *R. monacensis* (51.7%) infection rate. Similar prevalence for both agents was found in studies from Bulgaria (59%), Northeastern part of Italy and Slovenia (55.8%-58.5%) (Christova et al. 2003, Floris et al. 2008). *R. helvetica* was detected for the first time in Swiss *I. ricinus* in 1979, and confirmed as a new member of SFG Rickettsia in 1993 (Beati et al. 1993a). Since then it has been

45.4% in Hungary and Switzerland, respectively (Beati et al. 1994, Lakos and Raoult, 1999). In our study *D. marginatus* ticks showed high prevalence of infection with *R. slovaca* (41.5%), very similar to the prevalence found in previous studies from Portugal (34.5%), Switzerland (45.4%), La Rioja, Spain (40.6%) (Vitorino et al. 2007, Beati et al. 1994, Oteo et al. 2006). In Portugal *D. marginatus* is present in all districts with higher density from September to April. Surprisingly, to this day no cases of TIBOLA/DEBONEL have been described or notified in Portuguese patients. Based on the high prevalence of *R. slovaca* in *Dermacentor* ticks in our country we assume that the cases in Portuguese patients are probably misdiagnosed or diagnosed as SFG *Rickettsia* infection based only on serology. As there is cross-reaction between SFG rickettsiae, species identification is not possible.

The different conditions (Vero and C6/36 cells, maintained at 28 and 32ºC) used in this study for isolation attempts of *Rickettsia* had the intent of increasing the chances of successful isolation. In this study, *R. monacensis* was easily propagated and isolated in Vero cell lines at 28ºC. This strain was isolated for the first time in Germany using I. scapularis cell line (ISE6), and was later replicated in other tick (IRE11, DAE100) and mammalian (L-929 and Vero) cell lines at 34ºC (Simser et al., 2002). In Simser and collaborators study *Rickettsia* cocobacillus were observed after 26 days (ISE6 cell line), a longer period of time compared to the present study, where rickettsias were detected after a mean of 14 days. The authors also mention that *R. monacensis* took longer than 26 days to grow in the mammalian cell line. In our study the incubating temperature seemed more determinant for rickettsial growth than the type of cell line. More isolates of some species of *Rickettsia* were obtained at 28ºC, when compared to their counterparts cultivated at 32ºC, as was the case in three isolates of *R. helvetica* in C6/36 cell line. *R. slovaca* seemed to have an optimal growth at 32ºC. Based on the high

prevalence of infection with *R. raoultii* by PCR, we would have expected to have isolated this species; however, for reasons unbeknownst to us, this was not the case.

B. lusitaniae is the most prevalent *Borrelia* strain in Portugal and was isolated for the first time in *I. ricinus* ticks collected in the Alentejo region (Nuncio et al. 1993). In this study the prevalence of infection with *B. lusitaniae* in *D. marginatus* was similar to that found in *I. ricinus* ticks, the main vector of *B. burgdorferi* s.l. However, we have only been able to detect *B. lusitaniae* in *D. marginatus* by PCR, because unfortunately, *D. marginatus* could not be used for *Borrelia* isolation attempts as they were immediately frozen at -80ºC. It still do not know if the *D. marginatus* is a competent vector for *Borrelia* and further studies need to be address. Previous studies conducted by other researchers using mice models and *D. variabilis* ticks showed that this species can acquired the spirochetes of *B. burgdorferi* but transmission could not be demonstrate as compared with *I. scapularis* the main vector in United States (Soares et al. 2006). However, our results could be explained by the fact that in some ecosystems *D. marginatus* may be implicated as secondary vectors in *B. burgdorferi* s.l. transmission, as has been the case in other countries (Angelov et al. 1996). Furthermore, in another study conducted in Portugal, this hard tick species was also highly infected with *B. burgdorferi* s.l. in Grândola region (Baptista et al. 2004). Interestingly, Grândola is approximately 34 Km from the Safari park where the ticks analysed in this study were collected. Another important finding achieved by using the informatics "Recombination Detection Program, version 3" (Heath et al. 2006) was the fact that the sequence analysis obtained here seems to indicate that the positive isolates from *I. ricinus* may be recombinant strains, as was recently described for another *B. lusitaniae* strain found to have an intersection in the allelic profiles of the *B. lusitaniae* populations from two regions in Portugal, Mafra and Grândola (Vitorino et al. 2008). Moreover, an isolate of

this genospecies was recently obtained from an Apodemus sylvaticus also captured in Alentejo region, indicating that this mammal could be a competent reservoir for *B. lusitaniae* (Lopes de Carvalho et al. 2009). Further studies using housekeeping genes should be undertaken to clarify the phylogeographic structure of these *B. lusitaniae* strains.

Previous studies have confirmed the presence of several species of *Borrelia* (*B. lusitaniae*, *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. burgdorferi* s.s.) in ticks from mainland Portugal and Madeira island, and the infection prevalence could vary between 2.7% and 34.7% (de Michelis et al. 2000, Baptista et al. 2004, Lopes de Carvalho et al. 2008).

Studies on the frequency of co-infection of ticks with several pathogens vary depending on tick species and countries. However, it is always very important to evaluate the existence of co-infections as it may facilitate the prognosis of simultaneous infections in humans. To our knowledge, this is the first report of dual infections of *R. helvetica* and *R. slovaca*, each of which infected with *B. lusitaniae*. The most common bacterial co-infections in *Ixodes* ticks reported in previous studies include dual or multiple agents such as *B. burgdorferi* s.l, *Anaplasma phagocytophylum*, *Babesia* spp and *R. monacensis* (Fernandez- Soto et al. 2004, Swanson et al. 2006). To date, no description of dual infection with *R. helvetica* has been made, with the exception of the report by Fernández-Soto, which mentions infection with *R. helvetica* and *B. burgdorferi* s.l. in an *I. ricinus* collected from an asymptomatic patient (Fernandez- Soto et al. 2004). In Portugal co-infections of *B. lusitaniae* and *R. monacensis* were found in *I. ricinus* ticks in Madeira island, and previous studies based on serological data have also suggested dual infections in LB patients with *A. phagocytophilum* (Lopes de Carvalho et al. 2008, Santos et al. 2006).

Although reports have shown an exacerbated illness in patients co-infected with *A. phagocytophilum* and *Borrelia* compared with LB patients, little is known about the dynamics and transmission of dual infections (Thomas et al. 2001, Krause et al. 2002). Animal models have also shown that co-infection can enhance bacterial burden, transmission and disease progression (Thomas et al. 2001). The occurrence and implications of a simultaneous infection by *R. helvetica* or *R. slovaca* with other pathogenic agents, such as *B. lusitaniae*, in patients has to be further evaluated, and clinicians need to be aware of these possible differential diagnoses in Portuguese patients exposed to tick bites. Co-infections may show variable clinical symptoms.

Considering the prevalence of single and dual rates of infection found in this study, there is a considerable risk of tick exposure to both staff and visitors to this park. The Safari Park has a restricted area, where visitors may only circulate in a vehicle and also areas outside of this restricted area where people are allowed to circulate on foot and have contact with domestic animals. Moreover, the park has a new area with an artificial river for rafting activities surrounded by vegetation. Our flagging locations encompassed both areas (restricted and not restricted areas), where infected ticks were found. Areas are separated by fences; however it is possible that rodents and birds may pass those fences carrying ticks with them. We therefore think it would be advisable and instructive to institute flyers mostly for children, adapted to different ages, informing visitors of the Safari Park about preventive measures for tick-borne diseases.

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Table 1- Prevalence and isolates of *Rickettsia* spp and *Borrelia lusitaniae* from questing ticks of a Safari Park, Portugal

			Rickettsia				***Borrelia***			
			PCR		**Culture**		**PCR**		**Culture**	
Tick species	**No. of ticks collected**	**Gender/stage**	**No. pos/ analyzed**	**Rickettsia spp (Infection rate in positive tick species)**	**No. pos/ analyzed**	**Isolated species (n)**	**No. pos/ analyzed**	**Borrelia spp (Infection rate in positive tick species)**	**No. pos/ analyzed**	**Isolated species**
D. marginatus	291	161 F 130 M	106/146	*R. slovaca* (41.5%) *R. raoultii* (58.5%)	13/73	*R. slovaca* (13)	59/280	*B. lusitaniae* (21%)		*NP*
R. sanguineus	168	75 F 72 M 21 N	1/36	*R. massiliae*	3/15	*R. massiliae* (3)	0/16			
I. ricinus	163	101 F 62 M	29/35	*R. monacensis* (51.7%) *R. helvetica* (48.3%)	10/78	*R. monacensis* (5) *R. helvetica* (5)	8/35	*B. lusitaniae* (22,9%)	6/39	*B. lusitaniae*
H. lusitanicum	30	11 F 19 M	0/9				1/17	*B. lusitaniae*		*NP*
H. punctata	17	8 F 9M	0/13				0/15			
H. marginatum	8	2 F 4 M 2 N	0/8				0/8			
Total	677		136/247		26/166		68/371		6/39	

*NP – Culture was not performed; F=female, M=male, N=nymph

Table 2. *Rickettsia* isolates, culture conditions and GenBank accessing numbers

***Rickettsia* species isolates**	**Studied genes**	**Accession no.**	**Number of isolates and culture conditions**			
			Vero cells 28ºC	**Vero cells 32ºC**	**C6/36 cells 28ºC**	**C6/36 cells 32ºC**
R. monacensis strain IRS3 (PoTiR634)	*glt*A;*omp*A	HM149283;HM149288	4	-	1	-
R. slovaca (PoTiR443)	*glt*A;*omp*A	HM149281;HM149286	4	8	-	1
R. helvetica (PoTiR32)	*glt*A;*omp*B	HM149280;HM149285	-	1	3	1
R. massiliae (PoTiR600)	*glt*A;*omp*A	HM149282;HM149287	2	1	-	-

Chapter VIII. Molecular characterization of a new isolate of *Borrelia lusitaniae* derived from *Apodemus sylvaticus* in Portugal

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Molecular Characterization of a New Isolate of *Borrelia lusitaniae* Derived from *Apodemus sylvaticus* in Portugal

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Abstract

A total of 196 small mammals were collected in Portugal and tested for *Borrelia burgdorferi* sensu lato. Tissue samples were taken from each animal and cultured in Barbour-Stoenner-Kelly (BSK)-II medium. The single strain of spirochete isolated was confirmed as *Borrelia lusitaniae* by genetic analyses. This is the first report of *B. lusitaniae* isolated from *Apodemus sylvaticus*.

Key Words: *Apodemus sylvaticus*—*Borrelia lusitaniae*—Portugal—Reservoir.

Introduction

Lyme borreliosis is an emerging tick-borne disease with public health implications in countries such as Portugal (Lopes de Carvalho and Núncio 2006). The disease is caused by bacteria belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex, and is transmitted by *Ixodes* spp. ticks. *Borrelia lusitaniae* is the most prevalent *B. burgdorferi* s.l. species in Portugal (Baptista et al. 2004, Lopes de Carvalho et al. 2008b) and was first isolated from ticks collected in the southern regions of the country (Núncio et al. 1993). In 2004 and in 2008, two strains of *B. lusitaniae* were isolated from human patients, indicating its pathogenicity in humans (Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008a). Recent studies pointed out that regional strains of *B. lusitaniae* isolated in Portugal constitute genetically distinct populations (Vitorino et al. 2008). Other species of *B. burgdorferi* s.l., such as *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana*, had already been detected in ticks in mainland Portugal as well as in Madeira Island (Baptista et al. 2004, Lopes de Carvalho et al. 2008b). To our knowledge, these other *Borrelia* genospecies have not been detected in human samples from Portugal.

Different *Borrelia* genospecies are associated with distinct ecologies and enzootic cycles, specific pathogenicity, and clinical symptomatology in patients. The list of reservoirs for *B. burgdorferi* s.l. in endemic areas of Europe is extensive. Some associations, however, between *Borrelia* genospecies and vertebrate hosts have been identified, such as *B. garinii* and *B. valaisiana* in birds, *B. afzelii* within small rodents, and *B. burgdorferi* s.s. in red squirrels and hedgehogs (Humair and Gern 1998); lizards may be the principal reservoir hosts of *B. lusitaniae* (Richter and Matuschka 2006, Amore et al. 2007). Although *B. lusitaniae* circulates between a range of ticks and host vertebrate species such as lizards, birds (Poupon et al. 2006), and small mammals, the vertebrate reservoir of *B. lusitaniae* had yet to be identified. In a study performed in the Grandola region, *B. lusitaniae* DNA was detected by polymerase chain reaction in all small mammal species captured (*Mus spretus*, *Apodemus sylvaticus*, *Rattus norvegicus*, and *Crocidura russula*), but it was not possible to isolate spirochetes. These observations indicate that there may exist a sylvatic cycle specific to this *Borrelia* genospecies, maintained by a variety of hosts and reservoirs (Baptista 2006). In Portugal, to address this question we initiated a study to determine whether small mammals can contribute to the maintenance of *B. lusitaniae* in natural foci in Portugal.

Materials and Methods

One hundred ninety-six small mammals (22 *A. sylvaticus*, 160 *M. spretus*, and 14 *Rattus rattus*) were captured using Sherman traps, between July 2002 and October 2004 in three different National Parks of Portugal: Vale Guadiana (W 8°7′19″, N 41°47′19″), Peneda Gerês (W 9°2′13″, N 38°27′28″), and Arrábida (W 7°39′50″, N 37°41′21″).

The rodents were brought to the laboratory and identified by external morphology and skull features. Animals were then anesthetized with ketamine hydrochloride (Imalgene; Merial,

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FIG. 1. Neighbor-joining tree inferred from partial *OspA* gene sequences from the isolate obtained in this study (bold) compared to GenBank sequence data. Distance matrices were calculated using the Kimura 2-parameter model to correct for multiple substitutions. Bootstrap values were obtained from 1000 replicate trees and are indicated at the nodes (>50%). Po, Portuguese strains; ITAh, Italian strains; MT, Morocco strains; TT, Tunisia strains; RB, German strains.

Lyon, France) at a dose of 10 mg/kg, bled by cardiac puncture for serodiagnostics, and euthanized by exposure to CO_2 gas. Serum samples were then tested for the presence of immunoglobulin G antibodies against *Borrelia* by indirect immunofluorescence assay (Doby et al. 1991), using a strain of *B. garinii* as antigen. Positive serology indicated by a titer of 32.

Target organs (skin, bladder, and heart) were aseptically harvested for culture.

The organs were first disinfected by successive immersion in iodine, 70% ethanol, and phosphate-buffered saline; tissues were then minced and placed directly into 8 mL of Barbour-Stoenner-Kelly (BSK)-II medium. All cultures were main-

tained at 34°C for 3 months and examined weekly by dark-field microscopy to monitor the presence of live spirochetes. Heart tissue from a single *A. sylvaticus* mouse captured in Vale Guadiana demonstrated viable spirochetes 8 weeks after culture in BSK-II medium; 1 mL of culture was centrifuged for 5 min at 7000 *g*, and the sediment washed twice with sterile phosphate-buffered saline and frozen at −20°C until DNA extraction. Total DNA was extracted using the QIAamp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Three genes, *flaB* (9), *rrf (5S)-rrl* and *(23S)* intergenic spacer region of *B. burgdorferi* s.l. (Johnson et al. 1992, Rijpkema et al. 1995), and the outer surface protein A (*OspA*) gene, were used to characterize this isolate (Lopes de Carvalho et al. 2008a). The sequences were assembled by combining the sequences generated by each primer, using the BioEdit software. For phylogenetic inference the alignments were made using amino acid sequences and converted to DNA sequences using BioEdit software. All alignments were made using ClustalX program (Thompson et al. 1997) and manually inspected for misalignments. Primer sequences except for the last two (5′) to six (3′) nucleotides were removed from the alignment before phylogenetic analyses, regarding the polymorphisms observed in the remaining sequences. Neighbor-joining tree of DNA sequence alignment was conducted in PAUP* 4.0b10 software. Distance matrices were calculated using the Kimura two-parameter model to correct for multiple substitutions. Bootstrap analysis was obtained with 1000 replicates.

Results and Discussion

Of the 169 small mammals captured, 17 (10.6%) *M. spretus* and 1 (5.6%) *R. rattus* showed antibodies to *B. lusitaniae*; however, no positive cultures were obtained from these host species. Despite isolation of spirochetes from one *A. sylvaticus* captured in the Vale Guadiana National Park, no seropositive samples were obtained from this species. The *flaB* gene, the *rrf* (5S)–*rrl* (23S) intergenic spacer region of *B. burgdorferi* s.l., and the *OspA* gene were amplified from DNA extracted from the *A. sylvaticus* isolate, and further analysis of this isolate identified it as *B. lusitaniae.* Phylogenetic analyses based on the *OspA* gene grouped this new isolate near other *B. lusitaniae* isolated in Portugal and most closely aligned to the North African clade (Grego et al. 2007) (Fig. 1). OspA clustering via the Neighbor-joining algorithm was confirmed by Maximum Likelihood (ML) and MrBayes analysis (data not shown). Intergenic Spacer (IGS) sequences also confirmed the PoAnB1 strain as *B. lusitaniae* as the mean intra–*B. lusitaniae* group sequence identities vary between 95.8% and 91.6% and are below 90% with other *Borrelia* genospecies (89.8% with *B. garinii*, 86.9% with *B. afzelii*, 86.5% with *B. valaisiana*, and 85.1% with *B. burgdorferi* s.s.). The eco-epidemiological studies performed to date demonstrate that *B. lusitaniae* presents different characteristics to existing *B. burgdorferi* genospecies in other European countries (Zeidner et al. 2002, Grego et al. 2007). Previous serology results (Núncio 2002) as well as the DNA detection (Baptista 2006) and the isolation and molecular identification of *B. lusitaniae* indicate that small mammals, particularly, *A. sylvaticus*, may play a role in the maintenance of *B. lusitaniae* in Portugal. To our knowledge, this is the first report of live *B. lusitaniae* spirochetes isolated from *A. sylvaticus*, an indication that this mammal is a competent reservoir of *B. lusitaniae*.

Previous studies have demonstrated that *B. lusitaniae* is not as immunogenic as *B. burgdorferi* s.s. (Zeidner et al. 2002). Even in human cases, isolates derived by culture were achieved in a seronegative and in a borderline seropositive patient (Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008a). Thus, the low titer (Ig = 16) antibody detected in the *A. sylvaticus* mouse from which the borrelia was isolated seems to confirm a pattern regarding *B. lusitaniae*. Future studies, using an autochthonous borrelia strain as antigen, should improve the sensitivity of our serological testing.

Despite evidence in several other studies indicating lizards as main reservoir host of *B. lusitanie* in some geographical areas (Richter and Matuschka 2006, Amore et al. 2007), our findings demonstrate that more attention be given to small mammals as potential reservoirs. In addition, parallel studies in birds and lizards are currently ongoing to gain a more detailed assessment of both the ecology and pathogenicity of borrelia strains circulating in Portugal.

Nucleotide sequence accession number: The *rrf* (5S)-*rrl* (23S) intergenic spacer region of *B. burgdorferi* s.l., *flaB*, and *OspA* gene from PoAnB1 have been deposited in GenBank with accession numbers EU647595, EU122385, and FJ200368, respectively.

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Disclosure Statement

No competing financial interests exist.

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Chapter IX. *Borrelia garinii* and *Francisella tularensis* subsp. *holarctica* detected in migratory shorebirds in Portugal

In submission

Borrelia garinii and *Francisella tularensis* subsp. *holarctica* detected in migratory shorebirds in Portugal.

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Abstract

Migratory birds are important in the global spread of zoonotic diseases transmitted by ticks, including Lyme borreliosis and tularemia, two zoonotic diseases previously detected in Portugal. A total of 237 migratory shorebirds were captured in the Tagus and Sado estuaries, Portugal. Ninety blood samples were studied for the presence of *Borrelia burgdorferi* s.l. and *Francisella tularensis*. In the present study *B. garinii* was identified in seven (3 European black-tailed godwits *Limosa limosa limosa,* 2 Icelandic black-tailed godwits *Limosa limosa islandica,* 1 common redshank *Tringa totanus* and 1 little stint *Calidris minuta)* (9%), whereas *F. tularensis* subsp. *holartica* was identified in one (*C. minuta*) (1%) bird-blood sample, respectively. To our best knowledge, this work is the first evidence that shorebirds that migrate through or winter in Portugal, may contribute to the introduction of these pathogens.

Keywords: migratory shorebirds, *B. garinii*, *F. tularensis* subsp. *holarctica*, Portugal.

Introduction

Emerging infectious diseases are a key threat to conservation and public health, yet predicting and preventing their emergence is notoriously difficult. The increasing incidence of some of these diseases, namely the tick-borne ones, may be partially associated to migratory birds and with their migratory routes. Effectively, migratory birds are increasingly considered important in the global dispersal of zoonotic pathogens (Hubálek, 2004, Poupon et al. 2006, Dubska et al. 2008). In Europe, at least seven *Borrelia* species belonging to the complex of *Borrelia burgdorferi* sensu lato (s.l.), *B. valaisiana*, *B. garinii*, *B. lusitaniae*, *B. afzelii*, *B. burgdorferi* sensu strict (s.s.), *B. bissetti* and *B. spielmani* have been detected in the *Ixodes ricinus*, the main vector of this agent. *B. burgdorferi* s.l. complex patterns of zoonotic transmission implies that they are maintained in nature by ixodid (hard) ticks and a broad spectrum of competent vertebrate hosts (Kurtenbach et al. 2002). Birds play an important role in the transmission of Lyme borreliosis as carriers of the arthropod vectors of *Borrelia* (Humair, 2002). For Europe, pheasants (*Phasianus colchicus*), a few seabird species, and some passerine bird species have been shown to be reservoir competent for *B. garinii* and *B. valaisiana* but not for *B. afzelii*, a rodent specialist (Humair 2002, Hanincova et al. 2003, Poupon et al. 2006, Taragel'ová et al. 2008). However, little is known about the other migrating bird species with respect to their competence as Lyme borreliosis reservoirs and transmission abilities (Dubska et al. 2009). Host diversity and host associated selection driven by the immune system are likely to greatly affect the spatio-temporal structure of bird-borne zoonotic agents such as Lyme borreliosis spirochetes (Kurtenbach et al. 2002). *Francisella tularensis* is an important etiologic agent that may be transmitted during tick feeding, causing tularemia in humans. In Portugal, the presence of *F. tularensis* subsp *holarctica* has been confirmed in humans and in *D. reticulatus* ticks (1.1%), and also *Francisella*-like endosymbionts in the same tick species (39%) (Lopes de Carvalho et al. 2007, Lopes de Carvalho et al. 2010). A recent study confirmed the presence of *F. tularensis* subsp. *holarctica* in birds and occurrence of transmission this pathogen from birds to man (Padeshki et al. 2010). *F. tularensis* has been isolated from rodents, lagomorphs, deer and other ground and water mammals which can explain some water and foodborne outbreaks. The role of birds in dissemination of the disease, however, is not very clear and has often been neglected (Padeshki et al. 2010). The potential for transport and dissemination of certain pathogenic microorganisms by migratory birds is of concern (Hubálek 2004). Moreover, many migratory birds fly across Portugal and could disperse arthropods vectors that may carry pathogens with them. Since information about the role of migratory birds on the dispersion of these tick-borne pathogens is scarce, in the present study we investigated the role of several

shorebird species as a route of introduction of different genotypes of *B. burgdorferi* s.l. and *F. tularensis* in the Portuguese wetlands that could influence the epidemiology of Lyme borreliosis and tularemia in Portugal.

The purpose of the present study was to assess the presence of *B. burgdorferi* s.l. and *F. tularensis* in a group of birds that is understudied, the migratory shorebirds.

Material and Methods

Study Area

Rice-fields are large areas with inland freshwater that represent 15% of the world´s wetlands today and being considered a very important agricultural habitat for birds in areas were human development have forced them away from their original habitat (Lawler 2001, Tourenq *et al.* 2001). Estuaries in the central western coast of Portugal are the two main rice production areas with significance in the context of southern Europe (Lains and Sousa 1998), also harboring the highest amounts of waste rice, an important food source for some bird species (Lourenço and Piersma 2008).Coastal saltpans can be seen in several geographic regions and in the Western Europe they occur from southern France to the southern of Iberian Peninsula (Rufino *et al.* 1984). Classified as functional wetlands with high biological richness, they can provide alternative stopover foraging and roosting areas to high numbers of waterbirds during their migration movements, wintering areas and also as important nesting habitats during reproduction (Sadoul *et al*. 1998, Elphick and Oring 1998, Pedro and Ramos 2009). In this study the field investigation was conducted at the rice-fields of Samora Correia (38°57´N, 8°50´W), at the saltpans of Samouco (38°44´N, 8°59´W), Seixal (38°38´N, 9°05´W) and Alhos Vedros (38°39´N), at the saltmarshes of Seixal, Alhos Vedros and Moita (38°39´N, 8°59´W).

Bird catching and sampling

Continental black-tailed godwits (*Limosa limosa limosa*) were caught at night, using mist-nets and decoys, on the rice-fields in March 2008 and February 2009/2010, prior to their northern migration to the breeding grounds. Common redshanks (*Tringa totanus*) and little stints (*Calidris minuta*) were also caught at night with mist-nets and decoys during both migration movements (southward and northward), between September and March of 2008, 2009 and 2010 on the saltpans. Icelandic black-tailed godwits (*Limosa limosa islandica*) were caught

during the day using canon-netting in November 2008, February 2008/2009 and March 2010 in the saltmarshes. All the birds were marked with metal rings, aged and measured. A small blood sample was then taken from the bracial vein with a heparinized microhematocrit capillary tube. For detection of *Borrelia* and *Francisella* the blood sample was transferred to a FTA card (Whatman). The cards were left to dry out and then kept at room temperature until analysis. After sampling, the birds were released unharmed.

DNA extraction and PCR

A disk containing the sample area to be tested was punched out of the FTA card. Each disk was placed in a PCR tube, washed three times with FTA Purification Reagent (Whatman) and washed twice with TE-1 buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). The disk was dried in the PCR tube after which the PCR master mix was added, directly to the disk. A nested PCR, was then performed, targeting the 5S (rrf)-23S (rrl) intergenic spacer (Rijpkema et al. 1995) to detect the presence of *B. burgdorferi* s.l. The specific primer set FT393 and FT642, amplifying a 250 bp fragment of the gene coding the 17 kDa lipoprotein (*tul4)* of *Francisella* spp. was used as previously described (Karhukorpi and Karhukorpi 2001). Negative controls were included during extraction and PCR.

Identifying and Genotyping of *Borrelia* and *Francisella* species

Bacteria detected in birds were assigned by the DNA sequencing. The sequences of the positive samples were assembled by combining the sequences generated by each primer, using the BioEdit software. Homology searches within the GenBank data set were performed using the BLASTN algorithm (Altschul et al. 1997). For phylogenetic inference the alignments were made using DNA sequences and in ClustalX program (Thompson et al. 1997) and manually inspected for misalignments. Primers sequences were removed from the alignment before phylogenetic analysis.

All sequences used in this analysis are presented in Table 1. Recombination events were screened using Recombination Detection Program, version 3 (RDP3; Heath et al. 2006), using several methods that are implemented simultaneously, namely RDP (Martin and Rybicki, 2000, GENECONV (Padidam et al. 1999), Maximum Chi Square (MAXCHI; Maynard 1992), CHIMAERA (Posada and Crandall 2001), Sister Scanning (SISCAN; Gibbs et al. 2000) and 3SEQ (Boni et al. 2007). As recommended (RDP3 Instruction Manual) all sequences except one in groups of identical sequences were discarded from the analysis. So, the sequences checked for recombination events were from *B. garinii* strains PoAnB2, PoAnB8, PoTiBG4, PoTiBG86, IPT114, IPT130, IPT139, PBi (proposed *B. bavariensis* sp. nov., Margos et al., 2009) and *B.*

lusitaniae PoAnB1. The general settings were 0.05 for the highest acceptable P-value and Bonferroni corrections were enabling. In RDP the window size was set to 30 and the setting 'internal and external references' was chosen as recommended for small datasets (RDP3 Instruction Manual). In MAXCHI the'strip gaps' was switched on and 'variable site per window' was set to 70. In CHIMAERA the 'variable sites per window' was set to 60; and in SISCAN the window size was set to 150 and a step size of 40.

Neighbour-Joining (NJ) tree of DNA sequence alignment was conducted in PAUP* 4.0b10 software. Distance matrices were calculated using the Kimura two-parameter model to correct for multiple substitutions. IGS sequence from *B. lusitaniae* PoAnB1 was used as outgroup. Bootstrap analysis was obtained with 1000 replicates (Figure 1).

Results

A total of 237 migratory shorebirds were captured and 90 (38%) blood samples (29 black-winged stilts *Himantopus himantopus,* 20 common redshanks *Tringa totanus,* 17 nominate black-tailed godwits *Limosa l. limosa,* 13 Icelandic black-tailed godwit *Limosa l. islandica,* eight avocets *Recurvirostra avosetta,* two little stints *Calidris minuta* and one greenshank *T. nebularia*) were tested for the presence of *B. burgdorferi* s.l. and *F. tularensis*. In the 90 samples tested, *B. garinii* was identified in seven individuals (9%) from three different genera (5 black-tailed godwist: 3 belonging to the nominate population and 2 from the Icelandic population, 1 common redshank, 1 little stint) and *F. tularensis* subsp. *holarctica* in one (1%) little stint.

No recombination events were detected using RDP3 program. In Figure 1 an NJ tree based on Kimura two-parameter distances is shown with bootstrap values. Phylogenetic analysis inferred from *rrf-rrl* intergenic sequences (Fig. 1) clustered most of the *B. garinii* strains detected in Portugal, PoAnB2, PoAnB3, PoAnB4, PoAnB5, PoAnB7 and PoAnB8 in little stint, Icelandic black-tailed godwit and common redshank with *B. garinii* detected from diverse biological sources and geographical origins, namely in ticks (*I. ricinus and I. persulcatus*) and rodents, from China, France, Portugal and Russia (bootstrap support 54%).

Common redshank populations breed from Fennoscandia to the Baltic and west-central Europe, and winters along the Atlantic coast of Western Europe and Western Africa (Stroud et al. 2004). Strain PoAnB8 detected in one common redshank clusters with two *B. garinii* detected in China (bootstrap support 61%). Curiously, the only other available *rrf-rrl* intergenic sequences of *B. garinii* from birds cluster together (99% and 81% bootstrap values) but apart from the Portuguese strains. The *rrf (5S)-rrl (23S)* intergenic spacer region of *B. burgdorferi* s.l.

from PoAnB2-PoAnB8 have been deposited in Genbank with accession numbers HM588687-HM588693, respectively. The sequence accession number for partial sequences of 17 kDa lipoprotein gene generated in this study is HM588694 for PoAnF1.

Discussion

Wild birds are significant to public health because they carry emerging zoonotic pathogens, either as reservoir host or by dispersing infected arthropod vectors (Reed et al. 2002). However, the importance of birds as reservoir of tick-borne pathogens is still poorly understood. Lyme borreliosis is a zoonosis and the bacteria are maintained in nature by different vertebrate reservoir hosts. At least seven *Borrelia* species are associated with human disease: *B. burgdorferi* s.s., *B. valaisiana*, *B. afzelii*, *B. lusitaniae*, *B. spielmanii, B. bissetti* and *B. garinii* (Baranton et al. 2001, Richter et al. 2006, Lopes de Carvalho et al. 2008). The association between birds and some *Borrelia* species, namely, *B. valaisiana* and *B. garinii* is well documented (Hanincova et. al, 2003; Poupon et al, 2006). *B. garinii* have been found in a marine enzootic infection cycle worldwide and also among terrestrial animals which primarily involve birds, including seabirds and migratory passerines (Hanincova et al. 2003, Comsted et al. 2006, Taragel'ová et al. 2008, Duneau et al. 2008, Dubska et al. 2009). In accordance with other studies this study suggests that migratory shorebirds could be also an important reservoir for *B. garinii*. Contrarily to other study in Subarctic Eurasia involving strains of *B. garinii* (Comstedt et al. 2006) that suggest the existence of two monophyletic lineages with different levels of genetic diversity, this study suggest the existence of closely related strains found in *Ixodes* ticks, rodents, vole and birds from Eurasia to Southern Europe (Table 1, Figure 1). This scattered diversity can be associated with migratory routes, since birds are reservoirs of *B. garinii* and can disseminate infected ticks (Comstedt et al. 2006). Interestingly, the birds studied were mainly youngsters that according to Dubska et al. (2009) are more prone to infection by *B. garinii*.

Considering the positive studied migratory birds species, namely the black-tailed godwit (*L. limosa limosa*) and little stint (*C. minuta*) that winter in sub-Saharan Africa (Piersma et al. 1996), it is intriguing why *B. garinii* is not reported in this geographic region. The great majority of the members of the Scolopacidae family are migratory species that breed at northern latitudes and winter further south (Piersma et al. 1996). These species use the Portuguese wetlands either to refuel in their way to the breeding grounds during northward migration (nominate population of black-tailed godwit *Limosa limosa limosa*), to the wintering areas

during the southward migration, or simply as a wintering area (Icelandic black-tailed godwit *L. limosa islandica*).

Our findings are in agreement with previous studies describing the distinct clade (bootstrap support 92%) formed by PBi strain, that is a bird associated ecotype, and that it was recently proposed to named *B. bavariensis* sp.nov., and other OspA serotype 4 strains (Wilske et al. 1993) related to rodents ecotype The MLSA studies performed suggest that the specialization of OspA serotype 4 to birds were acquired more recently than to rodents (Margos et al. 2009). Furthermore, this study allowed the detection of *F. tularensis* in bird blood sample for the first time in Portugal, confirming the presence of these bacteria in migratory shorebirds. The role of birds in the epizoology and dissemination of *Francisella* spp. is not complete understood, although several reports suggesting bird-related transmission have been published since the past century (Bell, 1979, Morner et al. 1988, Padeshki et al. 2010). Due to the capacity that this bacterium has to survive in water and the fact that these bird species lives mainly in wet environmental, further studies are needed to assess the risk of a water-borne outbreak of tularemia in this region.

To our knowledge, this is the first evidence that shorebirds that migrate through or winter in Portugal, may contribute to the dispersion of *Borrelia* and *Francisella* in this southwest European country.

Hence the presence of both pathogens in migratory birds that cross Portuguese territory is proved, in future studies, efforts to achieve samples that enable tentative isolation of these bacteria will be made in a way to assess the real importance of these migratory shorebirds in borrelia and tularemia dissemination.

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Table 1. *Borrelia garinii* strains used in the phylogenetic analysis.

Strain	Biological source*	Geographic source	GenBank accession no.	Reference
20047[T]	*Ixodes ricinus*	France	L30119	Postic et al. 1994
PoAnB2	*Calidris minuta* N4118	Samouco, Portugal	HM588687	This study
PoAnB3	*Limosa limosa islandica* I012924	Seixal, Portugal	HM588688	This study
PoAnB4	*Limosa limosa islandica* J013994	Alhos Vedros, Portugal	HM588689	This study
PoAnB5	*Limosa limosa islandica* J005990	Samora Correia, Portugal	HM588690	This study
PoAnB6	*Limosa limosa islandica* J006000	Samora Correia, Portugal	HM588691	This study
PoAnB7	*Limosa limosa islandica* J013203	Samora Correia, Portugal	HM588692	This study
PoAnB8	*Tringa totanus* I013248	Samouco, Portugal	HM588693	This study
PoTiBG4	*Ixodes ricinus*[§]	Mafra, Portugal	AY463164	Collares-Pereira et al. 2004
PoTiBG20	*Ixodes ricinus*[§]	Mafra, Portugal	AY463166	Collares-Pereira et al. 2004
PoTiBG86	*Ixodes ricinus*[§]	Mafra, Portugal	AY463168	Collares-Pereira et al. 2004
PoTiBG163	*Ixodes ricinus*[§]	Mafra, Portugal	AY463169	Collares-Pereira et al. 2004
CFC1E	*Myodes glareolus*	Russia	AB178379	Masuzawa et al. 2005
JLHCH	-	China	DQ150549	Unpublished
Ip-5831	*Ixodes persulcatus*	Middle Urals, Russia	AM748065	Unpublished
Ipl-4845	*Ixodes persulcatus*	Middle Urals, Russia	AM748060	Unpublished
IPT28	*Ixodes ricinus*	Alsace, France	FJ546499	Margos et al. 2009
IPT114	*Ixodes ricinus*	Alsace, France	FJ546500	Margos et al. 2009
IPT130	*Ixodes ricinus*	Alsace, France	FJ546501	Margos et al. 2009
IPT139	*Ixodes ricinus*	Alsace, France	FJ546502	Margos et al. 2009
IPT156	*Ixodes ricinus*	Auvergne, France	FJ546504	Margos et al. 2009
IPT157	*Ixodes ricinus*	Limousin, France	FJ546505	Margos et al. 2009
IPT158	*Ixodes ricinus*	Limousin, France	FJ546506	Margos et al. 2009
IPT168	*Ixodes ricinus*	Limousin, France	FJ546509	Margos et al. 2009
IPT169	*Ixodes ricinus*	Auvergne, France	FJ546510	Margos et al. 2009
IPT172	*Ixodes ricinus*	Auvergne, France	FJ546512	Margos et al. 2009
IPT178	*Ixodes ricinus*	Auvergne, France	FJ546513	Margos et al. 2009
IPT189	*Ixodes ricinus*	Normandy, France	FJ546514	Margos et al. 2009
IPT195	*Ixodes ricinus*	Normandy, France	FJ546543	Margos et al. 2009
Ki118	*Ixodes ricinus*	Sweden	HM173574	Unpublished
MDH3	-	China	DQ150537	Unpublished
PM72/2	*Ixodes ricinus* from *Parus major*[¶]	Central Europe	EU401779	Dubska et al. 2009
PM72/4	*Ixodes ricinus* from *Parus major*[¶]	Central Europe	EU401780	Dubska et al. 2009
PM72/5	*Ixodes ricinus* from *Parus major*[¶]	Central Europe	EU401781	Dubska et al. 2009
R63	Rodent (spleen)	China	EU160460	Unpublished
TP 65/1	*Ixodes ricinus* from *Turdus philomelos*[¶]	Central Europe	EU401777	Dubska et al. 2009
TP 66/1	*Ixodes ricinus* from *Turdus philomelos*[¶]	Central Europe	EU401778	Dubska et al. 2009
TP 394/1	*Ixodes ricinus* from *Turdus philomelos*[¶]	Central Europe	EU401776	Dubska et al. 2009
VL2	-	China	DQ188936	Unpublished
PBi	Human (CSF)	Ingolstadt, Germany	FJ546494	Margos et al. 2009
PFlk	Human (CSF)	Munich, Germany	FJ546495	Margos et al. 2009
PTrob	Human (skin)	Slovenia	FJ546496	Margos et al. 2009
PRab	Human (synovia)	Villach, Austria	FJ546497	Margos et al. 2009
POb	Human (skin)	Munich, Germany	FJ546498	Margos et al. 2009

***** In strains PoAnB2-PoAnB8 the ID ring number of each bird is presented.

[§]Data generously provided by Dr. Margarida Collares-Pereira. [¶]Data gently provided by Dr. L. Dubská.

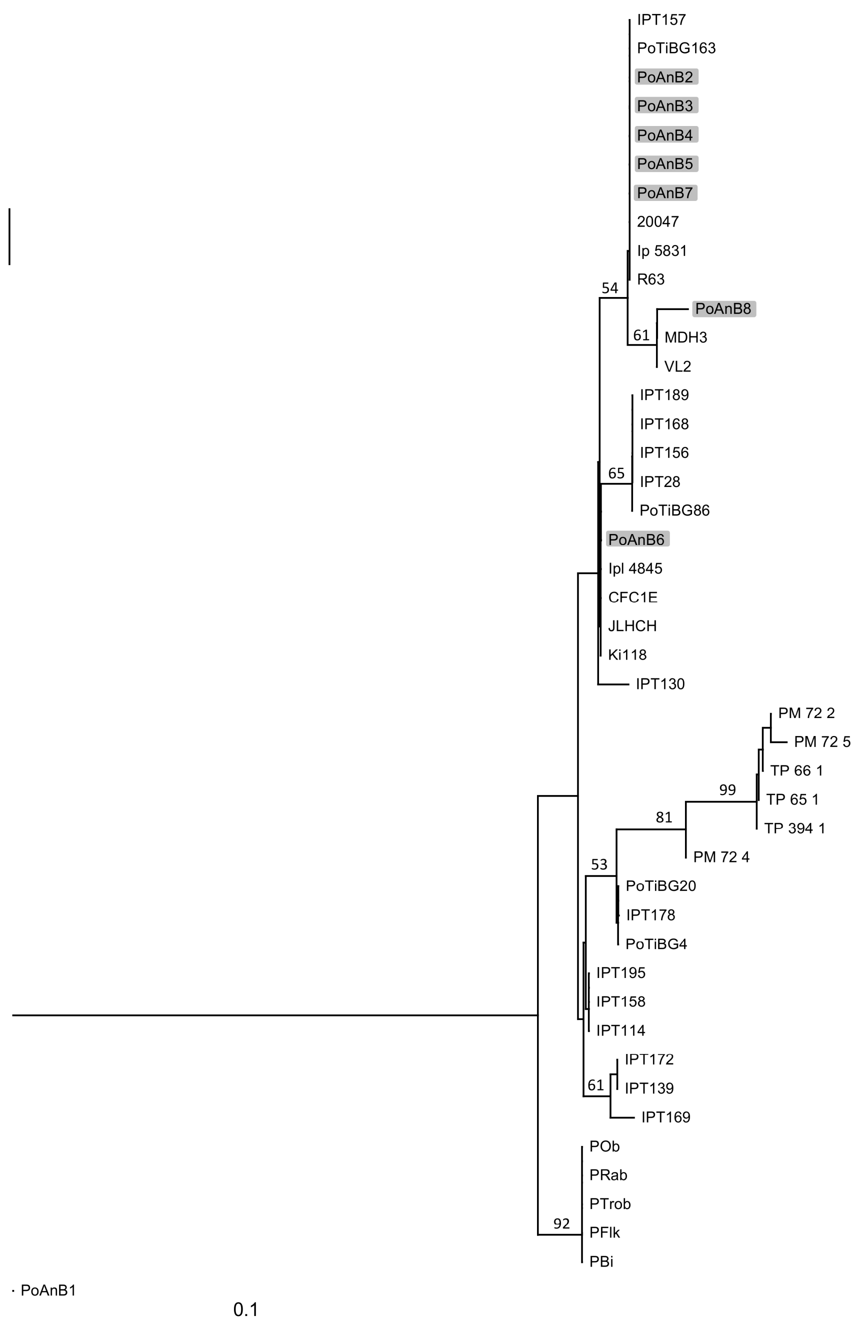

Figure 1: Neighbour-joining tree inferred from partial IGS sequences from the DNA detections obtained in this study (green boxes) compared to Genbank sequence data. Distance matrices were calculated using the Kimura 2-parameter model to correct for multiple substitutions. Bootstrap values were obtained from 1000 replicate trees and are indicated at the nodes (> 50%). *B. lusitaniae* PoAnB1 was used as outgroup.

Chapter X. *Borrelia burgdorferi* s.l. and other tick-borne agents in lizards (*Teira dugesii*) and their ticks on Madeira Island

In submission

Borrelia burgdorferi s.l. and other tick-borne agents in lizards (*Teira dugesii*) and their ticks on Madeira Island.

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Abstract

To investigate the role of the lizard *Teira dugesii* as a potential host for *Borrelia burgdorferi* sensu lato (s.l.) and two Rickettsial agents, *Anaplasma phagocytophilum* and *Rickettsia* spp., 151 lizards were caught from March 2009 to February of 2010 on Madeira Island, Portugal. From the total of tissue samples collected from lizard tails molecular-sequence-based methods have identified *B. lusitaniae* in seven (4.6%), *R. monacensis* in 10 (7.1%) and *A. phagocytophilum* in one (0.7%) lizards. No coinfections were detected in lizards. Culture of lizard tissues in BSK medium revealed seven (1.4%) positive isolations for *B. valaisiana*. To our knowledge this is the first time that this genospecies has been isolated from lizard tissues and rickettsial DNA were detected in the same type of specimens. One hundred lizards (66.2%) were infested by ticks and a total of 211 attached *I. ricinus* were removed. A mean infestation of 4.4 ticks (larvae and nymphs) per host was found. The prevalence of *B. burgdorferi* s.l., *Rickettsia* spp. and *A. phagocytophilum* in ticks detected by PCR was 11.8%, 45.1% and 8.6%, respectively. Two genospecies of *B. burgdorferi* s.l., *B. lusitaniae* and *B. valaisiana* and two *Rickettsia* species, *R. monacensis* and *R. helvetica* were identified in these ticks. Ten (5.7%) *I. ricinus* were co-infected with *B. lusitaniae* and *R. monacensis* and four (2.3%) with *A. phagocytophilum* and *R. monacensis*. These findings show the importance of *T. dugesii* and the *I. ricinus* that had fed on them, might have in the transmission cycle of *B. valaisiana* and *R. monacensis* on Madeira Island.

Keywords: Lizards, *Ixodes ricinus*, *Borrelia burgdorferi* s.l., *Rickettsia* spp., *Anaplasma phagocytophilum*, Portugal

Introduction

Lizards have been identified as vertebrate hosts of arthropod vectors such as ixodid ticks (Dantas-Torres et al. 2008). Ticks need to feed on one or more hosts to complete their life cycle and, in most cases, the immature stages usually prey on small animals such as rodents, birds, and reptiles. Upon pathogen transmission from infected ticks to the host during a blood meal, the latter may act as a competent reservoir by transmitting the pathogen to other ticks feeding on it. On the other hand, incompetent reservoirs are incapable of acquiring, maintaining or transmitting the pathogen to other ticks, or may even eliminate it (Ritcher and Matuschka, 2006). In Europe, *I. ricinus* ticks infest a wide variety of vertebrate hosts, such as mammals, birds and lizards.Most genospecies of *B. burdorferi* s.l. complex, responsible for LB have been shown to be incompetently transmitted by lizards, thereby reducing their ability to be maintained in an enzootic cycle (LoGiudice et al. 2008). It has been shown that the blood of lizards possesses borreliacidal properties which clear the bacteria from previously infected ticks, therefore acting zooprophylactic by reducing *Borrelia* prevalence in ticks (Lane et al. 1998, Ulmann et al. 2003). However, lizards were recently shown to be competently infect *I. ricinus* with *B. lusitaniae* both in the field and laboratory (Majlathova et al. 2006, Dsouli et al. 2006, Amore et al. 2007, Foldvari et al. 2009). Little is known about the role of lizards in the maintenance of other tick-borne agents such as *Rickettsia* spp. and *A. phagocytophilum*. The first report of a rickettsia in an arthropod ectoparasite associated with reptiles identified *Aponomma hydrosauri* as a reservoir of *R. honei*. Rickettsial DNA was detected in tick hemolymph, however none was found in lizard blood (Stenos et al. 2003). A few other studies have detected the presence of *Rickettsia* spp. in ticks from lizards although the authors did not mention the detection and the role of the lizards species in the maintenance of *Rickettsiae* (Reeves et al. 2006, Tijsse-Klassen et al. 2010). Recently, an experimental infection study performed on lizards using *A. phagocytophilum* suggested that lizards do not seem to act as reservoirs for this agent, demonstrated by the incapacity of tick larvae becoming infected after feeding on a PCR-positive lizard (Nieto et al. 2009). *T. dugesii*, a unique lizard species to Madeira archipelago (Madeira wall lizard) is endemic in Madeira Island, and has become one of the most prevalent vertebrate species (Matuschka et al. 1994). It occupies distinct habitats, ranging from rocky beaches to mountain tops, and favors an omnivorous diet, including fruit and insects. Studies have shown that *T. dugesii* is among the preferential hosts for *I. ricinus* and that this tick species is the most widely distributed tick species on Madeira Island (Matuschka et al. 1994).

Previous studies have detected the presence of *B. afzelii*, *B. burgdorferi* s.s., *B. garinii B. lusitaniae*, *B. valaisiana*, *R. monacensis* and *A. phagocytophilum* in *I. ricinus* collected in this island (Matuschka et al. 1998, Lopes de Carvalho et al. 2008). To date, only two clinical cases of Lyme borreliosis have been confirmed and so far no cases of human anaplasmosis or rickettsiosis have been identified in the island (Lopes de Carvalho et al. 2006). Given the abundance of both *T. dugesii* lizards and *I. ricinus* ticks in Madeira Island, the main aim of this study was to find out whether this lizard species can participate in the maintenance and transmission cycles of *Borrelia*, *Rickettsia* and *A. phagocytopiylum*, in natural foci and if an association between those *Ixodes*-borne agents exists.

Material and Methods

Study area

Field sampling was conducted monthly from March 2009 to February 2010 at the Calheta (32º 44′ 17N, 017º 10’ 29 O) and Campanário (32º 40' 00 N, 017º 01' 29 O) areas, on the Southwestern part of Madeira island, Portugal. Calheta and Campanário are semi-rural areas characterized by sharp cliffs and a grassland/farmland patchwork; they have a warm and humid temperate climate with moderate winters and a dry season during the summer months with an average temperature of 20ºC, ranging from 11ºC January to 31ºC in August and an average humidity of 68%. The average rainfall is 0.09 mm/year.

Lizard and tick collection

Lizards were captured along hiking and wall paths in which four bucket, filled with mashed banana and apple slices, were placed for one hour during early afternoon. Animals were characterized by species, sex and age (juvenile, adult) and examined for ticks. Before animals were released in the areas where they were captured, ticks were counted, removed with forceps and placed in plastic tubes with appropriate moisture to be brought alive to the laboratory for species and stage identification. Ticks were then frozen at -80ºC until being processed. A tissue specimen (2-cm of distal part of the tail) was also taken from each lizard. The distal part of the tail was removed via tail fracture (a natural escape mechanism in lizards) and cut longitudinally, half of the tail placed in a tube containing ethanol 70% and the other part kept in a tube at -80ºC without additives.

DNA extraction and polymerase chain reaction (PCR)

Tissue (25 mg) DNA from lizard's tail was extracted by using the DNeasy Tissue Kit, according to the manufacturer's instructions (Qiagen GmbH, Germany). Ticks were washed in 70% ethanol and sterile distilled water, dried on sterile paper and total DNA was extracted by alkaline lysis as described previously by Schouls et al. (1999). To monitor the occurrence of cross-contamination during DNA extractions, negative controls only composed of kit reagents were included (one control) per each extraction run of 15 tick and lizard samples. For *B. burgdorferi* s.l. detection was performed by a nested PCR using the intergenic spacer region *5S (rrf)-23S (rrl)*, primer pairs 23SN1/23SC1 and 5SCB/23SN2 (Rijpkema et al. 1995). The presence of rickettsial DNA in lizards and ticks was screened using specific primers targeting the gene for citrate synthase (*gltA)* and outer membrane protein B (*omp*B) as described previously (De Sousa et al. 2006, Choi et al. 2005). A simple PCR was used for testing tick's DNA using Rp Cs 415/ Rp CS1220 (gltA) and OR/OF (*ompB*) primers pairs and a nested PCR for lizard's DNA using for the first reaction the same primers used for ticks and in the second reaction the primer pairs RpCs.877p/RpCs.1258n and IF/IR as previously described (De Sousa et al. 2006, Choi et al. 2005). For *A. phagocytophilum* DNA amplification two primer sets were used in a nested reaction, HS1/HS6 and HS43/HS45 targeting heat-schock operon (*groESL*) and GE9f/GE10r GE2a/GE3a for 16S rDNA (*rrs*) (Sumner et al. 1997, Massung et al. 1998). Negative controls were included in all PCR reactions. To reduce the possibility of contamination of the PCR assays, DNA extractions, PCR set up master mix, PCR samples addition first reaction, nested PCR, and gel electrophoresis were performed in a separate rooms. Moreover all PCR set up were performed in a containment hoods sterilized by UV and clean regularly with bleach in each room.

DNA sequencing

Positive amplicons were purified using Jetquick PCR Purification Kit (Genomed GmbH, Germany) and sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 377 DNA sequencer, according to the manufacturer's recommendations. The sequencing reactions were performed with the forward and reverse primers used for the PCR amplifications. The sequences were assembled by combining the sequences generated by each primer, using the Lasergene software (DNASTAR). Sequence homology searches were performed using BLASTn algorithm in the NCBI database.

Isolation attempts of B. burgdorferi s.l.

Isolations were performed from lizard's frozen tissues or specimens preserved in 70% ethanol. The tissues were minced and placed directly into 8 ml of Barbour-Stoenner-Kelly (BSK) II medium (Sigma) with the following additions: 0.023% L-cysteine hydrochloride (Sigma), 0.015% DL- dithiothreitol (Sigma), 1 µg of L- glutamine per ml (GIBCO), 7% of gelatine (DIFCO), 50 µg rifampicin (Sigma) per ml, 25 µg of phosphomicyn (Sigma) per ml and 2.5 µg of amphotericin B fungizone (GIBCO) per ml (Sinsky and Piesman, 1989). Cultures were maintained at 34ºC for 3 months and examined weekly by dark-field microscopy to monitor the presence of spirochetes. In suspected cultures, 1 ml of each culture was centrifuged for 15 min at 12.000g, and DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen Hilden, Germany) according to the manufacturer's recommendations. Characterization of *Borrelia* isolates was performed by PCR and sequencing.

Results

Prevalence of infection in lizards

A total of 151 lizards belonging to the species *T. dugesii* including 50 females, 33 males and 68 juveniles were captured in Calheta and Campanário areas of Madeira Island. Of the total lizard tissue samples analyzed by PCR seven were positive for *B. lusitaniae* (4.6%), 10 for *R. monancensis* (7.1%) and one for *A. phagocytophilum* (0.7%), but no co-infection were detected. The 50 tails biopsies used for isolation attempts of *Borrelia* spp. resulted in seven isolates, which were identified as *B. valaisiana*.

Prevalence of infection in Ticks

One hundred lizards (66.2%), from Calheta, were parasitized by ticks and a total of 211 *I. ricinus* (65 larvae and 146 nymphs) were removed and identified. Nymphs were detected in all months studied, but larvae appear from early April to November (Fig.1). Ticks were mostly found in the forelimbs axial region. No other tick species were detected. An average /infestation index of 4.4 ticks per animal was determined and no statistical differences were found between sex and age in the group of parasitized lizards. The prevalence of *B. burgdorferi* s.l., *Rickettsia* spp. and *A. phagocytophilum* in lizard ticks, detected by PCR was 11.8%, 45.1% and 8.6% respectively (Table 1). Two genospecies of *Borrelia* spp. were detected, *B. lusitaniae*

(95%) and *B. valaisiana* (5%) and two *Rickettsia* species, *R. monacensis* (97.5%) and *R. helvetica* (2.5%). Ten (5.7%) *I. ricinus* were co-infected with *B. lusitaniae* and *R. monacensis* and four (2.3%) with *A. phagocytophilum* and *R. monacensis.* The overall prevalence of detected agents was higher in nymphs than in larvae. *B. lusitaniae* and *R. monacensis* infections were found in both tick stages however *A. phagocytophilum* was found only infecting nymphs (Table 1). In lizards captured in Campanário no attached ticks were found.

Genetic identities of ticks and lizards associated bacteria

Positive amplicons detected in ticks and in lizards tissue were sequenced and analyzed. Regarding *B. lusitaniae,* two genetic variants of IGS partial sequence were detected in tick samples. Variant 1 has 100% sequence identity to IGS sequence previously detected in Madeira island (PoTiB6 strain - GenBank accession no. EU078961) and variant 2 presents two polymorphisms, the deletion of one nucleotide (A) and one transversion (T→G) and shows >99% sequence identity with TT928 strain (GenBank accession no. AY575767). The lizard's isolates show > 99% sequence identity with *B. valaisiana* TN7 strain (GenBank accession no. DQ860262). Co-infection was confirmed for *B. valaisiana* in one lizard isolate and for *B. lusitaniae* in one tick sample. Concerning the tick sample 2644, the co-infection regards both detected IGS genetic variants of *B. lusitaniae*. IGS sequences from lizard isolates suggest the co-existence of several genetic variants of *B. valaisiana* since two polymorphisms were detected, one transition (C↔T) and one position presenting all four nucleotides. Sequence analyses of *Rickettsia* positive amplicons showed that two (2.5%) tick samples have 100% sequence identity with *gltA* of *R. helvetica* (GenBank accession number HM149280). Seventy-six fragments sequenced from ticks and ten from lizards tissues were 100% identical with *gltA* and *omp*B of *R. monacensis* (GenBank accession numbers HM149283 and EU078962), previously detected in Portuguese ticks (Lopes de Carvalho et al. 2008, Milhano et al. *in press*). Sequence analysis of *A. phagocytophilum* positive amplicons showed 100% sequence identity when compared to representatives *rrs* sequences of *A. phagocytophilum* strains and to previously described *rrs* sequences from Portuguese ticks (GenBank accession numbers EU098006 and EU098007) (Santos et al. 2009). However, when the 442 bp sequence of *groESL* were analyzed two main polymorphic positions were detected in the encoding sequence enabling the identification of three genetic variants, one 100% identical to North American strain isolated from humans (GenBank accession numbers U96728) and two other differing by 1-2 nucleotides. The sequences differing in one nucleotide were 100% identical to a previously

described *A. phagocytophilum groESL* partial sequence detected in *I. ricinus* from Madeira Island (GenBank accession numbers EU004826) (Santos et al. 2009).

Discussion

To our knowledge this is the first report of *B. valaisiana* isolation from lizard's tissues and detection on parasitizing ticks. In this study it was also possible to detect *B. lusitaniae* in lizards' tissues suggesting the importance of some lizards species in the maintenance of *B. lusitaniae* as previous reported by other authors (Dsouli et al. 2006, Majláthová et al. 2006, Amore et al. 2007). Studies conducted in Germany showed that immature ticks acquired *B. lusitaniae* through feeding in common wall lizards and sand lizards (Richter and Matuschka, 2006). In Tunisia, the reservoir competence of lizard (*Psammodromus algirus*) was demonstrated by xenodiagnosis and in Slovakia and Italy were also demonstrated that lizards play an important role as reservoir of this genospecies (Dsouli et al. 2006, Majláthová et al. 2006, Amore et al. 2007). *B. lusitaniae* has been also isolated from other reservoirs such as *A. sylvaticus* and from ticks of migratory birds (Poupon et al. 2006, Lopes de Carvalho et al. 2009). Moreover, these findings demonstrated that the previous associations of *Borrelia* genospecies for specific reservoirs hosts are not so restricted, at least concerning *B. lusitaniae*. As previously suggested, birds may be responsible for the geographic diffusion of *B. lusitaniae* and rodents and lizards could be responsible for perpetuating this genospecies at enzootic cycles where they are the major vertebrate hosts for *I. ricinus* (Richter and Matuschka, 2006, Lopes de Carvalho et al. 2009). The present work reinforces this hypothesis proving that both lizards and attached ticks were infected with *B. lusitaniae*. Sequence analysis was difficult, since most sequence data reveal the presence of more than one *Borrelia* genospecies. However, the presence of *B. lusitaniae* was clearly confirmed by PCR directly on lizard tails and ticks. Moreover in cultures inoculated with lizard tails co-infection is also suspected from sequence data but the presence of *B. valaisiana* is unequivocal. In one isolates the *OspA* gene was also amplified and the sequence analysis show 100% sequence identity with *B. lusitaniae* confirming co-infection (unpublished data). Interestingly, *B. valaisiana* was the main sequence detected in lizard tail-snips cultures but no molecular detection was achieved by direct PCR. A possible explanation for this finding relay in the fact that the number of borrelia is usually low in the vertebrate host, and thus the detection of DNA in skin biopsy specimens may not always be possible (Kurtenbach et al. 2002). However, more studies are essential since other factors might be involved limiting the isolation and/or detection. The presence of larvae infected with

B. lusitaniae feeding either in negative lizards or in *B. valaisiana* infected lizard (one case) can be explained by the cofeeding. Despite transestadial transmission is not considered relevant in *Borrelia* infection, lizards could be have an important role as reservoir of tick-borne pathogens once attached semi-engorged and engorged larvae infected with *Borrelia* were detected. Another important finding in this study is the presence of polymorphisms in some sequences that reveal the presence of genetic variants of *B. lusitaniae* and *B. valaisiana*. Since the sequences detected in mainland Portugal show differences on the variability, these findings suggest the presence of higher mutation rate on Madeira Island. Further studies to access the eventual evolution forces driven this genetic diversity and the eventual presence of *B. lusitaniae* and *B. valaisiana* subpopulations associated to specific reservoirs or the isolation of the island should be performed. Parallel studies are ongoing in questing ticks and to date it was already detected several *Borrelia* genospecies, *B. afzelii, B. garinii, B. lusitaniae* and *B. valaisiana,* as showed in other study conducted on Madeira Island (Matuschka et al. 1998). However, *B. lusitaniae* is the most prevalent as was showed in other study conducted for the authors (Lopes de Carvalho et al. 2008). The fact that the other *Borrelia* species were not detected in ticks or tissues from lizards could be suggest that these genospecies could be negatively selected against by *T. dugessi* and they may subsist in different biological systems, involving other vertebrate species as reservoirs. Concerning *Rickettsia* spp. this is the first description of *R. monacensis* in lizard tissues, which suggests not only that disseminated infections can occur in *T. dugesii* but also that this species may act as a reservoir or transitory reservoir, infecting ticks. Although, the viability of *R. monacensis* in the positive lizards were not confirmed; the recovery of bacterial DNA in a tail snip suggests that the infection were disseminated. Moreover, we believe that disseminated infections can occur since all the ticks collected from lizards were localized at the forelimbs axial area but sample tissue for PCR detection was removed from the tail. In this habitat *T. dugesii* seems to be a relevant host for *I. ricinus* to maintain the tick population, and might be a potential reservoir of *R. monacensis* on Madeira Island. Regarding *A. phagocytophilum*, the data obtained thus far suggest that Madeira Island lizards are exposed to infected ticks but they do not seem to be a primary reservoir hosts for this agent. In fact the sole *T. dugesi* positive tail-snip detected contrasts with the number of species studied, and with the much higher number of positive parasitizing nymphs found in the same area. Since *A. phagocytophilum* is not transovarial transmitted, the lack of *A. phagocytophilum* infection in all attached larvae suggest that lizards were not infectious for ticks and raises the hypothesis that positive nymphs could have resulted from previous infections as larvae, when feeding on other reservoir host. PCR analysis of available blood-prints is still ongoing to confirm the results of tail-snips screening. *A. phagocytophilum*

infection are generally not localized, affecting circulating peripheral neutrophils and although the tail snip fraction used for DNA extraction were from a highly vascularised region, its usefulness may be questionable and could have limited the detection of infected lizards. Although this was not a limitation for *Rickettsia* spp. detection, these agents present a tissue-tropism more marked than *A. phagocytophilum*. Further studies are essential to prove if *B. lusitaniae* and *B. valaisiana* can co-infect the same host or if the presence of one will influence the fitness of the other. Considering the difficulties inherent to these analyses, further studies on these findings are needed to clarify these preliminary results. Moreover, future experimental studies involving *R. monacensis* maintenance in lizards and its transmission to feeding ticks need to be addressed to evaluate if lizards are transitory reservoirs and how long they can sustained the infection. This study proves that a relationship exists at least between two tick-borne agents with lizard hosts, and preventives measures to reduce the infection on ticks have to include also some control of the lizard population.

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Table 1. Prevalence infection of *Borrelia burgdorferi* s.l., *Anaplasma* spp., and *Rickettsia* spp. in tick's lizards, detected by PCR.

Ticks		**No. infected ticks (%)**				
		B. burgdorferi s.l.		*Anaplasma* sp.	*Rickettsia* spp.	
Stage	No. examined	*B. lusitaniae*	*B.valaisiana*	*A. phagocytophilum*	*R.monacensis*	*R. helvetica*
Larvae	65	3 (4.6%)	0	0	19 (29.2%)	0
Nymph	146	21(14.4%)	1	15 (10.3%)	57(39%)	2 (1.4%)
		24	1		76	2
Total	211	25 (11.8%)		15 (8.6%)	78 (45.1%)	

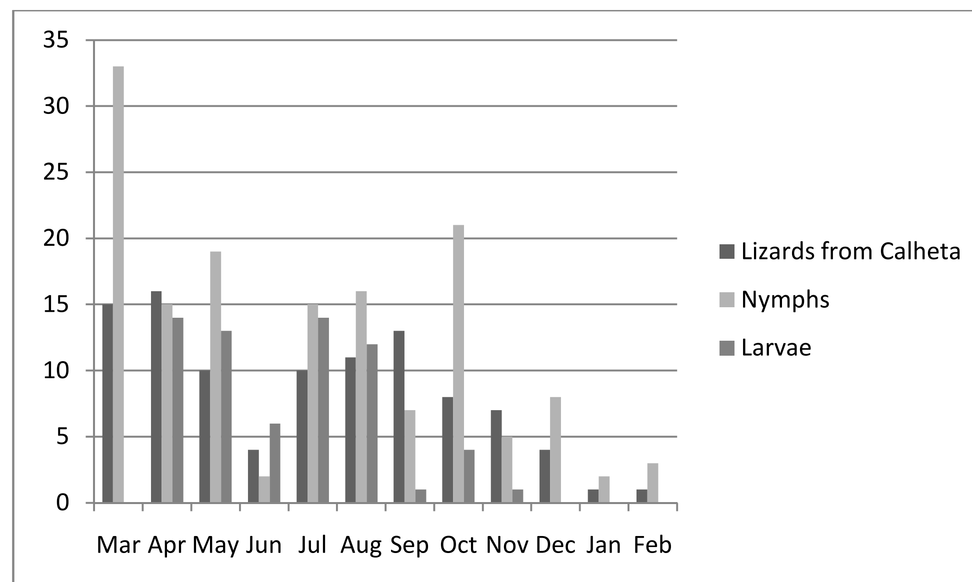

Figure 1. Number of lizards and their ticks (nymphs and larvae) collected in Calheta area.

Part IV – General Discussion and Concluding Remarks

Chapter XI. General Discussion

Lyme borreliosis is considered an emerging disease in some regions of the world, in particular Portugal. The first reported human case of LB in Portugal was in 1989 (David de Morais et al. 1989). Reference laboratory diagnosis is performed at the Centre for Vectors and Infectious Diseases Research (CEVDI), National Institute of Health. In 1999, LB became a mandatorily notifiable disease in Portugal, but only a few cases are currently reported each year, which does not allow consistent analysis of risk factors and the consequent assessment of the impact on public health.

The state of the art of LB in Portugal (**Chapter I**) confirm that several issues concerning LB and *B. burgdorferi* s.l. infection are still unknown or controversial, namely what concerns human infection and ecology of *B. lusitaniae*. To contribute to the clarification, several goals were established (**Chapter II**).

An epidemiological study to reveal the LB incidence in humans was performed. The evaluation of CEVDI data concerning human cases of LB and the number of notified cases during the 1990-2004 period was analysed in **Chapter III"Laboratory diagnosis of Lyme borreliosis at the Portuguese National Institute of Health (1990-2004)".** This study showed that LB, like other vector-borne diseases, such as boutonneuse fever (the most prevalent tick-borne disease in Portugal), is clearly underreported in our country (Sousa et al. 2003). Many experts admit that there is a significant underreporting of LB incidence rate that may be 2-3 times higher than reported (Hubalek, 2009). Between 1999-2004, we detected an average of 35 new cases of LB per year. The major problem of underreporting is the impossibility of conducting an epidemiological analysis of LB in Portugal. The districts that showing higher number of confirmed cases are Lisbon, Setubal and Évora, districts located in central and southern Portugal. It is possible that the results have been influenced by the proximity of the CEVDI's facilities to these regions. Nevertheless, the number of positive cases detected is undoubtedly higher than the number of cases reported. In most of the European countries it is possible to make only approximate estimates of LB incidence (Stanek et al. 2010). In this study it is possible to estimate an annual incidence of 0.04 per 100 000 inhabitants which is among the lowest reported incidence of LB in Europe. Although, a higher estimated rate of 0.4 per 100 000 inhabitants can be obtained if we take laboratory data into consideration.

Additional problems in LB notification are caused by different definitions of LB diagnosis and absence of laboratory confirmations. Due to the diversity of the possible clinical presentations that may be confused with other aetiologies, the benign course of the majority of clinical cases, and the usually very positive response to the timely application of antibiotics, a large

percentage of cases are never sent to the laboratory to confirm a clinical diagnosis. In this study, the positive cases which mention EM are very rare, probably because many clinicians are aware that this stage frequently does not evoke an antibody response and that laboratory confirmation cannot be expected, and therefore do not request a laboratory confirmation of their clinical diagnosis. Twenty years after the first human case of LB in Portugal the eco-epidemiology and the study of the impact and risk factors are still poorly understood. However, this study contributes for a more precise evaluation of the epidemiological situation of LB in Portugal.

To clarify the pathogenic effect of *B. lusitaniae* in humans several studies were undertaken. The main results are presented in **Chapters IV and V**.

Presently, the most common agents of human LB are *B. afzelii*, *B. burgdorferi* s.s. *B. garinii*, *B. lusitaniae* and *B. spielmanii* (Margos et al. 2009). In Portugal, the first strains of the complex *B. burgdorferi* s. l. were isolated from ticks captured in the south of Portugal and the molecular studies showed that they belong to a new species, *B. lusitaniae* (Núncio et al. 1993, Le fleche et al. 1997). Later, it became known that this genospecies causes disease in a C3H/HeN experimental mouse model, suggesting that some strains of this species could also be associated with human LB (Zeidner et al. 2001, Zeidner et al. 2002). The first human isolate of *B. lusitaniae* was published in 2004, from a Portuguese patient with a chronic skin lesion (Da Franca et al. 2005). The **Chapter IV "Vasculitis like syndrome associated with *Borrelia lusitaniae* infection"** reports the isolation of *B. lusitaniae* from a child presenting a vasculitis syndrome, representing the second isolation of this genospecies from a human patient.

Like other spirochete infections, borrelia infection may manifest a wide range of clinical manifestations, dermatological, neurological, cardiac, and musculoskeletal disorders (Aguero-Rosenfeld et al. 2005, Stanek et al. 2010). The clinical presentation was not very typical of LB; this case had features suggestive of vasculitis, which has been described as one of the characteristic psychopathological aspects of this disease (Moody et al. 1990). Moreover, acute ischemia of the digits had previously been demonstrated in the context of *Borrelia* infection (Bär et al. 2000). On the other hand, this patient had intermittent symptoms, which have also been pointed out as one of the possible characteristics of rheumatic symptoms associated with this condition (Sibilia et al. 2002). This clinical report pointed out the hypothesis that the clinical manifestations associated with *B. lusitaniae* infection are quite non-specific and may overlap with multiple clinical syndromes, which underlines the necessity to add laboratory diagnosis to clinical findings (Collares-Pereira et al. 2004).

The fact that specific antibodies are present in low titre may provide false negative laboratorial results. In the first human case of *B. lusitaniae* infection, serology was negative (Da Franca et al. 2005), while in the case presented here antibody titres were borderline positive. Detection of antibodies to *B. burgdorferi* s.l. by ELISA and immunoblot is currently the primary tool for laboratory diagnosis of LB in humans (Wang et al. 2010). However, in this case, both IFA and Immunoblot tests demonstrated more sensitivity than the ELISA. This may be due to different antigens used by each test, underscoring the importance of using multiple analyses when performing LB serology. Culture of spirochetes from patient samples is still the gold standard for laboratory diagnosis of LB (EUCALB) and was accomplished in this case. Interestingly, both human cases of *B. lusitaniae* already described were from patients residing near Lisbon, the first at Oeiras that is one of the administrative divisions of Lisbon district where *Ixodes* spp. are known to occur. The higher incidence in this district is also described in Chapter III and in other studies as having the highest number of laboratory confirmed cases of LB in Portugal (Da Franca et al. 2005, Baptista 2006). Furthermore, Lisbon district is mentioned in another study conducted as the residence place of at least three patients with LB clinical confirmed (Da Franca 2004).

This study confirmed that *B. lusitaniae* is pathogenic for humans, however, little is known regarding the progression of the disease associated with this genospecies.

As presented in **Chapter V** "**Clinical and laboratory data of Portuguese patients with diagnostic of Lyme borreliosis",** to achieve the complete characterization of the clinical picture of the disease caused by *B. lusitaniae*, twelve Portuguese patients with indicative serology and clinical diagnosis of LB were analyzed. Considering the major clinical manifestations, in this study fever prevailed among clinical symptoms, followed by dermatological lesions, neurological symptoms, and musculoskeletal manifestations (including arthritis) and at last cardiac signs. In Europe and in North America LB are similar in their main clinical features but the greater variety of genospecies in Europe leads to important differences in clinical presentation (Stanek et al. 2010). This study provides the first detection of *B. afzelii* DNA in a blood sample from a human patient. This patient presented fever, prostration and neurological injuries with mental confusion, although this genospecies was mostly associated with EM (Stanek and Strle, 2003). The circulation of more than one borrelia species may increase the complexity of the clinical picture and in many cases laboratory findings are very useful in order to confirm the clinical suspicion of LB. Neuroborreliosis appears to occur in a higher proportion of patients in Europe, and Lyme arthritis seems to be a more frequent manifestation in USA (Strle and Stanek, 2009). In the first study (**Chapter III**) neurological symptoms were the most detected. However, in this study, dermatological symptoms were the most frequent after the

reference to fever. These differences may be explained by the fact that in first study all the patients sent to the CEVDI were included, namely samples for differential diagnosis. Moreover, the majority of the samples were from the neurological hospital departments, so the origin of the samples could be the reason for these differences. These observations enhanced the necessity to improve the connection between laboratory experts and physicians, in order to achieve the correct diagnosis. On the other hand, in the present study, the patients analysed had already a clinical diagnosis of LB. This study also corroborated previous reports (**Chapter III and IV**) once the patients analysed here are mostly from central and southern regions.

All these studies indicate that an efficient diagnosis of LB has to be based on clinical and laboratory findings and the laboratory diagnosis should include whenever it is possible and necessary, a combination of various approaches such as serology, culture and DNA detection.

Concerning the eco-epidemiology of *B. lusitaniae*, the tick vector and the most probable reservoirs were studied (**Chapter VI-X**).

The first strains of *B. lusitaniae* were isolated from *I. ricinus* captured in the south of Portugal (Núncio et al. 1993, Le Fléche et al. 1997). Subsequent studies confirm the presence of several *B. burgdorferi* s.l. species (*B. lusitaniae*, *B. afzelii*, *B. garinii* and *B. valaisiana*) in ticks. *I. ricinus* has a restricted distribution occurring in the coastal region increasing from South to the North (Baptista, 2006).

To study the vector ticks involved in *B. burgdorferi* s.l. enzootic cycles transmission and the possibility of co-infection with other tick-borne agents, a higher number of *I. ricinus* and other tick species were collected by flagging vegetation in two main areas, Madeira Island and the southern region of mainland. Studies on the frequency of co-infection of ticks with several pathogens vary depending on tick species and regions. However, it is important to evaluate the existence of co-infections as it may facilitate the prognosis of simultaneous infections in humans.

In **Chapter VI "Detection of *Borrelia lusitaniae*, *Rickettsia* sp. IRS3, *Rickettsia monacensis* and *Anaplasma phagocytophilum* in *Ixodes ricinus* collected in Madeira Island, Portugal"**, *I. ricinus* were study for the presence of *B. burgdorferi* s.l. and also determined co-infection rates with *A. phagocytophilum* and *Rickettsia* spp. in Madeira Island. The first evidence of *B. burgdorferi* s.l. in Madeira Island was reported by Matuschka et al. 1998 and later by Núncio and coworkers (2001) that have detected *B. afzelli, B. valaisiana, B. garinii* and *B. burgdorferi* s.s. and *B. lusitaniae* in *I. ricinus* collected from vegetation. In this study the prevalence of *Borrelia* spp. found (2.7%), was lower when compared to the 31.2% rate demonstrated in a

previous study in Madeira (Núncio et al. 2001). However, it is similar to the prevalence found in the other study performed there (Matuschka et al. 1998). This might be due to a difference in the sensitivity of assays used in both studies.

Interestingly was the detection of *Rickettsia* sp. IRS3 and *R. monacensis,* which was recently associated to human cases, in *I. ricinus* ticks and their co-infection with *B. lusitaniae*, suggesting that simultaneous transmission of multiple tick-borne agents may occur among the population of the Island (Jado et al. 2007). *A. phagocytophilum* has also been detected in 4% of actively questing *I. ricinus* collected from vegetation in this island; however in this study lower percentage (0.3%) of infection was detected (Santos et al. 2004). These differences could be the result of the *Borrelia* infection, or the difference in the detection methods used. Previous studies based on serological data have suggested dual infections in LB patients with *A. phagocytophilum* (Santos et al. 2006). Although, only two human LB cases confirmed by laboratory were reported on Madeira Island probably due to a different clinical picture of this infections resulting in misdiagnosis. Moreover, in the study conducted in the southern region – **Chapter VII "Co-infections of *Rickettsia slovaca and R. helvetica* with *B. lusitaniae* in ticks collected in a Safari Park, Portugal"** three different species of ticks, *I. ricinus*, *D. marginatus* and *H. marginatum* were tested for the presence of *B. burgdorferi* s.l. and *Rickettsia* spp. In other studies conducted in Portugal, *I. ricinus* and *H. marginatum* ticks were found to be infected with *B. lusitaniae, B. afzelli, B. valaisiana, B. garinii* and *B. burgdorferi* s.s. (De Michelis et al. 2000, Kurtenbach et al. 2002). In this study, spirochete DNA of *B. lusitaniae* was detected with a prevalence of infection in *D. marginatus* similar to that found in *I. ricinus* ticks, the main vector of *B. burgdorferi* s.l.. These results raised the hypothesis that *B. lusitaniae* can survive in *D. marginatum* perhaps more than the other LB spirochete genospecies can, but the question of whether these ticks can actually transmit these spirochetes is not known and transmission studies has to be performed to clarify this fact. Some studies performed by another authors showed that *Dermacentor* ticks like *D. variabilis* can acquired the spirochetes, but not actually transmit the infection (Piesman and Happ, 1997, Johns et al. 2001, Soares et al. 2006). In this situation they should be called "carrier" species. Furthermore, six isolates of *B. lusitaniae,* from *I. ricinus,* were achieved and the molecular analysis revealed that could be recombinant strains, as was recently described for another *B. lusitaniae* strain found to have an intersection in the allelic profiles of the *B. lusitaniae* populations from two regions in Portugal (Vitorino et al. 2008). In this study it was also detected dual infections of *R. helvetica* and *R. slovaca*, each of which infected with *B. lusitaniae,* reinforcing the existence of co-infection of the two most important tick-borne agents in Portugal. All these findings raise a few questions concerning

whether these agents' life cycle is altered by their coexistence in the same vector and if the co-infection provoke a less typical LB clinical presentation or disease severity.

Several studies on the relation between *Borrelia* and their vertebrate host were performed. Results are presented in **Chapter VIII, IX and X.**

Several animal species, that can vary from place to place, are implicated and are susceptible to serve as reservoir hosts for *B*. *burgdorferi* s.l. Although, the reservoir of *B. lusitaniae* in nature had yet to be identify. Thus, to address this question, several animal populations that could serve as reservoirs (rodents, birds and lizards) were screened for active infections. To maximize resources, some biological specimens were obtained from ongoing projects at CEVDI.

Three rodent species (*A. sylvaticus*, *M. spretus* and *R. rattus*) were captured in Vale Guadiana, Peneda-Gerês and Arrábida, areas where *Ixodes* ticks are known to be present (McDonald and Barret, 1999, Santos-Silva personnel communication). One *A. sylvaticus* mouse captured in Vale Guadiana National Park demonstrated viable spirochetes that was identified as *B. lusitaniae* as presented in **Chapter VIII "Molecular characterization of a new isolate of *Borrelia lusitaniae* derived from *Apodemus sylvaticus* in Portugal"***;* however no seropositive samples were obtained from this species**.** This fact can be explained by the fact that this genospecies induced low immunoreactions (Zeidner et al. 2001, Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008). Antibodies against *B. lusitaniae* were detected in *M. spretus* and *R. rattus,* although no positive cultures were obtained from this species. In a previous unpublished study performed in Grandola region, *B. lusitaniae* DNA was detected by PCR in *M. spretus*, *A. sylvaticus*, *R. norvegicus* and *C. russula*; but no isolates were achieved (Baptista, 2006). The phylogenetic analyses based in *ospA* gene grouped the new isolate near other *B. lusitaniae* isolates from Portugal and most closely aligned to the North African clade (as showed in **Chapter IV**) (Grego et al. 2007). To complement the ecological study of *B. burgdorferi* s.l. and gain a more detailed assessment of pathogenicity of borrelia strains circulating in Portugal parallel studies in birds and lizards were conducted (**Chapter IX, X**). Concerning the possible infections in birds, samples from seven species of migratory shorebirds were obtained during an ongoing project at CEVDI with other institutional partners. These shorebirds species use the Portuguese wetlands either to refuel in their way to the breeding grounds during northward migration, to the wintering areas during the southward migration, or simply as a wintering area. As presented in **Chapter IX** "***Borrelia garinii* and *Francisella tularensis* subsp*. holarctica* detected in migratory shorebirds in Portugal"** *B. garinii* was detected, for the first time, in seven specimens of birds (9%) from three different

genus (black-tailed godwits *Limosa l. limosa,* Icelandic black-tailed godwit *Limosa l. islandica* and little stints *Calidris minuta*). Moreover, it was also detected one little stint infected with *F. tularensis* subsp. h*olarctica.* This bacteria was recently detected in human and tick samples in Portugal (Lopes de Carvalho et al. 2007).

The association between birds and *B. garinii* is well documented (Hanincova et al, 2003; Poupon et al, 2006). This genospecies have been found in a marine enzootic infection cycle worldwide and also among terrestrial animals which primarily involve birds, including seabirds and migratory passerines (Hanincova et al. 2003, Comsted et al. 2006, Taragel'ová et al. 2008, Duneau et al. 2008, Dubska et al. 2009). However, *B. garinii* OspA serotype 4 strains in Europe have been shown to infect rodents and, therefore, constitute a distinct ecotype within *B. garinii* (Huegli et al. 2002, Margos et al. 2009). This study is in accordance, describing the distinct clade formed by PBi strain and other OspA serotype 4 strains.

The findings obtained suggesting that migratory shorebirds could be also an important reservoir for *B. garinii* and confirmed the presence of *F. tularensis* DNA in these species of shorebirds. This is the first evidence that shorebirds that migrate through or winter in Portugal, may contribute to the dispersion of *Borrelia* and *Francisella* in this southwest European country.

To investigate the role of lizards as reservoir hosts of *B. burgdorferi* s.l. a study was conducted on Madeira Island, where the *Ixodes* ticks and *T. dugessi* are abundant as showed in **Chapter VI.**

Most genospecies of *B. burdorferi* s.l. complex have been shown to be incompetently transmitted by lizards, and it has been demonstrated that the blood of lizards possesses borreliacidal properties which clear the bacteria from previously infected ticks, therefore acting as a zooprophylactic host by reducing *Borrelia* prevalence in ticks (Lane et al. 1998, Ullmann et al. 2003). However, lizards were recently shown to be competently infecting *I. ricinus* with *B. lusitaniae* both in the field and laboratory (Dsouli et al. 2006, Majlathova et al. 2006, Amore et al. 2007, Foldvari et al. 2009). As demonstrated in **Chapter X "*Borrelia burgdorferi* s.l. and other tick-borne agents in lizards (*Teira dugesii*) and their ticks in Madeira Island"** the importance of lizards in the maintenance of *B. burgdorferi* s.l. was confirmed. *B. valaisiana* isolation from lizard tissues and detection in parasitizing ticks was reported for the first time. However, the presence of *B. lusitaniae* was clearly confirmed by PCR directly on lizard tails and ticks. Moreover in lizard tails cultures co-infection is also suspected from sequence data. In one isolate the *OspA* gene was also amplified and the sequences analysis show 100% sequence identity with *B. lusitaniae* (unpublished data).

Although, these data represents a preliminary results that have to be clarified, since most sequence data reveal the presence of more than one *Borrelia* genospecies.

Curiously, *B. valaisiana* was isolated from lizard tissue but no molecular detection was achieved by PCR. A possible explanation for this finding is the fact that the number of borrelia is usually low in the vertebrate host, and thus the detection of DNA in skin biopsy specimens may not always be possible (Kurtenbach et al. 2002).

In this study it was also possible to detect *B. lusitaniae* in lizard tissues suggesting the importance of some lizards species in the maintenance of *B. lusitaniae* as previous reported by other authors (Dsouli et al. 2006, Majláthová et al. 2006, Amore et al. 2007). These findings shows that the previous associations of *Borrelia* genospecies for specific reservoirs hosts are not so restricted, at least concerning *B. lusitaniae* (Kurtenbach et al. 2002, Poupon et al. 2006). As previously suggested, resident birds may be responsible for the geographic diffusion of *B. lusitaniae* and rodents (as showed in **Chapter VIII**) and lizards could be responsible for perpetuating this genospecies at enzootic cycles where they are a major vertebrate hosts for *I. ricinus* (Richter and Matuschka, 2006). In fact, *B. lusitaniae* shows a focal distribution in areas where the tick vector and the vertebrate reservoir coexist (Ragagli et al. 2010). The present work reinforces that hypothesis proving that both lizards and attached ticks were infected with *B. lusitaniae*.

Another important finding in this study is the presence of polymorphisms in some sequences that reveal the presence of genetic variants of *B. lusitaniae*. These IGS genetic variants detected on Madeira Island show differences from the *Borrelia* detected in mainland Portugal. Considering that *T. dugessi* is endemic on Madeira Island and evolution in the islands has specific constraints implies that the arrival of new diversity is reduced. The original gene pool, gene flow and the reproductive isolation caused by the geographic constraints is changed, comparing to continental areas. As pointed out in many other studies, colonizing populations tend to carry only a fraction of the parasites hosted by the originating population (Thorpe et al. 1994). Since *Borrelia* genospecies evolve in parallel with its reservoirs and hosts the constraints driving evolution on them will affect the *Borrelia* infection. Further studies addressing other gene sequences will be performed to clarify the specific situation of *B. burgdorferi* s.l. on Madeira Island.

The main conclusions of these studies are that *B. lusitaniae* circulates between a range of vertebrate host and small mammals and lizards could be also a potential reservoirs not only for *B. lusitaniae* but also for other *Borrelia* genospecies.

Chapter XII. Concluding Remarks

Twenty years after the first clinical case of Lyme borreliosis in Portugal some aspects of this disease still remain unclear.
The studies which are outlined in this dissertation are directed toward contributing to a better knowledge of the human infection and the eco-epidemiology of *B. burgdorferi* s.l., in particular of *B. lusitaniae*, the most prevalent genospecies present in Portugal.

Based on all studies performed it was possible to confirm the pathogenicity of *B. lusitaniae* for humans and support the hypothesis that in these patients the clinical symptoms may include some nonspecific symptoms for LB. In further studies, it will be interesting to continue the tentative culture and isolation of the clinical samples (skin biopsies, cerebrospinal fluid and blood), that most of the times are so difficult to obtain, in a way to achieve a better knowledge of the genetic diversity of this species.
Concerning *B. afzelii*, it was possible to detect DNA, for the first time, in a human patient with neurological symptoms.

The studies performed also highlight *B. lusitaniae* as the most prevalent species found in *I. ricinus* ticks, although other species such as *B. valaisiana*, *B. afzelii* and *B. garinii* are also present at least on Madeira Island. Moreover, co-infection was report for the first time between *R. monacensis*, *R. helvetica* and *R. slovaca*, respectively, each of which was infected with *B. lusitaniae.* The occurrence and implications of a simultaneous infection in patients has to be further evaluated, and clinicians need to be aware of these possible differential diagnoses in patients exposed to tick bites, since co-infections may show variable clinical symptoms.
The several isolates obtained from the *I. ricinus* tick confirm this species as the principal species in the vector transmission of *B. burgdorferi* s.l. However, as demonstrated, other species as *D. marginatus* may also act as a secondary vector but transmission studies have to be performed to clarify this hypothesis.

Another important finding was the detection of *B. garinii* DNA in some species of migratory shorebirds. The sequences obtained are diverse from the sequences detected in ticks from Madeira island, and could be related with other *B. garinii* associated to migratory birds. This is the first evidence that shorebirds that migrate through or winter in Portugal, may contribute to the dispersion of new genospecies in Portugal.

Ongoing studies may gain new insights on the potential reservoir host of *B. lusitaniae* with the first isolation from small mammals (*Apodemus sylvaticus*). Phylogenetic analyses based on *OspA* gene grouped this new isolate near other *B. lusitaniae* isolated in Portugal and most closely aligned to the North African clade. The same happened with other isolates obtained during the course of this work. Moreover, it was suggested that lizards are also implicated in the reservoir competence of *B. lusitaniae* but also of other *Borrelia* genospecies as the *B. valaisiana*. However, further studies such as cloning, are necessary to clarify if a co-infection of both genospecies is present, since the sequence data shows a suspicious of this co-infection. This is even more relevant since one isolate presented with *OspA* gene sequence 100% identical to *B. lusitaniae*. It was also confirmed the presence of polymorphisms in some sequences that reveal the presence of genetic variants of these both species. Further studies to access the eventual evolution forces driven this genetic diversity and the eventual presence of *B. lusitaniae* and *B. valaisiana* subpopulations associated to specific reservoirs should be performed in order to clarify this situation on Madeira Island.
The future use of a MLST approach based on chromosomal housekeeping genes could provide the genetic structure of these spirochetes and the level of diversity among each genospecies of *Borrelia* and namely the phylogeographic structure of *B. lusitaniae.*

The entry of the new system of obligatory notification that will include the clinical, epidemiological and laboratory notification will be a valuable instrument to reduce the underreporting of LB and to allow epidemiological analysis that is essential to implement adequate prevention programmes.

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